Summary

Objective: To determine erythrocyte phenotyping in blood donors and patients with sickle cell anemia (SS) treated at Hemocentro of Alagoas and describe the frequency and factors associated with erythrocyte alloimmunization. Methods: Cross-sectional study with 102 SS patients and 100 blood donors. The following tests were performed: erythrocyte phenotyping, Direct and Indirect antiglobulin test, and detection of irregular antibodies by panel of phenotyped red blood cells. Data were compared by Mann-Whitney, qui-square or Fisher’s exact tests. Factors associated with alloimmunization were studied by univariate and multiple logistic regression analysis. Results: The most frequent antigens found in patients and blood donors were: c, e, M, s, JK(a). Significant differences were observed between the frequency of the phenotype of patients and donors in regard to antigens s, FY(a) and JK(b). Of 79 transfused patients, 10 presented positive Indirect Coombs. Thirteen alloantibodies were found, 7 of the Rh system, 2 of Kell and 4 were not identified. Factors associated with alloimmunization were the period of time between the last transfusion and the date of the test and more than 10 red blood cell transfusions. Patients who received more than 10 transfusions were 16.39 (95% CI: 2.23-120.59) times more likely to be alloimmunized than patients with fewer transfusions. Conclusion: The prevalence of alloimmunization in SS patients was 12.7%, with 70% of antibodies belonging to the Rh and Kell systems. This study shows the importance of performing erythrocyte phenotyping in blood donors and receptors to decrease the risk of alloimmunization.

Keywords: Anemia, sickle cell; erythrocyte transfusion; donor selection; blood group antigens.
INTRODUCTION
Transfusion therapy with packed red blood cells (PRBCs) is a common practice in the treatment and prevention of complications in sickle cell anemia (SS). Approximately 50% of patients with SS receive transfusions of PRBCs at some stage of life and 5% to 10% of those enter the chronic transfusion program. The main objective of transfusion is to improve the capacity to transport oxygen and blood flow in the microcirculation by decreasing the percentage of hemoglobin S (HbS) and increasing the hematocrit level, which should not exceed 30%, preventing clinically significant vaso-occlusive events.

Among the immune reactions to red blood cell transfusions, alloimmunization to erythrocyte antigens is a relatively common complication and contributes substantially to the increase in disease comorbidities. The alloimmunization, which is characterized by the presence of alloantibodies, occurs in approximately 5% to 25% of patients with sickle cell anemia undergoing a chronic transfusion regimen.

The study of erythrocyte phenotypes of blood groups in patients and blood donors provides a comparison of the frequency of the most immunogenic genes in each system, being important to decrease the risk of alloimmunization, in addition to estimating the availability of compatible blood, especially in cases of previous delayed hemolytic transfusion reaction (DHTR). The use of blood products after the phenotyping of Rh (D, C, c, E, e) and Kell (K) systems reduces the cases of alloimmunization and hemolytic transfusion reactions in patients with SS, compared to non-phenotyped red blood cell transfusions.

In this context, this study aimed to study the prevalence of erythrocyte antigens in patients with SS in a sample of blood donors from Hemocentro de Alagoas (HEMOAL), in addition to detecting the rate of alloimmunization in patients, assessing the alloantibodies involved and factors associated with alloimmunization.

METHODS
This is a cross-sectional study of patients with SS and blood donors seen at HEMOAL. This project was approved by the Research Ethics Committee of Universidade Federal de São Paulo/Hospital São Paulo, protocol number 1416/08, and by the Ethics Committee of Universidade de Ciências da Saúde de Alagoas (UNCISAL) protocol # 1107.

The study included patients with SS detected by hemoglobin electrophoresis treated by the study researcher at HEMOAL in the period of December 2008 to April 2010, who signed the Free and Informed Consent (FIC) form. Also included in the study were 100 HEMOAL blood donors who signed the FIC. Patients who had received transfusions in the last three months were excluded. We collected demographic data from patients and donors (age, sex and self-reported ethnicity). In addition, we collected patients’ characteristics regarding the number of transfusions and date of last transfusion through interviews, confirmed by the review of patients’ medical records.

LABORATORY ASSESSMENT
A 15 mL blood sample was collected from patients and donors – 5 mL in a tube containing EDTA and 10 mL in a dry tube, for the following laboratory assessments: blood type, hematocrit, hemoglobin, antibody screening by direct and Indirect Coombs test and erythrocyte phenotyping. Erythrocyte phenotyping was performed for antigens A, B, D, C, c, E, e, K, M, N, S, s, Fy(a), Fy(b), JK(a) and JK(b). Samples that had irregular antibodies were submitted to antibody identification by the erythrocyte antigen panel (RBC antigen panel).

Blood donors were phenotyped for antigens A, B, D, C, c, E, e, K, M, N, S, s, Fy(a), Fy(b), JK(a) and JK(b) and compared to those of patients with SS.

The samples were tested to determine the direct and reverse blood typing using the tube technique. The Direct and Indirect Coombs test, red blood cell panel, autoantibody screening and phenotyping of other blood systems were performed by gel centrifugation technique using specific gel-test card (DiaMed Latino America AS, Brazil).

STATISTICAL ANALYSIS
To calculate sample size, we used the estimated frequency of 60% or more for erythrocyte antigens more frequently found in our population, given an absolute accuracy of 10% and obtaining the number of 92 subjects in each group.

Numerical variables were expressed as median and minimum and maximum values and compared using the Mann-Whitney test (non-normal distribution). Categorical variables were expressed as numbers and percentages and compared using the Chi-square or Fisher’s exact test. For the analysis of factors associated with alloimmunization, we used univariate and multiple logistic regression. Statistical significance level was set at p < 0.05. Statistical analysis was performed using SPSS for Win/v. 17.0, SPSS Inc., Chicago, IL.

RESULTS
Of the 116 patients with SS who came for consultation at the Hemocentro of Alagoas in the study period, 14 patients did not sign the FIC and were not included in the study. Thus, 102 (87.9%) patients were studied. The mean age at study enrollment was 16.0 ± 12.3 years with a median of 11.5 (range: 0.7 to 48.6); 52 patients (51.0%) were males and 82 (80.4%) were non-Caucasian.
The present study included 100 blood donors, with a median age of 32.05 (range: 19.6 to 60.5) years; 69% were males and 79% self-reported being Caucasians.

Regarding the ABO blood groups, of the patients included, 56 (54.9%) were type O, 37 (36.3%) were type A, 7 (6.9%) were type B and 2 (2%) were type AB. Of the 102 patients, 92 (90.2%) were Rh positive and 10 (9.8%) Rh negative. Blood donors had a similar distribution in relation to blood typing.

Table 1 shows the frequencies of erythrocyte antigens of SS patients and blood donors. The most common antigens in patients and donors were: c (93.1% vs. 83.0%), e (96.1% vs. 94.0%), M (82.4% vs. 82.0%), s (92.2% vs. 79.0%), JK(a) (88.2% vs. 82.0%). Statistically significant differences were observed between patients and donors regarding the frequency of antigen s, FY(b) and JK(b).

Of the 102 patients, 79 (77.5%) received at least one red blood cell transfusion prior to study enrollment and 39 (38.2%) received one to five transfusions, 10 (9.8%) received six to ten and 30 (29.4%) received more than ten transfusions.

The median age of patients who received at least one red blood cell transfusion was 13.0 years (range: 1.4 to 48.6), whereas nontransfused patients’ median age was 7 years (range: 0.7 to 40.1), p = 0.016. The proportion of males and females was similar in transfused patients (48.1% vs. 51.4%, p = 0.281).

Patients had similar hemoglobin and hematocrit levels at the time of enrollment. The median hemoglobin level was 7.4 g/dL (range: 5.0 to 11.2) and 7.7 g/dL (range: 6.1 to 10.5), p = 0.226 and hematocrit was 21.9% (range: 13.4 to 33.6%) and 22.5% (range: 17.7 to 29.5%), p = 0.225, respectively, in transfused and nontransfused patients.

Of the 79 previously transfused patients, 10 (12.7%) had a positive Indirect Coombs test. Thirteen antibodies were detected, of which 7 belonged to the Rh system (1 anti C, 1 anti c, 2 anti D, and 3 anti E), two belonged to the Kell system (one anti Kell, one anti Kpa) and four were not identified.

Alloimmunized patients tended to be older at the moment of study enrollment with a median age of 24.6 years (range: 1.7 to 47.6) years among alloimmunized individuals and 11.5 years (range: 1.36 to 48.6) years among those who had a negative Indirect Coombs test (Mann-Whitney test, p = 0.059). The frequency of alloimmunization was similar in the group of children and adolescents, when compared with those older than 19 years (40% vs. 60%, Fisher’s exact test, p = 0.179). Likewise, male and female individuals had similar rates of alloimmunization (50% vs. 50%, Fisher’s exact test, p = 1.000). Regarding ethnicity, 8 (12.7%) non-Caucasian and 2 (12.5%) Caucasian individuals had positive Indirect Coombs test (Fisher’s exact test, p = 1.000).

One alloimmunized patient had received three transfusions of red blood cells, another had received five transfusions and eight patients had received more than 10 transfusions. Patients who had received more than 10 transfusions had a higher frequency of alloimmunization, when compared to those that had received fewer than 10 (80% vs. 20%, Fisher’s exact test, p = 0.005).

### Table 1 – Erythrocyte phenotyping of patients with sickle cell anemia and blood donors

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Patients n = 102</th>
<th>Donors n = 100</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag D</td>
<td>92 (90.2%)</td>
<td>89 (89%)</td>
<td>0.781</td>
</tr>
<tr>
<td>Ag C</td>
<td>55 (53.9%)</td>
<td>58 (58%)</td>
<td>0.559</td>
</tr>
<tr>
<td>Ag Cw</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
<td>0.244 *</td>
</tr>
<tr>
<td>Ag c</td>
<td>95 (93.1%)</td>
<td>83 (83%)</td>
<td>0.026</td>
</tr>
<tr>
<td>Ag E</td>
<td>29 (28.4%)</td>
<td>27 (27%)</td>
<td>0.820</td>
</tr>
<tr>
<td>Ag e</td>
<td>98 (96.1%)</td>
<td>94 (94%)</td>
<td>0.535</td>
</tr>
<tr>
<td>Ag K</td>
<td>8 (7.8%)</td>
<td>4 (4%)</td>
<td>0.373*</td>
</tr>
<tr>
<td>Ag M</td>
<td>84 (82.4%)</td>
<td>82 (82%)</td>
<td>0.948</td>
</tr>
<tr>
<td>Ag N</td>
<td>64 (62.7%)</td>
<td>63 (63%)</td>
<td>0.970</td>
</tr>
<tr>
<td>Ag S</td>
<td>44 (43.1%)</td>
<td>44 (44%)</td>
<td>0.902</td>
</tr>
<tr>
<td>Ag s</td>
<td>94 (92.2%)</td>
<td>79 (79%)</td>
<td>0.008</td>
</tr>
<tr>
<td>Ag Fya</td>
<td>60 (58.8%)</td>
<td>53 (53%)</td>
<td>0.405</td>
</tr>
<tr>
<td>Ag Fyb</td>
<td>47 (46.1%)</td>
<td>66 (66%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Ag JKa</td>
<td>90 (88.2%)</td>
<td>82 (82%)</td>
<td>0.213</td>
</tr>
<tr>
<td>Ag JKb</td>
<td>51 (50%)</td>
<td>72 (72%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

p-value, chi-square; * Fisher’s exact test.
The interval between the last transfusion and date of the erythrocyte antibody screening was similar in the al-loimmunized and non-alloimmunized groups. This period was 1.2 (range: 0.3 to 13.5) years in alloimmunized and 1.3 (range: 0.3 to 11.4) years in non-alloimmunized individuals (Mann-Whitney test, p = 0.871).

The levels of hemoglobin and hematocrit were similar in patients with positive or negative Indirect Coombs. The median hemoglobin level was 7.1 g/dL (range: 6.6 to 11.2) and 7.4 g/dL (range: 5.0 to 10.7), respectively, in alloimmunized and non-alloimmunized individuals, through Mann-Whitney test, p = 0.929. The median level of hemoglobin in patients with positive Coombs was 20.6 g/dL (range: 18.7 to 33.6) and 22.0 g/dL (range: 13.4 to 31.9) in the other group (Mann-Whitney test, p = 0.929).

To study the factors associated with alloimmunization in patients with SS, we initially performed the univariate logistic regression, including possible associated factors described in the literature (Table 2). Variables such as patient age, interval between transfusions and more than 10 transfusions of red blood cells, which showed statistical significance < 0.20 in the univariate analysis, were included in the multivariate model.

Table 2 – Univariate logistic regression for factors associated with alloimmunization in patients with sickle cell anemia

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1.062</td>
<td>1.008-1.118</td>
<td>0.013</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.091</td>
<td>0.290-4.111</td>
<td>0.898</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.982</td>
<td>0.187-5.149</td>
<td>0.983</td>
</tr>
<tr>
<td>More than 10 transfusions</td>
<td>8.545</td>
<td>1.674-43.620</td>
<td>0.010</td>
</tr>
<tr>
<td>Interval between the last transfusion and antibody screening (years)</td>
<td>1.219</td>
<td>0.992-1.497</td>
<td>0.059</td>
</tr>
</tbody>
</table>

The final model of multiple logistic regression showed that: each additional year between the last transfusion and erythrocyte antibody screening increased by 36.9% the chance that the patient is alloimmunized (OR: 1.369, 95% CI: 1.059-1.771, p = 0.017) and patients who had received more than 10 red blood cell transfusions had a 16-fold higher chance of presenting alloimmunization (OR: 16.390, 95% CI: 2.228-120.586, p = 0.006) when compared to patients with fewer than 10 transfusions.

DISCUSSION

The erythrocyte immunophenotyping in patients with SS is important to prevent one of the major complications of transfusion therapy, as the presence of autoantibodies and alloantibodies make it difficult to obtain compatible blood and can cause acute or delayed hemolytic transfusion reactions, increasing morbidity.

In the present study, we observed that donors were mostly males (69%) and self-reported being Caucasians (79%), as described in the study by Sakhalkar et al.17.

Two studies reported differences in the frequency of antigen in blood donors and SS patients detected by erythrocyte phenotyping. The study by Moreira Jr. et al.1, showed significant difference in the C antigen frequency and the one by Matsuura18 showed difference in the frequency of D, Fy(a), Fy(b), S and s antigens. The comparative analysis of erythrocyte phenotypes in patients with sickle cell disease and blood donors detected differences did not influence erythrocyte alloimmunization, as no alloantibodies were detected against these antigens. Therefore, these data show that they are similar in the main phenotypes of blood group systems.

When evaluating the antibodies present in alloimmunized patients, we found 13 antibodies, and only three patients had more than one alloantibody. The alloimmunization rate was 12.7%, similar to that found by Moreira Jr. et al.3 in a study carried out in São Paulo (12.9%) and by Murao and Viana19 in Minas Gerais (9.9%).

In the present study, of 13 the antibodies found, seven belonged to the Rh system, two to the Kell system and four were not identified, without any agreement with the phenotypic difference of donors for antigens s, FY(b) and JK(B). Most alloantibodies found belonged to the Rh system, similar to what was observed in other studies showing that Rh system antigens are among the most immunogenic ones. Moreover, alloimmunization in SS depends on other factors such as immune response, the number and frequency of transfusions, antigen immunogenicity, the presence of HLA-B35 antigen and receiver’s gender19-21.

The demographic variables studied herein were not associated with alloimmunization (Table 2). It was observed that of 102 patients with SS, 79 (77.5%) received transfusions and 10 (12.6%) were alloimmunized, of which four were females and six were males. The alloimmunization rate is generally higher in women due to pregnancy and abortion history, which did not happen in this study, probably due to the predominance of children in the sample17,18,22,23.

In the present investigation, alloimmunization predominated in patients with more than 10 transfusions, similar to the study by Sakhalkar et al.17, who observed that most patients developed alloantibodies after 12 transfusions and that by Natukunda et al.8, in which 80.7% of alloimmunized patients had received more than 10 transfusions.
In the present study, the presence of several unidentified antibodies draws attention to limitations of the method used, in addition to the existence of other antibodies that have yet to be screened. In some cases, due to particular characteristics or very low titers, the alloantibody is only detected by using special techniques such as prolonged incubation, the use of enzyme-treated erythrocytes or with low ionic concentration media.

Our results are similar to those obtained in a study carried out in Saudi Arabia with retrospective analysis of clinical and transfusion history of 350 patients aged 2 to 75 years, who had received at least one transfusion. These authors identified alloimmunization in 48 (13.7%) patients and the alloantibodies detected were: anti-E (18.8%), unspecified (12.5%), inconclusive (12.5%), anti-K (10.4%) and anti-c (6.3%) and most cases with multiple antibodies. We concluded, as Bashawri20, that the prevalence of alloimmunization and its consequences are important for clinical management and laboratory practices of patients with SS.

The rate of alloimmunization found in this study was 12.7% and 30% of the antibodies were not identified. In the study by Matsuura18, of 72 patients with SS, previously transfused, the presence of irregular anti-erythrocyte antibodies was detected in 13 (18%) cases. Fourteen alloantibodies were detected, being 5 (32.7%) of the Rh system, 4 (28.6%) of Kell, 1 (7.1%) of the MNSs system, 1 (7.1%) of the Lewis system and 3 (21.5%) were not identified.

Screening for autoantibodies by DC was negative in all patients in the present study, whereas in the study by Matsuura18 in Manaus, 6 (8.3%) tests were positive. This study showed a statistically significant difference (p < 0.05) between patients and blood donors regarding the frequency of antigen D, S, Fy(a), Fy(b) and s, with the latter two antigens being also significant in the present study, in addition to JK(b). Therefore, the rate of alloimmunization and antigens involved in this research and in the study by Matsuura18 showed concordant results.

Erythrocyte phenotyping of patients and donors showed a statistical difference for the Ag c, reinforcing the importance of using blood phenotyped for the Rh system. We also verified a statistical difference of antigen s, Fy(b) and JK(b), which are less immunogenic than the Rh system, but it reflects the importance of the extended phenotyping of the MNSs, Kidd and Duffy blood systems to prevent alloimmunization in patients with SS, in agreement with Araújo et al.23, who recommend expanding the screening of erythrocyte antigens when indicating red blood cell transfusion in multitransfused patients. The study by Godfrey19 showed a decrease in the formation of autoantibodies and alloantibodies after introduction of C, E and K antigen phenotyping.

The final model of multiple logistic regression showed that for every additional year in the interval between the last transfusion and erythrocyte antibody screening, there was a 36.9% increase in the chance for the patient to be alloimmunized. Similarly, a retrospective multicenter study carried out in the United States emphasized that the time interval between transfusion and antibody detection was associated with antibody specificity and its early detection could reduce the risk of hemolytic transfusion reaction21. Fabron Jr.5 observed that within 10 months, 21% of previously documented alloantibodies had disappeared.

Patients who received more than 10 red blood cell transfusions had a 16-fold higher chance of being alloimmunized, compared to patients with fewer than 10 transfusions. It was observed that 80% of alloimmunized individuals had received over 10 units of red blood cells; as in the study by Fabron Jr.,5 most alloimmunized patients (61.5%) had previously received more than 10 units of PRBCs. According to Moreira Jr et al.3, the estimated risk of alloimmunization per unit of PRBCs transfused to Brazilian individuals with SS is approximately 1.15%.

In conclusion, phenotyping of erythrocyte donors and patients showed the importance of identifying the differences in phenotypes between donors and recipients, in order to avoid alloimmunization, a major complication of transfusion therapy. The prevalence of alloimmunization in patients was 12.7%, with 70% being from antibodies belonging to the Rh and Kell blood groups. Factors associated with erythrocyte alloimmunization in patients with sickle cell disease were the fact of receiving more than 10 red blood cell transfusions and longer time after the transfusion of red blood cells.

**References**