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Impact of Concordance Between Antinuclear Antibody Indirect Immunofluorescence Patterns and Myositis Antibodies in Idiopathic Inflammatory Myopathies: Study Protocol

Impacto da Concordância entre os Padrões de Imunofluorescência Indireta dos Anticorpos Antinucleares e os Anticorpos da Miosite nas Miopatias Inflamatórias Idiopáticas: Protocolo do Estudo

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ABSTRACT

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of systemic autoimmune rheumatic disorders in which chronic inflammation of skeletal muscle leads to muscle weakness. Many other organs, including the skin, heart, lungs, and joints, may be affected. Patients with IIM may be positive for myositis antibodies (MAs), including myositis-specific and/or associated antibodies. Although helpful for establishing the diagnosis of IIM in the appropriate clinical setting, the presence of MAs does not always predict the occurrence of connective tissue diseases. Additionally, commonly used techniques such as line blot are known to have high rates of false positivity, especially for rare MAs. The accuracy of MAs tests such as line blot may be improved by cross-checking their results with antinuclear antibody (ANA) patterns on HEp-2 indirect immunofluorescence (IIF). This study aims to examine the concordance between ANA IIF patterns and myositis-specific antibodies in a Portuguese cohort of patients with IIM. We will assess whether concordance between methods is associated with increased fulfillment of IIM classification criteria, greater disease severity, or distinct patterns of organ involvement among affected patients.

Keywords: Antibodies, Antinuclear; Fluorescent Antibody Technique, Indirect; Immunoblotting; Myositis

RESUMO

As miopatias inflamatórias idiopáticas (IIM) constituem um grupo heterogéneo de doenças reumáticas autoimunes sistémicas, nas quais a inflamação crónica do músculo esquelético conduz a fraqueza muscular. Vários outros órgãos, incluindo a pele, o coração, os pulmões e as articulações, podem ser igualmente afetados. Os doentes com IIM podem apresentar anticorpos de miosite (MAs), incluindo anticorpos específicos e/ou associados a miosite. Embora úteis para o diagnóstico de IIM num contexto clínico apropriado, a presença de MAs não prediz necessariamente a ocorrência de doenças do tecido conjuntivo. Adicionalmente, técnicas laboratoriais frequentemente utilizadas, tais como o *line blot*, podem apresentar elevadas taxas de falsos positivos, sobretudo no caso de MAs raros. A fiabilidade destes testes poderá ser melhorada através da comparação dos seus resultados com os padrões de anticorpos antinucleares (ANA) obtidos por imunofluorescência indireta (IIF) em células HEp-2. Assim, com este trabalho, pretendemos avaliar a concordância entre o padrão de ANA por IIF e os MAs numa coorte portuguesa de doentes com IIM, analisando se a concordância entre técnicas laboratoriais se correlaciona com uma maior taxa de cumprimento dos critérios de classificação para IIM, com maior gravidade da doença ou com envolvimentos de órgão distintos nos doentes afetados.

Palavras-chave: Anticorpos Antinucleares; Immunoblotting; Miosite; Técnica Indireta de Fluorescência para Anticorpo

INTRODUCTION

Background and rationale

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of systemic autoimmune disorders in which chronic inflammation of skeletal muscle leads to muscle weakness.[1] Apart from striated muscle, many other organs may be affected, such as the skin, heart, lungs, and joints.

Patients with IIM may be positive for myositis antibodies (MAs), conventionally divided into myositis-specific (MSAs) or myositis-associated (MAAs) antibodies.[2] Myositis-specific antibodies, including anti-Mi-2, anti-MDA5, anti-TIF1γ, anti-NXP2, anti-SAE, anti-synthetase antibodies, anti-SRP, anti-HMGCR, and anti-cN1A antibodies, help establish a

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diagnosis of IIM. They can also determine different risks of expressing different clinical phenotypes and have prognostic value.[3] Furthermore, they are often mutually exclusive, reinforcing their important role as disease biomarkers.[4]

Potentially found in IIM, MAAs can also be detected in other connective tissue diseases. These include anti-PM/SCL, anti-Ku, anti-Ro52, and anti-U1RNP antibodies.

Different laboratory techniques are available for the detection of MAs, each with its own unique characteristics, advantages, and limitations. Immunoprecipitation (IP) is often regarded as a gold standard in MAs detection, due to its high sensitivity and specificity.[5] Nevertheless, its methodological complexity and time-consuming nature limit its applicability in routine clinical practice.[6] In contrast, alternative approaches, such as line immunoassays, are more frequently employed due to their lower cost and widespread availability in most laboratories through commercial kits. Having low sensitivity for the detection of MSAs, indirect immunofluorescence (IIF) is commonly employed for the screening of antinuclear antibodies (ANA).[2,5] The International Consensus on ANA Patterns (ICAP) has provided a standardized framework for reporting the different patterns observed.[7] Notably, each autoantibody is associated with certain immunofluorescence patterns, which can facilitate an accurate interpretation.

The presence of circulating MSAs, especially in a low titer, can occur even without IIM or other connective tissue diseases.[1,8] Additionally, commonly used techniques such as line immunoassays are known to have high rates of false positivity and low specificity, especially for rare MAs.[9,10] It has been postulated that agreement with ANA patterns on HEp-2 IIF may improve the test accuracy of MAs.[11,12] Tables 1 - 3 summarize the most frequently associated ANA IIF patterns for each MA.

The primary objective of this project will be to determine whether agreement between MAs and ANA IIF is associated with a higher rate of 2017 European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria fulfillment for adult IIM and their major subgroups.[13] Secondly, we intend to analyze whether higher levels of agreement between MAs and ANA IIF are associated with a higher disease burden or specific organ involvement in affected patients.

METHODS

Study design

This is a retrospective analysis of prospectively collected multicenter data, based on the myositis protocol of the Rheumatic Diseases Portuguese Register (the Reuma.pt/Myositis) database.[14] The project will be open to all national Rheumatology centers willing to participate.

Eligibility criteria

Inclusion criteria

- Age of symptom onset of 18 years or older;
- Diagnosis of IIM according to the treating rheumatologist;
- Information regarding MAs and ANA IIF patterns;
- Patients registered in Reuma.pt/Myositis, who have provided written informed consent and have at least one clinical characterization.

Exclusion criteria:

- Other concomitant inflammatory connective tissue disease;
- No available information regarding ANA IIF pattern and/or MAs.

Table 1 – Myositis-specific antibodies and compatible antinuclear antibodies indirect immunofluorescence patterns. The suggested patterns were compiled from both the International Consensus on ANA Patterns and existing scientific literature.

Myositis-specific antibodies	Compatible ANA IIF Pattern (full description)	Compatible ANA IIF Pattern (short description)	ANA IIF ICAP Code
Anti-SRP	Cytoplasmic dense fine speckled[16]	Cytoplasmic	AC-19
Anti-HMGCR	Negative[16]	Negative[a]	AC-0
Anti-Mi2	Nuclear fine speckled[7]	Nuclear speckled	AC-4
Anti-MDA5	Negative[16] Cytoplasmic speckled[17] Cytoplasmic dense fine speckled[18]	Cytoplasmic	AC-0 AC-19 AC-20
Anti-TIF1γ	Nuclear fine speckled[7]	Nuclear speckled	AC-4
Anti-NPX2	Nuclear fine speckled[16] and/or multiple nuclear dots[7]	Nuclear speckled or multiple nuclear dots)	AC-4 AC-6
Anti-SAE	Nuclear fine speckled[11] Nuclear coarse speckled[19]	Nuclear speckled	AC-4 AC-5
Anti-CN1A	Undefined[16]	-	-

ANA: antinuclear antibodies; IIF: indirect immunofluorescence; ICAP: International Consensus on ANA Patterns.
a: A novel indirect immunofluorescence pattern, known as the HMGCR Associated Liver Immunofluorescence Pattern, has been recognized in experimental research, but is not commonly searched for in clinical practice.[20]

Data collection and variables of interest

Process of data collection

A. Each center's designated local investigators will fill in the missing information in Reuma.pt/Myositis protocol and include every patient followed in the center fulfilling inclusion criteria, inserting in Reuma.pt the variables required for this study. Potential new IIM patients fulfilling our inclusion criteria should also be registered in Reuma.pt.

B. Export and exploratory analysis of the data included in the Reuma.pt database.

Variables of interest

General data:

- Date of birth;
- Sex;
- Type of myositis (categorical variable: dermatomyositis, amyopathic/hypomyopathic dermatomyositis, non-specific myositis, polymyositis, immune necrotizing myositis, inclusion body myositis);
- Fulfillment of 2017 EULAR/ACR classification criteria for IIM;
- Age at first symptom and at diagnosis;
- Quantified tobacco use/alcohol use.

Presence of certain disease manifestations:

- Myalgia/ myositis;
- Gottron's papules;
- Heliotrope rash;
- Raynaud's phenomenon;
- Digital ulcers;
- Edema;
- Calcinosis;
- Periungual changes;
- Lipoatrophy;
- Arthralgia/ arthritis;
- Esophageal, gastric, intestinal, cardiac, or pulmonary involvement.

Positivity for myositis-specific and myositis-associated autoantibodies:

- ANA;
- Anti-Mi2;
- Anti-TIF1γ;
- Anti-MDA5;
- Anti-NPX2;
- Anti-SAE1;
- Anti-Ku;
- Anti-Pm/Scl;
- Anti-PL7;
- Anti-PL12;
- Anti-EJ;
- Anti-OJ;
- Anti-RNP;

Table 2 – Myositis-specific anti-synthetase antibodies and compatible antinuclear antibodies indirect immunofluorescence patterns. The suggested patterns were compiled both from the International Consensus on ANA Patterns and existing scientific literature.

Myositis-specific anti-synthetase antibodies	Compatible ANA IIF Pattern (full description)	Compatible ANA IIF Pattern (short description)	ANA IIF ICAP Code
Anti-PL-7	Cytoplasmic dense fine speckled[7]	Cytoplasmic	AC-19
Anti-PL-12	Cytoplasmic dense fine speckled[7]	Cytoplasmic	AC-19
Anti-EJ	Cytoplasmic speckled[16]	Cytoplasmic	AC-19 AC-20
Anti-OJ	Cytoplasmic speckled[16]	Cytoplasmic	AC-19 AC-20
Anti-Jo-1	Cytoplasmic fine speckled[7]	Cytoplasmic	AC-20

ANA: antinuclear antibodies; IIF: indirect immunofluorescence; ICAP: International Consensus on ANA Patterns.

Table 3 – Myositis-associated antibodies and compatible antinuclear antibodies indirect immunofluorescence patterns. The suggested patterns were compiled both from the International Consensus on ANA Patterns and existing scientific literature.

Myositis-associated antibodies	Compatible ANA IIF Pattern (full description)	Compatible ANA IIF Pattern (short description)	ANA IIF ICAP Code
Anti-U1RNP	Nuclear coarse speckled[7]	Nuclear speckled	AC-5
Anti-Ku	Nuclear fine speckled[7]	Nuclear speckled	AC-4
Anti-Ro52	Negative[16] Nuclear fine speckled[7] Cytoplasmic speckled[16]	Nuclear speckled or cytoplasmic	AC-0 AC-4 AC-19 AC-20
Anti-PM/Scl	Nucleolar homogeneous[7]	Nucleolar	AC-8
Anti-mitochondrial	Cytoplasmic reticular/AMA[7]	Cytoplasmic	AC-21

AMA: antimitochondrial antibodies; ANA: antinuclear antibodies; IIF: indirect immunofluorescence; ICAP: International Consensus on ANA Patterns.

- Anti-SSA/SSB;
- Anti-Jo1;
- Anti-SRP;
- Another positive autoantibody. We will also include in this section information about ANA titer, IIF pattern, and date of initial sampling.

Diagnostic tests:
- Muscle biopsy or magnetic resonance imaging with evidence of myositis;
- Elevated muscle enzymes (creatinine kinase, lactate dehydrogenase, aldolase, aspartate transaminase, and/or alanine aminotransferase);
- Myopathic alterations in electromyography.

Disease activity scores (at baseline; worst value):
- Number of painful and swollen joints;
- Patient Global Assessment;
- Health Assessment Questionnaire Disability Index;
- Manual Muscle Testing – 8;
- Modified Skin Disease Activity Score.

Sample size

Based on prior research with Reuma.pt/Myositis, we estimate a sample size of approximately 300 patients.[15]

Missing data

In order to mitigate the effects of missing data, an initial export from Reuma.pt will be screened to identify incomplete variables, which will then be verified and completed by local investigators within a six-week period. A subsequent export will be performed to ensure that all essential variables are adequately recorded.

Statistical analysis plan

Statistical significance will be set at $p < 0.05$. Patients will be generally divided into two main groups that will be compared: with (Group A) and without (Group B) agreement between MAs and ANA IIF patterns. Additionally, a third group (undetermined agreement) will also be created (Group C). We will also group the patients in different datasets to perform sensitivity analyses, ensuring robustness.

A descriptive analysis of Groups A, B, and C will be performed. Secondly, a univariate analysis will be conducted, comparing groups A and B. Differences between groups regarding continuous variables will be analyzed using the independent samples *t*-test or the Mann-Whitney *U* test, as appropriate. Differences in categorical variables will be analyzed using the chi-square test.

Expected limitations

Given the retrospective nature of this study, certain limitations should be acknowledged. First, missing information in relevant variables and the potential for recall bias are possible. However, we believe that our planned strategy for identifying and addressing missing data will help to mitigate this risk.

Another potential limitation is related to the use of ANA pattern descriptions provided by different laboratories. Not all national laboratories systematically apply the ICAP classification to report ANA patterns, which may lead to variability in the data. To minimize this limitation, we will request that local investigators provide all available details regarding the ANA patterns reported, in an attempt to standardize and harmonize the information as much as possible. Additionally, we will perform a sensitivity analysis to test the consistency of our results. One of the items that will be assessed through sensitivity analysis is the completeness of the ANA IIF reporting.

The inclusion criteria requiring information regarding MAs and ANA IIF patterns inherently select for patients where clinicians had sufficient suspicion to order both tests. This creates a selection bias toward patients with higher clinical suspicion and potentially excludes seronegative patients or those with atypical presentations where complete antibody panels were not ordered. However, this study could not be performed without using this data.

This study is also not generalizable to all patients, since it will be geographically limited to Portugal. However, it should be representative of the Portuguese population, which may be an advantage for the regional impact of the study.

Finally, we will not be able to compare the IIF and line immunoassays data with the gold standard (IP) due to a lack of access to this method in Portugal.

Ethical considerations and data management plan

This study was approved by the Centro Académico Clínico Egas Moniz Health Alliance Ethics Committee (08-CE-ICVS/CAC-EMHA/12.03.2025) and follows the Declaration of Helsinki. It will also be evaluated by Reuma.pt Scientific Committee.

Only patients with previously written informed consent for introduction in Reuma.pt/Myositis for clinical research will be included. Participants' data will be provided in an anonymized form to the principal investigator, and stored in a secure, password-protected database. Access to the final dataset will be limited to the principal investigator. All used data will be deleted after five years of the end of the study, as instructed by the Portuguese National Commission for the Protection of Data.

DISCUSSION

Little is known about the impact of agreement between ANA IIF patterns and MAs antibodies in different grades of pretest probability of IIM. A study conducted in 2022 with an Australian cohort of 118 patients tested for MAs due to distinct clinical indications found that patients with concordant MA-ANA patterns had a higher positive predictive value for IIM compared with those with discordant MA-ANA (38.7% *vs* 6.7%, $p < 0.001$).[12] However, this study did not differentiate between MAs and ANA patterns in terms of different levels of concordance (namely, partial or total levels of agreement). Also, tested patients were not divided according to different pretest probabilities of IIM; in fact, the indication for ANA testing varied, with only 12.7% being initially tested for suspected IIM.

This study aims to deepen our understanding of the impact of concordance between these two laboratory techniques, using a population of high pretest probability for IIM – namely, patients with a clinical diagnosis of IIM, according to the treating rheumatologist. Our expected results will also demonstrate if ANA IIF-MAs agreement is associated with a higher disease burden or certain clinical manifestations in affected patients, with potential utility in daily clinical practice.

PREVIOUS AWARDS AND PRESENTATIONS

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The authors have declared that no AI tools were used during the preparation of this work.

AUTHOR CONTRIBUTIONS

CPO: Study conception and design.

ED: Study conception and design, critical review of the manuscript.

MPS, CV, GC, MS, MJG, PS, VF, TM, CA, ARP, AB, RCM: Critical review of the manuscript.

All authors approved the final version to be published.

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