Sialylation regulates migration in chronic lymphocytic leukemia

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Supplementary Material and Methods

Neuraminidase treatment

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation (Immunological Science) according to the manufacturer's instructions. To remove sialic acids from the cell surface, PBMCs ($25x10^6$) were treated/mock treated with neuraminidase from *Vibro Cholerae* (0.1 U/ml; Merck) in 500 µl of serum-free RPMI 1640 at 37°C for 45 minutes. During the incubation, cells were gently resuspended every 15 min. After incubation, cells were washed and used for flow cytometry, Western blot analysis or functional assay.

Western blot analysis

Cells were lysed in 1% Triton-X100 based buffer (50 mM Tris●HCl pH 8, 150mM sodium chloride, 1% Triton-X100, 5 mM ethylenediaminetetraacetic acid [EDTA], all from Merck) supplemented with protease and phosphatase inhibitors (1:100; ThermoFisher Scientific). Protein concentration in lysates was determined using the bicinchoninic acid (BCA) assay (ThermoFisher Scientific) and an equal amount of protein (20 µg) was resolved on a gradient sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane and blocked for 1 h with 5% (w/v) not fat milk in PBS. Membranes were probed overnight with anti-human α4 (clone EPR1355Y) and β1 (clone EPR16896) antibodies (all from Abcam; Cambridge, UK) and β actin (Merck) diluted 1:1000 in 5% (w/v) BSA in PBS/ 0.05% (v/v) Tween 20 (PBST). After incubation, membranes were probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; Bio-Rad Laboratories; Hercules, CA) for 1 h in 5% (w/v) not fat milk in PBST. Membranes were then incubated for 2 min with Immobilon ECL Ultra Western HRP substrate (Merck) and immunoreactive bands were visualized and acquired using the Uvitec Alliance chemiluminescence imaging system (Uvitec; Cambridge, UK).

Supplementary Figure legends

Supplementary Figure 1. Mutated and unmutated *IGVH* CLL cells express comparable levels of α2-3 Sia, α2-6 Sia and $SLe^{a/x}$. PB collected from untreated CLL patients were lysed and stained for flow cytometry. Identification of CLL cells and quantification of the expression levels of α2-3 Sia, α2-6 Sia and $SLe^{a/x}$ are described in the main text. Graphs display the percentages and the MFI of 2-3 Sia (A, B), α2-6 Sia (C, D) and $SLe^{a/x}$ (E, F) expression levels in mutated and unmutated *IGVH* CLL cells. Dots represent the individual

measurements. Horizontal lines depict the median and the interquartile range. The Mann-Whitney test was used to determine statistical significance. ns: non-significant.

Supplementary Figure 2. CD38-negative and -positive CLL cells express comparable levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$. PB collected from untreated CLL patients were lysed and stained for flow cytometry. Identification of CLL cells and quantification of the expression levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ are described in the main text. Graphs display the percentages and the MFI of 2-3 Sia (A, B), α2-6 Sia (C, D) and SLe$^{a/x}$ (E, F) expression levels in CD38-negative and -positive CLL cells (cut-off 30% positivity). Dots represent the individual measurements. Horizontal lines depict the median and the interquartile range. The Mann-Whitney test was used to determine statistical significance. *: $p<0.05$; ns: non-significant.

Supplementary Figure 3. CD49d-negative and -positive CLL cells express comparable levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$. PB collected from untreated CLL patients were lysed and stained for flow cytometry. Identification of CLL cells and quantification of the expression levels of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ are described in the main text. Graphs display the percentages and the MFI of 2-3 Sia (A, B), α2-6 Sia (C, D) and SLe$^{a/x}$ (E, F) expression levels in CD49d-negative and -positive CLL cells (cut-off 30% positivity). Dots represent the individual measurements. Horizontal lines depict the median and the interquartile range. The Mann-Whitney test was used to determine statistical significance. ns: non-significant.

Supplementary Figure 4. Migration of CLL cells on BSA, VCAM1 and FN coated transwell in response to SDF1α. CLL cells were seeded on top of transwells coated overnight with BSA, VCAM1-Fc chimera and FN. The bottom chambers of the transwells were filled with serum free media supplemented with SDF1α (200 ng/mL). Cells were allowed to migrate for 5 h at 37°C. After incubation, cells in the lower chambers were collected and mixed with 25 µl of counting beads. Migrated CLL cells were counted using a BD FACS Canto I flow cytometer by gating on the counting beads and acquiring, in this gate, 2000 events.

Supplementary Figure 5. The MFI of CXCR4 expression correlates with migration stimulated by SDF1α and FN-dependent migration but not with VCAM1-dependent migration. CLL seeding, migration and its quantification are described above. Correlation between the number of migrated cells in SDF1α-stimulated migration on BSA (A), FN (B)

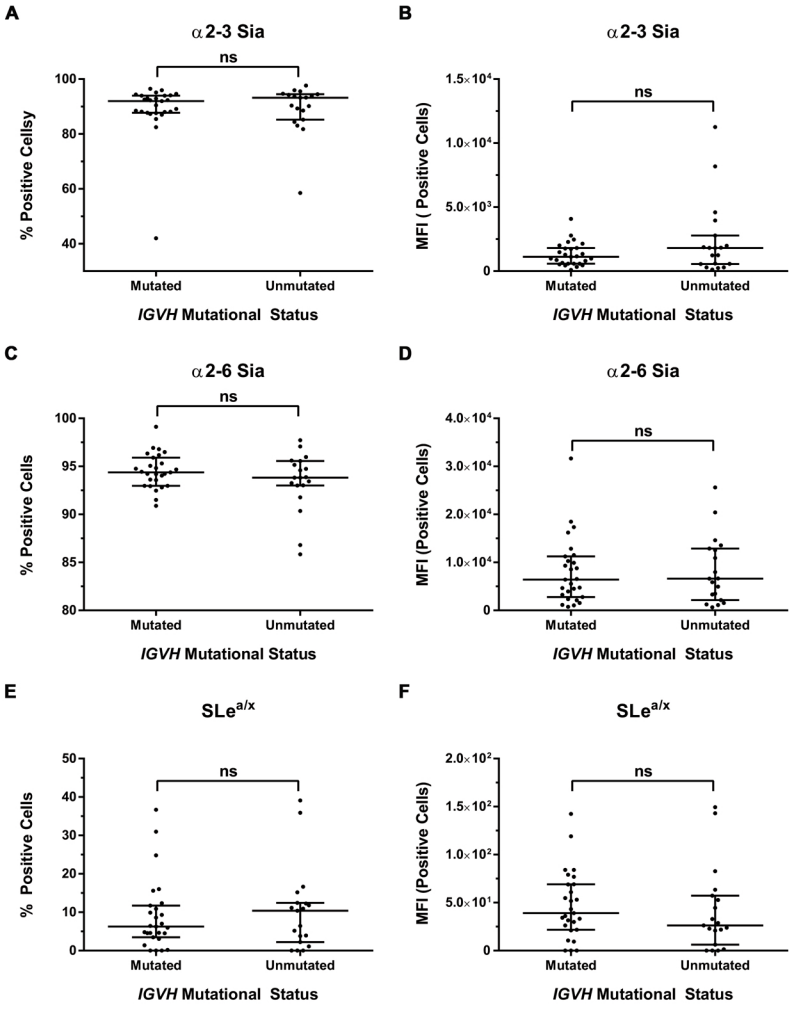
and VCAM1 (C) and the levels of CXCR4 expression (MFI). The p and the R2 values are displayed.

Supplementary Figure 6. Neuraminidase treatment results in depletion of α2-3 but not α2-6 linked sialic acids. CLL cells were treated/mock treated with neuraminidase from *Vibro Cholerae* (0.1 U/ml) for 45 min at 37ºC. After incubation, cells stained for flow cytometry as described in the main text. The Mann-Whitney test was used to determine statistical significance. ***: p<0.001; ns: non-significant.

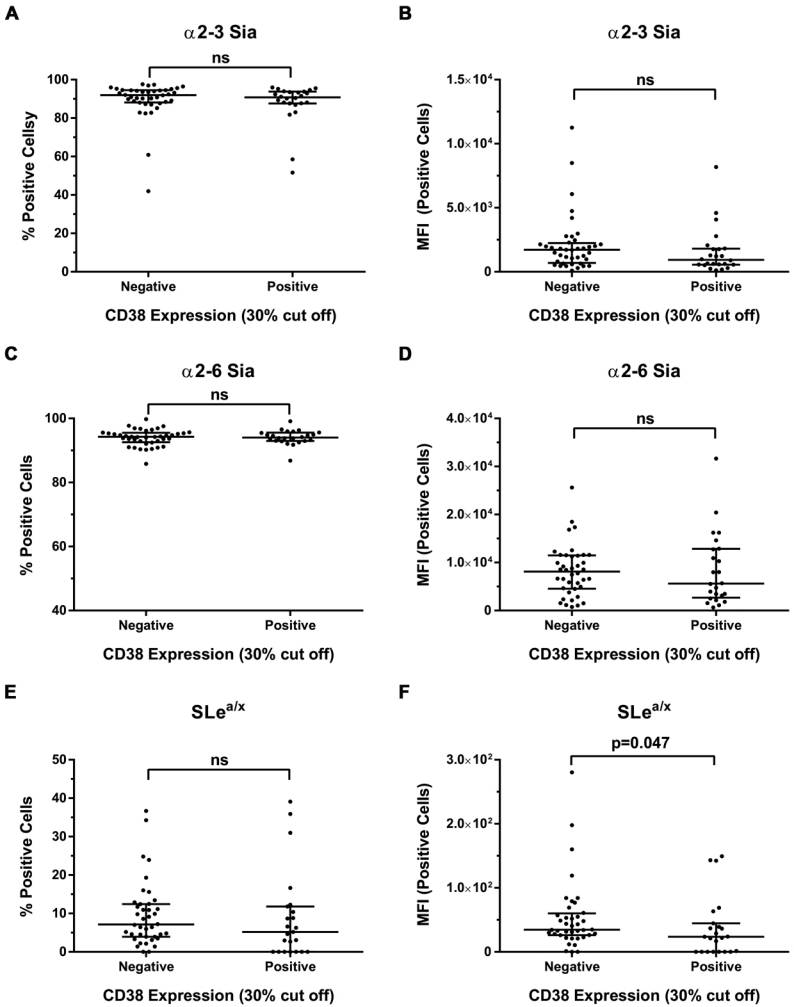
Supplementary Figure 7. Neuraminidase treatment does not significantly affects migration in CD49d-negative CLL cells. CLL cells obtained from 4 CD49d-negative patients were treated/mock treated with neuraminidase from *Vibro Cholerae* (0.1 U/ml) for 45 min and then seeded on top of transwells coated overnight with BSA, VCAM1-Fc chimera and FN. The bottom chambers of the transwells were filled with serum free media supplemented with SDF1α (200 ng/mL). Cells were allowed to migrate for 5 h at 37°C. After incubation, cells in the lower chambers were collected and mixed with 25 µl of counting beads. Migrated CLL cells were counted using a BD FACS Canto I flow cytometer by gating on the counting beads and acquiring, in this gate, 2000 events. Data are displayed as box and whiskers plot. The two-way ANOVA followed by Sidak's multiple comparison post-hoc testing was used to determine statistical significance. ns: non-significant.

Supplementary Figure 8. Expression of α2-3 Sia, α2-6 Sia and SLe$^{a/x}$ in CLL cells prior to and after ibrutinib treatment. Cryopreserved CLL cells collected from 13 ibrutinib treated patients prior to ibrutinib treatment were defrosted, stained for flow cytometry and compared to samples from the same patients taken after ibrutinib treatment. Graphs display the MFI of α2-3 Sia (A), α2-6 Sia (B) and SLe$^{a/x}$ (C) expression levels. Dots represent the individual measurements. The Wilcoxon matched-pairs signed rank test was used to determine statistical significance. *: p<0.05.

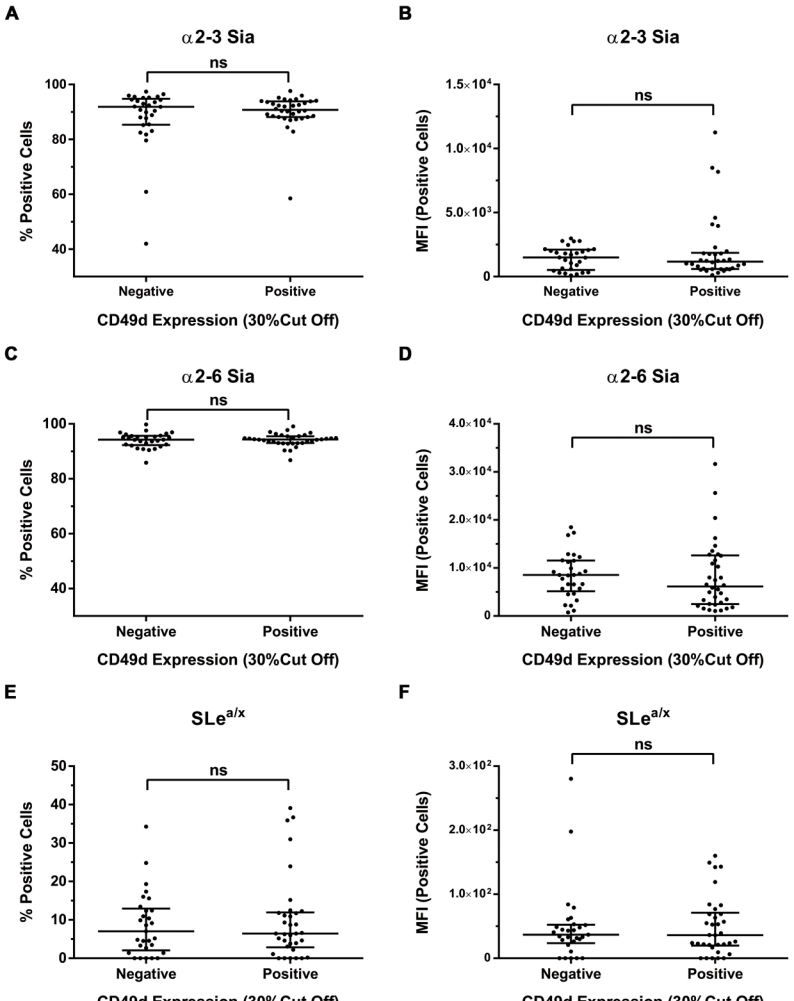
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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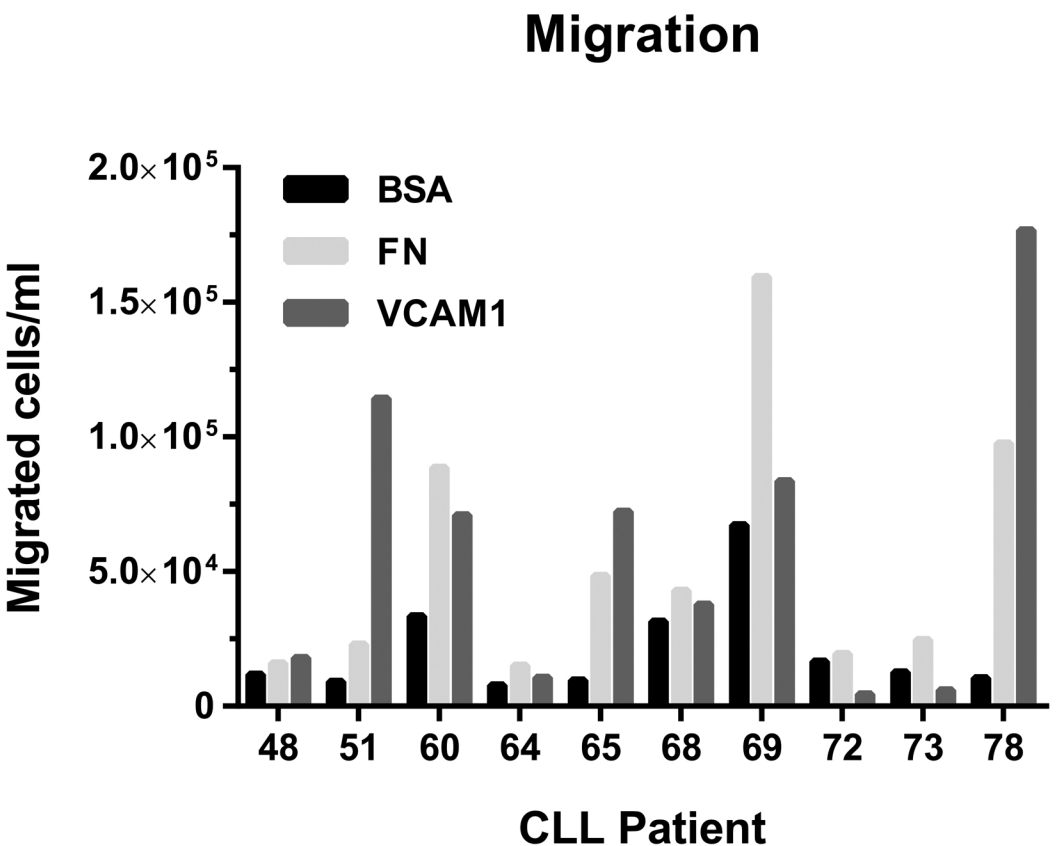

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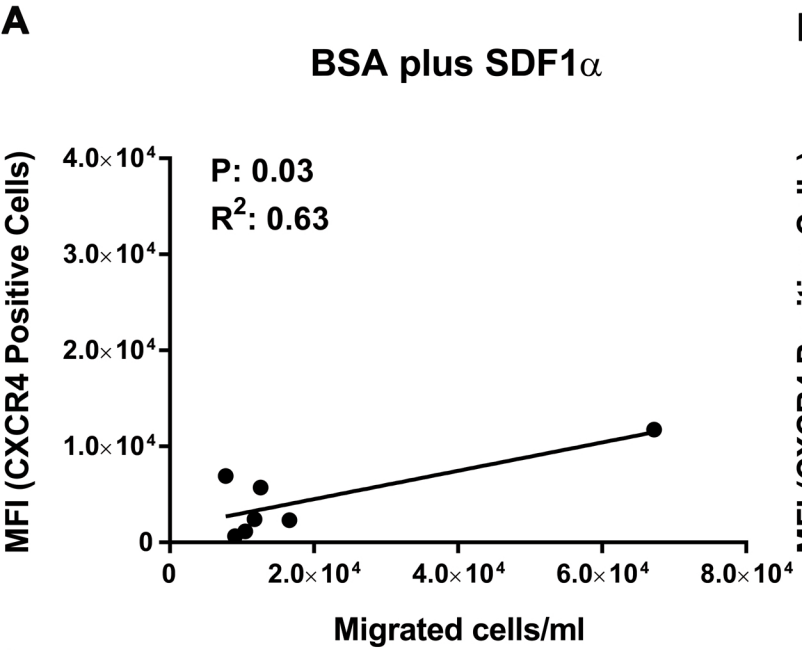

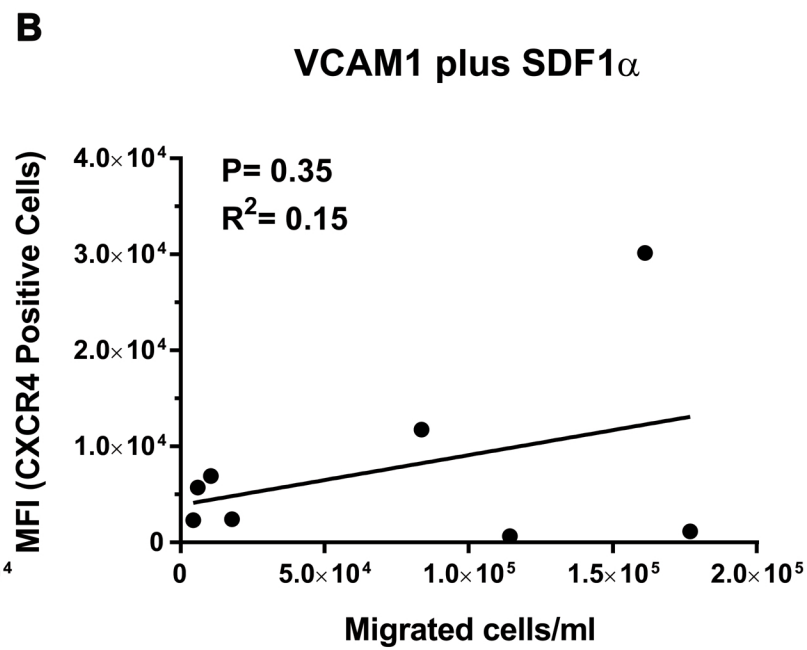

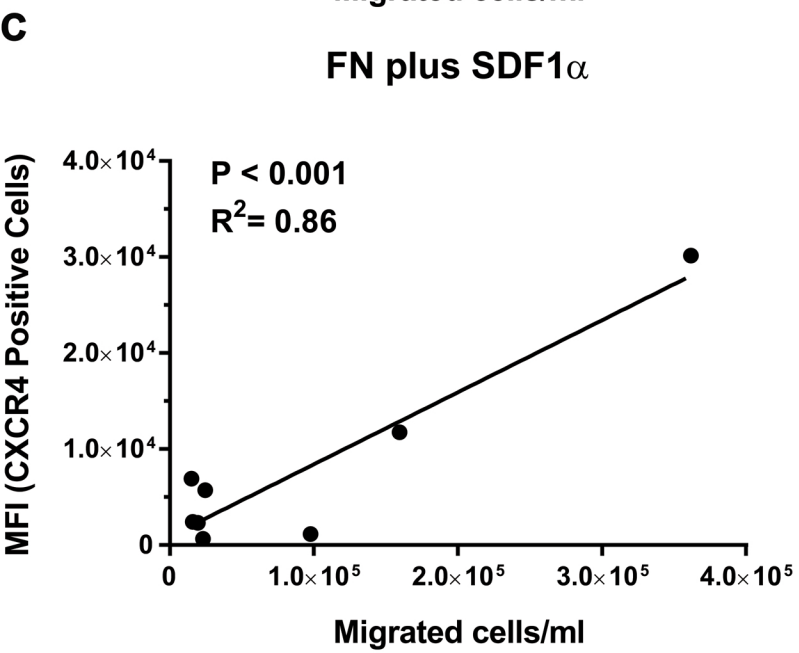

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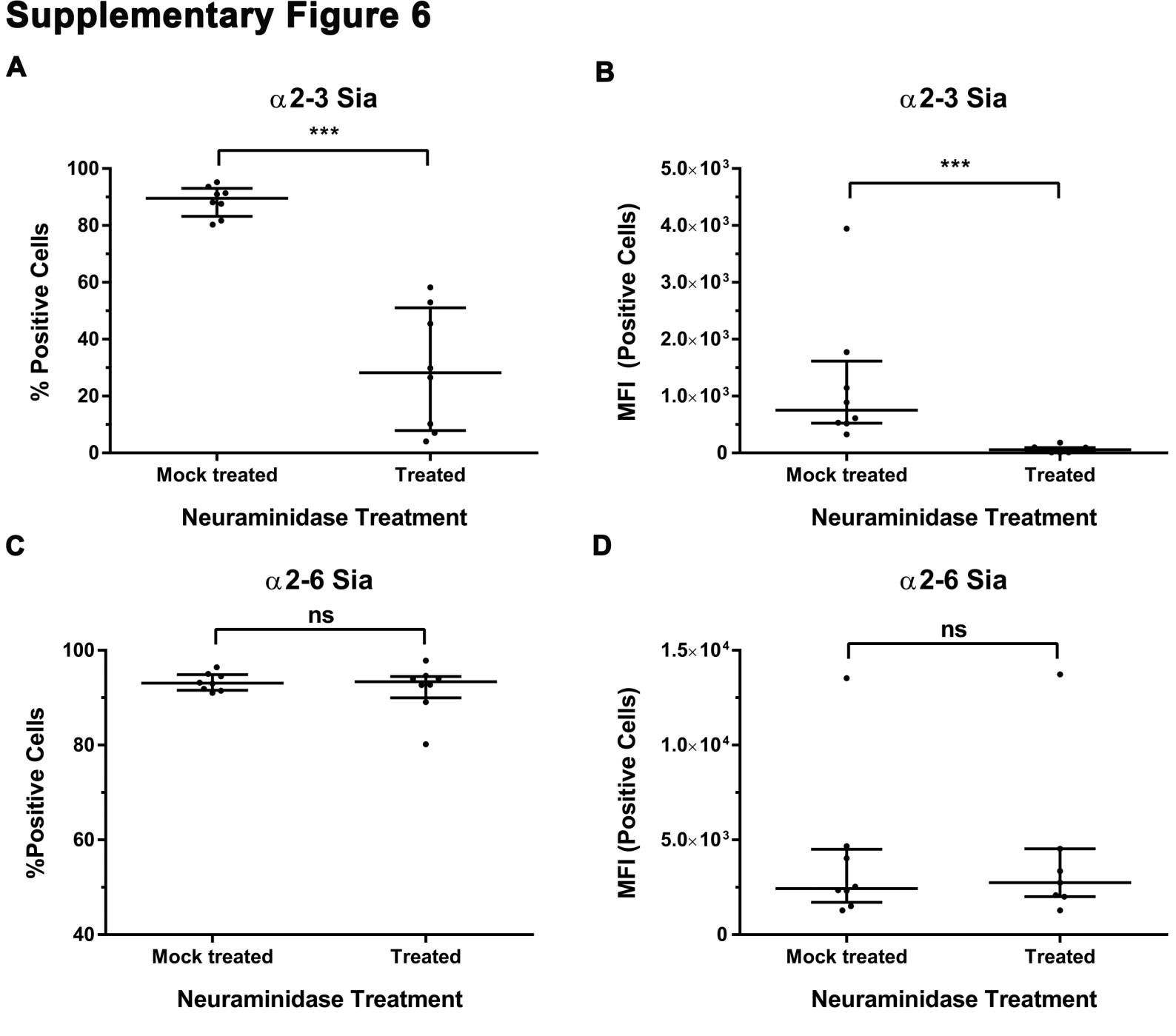

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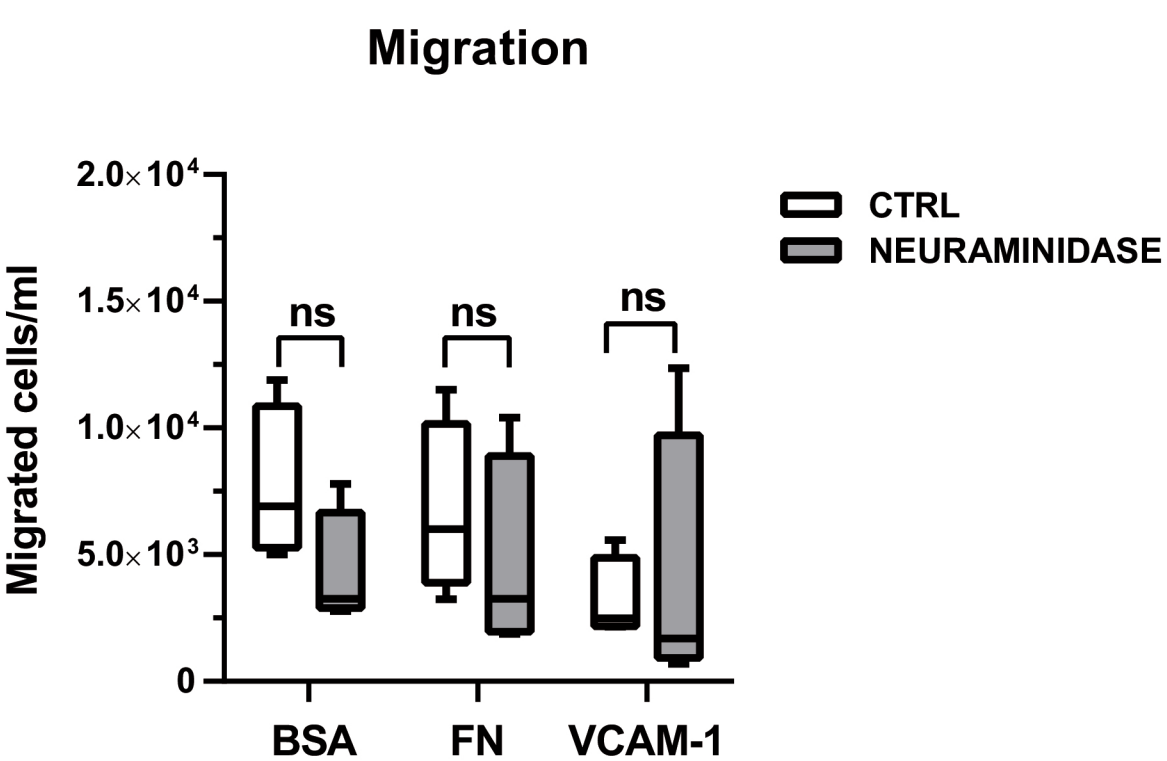

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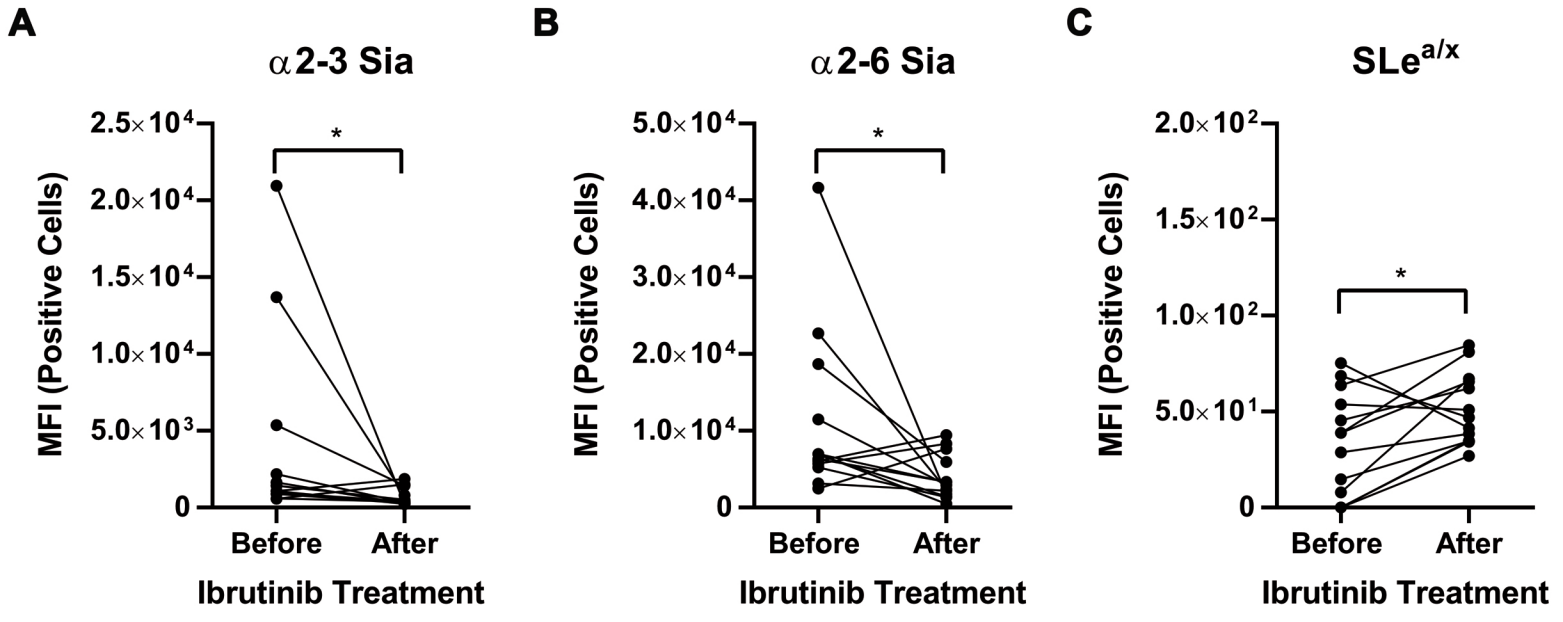

N	WBC (µl)	% Disease	CD49d (%)	CD38 (%)	*IGVH* Mutational Status
1	8500	63	NA	9	NA
2	30000	94	94	30	Unmutated
3	17260	77	100	100	Mutated
4	1102000	91	NA	NA	NA
5	12550	73	100	27	Unmutated
6	63630	93	100	100	Mutated
7	13940	76	NA	100	NA
8	92000	97	100	1	Mutated
9	21980	78	100	100	Mutated
10	13630	61	0	0	NA
11	21570	83	NA	0	NA
12	16350	71	NA	100	NA
13	1460	85	NA	10	NA
14	33690	85	NA	26	NA
15	19470	81	100	30	Unmutated
16	22760	84	100	100	Unmutated
17	13810	77	100	0	NA
18	10470	75	0	0	Mutated
19	13800	78	100	44	Mutated
20	13330	64	NA	NA	NA
21	14250	70	1	0	NA
22	27940	82	0	0	NA
23	21000	81	0	1	NA
24	12620	62	0	100	NA
25	23910	90	90	0	Mutated
26	35870	90	1	1	NA
27	7460	61	1	1	NA
28	45290	94	100	100	Unmutated
29	30640	90	100	100	NA
30	12060	78	NA	100	NA
31	13140	76	NA	100	NA
32	32830	92	0	0	Mutated
33	35280	84	100	0	Unmutated
34	11800	72	NA	NA	NA
35	21060	79	NA	6	NA
36	13850	79	NA	NA	NA
37	24350	82	NA	NA	NA
38	15570	72	100	100	NA
39	10410	62	100	0	NA
40	18590	78	NA	0	NA
41	24230	84	2	0	NA
42	13260	76	0	0	NA
43	39610	92	0	92	NA
44	17120	65	100	26	Mutated
45	17890	64	70	50	NA
46	14420	68	0	0	NA
47	292700	99	0	0	Mutated
48	109220	94	86	94	Unmutated
49	9540	86	3	0	Mutated
50	14580	79	1	0	Mutated
51	133400	94	100	94	Mutated
52	24000	85	0	0	Unmutated
53	19200	81	0	0	Mutated
54	72410	94	94	NA	Mutated
55	11140	65	0	0	Mutated
56	102400	91	19	92	Unmutated
57	13640	77	0	0	Mutated
58	17870	79	0	100	Unmutated
59	17950	84	0	0	Mutated
94	85820	94	94	0	Unmutated
61	25500	90	0	1	Unmutated

62	12670	81	6	0	NA
63	160420	97	97	32	Unmutated
64	78490	95	100	NA	Mutated
65	44960	92	95	40	NA
66	17170	80	2	NA	Mutated
67	10330	67	96	NA	Mutated
68	43460	87	94	NA	Mutated
69	84640	96	100	15	Mutated
70	71510	92	100	NA	Unmutated
71	NA	84	92	8	Mutated
72	22750	79	100	NA	Unmutated
73	NA	95	94	67	Unmutated
74	30400	86	43	NA	Unmutated
75	15450	76	100	6	Mutated
76	8770	73	27	9	Mutated
77	15040	75	94	0	Mutated
78	54400	91	100	52	Unmutated
79	58410	91	100	13	Unmutated

Supplementary Table 1. Clinical features of untreated CLL patients. NA: not available

N	% Disease	CD49d (% at baseline)	CD38 (% at baseline)	*TP53*	*IGVH* Mutational status
1	0.07	100	100	WT	Unmutated
2	1	100	100	WT	Unmutated
3	7	90	0	WT	Mutated
4	60	90	90	WT	Unmutated
5	4	14	53	WT	Mutated
6	22	2	8	WT	Unmutated
7	5	1	1	WT	Unmutated
8	6	100	56	WT	Unmutated
9	3	0	2	WT	Unmutated
10	2	NA	NA	WT	Unmutated
11	6	100	100	WT	Mutated
12	3	40	30	WT	Mutated
13	11	100	40	Mutated	Unmutated
14	1	100	18	WT	Unmutated
15	10	100	2	WT	Mutated
16	10	0	5	WT	Mutated
17	17	100	100	WT	Unmutated
18	17	0	2	WT	Unmutated
19	3	44	30	WT	Unmutated
20	30	6	7	WT	Mutated
21	10	2	8	WT	Unmutated
22	25	6	2	WT	Mutated
23	5	1	1	WT	Unmutated
24	28	2	1	WT	Mutated

Supplementary Table 2. Clinical features of ibrutinib-treated CLL patients. NA: not available.