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# Development of a Certified Reference Material for myeloperoxidase-anti-neutrophil cytoplasmic autoantibodies (MPO-ANCA)☆

Evanthia Monogioudi [a], Dana Petronela Hutu [a], Gustavo Martos [a], Joanna Sheldon [b], Heinz Schimmel [a], Pier Luigi Meroni [c], Ingrid Zegers [a,*]

[a] European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium
[b] Protein Reference Unit and Immunopathology Department, St Georges' Hospital, London, United Kingdom
[c] Department of Clinical Sciences and Community Health & Istituto Auxologico Italiano, Milan, Italy

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ABSTRACT

A serum Certified Reference Material (CRM) for supporting reliable autoimmune diagnostics was recently released by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre of the European Commission. It was produced in collaboration with a Working Group on the Harmonisation of Autoimmune Tests of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC WG-HAT). This material is aimed at facilitating the standardisation of measurements of anti-myeloperoxidase immunoglobulin G antibodies. The CRM could be used as a common calibrant by clinicians and manufacturers thereby significantly improving the comparability of results from commercial immunoassays used for IgG anti-MPO measurements. This paper provides information on the new CRM and its intended use.



## 1. Introduction

There are currently more than 70 diseases resulting from the body's immune system targeting its organs, tissues, cells and their components [1] rather than destroying invader cells or infectious organisms (viruses or bacteria). Many of these autoimmune diseases are characterised by the presence of specific autoantibodies. For example, anti-myeloperoxidase (anti-MPO) and anti-proteinase 3 (anti-PR3) are present in ANCA-associated vasculitides [2–5]. Clinicians rely on the detection and quantification of these autoantibodies for the diagnosis and treatment of the diseases. The need for quantitative information has led to the development of a variety of ELISA-based immunoassays, new assay principles are being developed and automation has become more common. Tests do not only need to be analytically specific and sensitive but should preferably also show small inter-method variation in order to establish common reference ranges, consistent "cut-off" values and clinical specificity and sensitivity [6]. Some issues are contributing to the variability of results, including molecular heterogeneity of the antigen and antibody target, performance variation between the different assays and also lot-to-lot variation within assays. There is currently no robust standardisation which contributes significantly to the variation of results obtained by different laboratories [7]. For instance, the UK National External Quality Assessment Service (UKNEQAS) has reported that the measurement interval for "negative" samples for IgG anti-PR3 antibodies can overlap with the values obtained for samples categorised as "positive" (UKNEQAS for ANCA distributions 132 and 134). A similar overlap in values for negative and positive samples has been shown in the UKNEQAS scheme for IgG anti-MPO.

A feasibility study by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group measured IgG anti-MPO in samples from 30 patients who were investigated for ANCA-associated vasculitis by 7 different methods. Nine of the samples were consistently classified as positive across the different methods and only one was consistently classified as negative. The remaining 20 samples gave contradictory classification depending on the type of assay used [8]. This highlights two problems - the extreme variation in the reported quantitative values but also in the classification of the samples in terms of positive and negative. It is likely that these two issues will need to be addressed separately. However, Hutu et al. have shown that the use of a common, commutable reference material would improve comparability of measurement results [8].

The challenges in establishing standards for complex biological molecules are well recognised. Antibodies are prone to degradation; they may show variations in affinity and avidity, may consist of different subclasses, and have high molecular diversity. The preparation of a serum based standard with multiple processing steps, including eventually purification and freeze-drying, may degrade the proteins making them behave differently from fresh serum samples. There are some possible sources of raw material for producing such reference materials, each

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* Corresponding author.

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with advantages and disadvantages which are summarised in Table 1 [9]. The most effective approach for developing reference materials is likely to use a combination of processes that link together. For example, pooled patient samples or plasmapheresis fluid would give a sufficient total volume of raw material and affinity-purified autoantibodies are likely to be used as a calibrant for assigning a property value to the prepared candidate reference material.

## 2. Standardisation efforts

The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre of the European Commission is participating in a Working Group on Harmonisation of Autoantibody Tests (WG-HAT) of IFCC [10]. The WG aims at evaluating the main causes of variability for clinically critical autoantibody measurements, identifying the autoantibodies for which a common calibrator would reduce the inter-assay variability and identifying and producing the materials that could be used for assay calibration. The final goal of this initiative is to prepare commutable certified reference materials with well-defined properties that would allow standardisation of autoantibody testing. If properly implemented the standardisation using certified reference materials should lead to comparable, harmonised measurement results.

## 3. First model system for developing procedures

The certified reference material (CRM) ERM-DA476/IFCC certified for its concentration of anti-MPO immunoglobulin G in human serum is the first outcome of the Working Group [11]. IgG anti-MPO is one of the two antibody specificities seen in ANCA-associated vasculitis, which include microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, initially known as Churg-Strauss Syndrome) [12] and granulomatosis with polyangiitis (GPA, formerly Wegener's Granulomatosis) [13]. These conditions mostly affect small arteries, such as arterioles [14], and although they are rare diseases, they can be very serious; symptoms can range in severity from minor to life threatening [14]. Early diagnosis and appropriate treatment are vital for the patient's survival but the disease course can be variable and there is still a high proportion of patients (almost 50%) who relapse [15].

ERM-DA476/IFCC is a material prepared from a plasmapheresis sample of a patient diagnosed with vasculitis, provided by Statens Serum Institute (DK). It was chosen because of its high titre of MPO ANCA autoantibodies and its commutability [8]. The term commutability refers to the characteristic of a material to behave in the same manner as clinical samples with regard to several measurement procedures [16]. The plasmapheresis material was transformed into serum (defibrinated) through a process described elsewhere [17] and processed according to a procedure similar to that used for other serum protein reference materials [17]. The serum was delipidated and filtered and three preservatives were added (sodium azide, aprotinin and benzamidine hydrochloride monohydrate). The material was then filter sterilised through a 0.22 μm PES filter (Corning Incorporated, USA), further diluted 1:6 with Zenalb® 4.5 (Bio Products Laboratory) and filled into 1 mL colourless, siliconised glass vials before lyophilisation under a low-pressure nitrogen atmosphere [11]. A concentration value for the IgG specific against myeloperoxidase was assigned to the material through an interlaboratory comparison involving ten laboratories. These laboratories were requested to use their own, routine MPO ANCA assays (ELISA, chemiluminescent and fluoroenzyme immunoassays) to measure dilutions of the reference material ERM-DA476/IFCC and dilutions of affinity-purified MPO ANCA. The solution of affinity-purified IgG had a value assigned using nephelometry and turbidimetry, calibrated with the reference material ERM-DA470k/IFCC, which has a certified value for total IgG [18]. The detailed process of the characterisation is published in the certification report of the CRM [11]. The measurement results for ERM-DA476/IFCC show that the 9 different assays perform well in terms of precision, but are using entirely different measurement scales (Fig. 1, without common calibrant). Even when using the same unit (IU/mL), the results varied from 40 to 356 IU/mL [11]. When the measured values for ERM-DA476/IFCC were recalibrated for each assay according to the response for affinity-purified IgG, the variation was reduced to 13% (Fig. 1, with common calibrant). Consequently it was possible to assign a certified value for MPO ANCA to ERM-DA476/IFCC, traceable to the value for the IgG concentration in ERM-DA470k/IFCC.

## 4. Intended use

The main purpose of this certified reference material is the calibration of immunoassay-based *in vitro* diagnostic devices or calibrators for MPO ANCA. It can also be used to maintain control charts and/or for validation studies of methods for which the CRM has been shown to be commutable. It is expected that the use of the CRM can put results from different methods on the same scale and could also help in reducing batch-to-batch variability.

It should be kept in mind that the complex nature of autoantibodies may still cause significant variation of the results for individual clinical samples between methods and over time. The characteristics of the "same" antibody from different patients may vary in terms of the subclass, affinity, avidity, and even properties such as glycosylation. Each of these factors will have a variable impact on the measured results from different methods. The use of a common reference material may improve the overall correlation between methods, but results will not be comparable among all patient samples, when different targets are measured.

Overall, the use of this CRM is likely to be a step towards a better understanding of the parameters that influence quantitative results for IgG MPO-ANCA.

## 5. Next autoimmune targets

The development of ERM-DA476/IFCC required extensive feasibility studies and the design of procedures for commutability assessment,

**Table 1**
Summary of advantages and disadvantages of the different types of starting material.

| | Pooled serum | Affinity purified | Monoclonal |
|---|---|---|---|
| Advantages | • Wide range of specificities within a particular antibody<br>• Polyclonal<br>• Possibly representative of most antibodies in most patients<br>• Small amounts from each patient needed<br>• Large total volumes can be collected | • Many potential interferences avoided<br>• Can blend material from patients or even of different isotypes<br>• Long term consistency | • Most potential interferences avoided<br>• Long term consistency |
| Disadvantages | • Other proteins in serum may interfere<br>• Interactions between antibodies in different samples *e.g.* rheumatoid factor<br>• Likely to be variable on long term | • Need *relevant* antigen in large amounts<br>• Need large volumes of raw material<br>• May not be representative of all antibodies in all patients<br>• Need for sophisticated facilities | • May not be representative of all antibodies in all patients<br>• Difficult to generate in large amounts<br>• Need for sophisticated facilities |

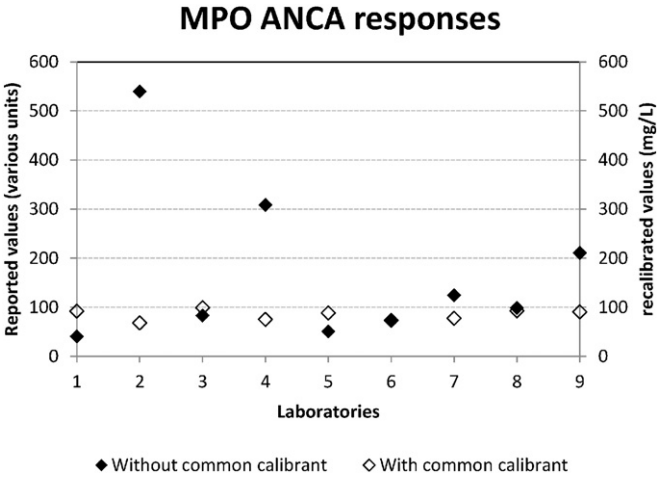


**Fig. 1.** Comparison of response values for each laboratory participating in the study before and after correction with the CRM. Samples of the CRM and purified MPO ANCA serving as calibrant, were sent to nine laboratories and tested with different ELISA-like techniques. The black diamonds represent the reported results from the laboratories using their own calibrant, while the white ones represent the results after being corrected with the use of the values for the purified MPO ANCA. The detailed procedure can be found elsewhere [11]. The participating laboratories were: Aesku Diagnostics, Bio-Rad Laboratories, Inc., Euro Diagnostica AB, Euroimmun AG, IMMCO Diagnostics, INOVA Diagnostics, INC., Phadia GmbH.

processing of the serum, and for the value assignment. These procedures will now be used for the development of other CRMs for autoimmune targets.

Presently, the IRMM is in collaboration with the IFCC in the process of preparing a CRM for anti-PR3 IgG autoantibodies. These autoantibodies are found in approximately 80% of patients with GPA and about 35% of patients with MPA and EGPA [19]. Additionally, the development of a CRM for anti-B2 glycoprotein has progressed significantly; the characterisation of this material is ongoing.

Other approaches may be needed for future materials. Therefore, the IFCC-WG HAT is also investigating different approaches for obtaining raw materials (*e.g.* pooled patient sera) and the possibility of using monoclonal calibration material. In Table 1, the advantages and disadvantages of using different raw materials is summarised. Altogether, the expertise developed should make it possible to produce CRMs for autoimmune testing more efficiently and to improve measurement comparability where possible.

## 6. Conclusions

New immunoassays, targeting different analytes and occasionally employing different principles are being produced. At the same time, the need for fast and reliable quantitative tests for the diagnosis of the various autoimmune diseases is increasing. However, as shown in some studies, there is still huge diversity in the results obtained by these immunoassays. Therefore, high-quality CRMs to be used as calibrants for *in vitro* diagnostics will help to foster comparability of measurement results. At the moment, a first material, for autoantibodies against anti-MPO, has been produced and is available to laboratories and manufacturers [20].

## Disclosure of conflicts of interest

The authors have no conflict of interest to declare.

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