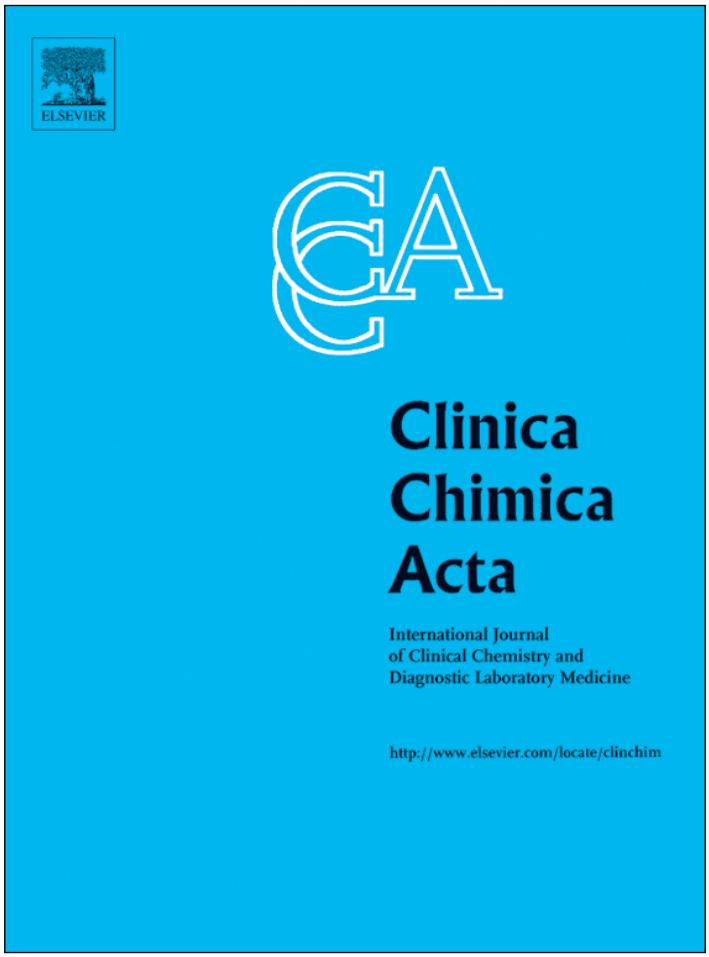

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Letter to the editor

Serum bikunin is a biomarker of linkeropathies

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Proteoglycans (PGs) are major components of extracellular matrices (ECM) and cell surfaces playing pivotal roles in cell interactions, cellular proliferation, ECM organization, cancer metastasis and immune responses. They are composed of a core protein linked to a glycosaminoglycan (GAG) chain starting by the common tetrasaccharide Xylose-Galactose-Galactose-Glucuronic Acid (Xyl-Gal-Gal-GlcA) further elongated by sulfated repeating disaccharide motif. Depending on this motif, chondroitin sulfate (CS), heparan sulfate and dermatan sulfate can be differentiated. Inherited defects in genes encoding for enzymes successively involved in the synthesis of the common tetrasaccharide linker, i.e., xylosyltransferases (XYLT1 or XYLT2), galactosyltransferases (B4GALT7 and B3GALT6) and glucuronyltransferase (B3GAT3), have been grouped as “linkeropathies” diseases. The clinical symptoms of linkeropathies are rather heterogeneous and notably share short stature, chondrodysplasia with multiple joint dislocations, fractures, developmental retardation and hypotonia [1]. When considering the very low prevalence of these diseases and the current absence of convenient blood screening test, there is a need for biomarkers of linkeropathies orientating their diagnosis and avoiding difficult and time-consuming biochemical/genetic investigations.

Our purpose was to evaluate the potentials of serum bikunin as a biomarker of linkeropathies. Bikunin is a circulating liver PG corresponding to a CS GAG chain (15 +/− 3 GlcNAc-GlcA sulfated disaccharide units) linked to a core protein, namely the free bikunin protein (fBkn; ~20 kDa), via the tetrasaccharide linker. Furthermore, as a unique feature of serum bikunin, the CS chain is mostly (> 98%) esterified by 1 or 2 glycoprotein(s) called “heavy chains” (HC). The main reported role of HC-linked bikunin isoforms would be, once extravasated from the blood, to exchange HC with hyaluronic acid notably leading to ECM stabilization [2]. Given the availability of an efficient polyclonal antibody (see figure legend for technical details), we undertook the Western-blot detection of fBkn (~20 kDa) and of Bkn-CS (~35 kDa) in 20-fold diluted sera from patients with mutations in *XYLT1*, *B4GALT7*, *B3GALT6* and *B3GAT3* as well as with mutations in *CHSY1* (encoding chondroitin sulfate synthase 1) [3], *PMM2* (phosphomannomutase 2), a sulfate transporter encoding gene (unpublished data) and *SLC35A2* (Golgi galactose transporter) [4].

As shown Fig.1, Western-blot duplicate analysis of one representative control serum showed one large and marked band (35 +/− 2 kDa) corresponding to bikunin linked to heterogeneous CS chain (Bkn-CS) as well as one minority band at 22 kDa corresponding to light free bikunin (fBkn). The analysis of 12 control sera further showed that Bkn-CS Mw landmarks ranged between 30 kDa and 45 kDa (Supplementary file). Profile of the serum from *XYLT1* mutated patient appeared similar to controls. By contrast, those from other patients with linkeropathies (B4GALT7, B3GALT6 and B3GAT3) showed marked % increase of light bikunin forms with average Mw rising from ~23 kDa to ~25 kDa. Since XYLT1 is not expressed in the liver [5], normal profile was not surprising in the related mutated case. For other linkeropathies, it should be suggested that observed abnormal accumulating light forms of bikunin from *B4GALT7*, *B3GALT6* and *B3GAT3* mutated patients mainly respectively correspond to Bkn-Xyl, Bkn-Xyl-Gal and Bkn-Xyl-Gal-Gal, in agreement with the stepwise action of related enzymes in the tetrasaccharide linker biosynthesis. For the patient with mutations in the *CHSY1* gene encoding for the dual enzyme adding GalNAc-GlcA disaccharide units, the band corresponding to Bkn-CS showed, despite faint staining, decreased average Mw (~31 +/− 2 kDa). Thus, this result is in accordance with a defect in the CS chain elongation. Furthermore, the accumulating bikunin light form of this sample appeared similar to the one of *B3GAT3* mutated patient, suggesting an associated defect in the GlcA addition at the end of the tetrasaccharide linker. As expected given to the small size of sulfate moieties, no abnormality

Abbreviations: B3GALT6, Beta-1,3-galactosyltransferase 6; B4GALT7, Beta-1,4-galactosyltransferase 7; B3GAT3, Beta-1,3-glucuronyltransferase 3; Bkn-CS, Bikunin linked to chondroitin sulfate chain; CDG, Congenital disorder of glycosylation; CHSY1, Chondroitin sulfate synthase 1; CS, Chondroitin sulfate; ECM, Extracellular matrice; fBkn, Free bikunin; GAG, Glycosaminoglycan; Gal, Galactose; GlcA, Glucuronic acid; GalNAc, N-Acetylgalactosamine; PG, Proteoglycans; PMM2, Phosphomannomutase 2; Xyl, Xylose; XYLT, Xylosyltransferase

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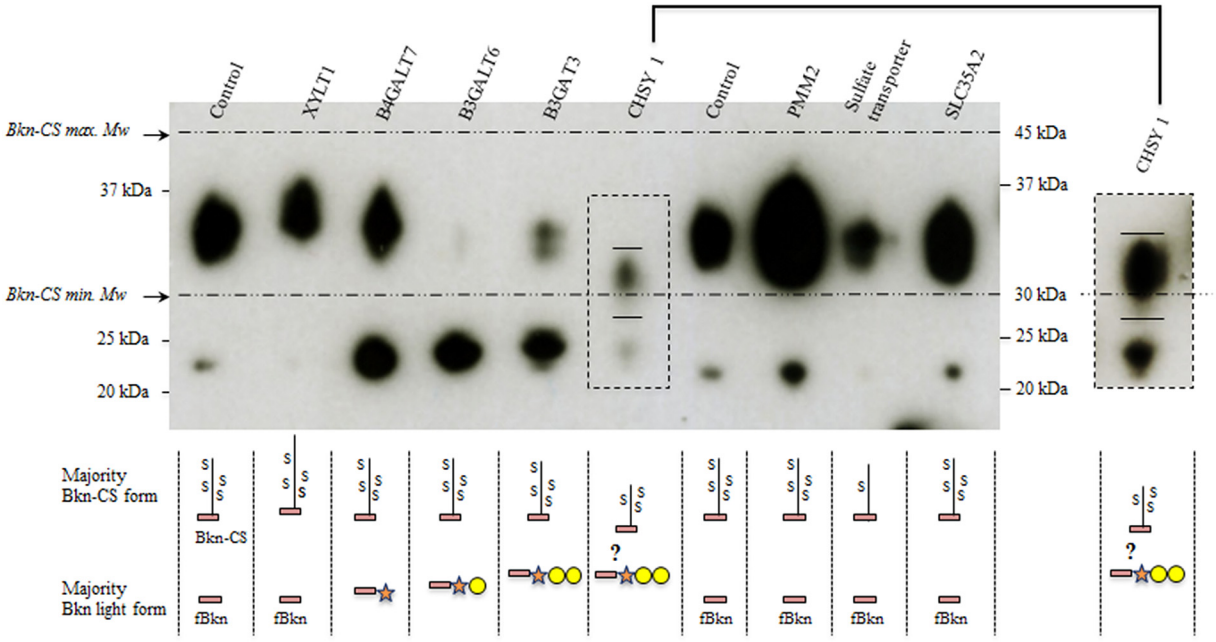

Fig. 1. Western-blot analysis of serum bikunin from patients with linkeropathies.
Serum bikunin from following samples (20-fold diluted in water) were analyzed: control, *XYLT1*, *B4GALT7*, *B3GALT6*, *B3GAT3*, *CHSY1*, *PMM2*, sulfate transporter encoding gene and *SLC35A2* mutated patients. PAGE (8 µL/well) was conducted using Nu-PAGE 4–12% bis-tris gels as recommended (Novex). After transfer on nitrocellulose (1 h, 100 V) and sheet cutting (Mw < 50 kDa), ECL film revelation was conducted after incubation with rabbit anti-bikunin primary antibodies (Millipore; 1/5000 v/v in TTBS-5% milk) and secondary HRP-linked anti-rabbit antibodies (Cell Signaling technologies; 1/5000 v/v). An additional profile of the *CHSY* serum (10-fold diluted) is presented (right) for better interpretation. Horizontal dot lines delimitate Bkn-CS Mw normal range. For each pattern, suspected majority Bkn-CS and light forms are schematized; rectangle, free bikunin (fBkn); line, CS chain; star, xylose; circles: galactose; S, sulfate.

could be evidenced in Bkn-CS from the patient with sulfate transporter encoding gene mutations. Serum of patient with *PMM2* mutations (PMM2-CDG), typically associated with partial absence of N-glycans on glycoproteins [6], did not show qualitative bikunin abnormality (even though in higher concentration) in agreement with the specific involvement of phosphomannomutase 2 in GDP-mannose biosynthesis. Although 2 galactose residues are incorporated in the linker tetrasaccharide, bikunin analysis of the *SLC35A2* mutated patient did not show galactosylation defects by contrast with N-glycans and mucin O-glycans abnormalities described elsewhere [7]. This could suggest either insufficient sensitivity of the bikunin analysis in this case or a preferential flux of residual galactose entering into the Golgi towards the synthesis of the PGs tetrasaccharide linker region.

In summary, we showed that, with exception of *XYLT1* mutations, marked % increase of bikunin light form could be specifically associated to linkeropathies with the ability to discriminate between causative mutations. Furthermore, potentially associated Mw decrease of Bkn-CS could further specifically orientate towards defect in CS GAG chain elongation. Although needing a stronger validation with additional (but very rare) samples, presented results are tightly consistent with related enzymatic defects already heavily suggesting the high potential of bikunin as a specific, simple and useful biomarker of linkeropathies.

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Appendix A. Supplementary data

Western-blot analysis of serum bikunin from 12 control patients.

Here, serum samples were more concentrated (i.e., 10-fold diluted sera) that in Fig. 1 in order to better determine Bkn-CS Mw normal range (defined by horizontal dot lines).Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.06.044.

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