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Evolution of autoimmune diagnostics over the past 10 years: lessons learned from the UK NEQAS external quality assessment EQA programs

https://doi.org/10.1515/cclm-2024-0781
Received July 3, 2024; accepted December 15, 2024;
published online January 9, 2025

Abstract

Objectives: External quality assessment (EQA) programs play a pivotal role in harmonizing laboratory practices, offering users a benchmark system to evaluate their own performance and identify areas requiring improvement. The objective of this study was to go through and analyze the UK NEQAS "Immunology, Immunochemistry and Allergy" EQA reports between 2012 and 2021 to assess the overall level of harmonization in autoimmune diagnostics and identify areas requiring improvement for future actions.

Methods: The EQA programs reviewed included anti-nuclear (ANA), anti-dsDNA, anti-centromere, anti-extractable nuclear antigen (ENA), anti-phospholipids, anti-neutrophil cytoplasm (ANCA), anti-proteinase 3 (PR3), anti-myeloperoxidase (MPO), anti-glomerular basement membrane (GBM), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), mitochondrial (AMA), liver-kidney-microsomal (LKM), smooth muscle (ASMA), APCA, and celiac disease antibodies.

Results: In the analyzed period, the number in participating laboratories showed an increase for almost all programs. Among solid phase methods, the use of ELISA techniques showed a progressive reduction, while new technologies, such as the fluoroenzymatic immunoassay, chemiluminescence immunoassay, Luminex and immunoblot showed an increased number of users. The number of results complying with the expected negative or positive target slightly increased for almost all antibodies in the last decade. A description of the most frequent causes of mistakes or misinterpretation for each specific test and method is also provided in this study.

Conclusions: Although numerous challenges need to be addressed in the area of autoantibody detection to enhance testing quality and attain higher harmonization, the period analyzed revealed that the ever-expanding range of autoantibodies, coupled with the introduction of new tests and methodologies and the advent of automated platforms, has brought about significant changes in autoimmune diagnostics.

Keywords: external quality assessment; harmonization; immunoassays; UK NEQAS

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Introduction

Over the last decades, the discovery of new antibodies and a better understanding of their clinical meaning led to relevant changes in autoimmune diagnostics [1]. In addition, methods for autoantibody measurement have undergone significant innovation. Multiplex technologies and automated platforms have been introduced in routine daily practice, affecting the comparability of results among laboratories [2–7]. The anti-nuclear antibody (ANA) test using indirect immunofluorescence (IIF) is probably the most challenging example. ANA-IIF is characterized by subjective visual reading limitations, high intra- and inter-laboratory variability, heterogeneity in pattern nomenclature, screening dilution and end-point titration, which hamper harmonization of the test [8–10]. Efforts for harmonization

should include multiple stakeholders involved in the process, such as laboratory professionals, assay manufacturers and External Quality Assessment (EQA) providers. The latter play a pivotal role in harmonizing laboratory practices, offering users a benchmarking system to assess their performance and identify areas requiring improvement.

EQA were introduced into laboratory medicine more than 60 years ago, evolving from educational tools to essential components of quality management systems. Initially addressing discrepancies in results among laboratories, now ensuring the consistency and accuracy of laboratory testing, often being a prerequisite for proficiency evaluation and accreditation in many countries [11–13]. To help in achieving this goal, EQA samples should include clear clinical information, be stable and mimic patients' samples in laboratory procedures. In order to ensure comparability of test results across different laboratories, EQA program purposes include evaluating performance for specific tests, identifying inter-laboratory discrepancies, and monitoring the success of harmonization/standardization efforts [9, 14]. Over recent years, in order to optimize EQA purposes, improvements were developed such as commutability and value assignment of control materials [15–17]. However, the continuous progress in autoimmune diagnostics calls for constant development and changes in EQA design, especially when rare antibodies and new methodologies are taken into account.

The objective of our study was to analyze the UK National External Quality Assessment Service (NEQAS) reports from 2012 to 2021 to assess the evolution in technology and improvement in the overall level of harmonization in autoimmune diagnostics. We also aimed to evaluate the performance of the various analytical methods for autoantibody detection and identify areas requiring improvement for future actions.

Materials and methods

Data source

Data were extracted from the UK NEQAS EQA program participant summary reports. We examined all reports between 2012 and 2021, consisting of four to six cycles a year, each encompassing two samples. The EQA reports summarized results returned by the participant laboratories within different EQA programs, related to the following antibody test: anti-nuclear (ANA), anti-dsDNA, anti-centromere and anti-extractable nuclear antigen (ENA), anti-phospholipid (aPL) including anticardiolipin (aCL) and anti-β2glycoprotein I (anti-β2GPI), anti-proteinase 3 (PR3), anti-myeloperoxidase (MPO), anti-glomerular basement membrane (GBM), anti-neutrophil cytoplasm (ANCA), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), mitochondrial (AMA), liver-kidney-microsomal (LKM), smooth muscle (ASMA) and parietal cell antibodies (PCA), and antibodies in celiac disease (anti-native gliadin [AGA], anti-tissue transglutaminase [tTG], anti-endomysial [EMA], anti-deamidated gliadin peptides [DGP]). According to the UK NEQAS requirements, participants distributed all over the world are instructed to treat the specimens as routine clinical samples and to report results with details of the analytical method used and its manufacturer. Results extracted from UK NEQAS reports were classified based on different commercially (or in-house) methods for each test. The designated responses for all qualitative UK NEQAS "Immunology, Immunochemistry and Allergy" EQA programs are based on consensus results. For some programs, a panel of expert laboratories may be involved. Also, the use of clinical information may be adopted to define the correct response for all programs, where this is possible.

Data analysis

In order to evaluate the evolution of technologies, the percentage of laboratories using the same method was computed for each analyte and compared over time. To evaluate harmonization among laboratories, the number of compliant answers with the expected target values was determined for each test and exercise. When the results were labeled by the UK NEQAS as equivocal, given the lack of a defined target, the exercises were not included in the analysis. When instead, we analyzed the grade of consensus returned for ANA exercises featuring different autoantibodies as target, the percentage of consensus among participants was computed for each report, including the exercises classified as equivocal. In this context, two different analyses for IIF and for solid-phase methods (SPA) were carried out.

Finally, for those antibodies for which an international quantitative standard is available and against which all manufacturers certify that they have calibrated their methods, i.e. anti-dsDNA antibodies and RF, the coefficient of variation (CV%) of the mean value of quantitative results of each method was calculated and compared for the 2012 and 2021 cycles.

Results

The number of participating laboratories within the UK NEQAS EQA "Immunology, Immunochemistry and Allergy" programs increased for almost all cycles analyzed between

2012 and 2021 (Tables 1 and 2). To note, there was no difference in terms of percentage of responses during the period of the COVID-19 pandemic as the EQA provider continued its service throughout the period with no disruptions [18–20].

In the context of technology and innovation, we explored the evolution of methods over the last decade. As shown in Table 1, despite the growing using of SPA, IIF on HEp-2 cell substrate remained the preferred method for ANA measurement for the majority of the participating laboratories (80 % in 2012; 78 % in 2021). Rat and mouse substrates which in 2012 were used by 2.3 % and 0.8 % of laboratories, respectively, completely disappeared in 2021. Among SPA, the fluoroenzyme immunoassay (FEIA), revealed a slight increase overtime in almost all exercises and became the predominant methodology for analyzing anti-dsDNA, ANCA, anti-ENA, anti-GBM, aCL and anti-β2GPI antibodies.

With the exception of AGA IgA and IgG (now performed by a limited number of laboratories) the use of the ELISA technique, once the most representative, showed a significant reduction, replaced by emerging technologies such as FEIA and chemiluminescence immunoassays (CLIA).

An opposite trend was observed for immunoblot (IB)-based assays (e.g. line-immunoassay and dot-immunoassay) showing an increased percentage of users for the detection of AMA, anti-LKM and anti-centromere antibodies, while their use was slightly reduced for anti-GBM antibodies (Tables 1 and 2).

Agreement among laboratories (any method) was slightly improved for almost all antibodies, not reaching however statistical significance (Supplementary Table 1). Conversely, it is noteworthy that the mean antibody CV level of immunometric methods measuring those antibodies for which an international quantitative standard is available, was 72 % in 2012 and 27 % in 2021 (p=0.021) for anti-dsDNA antibodies and 34 % in 2012 and 15 % in 2021 (p=0.08) for RF. In both cases, therefore, the improvement was significant. This improvement is likely the result of a combination of factors, such as the increasing use of new automated platforms and perhaps a drop in companies involved in producing the diagnostic systems (in 2012 there were more than 20 companies involved in the field, while in 2021 95 % of participants were employing assays from just five companies).

Table 1: Comparison of the number of autoimmunology laboratories participating in the UK NEQAS programs and percentage of the methods used to detect autoantibodies in 2012 and 2021.

	Year	No.	IIF%	ELISA%	FEIA%	CLIA%	Luminex%	IB%	Other%
ANA	2012	545	80.2	8.1	10.1	NA	1.6	–	–
	2021	566	78.2	4.2	14.2	1.3	2.1	–	–
dsDNA	2012	699	27.6	30.7	34.1	0.4	4.7	0.4	2.1
	2021	723	25.6	12.7	38.5	14.4	6.5	0.6	1.7
ENA	2012	208	–	36.1	29.2	1.0	8.7	25.0	–
	2021	418	–	7.9	38.7	14.4	13.4	25.6	–
Centromere	2012	206	23.1	14.2	36.5	1.1	10.8	6.3	8.0
	2021	239	32.8	3.9	33.4	6.4	10.7	12.0	0.8
ANCA	2012	666	44.4	23.4	21.8	4.7	3.0	0.5	2.2
	2021	799	42.9	10.9	28.8	10.9	5.1	0.5	0.9
GBM	2012	318	14.8	36.4	36.4	0	5.7	1.3	5.4
	2021	426	12.7	16.5	46.3	13.1	8.9	0.9	1.6
AMA	2012	375	88.4	8.0	NA	–	–	2.0	1.6
	2021	397	84.0	5.8	3.9	–	–	4.3	2.0
LKM	2012	370	89.0	7.3	–	–	–	2.3	1.4
	2021	380	87.1	5.4	–	–	–	5.7	1.8
ASMA	2012	366	92.4	5.1	–	–	–	1.4	1.1
	2021	365	94.5	3.1	–	–	–	1.6	0.8
APCA	2012	283	90.6	6.9	NA	–	–	0.0	2.5
	2021	297	80.7	7.0	9.1	–	–	1.7	1.5
aCL	2012	1,439	–	73.2	25.4	1.4	0.0	0.0	–
	2021	1,558	–	27.0	40.0	23.9	9.1	0.0	–
β2GPI	2012	1,992	–	33.8	15.0	1.2	50.0	0.0	–
	2021	2,664	–	21.5	42.2	26.0	10.3	0.0	0.0
RF	2012	343	–	15.2	–	0.3	1.2	0.0	83.3
	2021	310	–	20.6	–	2.3	0.3	0.0	76.8
ACPA	2012	322	–	32.9	56.5	6.8	0.0	1.6	2.2
	2021	409	–	13.0	49.9	29.3	0.0	7.1	0.7

ANA, anti-nuclear; dsDNA, anti-dsDNA; ENA, antiextractable nuclear antigen; ANCA, anti-neutrophil cytoplasm; GBM, anti-glomerular basement membrane; AMA, mitochondrial; LKM, liver-kidneymicrosomal; ASMA, smooth muscle; APCA; aCL, anticardiolipin; β2GPI, β2glycoprotein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies.

Table 2: Comparison between 2012 and 2021 of the number of autoimmunology laboratories participating in the UK NEQAS program and percentage of the methods used to detect autoantibodies in celiac disease.

		No.	ELISA%	FEIA%	CLIA%	Luminex%	Other%
AGA IgA	2012	90	46.7	41.1	0.0	4.4	7.8
	2021	39	46.2	46.2	0.0	2.5	5.1
AGA IgG	2012	76	48.7	40.8	0.0	3.9	6.6
	2021	35	45.7	45.7	0.0	2.9	5.7
DGP IgA	2012	127	33.1	48.0	2.4	2.4	14.1
	2021	178	16.3	48.9	23.0	9.6	2.2
DGP IgG	2012	146	31.5	54.8	1.4	1.4	10.9
	2021	246	14.2	54.1	22.8	6.9	2.0
tTG IgA	2012	417	47.5	44.4	3.4	0.9	3.8
	2021	489	18.2	51.9	21.1	7.4	1.4
tTG IgG	2012	NA	NA	NA	NA	NA	NA
	2021	277	16.2	57.1	17.3	7.6	1.8

AGA, anti-native gliadin; DGP, anti-deamidated gliadin peptides; tTG, anti-tissue transglutaminase.

Some ANA exercises designed as outliers, given the lower consensus achieved by participating laboratories, were observed. This may be attributed to different reasons such as assay limitations (i.e., failure in recognition of some cytoplasmic antigens not included in the profile of single SPA) or antigens not easily recognizable by the HEp-2 IIF method, like Jo1 and SSA/Ro60 (Figure 1).

In addition, even if UK NEQAS suggests that cytoplasmic patterns should be considered as positive, in line with the ICAP nomenclature system, some participants continue to classify cytoplasmic patterns as negative. Outlier values were also observed in anti-ENA results especially when the expected target was represented by antibodies to RNP, often mistaken with anti-Sm or anti-Jo1 (data not shown). Finally, in exercises related to CD diagnosis, consensus was not reached for several samples, especially those with borderline values of the involved analytes. Detailed information on the number of reports considered equivocal for CD exercises are given in Supplementary Table 2. Specifically, AGA IgA and anti-DGP IgA showed a high rate of equivocal results, each at 21.7 %. For EMA, the equivocal rate stood at 5 %, indicating a relatively lower frequency of discrepant results compared to AGA IgA and anti-DGP IgA. On the other hand, no equivocal results were observed for anti-tTG IgA, reflecting a high level of consistency for this analyte. Regarding the IgG isotype, the frequency of equivocal results was 11.7 % for AGA, 13.3 % for anti-DGP, 23.1 % for EMA and 15.4 % for anti-tTG.

Discussion

External quality assessment schemes provide the means for individual laboratories to compare their proficiency with that of their peers [21, 22]. Moreover, EQA are fundamental tools to harmonize laboratory practices and identify issues where improvements are needed. The EQA programs are also in a unique position to monitor trends in overall laboratory performance and the percentage of different technologies utilized.

Evolution of technologies

When looking at the methods used, although SPA using well characterized antigens gained widespread adoption in autoimmunity laboratories, they did not undermine the use

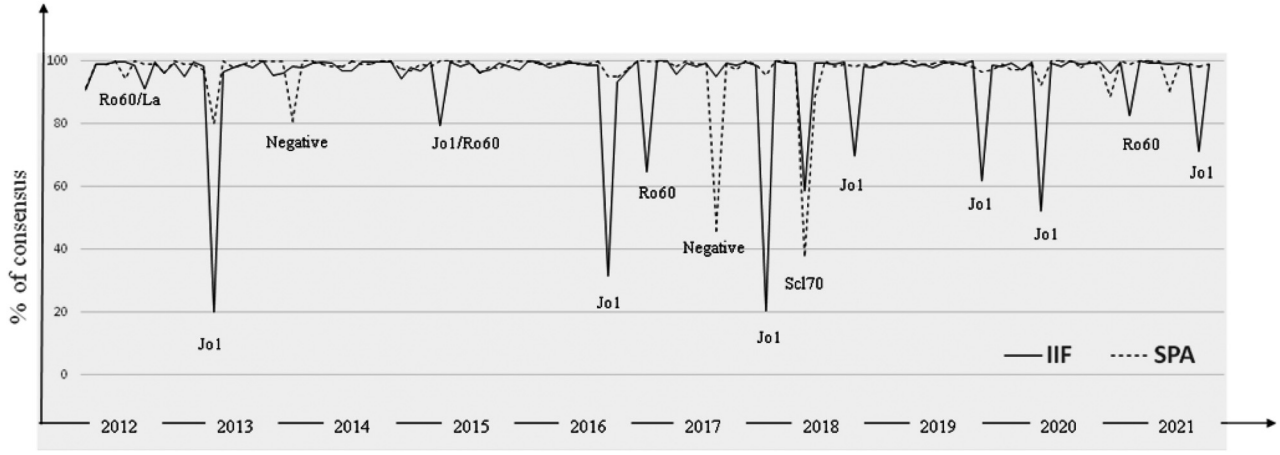


Figure 1: Line chart of consensus among participating laboratories in UK NEQAS ANA exercises from 2012 to 2021. The x-axis depicts the sequence of specimens per year to detect ANA using indirect immunofluorescence (IIF) (solid line) or solid-phase assays (SPA) (dotted line); the y-axis shows the percentage of consensus returned by laboratories. The spikes, identified by the corresponding autoantibodies, show the lower level of consensus achieved.

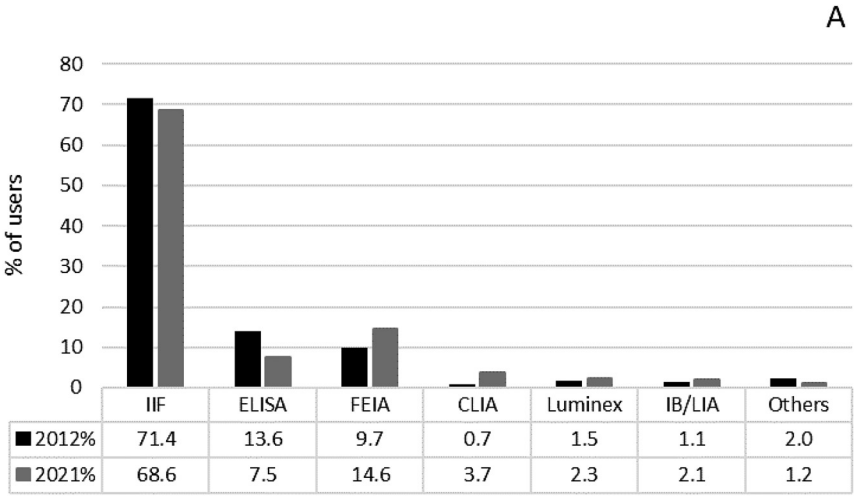
of IIF (Figure 2A). IIF on HEp-2 cell substrate was confirmed as the method of choice for ANA measurement for the majority of participants. Murine substrates were completely abandoned. In contrast, a slight increase in the use of HEp-2000 cells was observed, likely to overcome the low sensitivity of the HEp-2 substrate for the detection of anti-SSA/Ro60 antibodies.

Among SPA, FEIA is currently the most used method to search for APCA, ACPA and for antibodies to MPO, PR3, GBM, ENA, centromere, aCL and β2GPI. ELISA use dropped significantly, and was replaced by new automated platforms (Figure 2B), probably because these technologies are faster, random-access, have a greater measurement range, and may provide simultaneous evaluation of multiple antibodies [23, 24]. In the decade considered, IB-based immunoassays increased, albeit slightly, their use in detecting almost all antibodies, probably because they are used to confirm positive findings in screening tests performed with IIF or with solid phase methods [22, 25]. Complications related to radioactivity have led to the reduction of immunoassay used to detect anti-dsDNA antibodies (Farr technique), which are the only antibodies still searched for by this method.

These data are consistent with a recent survey conducted in Italy by our group, showing ELISA rapidly losing ground while methods such as CLIA, FEIA and Luminex have gained popularity [26], confirming that new technologies have been globally integrated in the fast-changing world of autoimmune diagnostics. Hence, early analytical methods were replaced by multiplex platforms enabling the simultaneous detection of several antibodies and the quantitative measurement of serum antibody concentration, useful for clinical monitoring of patients [1, 3, 23, 27].

Diagnostic performance of the methods

Despite heterogeneity, the overall diagnostic performance of the technologies showed decent comparability with



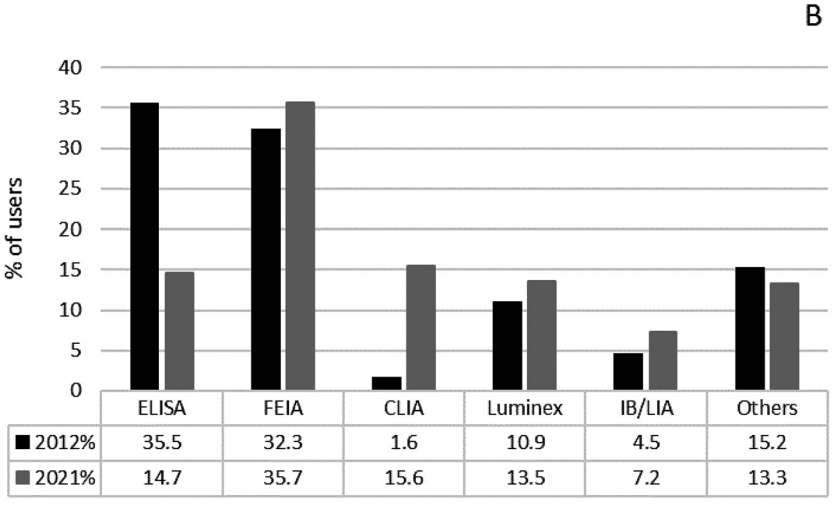


Figure 2: Overview of the changes in analytical methods used to detect autoantibodies by laboratories participating in the UK NEQAS programs from 2012 to 2021. Each column value represents the mean percentage of laboratories using a given method for all the antibodies studied. (A) Detailed comparison is provided for the various methods used, specifically focusing on those where IIF is an available method. These antibodies include ANA, anti-dsDNA, APCA, anti-LKM, AMA, ANCA and anti-GBM. (B) Focus shifts to changes in methodologies for analyses where IIF is not employed. These include tests for antibodies to ENA, centromere, aCL, β2GPI, tTG IgA and IgG, AGA IgA and IgG, anti-DGP IgA and IgG, RF and ACPA.

substantial inter-laboratory agreement. However, even if good consensus (compliance with the expected target) was observed for most of the EQA samples, some outliers showing lower consensus among participants, were evidenced in certain specific cycles. Such instances include the SSA/Ro60 antigen which is under-represented in the HEp-2 cells; this has limited sensitivity when the detection of antibodies against this antigen was performed by IIF. For the same reason, since most of the SPA miss most cytoplasmic antigens, their related antibodies were under-detected by laboratories using SPA. In addition, when antibodies to cytoplasmic antigens are involved, no uniform negative or positive classification emerged. Concurrently, strides have been made in harmonizing results following the introduction of the International Consensus for ANA Pattern (ICAP) nomenclature in 2016 within the UK NEQAS scheme. Though UK NEQAS reminds participants that mitotic and cytoplasmic patterns should be recorded as ANA positive, a number of laboratories still classify cytoplasmic patterns as ANA negative. Adding a comment underlining the importance of this shift may contribute to reducing the number or misclassified cytoplasmic patterns in the future. Similarly, in some EQA cycles, where the antigenic target was represented by RNP or Jo1, misinterpretation with Sm and SSA/Ro60, respectively, occurred. In the absence of a reference method for such antibodies, and the missing data about the manufacturing technique to obtain single antigens, how to classify discrepant results remains an issue.

Regarding antibodies that can be detected by IIF on liver-kidney-stomach triple tissue, no critical issues emerged but a slightly higher incidence of errors for ASMA and APCA, which proved to be the most complex tests at an interpretative level.

Interestingly, a high frequency of reports with lack of consensus on the target response was observed for celiac disease. This observation, which is not restricted to CD serology, offers the opportunity to address a controversial aspect in the reporting system. Managing how to report an EQA result which falls within the equivocal range of an assay has been the subject of frequently asked questions to the provider. To this end, in May 2023 UK NEQAS sent a communication instructing how to manage these cases since “equivocal” is not an available option when returning EQA results. The provider suggested submitting the quantitative data, selecting positive as the qualitative response and adding an appropriate comment in the comments box (e.g., result within equivocal range, reported as positive). Moreover, for all quantitative assays, it would be useful to know the cutoff value for each individual method to better interpret cases in which the sample has been considered equivocal. Finally, from our data, it is interesting to note how in 2021 as high as 10 % of laboratories still performed the AGA test even if these antibodies had clearly been declared obsolete [28] and were no longer recommended by the international guidelines for the diagnosis of celiac disease [29]. We were unsure whether their use by some laboratories was related to a particular clinical question (non celiac gluten sensitivity?) or to inappropriate requests by clinicians.

Since the results analyzed were anonymized, we were also unable to determine whether repeated inconsistent results were produced by the same laboratories. For the same reason, we were unable to analyze performance of the single countries. However, it is important to underline how this survey is mostly a snapshot of the European situation, as distribution of participants revealed that 31 % of laboratories were located in the UK, 54 % in the rest of Europe and only 15 % in the rest of the world.

In conclusion, data from this survey confirm, with few exceptions, that despite the evolution of technologies and in particular the introduction of CLIA methods, answers complying with expected responses have remained very similar (there was only a slight improvement) in the 10 years studied. Even if the standardization process still has a considerable distance to cover, the period analyzed revealed that the ever-expanding range of autoantibodies, coupled with the introduction of new tests, methodologies and automated platforms, has brought significant changes to autoimmune diagnostics.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Use of Large Language Models, AI and Machine Learning Tools: None declared.
Conflict of interests: The authors state no conflict of interest.
Research funding: None declared.
Data availability: Not applicable.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/cclm-2024-0781).