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## **Sialic acids rather than glycosaminoglycans affect normal and sickle red blood cell rheology by binding to four major sites on fibrinogen**

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without the currently accepted treatment with plasma exchange and corticosteroids would not have been considered without our patient's requests, and would not have been possible without our patient's intelligence, understanding and agreement. We could confidently manage her as an outpatient because she was reliable, she lived near to the medical center, and her family could help with her management and guarantee her safety. Also, we would not have considered this treatment if her thrombocytopenia had been severe or if she had any neurologic symptoms. But with all of these supportive circumstances, we were able to manage our patient successfully. Our experience illustrates the ability of caplacizumab and rituximab, without hospitalization, to achieve a remission in patients with TTP. More importantly, our experience illustrates the value of integrating shared decision-making into clinical practice and exemplifies how shared decision-making can facilitate changes of medical practice, including utilization of new treatments.

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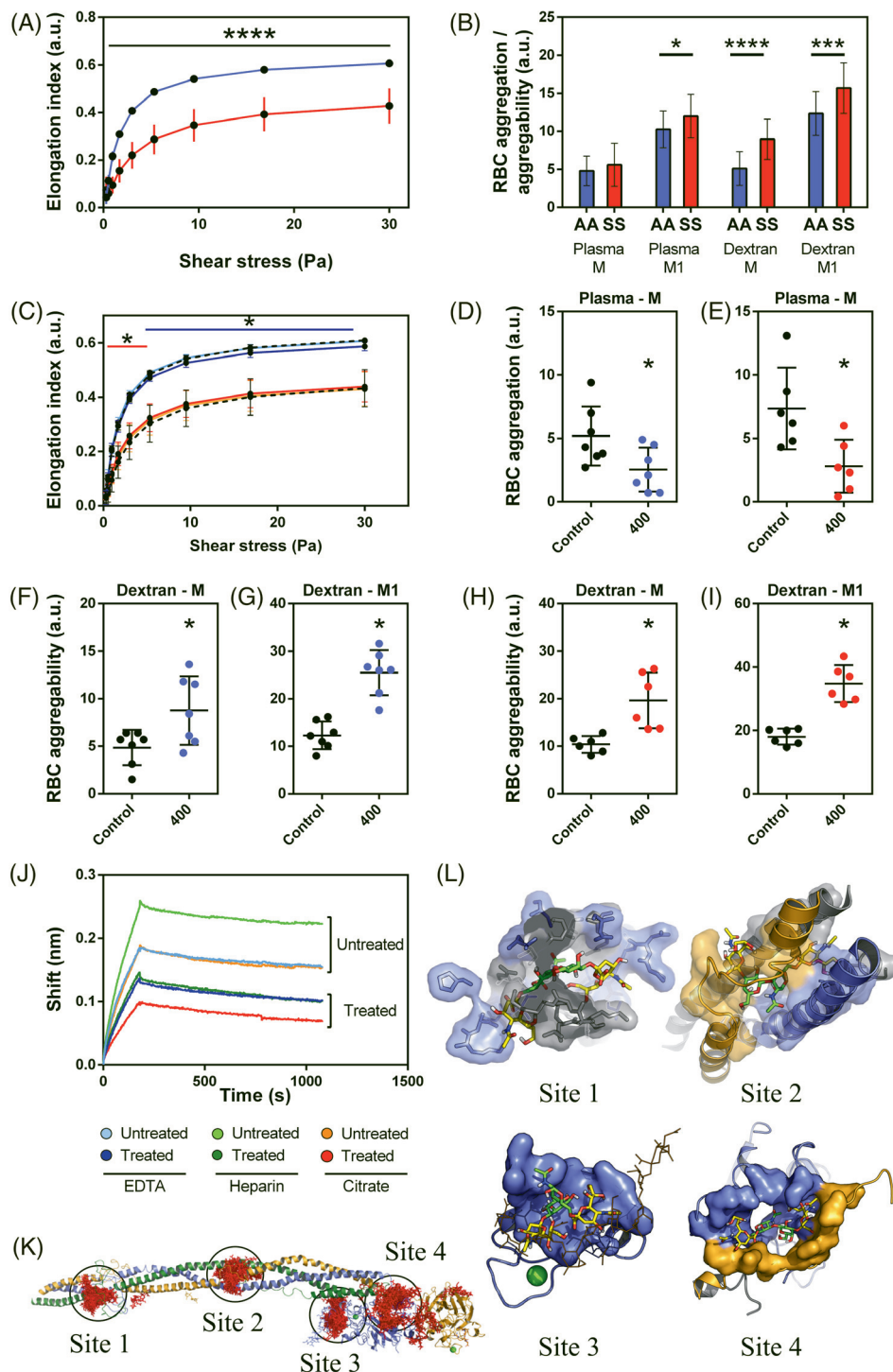
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## Sialic acids rather than glycosaminoglycans affect normal and sickle red blood cell rheology by binding to four major sites on fibrinogen

To the Editor:

Sickle cell anemia (SCA) is characterized by impaired red blood cell (RBC) rheology. The loss of RBC deformability plays a key role in the occurrence of painful vaso-occlusive events in SCA.<sup>1</sup> In addition, the strength of RBC aggregates correlates with the occurrence of acute chest syndrome, and RBC aggregation increases during acute vaso-occlusive crisis in adults with SCA.<sup>1</sup> However, the molecular mechanisms leading to abnormal RBC aggregation in SCA are currently unknown. The glycocalyx, covering the cell surface, contains glycoproteins with sialic acids (SA) and proteoglycans made of glycosaminoglycans (GAGs) covalently attached to core proteins.<sup>2</sup> The glycocalyx allows the adhesion of circulating cells to the endothelium, but its role in the modulation of the RBC rheological properties in healthy or SCA individuals has not been investigated so far. The aim of this study was to find the role of GAGs and SA in the deformability and aggregability of RBCs from healthy subjects and SCA patients. Also, the study was to confirm the binding of fibrinogen to SA displayed at the RBC surface, and to identify the binding sites of SA on fibrinogen.

For RBCs deformability, aggregability and aggregation were measured after enzymatic treatment with GAG- and SA-degrading enzymes. Blood was collected on EDTA from healthy subjects and SCA patients in clinical steady state. Informed consent was obtained for every individual. The protocol was approved by the "Hospices Civils de Lyon-CPP Est" Ethics Committee (L14-127) and was carried out in accordance with the declaration of Helsinki. Blood cells were washed three times (500 g, 4°C, 10 minutes) in phosphate buffered saline (PBS). Healthy and sickle RBCs were incubated for 60 minutes in PBS at 37°C at a hematocrit of 40% with four enzymes from Sigma-Aldrich: heparinase III (H8891), chondroitinase ABC (C2905), hyaluronidase (H3631), and neuraminidase (N2876). The RBCs were then centrifuged at 500g for 5 minutes, washed



**FIGURE 1** (A) Deformability of healthy (blue,  $n = 24$ ) and sickle RBCs (red,  $n = 24$ ). (B) Aggregation and aggregability of healthy (blue,  $n = 23$ ) and sickle RBCs (red,  $n = 23$ ) (M and M1 modes). Statistical analyses were performed with a Mann-Whitney unpaired  $t$  test (\* $P < .05$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ ). (C) Deformability of untreated healthy and sickle RBCs (black dotted lines), and of treated healthy (blue,  $n = 7$ ) and sickle (red,  $n = 6$ ) RBCs. Bright and dark colors: 400 and 600 mU/mL of neuraminidase respectively. (D) Aggregation of healthy (blue,  $n = 7$ ) and (E) sickle RBCs (red,  $n = 6$ ) in autologous plasma after treatment with 400 mU/mL of neuraminidase (M mode). (F-I) Aggregability of healthy (blue,  $n = 7$ ) and sickle RBCs (red,  $n = 6$ ) in dextran after treatment with 400 mU/mL of neuraminidase (M and M1 modes). A one-way Friedman ANOVA with repeated measurements followed by Dunn post hoc test was used to assess the effects of enzymes on RBC rheological properties (\* $P < .05$ ). (J) Bio-layer interferometry binding assays: binding of soluble fibrinogen to neuraminidase-treated and untreated healthy RBCs collected on EDTA, heparin and citrate captured on AR2G sensors. (K) Docking of the glycophorin O-glycosylation motif (O-GPA) containing two NANA residues on a fibrinogen monomer (green, blue and orange:  $\alpha$ ,  $\beta$ , and  $\gamma$  chains respectively). The 200 superimposed solutions are in red and calcium ions in green. (L) The four binding sites of O-GPA on fibrinogen: the best solution for each site is in green and NANA residues in yellow (chains are color-coded as in K)

twice with PBS and resuspended at a final hematocrit of 40% in autologous plasma or in 3% (w/v) dextran (70 kDa, Sigma-Aldrich, 31 390) in PBS. Treated RBCs were oxygenated for at least 10 minutes before measurements.<sup>3</sup> The RBC aggregation was measured with a Myrenne aggregometer (GmbH, Roetgen) in the stasis (M) and low shear (M1) modes.<sup>3</sup> Measurements in autologous plasma reflected RBC aggregation while the measurements made in dextran reflected RBC aggregability (ie, the cell intrinsic properties to form RBC aggregates). So, RBC deformability (elongation index EI) was measured by ektacytometry at 9 shear stresses (from 0.3 to 30 Pa).<sup>3</sup>

Red blood cell deformability was lower in SCA than in healthy individuals (Figure 1A). Both RBC aggregation, and aggregability were higher in SCA than in healthy subjects (Figure 1B). The difference in aggregability between sickle and healthy RBCs was higher than the difference in aggregation: +43.1% for M and +21.3% for M1 in dextran vs +14.6% for M and +14.5% for M1 in plasma. The treatment of healthy and sickle RBCs with heparinase III (heparan sulfate digestion) or chondroitinase ABC (digestion of chondroitin sulfate A, B and C) did not affect RBC deformability, aggregation or aggregability (data not shown). The digestion of hyaluronan (HA) had no impact on healthy and sickle RBC deformability (data not shown) or on RBC aggregation in both groups (data not shown). But, it induced a significant increase in aggregability of healthy RBCs (M mode, from  $3.54 \pm 1.5$  to  $4.7 \pm 1.3$  a.u. - arbitrary unit) and of sickle RBCs (M1 mode, from  $13.4 \pm 2.4$  to  $15.1 \pm 2.2$  a.u.). Note, SA digestion by neuraminidase at 600 mU/mL decreased the deformability of healthy RBCs from mid (5.33 Pa) to high (30 Pa) shear stresses but increased sickle RBC deformability at low to mid shear stresses (0.95 to 5.33 Pa) (Figure 1C). The use of a lower neuraminidase concentration (400 mU/mL) did not affect deformability in healthy RBCs but increased it in sickle RBCs (Figure 1C). Red blood cell aggregation decreased in both groups after SA digestion (Figure 1D,E). Aggregability increased in both healthy and sickle RBCs after incubation with 400 mU/mL of neuraminidase (Figure 1F-I). The increased aggregability and decreased aggregation after SA digestion suggest that a plasma factor could bind to SA and facilitate RBC aggregation. Fibrinogen, which promotes RBC aggregation,<sup>4</sup> and binds to RBCs via possible contribution of SA,<sup>5</sup> is a likely candidate.

Bio-layer interferometry assays were carried out in an OctetRed96 (Molecular Devices) to investigate fibrinogen binding to healthy RBCs. Blood was collected in EDTA, heparinized and citrated tubes because fibrinogen binds to heparin, zinc and calcium, and centrifuged for 10 minutes at 500g. Red blood cells were washed three times in 10 mM HEPES, 133 mM NaCl and 4.5 mM KCl pH 7.4 (HBS), and resuspended at 40% hematocrit in HBS. Poly-D-lysine (100  $\mu$ g/mL in 1 M NaCl) was covalently bound to amine reactive sensors (AR2G, Molecular Devices). The coated sensors were dipped in  $1 \times 10^6$  RBCs/ $\mu$ l HBS for 20 minutes. The sensors with captured RBCs were dipped in fibrinogen (F3879, Sigma, 100  $\mu$ g/mL in HBS + 1 mM  $\text{CaCl}_2$ ) for 180 seconds and then in HBS for 360 seconds to dissociate RBC-fibrinogen complexes. The binding levels of fibrinogen


to RBCs were normalized to the levels of captured RBCs. Fibrinogen bound to 2.9 to 4.5-fold more to untreated than to treated RBCs depending on collection conditions (Figure 1J). The binding sites of SA on fibrinogen were identified by docking using the 3D structure of human fibrinogen (PDB 3GHG), and a single N-acetylneuraminic acid (NANA), the most common SA at the RBC surface, or the O-glycan of glycophorin-A (O-GPA) including two NANA residues because 75% of the RBC SA are attached to GPA.<sup>6</sup> The NANA and O-GPA structures were created with CarbohydrateBuilder (Glycam.org). Fibrinogen is a homodimer but a single monomer ( $\alpha$ ,  $\beta$  and  $\gamma$ -chains) was used for docking because monomers associate in a head-to-tail manner. The monomer was divided in five overlapping grids (cubes with sides of 90 Å each, spacing 1 Å) including the interface between the monomers. Autodock Vina (Autodock suite 4.2.6) was run with an exhaustiveness of 32, and 40 models were generated per grid (200 models in total). Both the single NANA residue and O-GPA were docked on the same seven sites on human fibrinogen (Figure 1K), with binding energies from  $-4.4$  to  $-6.2$  kcal/mol for NANA and from  $-6.2$  to  $-8.5$  kcal/mol for O-GPA. Four out of the seven sites clustered 187 poses for O-GPA and 160 poses for NANA over the 200 computationally determined. The most favorable binding sites for O-GPA were located at the interface between both fibrinogen monomers in a pocket formed by the N-termini of  $\alpha$  and  $\beta$  chains (Figure 1L, site 1) or in a pocket of the coiled-coil domain. This is where O-GPA is wrapped around the helix formed by the  $\alpha$  chain and maintained by the  $\beta$  and  $\gamma$  chains (Figure 1L, site 2) with binding energies of  $-8.3$  and  $-8.5$  kcal/mol, respectively, corresponding to a calculated  $K_d$  of 1.0  $\mu$ M ( $K_d = \exp(\Delta G/R \cdot T)$ ,  $T = 310$  K). The second site encompasses the RGD tripeptide (95-97) involved in the binding of fibrinogen to RBCs, supporting the involvement of the sialylated RGD-dependent  $\alpha v \beta 3$  integrin in fibrinogen binding to RBCs.<sup>5,7</sup> The third and fourth binding sites, with binding energies of  $-7.2$  and  $-7.5$  kcal/mol, respectively, were located on  $\beta$  (mainly) and  $\gamma$ -globular heads of fibrinogen.

This is the first report showing that both RBC aggregation and aggregability are increased in sickle RBCs, suggesting that both plasma and cellular factors could be involved in this process. Sulfated GAGs, present in low amount on the RBC surface, do not play a significant role in the deformability and aggregability of healthy or sickle RBCs, but the digestion of a non-sulfated GAG, hyaluronan, increases the aggregability of healthy and sickle RBCs. In contrast, SA play a major role in RBC aggregation, likely through antagonist effects, namely strong electrostatic repulsion vs fibrinogen binding. The increased aggregation in sickle RBCs suggests that the decreased electrostatic repulsion caused by the lower amount of SA at their surface compared to healthy RBCs could play a prominent role compared to the pro-aggregant effect of fibrinogen.

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## Intravenous methotrexate at a dose of 1 g/m<sup>2</sup> incorporated into RCHOP prevented CNS relapse in high-risk DLBCL patients: A prospective, historic controlled study

To the Editor:

Central nervous system (CNS) relapse is a devastating event of diffuse large B cell lymphoma (DLBCL). It occurs in 4%-7% of DLBCL in general and the rate is considerably higher in high-risk patients, resulting in a poor outcome.<sup>1</sup> Effective methods of CNS prophylaxis have not yet been developed. In the past, intrathecal (IT) therapy had been widely used but its role has been questioned nowadays.<sup>2</sup> Systemic high-dose MTX (methotrexate, HD MTX) has been proven effective in several studies. With IV MTX at a dose of 3-3.5 g/m<sup>2</sup> is recommended and is often delivered on day 15 of RCHOP.<sup>3</sup> This routine was efficacious but may delay or interrupt subsequent chemotherapy due to increased toxicity, especially for elderly patients. Although IV MTX could also be delivered every 2 to 3 weeks after the last R-CHOP course, it is better to adopt prophylactic measures early in the treatment course since CNS relapse mainly occurs within the first year (median: 6 months).<sup>2,4</sup>

We herein report the result of our prospective, historic controlled study demonstrating that intravenous MTX at 1 g/m<sup>2</sup> could significantly decrease the CNS relapse rate in high risk DLBCL patients. As IV MTX was delivered during RCHOP, it was a convenient regimen which did not increase admission times.

From July 2015 to February 2017, 90 patients diagnosed of DLBCL with high CNS relapse risk were enrolled in our study and given IV MTX at a dose of 1 g/m<sup>2</sup> to prevent CNS recurrence. A historical group of 91 patients with comparable age, sex, stage, ECOG and IPI who only received IT prophylaxis were used as controls. All these patients were similar in the following aspects: (a) age  $\geq$  18 years; (b) with high CNS risk, which was defined as involvement of more than one extranodal site, or involvement of particular extranodal sites such as bone marrow, breasts, testes, paranasal sinuses, epidural space, adrenal glands, kidney and female genital system; (c) first-line treatment with RCHOP; (d) complete staging work-up; and (e) absence of CNS involvement at presentation, confirmed by cerebral spinal fluid (CSF) examinations and MRI. Patients with primary mediastinal lymphoma, primary CNS lymphoma, intravascular large B-cell lymphoma, plasmablastic lymphoma, DLBCL leg-type, Burkitt lymphoma, gray zone or high-grade lymphomas