Potential of molecular diagnostic methods in the early identification of multiple myeloma: an integrative review


ABSTRACT: Multiple Myeloma (MM) is a malignant and incurable neoplasm that affects the hematological system. It promotes the production and secretion of monoclonal IgG immunoglobulins or part of them, called M proteins. Its classic signs are represented by the acronym “CRAB” (C: hypercalcemia, R: renal failure, A: anemia, and B: bone lesions). The laboratory finding associated with the disease is a monoclonal peak in the patient’s serum and/or urine, most often evidenced by protein electrophoresis or immunofixation of serum proteins. These methods allow the staging of the disease, mainly against other monoclonal gammopathies, and aid in the investigation of the early diagnosis of MM. In this study, the main molecular diagnostic methods for MM were reviewed, showing the potential sensitivity and specificity of detecting abnormalities related to the disease by the methods studied. Furthermore, the study highlights the importance of molecular diagnostic methods in the early identification of MM. Detection of microRNAs by real-time PCR (qRT-PCR) proved to be the most sensitive diagnostic method (97.4%) and Mass Spectrometry, associated with Neural Networks, proved to be the most specific method (95%). In addition, Serum Immunofixation (SIF) and Protein Electrophoresis, the most commonly used methods today, proved to be sensitive and specific; however, they had a precocity of about 6.6 months for SIF. The other methods also showed potential for diagnosing and monitoring the disease, but further studies involving the methods studied are needed to further validate the data identified and make it available to the scientific community.

KEY WORDS: Gamopathies; Hematologic Neoplasms; Molecular Diagnostics; Mass Spectrometry; RT-qPCR.

RESUMO: O Mieloma Múltiplo (MM) é uma neoplasia maligna e incurável que afeta o sistema hematológico. Promove a produção e secreção de imunoglobulinas IgG monoclonais ou parte delas, chamadas proteínas M. Seus sinais clássicos são representados pela sigla “CRAB”, formada pela junção dos termos em inglês: “hyperCalcemia, Renal failure, Anemia and Bone lesions”. O achado laboratorial associado à doença é um pico monoclonal no soro e/ou urina do paciente, mais frequentemente evidenciado por eletroforese de proteínas ou imunofixação de proteínas séricas. Esses métodos permitem o estadiamento da doença, principalmente contra outras gamopatias monoclonais, e auxiliam na investigação do diagnóstico precoce do MM. Este estudo se trata de uma revisão integrativa, de forma a evidenciar os principais métodos de diagnóstico molecular do MM, mostrando a potencial sensibilidade e especificidade da detecção de anormalidades relacionadas à doença pelos métodos estudados. Além disso, o estudo destaca a importância dos métodos de diagnóstico molecular na identificação precoce do MM. A detecção de microRNAs por PCR em tempo real (qRT-PCR) mostrou-se o método diagnóstico mais sensível (97,4%) e a Espectrometria de Massas, associada a Redes Neurais, mostrou-se o método mais específico (95%). Além disso, a Imunofixação Sérica (IFS) e a Eletroforese de Proteínas, os métodos mais utilizados atualmente, mostraram-se sensíveis e específicos; entretanto, tiveram precocidade de cerca de 6,6 meses para IFS. Os demais métodos também demonstraram potencial para diagnóstico e monitoramento da doença, mas são necessários mais estudos envolvendo os métodos estudados para validar ainda mais os dados identificados e disponibilizá-los à comunidade científica.

PALAVRAS-CHAVE: Gamopatias; Neoplasias Hematológicas; Diagnóstico Molecular; Espectrometria de massa; RT-qPCR.
INTRODUCTION

Multiple Myeloma (MM) is an incurable malignant neoplasm of the hematological system, characterized by clonal proliferative dysfunction of plasmocytes in bone marrow. This dysfunction promotes the production and secretion of monoclonal IgG immunoglobulins, or parts of them, know M proteins, which can be identified in serum, urine, extramedullary sites, or peripheral blood during disease progression.

As the second most common hematological neoplasm, constituting about 1% of all malignant neoplasms globally, MM often presents nonspecifically, complicating its diagnosis and worsening the prognosis for affected patients. The presence of a monoclonal protein, evidenced by a “monoclonal peak” in the patient’s serum and/or urine, is a key finding identified through molecular methods, with protein electrophoresis and 24-hour urine immunofixation being the most commonly used methods to stage the disease against other monoclonal gammopathies accurately and for the investigation and diagnosis of MM.

SIGNS AND SYMPTOMS ASSOCIATED WITH MM

Patients with MM typically present extensive and often nonspecific symptoms; however, significant symptoms include fatigue, bone pain, commonly in the lumbar region, anemia, renal failure, hypercalcemia, and bone fractures unrelated to physical trauma or other underlying diseases. The classic symptomatology is commonly referred to by the acronym “CRAB” (C: hypercalcemia, R: renal failure, A: anemia, and B: bone lesions). As a hematological system pathology with a systemic nature, MM can also cause extramedullary dysfunction, occurring in 3-5% of cases, typically involving the skin, nasopharynx, larynx, upper respiratory tract, and central nervous system.

Figure 1 – Multiple Myeloma: classic signs and symptoms represented by the acronym “CRAB”. Created with BioRender.com

EPIDEMIOLOGY

Approximately 28.6% of MM cases are diagnosed between the ages of 65 and 74 years, and about 3.5% are diagnosed under the age of 44 years. Its incidence is higher in individuals of black ethnicity than white, and its is more common in males than females. In 2020, it was estimated that there were 12,380 deaths due to MM in the United States, and around 31,000 annual deaths in Europe.

MM DIAGNOSIS AND PRECURSOR STAGES

MM is preceded by asymptomatic precursor stages, most commonly beginning with Monoclonal Gammopathy.
of Undetermined Significance (MGUS), characterized by monoclonal presence in serum at a concentration of <30g/L, proliferation of clonal cells in the bone marrow, and absence of the clinical features of target organ damage	extsuperscript{a}. Between MGUS and MM, the smoldering multiple myeloma (SMM) stage occurs. In 2010, SMM was stratified according to the risk of progression to MM, ranging from low risk (5% chance of progression), to ultra-high-risk (40% chance of progression). Ultra-high-risk SMM is already considered and requires treatment	extsuperscript{b}. Figure 2 gathers data on the main precursor stages that evolve into MM.

Figure 2 - Definition of Multiple Myeloma and precursor plasma disorders. MGUS – Monoclonal Gammopathy of Undetermined Significance; SMM – Latent Multiple Myeloma; MM – Multiple Myeloma; CRAB- Hypercalcemia, Renal Failure, Anemia, and Bone Lesions; SFLC – Free Light Chain Fraction; MRI – Magnetic Resonance Imaging.

A diagnosis of MM is confirmed when there is clonal infiltration of $\geq 10\%$ in the bone marrow, associated with an organic lesion and detectable monoclonal protein in serum or urine	extsuperscript{c}. The first finding compatible with the diagnostic protocol is usually the presence of monoclonal protein or a monoclonal peak in serum or urine, resulting from the production and secretion of abnormal immunoglobulins by abnormal plasma cells.

MOST COMMONLY USED DIAGNOSTIC METHODS

Bence Jones (BJ) proteins is the denomination name given to light chain immunoglobulins that, when detected in the urine, receive this denomination that was first described by Dr. Henry Bence Jones. Despite being discovered in 1847, and having undergone multiple evolutions and scientific improvements over the years, the BJ protein test is becoming less common than more sensitive tests, even though it is a simple and low-cost method	extsuperscript{10}. Another potential technique is protein electrophoresis, which originated in studies by Michaelis in 1909	extsuperscript{11}. Today, it can be used to separate blood, urinary, and cerebrospinal fluid, and other solutions. However, for MM, as well as other monoclonal gammopathies, the most important protein component used in electrophoresis is gamma globulins. The molecular structures of immunoglobulins are heterogeneous; however, their migration in electrophoresis occurs homogeneously and forms very compact and delimited bands, called polyclonal bands. However, when faced with monoclonal gammopathies such as MM, electrophoretic tracing is distinct and occupies a wide and delimited position	extsuperscript{12}.

Serum immunofixation is around 10 times more sensitive than protein electrophoresis in identifying the presence of
monoclonal proteins, as well as the light or heavy chains involved, and can be used exclusively or in conjunction with electrophoresis. The technique involves using an antigen-antibody reaction to verify the formation of precipitin in gels or membranes and visualize the presence of specific proteins. The gel or membrane is washed to eliminate anything that does not belong to the immunoprecipitate. Following this, the material is stained with fluorescein, for example, allowing a better evaluation of the immunological reaction.

Other diagnostic methods can also be used. These include skeletal radiography, whole-body computed tomography, myelogram, and bone marrow biopsy—these methods are widely used to confirm MM and are also employed to rule out possible cases of MGUS or SMM. The myelogram and biopsy from bone marrow are of great practical importance in the diagnosis of MM because they allow the quantification of infiltrating plasmocytes and cytogenetic studies. It is estimated that bone marrow biopsies have correctly identified MM in 95% of symptomatic cases and are a recommended method for abnormal plasma cell evaluation. However, importantly, the most accurate and detailed assessment of marrow content can only be obtained by assessing the performance of the marrow aspirate added to the biopsy of this content. Due to its malignancy and vast symptomatology, MM has been widely researched, mainly in the therapeutic and diagnostic fields. Scientific advancement is necessary as there is a direct correlation between prognosis and early diagnosis; therefore, the search for more sensitive and specific techniques will promote early diagnosis and result in better outcomes and quality of life for patients.

Molecular diagnostic techniques have been the subject of much research and have been suggested as possible alternatives for early MM diagnosis because they are more sensitive and specific. Furthermore, they are less invasive and, in some cases, are more available and demand fewer labor resources, facilitating their availability.

**METHODOLOGY**

This is an integrative review of scientific literature, characterized by a comprehensive analysis of the literature encompassing discussions on methods, results, and general conclusions within a specific area of study, primarily focusing on clinical practice. Accordingly, data sources were explored to extensively examine molecular diagnostic methods for detecting Multiple Myeloma.

Studies published between January 2012 and December 2021, written in English, Spanish, or Portuguese, and available in the following databases were analyzed: PubMed, Latin American and Caribbean Literature in Health Sciences (LILACS), Scientific Electronic Library Online (SciELO), and Cochrane.

Through the DeCS/MeSH (Health Sciences Descriptors) platform, the following descriptors were chosen: “Molecular diagnostic techniques,” “Multiple myeloma,” “Early diagnosis,” and “Molecular diagnosis.” These descriptors were combined and used to search the relevant databases.

Cross-sectional observational, case-control, and cohort studies that assessed the accuracy of molecular diagnostic methods were included. Studies such as case reports, literature reviews, and those not meeting the recommendations outlined in the STROBE checklist for titles and abstracts were excluded. The STROBE form comprises 22 items pertaining to the information requisite in the title, abstract, introduction, methodology, results, and discussion sections of scientific articles delineating observational studies. These criteria are instrumental in assessing the quality of these sections, including the clarity of the title and abstract, the contextual framework and objectives, the robustness of the study design, the adequacy of the sample size, and the coherence of data interpretation. Consequently, studies failing to meet the minimum threshold of 50% adherence to these criteria were omitted from this review. The aforementioned adherence was calculated based on the presence of criteria items, as outlined in the form, relative to the total number of items within the form.

Initially, studies were selected based on their titles, followed by an evaluation of their abstracts and full texts to determine their suitability for the research topic and their potential to address the research questions.

**RESULTS**

After conducting searches using descriptors in the PubMed, Scielo, LILACS, and Cochrane databases, varying numbers of studies were obtained. A search for ‘Molecular Diagnostic Techniques and Multiple Myeloma’ yielded 53 studies selected by title. However, the Scielo database did not yield any results. Similarly, a search using the descriptor ‘Multiple Myeloma and Early Diagnosis’ resulted in 41 studies selected from all mentioned databases except for Cochrane. Additionally, a search for ‘Molecular Diagnostic and Multiple Myeloma’ produced 20 selected articles, with again no results from the Scielo database. In total, this initial title-based selection yielded 114 studies.

Following the search stage, studies that appeared repeatedly in the searches with all three combinations used or in just two of them were excluded from the initial selection. Out of the 41 studies selected from the second descriptor combination, 29 were excluded due to repetition, while in the third combination, 8 were excluded. Consequently, 77 articles proceeded to the next stage.

After the exclusion of repeated studies, the abstracts of the articles were read to verify potential suitability for the bias of molecular diagnostic methods in the context of Multiple Myeloma. Thus, 65 studies were excluded for not addressing molecular diagnostic methods, not being directed to Multiple Myeloma, but rather to other hematologic dysfunctions, or being other literature reviews that did not aim to measure the accuracy of diagnostic methods. At the end of this stage, 12 studies proceeded to full-text reading.

This stage involved the full-text reading of the selected studies, with a complete evaluation of the studies and verification of their quality by the presence or absence of the items indicated in the STROBE form. Of the 12 studies participating in this stage, 6 were excluded for not meeting the expected criteria after
reading their abstracts, as they did not explicitly state the stages that composed the research. In these studies, there was a low presence of items in the evaluative form used, so they did not even reach 50% of what was expected.

All 6 remaining studies, also evaluated by STROBE, scored above 80%. This percentage was calculated based on the number of items recommended by the evaluative form that were present in the analyzed studies, relative to the total number of items that comprise it. They were included in this review for their good quality and alignment with the researched topic. Therefore, 6 studies proceeded to data extraction, analysis, and formulation of the data to be presented in the results and discussion of this work, as well as serving as a source for conclusive ideas. The methodology application and the results of the selection, including the aforementioned exclusions, are schematically described in Figure 3.

The six selected studies are cohort studies with a retrospective nature, with samples from healthy donors, individuals with other Monoclonal Gammopathies (such as Monoclonal Gammopathy of Undetermined Significance and Smoldering Multiple Myeloma), and patients already diagnosed with Multiple Myeloma. Their objective is to evaluate diagnostic methods for their sensitivity and potential for disease detection, with the aim of enhancing diagnostic guidelines and positively impacting patient prognosis.

As delineated in Table 1, four studies were conducted in Europe (studies 1, 3, 4, and 5), one in Asia (study 6), and one originated from South America (study 2). The countries involved include the Czech Republic, England, China, Brazil, Turkey, and Italy. In terms of publication years, the studies ranged from 2012 to 2020, with the latter being the most recent.

Table 2 compiles information pertaining to the molecular methods emphasized by the selected studies, along with descriptions of sampling technique, and the results and conclusions derived from the research.
Table 1 - Description and reference of evaluated studies

<table>
<thead>
<tr>
<th>N</th>
<th>Title</th>
<th>Author</th>
<th>Study</th>
<th>Country</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Identification of circulating microRNAs, as diagnostic biomarkers for use in multiple myeloma</td>
<td>JONES, C I. et al.</td>
<td>Cohort</td>
<td>England</td>
<td>2012</td>
</tr>
<tr>
<td>2</td>
<td>Comparison between immunofixation and electrophoresis for the early detection of relapsed multiple myeloma</td>
<td>AITA, M H C. et al.</td>
<td>Cohort</td>
<td>Brazil</td>
<td>2015</td>
</tr>
<tr>
<td>3</td>
<td>Combined use of free light chain and heavy/light chain ratios allow diagnosis and monitoring of patients with monoclonal gammopathies: Experience of a single institute, with three exemplar case reports</td>
<td>GAGLIARDI, A. et al.</td>
<td>Cohort</td>
<td>Italy</td>
<td>2016</td>
</tr>
<tr>
<td>4</td>
<td>Rapid discrimination of multiple myeloma patients by artificial neural networks coupled with mass spectrometry of peripheral blood plasma</td>
<td>DEULOFEU, M. et al.</td>
<td>Cohort</td>
<td>Czech Republic</td>
<td>2019</td>
</tr>
<tr>
<td>5</td>
<td>Pros and Cons for Fluorescent in Situ Hybridization, Karyotyping and Next Generation Sequencing for Diagnosis and Follow-up of Multiple Myeloma</td>
<td>ALTI, E.I. et al.</td>
<td>Cohort</td>
<td>Turkey</td>
<td>2020</td>
</tr>
<tr>
<td>6</td>
<td>Circulating miRNAs as diagnostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance</td>
<td>LI, J; ZHANG, M; WANG, C.</td>
<td>Cohort</td>
<td>China</td>
<td>2020</td>
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</table>

Table 2 – Diagnostic method studied, sample, results and conclusions of the evaluated studies

<table>
<thead>
<tr>
<th>N</th>
<th>Author</th>
<th>Diagnostic Method</th>
<th>Sample</th>
<th>Results and Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JONES, C I. et al.</td>
<td>Detection of MicroRNAs by Real-Time PCR</td>
<td>Normal healthy donors: 13 People hospitalized donors: 20 People with MGUS: 15 People with MM: 24</td>
<td>Some microRNAs with biomarker potential were identified. Three miRNAs, miR-720, miR-1246, and miR-1308, were validated in individual patient samples. The combination of miR-1308 and miR-720 ([miR-1308]/[miR-720]) demonstrated excellent specificity and selectivity in distinguishing MGUS/myeloma patients from normal and healthy controls, with a sensitivity of 97.4% and specificity of 92.3%, resulting in only 7.7% false positives and 2.6% false negatives. Additionally, the combination of miR-1246 and miR-1308 ([miR-1246]/[miR-1308]) can differentiate myeloma from MGUS patients, with a sensitivity of 79.2% and specificity of 66.7%. The miRNA signature identified for myeloma also holds other potential benefits for myeloma patients.</td>
</tr>
<tr>
<td>2</td>
<td>AITA, M H C. et al.</td>
<td>Serum Immunofixation and Protein Electrophoresis</td>
<td>52 patients diagnosed with Multiple Myeloma who were followed from 2012 to 2014 at the Santa Maria University Hospital (HUSM).</td>
<td>In all nine patients, IFS detected the monoclonal component earlier than EFS, with an average lead time of 6.6 months. In five out of the nine patients, EFS failed to detect the monoclonal component, despite the patients presenting clinical characteristics. The findings suggested that IFS was more effective than EFS in detecting recurrences. Therefore, employing IFS enables better monitoring of MM patients, particularly in relapse detection, which aids in selecting the most suitable therapy and contributes to extending disease-free survival time.</td>
</tr>
<tr>
<td>N</td>
<td>Author</td>
<td>Diagnostic Method</td>
<td>Sample</td>
<td>Results and Conclusion</td>
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<tr>
<td>3</td>
<td>GAGLIARDI, A. et al.</td>
<td>Free Light Chain Fraction</td>
<td>300 samples were collected from 90 patients (49 MM, 6 SMM and 35 MGUS). In patients with MM, FLC analysis was repeated every 3 months for evaluation during treatment.</td>
<td>The free light chain fraction was detected in 82% of all patients when compared to the first sample received, which served as the diagnostic sample. Among the remaining 18% without FLC abnormality, 37.5% were patients with MM. Notably, approximately 70% of intact MM immunoglobulin samples exhibited abnormal FLC, indicating its utility in diagnosing and monitoring MM.</td>
</tr>
<tr>
<td>4</td>
<td>DEULOFEU, M. et al.</td>
<td>Mass Spectrometry Associated with Artificial Neural Networks</td>
<td>84 peripheral blood plasma samples were used, 44 obtained from patients with Multiple Myeloma (MM) and 40 from healthy donors (DS). A random cohort was carried out, divided into two groups: Training (20 MM and 20 DS) and Validation (24 MM 20 DS).</td>
<td>In the training group, sensitivity, specificity, and accuracy were 100%. In the validation group, sensitivity was 95.83%, specificity was 95%, and accuracy was 95.45%. In conclusion, we anticipate that disease-related spectral fingerprinting, in conjunction with artificial intelligence, can furnish a complementary and minimally invasive tool for the diagnosis and monitoring of MM patients.</td>
</tr>
<tr>
<td>5</td>
<td>ALTI, E.I. et al.</td>
<td>Conventional Karyotyping, FISH and Next-Generation Sequencing (NGS)</td>
<td>35 patients with Multiple Myeloma from 2018 to 2019</td>
<td>Conventional cytogenetic analysis failed in 10 (28.0%) of 35 patients, while 25 (71.4%) of the 35 patients with MM exhibited a normal karyotype. Abnormal FISH results were observed in eight (22.8%) of the 35 cases. Next-generation sequencing analysis was applied to all cases, leading to the detection of pathogenic or likely pathogenic variants in six of the 25 cytogenetically normal cases. Additionally, a pathological variant was identified in five of the eight cases with abnormal FISH results. In this study, conventional cytogenetic analysis was successfully conducted in 71.4% (25/35) of samples. The success rate of conventional cytogenetics in MM patients varies between 29.5% and 64.0% in the literature. Hence, technological advancements play a crucial role in classifying MM at the molecular level. It is essential to perform traditional cytogenetic analyses and FISH to uncover new specific recurrent chromosomal abnormalities or other genetic anomalies in MM patients.</td>
</tr>
<tr>
<td>6</td>
<td>LI, J; ZHANG, M; WANG, C.</td>
<td>Detection of MicroRNAs by Real-Time PCR</td>
<td>23 blood samples from MM patients, 16 samples from MGUS patients, and 18 samples from healthy individuals.</td>
<td>In this study, the relative expression levels of miRNA between MGUS and healthy donors (DS) were compared. Abnormal expression was observed in 175 miRNAs in MM compared to both MGUS and DS. Among these, 26 miRNAs exhibited aberrant expression when compared with either MGUS or DS. Multivariate logistic regression analysis in the present study identified miR-107, miR-15a-5p, and Hb as potential diagnostic biomarkers for MM and MGUS identification. The AUC (area under the curve) increased to 0.954, with sensitivity and specificity reaching 91.3% and 93.7%, respectively. This significantly enhances the diagnostic value of MM. Moreover, the combination of miR-107 and miR-15a-5p with Hb enables the discrimination of MM from MGUS, facilitating early treatment and improving prognosis.</td>
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</table>
Molecular Methods Highlighted

Methods Based on Protein Analysis

Mass Spectrometry Associated with Artificial Neural Networks

This method of analytical chemistry allows for both the identification of potential biomarkers and the detection of many peptides and proteins in serum. It has been applied to detect many types of cancer, such as kidney, lung, and liver. The method was developed in the late 1890s when J. J. Thomson determined the mass-to-charge ratio (m/z) of the electron. It is based on measuring the analyte molecules – substances or chemical components of the sample that are the target of analysis in a test – by separating ions in the gas phase according to their different m/z.

Artificial Neural Networks are mathematical tools capable of working with nonlinear data where the relationship between variables is unknown or very complex. They provide generalizations and predictions that are highly suitable for pattern recognition and classification, and consequently, they have been widely applied in different fields of clinical diagnosis.

Based on this, Deulofeu et al. segmented 84 samples into two groups: “training,” which contained 20 healthy donors and 20 MM patients, and “validation,” which included 20 healthy donors and 24 MM patients. In the training group, the sensitivity, specificity, and accuracy of the method in distinguishing MM samples were 100%, while in the validation group, the sensitivity was 95.83%, specificity was 95%, and accuracy was 95.45%.

Free Light Chain Fraction

The free light chain fraction (FLC) is a type of serum-free light chain polyclonal assay that identifies epitopes of the Kappa (κ) or Lambda (λ) light chain component of immunoglobulins, which are exposed when they are not bound to their heavy chain pair. By quantifying the κ and λ chains, it is possible to calculate the ratio between the concentrations found, which is a solid indicator of monoclonality. When one chain is in a higher proportion than the other, the κ/λ ratio is considered abnormal and a strong predictor of tumor. Currently, this quantification is most commonly performed using SPE and SIF, but FLC detected with Freelite® (The Binding Site Group Ltd., Birmingham, United Kingdom) is gaining increasing space due to the higher measurement accuracy of FLCs.

Gagliardi et al. evaluated the detection of FLC in patients with MM, MUGS, and SMM, who provided samples every 3 months for the study. They were found in 82% of all patients when compared to the first sample received, which was considered the diagnostic sample. Of the other 18% that did not have FLC abnormalities, 37% were MM patients. Therefore, approximately 70% of MM samples showed abnormal FLC, supporting its use in MM diagnosis and monitoring.

Serum Immunofixation and Protein Electrophoresis

Serum immunofixation (SIF) and protein electrophoresis (SPE) are part of the current diagnostic protocol for MM and are usually requested concurrently, with a sensitivity close to 97% and specificity of 93%. Aita et al. conducted a comparative analysis between the dates of monoclonal peak detection in patients with MM recurrence by SIF and SPE. In all 9 patients, SIF detected the component earlier than SPE, with an average of 6.6 months earlier. Furthermore, SPE did not detect the monoclonal component in five patients, even in those with clinical characteristics compatible with the disease, demonstrating the higher accuracy of SIF in terms of diagnostic earliness.

Methods Based on Nucleic Acid Analysis

Detection of MicroRNAs by Real-Time PCR

MicroRNAs are small non-coding RNAs that regulate gene expression. They typically bind to mRNAs (messenger RNAs) in their 3’ untranslated region and cause negative regulation of protein expression by translational repression or cleavage and degradation of the target mRNA. The use of miRNAs as biomarkers has increased significantly as it was discovered that they are present in circulating blood and can be detected in human serum or plasma, where they are believed to be protected from degradation by encapsulation in microvesicles or exosomes and/or by binding to RNA-binding proteins. Changes in circulating blood miRNA levels have been associated with various types of cancer. These data thus show that miRNAs derived from blood samples could be used as a minimally invasive diagnostic tool for cancer patients.

Jones et al. identified three microRNAs as biomarkers: miR-720, miR-1246, and miR-1308. From these data, it was identified that a combination of miR-1308 and miR-720 showed more promising results. This combination had a sensitivity of 97.4% and specificity of 92.3%, providing only 7.7% false positives and 2.6% false negatives in distinguishing between MM patients, MUGS, and healthy donors.

Additionally, the combination of miR-1246 and miR-1308 showed a sensitivity of 79.2% and specificity of 66.7%, lower than those presented by the previous combination. However, this combination was considered capable of distinguishing MM from its precursor stages.

Another line of research identified levels of miR-107, miR-15a-5p, and serum hemoglobin (Hb) as potential biomarkers. This method showed a sensitivity of 91.3% and specificity of 93.7%, thus improving the diagnostic value of MM.

Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) consists of recent DNA and RNA sequencing technologies and allows for the detection of variants and mutations. By combining unique sequencing chemistry advantages, sequencing arrays, and bioinformatics technology, NGS enables massive parallel sequencing of multiple lengths of genetic material sequences.
or even the entire genome in a relatively short time. NGS also allows for the analysis of cancer-related mutations, such as MM, in less time and requires a smaller sample amount to perform comprehensive genomic analysis.

Alti et al. compared the detection of MM-related abnormalities in this method compared to data obtained from sample analysis by Conventional Karyotyping (CC) and Fluorescence in Situ Hybridization (FISH). Thirty-five samples from MM patients were used, of which 25 had a normal karyotype, and pathogenic or potentially pathogenic variations were found by NGS in 6 samples (24%) in this group. Regarding FISH, 8 samples showed alterations. Among these, pathogenic variations were evidenced by NGS in 5 samples (62.5%).

**Methods Based on Chromosome Analysis**

**Conventional Karyotyping and FISH**

Conventional karyotyping (CC) and Fluorescence in Situ Hybridization (FISH) are methods based on cytogenetic techniques that analyze the structure, functionality, pathologies, and hereditary issues of chromosomes. Therefore, CC analyzes the karyotype, a study of the representation of chromosomes present in cells, which allows for the analysis of the presence of chromosomal alterations, with translocations and deletions being the main targets of recent research focused on MM.

FISH is a cytogenetic procedure that allows for the identification of a specific stretch of DNA or RNA. A probe labeled with a fluorescent dye is hybridized to indicate the complementary sequence of the probe. This technique is performed on slides, hence the term in Situ, using tissues and cytological preparations in which chromosomes are spread, denatured, and hybridized to display fluorescence.

Cytogenetic abnormalities have prognostic significance in MM, with approximately 30 to 50% of cases demonstrating abnormal karyotypes. The frequency of abnormalities also decreases in newly diagnosed patients. Cytogenetic analysis can provide useful prognostic information; however, the particularly low spontaneous proliferative activity of tumor cells in the early stage of the disease is considered an important limiting factor.

Alti et al. analyzed 35 samples from MM patients by CC, FISH, and NGS, with the results of the latter already mentioned earlier. CC failed in this study in 10 out of 35 patients (28.5%), and 25 patients had a normal karyotype (71.4%). FISH, on the other hand, was abnormal in 8 out of 35 samples (22.8%).

Figure 4 gathers data related to the sensitivity, specificity, positive predictive value, and negative predictive value of the molecular methods used in the diagnosis of MM identified in the literature.

**Sensitivity, Specificity, PPV, and NPV of the Evidenced Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectrometry associated with artificial neural networks</td>
<td>95.83%</td>
<td>95%</td>
<td>95.83%</td>
<td>95%</td>
</tr>
<tr>
<td>Free light chain fraction analysis</td>
<td>69.9%</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Comparison between immunofluorescence and protein electrophoresis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Identification of circulating microRNAs as diagnostic biomarkers for use in multiple myeloma</td>
<td>97.4%</td>
<td>92.3%</td>
<td>92.6%</td>
<td>97.26%</td>
</tr>
<tr>
<td>Circulating miRNAs as diagnostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance</td>
<td>91.3%</td>
<td>93.7%</td>
<td>93.83%</td>
<td>88.98%</td>
</tr>
<tr>
<td>Conventional karyotyping, FISH and NGS</td>
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</table>

**DISCUSSION**

The selected studies demonstrate variations in several aspects. Sampling differed not only in terms of the total number of samples used but also in the types of samples utilized. Three studies (2, 4, and 5 – table 2) exclusively utilized samples from multiple myeloma (MM) patients and healthy donors, while the other studies (1, 3, and 6 – table 2) also included samples from smoldering MM (SMM) and monoclonal gammopathy of undetermined significance (MGUS) patients. This is significant because these early stages often progress to MM, with approximately a 10% chance within the first 5 years of diagnosis. Therefore, their detection requires diligent monitoring, leading to early MM diagnosis should the condition progress to this outcome.
The diagnostic methods highlighted in the selected studies exhibited variability, both in terms of the methodologies employed and the sensitivity and specificity observed. Studies 4 and 5 utilized Mass Spectrometry associated with Artificial Neural Networks and karyotyping, in situ hybridization, and next-generation sequencing, respectively. These represent more complex molecular methods requiring advanced technology and specialized professionals for execution, and evidently, higher associated costs. In terms of statistical data, study 4 demonstrated higher sensitivity and specificity compared to study 5. Thus, Mass Spectrometry associated with Artificial Neural Networks exhibited the capability to detect MM in 95 out of 100 individuals with the disease, rendering it statistically more relevant than the methodology employed by study 5.

Real-time PCR detection of microRNAs was the focus of analysis in studies 1 and 6. Using a method initially developed in 1983 by Kary Mullis and widely used in the detection of SARS-CoV-2 during the recent COVID-19 pandemic, the two studies in question diverged in some ways. The two studies differed in several aspects. Initially, both studies necessitated prior analysis of potentially MM-associated microRNAs, representing a potential point of failure as the optimal disease-related biomarkers are not clearly established in the literature. Only after this preliminary analysis could real-time PCR be conducted. The primary point of divergence lay in the biomarkers utilized: while study 1 examined the combination of miR-1308 and miR-720, study 6 analyzed miR-107 and miR-15a-5p alongside serum hemoglobin levels.

In terms of statistical data, study 1 exhibited a sensitivity 6.1% higher than study 6. Regarding specificity, the percentage difference was smaller (1.4%). Thus, qPCR holds potential in MM diagnosis, but its performance is contingent upon the biomarkers employed, which are still under investigation, lacking consensus in the literature.

Study 2 compared two widely used methods in diagnosing various pathologies: Immunofixation (IFS) and serum protein electrophoresis (SPE), both integral to the diagnostic toolkit for MM and often requested individually or in combination. The aim of study 2 was to determine which method was more sensitive in detecting the monoclonal peak associated with myeloma. However, this study had some limitations. It failed to provide individual sensitivity and specificity data for each method, only citing the average lead time of 6.6 months for IFS in detecting the monoclonal component compared to SPE. Additionally, there was a reduction in sample size during the study, with only 9 out of 52 patients experiencing potential relapse, resulting in this reduced number forming the basis for the lead time data. As a result, study 2 had limitations for statistical analysis, with a significant decrease in sample size and a lack of sensitivity and specificity percentages for the studied methods. This omission hindered a thorough comparison not only between IFS and SPE but also with other methods identified in the literature review.

Study 3 analyzed the free light chain fraction, recognizing its potential due to its ability to measure and differentiate the involved immunoglobulin chains, crucial for accurate pathology classification, therapeutic management, and prognosis. However, statistically, study 3 demonstrated a lower sensitivity percentage compared to the methods mentioned previously. This numerical decline could be attributed to the sampling used, as the samples underwent changes over time and circumstances. Notably, the samples utilized in testing the FLC detection method had also received treatment for the disease, potentially affecting the method’s sensitivity due to the reduction of free light chains during disease remission. Despite this, the study did not consider therapeutic effectiveness as a factor influencing the obtained data, underscoring FLC as a potential tool for diagnosing and monitoring MM.

Considering all the aforementioned factors, the methods highlighted in studies 1, 4, and 6 demonstrated the highest sensitivity in MM detection, with percentages exceeding 90%, compared to studies 3 and 5, which reported sensitivity levels around 70%. The study 2 did not provide statistical data in this regard. However, all studies included in this literature review concluded their analyses with a positive perspective, suggesting that all methods can be utilized for MM diagnosis and/or monitoring. Consequently, early disease detection enables therapeutic intervention, thereby offering improved outcomes for patients. The choice of a specific method remains at the discretion of healthcare professionals, patients, and the availability of resources.

It is essential to note that the scarcity of research and publications in this field acted as a limiting factor for the development of this study, hindering data comparison and a comprehensive statistical analysis. Additionally, the study 2 presented statistical data that differed from those found in the other studies, complicating its interpretation and comparison with others.

Comparing the advantages and disadvantages of the highlighted methods, studies 1, 4, and 6 have the advantage of appearing to be the most sensitive methods for diagnosing MM, presenting potential for the early identification of the disease. However, Mass Spectrometry associated with Artificial Neural Networks (study 4) is a highly complex and high-cost technology that is not widely available in any location, which may interfere with the applicability of the method in the MM diagnostic flow.

The studies 1 and 6, which are based on the detection of miRNA by real-time PCR, can be more easily carried out, given the greater availability of access to the PCR technique. However, they face the disadvantage that the miRNA biomarkers for the disease have not yet been consolidated, requiring scientific development in this scenario to better utilize the technique.

When it comes to Conventional Karyotyping, FISH, and NGS, evidenced in study 5, despite being methods already used in the diagnosis of other diseases with genetic alterations, they have a high cost and low availability, being more accessible in large centers. Furthermore, there is still a lack of further publications related to chromosomal and nucleic acid changes found in patients with MM to improve the diagnostic potential of the methods in question.

The study 3, when dealing with the measurement of FLC, is advantageous because, in addition to being a diagnostic method, it also contributes to the characterization of MM as it allows evaluating which light chain is in the highest concentration in plasma, stratifying the disease into MM of kappa light chain or lambda light chain MM. However, it has a high cost added to...
the technique and low availability, requiring the search for larger centers to carry it out.

Finally, the study 2 highlights the techniques of Serum Immunofixation and Protein Electrophoresis which have the advantage of being more widely available, a lower associated cost compared to the other methods mentioned above, in addition to already being part of the current diagnostic routine. However, further investigation is still needed regarding the early detection nature of MM.

LIMITATIONS ON THE STUDY

The number of available studies acted as a limiting factor in this manuscript. A low number of studies related to MM was found in databases, and when applying temporal filters and targeting molecular methods for diagnosing the disease, this quantity was further reduced. Consequently, the studies included in this review were diverse, hindering effective comparisons between highlighted techniques.

Some studies presented results that did not allow for direct statistical comparison, such as specificity and sensitivity. Additionally, studies focusing on miRNAs faced challenges in comparison due to the lack of solid biomarkers for MM in the literature.

Considering the above, the scarcity of published studies in this manuscript’s subject area, coupled with the outlined limitations, may lead to an overestimation of statistical data related to the highlighted techniques. Further research is necessary to compare the presented data effectively.

CONCLUSIONS

The results were promising for advances in the early diagnosis of MM; however, they also indicate the need for greater scientific progress. From the analysis of the studies, it can be concluded that mass spectrometry associated with artificial neural networks and the detection of microRNAs by real-time PCR seem to be the most sensitive methods for the early diagnosis of MM.

Despite having lower percentages, CC, FISH, NGS, and FLC were also useful for diagnosing and monitoring the course of the disease. SIF and SPE, methodologies already widely used in diagnosis, accurately detected the monoclonal peak, even if concise statistical data were not presented.

However, further studies involving these methods are needed to verify existing data and compare it with future data. Based on this, it will be possible to improve the diagnosis of MM. This will bring further positive impacts to therapeutic approaches and medical-professional knowledge, and, above all, it will be possible to offer better outcomes and quality of life to patients and their families.

Finally, the data presented may be useful for selecting diagnostic methods when faced with possible MM patients. They also provide a source for future research and analysis about MM, monoclonal gammapathies, or even many other hematologic malignancies.

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