Sickle cell disease and α-thalassemia in Curaçao

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The general introduction provides an overview of present knowledge regarding hemoglobinopathies and thalassemias, with special attention to sickle cell disease (SCD), α-thalassemia and the screening and diagnosis of these disorders. The hemoglobin (Hb) disorders are a group of autosomal recessive diseases characterized by either the synthesis of a structurally abnormal globin chain or reduced synthesis of one or more normal globin chains. Both Hb structural and synthesis variants may interfere with Hb function, erythrocyte survival and blood flow (rheology) and thereby cause a variety of diseases collectively referred to as hemoglobinopathies and thalassemias. The quantitatively most important structural Hb variants encountered in the Curaçao population are sickle cell HbS (β6Glu→Val; 5.03% heterozygotes) and HbC (β6Glu→Lys; 6.70% heterozygotes), while α-thalassemia-2 (22.7% heterozygotes) is the major Hb synthesis variant.

SCD (i.e. HbSS, HbSC, HbSβ-thalassemia) is a heterogenic disease that is characterized by hemolytic and vasoocclusive components. The vasoocclusive component adds most to morbidity and mortality. The pathophysiological cascade leading from the mutation to its clinical presentation is as yet unclear. HbS is poorly soluble at low oxygen tension and solutions with deoxy HbS above 120 g/L are fully in the gel-state. The gel-state changes red blood cell (RBC) shape from biconcave into that of the typical sickled RBC that tends to occlude the microcirculation. The present notion is that the cascade is initiated by a combination of genetic and environmental factors that ultimately cause a pro-inflammatory, pro-adhesive and hypercoagulable phenotype. SCD should be considered as an endothelial disease in which interaction between the hemolytic component and the abnormal theological properties of sickle RBC gives rise to chronic endothelial activation, chronic damage to virtually all organs and occasional precipitation to painful vasoocclusive crisis. Crisis precipitating factors include infection, dehydration, cold, low-oxygen tension, acidosis and others. The heterogeneity of the disease is largely explained by a number of disease-ameliorating or aggravating genetic and environmental factors. Disease severity is related to Hb concentration, propensity of RBC to adhere to vascular endothelium, co-existing α- or β-thalassemia, trans (e.g. HbC) and cis acting β-structural variants, hereditary persistence of HbF (HPFH), haplotype, social class and psychosocial factors. Nitric oxide (NO) has recently been shown to play a central role in SCD vascular homeostasis, by maintaining basal and stimulated vasomotor tone, limiting platelet aggregation and ischemia-reperfusion injury, modulating endothelial proliferation, reducing VCAM-1 gene transcription and HbF reinduction. Low plasma arginine (the precursor of NO) and NO-metabolites during vasoocclusive crisis suggests that arginine might be a limiting factor in life-threatening situations in which NO-availability might be of crucial importance. SCD requires specialized comprehensive care to achieve optimal outcome in the sense of morbidity and mortality. Genetic counseling and family
education includes the education of its autosomal recessive inheritance and the provision of accurate information about genetic risk, clinical course, medical complications and treatment. A number of therapies have been proposed and developed. Stem-cell transplantation and chronic transfusion can cure SCD or dramatically lessen its severity. Other, mostly experimental, treatment modalities include red cell rehydration, antiadhesion therapy, antioxidant therapy, antithrombotic therapy, antisickling therapy and HbF reinduction (notably through hydroxyurea). Gene therapy offers promise, but progress has been slow. Modulation of environmental influences by institution of supportive measures is feasible for all. Avoidance of crisis precipitating factors for the prevention of acute effects and maintenance of a healthy endothelium for the prevention of both acute and long-term effects are ensuing consequences of the current pathophysiological concept. A healthy endothelium benefits from good nutrition, including sufficient calories, folate, zinc, low iron, antioxidants, fish oil and possibly arginine.

Mutations that result in impairment or complete abolition of α-chain (α-thalassemia) or β-chain (β-thalassemia) production are the most commonly encountered causes of the thalassemia syndromes. The two major types of α-thalassemia derive from loss of 1 to 4 α-genes. They are denoted as type 1 (heterozygous: αα/-, and homozygous: --/--) and type 2 (heterozygous: αα/α - and homozygous: -α/-α). The double heterozygous mixture of α-thalassemia types 1 and 2 is denoted as -α/-. α-Thalassemias have high incidences in Mediterraneans and South-East Asians (α-thalassemia-1 and 2), and blacks and Arabs (α-thalassemia-2). Hematologic parameters of subjects with heterozygous α-thalassemia-2 (αα/-α) are frequently within normal limits. Homozygous α-thalassemia-2 (-α/-α) and heterozygous α-thalassemia-1 (αα/--α) lead to hypochromia, microcytosis and accumulation of unpaired Hb chains. Usually these conditions do not require clinical attention, but loss of 3 (HbH disease) or 4 α-genes causes moderately severe hemolytic anemia and death (hydrops fetalis), respectively.

The diagnosis and screening of hemoglobinopathies and thalassemias may either be based on phenotypic- or molecular diagnostic testing. The present cornerstone is Hb-profiling by high performance liquid chromatography (HPLC). Preventive measures in Curaçao through genetic counseling and early diagnosis after birth may be accomplished by screening of all newborns for HbSS, HbSC and HbSβthalassemia. From an index patient, investigations may be extended to family members, notably for sake of genetic counseling. With respect to adults, it may especially be worth while to screen all pregnant women for hemoglobinopathies. Distinction between α-thalassemia and iron deficiency is important for the need to treat iron deficiency, but not for the establishment of the widely occurring α-thalassemia-2. Proper distinction may prevent treatment of α-thalassemia with iron supplements. Additional measurement of the soluble transferrin receptor.
(sTfR) concentration and the sTfR/ferritin ratio may be useful for distinction between iron deficiency and anemia of chronic disease.

In **chapter II.1**, we evaluated the use of an HPLC method for screening hemoglobins in cord blood. Genotype frequencies of HbS and HbC and α- and β-thalassemia were established in babies born in Curacao. During three months, 67.2% of all (748) newborns were screened: 122 (24.3%) had abnormal Hb pattern: 53 (43.4%) had a hemoglobinopathy (HbS or HbC), 64 (52.2%) α-thalassemia (HbBart's>0.5%, heterozygous or homozygous α-thalassemia-2), and 5 (4.1%) a hemoglobinopathy plus α-thalassemia. None of the newborns with heterozygous HbS and HbC had concomitant β*-thalassemia. The population genotype frequency of heterozygous α-thalassemia-2 was calculated to be 30.7%. Based on the HPLC results, we estimate that 67.1% of newborns with heterozygous α-thalassemia-2 remain undetected. A coincidental finding was a relation between demonstrable alpha-thalassemia and short gestation. The HPLC method was preeminently suitable for screening cord-blood samples.

Cord blood hemoglobin Barts (HbBarts) and hemocytometric indices may be used for classification of newborns into those without α-thalassemia-2 (αα/αα) and with heterozygous α-thalassemia-2 (-α^3.7/αα). In **chapter II.2** we investigated by logistic regression analysis whether the combination of HbBarts and hemocytometric indices improves classification compared with classification based on a single analyte. HbBarts percentages and hemocytometric indices were determined in cord blood of 208 consecutive newborns in Curacao (Netherlands Antilles). Of these, 157 had αα/αα and 51 had -α^3.7/αα, as established by DNA analysis. Between-group differences were significant for erythrocytes, mean cell volume, mean cell hemoglobin (MCH), mean cell hemoglobin concentration, platelets, Hbf₀, and HbBarts. The Logit equation of the logistic regression model, using MCH (pg) and HbBarts (%), was: 42.7164 + 5.7916(HbBarts) - 1.3110(MCH). A sensitivity of 100% was reached at a Logit value of -3.70. The corresponding specificity was 62.2%, and the predictive value of a positive test (PV⁺) was 46.3% (95% confidence interval, 37.0-55.7%). The relative information gains were as follows: 88% for the HbBarts-MCH combination, 26% for MCH (not significant), and 0% for HbBarts compared with the 24.6% -α^3.7/αα, prevalence. We conclude that the combined use of cord blood HbBarts and MCH improves classification compared with classification based on single hemocytometric indices.

In **chapter II.3** we investigated whether the previously established higher prematurity frequency of newborns with 0.5%<HbBarts≤2.0% and HbBarts>2.0%, compared with HbBarts≤0.5%, is associated with genotypically demonstrable by α-thalassemia-2. Cord blood was collected from 211 consecutive spontaneous life births in Curacao (The Netherlands Antilles). HbBarts percentages and α-
thalassemia-2 genotypes (i.e. αα/αα, -α/αα and -α/-α) were established and related to their prematurity (<37 weeks) frequencies and gestation lengths. Newborns with 0.5%<HbBarts<2.0% (n= 30; odds ratio for prematurity: 1.34 (1.34-18.58); mean gestational age 38.3 weeks) and HbBarts>2.0% (n=6; odds ratio: 41.50 (4.79-487.8); 33.5 weeks) had higher prematurity frequencies and shorter gestation lengths, compared with newborns with HbBarts≤0.5% (n=174; 39.3 weeks). Newborns with -α/αα (n= 51; odds ratio for prematurity: 3.55 (1.16-10.73); mean gestational age 39.5 weeks) had higher prematurity frequencies compared with newborns with αα/αα (n= 158; 39.0 weeks). From the 18 prematures 10 had HbBarts>0.5% and 9 had -α/αα. Classification into αα/αα, -α/αα and -α/-α coincided to a large extent with classification into HbBarts<0.5%, 0.5%<HbBarts<2.0% and HbBarts>2.0%, respectively. Misclassification was partially due to the gestational age dependency of HbBarts. We conclude that newborns with heterozygous α-thalassemia-2 have higher prematurity frequency than their unaffected counterparts.

In chapter III.1 we determined glycohemoglobin (gly-Hb) reference ranges of non-diabetic adults with HbAA (n = 17), HbAS (n = 37), HbAC (n = 22), HbSC (n = 8), HbSS (n = 6) and HbCC (n = 3) by 13 methods, based on affinity chromatography, HPLC, electrophoresis and immunoassay. Gly-Hb of subjects with HbAS and HbAC can be measured without major difficulties by most methods. Some give rise to absolute gly-Hb differences ≥1% compared with subjects with HbAA. Measurement of HbA1c/total Hb cannot be recommended. Some HPLC and immunoassay methods cannot measure gly-Hb in subjects with HbSC, HbSS and HbCC, whereas others may suffer from interference. Most methods showed low gly-Hb, reflecting increased erythrocyte turnover. Use of special reference ranges requires previous knowledge of the condition (affinity chromatography and immunoassay) or separation of gly-Hb and its precursor Hb (HPLC and electrophoresis). Interpretation is, however, not recommended because of the numerous factors that determine erythrocyte turnover.

Chapter III.2 reports on a study of parameters of calcium homeostasis and vitamin D status in HbSS patients (median age 8 years, range 3-19; 8 females, 10 males) and matched HbAA controls living in the tropical island of Curaçao. Serum calcium concentration in HbSS patients (2.32(0.07) mmol/L) was lower (ANCOVA, P = 0.002) than that of HbAA controls (2.44(0.14)). None of the subjects had hypocalcemia. There were no differences in serum concentrations of phosphate, total protein, albumin, intact parathyroid hormone (PTH), 25-hydroxyvitamin D (87(27) nmol/L in patients, 86(15) nmol/L in controls) and 1,25-dihydroxyvitamin D. There were no significant relations between PTH and 25(OH)D. We conclude that vitamin D status of HbSS patients in Curaçao is adequate.
In chapter III.3 we emphasize the limitations of painful crisis frequency as a parameter of disease severity. We also discuss the potential of immunological, hematological and biochemical factors to serve as parameters of disease severity. Vasoocclusion leads to pain, chronic organ damage, and a decreased life expectancy in patients with sickle cell disease. Therapeutic options for sickle cell disease have usually been evaluated according to their capacity for reducing the frequency of vasoocclusive crises requiring clinical attention. However, the frequency of vasoocclusive crises is not representative for the rate of accumulating organ damage in most sickle cell patients. This implies that the frequency of vasoocclusive crises needn't correlate with disease severity and, although being of importance, cannot solely serve as a parameter of treatment efficacy. Therefore, additional new objective parameters are needed to effectively study the vasoocclusive process in sickle cell disease. Several studies show that intricate adhesive interactions between (red) blood cells, plasma components, and endothelium play a crucial role in the pathophysiology of sickle cell vasoocclusion, offering new potential parameters to effectively assess disease severity as well as new therapeutical targets in the near future. Whether these adhesive mechanisms involve the causes or the effects of vasoocclusion will be determined if their inhibition, by interventive measures, results in therapeutic benefits.

In chapter IV.1 we investigated whether pediatric patients with sickle cell disease (SCD) (9±4 years; 27 homozygous SCD (HbSS); 19 sickle-C disease (HbSC)) have different folate status compared with age-, sex-, and race-matched normal hemoglobin (HbAA) controls (n = 20), and whether their folate status can be improved by folate supplementation. The patients were supplemented with vitamins B_6 and B_{12} during one week and with folate during the following week. Circulating folate, homocysteine, vitamin B_6 and vitamin B_{12} levels were measured at baseline (patients and controls), after one week and after two weeks (patients). The patients had similar folate, vitamin B_6, and vitamin B_{12}, but higher homocysteine levels compared with HbAA controls (12.7±4.5 vs. 10.9±3.5 μmol/l; P = 0.04). Vitamin B_6 and B_{12} supplementation did not change their homocysteine levels, but folate supplementation caused a 53% reduction (to 5.7±1.6). We conclude that patients with SCD have adequate vitamin B_6 and B_{12} status, but suboptimal folate status, leading to elevated plasma homocysteine levels. They may therefore benefit from folate supplementation to reduce their high risk for endothelial damage.

In chapters IV.2, IV.3 and IV.4 we embarked into a discussion on the adequate folate status of SCD patients as reported by Rodriguez and coworkers (chapter IV.2), the value of RBC folate as a parameter for folate status assessment in SCD patients (chapter IV.3) and the high number of pediatric SCD patients with low
RBC folate found by Kennedy and coworkers (chapter IV.4). Rodriguez and coworkers in the USA concluded that folic acid supplements are not required in most SCD patients. This conclusion was based on similar plasma homocysteine in pediatric SCD patients and controls, and differs from our observation in Curacao, as described in chapter IV.1. The disparity might result from the fortification of many food products with folic acid in the USA. Interestingly at least 10% of their patients exhibited plasma homocysteine levels above 9.4 μmol/l. This level is consistent with the upper limit of homocysteine after folate supplementation in our study and suggests that at least part of their patients might still benefit from folic acid supplementation. In chapter IV.3 we report on the coincidental finding that RBC folate is not to be taken as an adequate reflection of folate status in SCD patients. RBC folate is largely established during erythropoiesis and subsequently declines during RBC circulation. SCD patients therefore have higher RBC folate at a given folate status, since their RBC have much shorter half-life compared with unaffected controls. Use of special reference ranges for RBC folate for SCD patients does not seem to be a genuine solution because of the many (genetic and environmental) factors that influence their RBC half-life. Plasma homocysteine might be a better parameter for SCD folate status, although it should be kept in mind that homocysteine is not merely dependent on folate status. In the light of the inadequacy of RBC folate to establish folate status in SCD we conclude (chapter IV.4) that the finding of 15% SCD patients with below-normal RBC folate levels by Kennedy in the USA might be a conservative estimate. Since SCD patients should exhibit higher RBC folate to have a folate status comparable with controls, it might be expected that many of their SCD patients with normal RBC folate actually have low-folate status. That these high numbers occur despite the prescription of 1 mg folic acid/day, should, in our opinion, not be regarded as an indication for higher daily supplemental dosages. This notion derives from data reported in chapter IV.5 in which we show that all SCD patients reach lowest homocysteine levels from 700 μg folic acid daily.

Using homocysteine as a functional marker, we determined optimal folic acid, vitamin B₁₂, and vitamin B₆ dosages in 21 pediatric sickle cell disease (SCD) patients (11 HbSS, 10 HbSC; 7-16 years). Chapter IV.5 reports on the results of this study. Daily supplements of folic acid (400, 700, or 1,000 μg), vitamin B₁₂ (1, 3, or 5 U.S. 1989 RDA), and vitamin B₆ (1 or 3 U.S. 1989 RDA) were gradually increased in an 82-week dose-escalation study. Blood was taken at 9 occasions for measurements of erythrocyte (RBC) and serum folate, plasma vitamin B₁₂, whole-blood vitamin B₆, and plasma homocysteine. Augmentation of folic acid from 700 to 1,000 μg and vitamin B₁₂ from 3 to 5 RDA did not further decrease homocysteine. Percentages of patients exhibiting significant individual homocysteine decreases amounted to 43% (folic acid from 0 to 400 μg, vitamins B₁₂ and B₆ from 0 to 1 RDA), 14% (folic acid from 400 to 700 μg), 24% (vitamin B₁₂
from 1 to 3 RDA), and 18% (vitamin B₆ from 1 to 3 RDA). The lowest plasma homocysteine at 82 weeks was 5.9 +/- 2.2 μmol/L. Patients with HbSS had higher RBC folate than HbSC. The entire group exhibited an inverse relation between RBC folate and hemoglobin. We conclude that RBC folate is less valuable for folate status assessment in SCD patients. Optimal dosages are as follows: 700 μg folic acid (3.5-7 U.S. 1989 RDA), 3 U.S. 1989 RDA vitamin B₁₂ (4.2-6.0 μg), and 3 U.S. 1989 RDA vitamin B₆ (4.2-6.0 mg). A practical daily combination is 1 mg folic acid (4.3-8.5 U.S. 1998 RDA when taken with meals), 6 μg vitamin B₁₂ (2.5-5 U.S. 1998 RDA), and 6 mg vitamin B₆ (4.6-10 U.S. 1998 RDA). This combination may by simple and relatively inexpensive means reduce these patients' inherently high risk of endothelial damage.

Hyperhomocysteinemia is a modifiable risk factor for endothelial diseases. In chapter IV.6 we review the current reports on plasma homocysteine levels in patients with sickle cell disease (SCD). Several of these showed that these patients have increased homocysteine. Folate, but also vitamin B₁₂ and vitamin B₆, are determinants of their homocysteine. We established optimal supplemental dosages of these vitamins in 21 pediatric SCD patients by slowly increasing their intakes in 82 weeks. These were estimated at: 700 μg folic acid, 4.2-6.0 μg vitamin B₁₂ and 4.2-6.0 mg vitamin B₆. This combination is safe, relatively inexpensive and might reduce their inherently high risk of endothelial damage caused by both the hemolytic and vasoocclusive components of the disease.

Future perspectives
SCD is nowadays considered to be an endothelial disease that is characterized by a pro-adhesive, pro-inflammatory and pro-coagulant phenotype. Any affordable supportive measure that prevents this fragile 'steady state' from precipitation into painful crisis and chest syndrome, is likely to contribute to reduced morbidity and mortality. Most importantly, supportive measures are likely to limit silent chronic obstruction, which probably contributes most to chronic organ damage and its ultimately derived mortality. Supportive measures, as opposed to generally unaffordable and potentially risky or harmful therapies (e.g. bone marrow transplants, chronic blood transfusions and hydroxyurea), are to be found in family education (symptom recognition like splenic sequestration and febrile disease, maintenance of hydration, avoidance of adverse climatic conditions), prophylactic immunization, prompt diagnosis and treatment of infection, and last but not least good nutrition. The latter may especially aim at the endothelium. A healthy endothelium benefits from sufficient calories, folate (and vitamins B₁₂ and B₆), zinc, low iron, antioxidants (vitamins E and C; Se and Zn), fish oil and arginine.
The higher RBC turnover in SCD requires higher intake of a variety of micronutrients and possibly also macronutrients. Folate (preferably accompanied by vitamins B₁₂ and B₆) reduces plasma homocysteine, which is a risk factor for endothelial damage. Plasma homocysteine in SCD is e.g. associated with stroke. In an open-label study design we (1) have established the optimal dosages of folic acid, and vitamins B₁₂ and B₆ to reach the lowest homocysteine levels in patients with SCD. Many SCD patients have marginal zinc status, which relates to poor growth, delayed maturation and susceptibility to infection. In a previous study (2) we found that zinc supplementation reduces the percentage irreversibly sickled cells (ISC), but the percentage ISC has no established relation with morbidity or mortality. Iron supplementation and repeated blood transfusions are contraindicated, since they augment oxidative stress. A slightly hypochromic anemia may be beneficial because of the substantial delay in HbS polymerization with only small decreases of the MCHC. Oxidative stress seems to be a common end stage of many, if not all, pathophysiological pathways leading to cell damage or death, but it is still questionable whether it can be controlled by augmentation of antioxidant status. The latter is no simple concept, because it is determined by a variety of synergistically acting antioxidants (e.g. vitamins E and C, carotenes, flavonoids) involved in the limitation of the acute devastating effects of reactive oxygen species, and also by many cofactors (e.g. Se, Zn) of enzymes involved in their ultimate detoxification. In one of our previous studies (2) we found that fish oil supplementation reduces serum triglycerides, which may notably be caused by the expression of genes that govern intermediary metabolism (so called diet-gene interaction). By then there were no available soft endpoints to investigate whether the supplement also modulated the nowadays frequently used parameters of endothelial activation, inflammation or coagulation (3). In a small recent study by others, fish oil has been shown to reduce the frequency of pain episodes, possibly by reducing prothrombotic activity. Arginine supplementation during crisis augmented nitric oxide (NO) metabolite formation by 78% and seems to improve pulmonary hypertension. In a recent study (4) we found that patients with steady state SCD in CuraÇao have low plasma arginine and concomitantly increased proline, which points at augmentation of arginine flux via the cytokine-activated pathway of arginase. Activation of this pathway limits conversion of arginine to NO and thereby contributes to the low availability of NO for vasodilation in SCD. It is our aim to investigate in the near future the effect of supplementation of SCD patients with a combination of folic acid, zinc, antioxidants (vitamins E and C), fish oil and arginine. The study design is based on an open label trial that makes use of contemporary laboratory end points of endothelial activation, inflammation and coagulation.
References


