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## LETTERS TO THE EDITOR

### Regulation of serum hepcidin levels in sickle cell disease

The peptide hormone hepcidin exerts its function by binding to the transmembrane cellular iron exporter ferroportin and inducing its internalization and degradation, resulting in decreased intestinal iron uptake and iron retention in the reticulo-endothelial (RE) macrophages. Inflammatory cytokines and iron loading increase hepcidin production, while increased bone marrow activity, and anemia suppress hepcidin synthesis.<sup>1,2</sup> However, most of the evidence of these regulatory processes is obtained by molecular *in vitro* work and

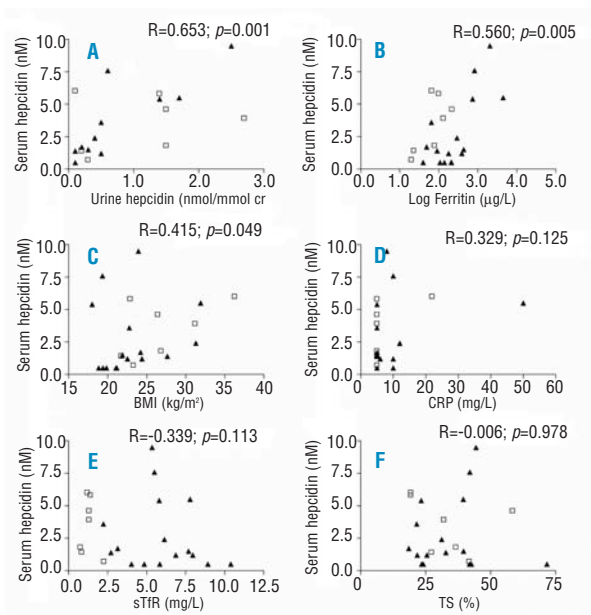
mice models, and much is still unknown about how these different stimuli interact in man.

Sickle cell disease (SCD) patients are characterized by chronic hemolytic anemia, increased erythropoiesis and a chronic inflammatory state with endothelial activation and enhanced red cell and leukocyte adhesion. Sickle cell patients have iron overload due to chronic blood transfusions in the treatment or prevention of the severe sickle cell-related complications such as stroke.<sup>3</sup> SCD has been associated with low urinary hepcidin levels in children.<sup>4</sup> However, serum hepcidin 25-amino acid isoform (hepcidin-25) levels, which are directly responsible for the biological effect, have not been documented and factors that contribute to hepcidin regulation in this disease have not been assessed.

**Table 1.** Characteristics of study populations of adult sickle cell disease patients in steady state of their disease.

Gender (M/F)	Age (years)	Transfused PCU (n)	Genotype	BMI (kg/m <sup>2</sup> )	Hb (mmol/L)	MCV (fL)	ALT (U/L)	Ferritin (µg/L)	TS (0/0)	CRP (mg/L)	sTfR (mg/L)	Reticulocytes (0/0)	Hepcidin-25 Serum (nmol/L)	Urine (nmol/mmol cr)	Hepcidin-25 Serum (pmol/µg)	Urine (pmol/mmol cr.µg)	
1	F	24	32	SS <sup>†</sup>	31.9	5.9	92.2	81	4470	39.5	50	7.79	11.00	5.5	1.7	1.2	0.4
2	M	22	15	SS <sup>†</sup>	19.4	6.0	—	21	213	71.7	5	8.93	—	<LLOD	0.1	2.3	0.5
3	F	52	16	SS	23.9	5.4	118.0 <sup>‡</sup>	23	2051	44.4	8	5.36	10.80	9.5	2.5	4.6	1.2
4	F	45	67	SS <sup>†</sup>	18.9	4.6	85.8	16	108	24.1	<5	5.85	8.80	<LLOD	0.1	4.6	0.9
5	F	18	2	SS <sup>†</sup>	21.1	6.0	96.2	16	140	42.6	<5	4.85	10.00	<LLOD	0.1	3.6	0.7
6	F	22	5	SS <sup>†</sup>	21.9	5.2	93.1	14	438	39.6	<5	7.69	19.20	1.5	0.3	3.4	0.7
7	F	33	51	SS	19.8	4.0	90.1	26	210	41.9	10	10.40	17.50	<LLOD	0.1	2.4	0.5
8	F	45	24	SS	18.0	6.1	99.9	68	739	23.3	<5	5.81	7.50	5.4	1.4	7.3	1.9
9	F	46	11	SS	31.3	5.2	125.0 <sup>‡</sup>	10	293	31.2	12	6.14	13.10	2.4	0.4	8.2	1.4
10	F	19	8	Sβ <sup>0</sup>	22.5	5.4	64.2	52	180	25.5	10	6.87	8.30	1.2	0.5	6.7	2.8
11	F	41	43	Sβ <sup>0</sup>	19.3	4.9	68.9	49	826	42.0	10	5.50	7.80	7.6	0.6	9.2	0.7
12	F	33	30	Sβ <sup>0</sup>	24.4	6.0	65.5	7	392	21.8	6	7.96	6.30	1.2	0.5	3.1	1.3
13	F	24	n.a.	SC	21.2	5.9	74.7	7	40	23.5	<5	4.03	3.00	<LLOD	0.1	12.5	2.5
14	M	32	19	SC	27.6	8.6	85.7	26	91	32.8	<5	2.72	2.70	1.4	0.1	15.4	1.1
15	F	29	4	SC	22.8	6.6	73.1	13	65	21.6	<5	2.23	1.60	3.6	0.5	55.4	7.7
16	F	40	n.a.	SC	24.2	7.3	71.1	6	49	18.5	<5	3.15	1.60	1.7	0.2	34.7	4.1
Med.n.a. (range)	33 (18-52)	18 (0-67)	n.a.	22.2 (18.0-31.9)	5.9 (4.0-8.6)	85.8 (64.2-125.0)	19 (6-81)	212 (40-4470)	32.0 (18.5-71.7)	10 (5-50)	5.83 (2.23-10.40)	8.30 (1.60-19.20)	1.5 (<0.5-9.5)	0.4 (0.1-1.7)	5.7 (1.2-55.4)	1.2 (0.4-7.7)	
Control <sup>^</sup>																	
17	M	27	n.a.	AS	26.4	7.8	93.0	16	219	58.5	<5	1.32	0.80	4.6	1.5	21.0	6.8
18	F	37	n.a.	AS	36.3	7.2	65.3	18	66	19.4	22	1.21	1.10	6.0	0.1	90.9	1.5
19	F	61	n.a.	AS	31.2	9.0	81.0	46	132	32.1	<5	1.32	1.00	3.9	2.7	29.6	20.5
20	M	42	n.a.	AS	22.9	8.9	78.5	17	100	19.4	<5	1.40	0.90	5.8	1.4	58.0	14.0
21	F	27	n.a.	AC	21.8	8.5	81.2	19	23	27.3	<5	0.85	0.70	1.4	0.2	60.9	8.7
22	F	38	n.a.	AS	23.3	9.4	76.7	19	20	41.8	<5	2.27	2.20	0.7	0.3	35.0	15.0
23	F	25	n.a.	AS	26.9	8.6	85.4	6	79	36.7	<5	0.76	1.70	1.8	1.5	22.8	19.0
Med. n.a. (range)	37 (25-61)	n.a.	n.a.	26.4 (21.8-36.3)	8.6 (7.2-9.4)	81.0 (65.3-93.0)	18 (6-46)	79 (20-219)	32.1 (19.4-58.5)	5 (5-22)	1.32 (0.76-2.27)	1.00 (0.70-2.20)	3.9 (0.7-6.0)	1.4 (0.1-2.7)	35.0 (21.0-90.9)	14.0 (1.5-20.5)	

<sup>†</sup>Patients 3 and 9 receive maintenance therapy with hydroxyurea; <sup>‡</sup>patients with co-inherited β-thalassemia; <sup>^</sup>these controls are race matched carriers and do not have anemia, enhanced hemolysis or inflammation; — indicates lab results are not available; n.a. not applicable; LLOD: lower limit of detection (0.5 nM). CRP is set <5 mg/L when there is no indication of inflammation. Reticulocytes are expressed as the % reticulocytes of the population of red blood cells and reticulocytes. PCU, packed cell units; BMI: body mass index; Hb: hemoglobin; MCV: mean corpuscular volume; ALT: alanine aminotransferase; TS: transferrin saturation; CRP: C-reactive protein; sTfR: soluble transferrin receptor, is a direct measure of the total transferrin receptor in the body and reflects both the cellular need for iron and the rate of erythropoiesis; cr: creatinine. Reference range Caucasian controls hepcidin-25 (n=24), serum 0.5-13.9 nM and urine 0.01-10.6 nmol/mmol creatinine (www.hepcidinanalysis.com); MCV 80-98 fL; serum TS female 15-50%, TS male 20-50%; ferritin female premenopausal 6-80 µg/L; ferritin female postmenopausal 6-190 µg/L; ferritin male 15-280 µg/L; sTfR 0.76-1.76 mg/L.



**Figure 1.** Spearman's correlation analysis of serum hepcidin-25 with (A) urine hepcidin-25 (B) Log Ferritin (C) BMI (D) CRP (E) sTfR, (F) TS. Data represent the whole study population of 16 SCD patients (HbSS, HbS $\beta^0$ -thalassemia and HbSC) (triangle) and 7 controls (square). BMI: body mass index; CRP: C-reactive protein; sTfR: soluble transferrin receptor; TS: transferrin saturation.

Samples were collected from adult steady state SCD patients<sup>5</sup> with various hemoglobin (Hb) genotypes (9 HbSS, 3 HbS $\beta^0$ -thalassemia and 4 HbSC, Table 1) between February 2005 and February 2006, and stored in polypropylene tubes at  $-80^{\circ}\text{C}$  until analysis. Patients received no transfusions or chelation therapy for two months prior to sampling. Race matched controls were heterozygous for HbS or C.<sup>5</sup> Serum and urinary hepcidin-25 measurements were performed in November and December 2007 by use of surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) as previously described.<sup>6,7</sup> The hepcidin regulators' inflammation, iron store and erythropoiesis (reflected in the serum markers C-reactive protein (CRP), ferritin and soluble transferrin receptor (sTfR), respectively) were assessed to delineate the regulatory pathways of hepcidin.<sup>8</sup> Approval for the study was obtained from the Medical Ethics Committee of the Academic Medical Center in Amsterdam.

We found the various serum parameters to vary widely within this population (Table 1). Of note is the pattern of the serum iron parameters, which shows highly variable ferritin levels, not simply related to the transfusion history and in the presence of normal transferrin saturation (TS). This suggests an iron distribution pattern of the anemia of chronic disease, with relatively more iron in the RE system.

Serum hepcidin-25 levels were below the lower limit of detection (LLOD  $<0.5$  nM) in 5 SCD patients, while in the rest, the levels were between 1 and 10 nM, which is considered to be the normal range (Table 1). The median serum and urine hepcidin-25 levels were similar for patients and controls ( $p>0.2$ ), but hepcidin-25/ferritin ratio's as a measure of appropriateness of hepatocyte-produced hepcidin for the iron burden,<sup>4</sup> were significantly lower for patients ( $p<0.01$ ) (Table 1). However, as ferritin in SCD might be increased by

inflammation and iron loading of RE cells by transfusions, this ratio might not be suitable in the evaluation of the adequacy of hepcidin in response to hepatocyte iron loading.<sup>9</sup>

Results confirm that erythropoiesis down-regulates hepcidin-25, i.e. when only sTfR is increased, serum hepcidin-25 levels are in the lower normal range or even not detectable ( $<\text{LLOD}-3.6$  nM; patients 2, 4, 5, 13-16). In cases where next to a substantially increased sTfR inflammation and/or high iron stores are also present, serum hepcidin-25 levels are in the normal range (1.2 - 9.5 nM; patients 1, 3, 6, 8-12) confirming the induction of hepcidin by inflammation and elevated iron stores in sickle cell patients. Interestingly, in patient 7 the low hepcidin-25 level due to increased erythropoiesis (highly elevated sTfR) is not compensated by low grade inflammation (CRP of 10 mg/L) and a slightly elevated iron store (ferritin of 210  $\mu\text{g/L}$ ), resulting in undetectable serum hepcidin-25 levels.

While this is a small study, the results only describe the qualitative contribution of the various parameters to hepcidin-25 levels. Nevertheless, Spearman's correlation analysis showed that serum hepcidin-25 levels were significantly correlated with urine hepcidin-25, log ferritin, Body Mass Index (BMI)<sup>10</sup> (Figure 1A-C) and age, but not with CRP, sTfR, TS (Figure 1D-F) and hemoglobin.

In conclusion, this proof of principle study in a heterogeneous group of SCD patients indicates that: (i) previous results obtained *in vitro* and mice studies of hepcidin-25 suppression by increased erythropoietic activity that is counterbalanced by iron stores and (low grade) inflammation are also valid in man; (ii) larger studies are needed to determine the quantitative contribution of various factors to hepcidin-25 regulation in this disease.

The insights gained in this study could be clinically beneficial in the identification and treatment of patients most at risk of iron mediated tissue damage.

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### Predictive value of $\beta$ 2-microglobulin ( $\beta$ 2-m) levels in chronic lymphocytic leukemia since Binet A stages

We read with interest the study by Rossi and co-workers, reporting CD49d expression as risk factor of treatment free survival (TFS) in Binet A CLL patients.<sup>1</sup> In this paper, a close association between CD49d and CD38, LDH and  $\beta$ 2-m is also described. We would like to add further information about the prognostic power of  $\beta$ 2-m. It is generally believed that  $\beta$ 2-m is released constitutively by CLL cells and that its level approximately correlates with tumor mass.<sup>2</sup> Based on these premises the predictive value of  $\beta$ 2-m serum concentration could vary in the course of the disease and be relatively low in the early disease stages, when tumor mass is low, irrespective of the subsequent clinical outcome. Therefore,  $\beta$ 2-m determination could exhibit a lower predictive power particularly at the early disease stages compared to the newer biological markers, such as IgVH gene status, ZAP-70 and CD38, which represent intrinsic cell features that can be determined since the earliest disease stages and never (IgVH) or rarely (ZAP-70 and CD38) change in the course of the disease.<sup>3</sup>

In order to explore this issue, we have measured  $\beta$ 2-m value in 222 Binet stage A patients at diagnosis. IgVH gene status and CD38 expression were also determined in all cases studied. Unlike  $\beta$ 2-m, which was measured at diagnosis, these markers were determined in the course of the disease when marker determinations became available. This approach, although irrelevant for the IgVH gene status, may introduce some, albeit minor, biases for CD38 for the reasons alluded to above. The median  $\beta$ 2-m value was 2 mg/dL (range 0.4-19). ROC analysis determined that the cut-off value capable of discriminating between patients whose disease progressed and required treatment from those with stable disease was 2.4 mg/dL (AUC:0.67,  $p=0.005$ ). Accordingly 149/222 patients (67%) were  $\beta$ 2-m<sup>neg</sup> and 73/222 (33%) as  $\beta$ 2-m<sup>pos</sup>. Overall, the results did not substantially change when arbitrary cut offs used by other authors<sup>4-7</sup> were employed.

The patients' features are summarized in Table 1.  $\beta$ 2-m levels overlap with CD38 expression in 128/219 cases (63%) [ $\beta$ 2-m<sup>pos</sup>/CD38 $\geq$ 30% cases: 23/55 (41.8%),  $\beta$ 2-m<sup>neg</sup>/CD38<30% cases: 115/164 (70.1%)], while  $\beta$ -m

levels overlap with IgVH status in 125/195 cases (64.1%) [ $\beta$ 2-m<sup>pos</sup>/IgVHunmutated cases: 29/62 (46.8%),  $\beta$ 2-m<sup>neg</sup>/IgVHmutated: 96/133 (72.2%)]. Finally, the concordance between CD38 expression and IgVH mutational status was 77.6% (149/192 cases) [IgVHunmutated/CD38 $\geq$ 30% cases: 35/52 (67.3%), IgVHmutated/CD38<30% cases: 114/140 (81.4%)].

After a median follow-up of 3.5 years, 55 of 222 Binet stage A (25%) required treatment.  $\beta$ 2-m<sup>neg</sup> cases showed a significantly longer TFS than  $\beta$ 2-m<sup>pos</sup> cases; in particular the projected median TFS was 5.3 years for  $\beta$ 2-m<sup>pos</sup> versus not reached for  $\beta$ 2-m<sup>neg</sup> (Figure 1A). TFS represented a reliable measure of disease progression since all centers agreed to follow NCI guidelines for treatment start.

In order to ascertain whether  $\beta$ 2-m identifies a patient subset of those with good prognostic markers, we calculated TFS of both CD38<30% and IgVHmutated CLL cases grouped according to the  $\beta$ 2-m expression.  $\beta$ 2-m<sup>pos</sup> CD38<30% cases exhibited a TFS which was significantly lower than that of  $\beta$ 2-m<sup>neg</sup> CD38<30% cases (3.5-years TFS probability:  $\beta$ 2-m<sup>neg</sup> vs.  $\beta$ 2-m<sup>pos</sup> 91% vs. 83%;  $p=0.05$ ). However, these differences were not seen in the IgVHmutated cases (3.5-years TFS probability:  $\beta$ 2-m<sup>neg</sup> vs.  $\beta$ 2-m<sup>pos</sup> 89% vs. 84%;  $p=ns$ ).

At Cox univariate analysis,  $\beta$ 2-m<sup>pos</sup> (HR:2.3,  $p=0.003$ ), CD38>30% (HR:3.9,  $p<0.0001$ ) and IgVHunmutated (HR:3.2,  $p<0.0001$ ) showed a statistically significant impact on TFS. At Cox multivariate analysis, all the three markers maintained an independent prognostic impact ( $\beta$ 2-m<sup>pos</sup>, HR:1.8,  $p=0.047$ ; CD38>30%, HR:2.0,  $p=0.03$ ; IgVHunmutated, HR:2.7,  $p=0.022$ ). When a scoring system in which one point was assigned to each unfavorable prognostic marker was utilized, the risk of an early treatment was highest (Figure 1B) in patients presenting all the three adverse prognostic markers. Cases with two, one or none of the unfavorable prognostic factors showed lower risk for an early treatment (Figure 1C).

Collectively, this study shows that  $\beta$ 2-m levels represent valuable predictors in early CLL stages, when the neoplastic cell burden is low. This finding raises a number of questions regarding the mechanisms governing the  $\beta$ 2-m levels. This molecule is constantly shedded<sup>8</sup>

**Table 1.** Comparisons of clinical and laboratory features among chronic lymphocytic leukemia patients devised according to  $\beta$ 2-m expression.

	All patients	$\beta$ 2-m <2.4 mg/d	$\beta$ 2-m $\geq$ 2.4 mg/dL	p
N. of patients	222	149 (67)	73 (33)	
Age (years)				
≤65	124 (56)	94 (63)	30 (41)	0.002
>65	98 (44)	55 (37)	43 (59)	
Gender				
Female	82 (37)	60 (40)	22 (30)	0.14
Male	140 (63)	99 (60)	51 (70)	
IgVH mutational status (n=195)				
Mutated	133 (68)	96 (74)	37 (56)	0.014
Germline	62 (32)	33 (26)	29 (44)	
CD38 expression (n=219)				
<30%	164 (75)	115 (78)	42 (58)	0.02
$\geq$ 30%	55 (25)	32 (22)	30 (42)	
Therapy				
no	167 (75)	123 (83)	44 (60)	<0.0001
yes	55 (25)	26 (17)	29 (40)	