Ann Hematol (2004) 83:371–375
DOI 10.1007/s00277-004-0856-9

ORIGINAL ARTICLE

John-John B. Schnog · Esther H. Jager · Fey P. L. van der Dijs · Ashley J. Duits · Han Moshage · Fred D. Muskiet · Frits A. J. Muskiet

Evidence for a metabolic shift of arginine metabolism in sickle cell disease

Received: 13 December 2003 / Accepted: 25 January 2004 / Published online: 31 March 2004

Abstract Over the last few years, a pivotal role has been ascribed to reduced nitric oxide (NO) availability as a contributing factor to the vaso-occlusive process of sickle cell disease. We investigated whether arginine metabolism in sickle cell patients is different from healthy controls. Blood samples were drawn by venipuncture in the fasting state from 8 clinically asymptomatic HbSS patients and 14 race-matched HbAA controls. HbSS patients had decreased plasma arginine (p=0.001) and increased proline (p=0.015) levels as compared to controls. Ratios of arginine to ornithine (p<0.001), proline (p<0.001), glutamate (p=0.003), and citrulline (p=0.026) were lower in HbSS patients. There were significant correlations of ornithine (r_s=–0.71, p=0.047), citrulline (r_s=–0.79, p=0.021), arginine/ornithine (r_s=0.93, p=0.001), and arginine/citrulline (r_s=0.81, p=0.015) to hemoglobin and of arginine/proline (r_s=–0.76, p=0.028) and citrulline (r_s=0.71, p=0.048) to leukocyte counts. These data indicate that in clinically asymptomatic sickle cell patients increased arginine metabolism is shifted to the arginase pathway and that this seems to be more profound in patients with higher hemolytic rates and leukocyte counts.

Keywords Sickle cell disease · Arginine · Nitric oxide · Hemolysis · Endothelium

J.-J. B. Schnog (✉)
Department of Internal Medicine (9B),
Slotervaart Hospital,
Louwesweg 6, 1066 EC Amsterdam, The Netherlands
e-mail: jschnog@wanadoo.nl
Tel.: +31-20-5129333
Fax: +31-20-5124783

J.-J. B. Schnog · A. J. Duits
Red Cross Blood Bank Foundation,
Curaçao, Netherlands Antilles

E. H. Jager · F. A. J. Muskiet
Department of Pathology and Laboratory Medicine,
Groningen University Hospital,
The Netherlands

E. H. Jager · F. P. L. van der Dijs
Analytical Diagnostic Center,
Curaçao, Netherlands Antilles

H. Moshage
Division of Gastroenterology and Hepatology,
Groningen University Hospital,
The Netherlands

F. D. Muskiet
Department of Pediatrics,
St. Elisabeth Hospital,
Curaçao, Netherlands Antilles

Introduction

Vaso-occlusion leads to organ damage, a decreased quality of life, and a decreased life expectancy in patients with sickle cell disease (SCD) [1]. The pathophysiological mechanism of vaso-occlusion in SCD is characterized by a proinflammatory state, involving adhesive processes between sickle red blood cells, leukocytes, and the activated endothelium, as well as hypercoagulability [2, 3]. Over the last few years, a pivotal role has been ascribed to reduced nitric oxide (NO) availability as a contributing factor to the vaso-occlusive process [4]. NO is a major endothelial relaxing factor with a central role in vascular homeostasis by maintaining basal and stimulated vasomotor tone. NO also limits platelet aggregation and reduces endothelial activation, as well as adhesion of sickle red blood cells to the activated endothelium [5, 6, 7]. Elegant studies have demonstrated that sickle cell patients are characterized by upregulation of NO synthesis, but that the ability to further increase NO production during acute vaso-occlusive events is limited, albeit perhaps more markedly in men than in women [8, 9]. Arginine availability for NO synthesis (through NO synthase) is dependent on cellular uptake via cationic amino acid transporters (CAT) and arginine recycling from citrulline. The metabolism of arginine is determined by the activity of two enzyme systems: the nitric oxide synthetase enzymes (inducible, neuronal, and endothelial nitric oxide synthase or iNOS, nNOS, and eNOS) that lead to NO

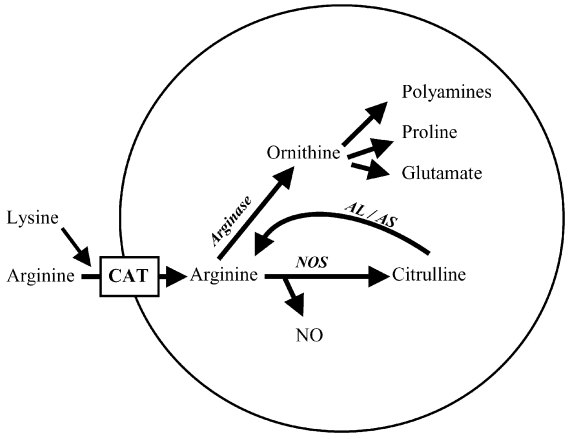

Fig. 1 Simplified scheme of arginine metabolism. Plasma arginine competes with lysine to enter the cell via a specific cationic amino acid transporter (*CAT*). Intracellular arginine can than either be metabolized to citrulline via nitric oxide synthetase (*NOS*) or to ornithine via arginase. Argininosuccinate synthetase (*AS*) and argininosuccinate lyase (*AL*) recycle citrulline to arginine. Ornithine can be converted to putrescine (and the higher polyamines spermidine and spermine), proline, and glutamate [10, 11, 12]

formation, and arginase enzymes, consisting of two isoforms (arginase I and II) that lead to ornithine formation. Ornithine can subsequently be converted into glutamate, proline, and the polyamine precursor putrescine (Fig. 1) [10, 11, 12]. CAT and many of the enzymes involved in intracellular arginine homeostasis are inducible by cytokines, and an upregulation of arginase may cause a metabolic shift towards increased production of ornithine at the expense of NO production. It is possible that increased arginase activity may also contribute to the reduced NO availability in SCD.

We investigated whether arginine metabolism in sickle cell patients is different from healthy controls. We show that amino acid levels and profiles are indicative of a metabolic shift to the arginase pathway in clinically asymptomatic sickle cell patients and that this is related to the degree of hemolysis and leukocytosis.

Materials and methods

Patients and controls

For this case control study, blood samples were drawn via venipuncture in the fasting state from eight consecutive HbSS patients at the outpatient Department of Internal Medicine (Sint Elisabeth Hospital, Curaçao). None of the patients were on any kind of treatment (apart from folic acid intake by some), nor had they received blood transfusions during the 4 months prior to sample collection. Patients were in the clinically asymptomatic state during sample collection (no overt painful crises or other acute vaso-occlusive events). Fourteen race-matched subjects with HbAA served as controls (see Table 1 for patient and control characteristics). Written informed consent was obtained from all patients and controls, and this study was approved by the local ethical review board.

Samples and analysis

Samples were immediately centrifuged at 1700 *g* for 10 min. Aliquots were stored at −70°C until further analysis. Ethylenediaminetetraacetate (EDTA) blood was used for determination of standard blood counts with a MAXM Hematology Analyzer (Beckman Coulter, Fullerton, Calif., USA) and confirmation of hemoglobin phenotype was obtained with high performance liquid chromatography [13]. Serum was used for determination of creatinine and lactate dehydrogenase (LDH) levels with a Vitros 950 system (Johnson & Johnson, New Brunswick, N.J., USA). In order to exclude other causes of anemia, serum ferritin, vitamin B_{12}, folate, and total homocysteine were determined with an immunochemistry analyzer (AxSym, Abbott Laboratories, Abbott Park, Ill., USA) [14, 15]. Plasma amino acids were determined by ion exchange chromatography with ninhydrin postcolumn derivatization and colorimetric detection. Plasma NO_x, which is the sum of nitrate and nitrite, was determined as previously described [16].

Data analyses and statistics

For relative plasma amino acid contents (in mol%) we took the sum of the 19 protein amino acids (tryptophane not included) and subsequently calculated the percentage of each amino acid. For between-group comparisons the Mann Whitney U test was employed and for correlation studies we determined the Spearman's rank correlation coefficient. The statistical software package SPSS 10.0 (SPSS Inc., Chicago, Ill., USA) was used and $p \leq 0.05$ was considered statistically significant.

Table 1 Laboratory data in patients and controls. Data are presented as median with interquartile ranges. *NS* not significant, *MCV* mean corpuscular volume, NO_x nitric oxide metabolites

	HbSS (8)	HbAA (14)	*p*
Age (years)	39 (35–44)	43 (33–48)	NS
Hemoglobin (g/dl)	8.7 (5.7–9.6)	13.8 (12.6–16.6)	<0.001
Leukocytes ($\times 10^9$/l)	11.7 (10.8–14.6)	6.3 (4.5–8.0)	<0.001
MCV (fl)	97 (91–100)	90 (86–92)	0.007
LDH (U/l)	1237 (740–1506)	408 (350–471)	<0.001
Creatinine (μmol/l)	61 (49–64)	69 (60–85)	0.034
NO_x (μmol/l)	40 (23–53)	36 (31–58)	NS
Arginine (μmol/l)	50 (41–52)	82 (63–92)	0.001
Ornithine (μmol/l)	45 (41–48)	50 (41–54)	NS
Proline (μmol/l)	208 (166–253)	151 (116–180)	0.015
Citrulline (μmol/l)	20 (19–32)	27 (21–29)	NS
Arginine/ornithine (mol/mol)	1.10 (0.95–1.18)	1.68 (1.46–1.77)	<0.001
Arginine/proline (mol/mol)	0.24 (0.18–0.31)	0.55 (0.43–0.64)	<0.001
Arginine/glutamate (mol/mol)	1.55 (1.42–1.71)	2.79 (1.98–3.46)	0.003
Arginine/citrulline (mol/mol)	2.47 (1.54–2.68)	3.05 (2.51–3.32)	0.026

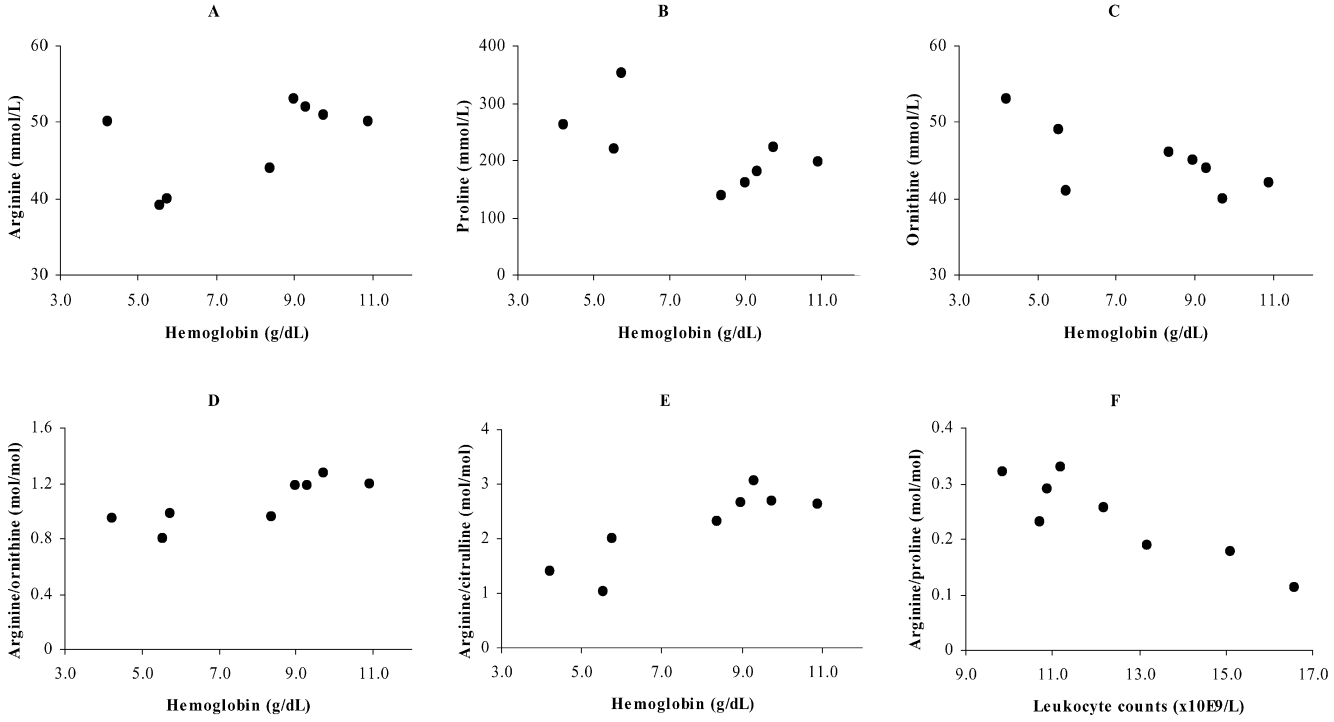

Fig. 2A–F Correlations of hemoglobin levels to amino acids and amino acid ratios in HbSS patients. **A** r_s=0.54, NS; **B** r_s=–0.36, NS; **C** r_s=–0.71, p=0.047; **D** r_s=0.93, p=0.001; **E** r_s=0.81, p=0.015; **F** r_s=–0.76, p=0.028

Results

The characteristics of patients and controls are shown in Table 1. Patients had a lower body mass index than controls (p=0.02). No differences were detected between folate, vitamin B_{12}, homocysteine, and ferritin levels between patients and controls (data not shown). HbSS patients had lower arginine levels as compared to controls. The ratios of arginine to ornithine, proline, glutamate, and citrulline were lower in HbSS patients as compared to HbAA controls. As sickle cell patients have an increased plasma volume due to their hemolytic anemia (possibly depicted by lower creatinine levels), we also compared relative amino acid levels in order to correct this potential source of error. When expressed as percentage of the total plasma amino acids, the differences between arginine [HbSS: median 2.3% (interquartile range: 2.0–2.7) and HbAA: 3.6% (3.2–3.8), p=0.001] and proline [HbSS: 9.4% (8.4–13.2), HbAA: 6.5% (5.7–7.8), p=0.002] were also evident. Of all other amino acids, only alanine [HbSS: 204 μmol/l (183–261), HbAA: 292 μmol/l (248–359), p=0.005] and histidine [HbSS: 62 μmol/l (53–67), HbAA: 79 μmol/l (69–90), p=0.002] differed significantly between patients and controls.

Correlation studies revealed a statistically significant correlation of the arginine/ornithine ratio to hemoglobin levels, with higher ornithine levels in patients exhibiting lower hemoglobin levels (Fig. 2C and D). In HbSS patients, a significant negative correlation between citrulline levels and hemoglobin (r_s=–0.79, p=0.021) and positive correlation between arginine/citrulline and hemoglobin (Fig. 2E) was detected. Also, in HbSS patients, taurine levels were significantly correlated to hemoglobin (r_s=–0.72, p=0.045). In HbAA controls, only proline was directly related to hemoglobin levels (r_s=0.78, p=0.013). All amino acids were correlated to hemoglobin levels and LDH, and no other significant correlations were detected. The arginine/proline ratio correlated significantly to the leukocyte count (Fig. 2F), and there was also a significant correlation of citrulline to the leukocyte count (r_s=0.71, p=0.048). No statistically significant correlations were observed between amino acids, their ratios, and platelet counts.

Discussion

Sickle cell patients are characterized by a decreased NO availability, and even though a major cause is the scavenging of NO by free hemoglobin, other mechanisms may be involved [8, 9, 17]. Upon cytokine stimulation, intracellular arginase activity may increase leading to a metabolic shift whereby arginase is metabolized to ornithine instead of citrulline, with a reduced NO synthesis as a result [10, 11, 12]. By measuring plasma amino acid levels in clinically asymptomatic sickle cell patients, we investigated whether arginine metabolism in SCD is shifted to the arginase pathway.

We show that our adult HbSS patients are characterized by decreased plasma arginine levels in the clinically asymptomatic state, but that they have normal levels of nitric oxide metabolites (NO_x). The latter finding is in

agreement with previous studies, and lower arginine levels in the clinically asymptomatic state have been previously described in adults, but not in children [18, 19, 20, 21]. Citrulline levels were similar between patients and controls, as were ornithine levels, but proline levels were higher in patients. The lower ratios of arginine to the other amino acids involved in arginine metabolism, as well as the elevated proline levels in patients, are highly suggestive of an increased rate of arginine metabolism via the arginase pathway. This is in accordance with a recent study by Morris et al. and may explain the relatively high proline levels previously reported by Van der Jagt and colleagues in Nigerian sickle cell patients [20, 22]. As continuous vascular endothelial damage occurs in patients with SCD, this could be the result of an increased demand for polyamines for vascular "healing" [23, 24]. The comparable NO_x and citrulline levels between patients and controls, with a reduced arginine to citrulline ratio and lower arginine levels in patients, suggests that NO synthesis from arginine, as well as arginine recycling from citrulline, could be increased. Augmentation of both CAT and NOS activity could also contribute to the maintenance of normal NO_x levels, but it is also possible that the remaining intracellular arginine pool is still sufficient for NO synthesis in clinically asymptomatic patients and becomes insufficient for NO synthesis during acute vaso-occlusive events [19, 21]. Both increased urinary losses of amino acids and insufficient dietary supplementation may contribute to the decreased arginine levels [18, 20]. However, contrary to previous studies, most other amino acids levels were comparable to healthy controls, making dietary insufficiency unlikely in our patients on Western diets.

There was a significant correlation between the arginine/ornithine ratio and hemoglobin, with a negative correlation between ornithine levels and hemoglobin, in HbSS patients. A significant negative correlation of citrulline with hemoglobin levels and a significant positive correlation of the arginine/citrulline ratio with hemoglobin levels was also detected. In the absence of other causes of anemia (patients had similar vitamin B_{12}, folate, homocysteine, and ferritin levels as compared to controls), this suggests that in patients with a higher hemolytic rate, more arginine is metabolized via the arginase pathway and that relatively more arginine is needed to maintain basal NO levels in patients with lower hemoglobin levels. The higher the hemolytic rate, the more free hemoglobin circulates, which is known to induce endothelial activation and reduce NO bioavailability, also implicating that the comparable NO_x levels between our patients and controls are not synonymous with comparable NO bioavailability [17, 25]. This may therefore reflect a response to continuous endothelial activation and damage that seems to be more profound in patients with lower hemoglobin levels [26]. The more profound arginase activity in patients with lower hemoglobin levels could also be the result of a higher demand for polyamines to support the stress erythropoiesis characteristic of HbSS patients [27]. Lower arginine/proline ratios and higher citrulline levels were significantly correlated to higher leukocyte counts, indicating that leukocytosis, which is associated with poor outcome in SCD, is also associated with increased arginase activity and higher rate of NO synthesis [28]. Leukocytes, which are activated in clinically asymptomatic sickle cell patients, may directly or indirectly contribute to activation of the arginase pathway [29].

Plasma levels and profiles of amino acids are a reflection of complex metabolic processes, and activity of enzymes such as NOS and arginase vary in different tissues [10, 30]. Our results therefore provide an "overall" view of arginine metabolism in SCD. Conceding the above and our small sample size, we provide evidence for a shift in arginine metabolism to the arginase pathway in clinically asymptomatic sickle cell patients. Current therapeutic studies of arginine supplementation largely focus on specific and acute complications [4]. As vaso-occlusion is a continuous process resulting in organ damage irrespective of the frequency of acute manifest clinical vaso-occlusive events, the potential beneficial effect of chronic arginine supplementation should also be explored [23].

References

1. Serjeant GR (2001) The emerging understanding of sickle cell disease. Br J Haematol 112:3–18
2. Frenette PS (2002) Sickle cell vaso-occlusion: multistep and multicellular paradigm. Curr Opin Hematol 9:101–106
3. Francis RB Jr (1991) Platelets, coagulation, and fibrinolysis in sickle cell disease: their possible role in vascular occlusion. Blood Coagul Fibrinolysis 2:341–353
4. Reiter CD, Gladwin MT (2003) An emerging role for nitric oxide in sickle cell disease vascular homeostasis and therapy. Curr Opin Hematol 10:99–107
5. Schechter AN, Gladwin MT (2003) Hemoglobin and the paracrine and endocrine functions of nitric oxide. N Engl J Med 348:1483–1485
6. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM (1996) Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc Natl Acad Sci U S A 93:9114–9119
7. Space SL, Lane PA, Pickett CK, Weil JV (2000) Nitric oxide attenuates normal and sickle red blood cell adherence to pulmonary endothelium. Am J Hematol 63:200–204
8. Belhassen L, Pelle G, Sediame S, Bachir D, Carville C, Bucherer C, Lacombe C, Galacteros F, Adnot S (2001) Endothelial dysfunction in patients with sickle cell disease is related to selective impairment of shear stress-mediated vasodilation. Blood 97:1584–1589
9. Gladwin MT, Schechter AN, Ognibene FP, Coles WA, Reiter CD, Schenke WH, Csako G, Waclawiw MA, Panza JA, Cannon RO 3rd (2003) Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation 107:271–278
10. Mori M, Gotoh T (2000) Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun 275:715–719
11. Hallemeesch MM, Lamers WH, Deutz NE (2002) Reduced arginine availability and nitric oxide production. Clin Nutr 21:273–279
12. Morris SM Jr (2002) Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 22:87–105

13. Van der Dijs FP, van den Berg GA, Schermer JG, Muskiet FD, Landman H, Muskiet FA (1992) Screening cord blood for hemoglobinopathies and thalassemia by HPLC. Clin Chem 38:1864–1869
14. Van der Dijs, Schnog JJ, Brouwer DA, Velvis HJ, van den Berg GA, Bakker AJ, Duits AJ, Muskiet FD, Muskiet FA (1998) Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients. Am J Hematol 59:192–198
15. Vichinsky E, Kleman K, Embury S, Lubin B (1981) The diagnosis of iron deficiency anemia in sickle cell disease. Blood 58:963–968
16. Moshage H, Kok B, Huizenga JR, Jansen PL (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem 41:892–896
17. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO 3rd, Schechter AN, Gladwin MT (2002) Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med 8:1383–1389
18. Enwonwu CO, Xu XX, Turner E (1990) Nitrogen metabolism in sickle cell anemia: free amino acids in plasma and urine. Am J Med Sci 300:366–371
19. Lopez BL, Kreshak AA, Morris CR, Davis-Moon L, Ballas SK, Ma XL (2003) L-arginine levels are diminished in adult acute vaso-occlusive sickle cell crisis in the emergency department. Br J Haematol 120:532–534
20. Van der Jagt DJ, Kanellis GJ, Isichei C, Patuszyn A, Glew RH (1997) Serum and urinary amino acid levels in sickle cell disease. J Trop Pediatr 43:220–225
21. Morris CR, Kuypers FA, Larkin S, Vichinsky EP, Styles LA (2000) Patterns of arginine and nitric oxide in patients with sickle cell disease with vaso-occlusive crisis and acute chest syndrome. J Pediatr Hematol Oncol 22:515–520
22. Morris CR, Morris SM Jr, Hagar W, Van Warmerdam J, Claster S, Kepka-Lenhart D, Machado L, Kuypers FA, Vichinsky EP (2003) Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? Am J Respir Crit Care Med 168:63–69
23. Schnog JB, Lard LR, Rojer RA, van der Dijs FP, Muskiet FA, Duits AJ (1998) New concepts in assessing sickle cell disease severity. Am J Hematol 58:61–66
24. Li H, Meininger CJ, Hawker JR Jr, Haynes TE, Kepka-Lenhart D, Mistry SK, Morris SM Jr, Wu G (2001) Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. Am J Physiol Endocrinol Metab 280:E75-E82
25. Wagener, Feldman E, de Witte T, Abraham NG (1997) Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. Proc Soc Exp Biol Med 216:456–463
26. Schnog JB, Rojer RA, Mac Gillavry MR, ten Cate H, Brandjes DP, Duits AJ (2003) Steady-state sVCAM-1 serum levels in adults with sickle cell disease. Ann Hematol 82:109–113
27. Serjeant G, Serjeant B, Stephens A, Roper D, Higgs D, Beckford M, Cook J, Thomas P (1996) Determinants of haemoglobin level in steady-state homozygous sickle cell disease. Br J Haematol 92:143–149
28. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP (1994) Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med 330:1639–1644
29. Lard LR, Mul FP, de Haas M, Roos D, Duits AJ (1999) Neutrophil activation in sickle cell disease. J Leukoc Biol 66:411–415
30. Cynober LA (2002) Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition 18:761–766