Our aim was to evaluate the influence of the hemodialysis (HD) procedure in red blood cells (RBC) membrane protein composition. We evaluated hematological data (RBC count, hemoglobin concentration, and hematometric indices) and RBC membrane protein composition (linear and exponential gradient polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [SDS-PAGE] followed by densitometry analysis of RBC membrane proteins) before and immediately after the HD procedure in 20 patients (10 responders and 10 non-responders to recombinant human erythropoietin therapy [rhEPO]) and 26 healthy controls. Before HD, patients presented anaemia and significant changes in membrane protein composition, namely, a statistically significant reduction in spectrin associated with a significant increase in bands 6, as well as an altered membrane protein interaction (protein 4.1/spectrin, protein 4.1/band 3, protein 4.2/band 3 and spectrin/band 3). After HD, we found that patients showed a statistically significant increase in RBC count and hemoglobin, a further and statistically significant decrease in spectrin, an increase in band 3, and an altered spectrin/band 3 ratio.
When comparing responders and non-responders patients after HD, we found that the non-responders presented a trend to a higher reduction in spectrin. Our data suggest that HD procedure seems to contribute to a reduction in spectrin, which is normally associated with a reduction in RBC deformability, being that reduction in spectrin is higher in non-responder patients.

**Keywords** chronic kidney disease, chronic renal failure, hemodialysis, spectrin, erythrocyte membrane proteins, resistance to rhEPO

**Introduction**

The red blood cell (RBC) membrane is a complex structure comprising a lipidic bilayer, transmembrane or integral proteins, and peripheral proteins, including the cytoskeleton proteins. Modifications in RBC membrane protein composition may account for changes in the deformability of the cell, compromising its circulation in the microvasculature and its survival.[1-3]

Spectrin is the major protein of the cytoskeleton, and therefore most responsible for RBC shape, integrity, and deformability. It links the cytoskeleton to the lipid bilayer by vertical protein interactions with the transmembrane proteins, band 3, and glycophorin C.[2,3] In the vertical protein interaction of spectrin with band 3 are also involved ankyrin (also known as band 2.1) and protein 4.2. A normal linkage of spectrin with the other proteins of the cytoskeleton assures normal horizontal protein interactions, and its linkage with the transmembrane proteins assures normal vertical interactions.[2,3]

Anaemia is a common complication in hemodialysis (HD) patients, due mainly to a failure in erythropoietin kidney production.[4] Moreover, the lifespan of RBCs of HD patients being shortened is an additional cause of anaemia in these patients.[5,6]

We recently reported[7] that HD patients present changes in RBC membrane protein composition, the decrease in spectrin being the most significant change. This spectrin deficiency may account for a poorer linkage of the cytoskeleton to the membrane, favoring membrane vesiculation and probably a reduction in the RBC lifespan of HD patients. We also found that HD patient non-responders to recombinant human erythropoetin (rhEPO) therapy, receiving high doses of rhEPO compared to responder’s patients, presented a trend to lower values of spectrin, though without statistically significance, and a significant increase in ankyrin that resulted in a significant disturbance in horizontal and vertical protein interactions, as suggested by the altered ankyrin/band 3 and spectrin/ankyrin ratios. We have hypothesized that alterations in RBC protein membrane structure in HD patients could be related to the chronic renal failure and/or to the HD procedure itself, and that in non-responders, it could be also related to the higher doses of rhEPO received by these patients. The aim of our work was to evaluate the effect of HD procedure in RBC membrane protein composition in HD patients, including responders and non-responders to rhEPO therapy.

**Materials and methods**

**Subjects**

We studied 20 HD patients (8 males, 12 females; mean age 60.3 ± 16.7 years) under rhEPO treatment, including 10 responders and 10 non-responders to rhEPO therapy. Classification of the patients, as responders or non-responders, was performed in accordance with the European Best Practice Guidelines,[8] which defines resistance to rhEPO as a failure to achieve target hemoglobin (Hb) levels (between 11 and 12 g/dL) with maintained doses of rhEPO higher than 300 IU/Kg/week of epoetin or 1.5 μg/Kg/week of darbepoeitin-α. The two groups of patients were matched for age, gender, weight, body mass index, mean time under HD, urea reduction ratio, urea Ktv, and parathyroid hormone serum levels.

HD patients were under therapeutic HD three times per week, for 3 to 5 h, for a median period of time of 41 months. All patients used the high-flux polysulfone FX-class dialyzers of Fresenius, 12 with FX60 and 8 with FX80 dialyzer type. The causes of renal failure in patient’s population were as follows: diabetic nephropathy (n = 5), chronic glomerulonephritis (n = 3), hypertensive nephrosclerosis (n = 2), obstructive nephropathy (n = 1), nephrolithiasis (n = 1), chronic interstitial nephritis (n = 1), and chronic renal failure of uncertain etiology (n = 7). Patients with autoimmune disease, malignancy, hematological disorders, and acute or chronic infection were excluded. All patients gave their informed consent to participate in this study.

Besides rhEPO therapy, all patients were under iron and folate prophylactic therapies, in accordance to the recommendations of European Best Practice Guidelines,[8] to avoid nutrient erythropoietic deficiencies.

The control group included 26 healthy volunteers presenting normal hematological and biochemical values, with no history of renal or inflammatory diseases, and, as far as possible, age- and gender-matched with HD patients. Controls did not receive any medication known to interfere with the studied variables.
Blood samples (using EDTA as anticoagulants) were drawn from fasting controls or before and immediately after the second dialysis session of the week in HD patients. We evaluated hematological data (RBC count, Hb concentration, and hematimetric indices) by using an automatic blood cell counter (Sysmex K1000; Sysmex, Hamburg, Germany) and RBC membrane protein composition using linear and exponential gradient polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) followed by development with Coomassie brilliant blue R-250 (Sigma) and densitometric analysis, as reported previously.\(^7\)

**Results**

The results were analyzed in order to study the differences between healthy controls and HD patients, to study changes imposed by the HD procedure, and to study the differences between responders and non-responders HD patients. In Table 1, we present the hematological data and RBC membrane protein profile for controls and HD patients (before and immediately after HD).

Before HD, the patients presented anaemia, as shown by the significantly decreased RBC count and Hb concentration, with a significant increase in the mean cell volume (MCV), mean cell Hb (MCH), and RBC distribution width (RDW); statistically significant changes in membrane protein composition were also observed.

### Table 1

Hematological data and RBC membrane protein profile for controls and HD patients

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 26)</th>
<th>Before HD</th>
<th>After HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>14.12 ± 1.27</td>
<td>11.94 ± 1.67*</td>
<td>12.98 ± 2.20†</td>
</tr>
<tr>
<td>RBC (×10(^12))/L</td>
<td>4.72 ± 0.59</td>
<td>3.78 ± 0.61*</td>
<td>4.13 ± 0.84†</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>92.00 (90.00–94.00)</td>
<td>93.00 (90.25–98.00)*</td>
<td>93.35 (93.00–98.00)*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.83 ± 1.39</td>
<td>31.83 ± 2.05*</td>
<td>31.60 ± 1.94*</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.47 ± 0.58</td>
<td>32.39 ± 0.71</td>
<td>32.13 ± 0.79</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.79 ± 0.52</td>
<td>14.84 ± 1.11*</td>
<td>14.77 ± 1.19*</td>
</tr>
<tr>
<td>Ankyrin (%)</td>
<td>6.97 ± 1.62</td>
<td>6.39 ± 1.55</td>
<td>6.23 ± 1.28</td>
</tr>
<tr>
<td>Band 3 (%)</td>
<td>38.57 ± 3.99</td>
<td>38.10 ± 3.78</td>
<td>41.13 ± 2.44*†</td>
</tr>
<tr>
<td>Protein 4.1 (%)</td>
<td>7.56 ± 1.45</td>
<td>6.48 ± 1.60</td>
<td>6.39 ± 1.69</td>
</tr>
<tr>
<td>Protein 4.2 (%)</td>
<td>5.51 ± 0.72</td>
<td>4.34 ± 0.99</td>
<td>4.84 ± 1.04</td>
</tr>
<tr>
<td>Band 5 (%)</td>
<td>6.82 ± 0.86</td>
<td>6.56 ± 0.91</td>
<td>6.71 ± 0.59</td>
</tr>
<tr>
<td>Band 6 (%)</td>
<td>5.19 ± 1.04</td>
<td>6.46 ± 0.87*</td>
<td>6.17 ± 1.15*</td>
</tr>
<tr>
<td>Band 7 (%)</td>
<td>2.20 ± 0.65</td>
<td>2.09 ± 0.43</td>
<td>2.37 ± 0.34</td>
</tr>
<tr>
<td>Protein 4.1/spectrin</td>
<td>0.276 ± 0.624</td>
<td>0.243 ± 0.070*</td>
<td>0.251 ± 0.081*</td>
</tr>
<tr>
<td>Protein 4.1iband 3</td>
<td>0.192 (0.154–0.227)</td>
<td>0.170 (0.138–0.206)*</td>
<td>0.163 (0.121–0.202)*</td>
</tr>
<tr>
<td>Protein 4.2/iband 3</td>
<td>0.149 (0.125–0.162)</td>
<td>0.114 (0.101–0.133)*</td>
<td>0.118 (0.101–0.147)*</td>
</tr>
<tr>
<td>Spectrin/iband 3</td>
<td>0.707 (0.649–0.822)</td>
<td>0.685 (0.626–0.796)*</td>
<td>0.647 (0.566–0.689)*†</td>
</tr>
<tr>
<td>Ankyrin/iband 3</td>
<td>0.185 ± 0.585</td>
<td>0.171 ± 0.049</td>
<td>0.152 ± 0.330*</td>
</tr>
<tr>
<td>Spectrin/ankirin</td>
<td>4.18 ± 1.07</td>
<td>4.48 ± 1.361</td>
<td>4.45 ± 1.49</td>
</tr>
</tbody>
</table>

*\(p < 0.05\) vs. controls, †\(p < 0.05\) vs. before HD. Results are presented as mean ± standard deviation and as median (interquartile ranges).

Abbreviations: Hb = hemoglobin concentration, RBC = red blood cell count, MCV = mean cell volume, MCH = mean cell hemoglobin, MCHC = mean cell hemoglobin concentration, RDW = red cell distribution width.
protein composition were observed, namely, a reduction in spectrin and an increase in band 6. Though only slight changes were observed in the other proteins, the ratios between them, reflecting altered horizontal and vertical protein interactions—protein 4.1/spectrin, protein 4.1/band 3, protein 4.2/band 3 and spectrin/band 3—were observed.

After dialysis, HD patients showed a statistical significant increase in RBC count and Hb concentration; no statistical significant changes were found in the other hematological parameters (see Table 1). Concerning the RBC membrane protein profile, a statistically significant decrease in spectrin, an increase in band 3, and an altered spectrin/band 3 ratio were observed after HD procedure. We also observed after HD some trends toward the control profile (band 6 and band 7) and some trends in opposition to control profile (a further decrease in ankyrin, protein 4.1, protein 4.2, and band 5). We must notice that band 3 increased and reached a statistically significantly higher value than that presented by the control. Most of the ratios, reflecting protein interactions, presented values that were still different from those of the control.

When comparing the two groups of HD patients (responders and non-responders), we did not find significant changes in membrane protein profile, excepting that non-responders presented a trend ($p = 0.124$) to a higher reduction in spectrin membrane content (see Figure 1).

**Discussion**

It is accepted that the HD procedure is able to promote a complex biological response when the patient’s blood interacts with the artificial HD membranes.[9–13] However, the effect of HD procedure in RBC membrane protein content has not been adequately defined.

In the present study, despite rhEPO therapy, anaemia was a consistent finding in our HD patients. This anaemia was associated with a statistically significant increase in RDW, suggesting the presence of anisocytic RBCs. Higher Hb levels and RBC counts were found after HD. This increase in circulating RBCs has been described[14,15] to be associated with a translocation of RBCs from the splanchnic circulation (and possibly from the splenic circulation) in order to compensate the hypovolemic stress during dialysis ultrafiltration.

The changes observed in the RBC membrane protein composition of HD patients (see Table 1), when compared to controls, were similar to those we have recently reported,[7] namely, a statistically significant reduction in spectrin in band 6 (no significant change was observed for band 7). The lower number of patients enrolled in the present study may explain the slighter differences in protein profile and in the ratios reflecting membrane protein interactions. We still found in the present study disturbed horizontal (protein 4.1/spectrin) and vertical (protein 4.1/band 3; protein 4.2/band 3; spectrin/band 3) interactions, suggesting a disturbance in membrane deformability and integrity. Spectrin deficiency may account for a poorer linkage of the cytoskeleton to the membrane, favoring membrane vesiculation and probably a reduction in the RBC lifespan of HD patients.[1,2] The increase in protein band 6 may further reflect an altered membrane protein interaction and destabilization of membrane structure.

When studying the differences in membrane protein composition imposed by the HD procedure, the results showed some trends toward a control pattern profile for some of the membrane proteins—bands 3, 6, and 7. Spectrin showed an even lower value after HD, and ankyrin, protein 4.1, protein 4.2, and band 5 also present a trend toward decreasing. The analysis of the ratios reflecting the protein interactions remained altered, when compared to controls; however, when comparing the ratios before and after HD, only the ratio spectrin/band 3 showed a statistically significant value, reflecting a vertical membrane protein disturbance. However, no statistically significant correlation between the RBC membrane protein alterations and the RBC counts and hemoglobin levels were found, which can be due to the lower patients number included in this study.

Moreover, we also found that this spectrin reduction is enhanced in the non-responder group (see Figure 1), which can be associated with an enhanced inflammation process. Indeed, results from our and other groups[16] have demonstrated that HD and particularly resistance to
rhEPO therapy are associated with a pronounced inflammatory response, demonstrated by higher levels of C-reactive protein (data not shown). However, no statistically significant differences were found between C-reactive protein levels and RBC membrane protein modifications found during HD procedure. We also should not exclude the possibility that the much higher doses of rhEPO used in non-responders, can be also involved in RBC membrane protein alterations.

In conclusion, the HD procedure seems to contribute to a reduction in spectrin, which is normally associated with a reduction in RBC deformability, being enhanced in non-responder patients. Therefore, we cannot exclude that the inflammatory product, being enhanced in HD patients (especially in non-responders) may play a role in RBC membrane protein disturbance.

Acknowledgments

We are very grateful to FMC, Dinefro—Diálises e Nefrologia, SA, and Uninefro—Sociedade Prestadora de Cuidados Médicos e de Diálise, SA, and to their nurses for the technical support. This study was supported by a PhD grant (SFRH/BD/27688/2006) attributed to E. Costa by FCT and FSE.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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