

## “IN VIVO” AND “IN VITRO” DEMONSTRATION OF HEMOGLOBIN C CRYSTALS IN NON-SPLENECTOMIZED PATIENTS

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### SUMMARY

We studied 12 Hb C carriers: 4 homozygotic Hb CC and 8 heterozygotic. We observed the presence of free crystals in the peripheral blood of the homozygotes but in none of the heterozygotes. However, after incubation with 3% NaCl we were able to detect crystals in the heterozygotes (Hb AC and Hb SC), and in the homozygotes (Hb CC). In patient 04 (P04) less crystals formation occurred due to inhibition of the process by the presence of elevated levels of Hb F (12.2%). All the homozygotic patients had a splenomegaly of 3 to 6 fingerbreadths. We believe that the spleen wears off with time, thus allowing the passage of crystals to the peripheral blood. This finding might be associated with splenic insufficiency without a reduction of its dimensions. Finally, the finding of crystals in the peripheral blood permitted the diagnosis of Hb C obviating the need for electrophoresis.

**KEYWORDS:** Hemoglobin C; Crystal; Non-splenectomized

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### INTRODUCTION

In 1950 ITANO & NEEL<sup>10</sup> described a new hemoglobin and named it hemoglobin C (Hb C). In 1953 SPAET *et al.*<sup>16</sup> described a second case and soon thereafter RANNEY *et al.*<sup>13</sup> report the third one with the curious observation of many target red blood cells in the patient's peripheral blood smear.

DIGGS *et al.*<sup>6</sup> in 1954 described intra-erythrocytic crystals in the peripheral blood of Hb C bearers, thus emphasizing that target cells were in reality crystals of Hb C and the presence of free rods identified as crystals also caught his attention.

The main defect of Hb C is the substitution of the glutamic acid residue at position 6 of the N-terminal of the beta chain by a lysine, whilst in sickle cell disease the presence of Hb S is due to the substitution of this same residue by a valine.

This phenomenon explains the morphological variations of the erythrocytes: forming crystals in Hb C, and sickle cells in Hb S, thus yielding it possible to identify these two different diseases by simply looking at peripheral blood smears.

In our opinion, the red cells described by RANNEY *et al.*<sup>13</sup> contained Hb C crystals within them and were morphologically different from the ones seen in Hb E, thalassemia, and certain liver diseases which are real target cells. Based on our experience it is possible to identify whether an Hb is of the C or E type simply by analyzing peripheral blood smears

using a common optical microscope. In Hb E, thalassemia, and certain liver diseases typical target cells can be seen which differ from the ones observed in Hb C were both intra and extra-erythrocytic crystals are found even with the spleen present. FABRY *et al.*<sup>7</sup>, mention that only in smears of splenectomized Hb C patients can the crystals be seen, as these crystals would be all removed by the spleen. Therefore, when splenectomy is performed, the resistance of the splenic network certainly disappears and consequently liberates these crystals. It is also hypothesized that as the splenic network wears off with time, splenic function in Hb C patients diminishes which leads to increasing amounts of the crystals in the peripheral blood with age. In sickle cell disease patients we know that fibrosis followed by splenic involution does occur, many times reducing the spleen to a single nodule. However, in patients homozygotic for Hb C, although a reduction in splenic size does not occur, the progressive decrease in function is a fact that explains the finding of crystals in peripheral blood.

ADACHI & ASAKURA, 1979<sup>1</sup> studied Hb C crystals using a concentrated phosphate buffer and were able to identify numerous intra and extraerythrocytic crystals of this hemoglobin in the blood of Hb C carriers. FABRY *et al.*, 1981<sup>7</sup> performed the same study using a 3% NaCl solution, and the resulting crystals were named by ROZENBERG<sup>14</sup> as sickling for Hb C, in contrast to red cells containing Hb S.

Although both Hb C and Hb S arise from the African continent, SUTCHARITCHAN *et al.*<sup>15</sup> performing molecular studies, were able to identify the Hb C gene in Thai natives, thus demonstrating a non-African origin for Hb C.

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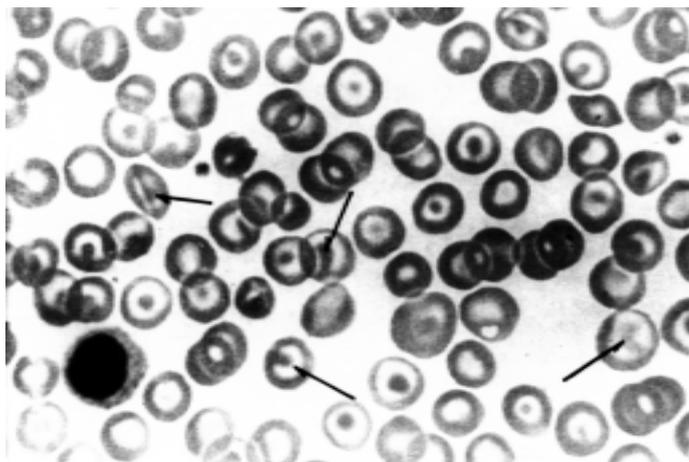


Fig. 1 - Hemoglobin C crystals in different erythrocytes, resembling target cells.

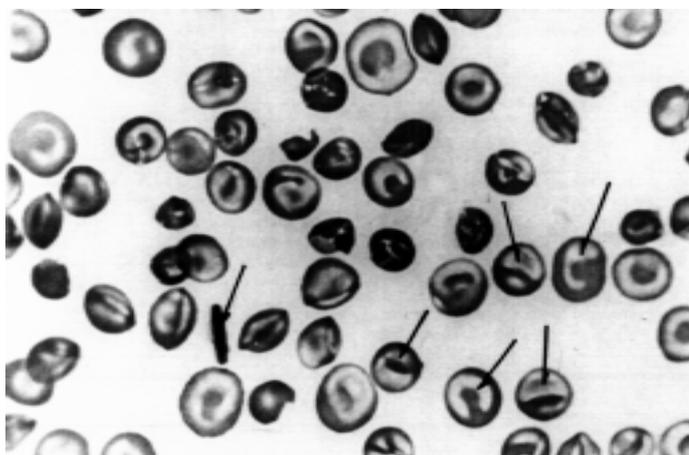


Fig. 2 - “In vitro” demonstration of tetrahedral crystals of Hb CC in the peripheral blood of non-splenectomized patients. Arrows indicate one crystal out of erythrocyte and many crystals inside erythrocytes.

The incidence of Hb C is 17 to 28 % in Western Africa, in the vicinities of Northern Ghana. Up to the present date, no reasonable explanation accounts for this high occurrence<sup>17</sup>.

In Brazil, ARAUJO *et al.*<sup>3</sup>, reported an incidence of 0.6 to 1.0% of Hb C by means of crystals demonstration. He also described rare cases of Hb C in Italian and Portuguese descendants.

#### METHODS AND MATERIALS

During our analysis we identified 4 non-splenectomized carriers of Hb CC, 4 of Hb AC, and also 4 of Hb SC. We studied Hb C crystals in all the carriers, both after incubation with a phosphate buffer and a 3% NaCl solution.

We also analyzed 50 normal blood samples as a negative control for the crystals.

The blood samples were collected with anticoagulant (EDTA), washed 3 times in saline, hemolysed to a 10 g% hemoglobin

concentration, and then analyzed by pH 8.6 cellulose acetate electrophoresis, and pH 6.2 agar gel. The fractions were measured after elution<sup>15</sup>, and Hb F was quantitated by the Betke method<sup>5</sup>.

In order to demonstrate the crystals, we used a hypertonic 3% NaCl solution, and also a phosphate buffer with a pH of 7.4 and 1.8M<sup>9</sup>.

The technique consisted of placing one drop of total blood and 2 drops of the 3% NaCl solution<sup>7</sup> in a test tube and then incubating at 37 °C for 4 hours. An identical procedure was performed using the phosphate buffer with a pH of 7.4 and 1.8M<sup>9</sup>.

The blood smears were stained with the Leishman method, and the crystals could be easily identified with the common optical microscope (Figure 1).

#### RESULTS

In the 50 samples of normal donors (negative controls) no intra or extra-erythrocytic crystals formation was observed.

The highest amount of crystals formation was seen in the homozygotes for Hb C, whilst the lower amount occurred in the heterozygotes for Hb AC. An amount of crystals intermediate between Hb CC and Hb AS was found in the Hb SC cases (Fig. 1 and Fig. 2).

Also, a lower amount of crystals was present in those homozygotes for Hb C that had higher levels of Fetal Hb (Table 1).

**Table 1**  
Concentration of hemoglobin in both states homozygotic and heterozygotic, and the presence of crystals

		% Hb A	% Hb C	% Hb S	% Hb Fetal
P1	Hb CC		98.7		1.3
P2	Hb CC		98.3		1.7
P3	Hb CC		98.4		1.6
P4	Hb CC		77.8		12.2
P5	Hb AC	58.8	40.0		1.2
P6	Hb AC	57.8	40.6		1.6
P7	Hb AC	58.9	39.8		1.3
P8	Hb AC	58.5	40.1		1.4
P9	Hb SC		45.5	51.1	3.4
P10	Hb SC		48.9	50.3	0.8
P11	Hb SC		48.3	51.0	0.8
P12	Hb SC		44.6	54.5	0.9

Visualization of the crystals was better with the phosphate buffer at pH 7.4 and 1.8M than with the 3% NaCl solution.

The concentration of Hb C measured by electrophoresis was of 30 to 40% for AC heterozygotes and of 40 to 50% for the SC. As for the homozygotes the concentration of Hb C was of 98% with the remaining 2% consisting of Fetal Hb, excepting those cases where there is an increase in Hb F which reaches a concentration of 12.2% (Fig. 3).

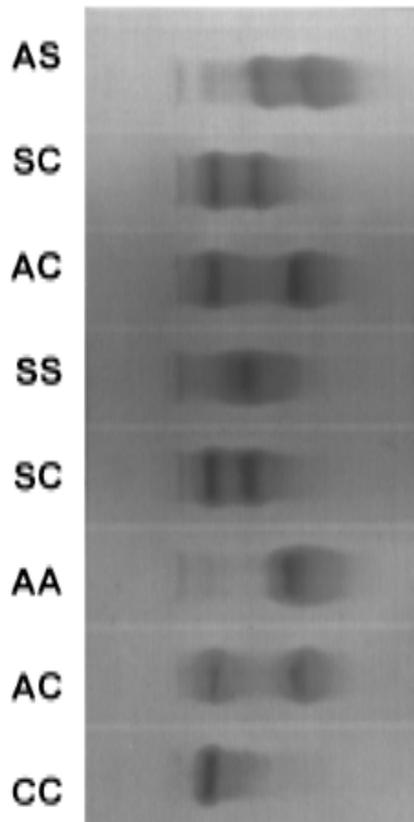


Fig. 3 - Hemoglobin electrophoresis by pH 8.6 agar gel demonstrating the differences in mobility of the hemoglobin fraction.

## DISCUSSION

The spleen's clearing function, also known as “culling”, refers to the organ's capacity of removing aged, dysmorphic, deformed, agglutinated, antibody or complement coated erythrocytes, and erythrocytic corpuscles (or bodies)<sup>11</sup>.

This is the reason why a normal spleen would remove any free Hb C crystals occasionally present in the circulation. However, even a normal splenic function might permit a few crystals containing erythrocytes to reach the circulation, and this accounts for the finding of these crystals in the peripheral blood of homozygotic Hb C carriers.

When an individual undergoes splenectomy, the peripheral blood may show red cells with crystals similar to sickling forms, free crystals, and also Heinz and Howell-Jolly bodies<sup>11</sup>.

DIGGS *et al.*<sup>6</sup> in 1954, and posteriorly FABRY *et al.*, 1981<sup>7</sup> demonstrated the presence of Hb C crystals in the peripheral blood of splenectomized and non-splenectomized patients. This is probably due to the fact the splenic network is many times unable to retain all the red cells with crystals, thus permitting their identification in the peripheral blood.

ROZENBERG<sup>14</sup>, using photographs, demonstrated the presence of crystals in the peripheral blood of individuals with Hb C, and also

confirmed the presence of these in the non-splenectomized carriers of Hb C by means of the 3% NaCl solution technique.

At our laboratory, we routinely examine the peripheral blood smears without prior knowledge of the diagnosis. In all cases, the crystals allowed us to identify the smears as proceeding from Hb C patients, which was further confirmed by electrophoresis. Due to this fact, we concluded that the spleen is unable to retain all the crystals containing red cells or even the free crystals (Figure 2). We believe that over time, as the spleen retains the crystals of Hb C the network becomes insufficient and thus allows the crystals to appear in the peripheral blood.

The spleen undergoes regression in sickle cell disease, in contrast to homozygotic Hb C disease where the spleen remains enlarged, and no regression occurs. This does not mean, however, that damage due to crystals deposition does not occur. In fact, the crystals can be seen in simple X-Rays of the spleen.

Also, lower amounts of crystals are formed when comparing patients with Hb C that present with high levels of Fetal hemoglobin in relation to those with normal levels.

HIRSCH *et al.*<sup>8</sup> demonstrated that Hb F inhibits the crystallization of Hb C when compared to Hb A. The same is true for A<sup>2</sup> ( $\alpha^2\delta^2$ ) although to a lesser extent than Hb F ( $\alpha^2\gamma^2$ ). These authors performed a study comparing the inhibitory potentials of the gamma ( $\gamma$ ) delta ( $\delta$ ) chains. These chains both differ from the beta ( $\beta$ ) chain at 12 substitution sites, whilst having 4 residues in common located at positions 9, 50, 22, and 87. The two former residues are probably not responsible for the phenomenon, as they involve substitutions exhibiting similar properties. Position 22, however, although a common point in both gamma ( $\gamma$ ) and delta ( $\delta$ ) chains, involves different substitutions. The gamma ( $\gamma$ ) chain possesses an Asp substituting a Glu, whilst at the delta ( $\delta$ ) chain the Glu is substituted by a Val. We are left with position 87 where Thr is substituted for Gli in both the gamma ( $\gamma$ ) and delta ( $\delta$ ) chains, and is therefore responsible for the crystallization of Hb C. In addition, this is the same residue that inhibits the polymerization of Hb S by Hb A<sup>2</sup> and Hb F<sup>8</sup>.

Hb Lepore-Washington originates from a mutation where a fusion between the beta and delta chains occurs, with substitution of only 6 residues. The resulting Hb is also able to inhibit Hb C crystals formation albeit in lesser degrees if compared to Hb A<sup>2</sup> and Hb F. Consequently, this observation suggests that the Gli 87 residue is in fact responsible for the inhibition of Hb C crystals formation. Nevertheless, in agreement with HIRSCH *et al.*<sup>8</sup>, the varied intensities of inhibition among the different hemoglobins enable us to conclude that there may be two possible explanations: the first is that this variation is probably due to the differences in hemoglobin molecular conformations, and the second is that residue 87 is not the only culprit. In this case, the other residues located between positions 88 and 146 might merit consideration.

Therefore, Fetal Hb with the  $\gamma$ Gli 87 residue partially inhibits Hb C crystallization, whilst Hb S speeds up the process. This fact definitely demonstrates the existence of intra-erythrocytic crystals in the oxygenated form of the red blood cells of Hb SC individuals. Hb S might speed up crystallization due to the fact the phosphate buffer renders it insoluble<sup>9</sup>.

The best visualization of the crystals occurred with the 1.8M

phosphate buffer at a pH of 7.4 using the common optical microscope, due to the fact Hb C is less soluble than Hb A in these conditions.

## RESUMO

### Cristais de hemoglobina C demonstráveis “in vivo” e “in vitro” em pacientes não esplenectomizados

Estudamos 12 pacientes portadores de Hb C, sendo 4 homozigotos Hb CC e 8 heterozigotos.

Observamos a presença de cristais livres no sangue periférico dos homozigotos e nenhum nos heterozigotos. Quando incubamos com NaCl 3%, detectamos cristais quer nos heterozigotos, Hb AC e Hb SC, quanto nos homozigotos, Hb CC. No paciente 4 (P04) houve menor formação de cristais devido a inibição pela Hb Fetal que se encontrava aumentada (12,2%).

Todos os pacientes homozigotos mostraram esplenomegalia de 3 a 6 dedos. Emitimos a hipótese de que ocorra no baço um desgaste com o progredir da idade, permitindo a passagem dos cristais para o sangue periférico. Este fato poderia estar associado a uma insuficiência esplênica sem redução do baço.

A presença dos cristais no sangue periférico permitiu o diagnóstico de Hb C mesmo sem a realização da eletroforese de Hb.

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