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Present and future in the diagnosis and treatment of autoimmune diseases

THE VIEW FROM THE LAB

Conference on Autoimmunity organized by Grifols, April 13, 2015, Santiago de Chile

GRIFOLS

PRESENT AND FUTURE FOR THE DIAGNOSIS AND TREATMENT OF AUTOIMMUNE DISEASES

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PRESENT AND FUTURE FOR THE DIAGNOSIS AND TREATMENT OF AUTOIMMUNE DISEASES

THE VIEW FROM THE LAB

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Challenges and outlooks in the diagnosis of autoimmune diseases

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MARKET PROSPECTS FOR DIAGNOSTIC MEDICINE

The field of diagnostic medicine has developed considerably in recent years and now includes a large number of methods and techniques for improving patient care. Diagnostic medicine includes screening tests, diagnostic confirmation and prognosis, disease monitoring and determining therapeutic drug levels. Although such work has been carried out in clinical laboratories for some time, the challenge has grown with respect to therapeutic drug monitoring, which is set to be fastest developing area of diagnostic medicine in the near future.

CHALLENGES FOR THE LAB AND IN VITRO DIAGNOSTIC INDUSTRY

One of the key trends facing the health and diagnostic markets in many countries is ageing population and the consequent appearance of chronic and other diseases of varying prevalence, all likely to become an increasingly frequent reality (**Table 1**). At the same time, clinicians in many countries are under increasing pressure to either control or reduce medical costs. For example, the Fondo Nacional de Salud (Chilean National Health System (FONASA)) has seen its percentage expenditure steadily cut, reducing the budget for patient care. On top of this, the demand for lab testing has risen considerably, mainly due to clinicians' growing reliance on such tests, as well as imaging and pathological anatomy studies. Numerous publications show that 60-80% of medical decisions are based on lab studies.[1] There is also a move towards testing for early detection, a significant advance as it keeps costs under control, while preventing illness and long-term complications for patients. Finally, one of the latest trends in healthcare is personalized medicine.

According to data from the Departamento de Estadísticas e Información de Salud (Department of Health Statistics and Information (DEIS)) in the Chilean Government's Ministry of Health, life expectancy for women in Chile in 2015 was over 80, slightly lower for men.[2] However, by 2025 the average age will be approaching 80, making Chilean life expectancy one of the highest in the region. Furthermore, according to the graph based on the United States Census Bureau International Database, the population pyramid forecast for 2050 in Chile shows the 55-60 age group as the largest and predicts a steadily ageing population, even exceeding 100.[3] This is the reverse of the current pyramid, whose largest population group is younger (from 20 to 30). Such a scenario brings with it a number of highly significant challenges, not just for test labs, but for all health departments.

Figures from the Chilean Government's Health Ministry DEIS Remsas database show that in 2012 the Chilean private sector performed around 28 million clinical lab tests on approximately 20% of the population.[4] This total has been growing steadily and is expected to continue in the future, particularly in the fields of hormone, genetic and immunological testing.

Table 1. Trends in the health-diagnostics market

Ageing population/rising life expectancy • Chronic diseases
Cost reduction/control
Rise in laboratory tests • 60-80% of medical decisions are based on tests • Increase in early detection tests
Personalized medicine

It is worth reiterating how the clinical approach in medicine has changed in recent years (**Figure 1**). Up to the 1990s, medicine was largely empirical; few studies were based on objectives-related methods. At that time, therapeutic and diagnostic decisions were largely based on tradition, theoretical reasoning and occasionally just anecdote, one-off cases and highly personalized activities supported essentially by physicians' experience. The year 1990 saw the beginning of revolutionary movement in medicine: evidence-based medicine (EBM). This spread during the following 20 years up to 2010 and has remained an extremely useful tool for improving efforts in healthcare and multidisciplinary work. In EBM, all decisions and protocols are based on clinical trials, the most frequent being randomized, double-blind multicenter trials, which continue to contribute enormously to healthcare. Finally, 2010 saw a new trend develop known as personalized medicine, an answer to a number of problems yet unsolved by EBM. Personalized medicine splits individuals into smaller categories to provide more individualized diagnoses and therapy. These three approaches to medicine will coexist in the future, and significant improvements will be seen in each as their use continues.

One of the greatest achievements of EBM is the publication of a large number of guidelines that have improved morbidity and mortality in many patients, most notably the 1990 guidelines on asthma treatment[5,6] or the guidelines on preventing thrombembolism in post-surgical patients. In addition, numerous protocols, studies and other guidelines including diagnostic tests have been published, helping improve medical practice. In Chile, for instance, through its Explicit Health Guarantees (Garantías Explícitas en Salud (GES)) plan, whose aim is to provide the public with four important health guarantees, the Ministry of Health has developed a number of guidelines for different diseases prioritized by the State due to their medical and social impact.

These clinical guidelines, known as GES (formerly AUGE), currently cover three immunological diseases: rheumatoid arthritis, juvenile idiopathic arthritis

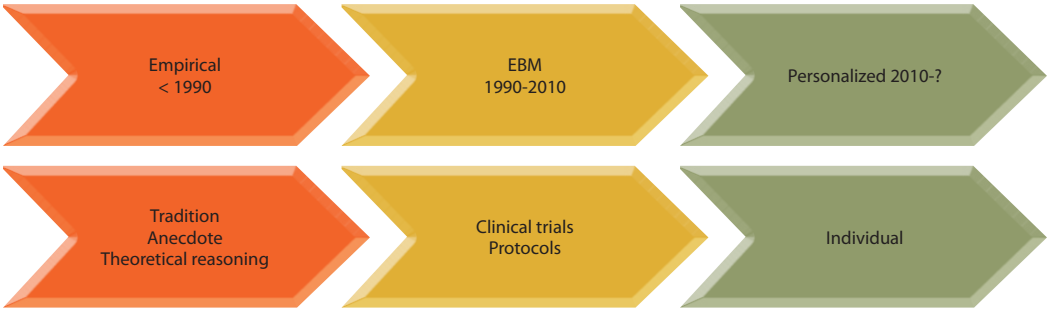

Figure 1. Changing trends in health and diagnostic medicine.
EBM: evidence-based medicine.

and systemic lupus erythematosus, included in the GES list in 2007, 2010 and 2013, respectively.[7-9] Thus, the GES guidelines on diagnosing rheumatoid arthritis provide a list of tests for a minimum diagnosis. They also mention tests to establish and assess prognosis, including a list of clinical elements as well as lab tests, covering general tests and others more specific to autoimmune diseases and genetic markers, which are likely to increase in coming years. Finally, the guidelines include a table of general tests, mainly for therapeutic monitoring, facilitating greater personalization in medicine for rheumatic patients.

However, EBM guidelines become obsolete at an ever faster rate. As in other areas of science, medical knowledge multiplies increasingly quickly and our current knowledge is expected have doubled by 2020 or 2050.

Despite its undoubted scientific impact, EBM has a number of problems and there are even signs suggesting it is in crisis, as discussed in a publication by the *British Medical Journal* in 2014.[10] The sheer volume of guidelines, publications and information has become an obstacle to their use in daily practice. Furthermore, the term "evidence-based" has been inappropriately used on a number of occasions, due to interests other than pure knowledge, which ought to be known when assessing studies. Another drawback is that statistical benefits for large populations are not necessarily useful for individuals. Equally, the volume of protocols means therapies and diagnostic methods are sometimes rather inflexibly employed, preventing personalization in clinical practice. Finally, there are some situations, such as patients with more than one illness, where evidence-based directives are of little help as studies often remove such cases.

In response to this crisis, the BMJ publication mentions a list of possible actions. It states that EBM must keep the patient as its central focus and clinical trials should ideally be free of interests or provide a detailed statement on conflicts of interests. Such studies should also be high quality, with their relevance and usefulness to diagnostic medicine clearly discernible.

The growing trend in personalized medicine is a response to these problems with EBM. It basically involves taking into consideration individual genetics and personal and family histories and using diagnostic medicine tools, such as lab and imaging tests, to make patients the focus of clinical and healthcare activity. Within personalized medicine, there are additional moves towards therapies supported by lab tests for monitoring. This is a significant change, which will continue to spread in the near future and one in which clinical labs must play their part, as it will considerably improve patients' functioning, quality of life and safety while ensuring they receive increasingly effective treatments with fewer side effects. For instance, the current market for cancer diagnostic tests and paired drugs was valued at $1.14 billion dollars in 2013 in the United States alone. At the same time, a large number of joint-ventures between large companies in both pharmaceutics and diagnostics have been set up to create such paired tests to the benefit of patients. In 2010, there were already 25 such joint-ventures, mainly involving Roche and Abbott and other large companies in the field of diagnostics.

PERSPECTIVES AND CHALLENGES OF AUTOIMMUNE DISEASES IN THE LABORATORY

The challenge posed by autoimmune diseases to laboratories is considerable, as they affect a large number of tissues and parenchymas. **Figure 2** summarizes different autoimmune diseases categorized by system: central nervous system and gastrointestinal diseases;

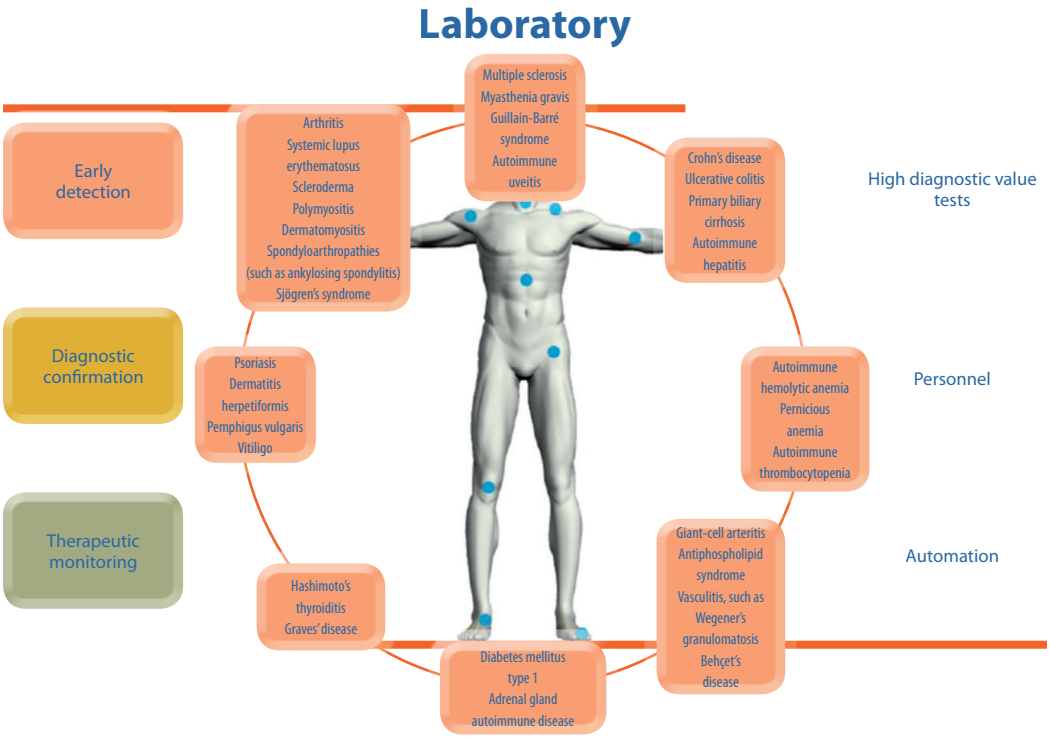

Figure 2. Autoimmune diseases: a challenge for labs.

blood diseases and others affecting the blood vessels (such as vasculitis); gland disorders which can be divided into two groups (highly significant and high prevalence worldwide); skin disorders; and finally connective tissue disorders. Autoimmune diseases represent a challenge not just for clinicians, rheumatologists in particular, but also for the lab, as large numbers of tests are required to support clinical activity. For instance, an early detection method is essential to permit both secondary prevention of autoimmune diseases and avoid the long-term complications that greatly limit people's quality of life and activity, especially at their most productive age. Secondly, tests are needed to resolve issues in diagnosis, which is often difficult to confirm. Establishing a diagnosis can take years for some diseases, posing problems for patients, clinicians and the lab. On top of this, there is now the added task of therapeutic drug monitoring.

APPROACHES TO THE CHALLENGES

Autoimmune diseases pose a number of additional challenges for the lab (**Figure 2**). One such challenge lies in choosing the test with the greatest diagnostic value out of the large number of test menus now available. The goal is to have a few good tests, although sometimes even defining or choosing them is not simple. In addition, it is essential to have highly qualified staff, experienced in these lab tests and who also take a holistic view of the patient, so that labs can offer advice and help in decision-making. Finally, another important issue is automation, a key factor in process standardiza-

tion, reducing the errors and meeting challenges such as a high workload. Automation also frees up highly qualified lab staff to work on more important tasks.

In short, evidence is required on the usefulness of diagnostic tests. There should also be assessments for prognostic tests, which play an important role in answering clinicians' concerns. The lab must also contribute to the safety and efficacy of clinical interventions. It is very important for diagnostic medicine to collaborate in this area, while also containing costs and assessing trends in the cost-effectiveness of diagnostic methods and therapies (e.g. establishing the most cost-effective frequency for therapeutic drug monitoring). Finally, it is worth noting the importance of multidisciplinary work, establishing further alliances in laboratory and clinical settings, while maintaining focus on the patient.

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Update on the diagnosis of autoimmune diseases

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INTRODUCTION: AUTOIMMUNE DISEASES

The diagnosis of autoimmune diseases represents a major challenge for clinical laboratories, as it requires definition of techniques, when to use them and their degree of complexity. Autoimmune diseases affect both organs and systems, thus there is a broad range of targets, further complicating lab diagnosis. Autoimmunity is produced by loss of tolerance to the body's own antigens and an adaptive autoantibody immune response. The causes are still the subject of research, but there are a number of sources known to eventually produce autoimmune disease, including hormones, drugs, infectious agents, genetic predisposition (sometimes associated with human leukocyte antigens (HLA)), all immunological factors related to hormone deficiencies and primary and secondary defects and environmental factors.

In autoimmune diseases, autoantibodies are continually active, attacking the individual. Some are specific and associated with a particular disease, but autoimmunity also includes another phenomenon known as multiple autoimmune syndrome and autoantibody overlap syndrome. Indeed, it is possible for patients to start with an organ-specific disease which develops into a systemic disease.

THE ROLE OF THE LABORATORY IN AUTOIMMUNITY

The importance of autoantibodies in the lab lies in their use as a diagnostic criterion in certain situations, while providing support for diagnosis in others;[1] hence correct detection in the lab is essential. There is a broad range of detectable autoantibodies and their numbers are increasing by the day. Consequently, laboratories and the market are moving towards more standardized processes, offering cost control, good reproducibility and coherent results.

Inadequate use of lab tests for antibody detection can lead clinicians to provide incorrect diagnoses or inadequate treatment, with the resulting increases in overall cost. As a result, expert committees are working worldwide to provide standardized guidelines for all fields of autoimmune disease and adequate lab testing for their study.

Given the challenge of autoantibody detection in autoimmune diseases, the first step in the lab is to choose the methodology. Today, the available options are the enzyme-ligand immunosorbent assay (ELISA), a quick, easily standardized technique, or immunofluorescence, which represents a major challenge to the lab.

THE USE OF IMMUNOFLUORESCENCE IN THE LAB

Whenever possible, labs have adopted the new, simpler diagnostic tests, such as Multiplex. Thus, while 20 years ago only four patterns were detectable by immunofluorescence, today many more can be detected, including homogeneous, membrane, speckled (coarse, fine, matrix) and cycle-dependent patterns, such as NuMA (nuclear-mitotic apparatus protein,

NuMA-1, -2 and -3), variation patterns with different cycles (as well as a PCNA (proliferating cell nuclear antigen)) and centromere, among others. The increasing availability of information pushed laboratories towards Multiplex technology. However, patients were being lost, because test sensitivity was being sacrificed. It is very important for the initial screening test to provide sufficient sensitivity; hence the American College of Rheumatology (ACR) position statement on the methodology of testing for antinuclear antibodies[2] states that the reference method is immunofluorescence, as it is the only one capable of simultaneously detecting between 100 and 150 possible antigens.

Immunofluorescence is a very valuable tool. It is the reference method and the technique to use when sensitivity is required, as it is much higher than in solid phase tests. The most frequently used cells for immunofluorescence assays are human epithelial type II cells (HEp-2 cells), which have approximately 100-150 possible autoantigens and permit the simultaneous description of nucleus and the whole cell pattern, as well as the antinuclear antibody titer. Unlike immunofluorescence, tests such as Multiplex and ELISA, even those that provide the highest number of antigens can only facilitate 10 antibodies. This mixture will never match those that can be found with HEp-2 cells. Clearly, lab results reports should also state the method used, as physicians need to know the power of the applied test, which should ideally match sensitivity with the lab cut-off values.

In the diagnostic process for autoimmune diseases, the initial screening test should be an antinuclear antibody in immunofluorescence followed by a second confirmation test using ELISA or another method.[3]

When performing the fluorescence test in the lab, it is important to bear in mind all factors affecting the result, such as the patient's antibodies (whose concentration, specificity, avidity and type must be measured), the substrate and the test conditions. Not all commercially available substrates are the same. Furthermore, it is necessary to guarantee that all phases of the cell cycle are present, as some patterns are only expressed in a particular phase; if the substrate lacks the right antigens, the correct patterns will fail to show up and the results will not be obtained as expected. Test conditions are also very important, so labs make every effort to ensure full standardization. These conditions include buffers, pH, incubation conditions and conjugate characteristics, along with other important variables such as origin, binding, storage, dilution, temperature and specificity. Previously, multi-purpose conjugates were used that included various types of immunoglobulins (Ig) in the same conjugate, specifically IgG-IgM-IgA, whereas now IgG alone is recommended, as specificity decreases when the others are used.

INTERNATIONAL GUIDELINES ON THE DIAGNOSIS OF AUTOIMMUNE DISEASES

A number of different international guidelines are now available for the diagnosis of autoimmune diseases in clinical laboratories. Previous recommendations were not sufficiently explicit; for instance the positions of the ACR[4] and other guidelines by the Clinical and Laboratory Standards Institute (CLSI) did not contain pattern descriptions.

Various countries started producing consensus documents, such as those of Europe,[5] Brazil[6] (a very thorough document now in its fourth edition), Argentina and Chile.[7] There are even local consensus documents for each country. These guidelines are extremely thorough and could provide the basis for developing ap-

propriate guidelines for each country that match local needs.

One of the most important documents currently available in this field is the International Recommendations for the Assessment of Autoantibodies.[8] This publication was produced by two expert committees from different places: the initiative known as the European Autoimmunity Standardisation Initiative (EASI) and the group including the International Union of Immunological Societies, World Health Organization, Arthritis Foundation and Centers for Disease Control and Prevention autoantibody standardization committee (IUIS/WHO/AF/CDC). The EASI initiative began over a decade ago with the aim of improving the diagnosis of systemic rheumatic autoimmune diseases and represents 15 European countries. The EASI expert committee not only included physicians and health specialists, but also lab professionals and scientists, all working exclusively on research into autoimmune diseases. The IUIS/WHO/AF/CDC group has been working since 1980 and covers all activities relating to autoantibody standardization.

The document provides 25 recommendations in 4 groups: 13 on antinuclear antibody immunofluorescence assays, 5 on deoxyribonucleic acid (DNA), 5 on extractable nuclear antigens and 2 on validations and local consensuses. **Table 1** summarizes the main recommendations in the International Recommendations document.[8] In general terms, the recommendations stress the importance of antinuclear antibodies for diagnosing autoimmune diseases, ensuring antigen differentiation and the use of an initial sensitive test, followed by a specific confirmation test.

Other important aspects covered in the document are appropriate terminology and communication between lab and clinician. The term "antinuclear antibody" is no longer considered technically correct, but it is proving difficult to change to the correct term, "anti-cell antibodies", which refers not only to the nucleus but to the rest of the cell as well: membrane, envelope, organelles, cytoplasm, and spindle apparatus. Also, when reporting results, labs should describe the nucleus and cytoplasm staining, including titers and patterns. Furthermore, labs should be capable of suggesting to physicians which test to carry out next, thus assisting diagnosis.

Table 1. Main recommendations for assessing cellular antigen antibodies known as antinuclear antibodies

The first-level test for diagnosing systemic rheumatic autoimmune diseases should be antinuclear antibody detection
Immunofluorescence is the reference method for antinuclear antibody screening. When another method is used, both negative and positive test results should be confirmed by immunofluorescence
Tests that are not based on this nuclear antigen principle should not be referred to as antinuclear antibody tests
All antinuclear antibody patterns, both nuclear and cytoplasmic, should be reported, in the latter case noting next to the result that it is cytoplasmic and stating the titer in both cases
Each lab should have established limits for the techniques they use. Hence the importance of local consensuses on cut-off values and lab standardization for the institution

THE INDIRECT IMMUNOFLUORESCENCE ASSAY FOR DETERMINING ANTINUCLEAR ANTIBODIES

The indirect immunofluorescence assay (IIFA), first described in 1950, is the reference method for deter-

Table 2. Sensitivity and specificity of the indirect immunofluorescence assay for detecting antinuclear antibodies in systemic lupus erythematosus (SLE)[12]

Disease	Dilution (cut-off value)	% Sensitivity	% Specificity
SLE	1:40	97.4	68.3
	1:80	97.4	87.6
	1:160	**94.7**	**95.0**
	1:320	86.8	96.7

Sensitivity: percentage of patients with the disease testing positive at the indicated cut-off value.
Specificity: percentage of normal individuals testing negative at the indicated cut-off value.
Adapted from Satoh et al. Mod Rheumatol 2009;19:219-28.

mining antinuclear antibodies, yet it has a number of limitations for the lab. Firstly, it is frequently performed manually, so the time required for the study is an important consideration. Secondly, it is limited by variables involved in the technique, such as demography, initial serum dilution, cut-off value, staff qualifications (they should be highly qualified) and the possibility of detecting healthy subjects with autoantibodies. Thirdly, it should be borne in mind that it is a less specific, though highly sensitive test, ideal for screening. Fourthly, the need for good interpretation and standardization in the lab is limiting. The cell lines used are HEp-2 from laryngeal cancer. HEp-2 cells are recommended, although a confirmation test must be performed after IIFA.

Choice of substrate is crucial, as each pattern can differ depending on the cell cycle and mitotic phase at the time. For example, only two dots can be clearly seen in the centriole pattern during the metaphase, whereas four can be seen when the cell is in the prophase or interphase. Furthermore, the location of the dots in the nucleus or cytoplasm is also significant. Thus, to ascertain whether the pattern is indeed as suspected, it is essential to know precisely which phases are relevant and to ensure that they will be visible with the chosen substrate. Although cells in the metaphase are very important, as it is the only phase in which all depolymerised antigens are available, it is not the only cell that should be assessed in interpretation.

With regard to titer, results are more likely to be clinically relevant when values of less than 1:160 are reported.[9] The recommendation for laboratories[10,11] is to make two dilutions simultaneously: 1:40 and 1:160, or 1:80 and 1:160. A 1:40 dilution can produce false positives in approximately 32% of the general population, while at a 1:160 dilution this figure drops to 5%.[11] **Table 2** shows how processing a sample at a 1:40 dilution produces high sensitivity (97.4%), but lower specificity (68.3%) in the result. If analyzed at 1:80, sensitivity is maintained but specificity increases to 87.6%. However, with a dilution of 1:160, both values are within the 95th percentile, thus providing sufficient sensitivity and specificity to guarantee detection of real patients with the disease and no false positives.[12]

The lab should also bear in mind the type of microscope used, as the lamps (halogen, mercury and LED

technology) and light intensity, available filters, objective quality and frequency of maintenance can all differ.

Consideration of the pH of the water used (ensuring it guarantees compliance for the whole procedure), strict observance of steps throughout the whole process, quality controls (both internal and external) and end-point control are also important.

Centers should check the values obtained under their conditions, as they can vary greatly from factory to laboratory, and such checks help in process standardization. Sample collection and storage is also a key point, and correct storage must be guaranteed. Substrate quality, conjugate isotope specificity, visualization of all phases of mitosis and using IgG as conjugate are all undoubtedly important.

To avoid discrepancies, each laboratory should always state the techniques and methodologies they use, thus enabling physicians to assess changes in their patients. Labs should also use a low positive control when performing the procedure manually. In fact, this is one of the most important controls as intensity is eventually assessed each day against this value. This prevents the false positives or negatives that can easily occur when technicians have been reading the microscope for a long time. In addition, the conjugate should be IgG marked with fluorescein or another previously validated new-generation fluorochrome. Dilution is equally important. The recommended cut-off value for dilution is 1:160 in the diagnosis of systemic rheumatic autoimmune diseases. However, negative results do not rule out the disease, so clinical assessment is required in the final diagnosis. There is no consensus for children under 16 years of age and existing literature is not sufficient to make a decision in this area; hence the dilution should be 1:40. It must be borne in mind that titer and disease are always related. Similarly, labs have information that could prove valuable to the clinician, so there should always be a way for them to write notes on suspicions and suggesting tests when delivering results.

RECOGNIZED ANTINUCLEAR ANTIBODY PATTERNS

Classic patterns identified by immunofluorescence in the nuclear group include homogeneous, thick speckled, fine speckled, centromere and nucleolar. Cytoplasm JO-1 ribosome-signal recognition particle (SRP) complex and mitochondria are the most common cytoplasmic patterns. However, other patterns are not so frequent but should be reported by the lab. These include peripheral, nuclear membrane, dense fine speckled, PCNA, granular nucleolar, multiple and few nuclear dots, centriole and mitotic spindle apparatus (MSA). These are all nuclear patterns and it should be remembered that they are sometimes difficult to differentiate in the lab. For example, dense fine speckled is often mistaken for homogeneous, but they have different characteristics. The dense fine speckled pattern is very frequent; it has a much greater occurrence than labs generally assume and can also occur among healthy subjects or in other inflammatory conditions, although specific tests are available for confirmation and ruling out the homogeneous pattern. Finally, less common cytoplasmic patterns include all dotted patterns, such as endosomes, peroxisomes and GW bodies (which contain protein GW182), the Golgi apparatus and cytoplasmic fibers, such as actin, cytokeratin, tropomyosin, vimentin, and desmin.

Figure 1 classifies some of the antinuclear antibody patterns by cell location, including some of their main observable characteristics. In this classification, the

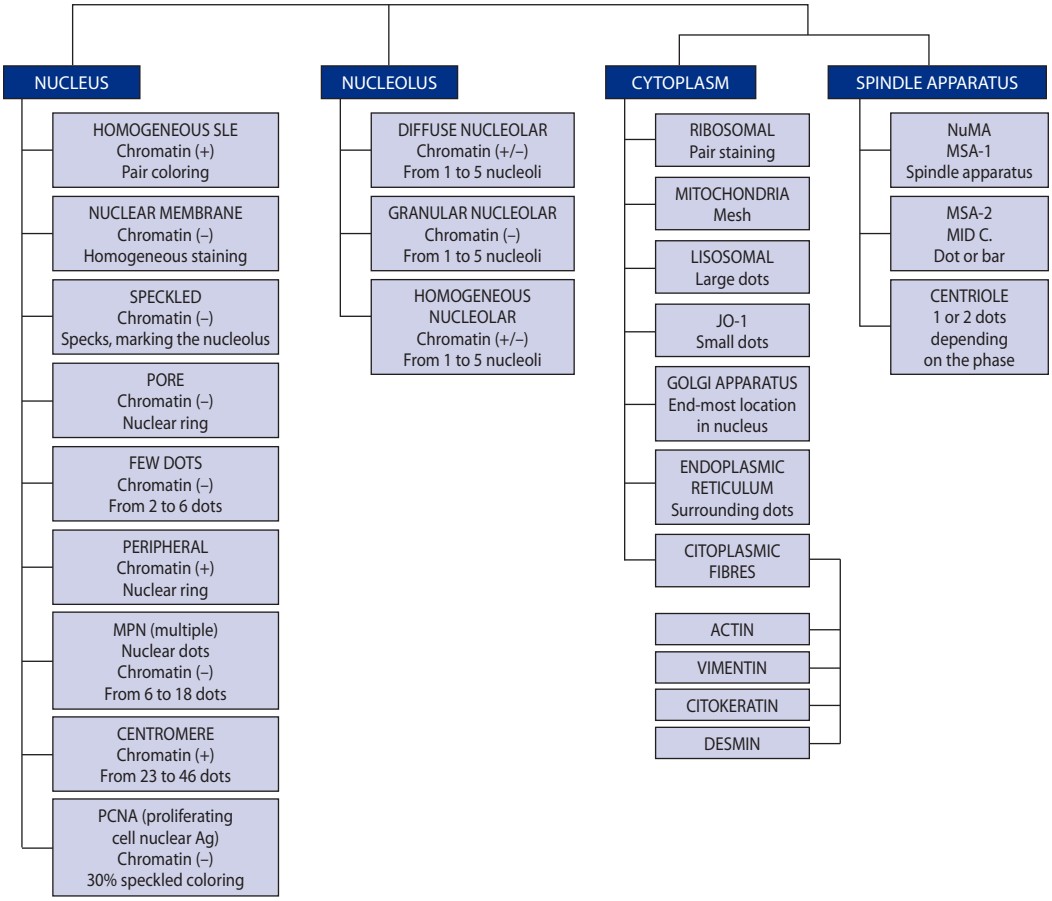

Figure 1. Diagram of the different nuclear and cytoplasmic patterns detectable by indirect immunofluorescence assay.

presence of the positive or negative metaphase can help greatly in nucleus-related aspects.

The heavier workload in labs after switching back to immunofluorescence led to the development of HELIOS analyzer, a fully automated device that can perform the whole analysis process without intervention from a technician, thanks to its complete hardware and software integration. HELIOS technology solves almost all the difficulties with immunofluorescence, such as transcription, time, reproducibility, traceability and, obviously, process standardization.

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HELIOS: the automated analyzer for reading and processing indirect immuno-fluorescence

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The HELIOS analyzer is the only fully automated device on the market that can perform the whole indirect immunofluorescence analysis process without the intervention of a technician, thanks to its complete hardware and software integration. Thus, sample processing and reading are integrated, avoiding the need for slide transfer, use of cover slips and reprogramming with both. The HELIOS analyzer was developed in Wendelsheim (Germany) from the complete platform already on the market, the HELMED, installed in 300 facilities worldwide.

The device has a processing capacity of 190 bar-coded samples and 20 slides with four different assays. It can simultaneously analyze antinuclear antibodies (ANA), anti-neutrophil cytoplasmic antibodies (ANCA) in ethanol and formalin, anti-deoxyribonucleic acid (DNA) antibodies in *Crithidia luciliae* and tissues. It includes camera, microscope, a barcode reader for samples and another for slides, thereby guaranteeing full traceability throughout the process. The barcode on commercial slides provides information on type of substrate, number of wells, batch and expiry date, which is all stored in the system thanks to the barcode reader. The LED microscope is motorized, so it is focused by the machine and not by the technician. Although the objective magnification is only 20×, it provides a zoom of up to 600×. In addition, the camera permits high-sensitivity digital images to be captured.

The analysis process consists of three simple steps. In the first, the technician just loads the slide processor, thus freeing up time for other lab activities. The machine estimates the required volume for each of the reagents and the processing time. When the machine completes the processing, it automatically switches to image capture and pre-classification, distinguishing between positive and negative samples. It can focus on up to 10 zones in each well when taking photos, although experience suggests three images are often enough to easily decode the pattern. Furthermore, reading times are very fast. Taking three photos per slide requires only five and a half minutes for ANA, AND and ANCA, while it is the only machine on the market able to capture tissue images, producing panoramic photos from 64 images. This process takes much longer, requiring approximately 20 minutes per slide. Thus, busy labs should analyze this substrate last or even leave the apparatus to take photos overnight. Automatic pre-classification is performed using the cut-off value, as set by the machine. This means the process is fully standardized, because although the value can be set to lab requirements, it guarantees that the same intensity of fluorescence is measured in each analysis. The end product is a file containing all the information required by the specialist to review the results, with the option of adding comments on required follow-up procedures.

The apparatus includes a comprehensive library with over 20 patterns which the expert can use for comparison and identification. It also recognizes all negative results at once so that over 100 samples can be classified in less than 10 seconds. This function is very useful in labs which generally analyze routine samples not exclusively from autoimmune patients, as this

produces a high rate of negatives. Remote access to results is also available for multicenter labs, as all the machines can be connected to a single platform. This means experts can access results from all centers for remote validation.

One of the main advantages of the HELIOS analyzer is that it does not require cover slips or slide transfers. Titer can be detected according to the cut-off point, which can be set by the lab, thereby permitting revision of the slides and as many confirmations as required during the process. In addition, the apparatus is small and light, facilitating installation wherever it is needed. As neither a darkroom nor secondary instruments are required (other systems normally consist of a processor and reader), not much space is required for the device.

The machine also provides high-definition on-screen images. All cells can be easily observed, with the different cell phases clearly distinguishable. The images are also a valuable and practical tool for exchanging results with colleagues and providing training in labs with specialists on rotation. Key features include the ability to distinguish between positive and negative antinuclear antibodies and ANCA and the ability to take photos of the *Crithidia luciliae* substrate for DNA, triple tissue and anti-endomysial antibodies (EMA).

The substrates available on the market for HELIOS and the antibodies they covers are: HEp-2 for ANA, *Crithidia luciliae* for DNA, granulocytes for ANCA, triple liver/kidney/stomach tissue (LKS) for anti-mitochondrial antibodies (AMA)/anti-smooth muscle antibodies (ASMA)/anti-parietal cell antibodies (APCA), anti-liver/kidney microsomal (LKM) antibodies and primate esophagus for EMA. The triple tissue LKS substrate, which permits autoantibodies to be detected simultaneously in the stomach, liver and kidney, comes in two presentations: rolled and separate; both permit excellent interpretation by HELIOS, but the rolled presentation is easier to use for photography.

The wide range of substrate cells offers the advantages of providing cells for all phases of the cell cycle, ensuring the cell size is suitable for analysis and that the mounting liquid supplied with each substrate and automatically added by the machine permits the nucleus and cytoplasm patterns to be defined while maintaining immunofluorescence for longer.

The development of HELIOS technology solves almost all the issues with immunofluorescence: transcription, with potential problems in writing; run time, which is shorter and thus frees up staff for other diagnostic areas; reproducibility, which is guaranteed; traceability; and, obviously, process standardization.

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Laboratory experience with the HELIOS analyzer

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INDIRECT IMMUNOFLUORESCENCE IN THE DIAGNOSTIC LAB

Indirect immunofluorescence is a key technique in immunological diagnosis of autoimmune diseases. Our lab carries out different tests in this field, such as for antinuclear antibodies (ANA), now known as cytoplasmic antinuclear antibodies and representing approximately 90% of the autoantibodies analyzed compared to others such as anti-double-stranded deoxyribonucleic acid (DNA) antibodies (anti-dsDNA), anti-endomysial antibodies (EMA), anti-smooth muscle antibodies (ASMA), anti-parietal cell antibodies (APCA), anti-liver/kidney microsomal type 1 antibodies (LKM-1), anti-neutrophil cytoplasmic antibodies (ANCA), anti-glomerular basement membrane antibodies (a-GBM) and anti-pancreatic islet cell antibodies (ICA).

Antinuclear cytoplasmic antibodies are the most frequently used and reflect the improvements made to the indirect immunofluorescence assay (IIFA), thanks to automated techniques implemented in recent years. Not only IIFA analysis of ANAs but also that of a-GBMs can be automated thanks to their incubation protocol.

Figure 1 provides a time line from 2006 to 2014 showing changes in indirect immunofluorescence work in the lab. Initially, all immunofluorescence techniques used manual dilutions, a time-consuming process for lab staff, and fluorescence observation by conventional fluorescence microscopy using mercury lamps. In 2008, aware of the potential harm caused by toxicity, the "Mercury-free Hospitals" campaign was launched, whereby all hospitals were required to gradually withdraw their devices containing mercury. The following year, Grifols launched the HELMED, which considerably optimized laboratory staff time by automating dilutions. Use of Evans blue contrast staining was phased out by the end of 2013 as HELMED does not require such stains, although the main reason was the highly teratogenic nature of Evans blue, posing a permanent risk to lab technicians. Finally, the HELIOS device was introduced in 2014, providing a number of additional advantages to lab staff.

THE HELIOS DEVICE

The first thing noticeable about the HELIOS device is that the microscope, with LED lighting technology, is part of the apparatus itself. This means the machine can take photos, whose number is chosen in direct relation to the required image resolution, as although the image is provided with a fixed magnification of 20×, the apparatus can print at a greater magnification with a digital zoom of up to 600×. Another new development over the HELMED is the sensor that counts test slides with a barcode containing the batch, so it can be related to the conjugate, reagent and quality control batches.

In addition, the equipment can produce dilutions from 1:1 to 1:25,600 in one or two steps. However, the most striking new feature of this equipment is the software, which includes a comparison pattern or model providing inexperienced operators or trainees learning

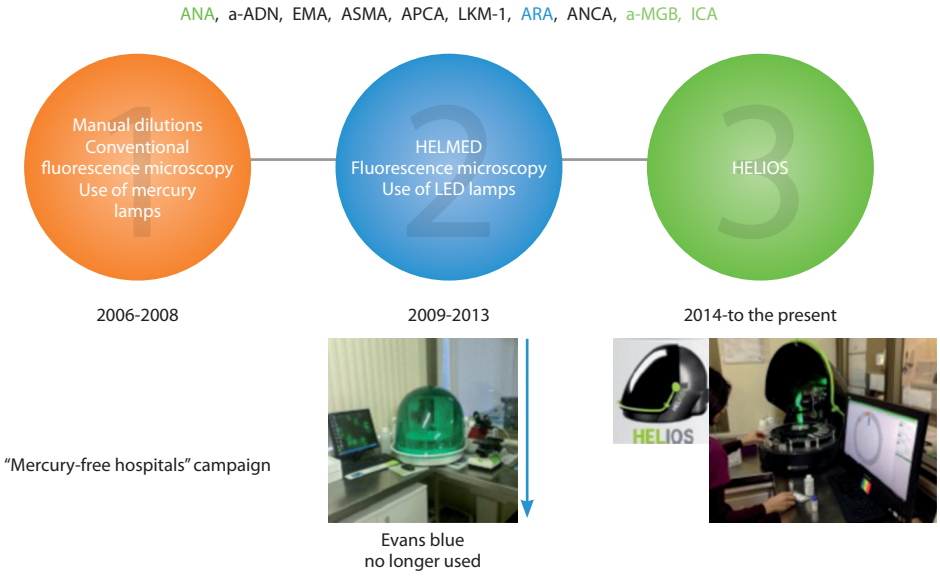

Figure 1. Time-line for working with indirect immunofluorescence in the diagnostic lab from 2006 to 2014.

a-DNA: anti-deoxyribonucleic acid antibodies; a-GBM: anti-glomerular basement membrane antibodies; ANA: antinuclear antibodies; ANCA: anti-neutrophil cytoplasmic antibodies; APCA: anti-parietal cell antibodies; ARA: anti-reticulin antibodies; ASMA: anti-smooth muscle antibodies; EMA: anti-endomysial antibodies; ICA: anti-pancreatic islet cell antibodies; LKM-1: anti-liver kidney microsomal antibodies type 1.

to read these autoantibodies by immunofluorescence with the opportunity to gain significant experience in a short space of time. The machine can also be operated remotely, loading work lists from elsewhere on the network, for subsequent image analysis and validation by a computer, mobile phone or other device.

OPERATIONAL ADVANTAGES OF HELIOS

One of the main advantages of the HELIOS system is shorter operator working times. Using HELMED, it took a day to produce the complete analysis, including loading samples, waiting for results and analyzing them with a conventional fluorescence microscope. Today, using ANA, DNA or ANCA techniques (the most frequently demanded tests), the process takes about half a day. Furthermore, the apparatus reduces response time, a feature that is highly valued by rheumatologists and ICU physicians, especially for hospitalized patients where ANA patterns and DNA results need to be known quickly.

The HELIOS incorporates an excellent automatic interpretation system; it projects an image and reports whether the result is positive or negative. Thus, when lab professionals have to provide a report urgently, they can view the image and simply corroborate the automatic interpretation. A minimal sample volume is required, which is particularly valuable for pediatric patients where punctures can prove difficult.

The immunofluorescence kit for HELIOS includes a code and quality control, the barcoded test slide and

a separate diluent to minimize the required amount of buffer solution (phosphate-buffered saline (PBS)). Another advantage over HELMED is that the amount of reagent is proportional to the amount of fluorescent stain used in each test; i.e. there will never be too much or too little reagent and control or conjugate will not have to be taken from other reagents, possibly from a different batch thereby rapidly reducing traceability. The machine also comes pre-programmed with all the kits; it is the apparatus and not the lab that uses them. Therefore, work can start in HELIOS on ANA and DNA, while the ANCA technique can be implemented at a later stage without the need for reconfiguration, as all the commercially available programs and kits for this system are already included in the software. Furthermore, if the internal microscope fails, the apparatus can work in HELMED mode.

HELIOS also facilitates internal quality control. Previously, when working with manual dilutions, all internal quality control data and variables relating to the interference with the antibody reaction, such as temperature, buffer solution pH (PBS) or its preparation, were entered manually. By contrast, the HELIOS machine does all this automatically. The data include operator name and all information stored in the barcode, such as kit batch number and expiry date, the slide batch number, the conjugate and the positive and negative controls. There is also a comments section to include all variables relating to internal quality control, such as room temperature, PBS pH and PBS preparation, among others. All this is logged in a database and the work sheet is printed out at the end of the process.

The software is intuitive and easy to use. It can be learned quickly by inexperienced operators, who can use the comparison model to quickly acquire adequate skill in fluorescence pattern recognition. This represents an excellent training tool in laboratories with a high turnover of recently qualified professionals and students.

The system delivers an image of the fluorescence pattern it detects so that the operator can superimpose a series of alternatives and identify the type of pattern. Although the machine has a database containing typical fluorescence patterns, labs can also create their own. Thus all the required patterns are available in a single folder, thereby facilitating correlation of fluorescence patterns with certain diseases, a particularly useful feature with regard to studies for publications. In addition, group analyses of fluorescence patterns can be performed, a particularly useful feature when dealing with mixed or confused patterns or ones that have not been found previously. Furthermore, when urgent or difficult to interpret tests are involved, the photo of the pattern can be sent to colleagues in the country's immunology network, thus speeding up the diagnostic process. Thus the system facilitates interaction with other colleagues and sharing experiences with other network users.

Apparatus maintenance is simple and similar to the HELMED. It only requires cleaning with isotonic solution at the start and end of the working day. Another key feature is that it is a desktop apparatus and easily installed in most labs, as it is small, weighs 33 kg and takes up minimal space. It also emits very little noise pollution, an important factor for professionals working in laboratories where numerous other machines are operating at the same time.

SPECIAL CASES WHEN WORKING WITH HELIOS

The HELIOS apparatus is well suited for working with ANA, ANCA and DNA. However, in the case of tissues, the manufacturer recommends taking a series of photos

(64, to be precise) to ensure good resolution. Due to the slowness in capturing tissue images, this type of photography requires longer than the other cases, taking up to several hours (e.g. loading the sample in the evening and obtaining the photos the following day), which is not ideal for a busy laboratory. The number of photos can be reduced to streamline the process, but at the expense of resolution, as the magnification and optical zoom are insufficient. In this case, backup from conventional fluorescence microscopy is required.

The mounting medium is provided by the device itself. This is a very low density medium differing somewhat from conventional media. Attention should be given to the air flow, as impurities can enter when adding the mounting medium. These generate fluorescence so the automatic microscope might stop taking automatic photos. If this occurs, the images come out completely green, the samples have to be reloaded and readings taken again of all the slides, with the resulting delay in results. Another drawback is that the views of the microscopic fields are predetermined for three fields and cannot be voluntarily modified in real time. The apparatus only permits the images to be viewed once they are all loaded.

One of the advantages of HELIOS is that when there is a defect in the internal microscope, it switches to the HELMED mode included in the apparatus, although the operator should be aware of activating the HELMED and HELIOS icons, as they are very similar and can easily be confused when working quickly. Finally, it is worth noting that technicians used to observing fluorescence in a darkroom with a conventional fluorescence microscope may require an adaptation period to accept there is no need for dark to observe the images provided by HELIOS, given that ambient lighting does not affect viewing.

In summary, implementing the HELIOS device is highly recommendable in a busy immunology lab. It optimizes response times for ANA, DNA and ANCA, although not to the same degree for tissues, where the feasibility of reducing the number of photos to improve response times should be assessed.

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