Original Article

JHSMR Journal of Health Science and Medical Research

Diagnostic Impact of ANA Interference on p-ANCA Interpretation in Autoimmune Testing

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Received 4 March 2025 ● Revised 21 August 2025 ● Accepted 2 September 2025 ● Published online 16 March 2026

Abstract:

Objective: Antineutrophil cytoplasmic antibodies (ANCA) are crucial for diagnosing ANCA-associated vasculitis (AAV). The indirect immunofluorescence assay (IIFA) is commonly used but may produce perinuclear ANCA (p-ANCA) positivity in non-AAV, especially in antinuclear antibody (ANA)-positive cases. Specific ANA patterns and high titers may increase the frequency of non-AAV–p-ANCA positivity. This study aims to assess the correlation and impact of ANA patterns and titers on p-ANCA interpretation by IIFA.

Material and Methods: This retrospective study analyzed ANA-positive serum samples collected between 2019 and 2024. IIFA tested ANA and ANCA, while antibodies to myeloperoxidase (MPO) and proteinase 3 (PR3) were assessed by enzyme-linked immunosorbent assay (ELISA). Only ELISA-negative cases were included. IIFA classified samples into non-MPO–p-ANCA-positive and p-ANCA-negative groups. ANA patterns and titers were interpreted using International Consensus on ANA Patterns (ICAP) guidelines. Chi-square and logistic regression identified factors associated with non-MPO–p-ANCA positivity.

Results: Non-MPO–p-ANCA positivity was identified in 23.9% of cases (n=32). The homogeneous ANA pattern showed a significant association (adjusted OR: 4.11, 95% confidence interval [CI]: 1.18–14.37, p-value=0.027). High ANA titer (≥1:1280) was also independently related to this outcome (adjusted OR: 4.47, 95% CI: 1.50–13.33, p-value=0.007), whereas intermediate titers were associated with a lower likelihood of positivity.

Conclusion: Homogeneous pattern and high titer were associated with non-MPO–p-ANCA positivity. Standardized ANCA testing, including ANA assessment and confirmatory MPO/PR3-ANCA ELISA, improves diagnostic accuracy and reduces misinterpretation in autoimmune serology.

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J Health Sci Med Res 2026;44(5):e20261330
doi: 10.31584/jhsmr.20261330
www.jhsmr.org

Keywords: autoimmune serology, diagnostic accuracy, myeloperoxidase antibodies (MPO-ANCA)

Introduction

Antineutrophil cytoplasmic antibodies (ANCAs) are essential serological markers for diagnosing and understanding the pathogenesis of ANCA-associated vasculitis (AAV)[1], a rare systemic necrotizing vasculitis that primarily affects small blood vessels and can lead to severe complications[2], including acute kidney failure and life-threatening diffuse alveolar hemorrhage[3]. AAV is classified into three major subtypes: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA)[2,4]. The two primary methods for ANCA detection are the indirect immunofluorescence assay (IIFA) and the enzyme-linked immunosorbent assay (ELISA)[5]. IIFA is widely used for detecting ANCA due to its high sensitivity and ability to classify ANCA into distinct fluorescence patterns[6], including cytoplasmic ANCA (c-ANCA), which primarily targets proteinase 3 (PR3); perinuclear ANCA (p-ANCA), mainly directed against myeloperoxidase (MPO); and atypical ANCA, associated with alternative autoantigens[5,7,8]. PR3-ANCA is strongly associated with GPA, while MPO-ANCA predominates in MPA. EGPA may show MPO-ANCA positivity in some cases, with reported ANCA positivity rates of approximately 30–38%, and the remaining patients are ANCA-negative[2]. While ELISA provides greater specificity, objective quantification, and the ability to differentiate PR3-ANCA from MPO-ANCA, it is recommended as both the primary screening and confirmatory test for AAV. However, in cases where clinical suspicion for AAV remains high despite negative ELISA results, IIFA may be considered as an adjunctive test, in accordance with the 2017 and 2020 International Consensus[2,7,9,10]. In addition, IIFA offers essential morphological insights that assist in distinguishing AAV from other autoimmune diseases[8,11]. It is particularly valuable in detecting low-titer or early-stage ANCA, where ELISA may yield false-negative results[8], reinforcing its role as an important screening tool in various clinical settings. Despite advancements in immunoassays, IIFA remains a critical screening tool, particularly in settings with limited access to ELISAs. Its ability to visually confirm ANCA patterns makes it an important complement to ELISAs, enhancing the overall diagnostic approach[7].

One limitation of IIFA is its susceptibility to interference from antinuclear antibodies (ANA)[8], which can produce fluorescence patterns resembling p-ANCA, leading to misinterpretation[8,12]. This challenge is particularly evident in samples with high ANA titers, where distinguishing between true MPO-ANCA positivity and ANA-induced fluorescence becomes challenging[8,13]. Homogeneous patterns are the most common cause of artefactual perinuclear staining on IIFA, which may be misinterpreted as p-ANCA positivity[14], followed by speckled and dense fine-speckled patterns; in contrast, the centromere pattern has minimal impact[15]. However, variations in ANCA testing methodologies, including laboratory protocols and interpretation criteria, may lead to inconsistencies in study outcomes, limiting the generalizability of the findings.

Given the clinical significance of ANA interference and the limitations of previous studies, this study evaluated the impact of ANA patterns and titers on non-MPO–p-ANCA positivity in IIFA detection among patients from Southern Thailand, following the established guidelines and manufacturer protocols. It also elucidates the limitations of IIFA in ANCA testing and evaluates the need for alternative diagnostic workflows to enhance diagnostic accuracy in autoimmune serology, especially for AAV.

Material and Methods

Study design and data collection

This retrospective observational study was conducted

at Songklanagarind Hospital, a tertiary care teaching hospital affiliated with the Faculty of Medicine, Prince of Songkla University, Thailand. Data were obtained from individuals who underwent ANA and ANCA testing using IIFA. MPO/PR3 testing was performed by ELISA. All tests were conducted between 2019 and 2024. The study included samples that were ANA-positive and had available ANCA results (either positive or negative), while the positive MPO-ANCA or PR3-ANCA were excluded. All included cases were classified as non-AAV–associated ANCA positivity according to the 2020 Revised International Consensus on ANCA Testing. For comparative purposes, samples were divided into two groups: (1) the non-MPO–p-ANCA-positive group, defined by ANA positivity, p-ANCA positivity, and MPO/PR3-ANCA negativity, representing cases with a tendency for non-AAV–associated ANCA positivity; and (2) the p-ANCA-negative group, comprising ANA-positive samples with non-p-ANCA patterns (ANCA-negative or atypical ANCA) and MPO/PR3-ANCA negativity.

ANA and ANCA testing procedures

ANA testing by IIFA was performed according to the manufacturer's instructions for the IIFT Mosaic: HEp-20-10/Liver (Monkey) test system (Euroimmun, Lübeck, Germany). The initial dilution titer was set at 1:80, followed by a two-fold serial dilution up to a final dilution of 1:1280. ANA pattern interpretation was based on the International Consensus on ANA Patterns classification[16,17].

ANCA testing by IIFA was performed according to the manufacturer's instructions for the IIFT: Granulocyte Mosaic 13 test system (Euroimmun, Lübeck, Germany). Serum samples were diluted at a titer of 1:10, and results were interpreted based on technical principles involving three cellular components: Hep-2 cells, ethanol-fixed human granulocytes, and formalin-fixed human granulocytes. ANCA-IIFA positivity was classified into three patterns: p-ANCA, c-ANCA, and atypical ANCA. MPO-ANCA and PR3-ANCA were tested using an ELISA (IgG) kit (Euroimmun, Lübeck, Germany) on an automated IF Sprinter analyzer. A result of less than 20 RU/mL was considered negative.

Statistical analysis

The chi-square test was used to assess variable associations, with statistical significance set at p-value<0.05, while logistic regression was applied for univariable and multivariable analyses to evaluate the factors influencing non-MPO–p-ANCA positivity. Results were expressed as odds ratios (ORs) with 95% confidence intervals (CIs), and statistical significance for all tests was set at p-value<0.05. All statistical analyses were conducted using Microsoft Excel and R Studio.

Ethical considerations

Patient data were anonymized using coded identifiers to ensure confidentiality. The study protocol was reviewed and approved by the Ethics Committee of Prince of Songkla University (REC.67-371-5-7).

Results

Demographics of the enrolled samples

A total of 155 ANA-positive serum samples with suspected autoimmune diseases were initially included. Of these, 21 cases with MPO-ANCA or PR3-ANCA positivity by ELISA were excluded to avoid potential overlap with AAV, as MPO/PR3 positivity can also occur in systemic lupus erythematosus (SLE), which was outside the scope of this study. Among ANA-positive cases, the speckled pattern was the most prevalent (42.5%), followed by the homogeneous pattern (23.9%) and the nucleolar pattern (11.9%). Regarding ANA titers, the low titer (1:80) was the most frequent (50.0%), while the high titer (≥1:1280) was observed in 17.9% of cases, as shown in Table 1. The remaining 134 cases, all negative for MPO and PR3 ELISA, were classified based on ANCA-IIFA results into three subgroups: non-MPO–p-ANCA positivity (n=32),

Table 1 Demographics of the enrolled samples (n=134)

Variable	Number (%)
ANA patterns	
Homogeneous	32 (23.9)
Speckled	57 (42.5)
Other patterns	
Nucleolar	16 (11.9)
Centromere	2 (1.5)
Multiple nuclear dots	3 (2.2)
Nuclear envelope	1 (0.7)
CENP-F-like	1 (0.7)
Centrosome	2 (1.5)
Spindle fibers	2 (1.5)
Intercellular bridge	4 (3.0)
Cytoplasmic	14 (10.4)
ANA Titer	
1:80	67 (50.0)
1:160	23 (17.2)
1:320	9 (6.7)
1:640	11 (8.2)
≥1:1280	24 (17.9)
ANCA (IIFA) results with MPO/PR3 ANCA negative	
Negative ANCA	88 (65.7)
Positive ANCA	46 (34.3)
p-ANCA	32 (23.9)
atypical ANCA	14 (10.4)

ANA=antinuclear antibody, ANCA=Antineutrophil cytoplasmic antibodies, IIFA=indirect immunofluorescence assay, p-ANCA=perinuclear ANCA, CENP-F=centromere protein F

atypical ANCA (n=14), and ANCA-negative (n=88). The classification of study participants and group allocation is summarized in Figure 1.

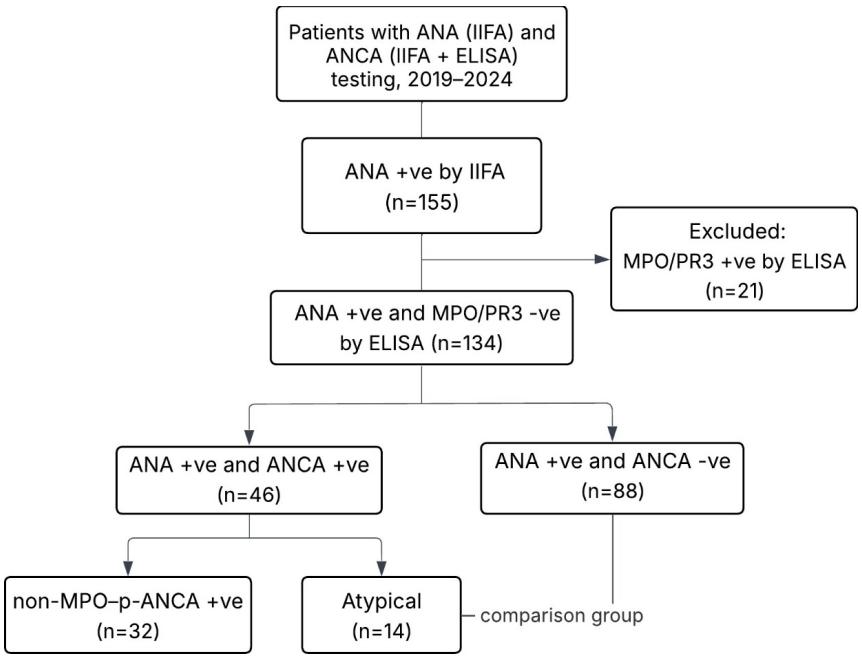
Correlation between ANA IIFA patterns/titers and p-ANCA interpretation

Table 2 highlights key observations regarding the association between ANA IIFA patterns, titers, and non-MPO–p-ANCA positivity. The homogeneous pattern was significantly associated with non-MPO–p-ANCA positivity (43.8% vs. 17.6%, p-value=0.004), whereas the speckled pattern exhibited no significant difference between groups. Other patterns, including nucleolar and centromere patterns, were more frequent in the p-ANCA-negative group (15.6% vs. 39.2%). High ANA titer (≥1:1280) was significantly associated with non-MPO–p-ANCA positivity (40.6% vs. 10.8%, p-value<0.001). The lowest ANA titer (1:80) and intermediate titers (1:160, 1:320, and 1:640) were more frequently observed in the p-ANCA–negative group, at 54.9% and 34.3% respectively, compared with 34.4% and 24.9% in the non-MPO–p-ANCA-positive group.

Influence of ANA IIFA patterns/titers on p-ANCA interpretation

Univariable and multivariable logistic regression analyses were conducted to assess the association between ANA IIFA patterns, ANA titers, and non-MPO–p-ANCA positivity (Table 3). The homogeneous ANA pattern was significantly associated with a higher likelihood of non-MPO–p-ANCA positivity, with a crude OR of 6.22 (95% CI: 1.95-19.90, p-value=0.002). After adjusting for potential confounders, it remained an independent factor (adjusted OR: 4.11, 95% CI: 1.18-14.37, p-value=0.027). In contrast, the speckled ANA pattern showed no statistically significant association with non-MPO–p-ANCA positivity, with an adjusted OR of 2.13 (p-value=0.207).

Regarding ANA titers, a high titer (≥1:1280) was strongly associated with non-MPO–p-ANCA positivity, with a crude OR of 6.02 (95% CI: 2.15–16.87, p-value<0.001) and an adjusted OR of 4.47 (95% CI: 1.50-13.33, p-value=0.007). In comparison, intermediate ANA titers (1:160, 1:320, 1:640) had lower odds ratios relative to the reference group (1:80), suggesting that non-MPO–p-ANCA positivity was more frequently observed at the high ANA titer (≥1:1280). These results indicate a notable association between ANA patterns, particularly homogeneous patterns, and high ANA titer with non-MPO–p-ANCA positivity.



ANA=antinuclear antibody, ANCA=antineutrophil cytoplasmic antibodies, IIFA=indirect immunofluorescence assay, p-ANCA=perinuclear ANCA, ICAP=International consensus on ANA patterns, MPO=myeloperoxidase, PR3=proteinase 3, ELISA=immunosorbent assay, AAV=ANCA-associated vasculitis

Figure 1 Patient selection and ANCA classification among ANA-positive, MPO/PR3-negative cases

Table 2 Correlation between ANA IIFA patterns/titers and non-MPO-p-ANCA positivity

Variable	Number (%)		p-value
	non-MPO-p-ANCA positive (n=32)	p-ANCA negative (n=102)	
ANA Patterns			0.004
Homogeneous	14 (43.8)	18 (17.6)	
Speckled	13 (40.6)	44 (43.1)	
Other patterns	5 (15.6)	40 (39.2)	
ANA titers			<0.001
1:80	11 (34.4)	56 (54.9)	
1:160	2 (6.2)	21 (20.6)	
1:320	4 (12.5)	5 (4.9)	
1:640	2 (6.2)	9 (8.8)	
≥1:1280	13 (40.6)	11 (10.8)	

ANA=antinuclear antibody, MPO=myeloperoxidase, p-ANCA=perinuclear ANCA

Table 3 Univariable and multivariable analyses of ANA IIFA patterns/titers associated with non-MPO–p-ANCA positivity

Variable	Univariate analysis		Multivariate analysis	
	Crude OR, 95% CI	p-value	adjusted OR, 95% CI	p-value
ANA patterns				
Homogeneous pattern	6.22 (1.95,19.90)	0.002	4.11 (1.18,14.37)	0.027
Speckled pattern	2.36 (0.77,7.22)	0.131	2.13 (0.66,6.93)	0.207
Other patterns	reference		reference	
ANA titer (ref: titer1:80)				
Titer 1:80	reference		reference	
Titer 1:160	0.48 (0.10,2.37)	0.371	0.51 (0.10,2.61)	0.415
Titer 1:320	4.07 (0.94,17.63)	0.060	3.75 (0.81,17.29)	0.091
Titer 1:640	1.13 (0.21,5.97)	0.884	0.92 (0.17,5.02)	0.923
Titer ≥1:1280	6.02 (2.15,16.87)	<0.001	4.47(1.50,13.33)	0.007

95% CI=95% confidence interval, ANA=antinuclear antibody

Discussion

This study highlights the significant influence of ANA patterns and titers on p-ANCA interpretation by IIFA, underscoring the diagnostic challenges and need for refined interpretation strategies. Among the 134 serum samples analyzed, 23.9% (n=32) were non-MPO–p-ANCA positive, which may reflect non-AAV–associated ANCA positivity attributable to ANA interference[14,18].

The strongest association was observed with the homogeneous pattern, where 43.8% of homogeneous ANA-positive samples tested positive for p-ANCA, compared to only 17.6% in the p-ANCA-negative group (p-value=0.004). Multivariate analysis confirmed the homogeneous pattern as an independent predictor of non-MPO–p-ANCA positivity (adjusted OR: 4.11, 95% CI: 1.18-14.37, p-value=0.027), reinforcing previous findings that the homogeneous pattern fluorescence can mimic perinuclear staining, leading to misinterpretation[18-20]. This observation may be attributed to the inherent constraints in interpreting the p-ANCA fluorescence pattern, which depends on evaluating fluorescence signals around the cell nucleus (ethanol-fixed human granulocytes). Misinterpretation can occur when this nuclear region exhibits fluorescence signals from anti-histone or anti-dsDNA, resulting in a homogeneous pattern in ANA (IIFA) testing[8,13,15]. Beyond homogeneous ANA, this study explored the impact of other ANA patterns on p-ANCA misinterpretation.

Speckled ANA patterns were observed in 40.6% of the non-MPO–p-ANCA positivity, but statistical analysis did not show a significant correlation (adjusted OR: 2.13, p-value=0.207). Other ANA patterns (such as nucleolar and centromere) were more frequently observed in the p-ANCA–negative group (39.2 %), indicating a lower likelihood of these patterns contributing to non-AAV–associated ANCA positivity, whereas prior research has reported that the dense fine-speckled pattern may also induce non-AAV p-ANCA positivity[15]. This discrepancy may arise from variations in ANA pattern classification, differences in ANA-IIFA methodologies, or diverse fluorescence intensities across different ANA patterns[14].

The role of ANA titers was also evident, with high ANA titer (≥1:1280) being significantly associated with non-MPO–p-ANCA positivity (40.6% vs. 10.8%, p-value<0.001). Multivariate analysis confirmed high ANA titer as an independent predictor (adjusted OR: 4.47, 95% CI: 1.50-13.33, p-value=0.007), reinforcing prior research linking ANA fluorescence intensity to increased misinterpretation[13,15].

In contrast, the lowest ANA titer (1:80) and intermediate ANA titers (1:160, 1:320, 1:640) exhibited a lower likelihood of non-MPO–p-ANCA positivity, reinforcing the concept that fluorescence intensity contributes directly to the risk of misinterpretation[15].

These findings highlight the importance of recognizing ANA pattern variability when interpreting p-ANCA results, as different patterns contribute to varying levels of diagnostic interference. The absence of MPO or PR3 reactivity in p-ANCA–positive samples should not necessarily be interpreted as analytical artefacts. ANA positivity, particularly with homogeneous patterns, can mimic p-ANCA through anti-dsDNA antibodies, resulting in atypical ANCA[8]. Moreover, antibodies against alternative neutrophil antigens, such as lactoferrin, elastase, and cathepsin G, may also account for non-MPO p-ANCA or c-ANCA positivity, which is observed in autoimmune conditions, including SLE, ulcerative colitis, and drug-induced autoimmunity[10,13,15]. Taken together, ANA positivity may give rise to three types of ANCA reactivities: (1) atypical ANCA derived from nuclear antigens[8], (2) true anti-MPO–p-ANCA, which may occur in overlap cases between ANA-positive diseases, particularly SLE, and AAV, and should therefore be confirmed by MPO-specific assays[10], and (3) non-MPO antibodies, including lactoferrin, elastase, or cathepsin G, that can mimic a p-ANCA pattern despite MPO negativity[8]. These possibilities highlight the importance of careful interpretation and confirmatory testing.

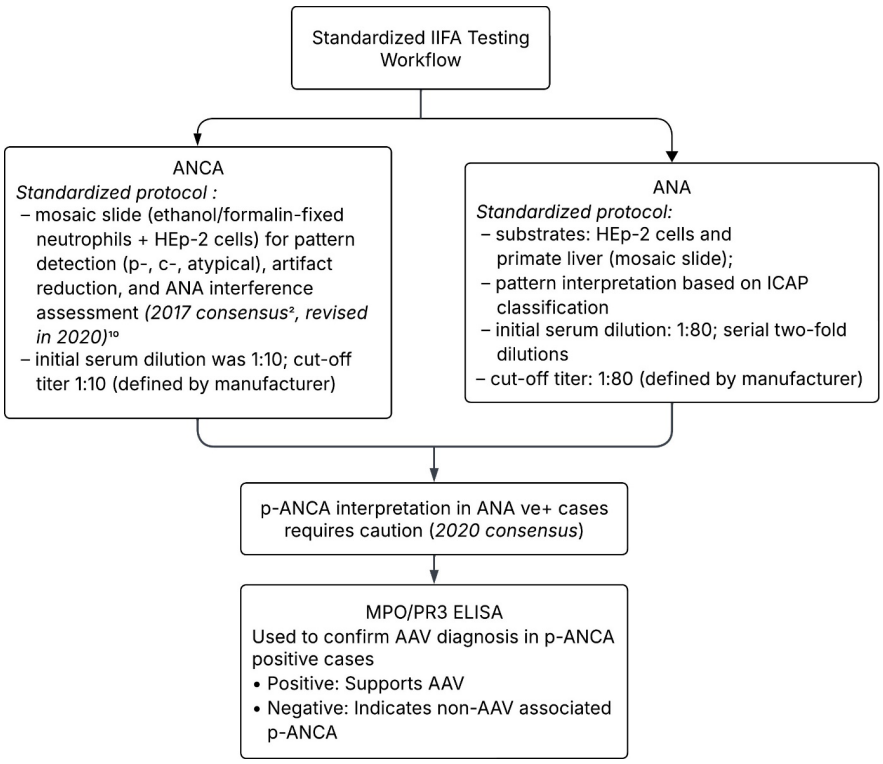
Confirmatory ELISA testing for MPO and PR3 remains essential, particularly when p-ANCA is positive but no antigen-specific reactivity is detected, as recommended by the 2020 Revised International Consensus on ANCA Testing[10,13,21]. Incorporating standardized ANA classification into ANCA testing protocols can improve diagnostic accuracy, especially when distinguishing AAV from other ANA-related autoimmune diseases[13].

To improve specificity in ANCA testing, several alternative diagnostic strategies should be considered. Routine ANA screening alongside ANCA testing may help identify patients at risk of interpretive interference in p-ANCA pattern recognition, especially those with a homogeneous ANA pattern or high titer (≥1:1280). Additionally, implementing dual-substrate IIFA testing using ethanol- and formalin-fixed neutrophils could minimize misinterpretation, as formalin fixation prevents MPO redistribution and eliminates false perinuclear staining[8]. Automated IIFA image analysis may enhance test accuracy by reducing observer variability in fluorescence pattern interpretation[15], The study underscores the diagnostic impact of ANA interference on p-ANCA interpretation in autoimmune testing, especially in settings where confirmatory ELISA is not routinely accessible. Integrating ANA pattern and titer evaluation with MPO/PR3-specific ELISA and advanced detection methodologies could significantly improve diagnostic precision in assessing ANCA-associated vasculitis. To support the clinical application, Figure 2 presents a proposed workflow that incorporates ANA characteristics into a standardized ANCA-IIFA testing protocol to improve accuracy and reduce misinterpretation.

Although this study focused on laboratory data, its retrospective design limited access to clinical outcomes. Future prospective studies that integrate both laboratory and clinical data are warranted to better define the diagnostic value of ANA interference in p-ANCA interpretation.

Conclusion

This study highlights the significant impact of homogeneous ANA patterns and high ANA titer (≥1:1280) on non-MPO–p-ANCA positivity, increasing the likelihood of misinterpretation. Confirmatory ELISA for MPO and PR3 is crucial for accurate diagnosis, as all p-ANCA-positive cases in this study were negative for MPO/PR3-ANCA. Standardizing ANA classification and incorporating advanced



ANA=antinuclear antibody, ANCA=antineutrophil cytoplasmic antibodies, IIFA=indirect immunofluorescence assay, p-ANCA=perinuclear ANCA, MPO=myeloperoxidase, PR3=proteinase 3, ELISA=immunosorbent assay, AAV=ANCA-associated vasculitis

Figure 2 Workflow for interpreting p-ANCA results using a standardized IIFA protocol, with integrated assessment of ANA interference based on international consensus

diagnostic strategies can enhance ANCA testing accuracy, minimize misinterpretation, and improve the differentiation between true ANCA-associated vasculitis and ANA-related autoimmune conditions.

Acknowledgement

The authors thank the Department of Pathology, Faculty of Medicine, Prince of Songkla University, and the Immunology and Virology Laboratory staff for their support. We also thank our colleagues for their valuable contributions. Lastly, we acknowledge the institutional resources that facilitated this study.

Funding sources

This research received no specific grants from any funding agencies in the public, commercial, or non-profit sectors.

Conflicts of interest

The authors declare no conflicts of interest regarding the content of this article.

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