Comparison between immunofixation and electrophoresis for the early detection of relapsed multiple myeloma

Comparação entre imunofixação e eletroforese na detecção precoce de recidivas do mieloma múltiplo

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ABSTRACT

Introduction: Multiple myeloma (MM) is an incurable progressive hematological neoplasia characterized by heterogeneous evolution and by relapses after therapy. Objective: Compare the effectiveness of serum immunofixation (SIF) and electrophoresis (SPE) techniques in the detection of relapses in MM patients undergoing treatment at the University Hospital of Santa Maria (HUSM). Material and methods: The study was conducted from January 2012 to July 2014 and included 52 patients from HUSM with confirmed diagnosis of MM. The retrospective monitoring based on laboratory analyses indicated that nine of these patients relapsed, in whom it was possible to compare the effectiveness of SIF and SPE techniques for detecting relapses. Results: For the nine patients, SIF always detected MM relapses earlier than SPE, with a precocity ranging from 2.0 to 18.8 months, for an average of 6.6 months. Discussion and conclusion: The results indicated that SIF was more effective than SPE for the early detection of relapses, regardless of the class and type of M component (mono/biclonal). Therefore, the use of SIF allows for better monitoring of MM patients, especially for the detection of relapses, thereby helping in choosing the most appropriate therapy and resulting in increased duration of survival period free of disease.

Key words: multiple myeloma; monoclonal immunoglobulins; immunofixation; electrophoresis; relapses.
relatively simple laboratory method for the detection of M-protein, the immunofixation serum (SIF) technique is considered the gold standard for confirming the presence of these proteins and to distinguish light and heavy chains in MM\textsuperscript{(18, 19)}. The combination of SPE and SIF techniques increases up to 97% sensitivity in the detection of M-protein in patients with MM\textsuperscript{(12, 20)}.

Whereas, following treatment there may occur complete remission of MM, but not its cure\textsuperscript{(21, 22)}, it is important monitoring these patients in order to be able to detect relapse as early as possible\textsuperscript{(23)}. In this context, a comparison between SIF and SPE techniques on its effectiveness in early detection of MM relapses, through the retrospective analysis of serum samples from nine patients, was the main objective of this study.

**MATERIAL AND METHODS**

**Patients**

The study population consisted of patients diagnosed with MM monitored at the Outpatient service of Hematology of the University Hospital of Santa Maria (HUSM), RS, Brazil, between January 2012 and July 2014. The evolution of 52 patients followed up during the study is shown at Figure 1. All patients had undergone routine laboratory tests before each visit and received the treatment standardized by HUSM.

For inclusion in the study were considered patients with a confirmed diagnosis of MM. Patients were excluded if: a) lost their clinical follow up and/or has incomplete data from the medical records; b) attended with disease relapsed and advanced state; and c) attended at the place where the research was conducted (HUSM) to chemotherapy and after performed clinical laboratory monitoring in the city of origin.

As the study of the patients was done retrospectively, the results of serum analysis (SPE and laboratory measures) were interpreted at each clinic visit, spreadsheets were developed for each study patient for better monitoring, especially for those who were in complete remission of the disease and could relapse.

**Laboratory analysis**

**Samples (pre-analysis)**

Blood samples were collected at the Hematologic-Oncology Laboratory of HUSM. To collect blood, the venipuncture standard technique was used and the material was transferred to Vacutainer\textsuperscript{®} tubes, BD Diagnostics, USA. Tubes without anticoagulant were used for serological tests, while tubes with 7.2 mg of anticoagulant dipotassium of ethylenediaminetetraacetic acid (EDTA) served for CBC analysis. After centrifuging the samples at 4,000 evolutions per minute (rpm) for 10 minutes, the patients’ serum was stored in aliquots (Eppendorf tubes) in a freezer at -70°C.

**SPE and SIF**

SPE technique was performed in electrophoretic tub/CELM, containing 80 ml of CELM buffer – pH 9.5. Serum was applied to agarose film (CELM Gel), according to the technique recommended by the manufacturer. The protein fractions reading were performed by a Software Program for Scanning Densitometry.

SIF analyzes were performed in Sebia\textsuperscript{®} HYDRASYS\textsuperscript{®} instrument with HydraGel IF Sebia 2/4, according to the manufacturer’s instructions. Such analyzes were performed at the Biochemistry Laboratory of the Hospital de Clínicas de Porto Alegre (RS), Brazil. Serum proteins were separated in an alkaline buffer (pH 9.1) for 9 minutes at 20 W (42 VH). The types of antisera to specific classes of immunoglobulins (immunoglobulins class G, A and M [IgG], [IgA] and [IgM], respectively) and light chains (κ and λ) were applied, and the identification was performed after antigens-antibodies complex staining, which resulted in immunoprecipitation. All reagents are included in the IF/Sebia kit.

Gels reading were performed according to the presence or absence of monoclonal and/or biclonal band(s) of immunoglobulin chains (IgG, IgA and IgM) linked to its light-chain κ and λ or only free light chain (κ and/or λ). Two patterns are considered, one normal (absence of monoclonal component), and the other abnormal (presence of monoclonal or biclonal component).

**Serological measures**

Serologic measures were performed in the Biochemistry Department of the Clinical Analysis Laboratory of HUSM, using
Siemens Dimension Pand Plus analyzers. Immunoturbidimetric method was used for immunoglobulin (IgG, IgA e IgM) analysis. For creatinine and albumin dosages, we used the colorimetric method; for lactate dehydrogenase (LDH) analysis, we used the ultraviolet (UV) method.

Blood counts were performed at Hematology Department of HUSM, using Sysmex XE 2100 equipment, while for serum β₂-microglobulin measuring samples were sent to Laboratório de Andilises Clínicas Álvaro (PR) for analysis by chemiluminescence method.

**Statistical analysis**

Because it is a case study, we only calculated the mean values and standard deviation for some of the variables under study, using the SPSS 15.0 software.

**RESULTS AND DISCUSSION**

The analysis of medical records of 52 patients with confirmed diagnosis of MM indicated that their average age was 59 years, ranging 28-83 years. The predominant race was white (56%), followed by the brown (23%) and black (21%), and the predominant sex was male (55.8%) (Table 1). The earlier onset of disease in this study, in relation to the 70-80 years age group, found around 1973(24), can be mainly attributed to advances in the diagnostic techniques, allowing to diagnose it earlier. Regarding race, our results differ from those reported by Kyle et al. (2002)(25) and by Klaus et al. (2009)(26), in which there was a higher incidence of the disease among black people. The MM relationship with race of the disease among black people. The MM relationship with the population's race is difficult to clearly establish for Brazilian populations, since the ethnicity of the population varies among the different regions of the country(27). The results obtained from the 52 patients who participated in the present study (Table 1) indicate predominance of IgG immunoglobulin (48%), followed by IgA (29%), free light chain (FLC) (23%) and IgM (2%). The presence of immunoglobulin class D (IgD) and class E (IgE) and nonsecretory MM were not detected. These results confirm those of other studies that the most common myeloma was IgG, with rare cases of IgD, IgE and nonsecretory myeloma(28, 29).

Several studies have reported the existence of risk factors that help the emergence of MM, especially exposure to high doses of ionizing radiation, occupational exposure to agricultural and petrochemical industries in the presence of benzene and other organic, and exposure to insecticides and herbicides(26, 29). Also lifestyle factors, such as socioeconomic status, smoking and alcohol, may predispose MM occurrence(29). In the population that constituted the present study, the most prevalent factors, in decreasing order, were: exposure to toxic agents, genetic alterations, smoking and alcohol consumption (Table 1).

In most cases, relapses in patients with MM develop aggressively(31), therefore the importance of its early detection through effective methods. SIF method is considered the gold standard(31, 32), with high sensitivity and specificity to detect the resurgence of a monoclonal protein and to distinguish the heavy chains from the light chains present in the serum and urine of patients with MM. From the 52 patients who were monitored in this study, nine relapsed during the study period, enabling to observe the evolution of the disease before and after relapse. The comparison between SIF and SPE techniques regarding their effectiveness in detecting relapses of patients in remission is shown in Figures 2, 3 and 4, in which the subjects were grouped according to immunoglobulin classes. One aspect to be highlighted refers to the fact that there was a predominance of IgA standard in the nine patients who relapsed, showing the aggressiveness and high relapse risk in this MM subtype.

Figure 2, shows the results of five relapsed patients (1, 2, 3, 4 and 7) with the same type of monoclonal/biclonal heavy chain (IgA), it is observed that in the first serum analysis of all patients, SPE was not sensitive in detecting the monoclonal component, for example, increased intensity of bone pain, asthenia and generally feeling unwell. When SPE results were negative in the same samples of these patients, SIF was applied, and the presence of monoclonal component was detected, confirming the suspicion of MM relapsed.

The higher sensitivity of SIF in relation to SPE in detecting MM relapse observed in patients with IgA standards also occurred in the two patients (5 and 9) type IgG protein (Figure 3), and in the two patients (6 and 8) that showed type FLC (Figure 4).

<p>| TABLE 1 – Features and general information about 52 patients with MM evaluated in the present study |</p>
<table>
<thead>
<tr>
<th>Features</th>
<th>General information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>29 (55.8%) male and 23 (44.2%) female</td>
</tr>
<tr>
<td>Race</td>
<td>29 (56%) white, 11 (21%) black and 12 (23%) brown</td>
</tr>
<tr>
<td>Age</td>
<td>Average 59 years ± 13.5 years, 15 (28.8%) 28-48 years, 23 (44.2%) 49-69 years, and 14 (26.9%) above 70 years</td>
</tr>
<tr>
<td>Monoclonal component</td>
<td>24 (48%) IgG, 15 (29%) IgA, 12 (23%) FLC, and 1 (2%) IgM</td>
</tr>
<tr>
<td>Risk factors</td>
<td>17 (33%) exposure to toxic agents, 10 (19%) genetic, 9 (17%) smoker/alcoholic, and 16 (31%) had no risk factor</td>
</tr>
</tbody>
</table>

**MM**: multiple myeloma; **IgG**: immunoglobulin G class; **IgA**: immunoglobulin A class; **FLC**: free light chains; **IgM**: immunoglobulin M class; **a**: radiation and toxic agents of occupations; **b**: first-degree relatives with hematological and/or autoimmune disorders; **c**: long-term of alcohol and/or tobacco use.
Comparison between immunofixation and electrophoresis in the early detection of relapse multiple myeloma

**Figure 2** - Comparative images between SIF and SPE in serum proteins analysis to detect mono/biclonal components in patients 1, 2, 3, 4, and 7, with the same IgA component, at different moments. MC+ and MC- indicate the presence and absence of mono/biclonal components, respectively.

SIF: serum immunofixation; SPE: serum electrophoresis; IgA: immunoglobulin A class; MC: monoclonal component; FLC: free light chains.
FIGURE 3 — Comparative images between SIF and SPE in serum proteins analysis to detect MC in patients 5 and 9, with the same IgG component, at different moments.

MC+ and MC- indicate the presence and absence of mono/biclonal components, respectively.

SIF: serum immunofixation; SPE: serum electrophoresis; MC: monoclonal component; IgG: immunoglobulin G class.

FIGURE 4 — Comparative images between SIF and SPE in serum proteins analysis to detect MC in patients 6 and 8, with the same FLC component, at different moments.

MC+ and MC- indicate the presence and absence of mono/biclonal components, respectively.

Table 2 was developed from the results presented in Figures 2, 3 and 4. It shows the date of relapse detection by SIF and SPE serum analysis, which allows quantitative comparison of the two techniques as its sensitivity in the early detection of relapses. In the nine relapsed patients, the early average of monoclonal pattern detection by SIF exceeded SPE in 6.6 ± 4.8 months, varying only 2.0 months in the worst case (patient 8) and 18.6 months in the most favorable situation (patient 9).

This greater sensitivity of SIF in relation to SPE in detecting monoclonal immunoglobulin, found in this study, confirms the results of several studies conducted mainly in the 1980s and 1990s(33-38). Working with a group of 101 patients with monoclonal gammopathy, Potdevin et al. (1983)(36) found that they were correctly identified by SIF in 97 patients, compared with only 50 cases when SPE was employed. For Vrethem et al. (1993)(39), the low sensitivity SPE is due to the inability of that technique to detect low concentrations of monoclonal immunoglobulins (< 1 g/l) when they are hidden or next to other protein bands. Working particularly with IgM monoclonal immunoglobulin, Keren (1990)(34) identified its presence by SIF in a concentration as high as 20 g/l, without that the IgM in question was detected by SPE. For the author, the negativity occurred by SPE is due to the fact that IgM molecules have large volumes and, therefore, they diffuse slowly in the agarose gel used in SPE. One thing to highlight in these works is that they have not been conducted with the specific objective of comparing the two techniques as its sensitivity in the early detection of relapses.

By comparing SIF and SPE techniques in a MM patient that underwent chemotherapy, Reichert et al. (1982)(47) found that it was wrongly considered free from gammopathy by SPE technique, since, when analyzing the same samples retrospectively using SIF, as it was done for the nine patients of this study (Figures 2, 3 and 4), the authors reported they found positive results for IgM monoclonal gammopathy. Marshall (1980)(49) also compared SPE and SIF techniques for identification of IgG, IGA and IgM monoclonal immunoglobulins in three serum samples with high concentrations and in three samples with low concentrations of such immunoglobulin, and found that SIF was able to identify immunoglobulins in all samples, while SPE showed ambiguous results in the three samples with lower concentration and also in one sample with high concentration. This was attributed to the greater capacity of SIF in determining the reaction of antibodies with monoclonal immunoglobulin.

This set of findings from the literature, added to the present study, proves that SPE, when used alone, is not a suitable technique for the early detection of MM relapses, and should always be performed in conjunction with other techniques more sensitive, such as SIF and FLC measurements, among others. Thus, patients can be monitored with greater safety, in both active and remission phase, and the relapses may be detected early and treatment may be quickly introduced.

Besides the determination of monoclonal immunoglobulin and their light chains and FLC-related (Figures 2, 3 and 4), we also analyzed the serum proteins $\beta_{2}$-microglobulin and albumin, which are important in monitoring MM relapses. According to Casaretto (2005)(39), measurement of $\beta_{2}$-microglobulin is a significant prognostic factor because it reflects the tumor mass and renal function of each patient affected by the disease. Based on the determination of $\beta_{2}$-microglobulin and albumin measurements, Greipp et al. (2005)(40) proposed an international classification for MM staging, in which patients are divided into I, II and III, whose median survival corresponds to 62, 44 and 29 months, respectively.

By analyzing these two serum proteins, it was possible to compare the staging in the nine patients studied at the moment MM relapse was detected by SIF and SPE (Table 3). It was observed that two of the nine patients (patients 2 and 7) were in the early stages of relapse when the monoclonal protein was detected by SIF technique (stage I) and were already at stage II when SPE detection occurred. Similar behavior was observed in patients 4 and 6, in which the relapse could have been detected earlier (stage II) by SIF than by SPE (stage III). This detection of relapses in less advanced stages of MM, using SIF, illustrates the advantage of this technique in relation to SPE in monitoring patients with the disease, since it enables to anticipate the indication of treatment schemes and/or autologous stem cells transplantation. Therefore, this anticipation in treatment could result in a more favorable outcome, since patients would be in better physical shape and health (lower bone pain intensity, regular humoral immunity and absence of anemia), increasing the chances of remission and, consequently,

<table>
<thead>
<tr>
<th>Patients $(n=9)$</th>
<th>Relapse detection by SIF (date)</th>
<th>Relapse detection by SPE (date)</th>
<th>Anticipate the detection of relapse by SIF (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/03/2013</td>
<td>22/10/2013</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>08/01/2014</td>
<td>09/07/2014</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>13/03/2014</td>
<td>02/07/2014</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>12/07/2012</td>
<td>02/01/2013</td>
<td>5.7</td>
</tr>
<tr>
<td>5</td>
<td>03/09/2012</td>
<td>10/01/2013</td>
<td>4.2</td>
</tr>
<tr>
<td>6</td>
<td>23/12/2013</td>
<td>30/06/2014</td>
<td>6.2</td>
</tr>
<tr>
<td>7</td>
<td>13/12/2012</td>
<td>14/06/2013</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>01/06/2014</td>
<td>31/07/2014</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>10/04/2013</td>
<td>27/10/2013</td>
<td>18.6</td>
</tr>
</tbody>
</table>

SIF: serum immunofixation; SPE: serum electrophoresis; MM: multiple myeloma.
increase of survival and improvement in quality of life of patients.

One of the analytical limitations of this study is that it does not offer the possibility of comparing SIF with SPE in detecting monoclonal immunoglobulins (heavy chains and/or FLC) in urine samples from the nine patients studied. This was due to the difficulty of collecting 24-hour urine sample in relapsed patients, since they were already weakened and living elsewhere. To overcome this difficulty, it would be important to perform serum free and heavy chains measurements in order to obtain the correlation between them. Through these analyzes, it would be possible to increase the sensitivity to detect the remission state of these patients, as well as their relapses.

Some laboratory parameters that assist in monitoring the nine patients in study were evaluated and are shown in Table 4, in which it is observed that there was worsening of anemia in the patients studied. In 66.6 % patients (1, 2, 4, 6, 7 and 8), this had already occurred even when the relapse was detected early by SIF. This result can be explained by the fact that such patients have MM for several years (average of six years) and, therefore, are already debilitated by the disease itself as well by the frequent use of therapies and/or chemotherapy in previous relapses.

Although the quantification of complete monoclonal immunoglobulins (IgG, IgA and IgM) assist in monitoring patients with MM, it must be used in conjunction with high sensitivity methods (32). As seen in Table 4, patients 1, 2, 3, 4 and 7 showed average normal values of IgA 345.8 mg/dl (128.9-775.0 mg/dl) when the relapse was detected early by SIF, and average values 3.6 times increased (787.3-2,240.0 mg/dl) when the relapse was detected late by SPE. In both relapsed patients with monoclonal component IgG (5 and 9), although this immunoglobulin values are at normal levels, we observed that there was an average increase of 989.6 mg/dl in SIF to 1497.5 (51.3 %) in SPE, which reinforces the advantage of using SIF in relation to SPE in the early detection of this monoclonal component in relapsed MM.

Serum creatinine reflects renal function and, therefore, patients 2 and 6 already had higher value (2.0 and 1.4 mg/dl, respectively), even when the relapse was detected by SIF. This occurred because these patients had MM type FLC at diagnosis, which caused damage to renal tubules. As relapse was detected later by SPE, the amount of creatinine level increased from 2.0 to 2.6 mg/dl in patient 2, and 1.4 to 2.1 in patient 6, indicating worsening of renal function.

### Table 3 - ISS of recurrent patients at early positive SIF and SPE

<table>
<thead>
<tr>
<th>Patients (n = 9)</th>
<th>Early positive SIF</th>
<th>Early positive SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₂₅ₐ (mg/dl)</td>
<td>Serum albumin (g/dl)</td>
<td>ISS</td>
</tr>
<tr>
<td>1</td>
<td>2.4</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
<td>3.2</td>
</tr>
<tr>
<td>5</td>
<td>1.9</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>4.3</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>4.1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>2.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>


### Table 4 - Laboratories parameters at early positivity by serum immunofixation (SIF) and at early positivity by serum electrophoresis (SPE) of relapsed patients

<table>
<thead>
<tr>
<th>Patients (n = 9)</th>
<th>Laboratories values at early positivity by SIF</th>
<th>Laboratories values at early positivity by SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBC</td>
<td>IgG (mg/dl)</td>
<td>IgA (mg/dl)</td>
</tr>
<tr>
<td>1</td>
<td>NC/NC</td>
<td>612.5</td>
</tr>
<tr>
<td>2</td>
<td>NC/NC</td>
<td>785.8</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>1242</td>
</tr>
<tr>
<td>4</td>
<td>NC/NC</td>
<td>1591</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td>1030.6</td>
</tr>
<tr>
<td>6</td>
<td>NC/NC</td>
<td>1549</td>
</tr>
<tr>
<td>7</td>
<td>NC/NC</td>
<td>717.7</td>
</tr>
<tr>
<td>8</td>
<td>NC/NC</td>
<td>1530</td>
</tr>
<tr>
<td>9</td>
<td>N</td>
<td>948.6</td>
</tr>
</tbody>
</table>

Reference values: IgG (681-1,648 mg/dl); IgA (87-474 mg/dl); IgM (49-312 mg/dl); LDH (81-234 UI/l); CRE (Male: 0.8-1.3; Female: 0.6-1.0 mg/dl).

SIF: serum immunofixation; SPE: serum electrophoresis; CBC: cells blood count; N: normal; NC/NC: normocytic normochromic anemia; IgG: immunoglobulin G class; IgA: immunoglobulin A class; IgM: immunoglobulin M class; LDH: lactate dehydrogenase; CRE: creatinine.
Besides providing early detection of MM relapses in relation to SPE, SIF also allows us to evaluate in general the evolution of clonality, in both intact immunoglobulins and FLC. In Table 5, it is observed that with the exception of patients 5, 7 and 9, there was a change in the type of monoclonal component during the course of the disease. It is likely that this heterogeneity clonal also noted by Magrangeas et al. (2013) (41), Ahn et al. (2014) (42) and Brioli et al. (2014) (43), is due to the prolonged use of chemotherapy drugs and maintenance after the relapses occurred. This change of MM clonality implies intrinsic cellular resistance to subsequent therapies, requiring new therapeutic approaches.

### CONCLUSION

The comparison between SIF and SPE techniques performed in this study, via retrospective analysis of MM relapsed patients, showed that SIF was more effective than SPE in the early detection of relapses, regardless of the class of monoclonal immunoglobulins present. The average of the nine relapsed patients, SIF has detected a monoclonal standard 6.6 months earlier than SPE, varying only two months in the worst cases to 18.6 months in a more favorable situation.

### REFERENCES


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