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Reflecting on a decade of the international consensus on ANA patterns (ICAP): Accomplishments and challenges from the perspective of the 7th ICAP workshop

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A B S T R A C T

The **I**nternational **C**onsensus on **A**NA **P**atterns (ICAP) is an ongoing international initiative dedicated to harmonizing technical and interpretation aspects of the HEp-2 IFA test. Comprised of internationally recognized experts in autoimmunity and HEp-2 IFA testing, ICAP has operated for the last 10 years by promoting accurate reading, interpretation, and reporting of HEp-2 IFA images by professionals involved in various areas related to autoimmune diseases, such as clinical diagnostic laboratories, academic research, IVD industry, and patient care. ICAP operates through continuous information exchange with the international community and encourages the participation of younger experts from all over the world. The 7th ICAP workshop has addressed several aspects that originated from this interaction with the international community and has effectively established objective goals and tasks to be delivered over the next two years. Some of these are outlined in this article, including the planning of three audio-visual educational modules to be posted at the www.anapattern.org website, the classification of two novel HEp-2 IFA patterns, the implementation of a project dedicated to continuously updating the information on the clinical and immunologic relevance of the HEp-2 IFA patterns, and the launch of two additional branches of the HEp-2 Clinical and Immunological (HEp-2 CIC) project.

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1. Introduction

Autoantibodies are serological biomarkers used worldwide as a screening tool with well-established utility in the research, diagnosis, classification, and phenotyping of systemic and organ-specific autoimmune diseases [1–3]. A wide spectrum of autoantibodies can be detected by the indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA), historically known as the antinuclear antibodies (ANA) test [4]. The HEp-2 IFA detects dozens of autoantibodies directed against numerous intracellular antigens and provides information on the serum levels and possible antigenic targets. The end-point titer obtained in classical immunofluorescence microscopy or the fluorescence intensity assessed by computer-assisted image analysis provides an estimate of the autoantibody serum levels [5,6]. The HEp-2 IFA staining pattern reflects the topographic distribution of the target autoantigens localized to various organelles and cellular domains across the cell cycle stages, which provides indirect evidence pointing to the possible molecular targets and the cognate autoantibody specificities in the sample. This feature of the HEp-2 IFA method has been instrumental in the characterization of a series of anti-cell autoantibodies, including those reacting with the centromeres [7], proliferating cell nuclear antigen (PCNA) [8,9], U3-ribonucleoprotein/fibrillarin [10,11], CENP-F [12], Cajal bodies [13], mitotic apparatus [14,15], rods and rings [16], and others.

The HEp-2 IFA test consists of a multi-stage methodology and is limited by considerable subjectivity in the interpretation. In addition, an increasing number of in vitro diagnostic (IVD) manufacturers offer products worldwide, with considerable heterogeneity in their intrinsic performance characteristics [17,18]. Therefore, there is an increasing need for recommendations that improve the harmonization of the procedures and reporting of the HEp-2 IFA test results [19–21]. Since the year 2000, several groups have elaborated recommendations on the harmonization of the HEp-2 IFA procedure and interpretation [22–26]. In response to these initiatives, the Autoantibody Standardization Committee [27], affiliated with the International Union of Immunology Societies (IUIS), established the International Consensus on Antinuclear Antibody Patterns (ICAP) initiative. The first ICAP workshop was held during the 12th International Workshop on Autoantibodies and Autoimmunity in São Paulo, Brazil in 2014 [28]. The ICAP activities are prioritized and implemented by an international team of basic and clinical scientists with expertise and interest in autoantibody testing. Recently, the ICAP team was enriched by the membership appointments of Lucile Musset (Groupe Hospitalier Pitié-Salpêrière, Paris, France), Maria Infantino (San Giovanni di Dio Hospital, Florence, Italy), and May Choi (University of Calgary, Calgary, Alberta, Canada), diversifying the geopolitical and sex representation of the committee. Through successive meetings, publications, educational modules, and other initiatives, ICAP has consolidated a comprehensive classification of the most relevant and prevalent HEp-2 IFA patterns and harmonized their nomenclature, where each pattern is assigned an alpha-numeric anti-cell (AC) code (AC-#) (https://www.anapatterns.org). Over the past 10 years, ICAP classification recommendations have attained widespread acceptance among academicians, physicians, laboratory technologists, and the IVD industry, thereby contributing to the improvement in the application of the HEp-2 IFA test in patient care and research [20,29–31].

The ICAP-hosted website (www.anapatterns.org) is available in 18 languages and in 2023 was accessed by more than 280,000 visitors from over 192 countries. The website contains a classification tree with a hierarchical organization of 30 HEp-2 IFA patterns defined by ICAP. A hyperlink from each pattern in the classification tree opens a respective subpage containing HEp-2 IFA images and descriptions of the pattern, information on the autoantibody targets, and clinical relevance, supported by selected literature. Of great interest for education and interaction with the community is the "Frequently Asked Questions (FAQ)" pages, where questions from HEp-2 IFA users worldwide are answered in detail by an international group of experts. All functionalities of the webpage are also available free of charge as application software for IOS and Android platforms.

The 7th ICAP Workshop was held on September 11th, 2023 in Dresden, Germany, as a pre-congress session of the 16th Dresden Symposium on Autoantibodies [32]. The agenda included two sessions with twelve presentations and an opportunity for debate with the audience (Suppl. Table 1). At the session's opening, Marvin Fritzler reflected on the prominent role of the late Allan Wiik in the field of laboratory medicine in autoimmunity. Among his many contributions, Dr. Wiik dedicated considerable effort to the serodiagnostics of systemic autoimmune diseases, especially to immunoassays for autoantibody determination. He was a pioneer in promoting initiatives focused on harmonization and standardization of laboratory procedures, interpretation, and reporting of diagnostic immunoassays. Dr. Wiik participated actively in many international initiatives on the harmonization of clinical autoantibody analyses [25,33] and was an inaugural member and Vice-Chair of the Autoantibody Standardizing Subcommittee of the International Union of Immunological Societies (IUIS) [27]. He was also a co-founder and president of the European Autoimmunity Standardization Initiative (EASI: https://www.gfid-ev.de/easi/) [34]. His work using advanced digital imaging and algorithms to harmonize HEp-2 IFA pattern nomenclature is of great value to ICAP [25]. In acknowledgment of his seminal contributions to the field of autoimmunity testing, Dr. Wiik was elected the first Honorary Member of ICAP. Following the recent passing of Professor Eng M. Tan, a founding member of the Autoantibody Standardization Committee (ASC), the parent committee of ICAP, the ICAP Executive has voted to posthumously honor him as an honorary member in recognition of his exceptional contributions to the ANA field spanning six decades.

Since the founding workshop in 2014, ICAP has based its agendas, work, and recommendations on open public discourse and debate involving all potentially interested stakeholders and participants attending the successive ICAP workshops held in international congresses. In addition, valued input comes from the interaction and participation of the world community through the ICAP website and personal communications with ICAP executive committee members. In response to the feedback and discussion with the community over the past few years and during the 7th ICAP Workshop, the Executive Committee held a decision-making post-workshop session addressing the most relevant topics, and the respective actions and recommendations as outlined below.

2. Updating information on the clinical and immunologic associations of HEp-2 IFA patterns (the Pattern Study Group Project)

The original ICAP classification of HEp-2 IFA patterns indicates specific immunologic and clinical associations, which are of great importance for the clinical interpretation of the test results and advice on further antigen-specific immunoassays. The extensive review performed by the ICAP Executive Committee addressed most of the information available at the time of the ICAP launch in 2014. The clinical relevance of each pattern was subsequently further defined in the following workshops and published in 2019 [35]. However, active surveillance of novel information is necessary to keep ICAP recommendations updated and clinically useful. Accordingly, the ICAP Executive has implemented the Pattern Study Group Project that will promote active surveillance of the literature aiming to identify peer-reviewed publications on novel clinical and immunologic associations of HEp-2 IFA patterns. This initiative will be led by separate teams coordinated by ICAP members; each team dedicated to a specific cell compartment. In the establishment of these teams, the coordinators will actively seek to identify young investigators with an appropriate scientific background to collaborate on the project. Novel information identified by the teams will be submitted in a structured protocol and reviewed by the ICAP Executive. Approved novel information will be uploaded to the ICAP

website. ICAP users are also encouraged to communicate relevant updates to ICAP for review and discussion, eventually facilitating fine-tuning and advances in pattern classification and clinical relevance.

3. Competent- versus Expert-level reporting

The ICAP classification tree (https://anapatterns.org/trees-2021.php) parses the HEp-2 IFA AC patterns into two main colour-coded categories, Competent- and Expert-level patterns. The rationale for this arrangement is the perception that pattern recognition is analytically subjective with variability in the inherent difficulty in the recognition of each pattern. The Competent-level pattern designation aims to indicate patterns considered relatively easy to identify; any analyst performing HEp-2 IFA should be able to recognize those HEp-2 IFA patterns. In contrast, the Expert-level pattern designation indicates patterns with an increasing degree of difficulty in accurate identification. Ideally, any HEp-2 IFA analyst should strive to master the recognition of Competent- and Expert-level patterns. At the individual analyst level, the ability to identify Expert-level patterns should be obtained through complementary educational activities such as surveying the literature, ICAP website review, attending workshops, interaction with experienced mentors, and meticulous examination of images at the microscope. ICAP provides collections of images for Competent- and Expert-level patterns on the website, with the acknowledgment that these represent a limited 'real-world' portfolio. In addition, the first Educational Module on the ICAP website (https://anapatterns.org/courses.php) provides recommendations on the technical performance of the HEp-2 IFA test and guidelines for the interpretation and recognition of IFA patterns. In acknowledgment of the unmet need for further didactic material, the Executive Committee is preparing three novel Educational Modules dedicated to the Functional Anatomy of the HEp-2 Cell, the identification of Compound Patterns, and Unclassified (AC-XX) Patterns, respectively (discussed in more detail below). The goal is to complete and upload these modules by the end of 2024.

It is of utmost importance that the designations of Competent- versus Expert- levels be not interpreted as an invariable status of an individual analyst. Instead, this classification aims to indicate the minimum (Competent) and the desirable (Expert) levels of expertise in the training of HEp-2 IFA interpretation. Therefore, along with training, learning, and routine operation as outlined above, any analyst should gradually be able to identify Expert-level patterns. Importantly, it should be appreciated that the HEp-2 IFA images seen at the microscope do not always fit perfectly into the classified ICAP patterns. Depending on the combination of autoantibodies in the sample, the resulting HEp-2 IFA pattern may not correspond to the discrete patterns currently classified by ICAP. As an example, an analyst may readily identify the bona fide Expert-level AC-4 and AC-5 patterns but may have difficulty in classifying a given speckled nuclear image where there are superimposed features of AC-4 and AC-5 and may choose to use the combined AC-4/5 Competent-level nomenclature. This example can be applied to other combinations of patterns that differ from each other in fine nuances, such as AC-6/7, AC-8/9/10, AC-11/12, AC-13/14, AC-15/16/17, and AC-19/20. Therefore, the "competency level" is a rather flexible and dynamic classification that should be judiciously exercised by each analyst upon each image.

4. Clinical and immunological characterization of HEp-2 IFA patterns

Since its launch in 2014, ICAP has achieved progressive international acceptance and adherence as documented by statistical parameters monitored on the website https://www.anapatterns.org. According to Google Analytics, the annual number of visitors has increased steadily, with over 280,000 visitors from 192 countries in 2023 (Fig. 1A). The number of affiliated members has steadily increased and is close to 5000 (Fig. 1B). The top 15 visiting countries were the USA, Brazil, Mexico, Spain, Italy, Chile, India, Germany, Taiwan, Colombia, Argentina, Canada, the United Kingdom, Switzerland, and Portugal, respectively. Importantly, in China, there is a parallel ICAP-translated site https://www.anapatterns.cn, which is not included in these statistics. There is a growing number of clinical and research publications that use ICAP nomenclature, most IVD companies have adopted ICAP nomenclature into their product guidelines inserts, marketing folders, and computer-aided diagnosis (CAD) systems, and an increasing number of clinical laboratories now refer to ICAP and ICAP staining patterns in their HEp-2 IFA test reports.

The HEp-2 IFA test has several methodological nuances and considerable subjectivity in the interpretation of the results. Therefore, specialists in various parts of the world may have different interpretations of some images/patterns and may take alternative approaches regarding several operational aspects of the test despite their adherence to some of the ICAP recommendations. Another important

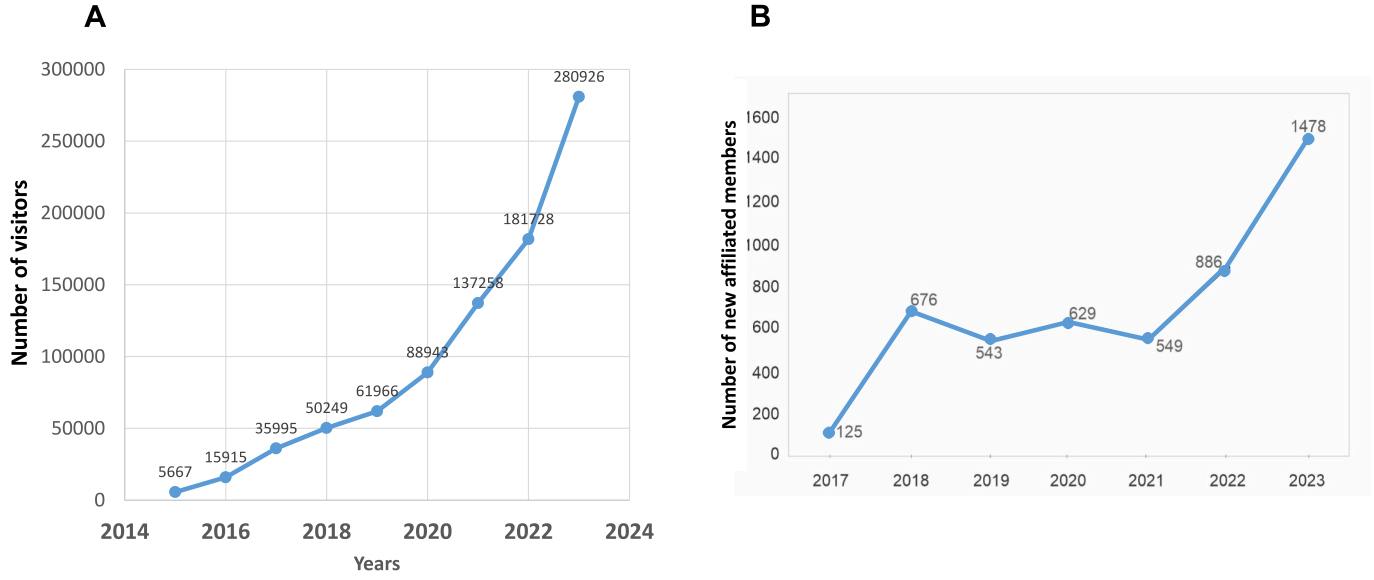

Fig. 1. Statistics on the use of the www.anapatterns.org website according to Google Analytics. (A) The overall number of visitors since the website launch; (B) Number of new affiliated members per year, totaling 4886 in 2023.

aspect is that ICAP is an ongoing initiative aiming for progressive adjustment and improvement. As detailed later in this manuscript, continuous improvement in the classification tree has been promoted over the years, with new modifications approved during the 7th ICAP Workshop. One major challenge is evidence-based consensus on the clinical and immunological relevance of all HEp-2 IFA patterns. The commonest patterns are well characterized in terms of immunological and clinical associations [35–37]. However, sustained effort is needed to keep the information updated as novel associations are reported (see section "Updating information on the clinical and immunologic associations of HEp-2 IFA patterns"). In contrast to the commonest ICAP AC patterns, the literature provides only preliminary and fragmentary evidence on the clinical and immunological associations of several patterns, such as the cytoplasmic discrete dots (AC-18), the cytoplasmic Golgi-like (AC-22), and the rods and rings (AC-23) patterns. The same applies to rarer patterns not currently incorporated into the ICAP classification. The rarity of these patterns limits well-controlled studies to determine their target specificity, as well as clinical and immunologic significance. The challenge of characterizing rare patterns could be addressed by the establishment of cooperative multicenter projects that allow the analysis of adequate numbers of serum samples with consistent clinical data, as well as the use of cutting-edge methodology (e.g., immunoprecipitation-mass spectroscopy: IP-MS) for the identification of the target autoantigens [38].

In response to these challenges, the 2019 ICAP Executive launched the **C**linical and **I**mmunologic **C**haracterization of HEp-2 IFA Patterns (HEp-2 CIC project), which comprises three branches. Branch #1 aims to determine the operational characteristics and the frequency of the ICAP patterns reported in several clinical laboratories worldwide. Preliminary data on this project was presented by Trischna Berger (Berlin, Germany) at the 7th ICAP Workshop [39]. Fifty laboratories from 35 countries participated in the study by providing all the HEp-2 IFA reports from their routine operation during 2019. Most laboratories used the ICAP nomenclature, although some did not report all the ICAP patterns. Considerable heterogeneity in some operational parameters (e.g., screening dilution, end-point titer) was observed. In addition, there was striking heterogeneity in the frequency of reported AC patterns, which varied (for example) from 5 % to 59 % for the nuclear homogeneous pattern (AC-1) and from 1 % to 28 % for the nuclear dense fine-speckled (AC-2). In contrast, the interpretation of some patterns showed less variability in the frequency among positive results, such as the centromere pattern (AC-3) which had a variance ranging from 1 % to 10 %. Interestingly, a reciprocal frequency relationship was noticed for some groups of patterns. For example, centers that did not report or reported a lower frequency of the AC-2 pattern tended to report a higher frequency of the AC-1 pattern. Considering the different clinical and immunologic associations of these two patterns, this observation indicates the need for the dissemination and additional education about the morphological characteristics of these patterns.

The two other branches of the HEp-2 CIC project are interrelated and mandate the establishment of clinical associations (Branch #2) and the characterization of the antigenic specificity (Branch #3) of selected ICAP patterns. These two initiatives require additional formal arrangements, especially concerning ethics committee approval by the participating centers and standard operating protocols (SOP) for collecting, storing, and shipping samples to the investigation laboratories in charge of target antigen identification (Branch #3). Implementation of these two branches of the HEp-2 CIC project was considered a priority. Three patterns chosen to be addressed initially included the cytoplasmic Golgi-like (AC-22) pattern, the so-called SG2NA (S/G2 cell cycle phase nuclear antigen) pattern [40,41], and the so-called nuclear matrix pattern [42–44] (Fig. 2). Three groups of ICAP Executive members will coordinate the subprojects dedicated to each of these patterns, respectively. These coordinators are responsible for elaborating SOPs, inviting collaborators, assembling and coordinating the respective teams, advising on institutional review board (i.e., ethics, legalities) issues, collecting and analyzing data, and producing final reports and publications. Voluntary participation from experts worldwide is welcome and candidate participants should contact the ICAP coordinators (www.anapatterns.org). Xavier Bossuyt from the Catholic University of Leuven, Belgium will provide support for novel immunoprecipitation-mass spectroscopy (IP-MS) approaches to identification of the target autoantigens associated with the patterns of interest (Branch #3).

5. Next educational modules at the ICAP website

The first ICAP educational module (https://anapatterns.org/courses.php) addressed four main points: 1) an introduction to ICAP history and goals, 2) a description of the ICAP patterns, 3) a guide on how to navigate the ICAP website, and 4) technical recommendations on how to perform the HEp-2 IFA test. This module has been successful, reaching circa 2000 users from several countries. It consists of an audiovisual presentation and an interactive self-administered exam to assess the learning achievements of the participants. A certificate from ICAP is provided to those who achieve the appropriate learning levels. In response to positive feedback, the ICAP Executive will implement the following educational modules: 1) The Functional Anatomy of the HEp-2 Cell; 2) Compound (multiple, mixed, and composite) Patterns; and 3) Unclassified (AC-XX) patterns.

The educational module *Functional Anatomy of the HEp-2 Cell* will help analysts understand how the HEp-2 IFA patterns originate. The conceptual basis for the association of HEp-2 IFA patterns and the target antigens recognized by the autoantibodies is based on evidence that each autoantigen tends to have a specific topographic distribution, which is often localized to several cellular domains and/or organelles. The topographic distribution of each antigen is related to its molecular

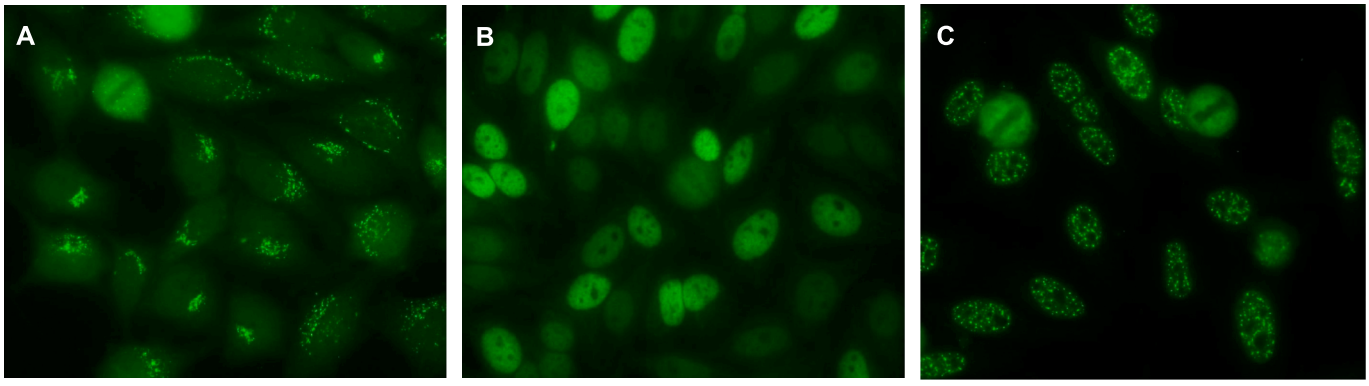

Fig. 2. HEp-2 IFA patterns selected for the clinical and immunologic characterization in the multicenter HEp-2 CIC project. (A) Cytoplasmic Golgi-like (AC-22) pattern; (B) Nuclear SG2NA AC-XX pattern; (C) Nuclear matrix AC-XX pattern.

nature and its function in one or more cellular domains. Therefore, the knowledge of the structure and function of the diverse cellular domains is useful in understanding how the autoantigens are represented across the several cellular domains in consecutive stages of the cell cycle. For example, Sm (U2-U6 RNP) and U1-RNP are crucial messenger RNA splicing factors and, therefore, are distributed along the spliceosome reticular network in the interphase nucleus. During mitosis, messenger RNA transcription and translation are quiescent, therefore the Sm/U1RNP network is disassembled and there is typically diffuse amorphous staining of the mitotic cell with no staining of the mitotic chromatin mass. In contrast, antibodies to dsDNA and to nucleosomes yield a diffuse homogeneous nuclear pattern in interphase cells that reflects the topographic distribution of heterochromatin. This pattern changes during mitosis when the staining is concentrated in the condensed chromatin mass of mitotic cells. The awareness of the molecular and cell biology of these two groups of autoantigens helps the analyst's interpretation of the characteristics of their respective HEp-2 IFA patterns. Similarly, the knowledge of the molecular and cell biology of other autoantigens provides clues to the understanding of their HEp-2 IFA patterns, such as the assembly and disassembly of the nucleolus and apparent reallocation of some autoantigens to the periphery of condensed chromosomes (e.g., fibrillarin) or the nucleolar organizing regions (NOR) of the metaphase chromatin mass (e.g., NOR-90 and RNA polymerase I) during mitosis. The educational module "Functional Anatomy of the HEp-2 cell" will provide a practical bridge between molecular and cell biology and the most relevant HEp-2 IFA patterns.

The educational module *Compound Patterns* covers three categories of patterns with more intricate information than the 'Simple Patterns'. A simple pattern refers to the characteristic staining of a single cellular domain with attributes that are distinct from those of other simple patterns. Most patterns in the ICAP classification tree are simple patterns (Table 1). In contrast, the Compound Pattern group (multiple patterns, mixed patterns, and composite patterns) exhibits more elaborate characteristics. Multiple patterns refer to the co-occurrence of two or more simple patterns that can be identified independently. An example of multiple patterns is the combination of AC-1 and AC-15 patterns, where the specific staining features of each HEp-2 IFA pattern, AC-1 vs. AC-15, are readily distinguishable from one another. Several similar combinations are possible, and some have a particular clinical interest because, in the appropriate clinical context, they indicate the possibility of more than one autoantibody associated with the same disease, thereby increasing the likelihood of that disease in that particular patient. For example, patients with primary biliary cholangitis (PBC) frequently have antibodies to mitochondria, which are associated with the cytoplasmic reticular AC-21 pattern. PBC patients may also have antibodies to Sp100, gp210, and centromere, represented by the nuclear multiple discrete pattern (AC-6), the nuclear discontinuous envelope pattern (AC-11), and the centromere pattern (AC-3), respectively [45]. Any combination of two or more of these four patterns strengthens the possibility of the cognate-associated autoantibodies and the diagnosis of PBC and can imply a risk of co-morbidity or more severe disease [45,46].

The category "Mixed Patterns" refers to the co-occurrence of two or more simple patterns staining the same cell compartment when it is not possible to identify any of the individual patterns. This is especially frequent in the nucleus compartment where patterns with different textures may overlap, yielding a mixed pattern with none of the characteristic textures of either of the original AC patterns. This situation is particularly relevant to systemic autoimmune diseases with multiple autoantibodies against certain nuclear antigens. In systemic lupus erythematosus, for example, there is frequently co-occurrence of autoantibodies against double-stranded DNA that yield the nuclear homogeneous pattern (AC-1), SS-A/Ro60 that can yield the nuclear fine speckled pattern (AC-4), and Sm/U1-RNP that can yield the nuclear coarse speckled pattern (AC-5) [47]. The combination of two or more of these autoantibodies frequently yields a diffuse nuclear and blurred or masked staining without the well-defined features of the original nuclear patterns, so that one cannot identify the characteristic texture of the nuclear patterns associated with the individual autoantibodies in the sample. In such cases, the distinctive autoantibody associations of the HEp-2 IFA patterns are hampered, but one can suspect the presence of multiple autoantibodies in the sample. However, if the concentrations of the autoantibodies in the sample are widely different, progressive

Table 1
Simple and Composite Patterns classified by the International Consensus on ANA Patterns – ICAP.

AC code	Designation	Cell domains/regions stained	Number of domains
AC-1*	Nuclear homogeneous	Nucleus and mitotic chromosome mass	2
AC-2*	Nuclear dense fine speckled	Nucleus and mitotic chromosome mass	2
AC-3	Centromere	Centromeres	1
AC-4	Nuclear fine speckled	Nucleus	1
AC-5	Nuclear large/coarse speckled	Nucleus	1
AC-6	Multiple nuclear dots	Nuclear PML bodies	1
AC-7	Few nuclear dots	Nuclear Cajal bodies	1
AC-8	Homogeneous nucleolar	Nucleolus	1
AC-9*	Clumpy nucleolar	Nucleolus and periphery of mitotic chromosome mass	2
AC-10*	Punctate nucleolar	Nucleolus and NORs	2
AC-11	Smooth nuclear envelope	Nuclear envelope	1
AC-12	Punctate nuclear envelope	Nuclear envelope	1
AC-13	Pleomorphic nuclear PCNA-like	Nucleus	1
AC-14*	Pleomorphic nuclear CENP-F-like	Nucleus, midbody, mitotic centromeres, nuclear envelope at prometaphase	4
AC-15	Cytoplasmic fibrillar linear	Cytoplasm cytoskeleton	1
AC-16	Cytoplasmic fibrillar filamentous	Cytoplasm cytoskeleton	1
AC-17	Cytoplasmic fibrillar segmented	Cytoplasm cytoskeleton	1
AC-18	Cytoplasmic discrete dots	Cytoplasmic G/W bodies	1
AC-19	Cytoplasmic dense fine speckled	Cytoplasm	1
AC-20	Cytoplasmic fine speckled	Cytoplasm	1
AC-21	Cytoplasmic reticular/AMA-like	Mitochondria	1
AC-22	Cytoplasmic polar / Golgi-like	Golgi apparatus	1
AC-23	Cytoplasmic rods and rings	Distinct cytoplasmic rods and rings structures	1
AC-24	Centrosome	Centrioles/pericentriolar region	1
AC-25	Spindle fibers	Mitotic spindle fibers	1
AC-26*	NuMA-like	Nucleus and mitotic spindle fibers	2
AC-27	Intercellular bridge	Intercellular bridge	1
AC-28	Mitotic chromosomes	Condensed chromatin only in mitotic cells	1
AC-29*	DNA topo I-like	Nucleus, mitotic chromosome mass, NORs, cytoplasm, nucleolus	5

NOR: nucleolar organizing region; PML: Promyelocytic Leukemia bodies; GW bodies: also known as P bodies. * Composite patterns.

dilution of the sample should allow the distinction of one or more individual simple patterns.

The category "Composite Patterns" refers to patterns localized to more than one cell compartment stained by a single autoantibody specificity (Table 1). Frequently, this is evident in cells in different phases of the cell cycle. Composite patterns are of particular interest because their unique assembly of patterns is virtually specific for one autoantibody specificity. Because of the strong association with a specific autoantibody, some composite patterns bear the primary target autoantigen in their names, such as the NuMA-like pattern (AC-26) [11,12], the CENP-F-like pattern (AC-14) [10], and the Topo I-like pattern (AC-29) [12,15,48] (also see www.anapatterns.org).

The educational module addressing *Unclassified (AC-XX) Patterns* will review relatively rare and unusual patterns that have not been fully characterized and, therefore, not included in the current ICAP classification tree. Even though their clinical and immunologic significance is uncertain, these patterns are observed in the routine HEp-2 IFA operation and are often the topic of informal discussion, anecdotes, and case reports by ICAP users. In recognition of this knowledge gap, ICAP has created the AC-XX code that can be provisionally used to classify these patterns [49]. The AC-XX category is an 'umbrella' designation that accommodates a variety of unusual and unclassified patterns staining the nucleus, cytoplasm, and mitotic apparatus. The educational module on Unclassified (AC-XX) patterns will describe several of these patterns and provide representative images as well as any supporting literature. It is hoped that this will generate a fulsome discussion and debate on the importance of these patterns resulting in a clear evidence-based clarification of how they are "actionable" (clinically useful) and eventually be correctly positioned in ICAP algorithms with their proper AC codes. In acknowledgment of the importance of the unclassified patterns, a dedicated AC-XX box will be added to the ICAP classification tree with a hyperlink directing the visitor to a separate page with representative images of AC-XX patterns (Fig. 3).

6. Implementation of new patterns in the classification tree – AC-30 and AC-31

During the 7th ICAP workshop, some presentations provided evidence for the recognition of two new independent nuclear patterns. These two novel patterns represent variations of the dense fine-speckled nuclear pattern (AC-2) and the fine-speckled nuclear pattern (AC-4), respectively.

The dense fine-speckled nuclear (AC-2) pattern is characterized by relatively small speckles that are heterogeneous in size, brightness, and density. There are fine and dim speckles, interspersed with coarser and brighter speckles, as well as some areas with a sparser distribution of speckles intermingled with more densely packed areas. Because of this heterogeneity, the periphery of the nucleus appears as an irregular and discontinuous rim. The metaphase chromosome plate is brighter than the interphase nucleus but holds the same morphological characteristics [50]. The AC-2 pattern has a strong association with anti-DFS70 antibodies [51–54] and, therefore, represents a preliminary indication for the interpretation of the significance of the HEp-2 IFA result as well as further testing for anti-DFS70 antibodies. The genuine AC-2 pattern is readily visible when anti-DFS70 is the only antinuclear antibody in the sample, as the presence of additional antinuclear antibodies obfuscates the AC-2 characteristics [54]. This is especially relevant because the isolated (also called 'monospecific') anti-DFS70 antibody is not associated with systemic autoimmune diseases such as systemic lupus erythematosus. The AC-2 pattern is readily distinguished from the fine (AC-4) and coarse (AC-5) speckled nuclear patterns because these are not associated with metaphase plate staining. However, the correct identification of the genuine AC-2 pattern may present some difficulty, because some non-DFS70 antibody samples also produce a fine speckled nuclear pattern with staining of the metaphase plate. In such cases, the fine-speckled nuclear pattern is more homogeneous regarding the size and brightness of the speckles, and it is more evenly distributed in the interphase nuclei, metaphase plate, and along the nuclear periphery

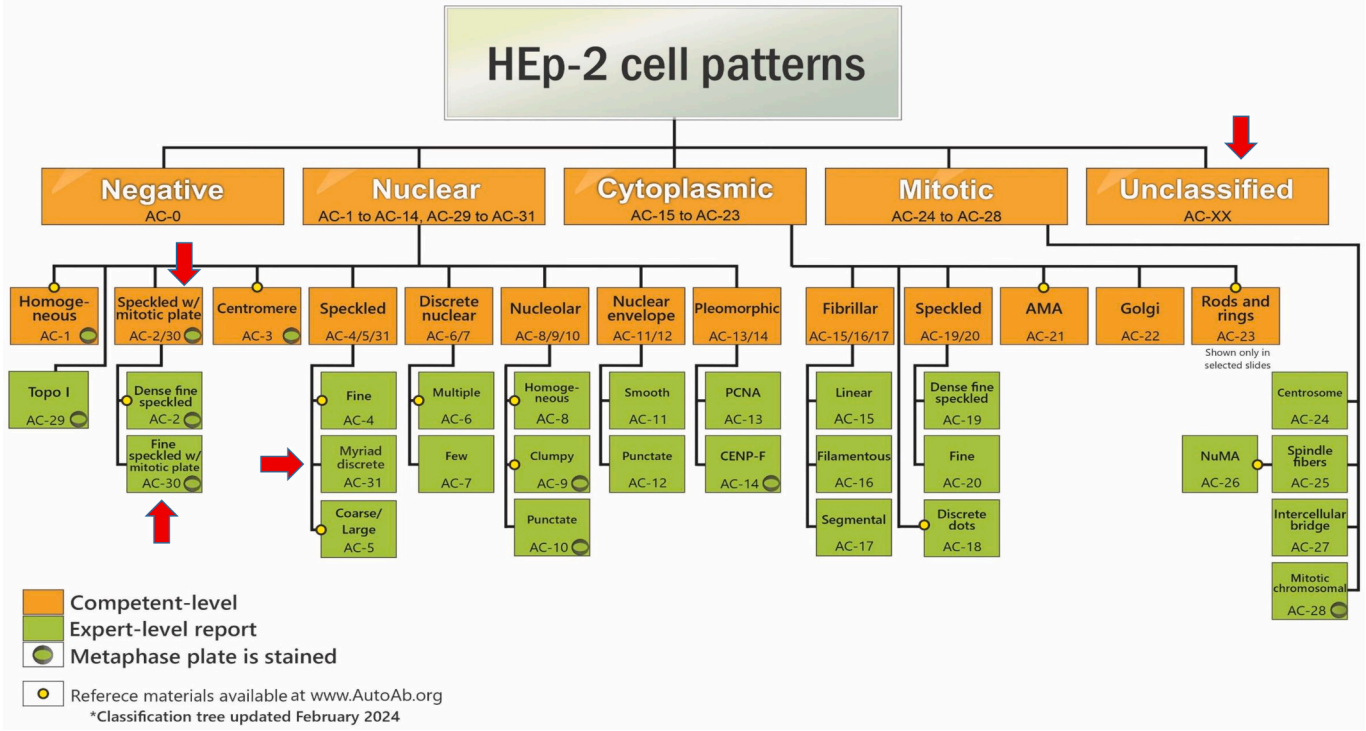

Fig. 3. Proposed novel ICAP pattern classification tree with the addition of the "Unclassified" (AC-XX) category and the AC-30 and AC-31 patterns. The AC-XX box directs the visitor to a separate page with representative images of AC-XX patterns. Red arrows indicate new boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

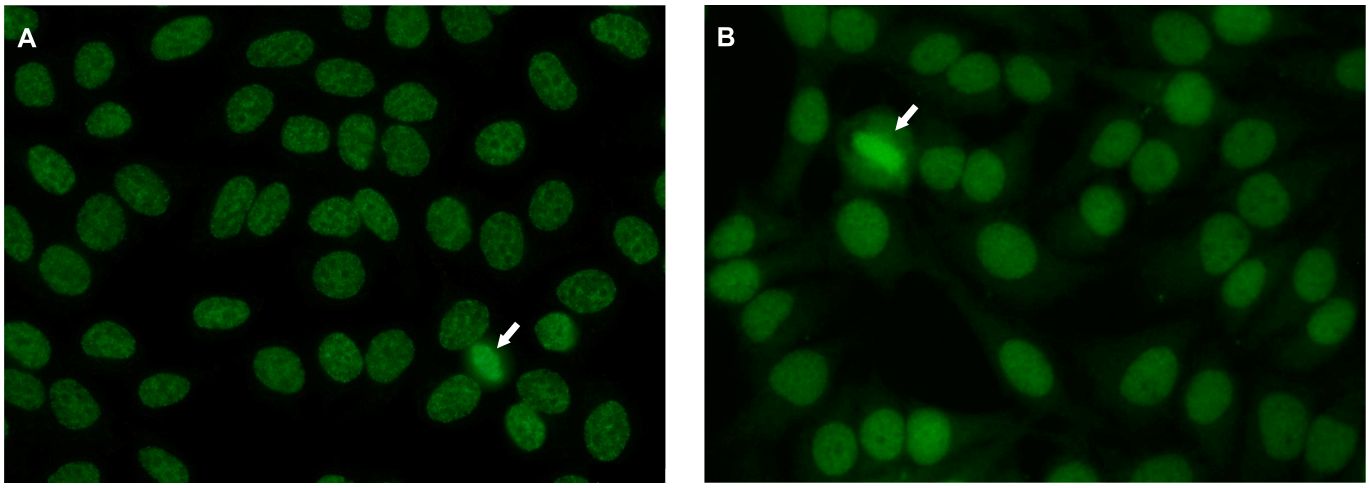

Fig. 4. Distinct HEp-2 IFA nuclear-speckled patterns staining the metaphase plate. (A) Nuclear dense fine speckled (AC-2) pattern produced with a sample with reactivity to DFS70 and no other known nuclear antigen; (B) Nuclear (smooth) fine speckled pattern with stained metaphase plate (AC-30) produced with a sample with no reactivity to DFS70. These two nuclear-speckled patterns stain the metaphase plate (arrow) but have different staining textures and different immunologic and clinical associations.

(Fig. 4). At the 7th ICAP workshop Executive meeting, after weighing the evidence for the impact of distinguishing possible variants of the AC-2 pattern, the non-AC-2 fine-speckled nuclear pattern with mitotic plate was assigned the ICAP AC-30 code. The presented evidence included a retrospective analysis of 2560 samples in a large clinical laboratory [55], where the presence of relevant antinuclear antibodies (dsDNA, nucleosome, Sm, U1-RNP, Scl-70, SS-A/Ro60, SS-B/La) was detected in 23.8 % of AC-30 samples compared to only 3.4 % of AC-2 samples (Fig. 5A). The

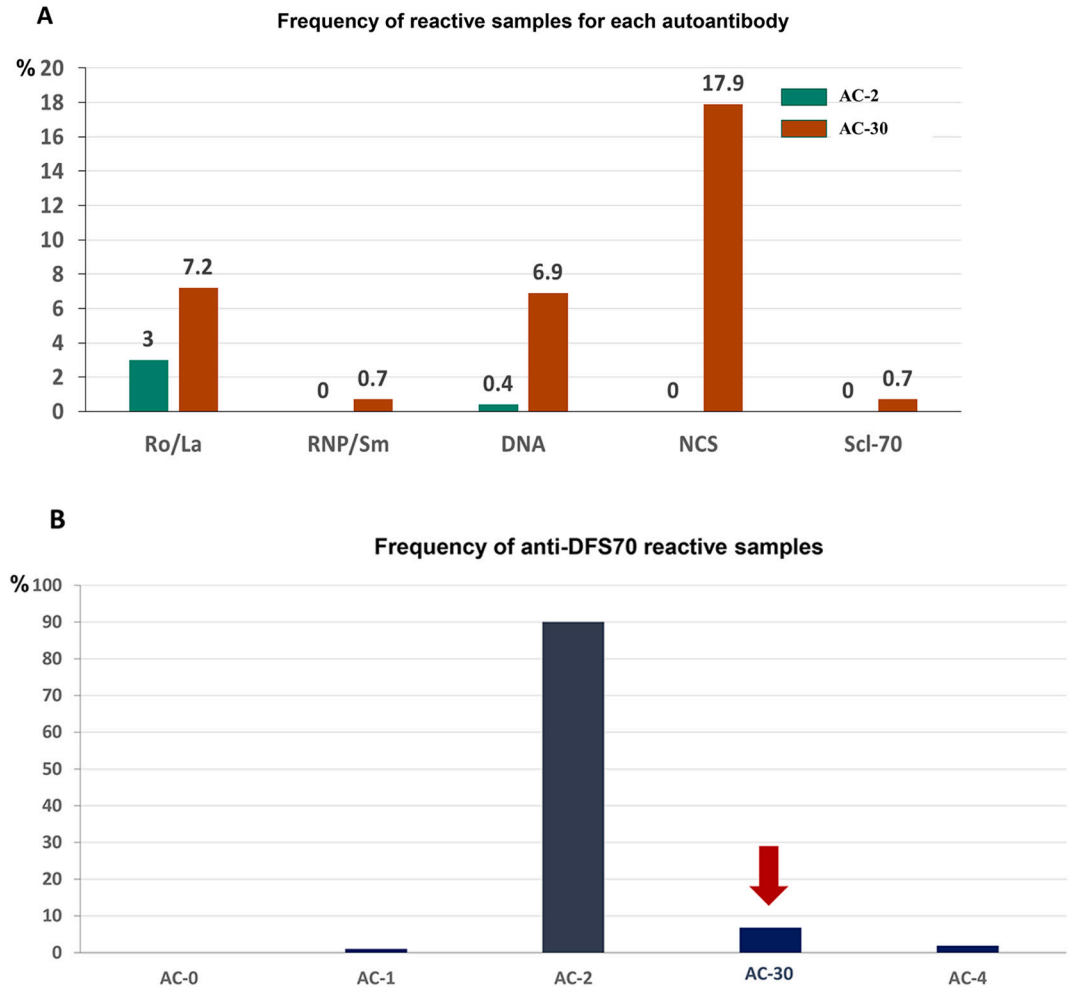

Fig. 5. The AC-2 and AC-30 nuclear patterns have distinct immunologic associations. (A) Retrospective analysis of 2560 samples concomitantly tested for HEp-2 IFA and seven antinuclear antibodies (dsDNA, nucleosome [NCS], Sm, U1-RNP, Scl-70, SS-A/Ro, and SS-B/La). (B) Retrospective analysis of 310 samples concomitantly tested for HEp-2 IFA and anti-DFS70 antibodies. The AC-2 nuclear pattern, but not the AC-30 pattern (red arrow), is strongly associated with anti-DFS-70 antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

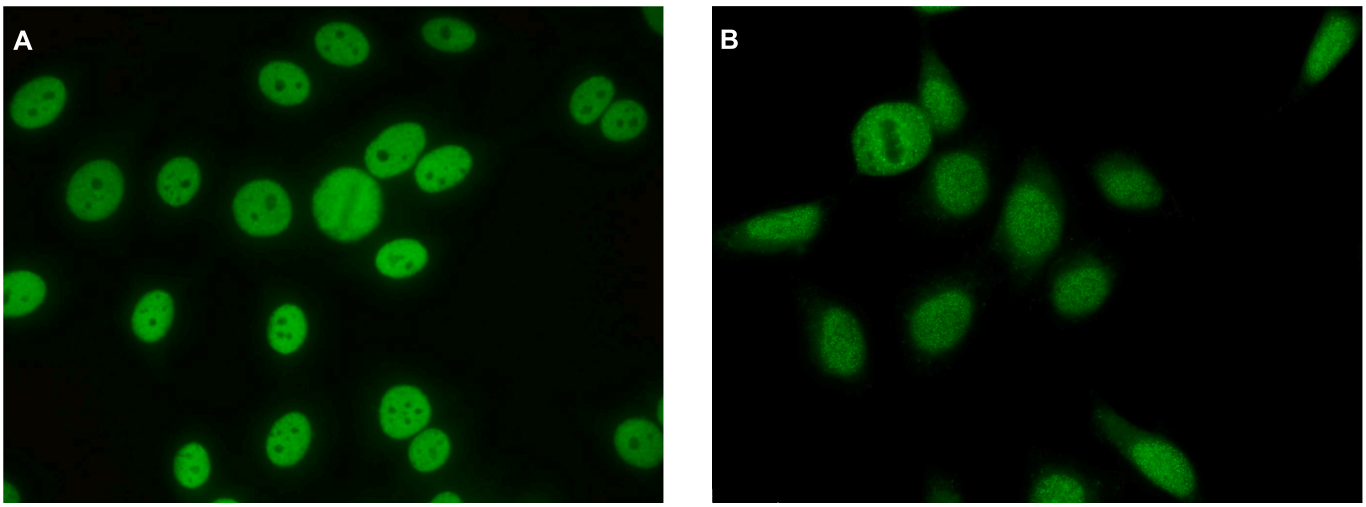

Fig. 6. The nuclear smooth fine speckled pattern (AC-4) and the nuclear myriad discrete tiny dots speckled pattern (AC-31) do not stain the metaphase plate but have different staining textures and different immunologic and clinical associations. HEp-2 IFA images of the AC-4 (A) and AC-31 (B) patterns were obtained with samples without (A) and with (B) reactivity to SS-A/Ro 60 kDa, respectively.

most frequent autoantibodies observed in the AC-30 samples were directed against nucleosomes, dsDNA, and SS-A/Ro60. Importantly, among 310 samples assayed for anti-DFS70 and HEp-2 IFA, anti-DFS70 reactivity was demonstrated in 92 % of the AC-2 samples and only 7 % of the AC-30 samples (Fig. 5B). Considering the subtlety in distinguishing the AC-2 and AC-30 patterns, both are to be considered Expert-level patterns and will be placed in the classification tree under the Competent-level overarching AC-2/AC-30 pattern, i.e., nuclear speckled with mitotic plate (Fig. 3). The AC-30 box will provide a hyperlink to the corresponding AC-30 subpage containing representative images and information on the clinical relevance.

The recognition of a variant of the AC-4 pattern characterized by myriad discrete tiny dots and the absence of metaphase chromatin mass staining was first reported in 2013 [56] and later confirmed by a multicenter international study [37]. These studies show that in reality, the overarching AC-4 pattern category comprises two distinct patterns, one with a smooth inconspicuous texture and another in which myriad discrete tiny dots are evident (Fig. 6). The authors showed they have distinct immunologic significance and designated the first as the AC-4 pattern and the latter as a novel AC-4 variant. The simple AC-4 pattern is very frequent in routine clinical laboratory operations and has broad autoantibody associations, including antibodies to SS-A/Ro60, SS-B/La, Mi-2, TIF1γ, TIF1β, and Ku. In contrast, the variant AC-4 pattern represents a minority of fine-speckled nuclear patterns in the laboratory routine and has a strong but rather restricted association with antibodies to SS-A/Ro60; an association that was confirmed using anti-SS-A/Ro60 monoclonal antibodies [37]. This novel AC-4 variant pattern was approved during the 6th ICAP Workshop in 2021 [29] and was provisionally designated the AC-4a pattern. Information on the characteristics and significance of this new variant was provided as a note on the AC-4 page of the ICAP website (https://anapatterns.org/view_pattern.php?pattern=4). Considering the clinical and immunologic relevance of this new pattern, the 7th ICAP Workshop decided to incorporate it into the ICAP classification tree, clarifying that it is an independent pattern and ascribing it to a unique AC code, AC-31 (Fig. 3). AC-31 is considered an Expert-level pattern and is named “myriad discrete speckled”. A corresponding web subpage will be created for the AC-31 pattern containing representative images and information on clinical relevance.

7. A hyperlink to AC-XX patterns on the website

As addressed in the session “Educational Modules”, there are unusual patterns observed in HEp-2 IFA routine testing that are not currently included in the ICAP classification tree. However, ICAP acknowledges that these patterns should not be dismissed just because their clinical significance is not fully determined or because they are not included in the ICAP classification. Accordingly, the general category AC-XX was established to accommodate these unusual and atypical patterns [49]. As mentioned earlier, efforts to establish the immunologic and clinical associations of these rare patterns are part of the ongoing HEp-2 CIC project. In the routine HEp-2 IFA operation, it is important to recognize these patterns to prevent their misclassification as a traditional ICAP AC pattern. For example, there are pleomorphic speckled nuclear patterns that resemble but are not exactly the PCNA-like (AC-13) or the CENP-F-like (AC-14) pleomorphic patterns. Therefore, it is appropriate that the users of the HEp-2 IFA methodology be acquainted with the images associated with the different AC-XX patterns. In recognition of this need, ICAP decided to insert a new category (AC-XX) into the classification tree (Fig. 3) and this box will contain a hyperlink to a separate page displaying images and descriptive characteristics of several AC-XX patterns.

8. Conclusions

ICAP, comprised of internationally recognized experts in autoimmunity and HEp-2 IFA testing, is an ongoing international initiative dedicated to the harmonization of the HEp-2 IFA, thereby fostering accurate reading, interpretation, and reporting of HEp-2 IFA images by analysts in clinical diagnostic laboratories, academicians and scientists in university laboratories, the IVD industry, and clinicians in charge of patient care, all in the context of autoimmune diseases. The 7th ICAP workshop has addressed several aspects that originated from the interaction with the international community and has effectively established objective goals and tasks to be delivered over the next two years. This article outlines the most relevant resolutions, including the classification of two novel and clinically relevant HEp-2 IFA patterns, the planning of three audio-visual educational modules to be posted at the www.anapattern.org website, the implementation of a project dedicated to continuously updating the information on the clinical and immunologic relevance of the HEp-2 IFA patterns, and the launch of two additional branches of the HEp-2 Clinical and Immunological (HEp-2 CIC) project. ICAP invites continuous information exchange with the international community and encourages the participation of younger experts from all over the world.

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Use of artificial intelligence

During the preparation of this work, the authors have not used any Artificial Intelligence tool or service.

Declaration of competing interest

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Luis E. C. Andrade reports a relationship with Inova that includes: consulting or advisory. May Choi receives consulting fees from Werfen, MitogenDx, AstraZeneca, GlaxoSmithKline, Celltrion, Organon, and Mallinckrodt Pharmaceuticals. Jan Damoiseaux is consultant for ThermoFisher/Phadia and Werfen/Inova and receives speaker fees from Euroimmun, ThermoFisher/Phadia, Werfen/Inova, and Menarini. Carlos Alberto von Mühlen is consultant for Euroimmun and ARTIVA Biotherapeutics. Marvin J. Fritzler was and/or continues to be a consultant to Inova/Werfen, Alexion Pharmaceuticals, and Bio-Rad. Marvin J. Fritzler and May Choi are directors of Mitogen Diagnostics Corporation. ICAP is partially supported by grants from the American Proficiency Institute, Grifols, Immunoconcepts, Bio-Rad, Aesku Group, Biosystems, Trinity Biotech, Mitogen Diagnostics, A.Menarini Diagnostics, Euroimmun, ThremoFischer Scientific, and Inova Diagnostics. The funding organizations played no role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the report for publication.

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Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autrev.2024.103608.

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