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1 **Humoral responses against BQ.1.1 elicited after breakthrough infection and SARS-
2 CoV-2 mRNA vaccination**

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21 **Abstract**

22

23 The Omicron BQ.1.1 variant is now the major SARS-CoV-2 circulating strain in many
24 countries. Because of the many mutations present in its Spike glycoprotein, this variant is
25 resistant to humoral responses elicited by monovalent mRNA vaccines. With the goal to
26 improve immune responses against Omicron subvariants, bivalent mRNA vaccines have
27 recently been approved in several countries. In this study, we measure the capacity of plasma
28 from vaccinated individuals, before and after a fourth dose of mono- or bivalent mRNA
29 vaccine, to recognize and neutralize the ancestral (D614G) and the BQ.1.1 Spikes. Before
30 and after the fourth dose, we observe a significantly better recognition and neutralization of
31 the ancestral Spike. We also observe that fourth-dose vaccinated individuals who have been
32 recently infected recognize and neutralize better the BQ.1.1 Spike, independently of the
33 mRNA vaccine used, than donors who have never been infected or have an older infection.
34 Our study supports that hybrid immunity, generated by vaccination and a recent infection,
35 induces higher humoral responses than vaccination alone, independently of the mRNA
36 vaccine used.

37

38 **Keywords:** COVID-19, SARS-CoV-2, mRNA bivalent vaccine, Hybrid immunity, Humoral
39 responses, BQ.1.1

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40 **Introduction**

41 The Omicron BQ.1.1 variant is a sublineage of the BA.5 variant that spreads very rapidly
42 and is now the major circulating lineage in several countries [1,2]. Recent studies have shown
43 that original SARS-CoV-2 mRNA vaccines, based on the ancestral Wuhan strain Spike (S),
44 lead to poor humoral responses against several Omicron subvariants, including the BQ.1.1
45 variant [3–5]. With the goal to improve immune responses against these subvariants,
46 Moderna and Pfizer bivalent vaccines have recently been approved by health authorities in
47 many countries [6–8]. These updated versions of the vaccines are composed of mRNA coding
48 for the expression of both the ancestral and an Omicron subvariant S [9,10]. However, the
49 continued evolution of SARS-CoV-2 has resulted in the emergence of multiple Omicron sub-
50 lineages showing signs of convergent evolution by the acquisition of the same immune
51 escape mutation in the RBD region of the Spike protein. Notably, all five recent convergent
52 mutations are present in BQ. 1.1: R346T, K444T, L452R, N460K, or F486V [3]. Because of
53 these newly acquired mutations, the benefits of bivalent compared to monovalent vaccines
54 against this lineage remain to be established.

55 It is well accepted now that hybrid immunity leads to better immune responses and
56 protection from severe outcomes than vaccination alone [11–17]. Because the original
57 mRNA vaccines poorly prevent viral transmission, an important part of the vaccinated
58 population have been recently infected by Omicron subvariants, leading to improved immune
59 responses in these individuals compared to SARS-CoV-2 naïve individuals who have just
60 been vaccinated.

61 In this study, we evaluated the capacity of plasma antibodies to recognize and neutralize
62 the original D614G and the Omicron BQ.1.1 subvariant S four weeks (W4-Va3) and four
63 months (M4-Va3) after the third dose and four weeks after the fourth dose (W4-Va4) of
64 mRNA vaccines (Figure 1A). These participants mainly received as their first three doses of
65 vaccine the Pfizer monovalent vaccine, and as the fourth dose either the Pfizer or Moderna
66 monovalent or Pfizer (BA.4/5) or Moderna (BA.1) bivalent vaccines. We also measured the
67 anti-nucleocapsid (N) level to determine if the donors had recent breakthrough infection

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68 (BTI), i.e., they have been infected between their third and fourth doses of vaccine by a
69 Omicron sublineage. Basic demographic characteristics of the cohort are summarized in
70 Table 1.

71

72 **Materials and Methods**

73 **Ethics statement**

74 The study was conducted in accordance with the Declaration of Helsinki in terms of
75 informed consent and approval by an appropriate institutional board. The protocol was
76 approved by the Ethics Committee of CHUM (19.381, approved on February 28, 2022) and
77 Héma-Québec (2022-016, approved on October 7, 2022).

78 **Human subjects**

79 The study was conducted in 63 individuals (25 males and 38 females; age range: 24-84
80 years). 20 of these individuals had recent breakthrough infection with an Omicron sublineage
81 (9 males and 11 females; age range: 24-67 years), i.e. as determined by the increase in anti-
82 N levels between W4-Va3 and M4-Va3 or between M4-Va3 and W4-Va4 using a recently
83 described analytical approach [18] (Figure S1). For the other donors (16 males and 27
84 females; age range: 31-84 years), we did not observe a significant increase of the anti-N
85 levels, although some of them have a history of infection. No other specific criteria such as
86 number of patients (sample size), sex, clinical or demographic were used for inclusion.

87 **Plasma samples and antibodies**

88 Plasma samples were either recovered from whole blood or directly obtained from the
89 PlasCov biobank [19], heat-inactivated for 1 hour at 56°C and stored at -80°C until use in
90 subsequent experiments. Pre-pandemic plasma samples were used as negative controls in
91 cytometry assays (data not shown). The conformationally independent S2-specific
92 monoclonal antibody CV3-25 was used as a positive control and to normalize Spike
93 expression in flow cytometry assays, as described [4,20–23]. Alexa Fluor-647-conjugated
94 goat anti-human antibodies (Abs) able to detect all Ig isotypes (anti-human IgM+IgG+IgA;
95 Jackson ImmunoResearch Laboratories, Cat # 109-605-064) were used as secondary Abs to
96 detect plasma binding in flow cytometry experiments.

97 **Plasmids**

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98 The plasmids encoding the SARS-CoV-2 D614G and BQ.1.1 Spike variants were
99 previously described [4]. The pNL4.3 R-E-Luc plasmid was obtained from the NIH AIDS
100 Reagent Program (Cat# 3418). The pIRES2-EGFP expressing plasmid was purchased from
101 Clontech (Cat# 6029-1).

102 **Cell lines**

103 293T human embryonic kidney cells (obtained from ATCC, Cat# CRL-3216) were
104 maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)
105 (Wisent) containing 5% fetal bovine serum (FBS) (VWR) and 100 µg/ml of penicillin-
106 streptomycin (Wisent). 293T-ACE2 cell line was previously reported [24].

107 **Enzyme-linked immunosorbent assay (ELISA)**

108 All samples were tested for anti-N total immunoglobulin levels using an in-house anti-
109 N ELISA. The assay protocol is similar to the anti-SARS-CoV-2 RBD ELISA previously
110 developed by our group [25], except that recombinant N (Centre National en Électrochimie
111 et en Technologies Environnementales Inc., Shawinigan, Canada) was used (0.25 µg/ml) in
112 lieu of the RBD antigen (2.5 µg/ml).

113 **Cell surface staining and flow cytometry analysis**

114 293T were transfected with full-length SARS-CoV-2 Spikes and a green fluorescent
115 protein (GFP) expressor (pIRES2-eGFP) using the calcium-phosphate method. Two days
116 post-transfection, Spike-expressing 293T cells were stained with the CV3-25 Ab (5 µg/mL)
117 as control or plasma (1:250 dilution) for 45 min at 37°C. AlexaFluor-647-conjugated goat
118 anti-human IgM+IgG+IgA (1/800 dilution) were used as secondary Abs. The percentage of
119 Spike-expressing cells (GFP + cells) was determined by gating the living cell population
120 based on viability dye staining (Aqua Vivid, Invitrogen). Samples were acquired on a
121 LSRFortessa cytometer (BD Biosciences), and data analysis was performed using FlowJo
122 v10.7.1 (Tree Star). The conformationally-independent anti-S2 antibody CV3-25, effective
123 against all Spike variants, was used to normalize Spike expression, as reported [4,20,22,23].
124 The Median Fluorescence intensities (MFI) obtained with plasma were normalized to the
125 MFI obtained with CV3-25 and presented as percentage of CV3-25 binding.

126 **Virus neutralization assay**

127 293T cells were transfected with the lentiviral vector pNL4.3 R-E− Luc and a plasmid
128 encoding the D614G or the BQ.1.1 S glycoprotein at a ratio of 10:1 to produce SARS-CoV-

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129 2 pseudoviruses. Two days post-transfection, cell supernatants were harvested and stored at
130 −80°C until use. For the neutralization assay, 293T-ACE2 target cells were seeded at a
131 density of 1×10^4 cells/well in 96-well luminometer-compatible tissue culture plates
132 (PerkinElmer) 24h before infection. Pseudoviral particles were incubated with several plasma
133 dilutions (1/50; 1/250; 1/1250; 1/6250; 1/31250) for 1h at 37°C and were then added to the
134 target cells followed by incubation for 48h at 37°C. Cells were lysed by the addition of 30 µL
135 of passive lysis buffer (Promega) followed by one freeze-thaw cycle. An LB942 TriStar
136 luminometer (Berthold Technologies) was used to measure the luciferase activity of each
137 well after the addition of 100 µL of luciferin buffer (15mM $MgSO_4$, 15mM KH_2PO_4 [pH
138 7.8], 1mM ATP, and 1mM dithiothreitol) and 50 µL of 1mM d-luciferin potassium salt
139 (Prolume). The neutralization half-maximal inhibitory dilution (ID_{50}) represents the plasma
140 dilution to inhibit 50% of the infection of 293T-ACE2 cells by pseudoviruses.

141 **Statistical analysis**

142 Symbols represent biologically independent samples from individuals. Statistics were
143 analyzed using GraphPad Prism version 8.0.1 (GraphPad, San Diego, CA). Each dataset was
144 tested for statistical normality and this information was used to apply the appropriate
145 (parametric or nonparametric) statistical test. p values < 0.05 were considered significant;
146 significance values are indicated as $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{***}P<0.0001$, ns, non-
147 significant.

148

149 **Results**

150 We first monitored the capacity of plasma to recognize the D614G and BQ.1.1 Spikes
151 after the third and fourth doses of mRNA vaccine by flow cytometry (Figure 1B-D). For the
152 D614G S, no significant differences were observed four weeks and four months after the
153 third dose of vaccine between individuals with or without recent BTI. In contrast, four weeks
154 after the fourth dose of mRNA vaccine, individuals with recent BTI recognized better the
155 D614G S than donors with no recent BTI regardless of the vaccine type received (Figure 1B).
156 For the BQ.1.1 S, at the M4-Va3 timepoint donors with recent BTI better recognized the S
157 than individuals with no recent infection, and this difference in recognition was more

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158 significant four weeks after the fourth dose (Figure 1C). The level of BQ.1.1 S recognition
159 was significantly lower compared to D614G S in donors without recent BTI (Figure 1D), in
160 agreement with recent reports [4,5]. In donors who had recently been infected, there was a
161 significant but smaller difference in the level of recognition between the two Spikes
162 compared to the other group.
163
164 We also measured the neutralizing activity of plasma against the D614G and BQ.1.1 S
165 (Figure 1E-G). We observed pattern similar to that measured for Spike recognition. No
166 significant differences were observed between the two groups at W4-Va3 and M4-Va3
167 timepoints (Figure 1E-F). In contrast, four weeks after the fourth dose, donors with recent
168 BTI had a significantly higher level of neutralizing activity against D614G and BQ.1.1 S. All
169 donors with recent BTI who received a fourth dose developed neutralizing antibodies against
170 BQ.1.1 S, while some donors who just received four doses of vaccine were still not able to
171 neutralize this Spike. As observed for S recognition (Figure 1D), BQ.1.1 Spike was
172 significantly less neutralized than D614G S, even after four doses of mRNA vaccine (Figure
173 1G). However, the difference in neutralization between the two S was smaller in the group
174 with recent BTI.
175
176 The Moderna BA.1 bivalent vaccine (blue points) tended to induce better recognition and
177 neutralization than the other vaccine platforms including the Pfizer BA.4/5 bivalent vaccine
178 with lesser decrease of recognition and neutralization of BQ.1.1 S (Figure 1B-G, Figure S2).
179 These differences did not reach statistical significance; whether this is due to the relatively
180 low number of samples tested remains to be determined.
181
182
183 **Discussion**

184 Since its emergence in late 2021, the Omicron variant continues to evolve into new
185 subvariants that are increasingly resistant to monoclonal antibodies and vaccination [5,26–
186 30]. To address vaccine resistance, bivalent mRNA vaccines, expressing both the original

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187 Spike and one of the parental lineages of Omicron (BA.1 or BA.4/5) Spike, have been
188 developed and are now being administered in several jurisdictions worldwide. However,
189 although the bivalent mRNA vaccine has been shown to increase the level of protection
190 against BA.5 variant in mice [31], evidence of its superior effectiveness in the human
191 population remains to be demonstrated, especially against sub-lineages with newly acquired
192 immune escape mutations. Recent studies showed that both monovalent and bivalent
193 vaccines induced low humoral responses against BQ.1.1, but recent breakthrough infection
194 before vaccination strongly improved these responses [32]. The results presented herein
195 support these observations.

196 As previously reported in numerous studies, including ours, hybrid immunity led to
197 better humoral responses against the BQ.1.1 and other recent variants than just vaccination
198 [4,5,32]. Also, we observed that after 4 doses of mRNA vaccine and no recent BTI, some
199 donors did not have neutralizing activity against pseudoviral particles bearing the BQ.1.1
200 Spike. Whether these changes of recognition and neutralization translate into greater risk of
201 severe disease is currently unknown. In contrast, BTI likely increased the breadth of
202 neutralizing antibodies since all donors had detectable levels of neutralization against
203 BQ.1.1.

204

205 These results indicate that further efforts have to be devoted to improve vaccines against
206 new SARS-CoV-2 variants of concern. Whether immune responses comparable to those
207 observed with breakthrough infections could be obtained with new vaccine formulations
208 remains to be determined.

209

210 **Author Contributions:** A.T., R.B. and A.F. conceived the study. A.T., M.B., H.M., J.P.,
211 G.G-L., R.B. and A.F. performed, analyzed, and interpreted the experiments. A.T. performed
212 statistical analysis. H.M., G.G-L., M.C., and A.F. contributed unique reagents. L.G., P.A.,
213 C.M., C.T., D.E.K., Y.G. and V.M.-L. collected and provided clinical samples. R.B., G.D.S.,
214 and I.L. provided scientific input related to VOCs and vaccine efficacy. A.T. and A.F. wrote
215 the manuscript with inputs from others. Every author has read, edited and approved the final
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232

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234 Declaration of Helsinki in terms of informed consent and approval by an appropriate
235 institutional board. The protocol was approved by the Ethics Committee of CHUM (19.381,
236 approved on February 28, 2022) and Héma-Québec (2022-016, approved on October 7,
237 2022).

238

239 **Informed Consent Statement:** Informed consent was obtained from all subjects involved in
240 the study.

241

242 **Data Availability Statement:** Further information, data reported in this paper, and requests
243 for resources and reagents should be directed to and will be fulfilled by the lead contact,
244 Andrés Finzi (andres.finzi@umontreal.ca) upon request.

245

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382 **Table 1. Characteristics of the SARS-CoV-2 vaccinated cohort**

		Entire cohort	No recent BTI	Recent BTI
Number		63	43	20
Age**		59 (24-84)	63 (31-84)	54 (24-67)
Sex	**Female (n)**	38	27	11
	Male (n)	25	16	9
Days between the third and fourth doses******		186 (101-313)	155 (101-271)	268 (134-313)
Fourth dose	**Pfizer monovalent**	28	22	6
	Moderna monovalent	21	18	3
	Pfizer BA.4/5	4	1	3
	Moderna BA.1	10	2	8
Days between the third and W4-Va3		26 (17-45)	25 (17-45)	28 (18-41)
Days between the third and M4-Va3		120 (90-194)	120 (90-194)	122 (92-150)
Days between the fourth and W4-Va4		28 (18-96)	28 (18-96)	25 (20-42)

383 Values displayed are medians, with ranges in parentheses. Continuous variables between
384 individuals with no recent and recent BTI were compared by using Mann-Whitney tests. $p < 0.05$
385 was considered statistically significant for all analyses. Statistical differences between
386 the two groups were found for the age of the donors and the interval between the third and
387 fourth doses of vaccine. (** $P < 0.01$; **** $P < 0.0001$).

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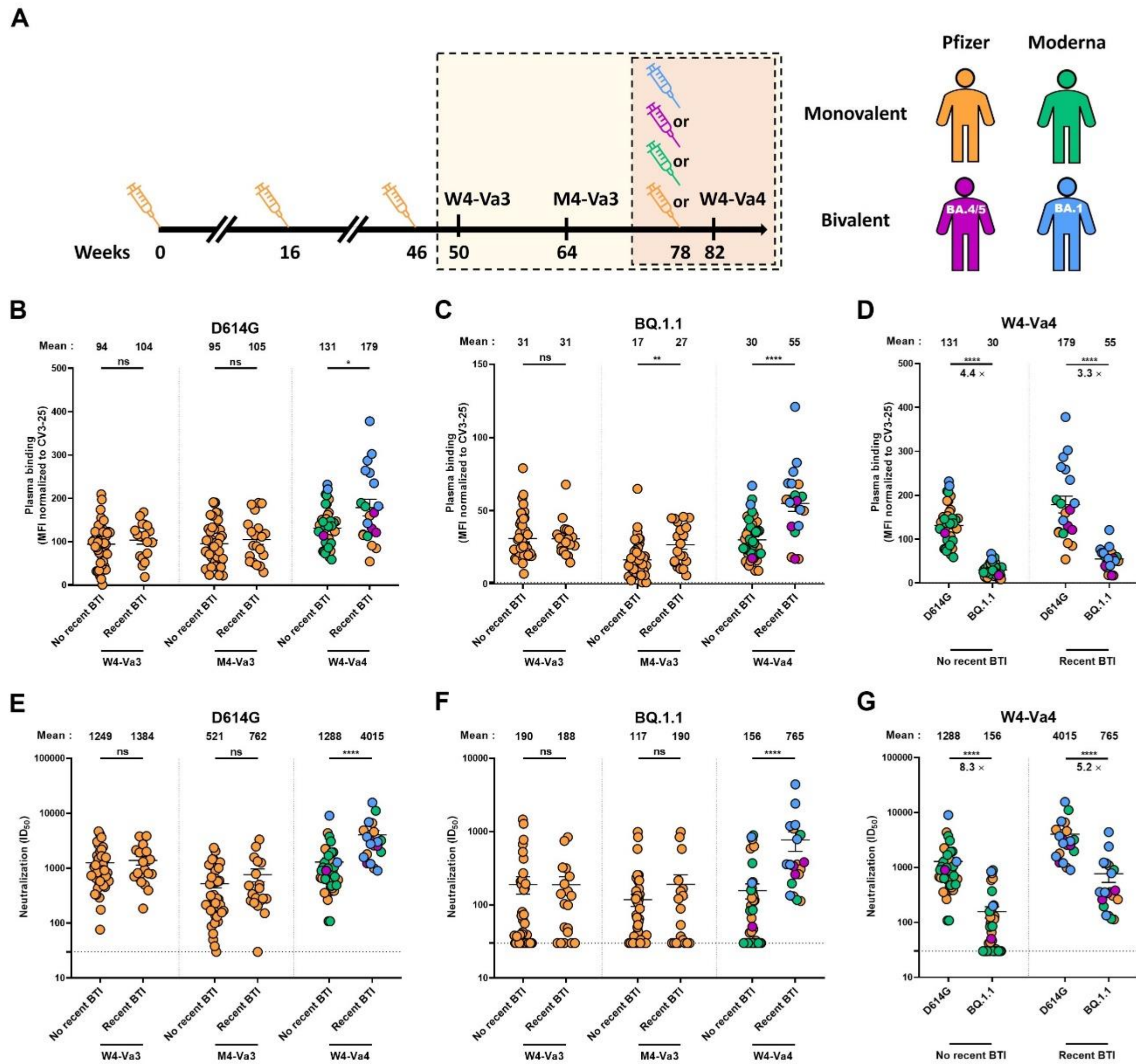

388

389 **Figure 1. Recognition and neutralization of the D614G and BQ.1.1 Spikes after the third**
390 **and fourth doses of SARS-CoV-2 vaccine in individuals with or without a recent**
391 **breakthrough infection.** (**A**) SARS-CoV-2 vaccine cohort design. The yellow box identifies
392 the three timepoints under study shown in panels B, C, E and F and the red box the period
393 presented in panels D and G. (**B-D**) 293T cells were transfected with the full-length D614G
394 or BQ.1.1 S, stained with the CV3-25 mAb or with plasma from vaccinated individuals and
395 analyzed by flow cytometry. The values represent the MFI normalized by CV3-25 mAb
396 binding. (**E-G**) Neutralization activity was measured by incubating pseudoviruses bearing
397 SARS-CoV-2 S glycoproteins, with serial dilutions of plasma for 1 h at 37°C before infecting
398 293T-ACE2 cells. Neutralization half maximal inhibitory serum dilution (ID_{50}) values were
399 determined using a normalized non-linear regression using GraphPad Prism software.
400 Individuals vaccinated with Pfizer monovalent, Moderna monovalent, Pfizer bivalent

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401 (BA.4/5) or Moderna bivalent (BA.1) fourth dose are represented by orange, green, purple
402 and blue points respectively. Limits of detection are plotted. Error bars indicate means ±
403 SEM. (* P < 0.05; ** P < 0.01; **** P < 0.0001; ns, non-significant).
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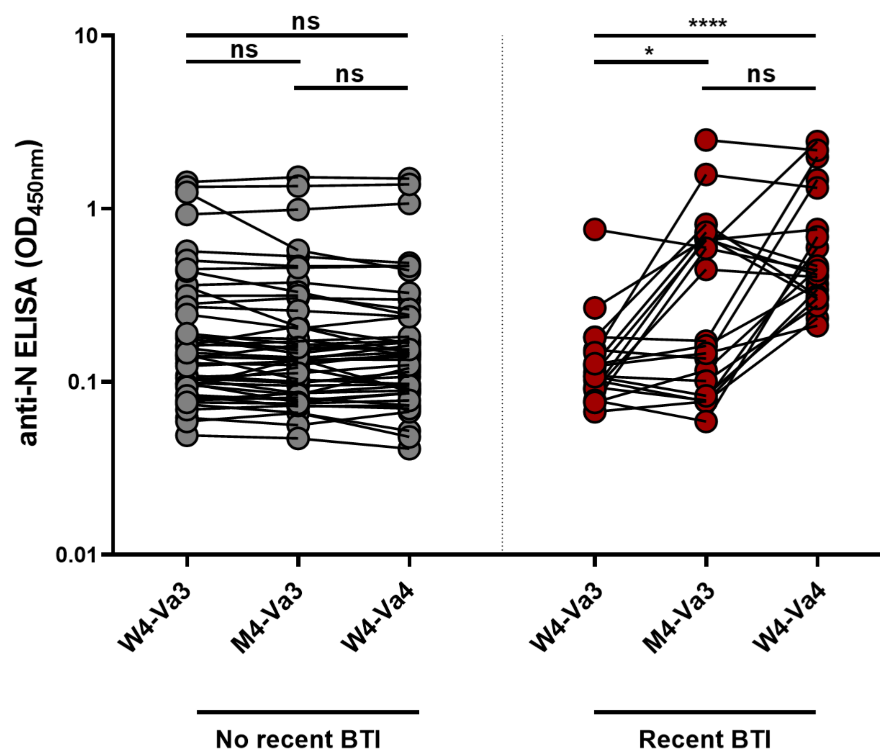

405

406 **Figure S1. Anti-N level measured after the third and fourth doses of SARS-CoV-2**
407 **vaccine.** Anti-N level was measured in plasma from vaccinated donors by ELISA. Donors
408 are considered to have a recent BTI when a significant increase of anti-N Abs level between
409 W4-Va3 and M4-Va3 or between M4-Va3 and W4-Va4 is observed, according to a recently
410 described analytical approach based on the ratio of anti-N absorbance. Individuals with no
411 recent BTI and recent BTI are represented by gray and red dots respectively. (* $P < 0.05$;
412 **** $P < 0.0001$; ns, non-significant).

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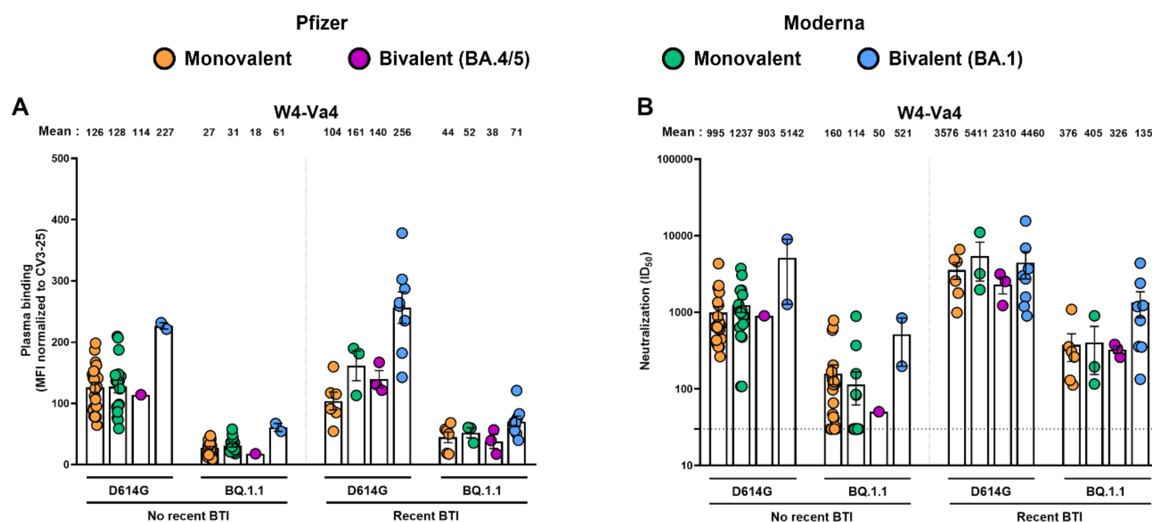

414

415 **Figure S2. Recognition and neutralization of the D614G and BQ.1.1 Spikes after the**
416 **fourth doses of SARS-CoV-2 vaccine in individuals with or without a recent**
417 **breakthrough infection.** (**A**) 293T cells were transfected with the full-length D614G or
418 BQ.1.1 S, stained with the CV3-25 mAb or with plasma from vaccinated individuals and
419 analyzed by flow cytometry. The values represent the MFI normalized by CV3-25 mAb
420 binding. (**B**) Neutralization activity was measured by incubating pseudoviruses bearing
421 SARS-CoV-2 S glycoproteins, with serial dilutions of plasma for 1 h at 37°C before infecting
422 293T-ACE2 cells. Neutralization half maximal inhibitory serum dilution (ID_{50}) values were
423 determined using a normalized non-linear regression using GraphPad Prism software.
424 Individuals vaccinated with Pfizer monovalent, Moderna monovalent, Pfizer bivalent
425 (BA.4/5) or Moderna bivalent (BA.1) fourth dose are represented by orange, green, purple
426 and blue points respectively. Limits of detection are plotted. Error bars indicate means ±
427 SEM.