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Standardized diagnostic approach for cold agglutinin disease: results from a Delphi-Based Expert Consensus

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Background - Cold agglutinin disease (CAD) is a rare autoimmune hemolytic anemia characterized by clonal immunoglobulin M (IgM) autoantibodies that activate complement at low temperatures, leading to hemolysis. Diagnostic practices remain inconsistent, with considerable interlaboratory variability and no established guidelines.

Objective - To develop a consensus-based diagnostic framework for CAD through a structured Delphi methodology, with the goal of harmonizing laboratory practices across medical centers.

Materials and methods - A total of 30 experts in classic hematology and transfusion medicine were invited to participate in a two-round, web-based modified Delphi survey; 27 (90%) completed both rounds. A scientific board developed 37 diagnostic statements covering five domains: CAD definition, clinical evaluation, immunohematological testing, and pre-analytical and analytical procedures. Statements were rated on a 5-point Likert scale, with consensus defined a priori as a score of 4 or 5 by ≥80% of respondents.

Results - All 37 statements achieved consensus, with 36 (97.3%) doing so in Round 1 and the remaining one in Round 2 after re-evaluation. The panel provided recommendations on when to suspect CAD and specified necessary diagnostic tests, including C3d positivity on direct antiglobulin test (DAT) with monospecific antisera and the detection of cold agglutinins in serum. They heavily emphasized the importance of sample handling at 37°C, alongside issuing recommendations on cold agglutinin titration, transfusion management, and immune-hematologic follow-up.

Discussion - This expert consensus provides a standardized diagnostic approach for CAD, addressing current gaps in clinical and laboratory evaluation. Implementing these recommendations is expected to improve diagnostic accuracy and consistency, facilitating better patient outcomes and national harmonization of practices.

Keywords: cold agglutinin disease, Delphi consensus statement, immunohematological testing, Coombs test, diagnostic standardization.



INTRODUCTION

Autoimmune hemolytic anemia (AIHA) is classified into warm, cold, mixed, and atypical types based on the isotype and the thermal characteristics of autoantibody reactivity. Cold agglutinin disease (CAD) is a rare form of autoimmune hemolytic anemia (AIHA), accounting for approximately 20–30% of all AIHA cases¹. CAD is recognized as a distinct clonal B-cell lymphoproliferative disorder, separate from cold agglutinin syndrome (CAS), the secondary form of cold-mediated AIHA associated with infections, autoimmune conditions, or lymphoproliferative malignancies^{2–5}.

CAD is caused by a monoclonal immunoglobulin M (IgM) autoantibody that binds to red blood cells (RBCs) at temperatures below 37°C, leading to agglutination and hemolysis. IgM antibodies, being pentameric, are highly effective at activating the classical complement pathway. This activation results in two central hemolytic mechanisms: extravascular hemolysis of C3d-opsonized RBCs in the liver, and intravascular hemolysis in cases of extensive complement activation until the terminal C5b-9 complex. The central role of complement in the pathophysiology of CAD has implications for both diagnostic approaches and treatment strategies. An accurate immunohematologic evaluation is crucial for distinguishing CAD from other AIHA subtypes, as each AIHA subtype requires a distinct therapeutic strategy to ensure appropriate patient management.

Diagnosis of CAD relies on demonstrating the presence of hemolytic anemia with evidence of complement-mediated erythrocyte destruction, typically accompanied by a strongly positive direct antiglobulin test (DAT) for C3d, with little or no IgG⁶. Most patients exhibit an anti-erythrocyte monoclonal IgMx antibody with a high titer ($\geq 1:64$ at 4°C) and a broad thermal amplitude. In practice, an agglutinin titer $\geq 1:64$ at 4°C is often considered a hallmark of primary CAD diagnosis⁷. In contrast, lower titers (e.g., $< 1:64$) are more often associated with secondary cold AIHA^{1,8} or are not associated with hemolytic pathology. The clinical presentation of CAD is usually insidious and chronic, frequently featuring symptoms of anemia (fatigue, weakness) alongside classic cold-induced acrocyanosis, Raynaud-like phenomenon, or livedo reticularis in peripheral extremities^{6,9}. A thorough diagnostic work-up must also exclude secondary causes

of cold AIHA (CAS) through serological testing for infection-related antibodies, imaging studies (to detect occult malignancy), and autoimmune screenings.

Despite advances in understanding its biology, the diagnostic approach to CAD remains heterogeneous^{8,10}. Considerable inter-center variability exists in pre-analytical sample handling, execution and interpretation of the direct antiglobulin test (DAT), assessment of cold agglutinin titers and thermal amplitude, and reporting practices^{7,9}. Diagnostic challenges such as sample agglutination, inconsistent temperature control, or misinterpretation of C3d-positive DAT results frequently contribute to delayed or uncertain diagnoses^{8,9}. Moreover, recent evaluations –including the BEST study– have highlighted substantial discrepancies in serologic testing for cold agglutinins across laboratories, with poor standardization and limited reproducibility^{10–12}. Although a few publications have addressed the diagnostic challenges of CAD^{8,10,13,14}, to date, no national or international guidelines provide a unified diagnostic algorithm for CAD.

Given these gaps, a structured expert consensus was proposed to harmonize diagnostic criteria and laboratory workflows. This study reports the results of a two-round modified Delphi process involving a multidisciplinary panel of Italian specialists in classic hematology and transfusion medicine. The aim was to develop consensus-based recommendations covering the entire diagnostic pathway for CAD, from clinical evaluation to pre-analytical procedures, immunohematologic testing, and analytical methodologies, to standardize practice and support timely and accurate diagnosis across Italian centers.

MATERIALS AND METHODS

Study design and objective

This project was designed as a prospective, two-round, web-based modified Delphi consensus to achieve expert agreement on the diagnostic approach to cold agglutinin disease (CAD). The two-round structure was defined a priori to allow assessment of response stability and to provide participants with the opportunity to reconsider their ratings after controlled feedback.

The initiative was coordinated by a scientific board of specialists in immunohematology and transfusion

medicine under the auspices of the Italian Society of Transfusion Medicine and Immunohematology (SIMTI). The scientific board, comprising 10 multidisciplinary experts in hematology, transfusion medicine, and immunohematology, conducted iterative meetings (both in person and virtual) and reviewed the literature. The board then drafted the initial set of diagnostic statements. These statements were derived from, and represent a formal consensus validation of the diagnostic recommendations previously published by SIMTI in the document “Raccomandazioni per la corretta diagnosi della Malattia da Agglutinine Fredde (CAD)”. This prior consensus was based on the Nominal Group Technique (NGT) and served as the background evidence base, providing the conceptual and operational foundations for the Delphi statements. NGT is a structured method for idea generation and consensus-building that promotes equal participation through individual input, round-robin sharing, and ranked voting, thereby minimizing bias and dominance in group decisions.

Having achieved agreement among board members, each recommendation was reformulated into a Delphi-appropriate statement for evaluation by the wider expert panel.

The objective was to develop a standardized, consensus-based framework for the diagnosis of CAD and to harmonize laboratory and clinical practices across Italian centers.

Expert panel and participant selection

A total of 30 experts in autoimmune hemolytic anemias and related immunohematologic disorders were invited to participate and 27 accepted (2 from hematology and 25 from transfusion medicine). The participants were from 27 hospitals across Italy, including university hospitals, reference transfusion centers, and regional laboratories. Experts were selected based on recognized clinical and scientific expertise in CAD or AIHA, authorship of relevant publications, and/or participation in national working groups. Panelists participated independently and anonymously. All interactions with participants were managed through an independent third-party organization to preserve anonymity and prevent any direct identification of individual responses by the scientific board. No financial compensation or industry input influenced the Delphi process.

Delphi methodology

The Delphi method is a structured, iterative process for obtaining consensus from a panel of independent experts through a series of controlled rounds with anonymized feedback. For this study, two sequential online survey rounds were conducted between September and October 2025, as predefined in the study design, each including a set of statements covering five diagnostic domains:

1. definition of CAD and CAS;
2. clinical evaluation of CAD;
3. indications for immunohematologic testing;
4. pre-analytical phase;
5. analytical phase.

Experts rated their level of agreement with each statement using a five-point Likert scale (1 = strongly disagree; 5 = strongly agree)¹⁵. The use of a 5-point Likert scale was agreed upon by the Scientific Board as it is widely used in consensus processes and allows balanced capture of agreement, disagreement, and intermediate positions without excessive complexity. After Round 1, the scientific board reviewed results and reformulated items with residual disagreement. Statements that did not achieve unanimous agreement were included in Round 2 to evaluate response stability and enhance methodological robustness.

After Round 1:

- aggregated responses (mean, median, and interquartile range) were shared in anonymized form;
- each participant also received their individual responses for self-appraisal;
- participants were invited to provide optional qualitative feedback.

No direct interaction between panelists occurred; “group feedback” was operationalized through the comparison of individual responses with anonymized aggregated data, in line with standard Delphi methodology.

The scientific board reviewed both quantitative results and qualitative comments.

Only one statement did not meet the predefined consensus threshold in Round 1 and was revised accordingly. In addition, three statements were reformulated based on qualitative feedback to improve clarity and clinical applicability, despite having already reached consensus.

To assess response stability, all statements that did not achieve 100% agreement in Round 1 (No.=24)

were re-submitted in Round 2, even if they had exceeded the predefined consensus threshold.

The revised statements and those included for stability assessment were re-rated in Round 2.

Definition of consensus

Consensus was defined a priori as a score of 4 or 5 by $\geq 80\%$ of respondents for each statement. This threshold was considered to represent strong consensus, in line with commonly adopted cut-offs in Delphi studies (typically ranging from 66% to 80%).

Accordingly, consensus in this study does not imply unanimity (100% agreement), and statements exceeding the $\geq 80\%$ threshold but with residual disagreement were considered to have achieved strong consensus.

Median and interquartile range values were used to assess the stability of agreement between rounds.

Data analysis and reporting

Descriptive statistics (mean, standard deviation, median, and interquartile range) were calculated for each statement to quantify the level of agreement (LoA). Statements meeting the consensus threshold were included in the final set of recommendations, grouped by the five domains. Changes in agreement between rounds were interpreted descriptively and cautiously, with emphasis placed on the maintenance of strong consensus and the stability of responses rather than on minor numerical variations.

RESULTS

A total of 37 statements were generated by the scientific board and submitted to Round 1 of the Delphi survey. These statements originated from a structured preliminary design and analysis phase based on the Nominal Group Technique (NGT). The collaborative work of the scientific board ensured that the initial 37 statements submitted in Round 1 comprehensively and systematically addressed the relevant clinical, immunohematologic, and procedural aspects of CAD diagnosis. A total of 30 experts were invited to participate; 27 (90%) accepted. All 27 experts completed both rounds (100% response rate). Consensus was defined as $\geq 80\%$ of panelists scoring a statement 4 or 5 on the Likert scale.

In Round 1, 36 of 37 statements (97.3%) reached consensus. One statement did not meet the consensus threshold and was revised in response to expert feedback. In addition, three statements were modified for clarity based on

qualitative feedback received after Round 1. To assess response stability, all statements that did not achieve 100% agreement in Round 1 were resubmitted in Round 2. A total of 24 statements were re-evaluated in Round 2, and all achieved the predefined consensus score of 4 or 5, with $\geq 80\%$ of respondents achieving this score. Across both rounds, median scores ranged from 4.0 to 5.0 with consistently narrow interquartile ranges (IQR=1.0), indicating stable agreement.

The statements are shown in **Table I**, and the results are summarized below according to the five predefined diagnostic domains.

Definition of CAD and CAS

In this domain, experts reached consensus on core definitions. After a reformulation between Round 1 and 2, consensus was achieved (96.3%) on describing cold agglutinin disease as a primary form of autoimmune hemolytic anemia mediated by clonal IgM autoantibodies, with a thermal optimum below 20°C but reactivity extending nearly to 37°C. The definition of CAS as a secondary form of cold AIHA associated with identifiable underlying conditions—such as infections (e.g., *Mycoplasma pneumoniae*, Epstein-Barr virus [EBV]), autoimmune diseases, and malignancies including non-Hodgkin lymphoma (NHL), Waldenström macroglobulinemia (WM), chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), or solid tumors reached 100% consensus in Round 1. Experts also agreed that hemolysis in both CAD and CAS is predominantly extravascular, primarily occurring in the liver, with intravascular hemolysis possible in acute settings.

Clinical evaluation of CAD

Consensus was consistently high across all clinical evaluation statements. Experts unanimously agreed in Round 1 that CAD assessment should consider current or past symptoms and signs commonly associated with hemolysis, including fatigue, dyspnea, jaundice, dark urine, gallstones, and acrocyanosis, particularly when these manifestations are exacerbated by cold exposure or concurrent infections. The presence of abnormalities in blood count parameters, biochemical markers of hemolysis, or evidence of in vitro agglutination was also considered sufficient grounds to initiate evaluation. This statement reached 100% agreement in Round 2.

Table 1 - Summary of diagnostic recommendations and consensus levels from the Delphi survey on cold agglutinin disease (CAD)

Statement/recommendation		LoA					
Round 1	Round 2	Round 1 (No.=27) LoA <100% → Round 2			Round 2 (No.=27)		
		Responders (No.)	% agreement	Median (IQR)	Responders (No.)	% agreement	Median (IQR)
Definition of CAD and CAS							
1. Cold Agglutinin Disease (CAD) is a primary form of autoimmune hemolytic anemia (AIHA), typically characterized by clonal IgM autoantibodies with high thermal amplitude that activate complement and induce hemolysis, usually with a reactivity lower than 20°C.	1. Cold Agglutinin Disease (CAD) is a primary form of autoimmune hemolytic anemia (AIHA), typically due to clonal IgM autoantibodies that activate complement and induce hemolysis, with temperature thermal optimum lower than 20°C and reactive up to nearly 37 °C.	27	88.89%	4.00 (1.00)	27	96.30%	5.00 (1.00)
2. Cold Agglutinin Syndrome (CAS) refers to secondary cold AIHA occurring in association with an identifiable underlying condition, such as infections (e.g., Mycoplasma pneumoniae, EBV), autoimmune diseases, or malignancies (NHL, WM, CLL, DLBCL, or solid tumors).		27	100.00%	5.00 (1.00)			
3. Both CAD and CAS involve predominantly extravascular hemolysis, mediated by complement and primarily occurring in the liver. In acute cases, intravascular hemolysis may also be present.	2. Both CAD and CAS involve predominantly extravascular hemolysis, mediated by complement and primarily occurring in the liver. In acute cases, intravascular hemolysis may also be present.	27	96.30%	4.00 (1.00)	27	92.60%	4.00 (1.00)
Clinical evaluation of CAD							
4. Clinical evaluation of CAD should take into account current or past symptoms and signs (e.g., fatigue, dyspnea, jaundice, dark urine, gallstones, acrocyanosis), which are often worsened by cold exposure or concurrent infections.		27	100.00%	4.00 (1.00)			
5. CAD evaluation is warranted in the presence of abnormalities in blood count parameters, hemolysis markers, and/or evidence of agglutination/hemolysis in the blood sample.	3. CAD evaluation is warranted in the presence of abnormalities in blood count parameters, hemolysis markers, and/or evidence of agglutination/hemolysis in the blood sample.	27	92.60%	5.00 (1.00)	27	100.00%	4.00 (1.00)
6. Incidental findings in immunohematologic testing (e.g., positive IAT, DAT, or difficulties in ABO typing) should also prompt clinical evaluation for CAD.	4. Incidental findings in immunohematologic testing (e.g., positive IAT, DAT, or difficulties in ABO typing) should also prompt clinical evaluation for CAD.	27	92.60%	4.00 (1.00)	27	96.30%	4.00 (1.00)

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Table 1 - Summary of diagnostic recommendations and consensus levels from the Delphi survey on cold agglutinin disease (CAD) (continues from previous page)

Statement/recommendation		LoA				
Round 1	Round 2	Round 1 (No.=27) LoA <100% → Round 2			Round 2 (No.=27)	
		Responders (No.)	% agreement	Median (IQR)	Responders (No.)	Median (IQR)
7. Clinical indications for immunohematologic testing should include: hemolytic anemia, signs/symptoms of hemolysis, acrocyanosis, and associated underlying diseases.		27	100.00%	4.00 (1.00)		
8. Laboratory indications should include: evidence of hemolysis in the biological sample, hemolysis after cold exposure, evidence of agglutination in the blood sample.	5. Laboratory indications should include: evidence of hemolysis in the biological sample, hemolysis after cold exposure, evidence of agglutination in the blood sample.	27	92.60%	4.00 (1.00)	27	81.90% 4.00 (1.00)
9. DAT (Direct Antibody Testing) is indicated in cases of panagglutination at IAT (Indirect Antibody Testing) or discrepancies between direct and indirect ABO typing.	6. DAT (Direct Antibody Testing) is indicated in cases of panagglutination at IAT (Indirect Antibody Testing) or discrepancies between direct and indirect ABO typing.	27	96.30%	4.00 (1.00)	27	100.00% 5.00 (1.00)
10. DAT should be performed using both polyspecific (IgG and C3d) and monospecific (IgG, C3d, C-) antisera.		27	100.00%	5.00 (1.00)		
11. Testing for cold agglutinins is warranted when DAT is positive for C3d (polyspecific or monospecific), regardless of IgG findings.	7. Testing for cold agglutinins is warranted when DAT is positive for C3d (polyspecific or monospecific), regardless of IgG findings.	27	81.50%	4.00 (1.00)	27	81.90% 4.00 (1.00)
12. Laboratories should define risk-based protocols for sample handling.	8. Laboratories should define risk-based protocols for sample handling.	27	81.90%	4.00 (1.00)	27	100.00% 4.00 (1.00)
13. If the sample arrives agglutinated, personnel should notify the requesting clinician and request a second sample kept at 37°C and proceed with analysis of the first sample.	9. If the sample arrives agglutinated, personnel should notify the requesting clinician and request a second sample kept at 37°C. The initial sample should be analyzed only if it disaggregates after a 15-30 minute incubation at 37°C.	27	81.50%	4.00 (1.00)	27	88.90% 5.00 (1.00)
14. Test conditions must be clearly stated in the laboratory report.	10. Test conditions must be clearly stated in the laboratory report.		96.30%	4.00 (1.00)	27	100.00% 4.00 (1.00)

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Table I - Summary of diagnostic recommendations and consensus levels from the Delphi survey on cold agglutinin disease (CAD) (continues from previous page)

Statement/recommendation		LoA				
Round 1	Round 2	Round 1 (No.=27) LoA <100% → Round 2			Round 2 (No.=27)	
		Responders (No.)	% agreement	Median (IQR)	Responders (No.)	Median (IQR)
15. For follow-up purposes, the second sample should be transported while maintaining a constant temperature of 37°C.		27	100.00%	5.00 (1.00)		
16. The sample must be kept in a thermostat at 37°C until separation of its components.	11. The sample must be kept in a thermostat at 37°C until separation of its components.	27	96.30%	5.00 (1.00)	27	4.00 (1.00)
17. Further studies are needed to determine whether additional clinical and/or laboratory evaluation is warranted in asymptomatic patients with incidental DAT positivity.	12. Further studies are needed to determine whether additional clinical and/or laboratory evaluation is warranted in asymptomatic patients with incidental DAT positivity.	27	85.20%	4.00 (1.00)	27	4.00 (1.00)
Analytical phase						
18. Cold agglutinin testing must be performed in a test tube at a controlled temperature of 20°C, using adult RBCs, cord blood ABO-compatible RBCs, and autologous RBCs. The test should be repeated at 4°C after overnight incubation.	13. Cold agglutinin testing should be performed in a test tube at a controlled temperature of 20°C, using adult RBCs, cord blood ABO-compatible RBCs, and autologous RBCs. The test should be repeated at 4°C after a minimum incubation period of 4 hours, and it is advisable to verify the titer at 4°C after an extended incubation period (overnight).	27	74.10%	4.00 (1.00)	27	4.00 (1.00)
19. If the DAT is positive also for IgG, an eluate should be performed to differentiate between IgG autoantibodies and alloantibodies.		27	100.00%	4.00 (1.00)		
20. The thermal amplitude of the antibody must be assessed at different temperatures to determine clinical significance. The required testing temperatures are: room temperature, overnight, and 37°C.	14. The thermal amplitude of the antibody must be assessed at different temperatures: room temperature, 4°C (2-3 hours or overnight), and 37°C.	27	88.90%	5.00 (1.00)	27	4.00 (1.00)
21. Special attention should be given to samples showing agglutination at 37°C, suggesting the presence of "warm" IgM antibodies.	15. Special attention should be given to samples showing agglutination at 37°C, suggesting the presence of "warm" IgM antibodies.	27	88.90%	4.00 (1.00)	27	5.00 (1.00)
22. Titration should be conducted in test tubes using serial two-fold dilutions of the patient's serum.		26	100.00%	4.50 (1.00)		

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Table I - Summary of diagnostic recommendations and consensus levels from the Delphi survey on cold agglutinin disease (CAD) (continues from previous page)

Statement/recommendation		LoA					
Round 1	Round 2	Round 1 (No.=27) LoA <100% → Round 2		Round 2 (No.=27)			
		Responders (No.)	% agreement	Median (IQR)	Responders (No.)	% agreement	Median (IQR)
23. Titration using adult, cord blood ABO-compatible, and autologous RBCs helps determine the antigen specificity of the antibody.	17. Titration using adult, cord blood ABO-compatible, and autologous RBCs helps determine the antigen specificity of the antibody.	27	81.50%	4.00 (1.00)	26	96.20%	4.00 (1.00)
24. A cold agglutinin titer is defined as the inverse of the highest serum dilution producing visible (1+) agglutination at a specific testing temperature.	18. A cold agglutinin titer is defined as the inverse of the highest serum dilution producing visible (1+) agglutination at a specific testing temperature.	27	96.30%	4.00 (1.00)	27	100.00%	4.00 (1.00)
25. A cold agglutinin titer $\geq 1:64$ at 4°C is generally considered clinically significant and should be reported with the thermal amplitude.		27	100.00%	5.00 (1.00)			
26. The report must specify whether cold agglutinins are detected; if positive, the titer and thermal amplitude must be reported.		27	100.00%	5.00 (1.00)			
27. In complex cases, bidirectional communication between the laboratory and the clinician is necessary to plan further investigations.		27	100.00%	5.00 (1.00)			
28. Differentiating anti-I from anti-i specificity can help distinguish primary CAD from secondary CAS, especially in infectious or lymphoproliferative contexts.	19. Differentiating anti-I from anti-i specificity can help distinguish primary CAD from secondary CAS, especially in infectious or lymphoproliferative contexts.	27	88.90%	4.00 (1.00)	27	88.90%	4.00 (1.00)
29. Patient typing for clinically significant antigens (RH, Kell, JK, FY, MNS) should be performed by serology or molecular testing, especially if transfusion occurred within the last 3 months.	20. Patient typing for clinically significant antigens (RH, Kell, JK, FY, MNS) should be performed by serology or molecular testing, especially if transfusion occurred within the last 3 months.	27	81.50%	4.00 (1.00)	27	92.60%	4.00 (1.00)
30. In urgent cases, compatible units for ABO-, Rh- and Kell- should be used.	21. In urgent cases, compatible units for ABO-, Rh- and Kell- should be used.	27	92.60%	4.00 (1.00)	27	100.00%	4.00 (1.00)

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Table 1 - Summary of diagnostic recommendations and consensus levels from the Delphi survey on cold agglutinin disease (CAD) (continues from previous page)

Statement/recommendation		LoA					
Round 1	Round 2	Round 1 (No.=27) LoA <100% → Round 2		Round 2 (No.=27)			
		Responders (No.)	% agreement	Median (IQR)	Responders (No.)	% agreement	Median (IQR)
31. When time allows, pre-transfusion testing should include autologous/ allogeneic adsorption or molecular typing, depending on laboratory capabilities.	22. When time allows, pre-transfusion testing should include autologous/ allogeneic adsorption or molecular typing, depending on laboratory capabilities.	27	81.50%	4.00 (1.00)	27	88.90%	4.00 (1.00)
32. Laboratories should adopt structured report templates with standardized terminology to enhance clarity and interdisciplinary communication.		27	100.00%	5.00 (1.00)			
33. The standard report should include: ABO blood group typing, DAT results with monospecific antisera, cold agglutinin test result, titer, thermal amplitude, and antigen specificity of the autoantibody.	23. The standard report should include: ABO blood group typing, DAT results with monospecific antisera, cold agglutinin test result, titer, thermal amplitude, and antigen specificity of the autoantibody.	27	96.30%	5.00 (1.00)	27	96.30%	5.00 (1.00)
34. Follow-up intervals should be tailored based on AIHA subtype (warm, cold, mixed), clinical course, transfusion dependency, and therapy status.		27	100.00%	4.00 (1.00)			
35. Follow-up strategies should be jointly planned by the immunohematology laboratory and the treating clinician.	24. Follow-up strategies should be jointly planned by the immunohematology laboratory and the treating clinician.	27	96.30%	5.00 (1.00)	27	100.00%	5.00 (1.00)

AIHA: autoimmune hemolytic anemia, CAD: cold agglutinin disease, CAS: cold agglutinin syndrome, DAT: direct antiglobulin test, IgM: immunoglobulin M, IQR: interquartile range, LoA: level of agreement, RBC: red blood cells.

Statements that did not achieve 100% consensus in Round 1 were resubmitted to Round 2.

Additionally, incidental findings arising during routine immunohematologic testing, such as a positive indirect antiglobulin test or DAT or difficulties in ABO typing, were recognized as relevant indicators. Agreement for this latter statement was 92.3% in Round 1 and increased to 96.3% Round 2, demonstrating stability of agreement across rounds.

Indications for immunohematologic testing

All eight statements in this domain achieved consensus by the end of Round 2. Experts agreed that immunohematologic testing is indicated in patients presenting with hemolytic anemia, signs or symptoms compatible with hemolysis, acrocyanosis, or underlying conditions associated with cold-reactive antibodies. They also endorsed testing when laboratory findings demonstrate evidence of hemolysis in the biological sample, hemolysis following cold exposure, or visible agglutination. Consensus was reached on performing a DAT using both commercial polyspecific (anti-IgG + anti-C3d) and monospecific antisera (anti-IgG and anti-C3d, separately). The recommendation that searching for cold agglutinins should be performed whenever the DAT is positive for C3d initially reached 81.48% consensus in Round 1 but achieved 88.89% agreement in Round 2. Full consensus was reached on the need for laboratories to define risk-based protocols for sample handling. Experts further agreed that agglutinated samples require immediate communication with the prescribing clinician and the collection of a new sample, which should be maintained at 37°C. The original specimen should only be analyzed if it completely disaggregates after 15-30 minutes of incubation at 37°C. The requirement to clearly document test conditions within laboratory reports also received full support in Round 2.

Pre-analytical phase

All statements concerning the pre-analytical phase achieved consensus in the 1st round. Experts agreed that follow-up samples must be transported at a constant 37°C temperature. They also supported the requirement that blood samples be kept at 37°C in a warming device until component separation is complete.

For thermal amplitude titration studies, participants emphasized the importance of using warm-washed autologous RBCs and maintaining temperature control during centrifugation and result interpretation, since

even slight cooling may affect test reactivity and lead to inconsistent results across laboratories. When available, the use of warmed centrifuges or alternative approaches aimed at avoiding sample cooling during reading procedures should be considered.

The statement indicating the need for further evidence to justify a clinical or laboratory evaluation in asymptomatic patients with incidental DAT positivity reached consensus in both rounds, reflecting the recognition of the existing uncertainty in this area.

Analytical phase

The analytical phase encompassed 20 statements, all of which achieved consensus after two rounds. Experts agreed that cold agglutinin testing should be performed in test tubes at 20°C, incubating the patient's serum with adult RBCs, cord blood ABO-compatible RBCs, and autologous RBCs for at least 1 hour. Testing should be repeated at 4°C after a minimum incubation period of two hours, with an additional overnight incubation recommended for confirmation. Consensus was reached on the requirement to assess thermal amplitude at room temperature, at 4°C for at least two to three hours or overnight, and at 37°C. Special attention was advised for samples demonstrating agglutination at 37°C, as this may indicate the presence of a warm IgM autoantibody, which characterizes a different form of AIHA¹⁶. The latter has been associated with massive acute hemolysis in vivo and requires clinical suspicion and urgent therapy¹⁷.

Statements concerning titration procedures also received consensus. The panel supported performing titration using serial two-fold dilutions to determine the potential clinical significance of cold agglutinins. They endorsed the use of adult, cord blood ABO-compatible, and autologous RBCs to identify antigen specificity. Consensus was reached on defining the cold agglutinin titer as the inverse of the highest serum dilution producing at visible (1+) agglutination, and on considering a titer of $\geq 1:64$ at 4°C as significant, to be reported together with its thermal amplitude, since even lower antibody titers, in the presence of clinical signs, can be compatible with a diagnosis of CAD. The panel also agreed that eluate testing should be performed when the DAT is positive for IgG to distinguish warm IgG autoantibodies from alloantibodies, particularly in recently transfused patients.

Further statements addressed diagnostic refinement and transfusion support. Experts endorsed the value of differentiating anti-I from anti-i specificity to help distinguish primary CAD from secondary CAS, especially in infectious or lymphoproliferative contexts. They also agreed to perform extended typing for clinically significant antigens –including RH, KEL, JK, FY, and MNS– using serology or molecular methods, particularly when transfusion has occurred within the previous three months. Compatible units for ABO, RH, and KEL antigens were recommended for urgent transfusion settings. When time allows, the use of autologous or allogeneic adsorption or molecular typing was endorsed as part of pre-transfusion testing, depending on laboratory capabilities.

Ultimately, experts reached a consensus on reporting and follow-up procedures. Structured report templates with standardized terminology were recommended, and laboratory reports should include ABO typing, DAT results with monospecific antisera, cold agglutinin testing results, titers, thermal amplitude, and antigen specificity. Follow-up intervals should be individualized based on the AIHA subtype, clinical course, transfusion requirements, and therapeutic context. Experts agreed that follow-up strategies should be jointly planned by immunohematology laboratories and treating clinicians.

DISCUSSION

The diagnosis of CAD presents substantial laboratory heterogeneity across clinical centers^{8,18}. Variability exists in pre-analytical procedures, DAT interpretation, cold agglutinin titration thresholds, and thermal amplitude assessment¹². Factors such as inconsistent temperature control during sample handling, lack of standardization in serological protocols, and diverse reporting practices contribute to diagnostic uncertainty and delay. These inconsistencies may result in underdiagnosis or misclassification, particularly in distinguishing CAD from other forms of AIHA, such as warm AIHA and mixed forms. The rarity of the disease and the heterogeneity in clinical presentation further complicate the picture. All in all, two crucial steps lead to CAD diagnosis: 1) the clinical suspicion in a patient with anemia and signs and symptoms of hemolysis, particularly in association with exposure to cold temperature, 2) the laboratory

assessment during routine blood counts with detection of agglutination or hemolysis and during transfusion medicine assessment in patients requiring blood units. Given the absence of universally adopted guidelines and the complexity of diagnostic parameters, harmonizing clinical and laboratory workflows is critical to ensuring timely and accurate CAD recognition¹.

This two-round modified Delphi study achieved complete consensus among 27 Italian experts on all 37 statements regarding the diagnosis of CAD, demonstrating high convergence across clinical, immunohematologic, and laboratory domains. The consistently elevated levels of agreement ($\geq 80\%$ for all statements), together with median scores ranging from 4.0 to 5.0 and narrow interquartile ranges, indicate strong stability of expert opinion across rounds and underscore the shared need for standardized diagnostic practices in Italy.

Experts unanimously agreed on the distinction between CAD and CAS. As reflected in the results, CAD was defined as a primary clonal IgM-mediated autoimmune hemolytic anemia, whereas CAS was recognized as secondary to infections, autoimmune diseases, or malignancies. This reinforces established evidence that CAD represents a unique clinicopathologic entity requiring specific diagnostic and therapeutic considerations¹⁻³. In any case, it should be remembered that the distinction between CAD and CAS may not be so obvious in clinical practice. For instance, during infections that may either “cause” a CAS or trigger the relapse of a “CAD”. The distinction is also influenced by the extent of the thorough clinical/laboratory assessment to evaluate and exclude associated conditions (serologies for viral/bacterial infections, organ- and non-organ-specific autoantibody assessment, bone marrow evaluation, including trephine and molecular testing for MYD88, and contrast-enhanced CT scan).

The panel reached complete consensus on the clinical features that should prompt evaluation for CAD, including hemolytic symptoms, cold-induced manifestations, and incidental abnormalities in immunohematologic testing. These findings align with real-world observations that CAD is often underrecognized, may present subtly, and is frequently detected through laboratory discrepancies rather than overt symptoms^{19,20}. The high consensus on these criteria supports their incorporation into a standardized diagnostic pathway to reduce diagnostic

delays, particularly in milder cases. The presence of nutrient deficiencies and comorbidities (i.e., chronic inflammatory conditions) that may confound CAD diagnosis in the elderly population should also be considered.

Experts strongly supported performing a DAT using both polyspecific and monospecific antisera (e.g., anti-IgG and anti-C3d). They agreed that testing for cold agglutinins is warranted whenever the DAT is positive for C3d, regardless of IgG findings. This is a critical recommendation, as complement positivity is a hallmark of cold antibody-mediated hemolysis²¹. This statement not only reinforces the diagnostic relevance of complement detection but also highlights the need for harmonized immunohematologic protocols, particularly the availability of monospecific tests for IgG and C3d, which have been established as essential tools in transfusion centers.

Strong consensus was reached on the need for strict temperature control at 37°C during sample collection, transportation, and processing. Panelists agreed that agglutinated samples should prompt clinician notification and the collection of a new, warmed specimen, and that the initial sample can be analyzed only if it fully disaggregates after incubation at 37°C. These recommendations directly address well-recognized pre-analytical pitfalls and align with the literature, which demonstrates that inadequate temperature control significantly compromises test accuracy in cold-reactive disorders¹¹. The panel also identified a knowledge gap regarding asymptomatic patients with incidental DAT positivity, regarding the need for a complete evaluation in the absence of anemia or hemolysis, reflecting the need for further evidence-based guidance in this scenario.

The panel endorsed detailed and specific laboratory procedures, including thermal amplitude assessment at multiple temperatures, extended incubation periods (i.e., overnight), standardized titration using adult and cord RBCs, and structured reporting of titer, thermal amplitude, and antigen specificity. The threshold of the cold agglutinin titer at 1:64 has been further debated and considered the usual hallmark of primary CAD. However, the panel recognized that lower titers in the presence of overt clinical manifestations may still be consistent with CAD diagnosis, and may be due to pre-analytical variables, prior treatment with immunosuppressive agents, or the presence of highly avid autoantibodies.

Together, these findings provide a comprehensive, standardized diagnostic pathway spanning the entire pre-analytical, analytical, and clinical evaluation process. The strengths of this study include the diverse expertise of the panel, the 100% response rate across both Delphi rounds, and the structured methodology that enables iterative refinement of the statements.

This study also carries limitations inherent to expert consensus methodologies. Delphi processes synthesize expert judgment rather than empirical validation, and therefore, the resulting recommendations should serve as a standardized framework rather than definitive clinical guidelines. Future research should evaluate the real-world impact of these recommendations, including their effect on diagnostic accuracy, inter-laboratory reproducibility, and time to diagnosis. Moreover, since this consensus focuses exclusively on diagnostic aspects, further collaborative efforts are warranted to develop standardized pathways for treatment and follow-up.

In summary, this Delphi consensus provides an integrated and evidence-supported diagnostic framework for CAD, aligned with current knowledge and tailored to address the variability observed among Italian centers. The recommendations generated through this process have the potential to improve diagnostic precision, enhance laboratory standardization, and support more timely and consistent evaluation of patients with suspected CAD.

CONCLUSIONS

The Delphi consensus statement presented here establishes a structured and evidence-based diagnostic framework for cold agglutinin disease. By defining standardized criteria for clinical evaluation, immunohematologic testing, and laboratory reporting, a panel of 27 experts has provided a practical tool to support clinicians and transfusion specialists in the timely and accurate identification of CAD. These findings are expected to enhance diagnostic uniformity, improve interdisciplinary communication, and ultimately optimize patient care for this rare hematologic disorder.

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Authors' contributions

All Authors contributed equally to this work. All Authors contributed to the study conception and design. All Authors contributed to the development and revision of the Delphi statements and interpretation of the results. All Authors critically revised the manuscript for important intellectual content and approved the final version for submission.

Conflicts of interest

SC serves as Editor in chief and Section editor of *Blood Transfusion*. BF received consultancy and speaker's bureau from Alexion, AstraZeneca, Janssen, Novartis, Recordati, Roche, and Sanofi. FF declares that he has received honoraria for lectures and teaching activities from Grifols. He also serves as Affiliated Society Editor of *Blood Transfusion*. AM serves as component of the International Editorial Board of *Blood Transfusion*. DP declares that he has received funding for travel and research projects, as well as honoraria for lectures and teaching activities from Cerus, Diasorin, Diamed, Diatech Pharmacogenetics, Grifols, Immucor, Macopharma, Ortho Clinical Diagnostics, Terumo. He also serves as Section Editor of *Blood Transfusion*. NR serves as component of the International Editorial Board of *Blood Transfusion*. GU serves as section Editor for *Blood Transfusion*.

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