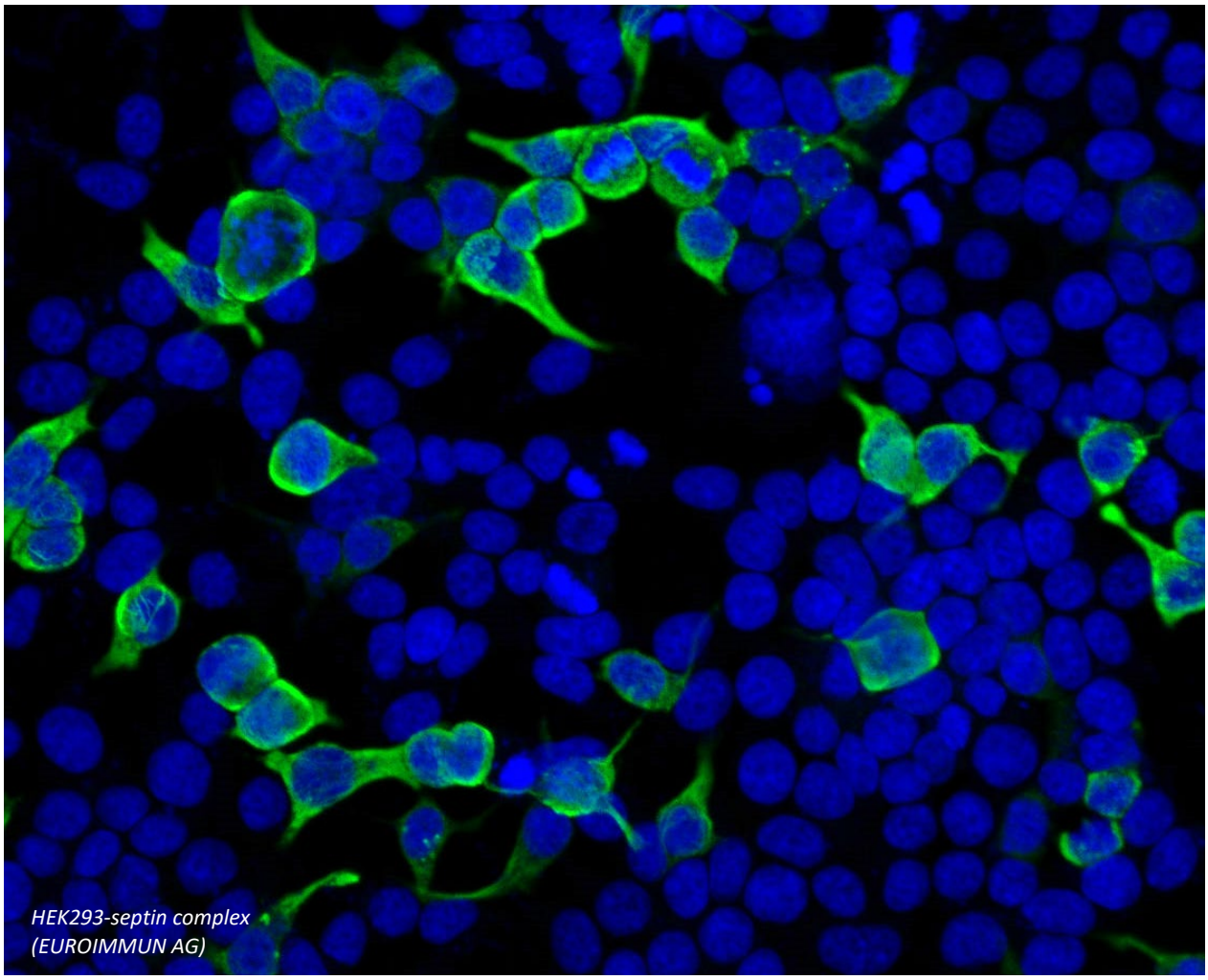
Karsten Conrad, Luis E. C. Andrade, Edward K. L. Chan, Jan Damoiseaux, Marvin J. Fritzler, Pier Luigi Meroni, Ger J. M. Pruijn, Yehuda Shoenfeld, Günter Steiner (Eds.)

Achievements and Challenges in Research, Diagnostics and Therapy of Autoimmune Diseases

Abstracts of the 16th Dresden Symposium on Autoantibodies, September 12-15, 2023



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Preface

Since the 3rd Dresden Symposium on Autoantibodies (1996), numerous aspects of the pathogenetic and diagnostic relevance of autoantibodies were presented and discussed. The main topics dealt especially with the role of autoantigens and autoantibodies in the pathogenesis of autoimmune diseases; the impact of genomics, ribonomics, proteomics on autoantigen and autoantibody research; animal models and human genetics in the research on the induction and pathogenicity of autoantibodies; the relevance of autoantibodies in the prediction of autoimmune diseases; infections, tumors and autoimmunity; immunodeficiency, infection and autoimmune diseases; precision medicine in the care of autoimmune diseases; and autoimmunity in the SARS-CoV-2 era. The current symposium entitled “Achievements and Challenges in Research, Diagnostics and Therapy of Autoimmune Diseases” includes a wide range of topics with special focus on rheumatoid arthritis, ANA associated rheumatic diseases, autoimmune neurological and liver diseases and highlights the role of autoantibodies in the prediction and classification of autoimmune diseases. As with all previous symposia, new pathologically or diagnostically relevant autoantibodies will be presented as well as methodical aspects of autoantibody analyses will be discussed. Special attention is paid to aspects that are of practical relevance for the serological diagnosis of autoimmune diseases like autoantibody profiling, identification of autoantibodies recognizing conformational epitopes, or incorporating likelihood ratios within medical practice. Key topics show the progress in the serology of rheumatoid arthritis (novel autoantibodies, anti-PAD antibodies, early diagnosis, personalized medicine), ANA associated rheumatic diseases (SLE, systemic sclerosis, idiopathic inflammatory myopathies, systemic autoimmune disease associated interstitial lung disease), autoimmune neurological diseases (potential clinical relevance of numerous novel autoantibodies in autoimmune encephalopathies and peripheral neuropathies), autoimmune liver diseases (autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis) and in the harmonization of autoantibody detection (including progress in the assessment of HEp-2 cell pattern).

Hopefully, the data and information described and discussed in this abstract book will further stimulate initiatives to improve the prediction, diagnosis and management of systemic and organ specific autoimmune diseases.

Karsten Conrad

1 In Memoriam

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As president of the Association for the Advancement of Immune Diagnostics e.V. (GFID e.V.) and on behalf of the members of the organizing committee of the 16th Dresden Symposium of Autoantibodies I would like to take this opportunity to commemorate important colleagues on the field of autoimmunology who have passed away since 2020:

Noel Richard Rose - The father of modern autoimmunology

Noel R. Rose (December 3, 1927 – July 30, 2020), an American immunologist, pathologist, and molecular microbiologist, is widely recognized as a father of autoimmune disease research. In 1957, he co-authored a paper which determined whether a disease entity could be regarded as an autoimmune disease (1). 36 years later, these "Witebsky's postulates" (also known as “Witebsky-Rose Criteria”) were revised based on direct evidence from transfer of pathogenic antibody or pathogenic T-cells, indirect evidence based on reproduction of the autoimmune disease in experimental animals and circumstantial evidence from clinical clues (2). Based on the revised autoimmune disease criteria it was shown in a large epidemiological study that autoimmune diseases as a group are a significant source of morbidity in the world (3). Noel R. Rose’s pioneering studies on autoimmune thyroiditis and myocarditis helped to initiate the modern era of research on autoimmune diseases (4-7). He and his colleagues published for the first time that the major histocompatibility complex contains the main genes that determine the risk for autoimmune diseases (8). Together with David Silverman he presented evidence that the thymus is important in the control of autoimmune pathology (9). In further investigations, Noel

R. Rose and his colleagues showed the influence of infection and environmental agents in the initiation of autoimmune diseases in genetically predisposed animals (10-12). Last but not least, he discussed new management strategies and the possibilities of prediction and prevention of autoimmune diseases (12,13).

Noel R. Rose published almost 900 scientific papers and was editor or co-editor of 23 books, most notably the classic textbook “The Autoimmune Diseases”. He was a consultant to the World Health Organization, chaired the Autoimmune Diseases Coordinating Committee at the National Institutes of Health and was a fellow of the American Association for the Advancement of Science. In 1991, together with Virginia T. Ladd he founded the American Autoimmune Related Diseases Association (AARDA: https://autoimmune.org/), a research and advocacy group for autoimmune diseases and he chaired the organization's scientific committee for 20 years. Noel R. Rose received numerous awards and prizes for his scientific work. e.g., the AESKU.AWARD for life contribution to autoimmunity at the 4th International Congress on Autoimmunity in Budapest November 2004.

Noel R Rose as the main speaker and in discussion with Walter van Venrooij at the 8th Dresden Symposium on Autoantibodies (2007).

As a sought-after talker he gave the opening speech “Autoantibodies as predictors of diseases” at the 8th Dresden Symposium on Autoantibodies (see pictures). In memory of him, it was decided to name a session in the Congress of Autoimmunity after Noel R. Rose.

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Alan Sture Wiik - The father of autoantibody harmonization

As a clinical rheumatologist heading the central Danish autoantibody laboratory in Copenhagen, Allan Wiik (December 1936 – October 2022) very early understood the needs of collaboration between physicians, clinical laboratories and patients to optimize the use of autoantibody analyses in health care (1). His main scientific contributions focus on the serodiagnostics of rheumatoid arthritis, systemic autoimmune disorders, and idiopathic vasculitides. He foresaw the future needs for alignment and standardisation of laboratory procedures, interpretation and reporting of laboratory results in clinical autoantibody diagnostics. He actively started and/or participated in many of the organizations which today work on harmonization of clinical autoantibody analyses (2,3). Alan Wiik was an inaugural member and Vice-Chair of the ANA Subcommittee of the International Union of Immunological Societies (IUIS) (4). He was also the main source of idea, co-founder and in the early years president of the European Autoimmunity Standardisation Initiative (EASI: https://www.gfid-ev.de/easi/) (5). Of outstanding importance was his work using advanced imaging and algorithms to standardize HEp-2 immunofluorescence pattern nomenclature and reporting (3), which became a basis for the following ICAP's work (12).

Being both a clinician and a clinical laboratory specialist he is a popular speaker. In June 2007, he was awarded with the EULAR Award for Meritorious Service to Rheumatology, and in May 2012 with the AESKU.AWARD for life contribution to autoimmunity at the 8th International Congress on Autoimmunity in Granada.

Alan Wiik was a member of the International Scientific Advisory Board at the 7th and 10th Dresden Symposium on Autoantibodies (2004 and 2011, see picture) and Co-Chair at the 8th Dresden Symposium (2007). As a main speaker, he presented "Cutting edge diagnostics' in rheumatology", "Strategies and tools to attain early diagnosis and estimate prognosis of autoimmune rheumatic

disorders", and "Multicentre collaboration is needed to reach unified classification of IIF ANA and clinical use of strictly defined findings".

Alan Wiik together with Edward K. Chan and Luis E.C. Andrade at the 10th Dresden Symposum on Autoantibodies (2011)

Allan Wiik was a grand personality, with an absolute pitch not only for the needs in clinical immunology, but also in music and in interpersonal relations.

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Pierre Youinou - A founder in the field of immunopathology of autoimmune rheumatic diseases

As a head of the hospital immunology department at Brest Medical School from 1989 to 2011, Pierre Youinou (1945 - August 2022) obtained recognition for this department as a European reference laboratory in autoimmunity. His clinical research has provided important insights in the role of B cells in the pathogenesis of systemic autoimmune rheumatic diseases. Pierre Youinou's laboratory was one of the most successful to study the pathogenesis of Sjögren's syndrome. He and his colleagues have shown that the role of B cells in disease extends far beyond autoantibody production. Thus, B cells could promote the deleterious effects of autoreactive T cells through autoantigen presentation, and even be amplified by B cell-derived cytokines. The result is that therapeutic targeting of B cells would ameliorate autoimmune conditions by acting on numerous disease-promoting pathways (1-4). Another focus of Pierre Youinous's work was the search on the function of anti-Fcγ receptor and anti-endothelial cell antibodies. He showed their deleterious effects in three ways: activation, induction of apoptosis and cytotoxicity (5-7). He characterised with his collaborators an interaction between TLR2 and β2GPI and showed that inhibition of this receptor decreased endothelial cell activation via β2GPI (8,9).

Pierre Youinou in discussion with Yehuda Shoenfeld at the International Congress on Autoimmunity

Pierre Youinou has written more than 700 scientific articles, and among them 200 book chapters, editorials and reviews. He was a corner stone of the Congresses of autoimmunity and received the life contribution prize for autoimmunity (AESKU.AWARD 2012) for that. He himself organized several symposia on autoimmunity and was the B cell person of the world regarding the involvement of B cells in autoimmune diseases. Furthermore, he was a member of the International Scientific Advisory Board at the 3rd (1996) and the 9th Dresden Symposium on Autoantibodies (2009). As a main speaker he presented "Genetic aspects of pathogenic idiotypes" and "Serological changes induced by B cell ablative therapy"

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See also: Jacques-Oliver Pers: In Memoriam Pierre Youinou, MD, DsC 1945-2022. Clin Exp Rheum 2022. https://www.clinexprheumatol.org › article.asp?a=19513

Detlef Zillikens – One of the leading experts in the field of autoimmune blistering diseases

Detlef Zillikens (15.07.1958-19.09.2022) was an internationally outstanding scientist in the field of autoimmune blistering diseases. First in Würzburg and since 2004 as the Chair of the Department of Dermatology at the University of Lübeck (Germany) he established completely new experimental models for the research on autoimmune skin diseases with the help of which clinical and immunopathological changes in epidermolysis bullosa acquisita, bullous pemphigoid and mucous membrane pemphigoid could be identified and reproduced. He and his coworkers

established protein A immunoadsorption as an effective tool for treatment. Furthermore, he described the new entity of anti-p200 pemphigoid for the first time. Clearly, these works had immediate effects on the treatment, diagnosis and management of patients with potentially life-threatening and debilitating skin disease of autoimmune origin.

Detlef Zillikens organized regular visiting professorships from international colleagues as well as two large international meetings on blistering diseases in Lübeck in 2013 and 2017 that are much remembered in the blistering diseases field. He was a main speaker at the 4th Dresden Symposium on Autoantibodies (1998) with his presentation "Autoantigens and autoantibodies in subepidermal blistering diseases".

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We will all remember and memorialize Noel R. Rose, Allan S. Wiik, Pierre Youinou and Detlef Zillikens, whose keen thinking, scholarly approach and statesmanship will be missed, but not forgotten.

2 Role of Autoantibodies in the Prediction and Classification of Autoimmune Diseases

The importance of autoantibodies for diagnosis and classification of rheumatoid arthritis

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Background

Rheumatoid factor (RF) and anti-cyclic citrullinated protein/peptide antibodies (ACPA) are included in the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria for RA (1). Several studies (2-6) demonstrated an increased diagnostic sensitivity (73.5% to 84%), but decreased diagnostic specificity (60% to 71%) of the ACR/EULAR 2010 criteria to the former ACR 1987 RA criteria (7). The drawback of decreased specificity is the risk of misclassification and over-diagnosis at baseline. Part of the lower specificity can be attributed to RF and ACPA since approximately 20% of 'false positive' patients were autoantibody positive (6).

In the ACR/EULAR 2010 classification criteria for RA, a low positive RF or a low positive ACPA contributes two points while a high positive (>3-times the upper limit of normal) RF or ACPA contributes 3 points; a score of ≥6 points allows classification of RA and is therefore indicative of definite RA (1). Thus, in the ACR/EULAR 2010 classification criteria RF and ACPA results are attributed the same scoring weight. However, it is well known that ACPA has a significantly higher specificity for RA than RF (9-13) as RF can be detected in other rheumatic disorders, infections and in apparently healthy individuals (14, 15). Moreover, the classification criteria do not consider a difference in scoring weight for combined positivity of RF and ACPA compared to single positivity (16-19). It has been recently suggested that the specificity of RA classification could be enhanced by changing the weight factors of the serological grades based on the different diagnostic performance of RF and ACPA (20 and unpublished data). Thus, by ascribing a higher weight to high antibody levels than to low antibody levels, a higher weight to ACPA than to RF and the highest

weight to combined RF and ACPA positivity a gain in specificity of the 2010 classification criteria can be expected.

Methods

To address this issue, we used the cohort of the SAVE (Stop Arthritis Very Early) multicenter trial (21), including 325 patients with early arthritis of which after a one-year follow-up period 131 were diagnosed as having RA while 194 patients had other rheumatic diseases (rheumatic disease control group, RDCG). The SAVE cohort had previously been used for validation of the ACR/EULAR 2010 criteria (6).

Results

The serology data are summarized in Table 1: positive RF results were obtained for 50.0% of the RA population and 8.3% for the RDCG (LR 6.0); positive ACPA results were obtained for 51.5% of the RA population and 3.6% for the RDCG group (LR 14.3); positive serology for both ACPA and RF was obtained in 45.4% of the RA cohort, but only in 2.1% of the RDCG cohort (LR 21.6). Further analysis of the data revealed that high RF in the absence of ACPA was only weakly specific (LR 1.1) and low RF was even more prevalent in RDCG patients (LR 0.6). In contrast, high ACPA in the absence of RF was moderately specific for RA (LR 2.8); however, specificity was considerably augmented by the presence of both low or high RF (LR 20.5). Low ACPA was rarely observed in RA patients and not at all in the RDCG and therefore its specificity could not be properly evaluated.

Table 1. Serology results of the rheumatoid arthritis cohort (RA, n=132) and inflammatory rheumatic disease control group (n= 193) of the SAVE trial (6).

ACPA	RF-IgM	RA (% pos)	RDCG (% pos)	LR
Total		51.5	3.6	14.3
	Total	50.0	8.3	6.0
pos	pos	45.4	2.1	21.6
neg	low	2.3	4.1	0.6
neg	high	2.3	2.1	1.1
low	neg	1.5	0.0	n.a.
high	neg	4.5	1.6	2.8
low	pos	2.3	0.0	n.a.
high	pos	43.1	2.1	20.5

Pronounced differences in diagnostic performance were not only seen between RF and ACPA but especially also between single and double positivity. Especially RF in the absence of ACPA showed rather low specificity for RA while ACPA in the absence of RF was moderately specific. Remarkably however, RF/ACPA double positivity was highly specific for RA being detectable in almost half of the RA patients but rarely in disease controls.

Therefore, we propose that different weights should be given based on the antibody type (RF versus ACPA), the antibody level and the combined positivity. A suggestion for refined weights of serological scores for RA classification is presented in Table 2: “0” for both RF and ACPA negative patients as well as for isolated low positivity for RF; “1” for isolated high positivity for RF; “2” for isolated and low ACPA positivity; “3” for low ACPA combined with RF positivity and for isolated high ACPA positivity; “4” for high ACPA combined with RF positivity.

Table 2. Suggestion for refined weights of serological scores for RA classification

Antibody	negative	RF low	RF hi	ACPA low	ACPA hi	ACPA low/RF	ACPA hi/RF
Score	0	0	1	2	3	3	4

Applying the refined score in RA classification reduced RA misclassification of seropositive RDCG patients from 79.0% to 47.4% without affecting diagnostic sensitivity (87.1% for both classification scores).

Conclusions

Thus, the major findings can be summarised as follows:

(i) a positive RF result in absence of ACPA is not specific for RA;
(ii) a positive ACPA result in absence of RF is moderately specific for RA;
(iii) the co-occurrence of ACPA and RF is highly specific for RA.

Since the proposed serological scoring system significantly reduced misclassification of (seropositive) RDCG patients without affecting sensitivity, we strongly suggest to modify the ACR/EULAR 2010 criteria accordingly. Giving the same weight to both antibodies is no longer justified and may lead not only to misclassification but also to a false diagnosis with potentially unfavourable consequences for the patient. Clearly, these data need to be confirmed in other cohorts before modification of the classification criteria can be seriously discussed.

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Prevention and early diagnosis of rheumatoid arthritis (RA) – The DigiPrevent project

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Background

Rheumatoid arthritis (RA) is a chronic and debilitating disease that affects over five million people in Europe and requires lifelong and expensive treatment. Patients with the condition often experience pain and comorbidities that influence their daily social and working lives.

Prevention and early curative treatment for RA is the ultimate solution for patients and society. But the current structure of healthcare in Europe rarely enables prevention or adequate early treatment.

Aim

The DigiPrevent project is a research collaboration between academia, healthcare providers, patient organizations, payors, and industry with the aim to improve the patient workflow to identify individuals with an increased risk for RA to be able to start treatment earlier which may lead to drug-free remission. The project also aims to improve the referral from primary care to secondary care.

Methods and results

The project includes a digital screening tool that is aimed to be used by individuals with musculoskeletal complaints at primary care level or even before approaching health care. The digital questionnaire aims to identify individuals which have symptoms that could be caused by a rheumatic disease at an early stage. The information from the questionnaire will be combined

with serologic testing and genetic information into an algorithm improving the patient workflow by supporting earlier diagnosis and better referral. The tools will be evaluated on patients from Sweden, Netherlands, and Germany.

The study is highlighting points in the patient workflow were new digital tools and additional serologic biomarker could improve the status quo and lead to earlier treatment of the patient, better prognosis for drug-free remission and lower costs for society.

Expected outcomes

The DigiPrevent team is working with patients and patient organizations, healthcare providers and payors to validate and implement the system and tools for transformation of healthcare.

DigiPrevent calculates that there is a potential for reduction of cases of chronic RA by an estimate of 20-50%, based on today's knowledge of modifiable lifestyle factors and effects of existing therapies in individuals at very high risk of developing RA.

This project has received funding from EIT Health. EIT Health is supported by the European Institute of Innovation and Technology (EIT), a body of the European Union that receives support from the European Union's Horizon 2020 Research and Innovation program.

Differentiation of idiopathic inflammatory myopathies by increasing number of myositis specific and myositis associated autoantibodies

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Abstract

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of conditions that have as hallmark a variable degree of muscle involvement. To date, more than fifteen autoantibodies have been described and different methods for detecting autoantibodies are available, though, not fully validated. Currently, there is an increasing interest in harmonizing and standardizing the use of such methods to detect autoantibodies. International research groups are collaborating to standardize the use of different assays in the daily clinical setting. Detecting autoantibodies in patients with IIM offer several advantages in the clinical setting. First, they may be useful to differentiate autoimmune myositis from other forms of neuromuscular myopathies. Second, autoantibodies may be helpful in stratifying patients according to their prognostic profile. Third, autoantibodies may serve as predictors for response to treatment and some specificities may even mirror disease activity. Autoantibodies are so clinically relevant that they are now included in the 2017 EULAR/ACR classification criteria for IIM and in other additional proposed criteria to improve the accuracy of such criteria. These criteria may offer not only a framework to identify a candidate for clinical trials but may also offer a system for sorting patients according to their prognostic profile.

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New developments in the classification of the antiphospholipid syndrome

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Abstract

Laboratory assays for anti-phospholipid antibodies (aPL) are crucial for both the classification/diagnosis of anti-phospholipid syndrome (APS). The same tests are also important for stratifying the risk of developing the clinical manifestations of the syndrome (1).

The new ACR/EULAR APS classification criteria include an entry criterion of at least one positive aPL test within three years after an aPL-associated clinical criterion followed by additive weighted criteria clustered into six clinical and two laboratory domains (Lupus Anticoagulant [LAC], and aCL and/or anti-β2glycoprotein-I (β2GPI) antibody IgG/M detected by ELISA). Patients accumulating at least three points from clinical and laboratory domains are classified as having APS (2).

LAC should be performed and interpreted according to the International Society of Thrombosis and Hemostasis guidelines.

While the techniques for LAC are relatively well standardized, the detection of aPL by solid-phase assays is still a matter of discussion despite the efforts for their standardization (3). aPL represent the example of a laboratory test that moved from dichotomous to quantitative/semiquantitative results consistent with the idea that aPL titer offers more diagnostic/prognostic information for both vascular and obstetric manifestations (1). The inclusion in the new classification criteria of two levels of aCL/aβ2GPI ELISA positivity (“moderate” and “high” titers) and the combined aCL IgG and aβ2GPI IgG positivity is consistent with the higher prognostic value of medium/high aPL levels and the main value of β2GPI-dependent antibodies. The definition of aPL “persistence” (two positive tests at least 12 weeks apart) was not changed in comparison with the previous criteria. The levels for “moderate” and “high” positivity apply to ELISA tests but not to others, e.g., new automated platforms (2). In particular, the higher sensitivity of chemiluminescence raises the issue of the real diagnostic/prognostic value of results close to the cutoff limits used for the other solid-phase assays (4). Comparison studies among the different aPL solid-phase techniques are limited and report a similar specificity of the assays even though discrepancies can be found (personal data).

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Sarcoidosis: a comprehensive review of clinical cases in Brazil, with focus on autoimmune features

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Introduction

Sarcoidosis is defined as a multisystem inflammatory disease of undefined etiology, characterized by the formation of non-caseating granulomas, which may involve almost any organ. Clinical manifestations are protean, which makes the diagnostic process challenging. We might view sarcoidosis as an autoimmune disease based on several characteristic features, such as the immune system involvement on the formation of granulomas, the presence of autoantibodies seen in some studies, genetic factors associated with HLA haplotypes, environmental triggers, chronic inflammation in response to an improperly regulated immune system, as well as the response to immunosuppressants.

Objectives

To evaluate the diagnostic difficulties, clinical manifestations, and possible complications in Brazilian patients with sarcoidosis.

Methods

Observational, cross-sectional and descriptive study. The research was composed of patients from Brazil with a self-reported diagnosis of sarcoidosis, over 18 years old, of any sex, ethnicity, and origin. Data collection was held from August 2022 to November 2022. Patients were asked to fill in a questionnaire on Google Forms. Data were tabulated in Excel and analysis was carried out using IBM Statistical Package for the Social Sciences (SPSS)® 20.0. The study complied with Resolution 466/2012 of the Brazilian National Health Council, with agreement of our protocol and methods by the local IRB.

Results

We received 125 questionnaires, with spontaneous participation from individuals belonging to private sarcoidosis groups on social media. The response rate was 17%, considering 725 members on the Facebook group. Patients were predominantly women (83%) with 42 ± 10 years old on average. Environmental exposure factors were present in 41% of the patients, and 26% had another concomitant autoimmune disease. It took more than three medical professionals, and 5 or more years of symptoms in 50% of the patients to reach a final diagnosis. A biopsy was the most frequently performed test to assist in the diagnostic definition (95%), all others with Löfgren's syndrome diagnosed by clinical findings and lung radiographs. Tuberculosis was the most frequent differential diagnosis (76%), followed by lymphomas, other neoplasias, and systemic inflammatory rheumatic diseases. Lung involvement was reported by 39% of patients, the majority with pulmonary fibrosis. Heart (arrhythmias), eye and CNS manifestations followed in prevalence. Tobacco was currently used by 5% of the patients, and illicit drugs by 8%. Most common treatment was the use of systemic corticosteroids in 70% of the participants, followed by immunosuppressants and biologic drugs.

Conclusions

Sarcoidosis remains a diagnostic challenge, considering the number of professionals and years to reach a conclusion. Symptoms are protean, many organs may be involved with a wide array of clinical manifestations. Invasive procedures are generally necessary, with adequate analyses by experienced pathologists. Autoantibodies have no place in the diagnostic evaluation, and laboratory tests are nonspecific. While there are compelling signs pointing to sarcoidosis having an autoimmune component, it doesn't unequivocally meet all the criteria for being classified as an autoimmune disease according to accepted definitions. Further research is needed for a deeper understanding of the complex nature of sarcoidosis.

3 Challenges and Advances in Autoantibody Detection

3.1 Harmonization of Autoantibody Determination

The International Consensus on ANA Pattern (ICAP) – update 2023

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Introduction

The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) is the reference method for autoantibody screening (1). The HEp-2 IFA titer or fluorescence intensity informs on the autoantibody serum level, which has clinical relevance as higher autoantibody levels have stronger association with systemic autoimmune diseases (SAID) (2) and increased likelihood to identify the autoantigen in follow-up testing (3). The HEp-2 IFA patterns provide useful information on the possible autoantibodies in the sample. Accordingly, the HEp-2 IFA patterns provide guidance to follow-up testing for antigen-specificity and different patterns may have distinct clinical and immunological relevance, such as the nuclear homogeneous (AC-1), the nuclear dense fine speckled (AC-2), and the centromere (AC-3) patterns (4).

Recognizing the importance of the harmonization of the HEp-2 IFA test, an international group of specialists launched the International Consensus on ANA Patterns (ICAP) initiative in a full-day workshop at the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA) held in 2014 in São Paulo, Brazil. ICAP has established a platform to promote consensus on the definition and nomenclature of the most relevant patterns. The nomenclature, classification tree, and a set of images for each pattern are available at the website www.ANApatterns.org, where the most relevant and usual patterns are assigned an alphanumeric code from AC-0 (negative result) to AC-29 (5). Six workshops were held since the launching of ICAP and the 7th workshop will take place on September 11, 2023 in Dresden immediately before the 16th Dresden Symposium on Autoantibodies (https://www.gfid-ev.de/index_eng.htm). In addition, ICAP

sessions occurred were held as parallel activities in some editions of the International Congress of Autoimmunity, the International Congress of Immunology, and various national scientific meetings.

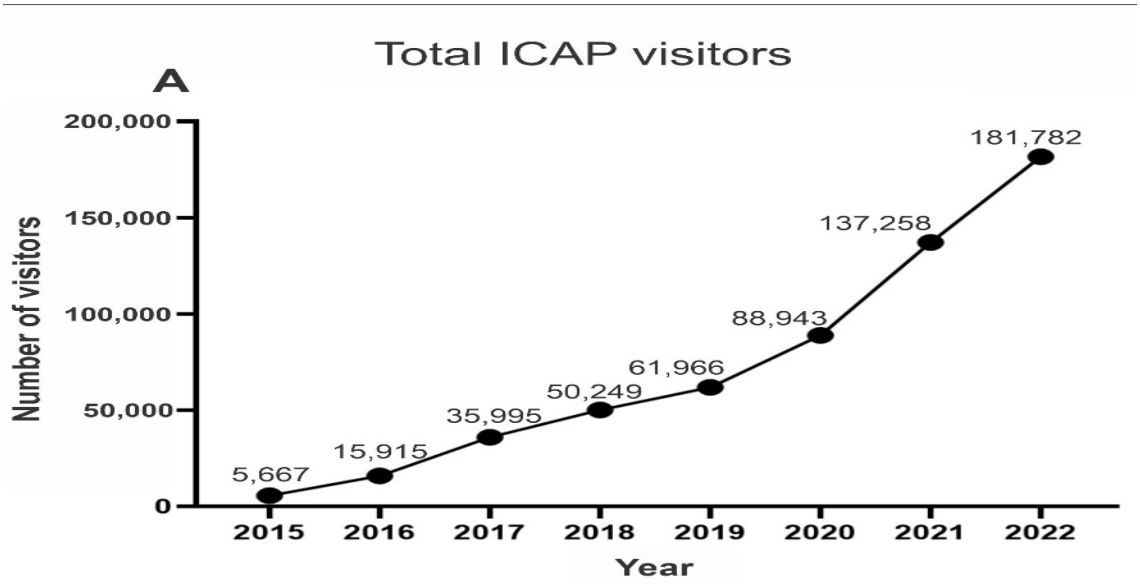

Figure 1. Annual frequency of users of the www.anapatterns.org website

From its start the website has been increasingly accessed by users from different countries across the world (Figure 1), reaching a total of 4,873 subscribed users up to July 2023. The majority of subscribed users of the website are physicians (47.02%), followed by technologists (18.19%), students (8.53%), and researchers (6.08%). These users belong to laboratory, clinical, academic, and business institutions: Clinical/Diagnostic Laboratory (42.07%), Hospital/Outpatient Clinic (32.64%), Academic institutions (15.61%), and In Vitro Diagnostic (IVD) Industry/Business (6.54%). The ICAP website is accessed by circa 200,000 users per year from 186 countries and is translated into 16 languages. Up to the end of July this year, 108,522 users accessed the website.

HEp-2 CIC project

Despite the wide acceptance of ICAP recommendations by clinical laboratories, academic institutions and IVD industries, it is generally recognized that there is still considerable heterogeneity across the world regarding the interpretation and reporting of HEp-2 IFA. The scientific publications regarding the prevalence of HEp-2 IFA are most frequently based on local/regional data, making it difficult to accurately register possible differences in the frequency of positive HEp-2 IFA and of each HEp-2 IFA pattern in different parts of the world. In response to this scenario, ICAP has launched the HEp-2 Clinical and Immunological Characterization (HEp-2 CIC) project that has, among other goals, the objective to investigate the frequency of positive HEp-2 IFA results and the respective patterns in laboratories worldwide. The preliminary results of this part of the initiative are presented in detail elsewhere in this edition of the 16th Dresden Autoantibody Symposium Book, with the participation of 50 laboratories from 34 countries on five continents, totalizing 531,241 HEp-2 IFA results during the year 2019. This uncontrolled

transversal analysis showed that the ICAP classification has been progressively incorporated into the interpretation and reporting of HEp-2 IFA patterns in laboratories across the world. However, there is still substantial heterogeneity regarding specific patterns, especially those considered expert-level patterns. These results emphasize the need for improvement in harmonization in the reporting strategy of the HEp-2 IFA test.

Another goal of the HEp-2 CIC project addresses HEp-2 IFA patterns not entirely characterized in terms of autoantibody association and clinical relevance. These patterns do not occur very often in the clinical laboratory routine and the insufficient number of cases necessary for definitive characterization usually limits research in individual centres. The HEp-2 CIC project aims to provide a framework for the establishment of multicentre collaborative studies to characterize these patterns in terms of autoantibody association and clinical relevance. During the 7th ICAP Workshop, the ICAP Executive Committee will discuss and define protocols to put forward this part of the HEp-2 CIC project.

ICAP App for smartphones

During the 6th ICAP workshop at the 15th Dresden Symposium on Autoantibodies in 2021, a survey was done to gauge the need for a smartphone App. There was overwhelming support from ICAP users that a smartphone App would be highly useful, especially among the younger users. Fund was raised to generate such an App including the majority of functions currently available on the ICAP website. Figure 2 illustrates many of the functions of the recently launched ICAP smartphone App, which is compatible with iOS and Android operating systems and available free of charge from Apple and Google Stores under the search words “ANA patterns” and select “ICAP Official App.” The App is now in beta testing, only in the English language, and is freely available to all users. Interested users should note that they must first register for a free login account at www.ANApatterns.org. It is expected that this new media will increase the reach of the ICAP initiative and the usage of ICAP HEp-2 IFA patterns.

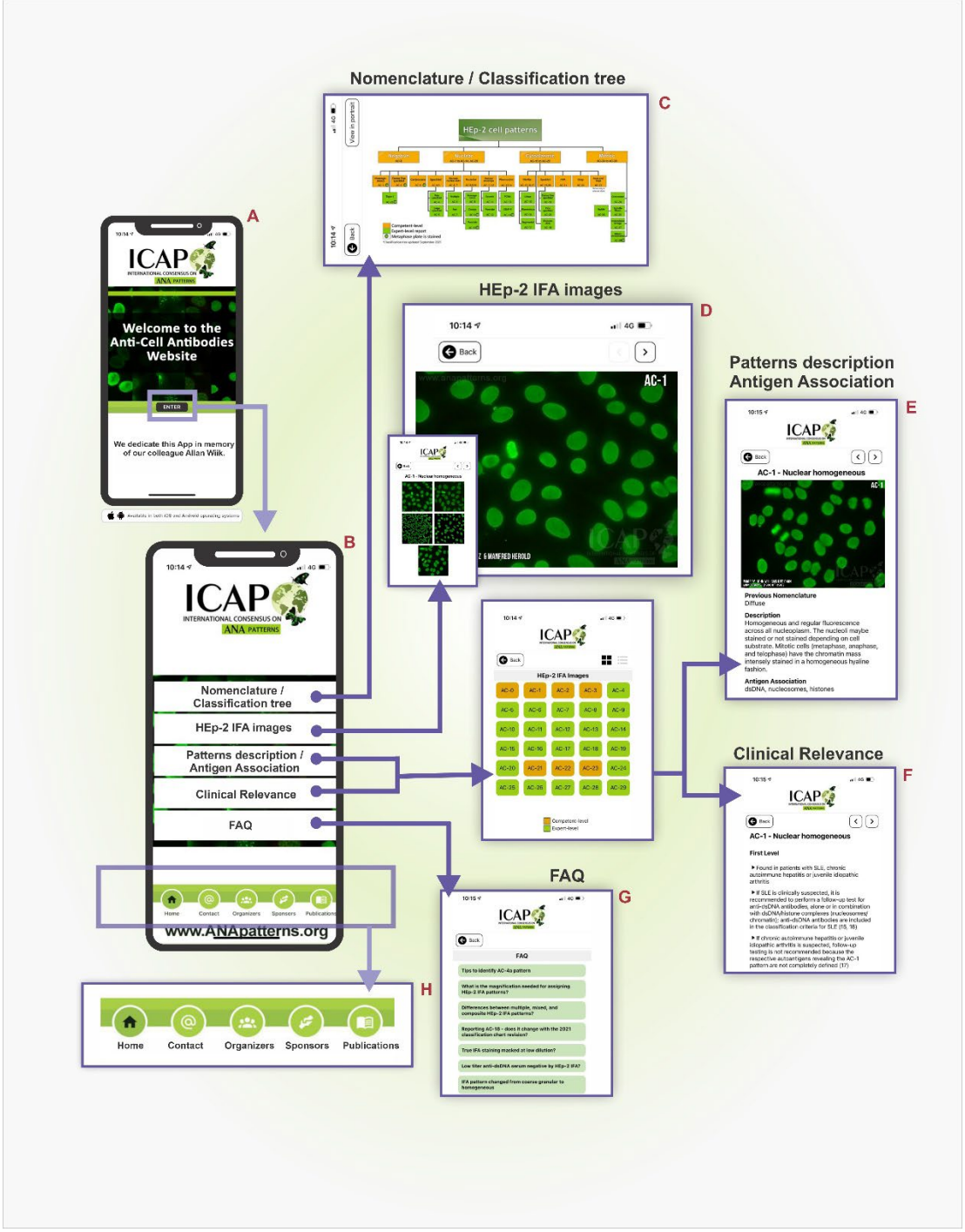

Figure 2. Outline of the functions available in the ICAP smartphone App with welcome page (A), main menu page (B) with five subheadings: Nomenclature/classification tree (C), HEp-2 IFA images (D), Patterns description/antigen association (E), Clinical Relevance (F), and Frequently Asked Questions (G). The bottom menu (H) shows contact access, organizers, sponsors, and publications. The free-of-charge ICAP App for smartphones is iOS/Android-compatible and conveys most of the functionalities of the www.anapatterns.org website.

Challenges and future developments

Despite the achievements of ICAP in contributing to harmonization in the HEp-2 IFA test, we acknowledge that there are still several unmet needs. Here we address some of the current challenges and limitations of ICAP and possible developments to solve them.

Heterogeneity of results obtained using HEp-2 slides from different commercial brands

One major issue in HEp-2 IFA testing is the heterogeneity in results obtained with different commercial brands of HEp-2 cell slides. It is well-documented that peculiarities in culturing, permeabilizing and fixing the cells can affect to various extension the topographic distribution of autoantigens and the availability of key autoepitopes (6). Accordingly, several groups have documented considerable discrepancy in the positivity, pattern, and titer of HEp-2 IFA using different HEp-2 cell slide brands (7-10.). The IVD industry has put considerable effort in providing good and cost-effective HEp-2 IFA kits, however, these are heterogeneous in many aspects, including the culturing, permeabilization, and fixation of cells, the isotype-specificity of the conjugate, and the F/P ratio of the conjugate, just to mention a few variables. These features contribute to the proprietary characteristics of the HEp-2 IFA kit trademarks and are very difficult to standardize. However, the current heterogeneity hinders the robustness of the HEp-2 IFA test in terms of inter-laboratory reproducibility of results. Therefore, efforts should be endeavored to promote a minimum degree of harmonization among commercial HEp-2 IFA kits. A tripartite initiative involving ICAP, IVD industry representatives, and official regulatory agencies would be a possible approach to promote improvement in this area.

HEp-2 IFA and Computer-Added Diagnosis and Artificial Intelligence

Computer-added diagnosis technology has emerged as a major trend in the HEp-2 IFA processing and interpretation, with several commercial CAD systems available. These systems are especially attractive for large laboratories with high throughput operation. To the moment, the CAD systems applied to HEP-2 IFA are able to identify only a minor proportion of patterns, but they are effective in discriminating positive and negative tests. While the CAD systems may contribute to harmonization in that they do not rely on human subjectivity, they introduce several variables that may contribute to heterogeneity in results among laboratories using different proprietary brand CAD systems, such as DNA counterstain, optical system and image acquisition, magnification power, run-time, types of recognized HEp-2 IFA patterns, and software, among others (11). Artificial Intelligence (AI) can be integrated to CAD systems in the progressive definition of imaging features that may allow for optimization of the system in defining HEp-2 IFA patterns. Additionally, IA also opens possibilities of integration of HEp-2 IFA data with other relevant tests into algorithms that may strengthen the accuracy of results. ICAP should find opportunities for contributing with these trends with respect to harmonization in HEp-2 IFA.

Updating the autoantibody/pattern association

One of the most useful pieces of information provided by the HEp-2 IFA patterns is clinical relevance. Accordingly, ICAP has reviewed the pertinent literature and organized the available information into bullet messages at the www.anapatterns.org website (4). However, this is a quite dynamic field, as continuous research activity continuously reports on novel autoantibodies or revisits previously established autoantibody/pattern associations. Accordingly, ICAP is working on a “modus operadi” for adequate updating, including the establishment of task-forces with the

help of young ICAP supporters worldwide. In addition, comments related to appropriate updates on clinical relevance are welcome from colleagues to help facilitate these laborious efforts.

Consolidation and expansion of the HEp-2 IFA pattern portfolio

The original ICAP classification tree contained 28 patterns and in the following years additional categories were created, namely AC-0 (negative result) (12), AC-XX (for atypical patterns) (12), AC-29 (Topoisomerase-like pattern) (13,14), and the splitting of AC-4 into AC-4a (myriad discrete fine speckled pattern associated with anti-Ro60 antibodies) and AC-4b (plain fine speckled nuclear pattern, not associated with anti-Ro60 antibodies) (15,16). This expansion results from interaction with the international community and internal discussions within the ICAP Executive Committee. New patterns should meet the following criteria: 1) Unique morphologic characteristics that allow the distinction from already classified patterns; 2) Published report of the candidate novel pattern by independent group(s); 3) Consistent images to be displayed in publications and at the website. Ideally, novel patterns should bear relevant autoantibody association and clinical relevance, but this has not been a mandatory criterion. Currently, the ICAP group is analyzing novel candidate patterns that could be a spinoff of the AC-2 and the AC-5 patterns, respectively.

The AC-2 pattern (nuclear dense fine speckled) is characterized by a peculiar staining of the whole interphase nucleus with heterogeneity in brightness, size, and density of the several speckles and a similar staining pattern at the metaphase chromatin plate (17). The AC-2 pattern is clinically relevant as this pattern is strongly associated with anti-DFS70 antibodies in the absence of other relevant antinuclear antibodies (18). Considering that anti-DFS70 antibodies are prevalent in the general population and in non-SAID patients, the AC-2 pattern speaks against the diagnosis of SAID (19). The positive staining of the metaphase plate in the AC-2 pattern clearly differentiates it from the AC-4 pattern, which shows no staining of the metaphase plate. However, there are samples yielding a fine speckled nuclear/ metaphase plate pattern with unique morphological features that allow the distinction from the AC-2 pattern. Preliminary evidence suggests that this variant also differs from the AC-2 pattern with respect to the association with anti-DFS70 antibodies. As of today, analysts around the world face difficulty in classifying samples depicting such “pseudo-DFS” pattern, as this variant is not in the ICAP classification tree (20). The ICAP group is currently investigating the feasibility and appropriateness of splitting the AC-2 pattern into AC-2a (nuclear dense fine speckled pattern) and AC-2b (other nuclear fine speckled patterns with stained metaphase plate).

A similar situation occurs regarding the AC-5 pattern, which is currently assigned as the coarse/large speckled nuclear pattern in the ICAP classification. However, there is heterogeneity within this group as the coarse speckled nuclear pattern is closely associated with antibodies to spliceosome antigens (Sm and U1-RNP) whereas the large speckled nuclear pattern has been designated by some groups as the nuclear matrix pattern, which is not particularly associated with anti-Sm/U1-RNP antibodies, as pointed out by Wiik et al (21). Further studies by independent groups are warranted in order to advance the proposal of splitting the AC-5 pattern into these two variants.

Another important aspect to be worked out refers to patterns that do not fit into the conventional single patterns displayed at the ICAP classification tree, i.e., cases in which more than one autoantibody in the sample causes overlay of the respective patterns. Currently, these are not addressed at the ICAP classification tree. When there are two or more autoantibodies in a sample, the respective patterns may interfere with each other in two different ways. When each of the

autoantibodies stains distinct cellular domains, it is possible to identify each one of the respective patterns and we tend to classify this sample as “multiple patterns”. A relevant example is the overlay of AC-6 (multiple nuclear dots, associated with anti-sp100 antibodies) and AC-21 (cytoplasmic mitochondria-like, associated with anti-mitochondria antibodies) and the combination AC-6/AC-21 is not infrequently seen in patients with primary biliary cholangitis. The recognition of these two patterns is possible because each one refers to distinct cell compartments. In contrast, when the antigens recognized by coexistent autoantibodies in a sample share to some extent the topographic distribution across cell domains it is frequently not possible to identify clearly any of the individual patterns associated with each of the autoantibodies. A frequent example is the coexistence of anti-SS-A/Ro or anti-SS-B/La (causing the AC-4 pattern) and anti-Sm or anti-U1-RNP (yielding the AC-5 pattern) antibodies. The close topographic distribution of these two patterns across the nucleoplasm frequently prevents the recognition of the individual AC-4 and AC-5 patterns. In such cases, we tend to classify the sample as a “mixed pattern”.

Updating and strengthening the website

A current challenge refers to the continuous development of the ICAP website www.anapatterns.org. Launched in 2015, the website has evolved extensively in content and in the number of visitors. Although the website has been working successfully, an upgrade in its framework will be necessary in order to guarantee full functionality and the possibility of further expansion. The reorganization of the website has inherent costs and ICAP has no income sources other than unrestricted educational funding voluntarily provided by several IVD industries and some clinical laboratories (www.anapatterns.org). Efforts are underway in order to match the budget for the website consolidation.

Novel Educational Courses at the Website

ICAP develops educational projects that are available free of charge on the website. The first module introduces the ICAP concept, the classification tree, guidance for navigating through the website, and presents technical recommendations on how to perform the HEp-2 IFA. After taking the training module, participants can take the final assessment and receive a certificate stating his/her successful completion of the course. This first module was released in July 2020 and to date 1,719 individuals have assessed the educational modules and 44.6% completed and obtained the certificate of training. Professionals from 91 countries participated so far. Translation of the training module to Chinese and Spanish has been completed and more than 300 users have already participated. Additional training modules are planned for the near future, including one focusing on basic (competent) and advanced (expert) patterns, and one exploring the interface between the HEp-2 IFA patterns and the “functional anatomy” of the HEp-2 cell.

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How consistent are the association of HEp-2 patterns and autoantibody specificities

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Abstract

1. Introduction: Autoantibodies are useful biomarkers in the diagnosis and monitoring of systemic autoimmune diseases (SAID). Indirect Immunofluorescence Assay (IFA) on HEp-2 cells (HEp-2 IFA) is widely used for autoantibody screening and the immunofluorescence pattern provides preliminary information on the possible autoantibody specificities. Associations between HEp-2 IFA patterns and autoantibodies have not been studied formally in large cohorts and most published studies are based on a small number of samples and frequently suffer from selection bias in referral autoimmunity centers. Personal experience indicates that HEp-2 IFA patterns do not always reflect the existence of autoantibodies to which they are presumably associated.

2. Objectives: to study the association between HEp-2 IFA patterns and specific autoantibodies in a large non-biased cohort of samples from a general clinical laboratory.

3. Methods: In this analytical, observational, retrospective study, all HEp-2 IFA tests requested between the years 2012 and 2019 were recruited from the database of the Fleury laboratory, São Paulo, Brazil. Only cases with a single HEp-2 IFA pattern were analyzed and patterns were expressed according to the ICAP classification. We looked specifically for cases with simultaneous determination of at least one of 21 selected autoantibodies (dsDNA, Nucleosome, Histone, DFS70, CENP-B, SS-A/Ro, SS-B/La, Mi-2, RNP, Sm, RNA Pol III, sp100, gp210, PMScl, Scl70, ASMA, actin, ribosomal P0, Jo-1, AMA and M2). The association between HEp-2 IFA patterns and autoantibody specificities was determined in a bidirectional analysis. Data is presented descriptively with absolute frequency and percentage parameters.

4. Results: From January 2012 to December 2019, 453,416 HEp-2 IFA reports were assessed, with 140,345 positive results. Among the nuclear patterns, AC-4 dominated, followed by AC-2b and AC-2, while AC-1 and AC-5 followed. The cytoplasmic patterns AC-21 and AC-15 were prevalent. In the first-tier analysis, traditional associations were confirmed; AC-1 was associated with antibodies to nucleosome (76.25%) and anti-dsDNA (35.66%). Unexpectedly, some AC-1 samples exhibited antibodies to DFS70 (14.29%), anti-CENP-B (5.88%), and anti-RNP (4.76%). Samples with the AC-2 pattern had a high frequency of antibodies to DFS70 (98.28%), but also against histone (11.61%) and RNA pol III (20.00%). Samples with the centromere pattern (AC-3) had 89.66% positivity rate for anti-CENP-B antibodies. Samples with the AC-4 pattern revealed rare reactivity against SS-A/Ro, SS-B/La, and Mi-2 antibodies, while RNA pol III, nucleosome, and histone displayed notable positivity. Second-tier analysis showcased diverse autoantibody-pattern associations. Anti-CENP-B predominantly appeared in AC-3 samples, while anti-SS-A/Ro, ASMA, anti-dsDNA, and AMA antibodies showed diverse pattern presence. These findings demonstrate that the known associations between HEp-2 IFA patterns and specific autoantibodies are true, but not absolute, emphasizing the complexity of the interactions of autoantibodies in any given sample with regard to the establishment of the HEp-2 IFA pattern.

Introduction

The immunological and clinical significance of autoantibodies is diverse and we know of three types of autoantibodies: a) Natural autoantibodies, present in all individuals at relatively low

titers; b) Infectious, arise as a result of inflammation and tissue destruction, are not associated with clinical manifestations of autoimmune disease and their titers fall when the infection is resolved; c) Autoimmune, which reflect the loss of immunological tolerance and may participate in the pathophysiology of systemic autoimmune diseases (SAID).

Autoantibodies are useful biomarkers in the diagnosis and follow-up of SAID1 and they may be detected before, during, or after the onset of clinical manifestations2. In the clinical laboratory, various methods are used to detect autoantibodies and the indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2 IFA), also known as anti-nucleus cytoplasmic (ANA) antibody test, is the standardized technique as the first step in laboratory algorithms to track the presence of autoantibodies (3,4). HEp-2 cells in monolayer (human epithelial cells derived from a laryngeal carcinoma) attached to glass slides are used as the antigenic substrate and they present advantages over other previously used substrates, as they distinguish more than 30 different morphological patterns, evidencing dozens of autoantibodies that are relevant for the diagnosis of a wide variety of SAID5. The HEp-2 IFA test is today considered the gold standard assay for the screening of autoantibodies.

When interpreting HEp-2 IFA, the titer and pattern must be assessed. The titer reflects the autoantibody concentration and higher titers indicate higher probability of a SAID6. On the other hand, the HEp-2 IFA pattern provides a preliminary indication of the autoantibodies in the sample which is of great relevance to the clinical practice. This is because the distribution of the autoantigens within the cell adopts specific morphological patterns of immunofluorescence in the HEp-2 IFA test and is an indirect indication of the possible autoantibodies present in the analyzed sample (Table 1). In recognition of the importance of the HEp-2 IFA patterns, the International Consensus on ANA Patterns (ICAP) initiative has defined 30 relevant patterns that are classified under an alpha-numerical code (AC) (see www.anapatterns.org) (3).

There are some clinically relevant associations between certain autoantibodies and SAID, some being strong enough to be used as diagnostic biomarkers and in classification criteria (Table 1). For example, AC-4 (nuclear fine speckled pattern) is associated with autoantibodies against: SS-A/Ro, SS-B/La, Mi-2, TIF1γ, TIF1β and Ku, and these autoantibodies can be found in several systemic SAID7. Other patterns have a more restricted autoantibody association, frequently relating to a single autoantibody, such as the AC-29 pattern which is strongly related to anti-Scl-70 (DNA topoisomerase I)8, a specific biomarker of systemic sclerosis (SSc) (8,9) and the AC-3 pattern, associated with anti-CENP-A/B present in SSc9, primary biliary cholangitis (PBC) and occasionally in patients with Raynaud's phenomenon.

However, the observation of any pattern should not be taken as the definitive determination of the specificity of the autoantibody. Even if a pattern is strongly associated with a given autoantibody, the specific confirmatory immunoassay for this autoantibody should be performed since, in daily laboratory practice, dissociations between a pattern and the expected autoantibody are not uncommon. In addition, clinically unexpected positive results have risen in frequency, as the HEp-2 IFA test is increasingly requested by a wide range of clinicians in a scenario of low pretest probability, often without clinical evidence of SAID5. Therefore, it is always necessary to confirm the autoantibody suggested by the HEp-2 IFA pattern using autoantigen-specific immunoassays (immunoblot, immunoprecipitation, ELISA, CLIA, immunodiffusion) (3,10).

Table 1. Associations between immunofluorescence patterns in HRp-2 IFA test. Autoantibodies and clinical manifestations.

Boss	**Associated autoantibodies**	**Clinical partnership**
AC-1	dsDNA, nucleosome	SLE, chronic HAI, juvenile idiopathic arthritis
AC-3	CENP-A, CENP-B	Limited cutaneous SSc
AC-4	SS-A/Ro, SS-B/La, Ku	SjS, SLE, cutaneous, neonatal LE, DM, SSc, congenital heart block
AC-5	Sm, ARN Pol III, U1-RNP	SLE, SSc, MCTD, UCTD
AC-8	PM/Scl, A/Th	SSc, SSc-AIM
AC-9	Fibrillarin, U3-RNP	SSc
AM-19	ribosomal protein P, PL-7, PL-12	SLE, anti-synthetase syndrome
AM-20	Jo-1	Anti-synthetase syndrome
AC-21	Mitochondria, E2-PDH	CBP, SSc, CBP-SSc, CBP-SjS
AM-29	Scl-70 (anti-topo I)	Diffuse cutaneous SSc and more aggressive forms of SSc.

SLE, systemic lupus erythematosus; HAI: autoimmune hepatitis; SjS, Sjögren's syndrome; LE, lupus erythematosus; DM, dermatomyositis; SSc, systemic sclerosis; MCTD, mixed connective tissue disease; UCTD, undifferentiated connective tissue disease; AIM, autoimmune myopathies; PBC: primary biliary cholangitis.

A critical analysis of the relevance of the HEp-2 IFA pattern indicates that: 1) it is an important parameter in the laboratory algorithm of autoantibody testing; 2) there is heterogeneity in the strength and specificity of the association of HEp-2 IFA patterns and specific autoantibodies; 3) daily practice shows frequent deviations from the expected associations. This evaluation suggests that there is room for further research in the area, with particular emphasis on quantifying the consistency of pattern/autoantibody associations. The associations between them have not been studied formally in large cohorts, as most published studies are based on small groups and usually are biased from retrieval from referral autoimmunity centers. There is evidence that HEp-2 IFA patterns do not always reflect the existence of autoantibodies to which they are presumably associated and erroneous assumptions about the immunological significance and clinical relevance of ANA patterns can lead to delays or errors in the diagnosis and therefore in the treatment of patients.

Objectives

General objective: to study the associations between HEp-2 IFA patterns and specific autoantibodies in a large cohort of samples from a general laboratory.

Specific objectives: determine the HEp-2 IFA positivity frequency, the frequency of each ICAP pattern, and determine the relationship of AC-1 to AC-29 patterns to specific autoantibodies.

Design and Methodology

An analytical, observational, retrospective study was carried out with a dataset obtained from a large clinical laboratory (Fleury Medicine and Health, Sao Paulo, Brazil) in the period between 2012 and 2019. The data extracted from each registry refer exclusively to the results of the HEp-2 IFA test and the autoantibodies against dsDNA, Nucleosome, Histone, DFS70, CENPB, SS-A/Ro, SS-B/La, Mi-2, RNP, Sm, RNA Pol III, sp100, gp210, PMScl, Scl-70, ASMA, actin, P0, Jo-1, AMA and M2. Along this 8-year interval, records from a total of 453.416 samples were analyzed.

The HEp-2 IFA and specific autoantibody test results registered in the database were produced by a team of expert analysts in the field. Analysts are trained, presenting daily sessions for the discussion of cases of difficult interpretation, as well as a weekly meeting to standardize interpretation and work methodology. The HEp-2 IFA were registered according to ICAP recommendations with the addition of the AC-2b variant, which refers to a regular nuclear fine speckled pattern (not dense fine speckled) with similar staining at the metaphase plate. Although not classified by ICAP, this variant is very frequent and was classified separately from the bona fide dense fine speckled pattern (AC-2) in this study. The autoantibody routine at Fleury laboratory is subject to an internal quality control process and two external quality assessment programs, the College of American Pathologists (CAP) and the Program of Excellence in Laboratory Medicine (PELM). These characteristics contribute to the consistency of the data over the years.

The analyses were bidirectional: Tier 1) pattern → autoantibody, in which for each HEp-2 IFA pattern the frequencies of the associated autoantibodies were analyzed; and Tier 2) autoantibody → pattern, in which, for each selected autoantibody, the frequencies of the associated IFA HEp-2 patterns were analyzed.

Tier 1 analysis (Pattern → autoantibody analysis): the reference parameter is the HEp-2 IFA pattern in which only records with a single pattern were used (excluding records with mixed patterns and multiple patterns). This decision was taken to allow for the establishment of clear associations between patterns and autoantibodies. For each pattern of interest, sequential analyses of autoantibody specificities possibly related to the pattern in question were made. This methodological strategy has been applied to the top 5 patterns of interest (AC-1 to AC-5).

Tier 2 analysis (Autoantibody → pattern analysis): the reference parameter is the autoantibody specificity and the objective was to verify the frequency with which an autoantibody produced reactivity in the different cell compartments, that is, what patterns were observed in association with each autoantibody (in this case, any result of the HEp-2 IFA test is relevant, whether negative or positive). From the specific selections for each autoantibody, the frequencies of the observed HEp-2 IFA patterns were evaluated.

The methods used to determine the analyzed autoantibodies include double immunodiffusion, indirect immunofluorescence on different substrates, ELISA, immunoblot and CLIA, all of which are consistently approved in the External Quality Assessment programs from the College of American Pathologists (CAP) and the Program of Excellence in Laboratory Medicine (PELM). Patient data is devoid of identification. The study was approved by the ethics committee of Fleury Group and UNIFESP. No additional testing has been performed on patient samples.

Here we present the preliminary analysis of frequency of patterns in association specific autoantibodies and, conversely, the frequency of autoantibodies associated with some HEp-2 IFA patterns.

Results

Between January 2012 and December 2019, there were 453,416 records of HEp-2 IFA reports and 140,345 had a positive result with a simple immunofluorescence pattern. Altogether, the nuclear patterns predominate in frequency and among them, the nuclear fine speckled (AC-4) was the most frequent, followed by the AC-2b and AC-2 patterns (Figure 1 and Table 2)). The nuclear homogenous (AC-1) and coarse speckled (AC-5) patterns came next in frequency. Among the cytoplasmic patterns, the reticular mitochondria-like (AC-21) and the fibrillar linear (AC-15) patterns were the most prevalent.

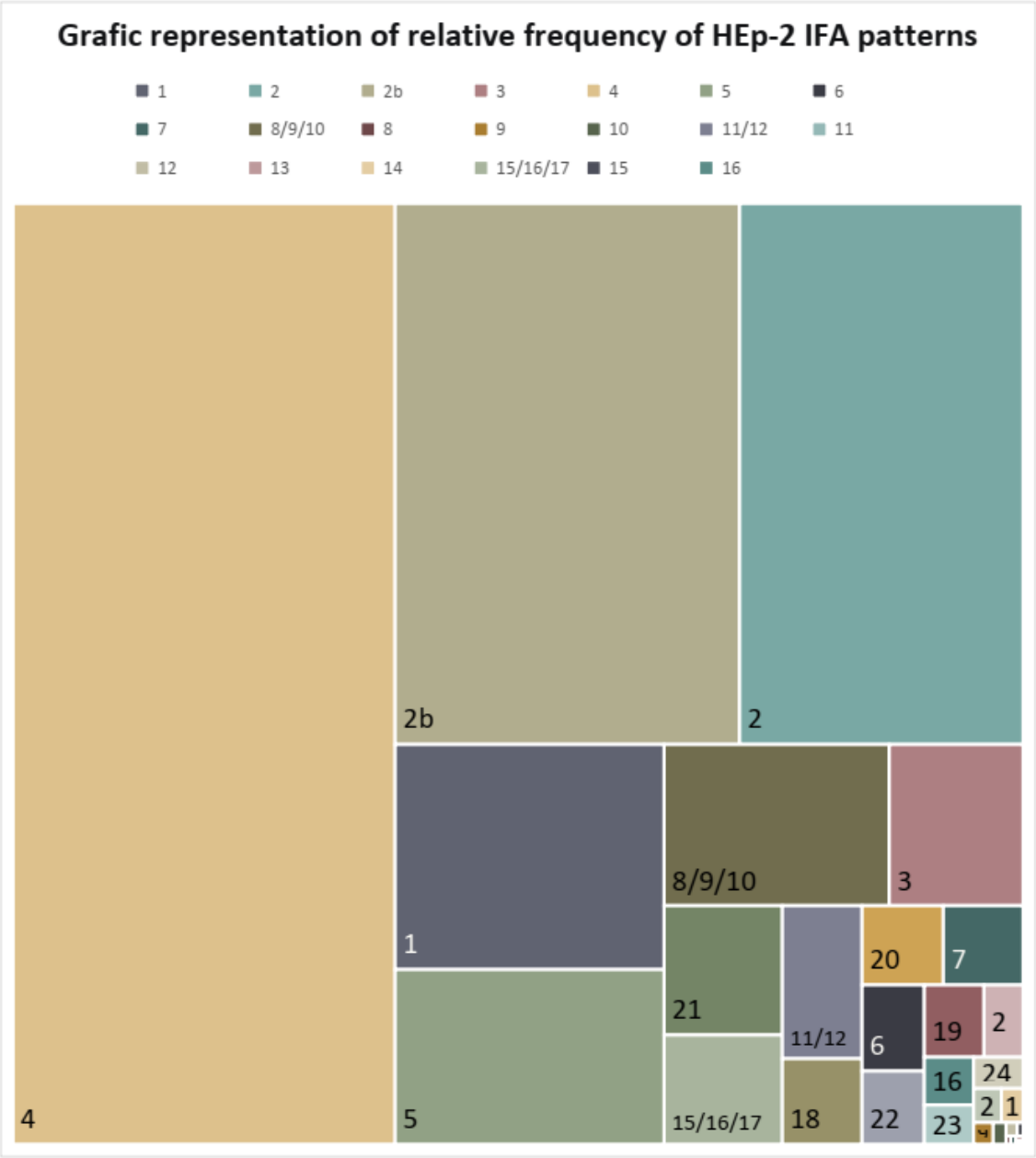

Figure 1. Relative frequency of HEp-2 IFA patterns. Numbers represent AC patterns. The areas of the rectanglesare proportional to the frequency of each pattern relative to the total number of positive HEp-2 IFA results. Some nucleolar and nuclear envelope patterns have been pooled as AC-8/9/10, and AC-11/12, respectively. The AC-2 refers to the standard nuclear dense fine speckled (DFS) pattern and AC-2b refers to the variant (non-DFS) nuclear fine speckled pattern with similar staining at the metaphase plate.

Table 2. Absolute and relative frequency of AC patterns relative to positive HEp-2 IFA tests. AC- 26 and AC-29 under revision, data not shown.

AC	Number of Observations	%
1	8.925	6,4%
2	22.636	16,1%
2b	27.513	19,6%
3	3.190	2,3%
4	53.132	37,9%
5	6.939	4,9%
6	787	0,6%
7	933	0,7%
8/9/10	5.366	3,8%
8	6	0,0%
9	63	0,0%
10	41	0,0%
11/12	1.802	1,3%
11	1	0,0%
12	23	0,0%
13	5	0,0%
14	110	0,1%
15/16/17	1.909	1,4%
15	1	0,0%
16	350	0,2%
17	-	0,0%
18	1.009	0,7%
19	630	0,4%
20	955	0,7%
21	2.250	1,6%
22	676	0,5%
23	285	0,2%
24	231	0,2%
25	417	0,3%
27	141	0,1%
28	11	0,0%
Total Samples	**140.345**	**100,0%**

In the first-tier pattern-driven analysis, several traditional associations were confirmed, but some interesting associations emerged. Notably, when selecting AC-1 (the homogeneous pattern), anti-nucleosome was positive in 76.25% of the 333 cases in which this autoantibody was requested, whereas anti-dsDNA was positive in 35.66% of the 4,767 cases with request for this autoantibody. Interestingly, some unexpected autoantibodies were detected in cases with the AC-1 pattern: anti-DFS70 was present in 14.29%, anti-CENP-B in 5.88%, and anti-RNP in 4.76% of AC-1 cases with request for each of these autoantibodies, respectively.

Upon analyzing AC-2, the predominant autoantibody observed was against DFS70, which was positive in 98.28% of AC-2 samples in which this autoantibody was requested. Interestingly, other

autoantibodies were present in a minority of AC-2 samples, namely anti-histone was present in 11.6% and anti-RNA pol III was positive in 20% of AC-2 samples in which these autoantibodies have been requested, respectively. In the case of the centromere pattern (AC-3), a high frequency of CENP-B (89.66%) was noted, as expected. Moreover, a considerable number of antibodies to nucleosome (15.22%), actin (50%), AMA (5.92%), and M2 (4.08%) in AC-3 samples for which these autoantibodies had been requested, respectively.

The most unexpected finding from the AC-4 analysis was the low frequency of autoantibodies to SS-A/Ro, SS-B/La, and Mi-2, suggesting that a variety of uncharacterized autoantibodies are prevalent among the samples yielding the AC-4 pattern. There were only 16 requests for Mi-2, and all were negative. On the other hand, there was a noticeable frequency of autoantibodies to RNA pol III (26.7%), nucleosome (8.3%), and histone (12.2%) in the AC-4 samples for which these autoantibodies had been requested, respectively).

Table 3: Frequency of AC patterns observed in samples positive for specific autoantibodies. All samples with a positive result for the relevant autoantibody and a concomitant request for HEp-2 IFA were analyzed (last row). The numbers in each column represent the frequency of each AC pattern in samples presenting a positive result for the respective autoantibody.

Association Results from Positive AAB																				
AC	dsDNA	NucIL	Histone	DFS70	CENP-B	Ro	La	Mi-2	RNP	Sm	RNA Pol III	sp100	gp210	PMScl	SCl70	ASMA	Actin	P0	Jo1	AMA	M2
M	1.244	134	36	34	16	1.253	26	-	526	171	1	9	2	2	289	373	5	6	12	260	275
0	93	79	121	-	-	347	-	-	10	1	6	1	-	1	2	243	4	2	4	70	30
1	1.700	254	42	1	1	427	3	-	86	22	-	-	-	-	4	24	-	5	-	1	2
2	7	11	13	229	-	51	-	-	4	-	1	-	-	-	-	18	-	-	1	3	3
2.b	460	110	47	17	-	342	3	-	38	1	-	-	-	-	1	49	1	-	-	9	3
3	11	7	-	-	26	37	-	-	6	1	-	-	-	1	-	5	1	-	-	9	6
4	515	63	25	2	1	4.723	168	-	79	13	4	-	-	-	6	98	1	3	3	27	7
5	369	24	9	1	1	359	2	-	1.430	295	-	-	-	1	1	8	-	2	1	3	1
6	-	1	-	-	-	6	-	-	-	-	-	2	-	-	-	4	-	-	-	5	-
7	-	-	1	-	-	1	-	-	7	-	-	-	-	-	-	1	-	-	-	-	1
8.9.10	23	1	1	-	-	19	-	-	-	-	-	-	-	-	-	14	-	-	1	2	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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15	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
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18	8	-	-	-	-	7	-	-	-	-	-	-	-	-	-	4	-	-	-	2	-
19	11	5	1	-	-	37	-	-	10	4	-	-	-	-	-	-	-	1	-	-	-
20	2	-	-	-	-	19	-	-	2	-	-	-	-	-	-	1	-	-	31	5	2
21	7	2	2	-	-	15	-	-	2	-	-	1	-	-	-	4	-	-	-	370	332
22	1	1	-	-	-	8	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-
23	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
24	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
25	3	-	-	-	-	5	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	1	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
Total Positives	9.826	1.170	370	348	65	9.342	244	-	2.769	623	17	19	4	6	374	1.224	22	43	87	1.045	862

The second-tier autoantibody-driven analysis, presents a myriad of intriguing findings, as outlined below. Table 3 depicts all positive tests for each specific autoantibody (last row), according to the AC pattern observed in the HEp-2 IFA test (upper rows). This tabular representation can be read as a heatmap, where cells in green with a dash denote no results, and cells in red signify the highest frequency of positive outcomes. For instance, for anti-dsDNA, the highest frequency of positive results was observed in association with the AC-1 pattern (1,700), followed by mixed patterns (M, 1,244), and so forth. A similar trend of AC pattern association was observed for anti-

nucleosome antibodies. A substantial number of samples positive for anti-dsDNA or anti-nucleosome showed AC-2b, AC-4 or AC-5 patterns, which may be caused by the concomitant presence of other autoantibodies that would disturb the pure AC-1 pattern. This rationale is supported by the observation of a high frequency of mixed patterns (first row) in association with autoantibodies to nuclear antigens that share widespread distribution across the nucleoplasm. Remarkably, a sizable frequency of negative HEp-2 IFA results (AC-0) was observed for several samples with a positive autoantibody test, including those to dsDNA, nucleosome, SS-A/Ro, and smooth muscle (second row). It is possible that epitope availability in different methodological immunoassay platforms may account for such discrepancy in some samples. This phenomenon was particularly evident for positive anti-histone results which were most frequently associated with AC-0 (negative HEp-2 IFA).

As expected, anti-SS-A/Ro and anti-SS-B/La antibodies were strongly associated with the AC-4 pattern. This contrasts with the observation from first tier pattern-driven analysis, in which reactivity to SS-A/Ro and SS-B/La was not prevalent in samples with the AC-4 pattern. This observation highlights the low specificity of the AC-4 pattern as an indicator of these two autoantibody specificities in unbiased samples from a general clinical laboratory. In such scenario, the most frequent pattern, i.e., AC-4, is mostly not associated with any clinically relevant autoantibody.

Although anti-CENP-B positive samples showed predominantly the AC-3 pattern, some samples presented AC-1, AC-4, and AC-5 pure patterns, with no hint of centromere staining, suggesting that high serum levels of other autoantibodies may override the AC-3 pattern in some cases. Notably, antibodies against SS-A/Ro and ASMA emerged as the ones appearing across the highest diversity of patterns, followed by dsDNA and AMA.

The second-tier autoantibody-driven analysis provides valuable insights into the intriguing interplay between autoantibodies in the assembly of corresponding HEp-2 IFA patterns, as portrayed in Table 3, which offers a comprehensive view of the results as a heat map. Several unexpected autoantibody/pattern associations highlight the complexity and diversity of the autoimmune responses and how this may affect the canonical associations of autoantibody specificities and HEp-2 IFA patterns.

Conclusion

Overall, the established associations between autoantibody specificities and HEp-2 IFA patterns were confirmed. However, the bidirectional analysis applied to a large cohort of samples with no bias selection in a general clinical laboratory showed that some of the known autoantibody/pattern associations hold true in the autoantibody-driven analysis (second tier) but not in the pattern-driven analysis (first tier). In other words, in a scenario of low pre-test probability (i.e., a general clinical laboratory) some of the AC patterns had poor correspondence with the expected autoantibody specificity. This was especially evident for some patterns (e.g., AC-4) and less evident for some other patterns (e.g., AC-1, AC-2, and AC-3), which were strongly associated with the respective autoantibodies even in a scenario of a general clinical laboratory.

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Heterogeneity on HEp-2 IFA reporting worldwide

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Introduction and aims

The presence of autoantibodies against cellular antigens, including nuclear, cytoplasmic, and mitotic apparatus antigens, is a hallmark of systemic autoimmune rheumatic diseases (SARD). Many of these autoantibodies are successfully screened by using the indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA), historically known as the antinuclear antibodies (ANA) test. HEp-2 IFA testing is usually performed as part of the initial diagnostic workup in patients under investigation for SARD and other systemic autoimmune disorders. In addition to information on the presence or absence of autoantibodies, HEp-2 IFA offers preliminary information on the possible identity of the targeted autoantigens according to the morphological immunofluorescence patterns. Historically, there is considerable heterogeneity in the recognition, classification, and reporting of HEp-2 IFA patterns in laboratories in different parts of the world. Starting in 2014, the International Consensus on ANA Patterns (ICAP) has developed a consensus classification and nomenclature of the most consistent HEp-2 IFA patterns.

Aiming to expand the efforts for international standardization in the HEp-2 IFA test, ICAP launched the HEp-2 CIC project: Clinical and Immunological Characterization of HEp-2 Patterns. Among other goals, the HEp-2 CIC project aims to collect information on the possible heterogeneity in methodology and result reporting in laboratories across the world. Herein we present the frequency distribution of HEp-2 IFA patterns in 531.241 results issued in 50 laboratories from 32 countries on five continents.

Methods

Laboratories from different countries were selected obeying a criterion of global geographical representation, including all continents. Invited participating laboratories should have expertise

in autoantibody testing, consistent scientific productivity in the field, and/or recommendation by members of the ICAP executive board.

Participating laboratories were asked to provide the HEp-2 IFA results (pattern and titer) for all samples over a one-year period. For the majority of samples, there was also information regarding the gender, and age of the patient, as well as the date of the blood draw.

Data obtained from the participating laboratories was transferred to a structured form that allowed the comparison and harmonization of data across the different laboratories. Comparison of frequency among laboratories was performed using the Chi-Square test. Statistical significance was established at 0.05.

Results

There were 50 participating laboratories from 34 countries spread over five continents (Figure 1). The overall number of samples with HEp-2 IFA results was 531,241.

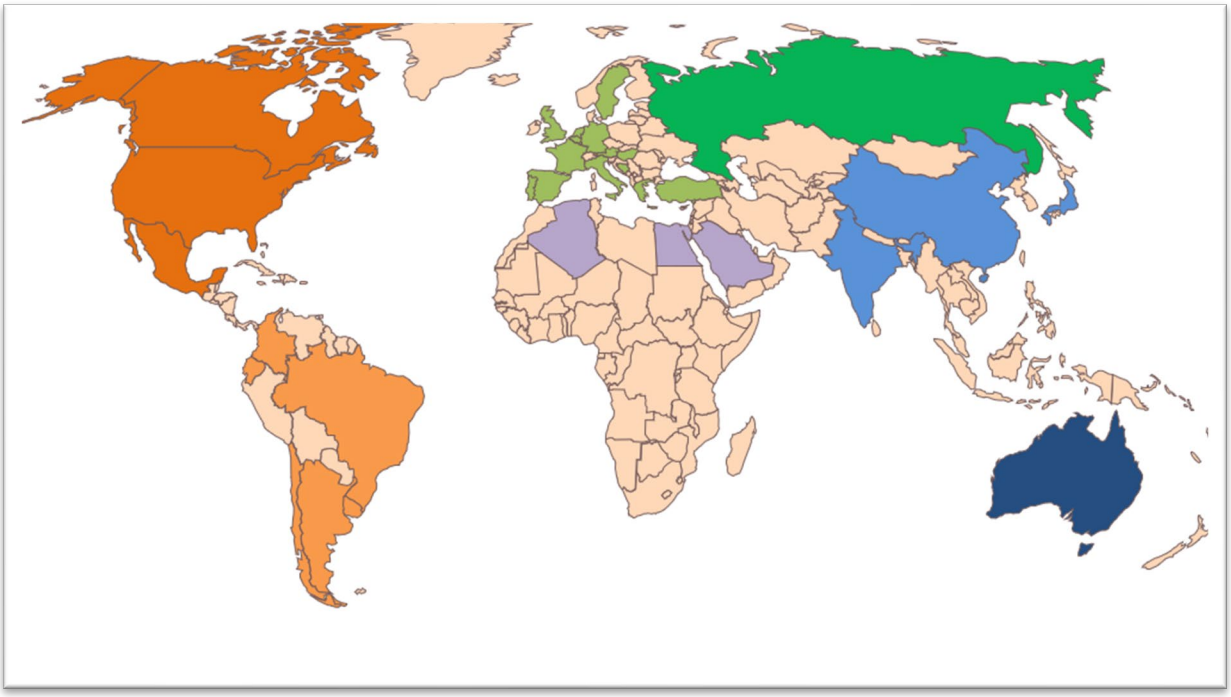

Figure 1. Geographic distribution of the participating laboratories. Colours indicate the continents where laboratories are located.

Thirty-four laboratories (68%) belong to the public sector and the other 16 laboratories (32%) are acting in the private sector. Approximately half of the laboratories (51%) are Immunology/Autoimmunity laboratories. General laboratories represented around one-third of the participants (34%) and referral laboratories comprised 15% of participating laboratories.

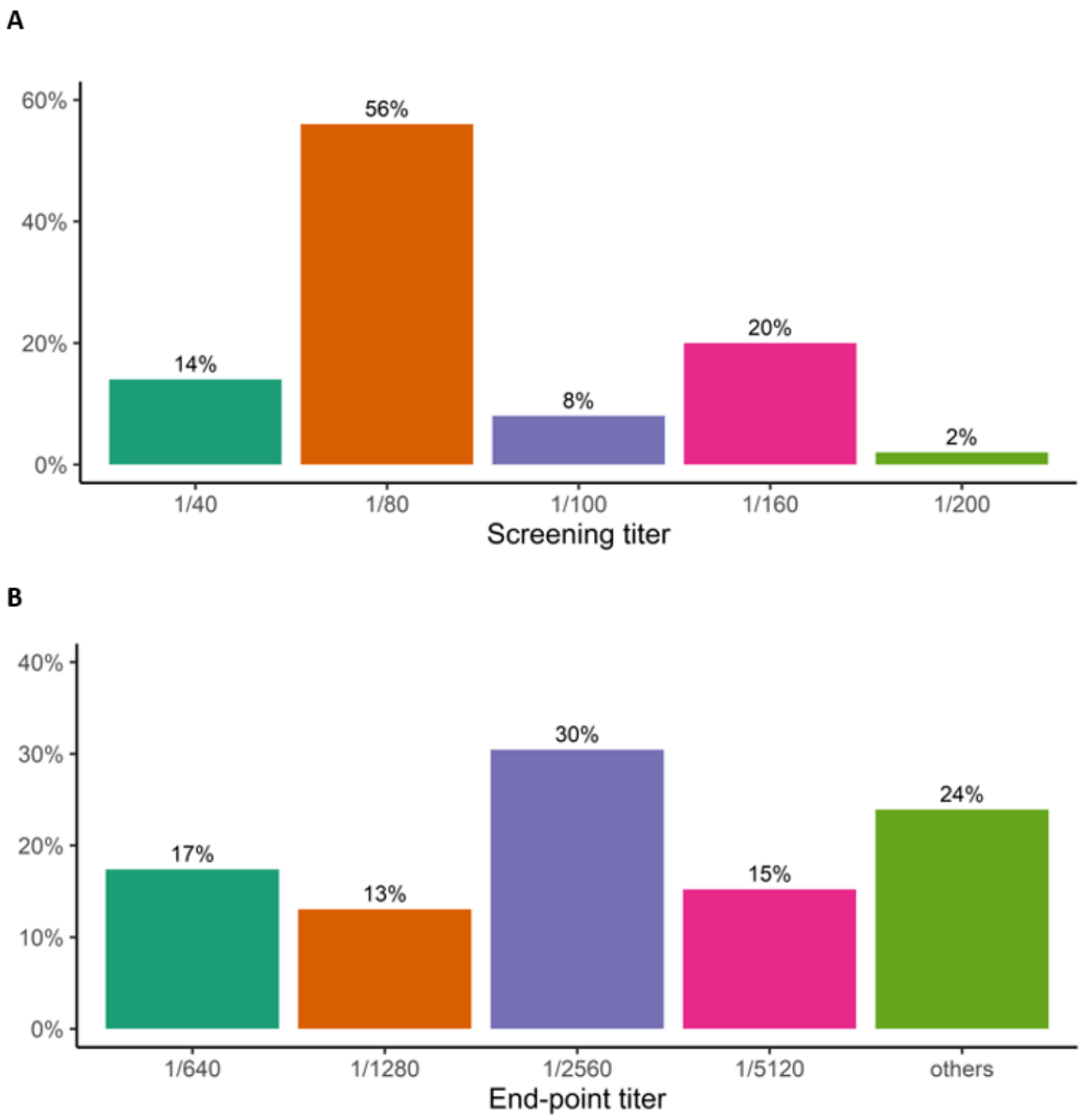

Figure 2. Screening titer (A) and end-point titer (B) used by the participating laboratories.

The most frequent screening titer was 1/80, reported by approximately half (56%) of the participating laboratories. The second most used screening titer was 1/160 and it was reported by 1/5 of the laboratories. Other screening titers, like 1/40, 1/100 and 1/200 were also reported in lower frequencies (respectively, 14%, 8% and 2%) (Figure 2A).

The results show a diversity of reported end-point titers, being the 1/2560 dilution the most frequently used as end-point titer (30%). Other end-point titers used in less than 20% of the participating laboratories were the 1/640, 1/5120, and 1/640 titers. Several other titers (e.g., 1/320, 1/1000, 10240) were also reported to be used in 24% of the laboratories (Figure 2B).

There was considerable difference in the frequency of positive results across participating laboratories. The highest frequency of positive results was 80% and the lowest frequency was 8%, with an average of 47% (Figure 3). Laboratories with the highest positivity rates tended to be referral and specialty laboratories.

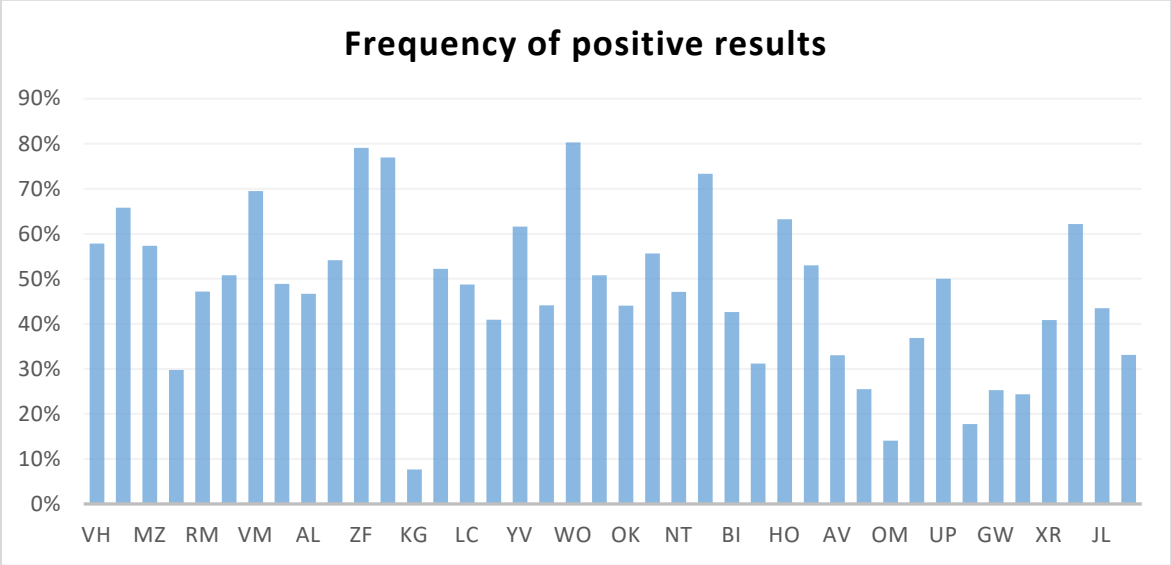

Figure 3. Frequency of positive results across participating laboratories. Each laboratory was assigned a random code.

Nuclear patterns

There is significant heterogeneity in the nuclear patterns recognized and reported by the participating laboratories. For example, all centers report the Homogeneous (AC-1) and the centromere (AC-3) patterns, but just 56% report the fine speckled (AC-4) and the coarse speckled (AC-5) patterns. In fact, 44% of the participating laboratories report nuclear speckled patterns with no further qualification (AC-4/5). The dense fine speckled (DFS) pattern (AC-2) was not reported or reported at a very low frequency in several laboratories. Notably, the laboratories that do not report or report this pattern at very low frequency showed a higher frequency of results with the Homogeneous (AC-1) and/or the Speckled patterns (AC-4/AC-5) (Figure 4).

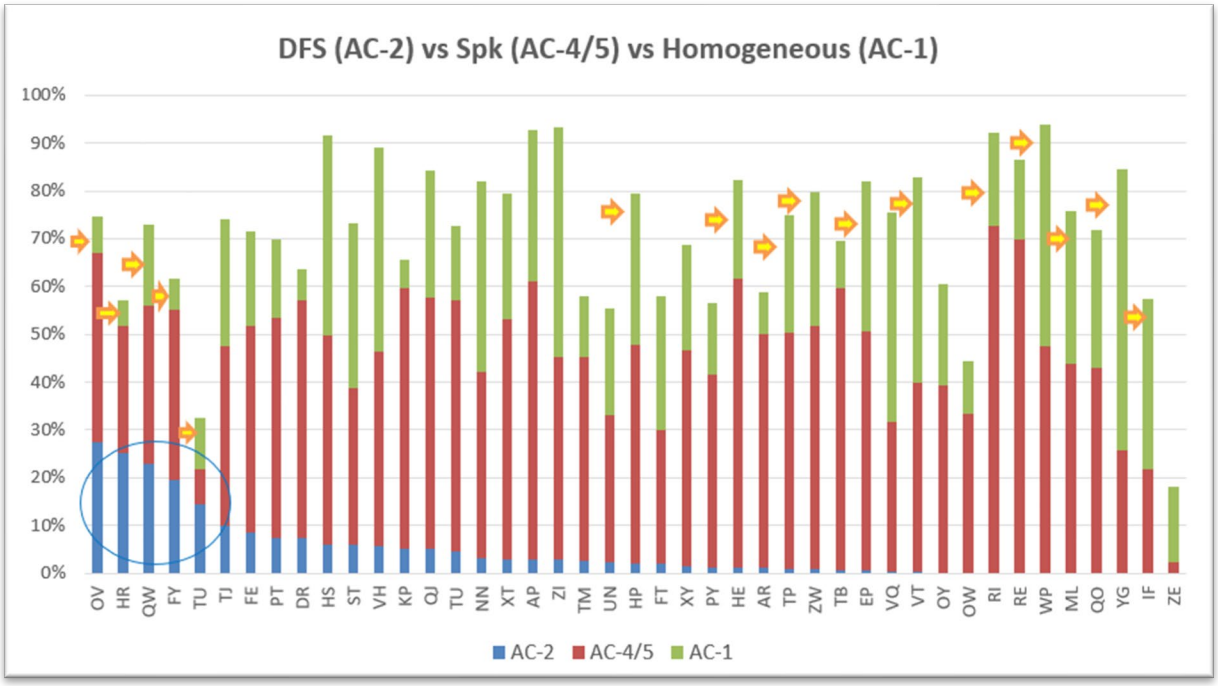

Figure 4. Frequency of positive results when reporting AC-1, AC-2 and AC4/5. The frequency of each pattern was calculated relative to the total number of positive HEp-2 IFA results in each laboratory. Each laboratory was assigned a random code.

In addition, there was considerable heterogeneity in the frequency of nuclear patterns across laboratories, except for the centromere (AC-3) pattern (Table 1).

Table 1. Top five nuclear patterns: frequency of laboratories reporting each pattern and the maximum and minimum frequency of each pattern across the participating laboratories.

	Top five nuclear patterns					
	AC-1 (Homogeneous)	**AC-2 (Dense fine speckled)**	**AC-3 (Centromere)**	**AC-4 (Fine speckled)**	**AC-5 (Large/coarse speckled)**	**AC-4/5 (Speckled)**
Laboratories reporting	100%	81%	100%	56%	56%	44%
Maximum frequency	59%	28%	10%	54%	26%	73%
Minimum Frequency	5%	1%	1%	1%	1%	3%
Average Frequency	25%	5%	3%	19%	3%	21%

There was also considerable heterogeneity among the participating laboratories regarding the distinction between multiple nuclear dots (AC-6) and few nuclear dots (AC-7), the differentiation of nucleolar patterns (AC-8, AC-9, and AC-10), the differentiation between the smooth nuclear envelope (AC-11) and the punctate nuclear envelope (AC-12), and the discrimination between pleomorphic patterns PCNA-like (AC-13) and CENP-F (AC-14) (data not shown).

Cytoplasmic patterns

The cytoplasmic patterns reported by the largest number of participating laboratories were the AC-18, AC-19, AC-20, AC-21, and AC-22 patterns, whereas the fibrillar patterns were reported by a lower number of laboratories. Twelve percent of the participating laboratories report cytoplasmic patterns with no differentiation across AC-15 to AC-23 (Table 2). Overall, the frequency of results assigning a cytoplasmic pattern was much lower than those with nuclear patterns (Table 2). The cytoplasmic reticular/AMA (AC-21) pattern was the most frequent cytoplasmic pattern and had a maximum frequency of 11%. Despite being reported in more than half of the laboratories, the AC-22 pattern was rather rare (1% of the positive samples).

Table 2. Cytoplasmic patterns: frequency of laboratories reporting, and the maximum frequency of each pattern observed among all laboratories.

	Cytoplasmic patterns (AC-15 to AC-23)									
	AC-15 (Fibrillar linear)	**AC-16 (Fibrillar filamen.)**	**AC-17 (Fibrillar segmental)**	**AC-18 (Discrete dots)**	**AC-19 (Dense fine spk.)**	**AC-20 (Fine spk.)**	**AC-21 (AMA)**	**AC-22 (Golgi)**	**AC-23 (RR)**	**AC-15 to AC-23**
Laboratories reporting	35%	42%	28%	58%	56%	58%	72%	65%	40%	12%
Maximum frequency	6%	3%	1%	3%	8%	7%	11%	1%	1%	22%

Mitotic patterns

As shown in Table 3, all the mitotic patterns were reported in more than 50% of the participating laboratories, except for the AC-28, which was reported in less than a third of the laboratories. The mitotic sub-group of patterns had in general a very low frequency (<4%).

Table 3. Mitotic patterns: frequency of laboratories reporting and the frequency of each pattern observed among all laboratories.

	Mitotic patterns (AC-24 to AC-28)				
	AC-24 (Centrosome)	**AC-25 (Spindle fibers)**	**AC-26 (NuMA)**	**AC-27 (Intercellular bridge)**	**AC-28 (Mitotic chromosomal)**
Laboratories reporting	63%	56%	51%	60%	28%
Maximum frequency	3%	2%	1%	4%	1%

Discussion and Conclusions

The HEp-2 IFA test is widely used as a screening test for autoantibodies; however, this assay bears considerable subjectivity in its interpretation and reporting. Therefore, standardization is strongly needed in order to promote the harmonization of results in different laboratories. The ICAP initiative has contributed substantially to this goal by defining and classifying the most relevant immunofluorescence patterns observed in the HEp-2 IFA. This study aimed to assess some

methodological characteristics and how frequently the HEp-2 IFA patterns are recognized and reported in clinical laboratories across the world. Our results show substantial differences in the methods and reporting characteristics amidst a sample of 50 laboratories across the world.

There was more consistency among the participants when reporting the competent-level nuclear patterns, like the Homogeneous (AC-1) and the Centromere (AC-3) than the Expert-level nuclear patterns. Differentiation of some patterns, such as the speckled (AC-4/5), the nucleolar (AC-8/9/10), and the nuclear envelope (AC-11/12) patterns is still not frequently done in most of the laboratories. In the peculiar case of the Dense Fine Speckled (AC-2) pattern, there is clearly a triangle effect, when compared with the frequency of nuclear patterns that might resemble the AC-2 pattern, i.e., the Homogeneous (AC-1) and Speckled (AC-4/5) patterns. The laboratories that do not report the AC-2 pattern or report a very low frequency of samples with the AC-2 pattern have a higher frequency of samples reported as AC-1 and/or AC-4/5. This observation suggests that, in these laboratories, some samples reported as AC-1 or AC-4/5 are indeed AC-2. Considering that the immunological and clinical relevance of the AC-2 pattern is completely distinct from AC-1 and AC-4/5, this misinterpretation may have undesired consequences in the clinical management of patients. Being the DFS pattern (AC-2) a competent level pattern, commonly found in apparently healthy individuals or in patients who do not have a SARD, there is a need to improve the ability in recognizing the DFS pattern (AC-2) across the world.

Another opportunity for improvement refers to the appropriate recognition of the Multiple nuclear dots (AC-6) and the Punctate nuclear envelope (AC-12), which indicate the putative presence of autoantibodies against sp100 and gp210, respectively, both of them considered biomarkers for the diagnosis of primary biliary cholangitis (PBC). Our data show that a substantial fraction of laboratories does not discriminate between the AC-12 and AC-11 patterns or between the AC-6 and AC-7 patterns (data not shown). Of interest, the Few nuclear dots (AC-7) and the Smooth nuclear envelope (AC-11) patterns are associated with autoantibodies with weak clinical relevance for autoimmune diseases. Although the differentiation of the nuances in these patterns is not easy, it is highly recommended that analysts in charge of the interpretation of the HEp-2 IFA test put continuous effort into acquiring the necessary expertise.

The present results show that the differentiation of close-related patterns at the expert level, such as the nuclear speckled patterns, the nucleolar patterns, the nuclear envelope patterns, and the nuclear discrete dots patterns is still a challenge. This scenario shows the need for expert training of the laboratory personnel, which can be done by assessing the corresponding pattern images in publications and at the ICAP website www.anapatterns.org, as well as by using reference material for autoantibodies associated with specific patterns provided by the Autoantibody Standardization Committee at www. https://asc.dental.ufl.edu/.

It should be recognized that the observed differences in frequencies of patterns may not be due exclusively to the interpretation of the analysts in each laboratory. As there were general and referral/specialty laboratories, it is probable that the distribution of patterns has been influenced partially by the sample selection bias characteristic of these two types of laboratories. We are currently analyzing the data to verify this possibility. In addition, one cannot exclude that ethnical, geographical, and sociocultural peculiarities associated with the laboratories may have a genuine influence on the frequency of certain autoantibodies, thereby affecting the frequency of associated HEp-2 IFA patterns.

In conclusion, this uncontrolled transversal analysis showed that the ICAP classification has been progressively incorporated into the interpretation and reporting of HEp-2 IFA patterns in laboratories across the world. However, there is still substantial heterogeneity regarding specific patterns, especially those considered expert-level patterns. These results emphasize the need for improvement in standardization and harmonization in the reporting strategy of the HEp-2 IFA test.

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Adopting the International Consensus on ANA Patterns (ICAP) classification for reporting: the experience of Italian clinical laboratories

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Abstract

The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) is still considered the reference method to detect anti-nuclear antibodies (ANA) because of its high sensitivity and represents a relevant tool for the diagnosis of autoimmune rheumatic diseases.

During the last decade, the International Consensus on ANA Patterns (ICAP) initiative promoted harmonization and understanding of HEp-2 IFA staining pattern nomenclature, as well as promoting their use in patient care by providing interpretation for HEp-2 IFA test results.

In conjunction with a nationwide survey on the evolution of autoantibody diagnostics in autoimmune rheumatic diseases, we focused on the adherence of the Italian laboratories to the ICAP nomenclature analyzing its lights and shadows. A standardized nomenclature for the HEp-2 IFA report is fundamental for appropriate diagnostic, therapeutic, and prognostic management of the patients. The recent ICAP-oriented report, largely used today among Italian laboratories, also represents a further step in harmonizing and improving communication with the clinicians, adding value to laboratory findings and helping with critical clinical decisions.

Harmonization in autoantibody testing over the past 10 years: lessons learned from the UK NEQAS external quality assurance

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Abstract

There are several challenges that need to be met in autoantibody detection to increase the quality of testing and meet an adequate level of harmonization. External quality assurance programs (EQA) are fundamental tools for standardization and harmonization of laboratory practices, providing users with a benchmarking system to evaluate their performance and identify issues where improvement actions are needed. Our aim was to analyze the UK NEQAS EQA reports of the last decade (2012 -2021) to assess the global level of harmonization in autoimmune diagnostics and to highlight the unmet needs. All reports of the UK NEQAS for Nuclear Antibodies and related antigens programs (six a year), each one including two exercises, were analyzed for anti-nuclear (ANA), anti-dsDNA, anti-centromere and anti-extractable nuclear antigen (ENA) antibody results. The indirect immunofluorescence (IIF) on HEp-2 cell substrate was confirmed as the method of choice for ANA measurement for the majority of participants (80% in 2012 and 79% in 2021), although solid phase immunoassays (SPA) using well characterized antigens have gained widespread adoption in autoimmunity laboratories. Among SPA, the fluoroenzyme immunoassay (FEIA) is the most used (10% in 2012 and 12% in 2021), while use of ELISAs has decreased from 7% in 2012 to 3% in 2021. New technologies such as chemiluminescence (CLIA) and Luminex have been adopted, gradually replacing ELISA.

As far as anti-ENA antibodies are concerned, the use of the ELISA technique has undergone a significant reduction from 36 to 8% between 2012 and 2021, with a considerable increase observed in the use of the CLIA methods (from 1% to 14%), FEIA (29% to 39%), immunodot (6% to 16%) and Luminex (6% to 13%). The lineimmunoassay, used by a lower number of participants,

did not show any significant changes (2% both in 2012 and 2021). Noteworthy is the average increase, starting from 2014, in the percentage of responses compliant with the expected target, always exceeding 90% for the positive targets and exceeding 95% for most of the exercises with a negative target.

Overall, the scenario of the last 10 years highlighted that, due to the great variety and ever-widening number of autoantibodies, the introduction of additional tests and methodologies together with the advent of automated platforms, brought significant changes in autoimmune diagnostics, but the harmonization process has still a long way to go. This is particularly true for exercises that use target antigens that are not included in the profile of single SPA or that are not easily recognizable by the IIF method (for example Jo1). At the same time, advances have been made in harmonizing results following the introduction of the ICAP nomenclature in 2016 within the UK NEQAS scheme. This change may have contributed to the reduced number of misclassification cases of cytoplasmic patterns that prior to 2016 were classified as a negative pattern. Finally, we cannot exclude that a higher harmonization has been caused by the sharp reduction in assay manufacturers: 95% of participants in 2021 used assays manufactured by only five companies, while there were more than 20 in 2012.

Harmonization of autoimmune serology in idiopathic inflammatory myopathies

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Abstract

Autoimmune serology for the idiopathic inflammatory myopathies (IIM) has gained over the years impact in the diagnostic work-up of these diseases. The myositisblot is offered by a restricted number of laboratories, but the way from request to interpretation of results is rather variable, as illustrated by the results of a Dutch questionnaire on this topic.

The 256th ENMC workshop (October, 2021) was a hybrid meeting about the role of myositis specific autoantibodies (MSA) in IIM.[1] Since standardization of autoantibody assays seems to be hardly impossible, the focus was on harmonization. Consensus was achieved on the clinical manifestations that warrant testing for MSA (Table 1). Applying such a gating strategy will prevent from testing patients with a low pre-test probability and reduce the number of false-positive results.

Table 1A. Myositis syndrome features that ask for detection of myositis specific autoantibodies

- The triad of myositis, interstitial lung disease, and arthritis, possibly accompanied by Raynaud's phenomenon, mechanic's hands, and fever
- Characteristic skin rash, including Gottron's papules, shawl sign, and heliotrope rash, most often in combination with symmetrical proximal muscle weakness
- Severe proximal muscle weakness with a sub-acute onset and without clinical extra-muscular manifestations
- Slowly progressive muscle weakness with an asymmetrical distribution involving both proximal and distal muscles, typically presenting after the age of 40 (only anti-cN1A*)

**Formally, anti-cN1A is considered not to be a myositis specific autoantibody.*

Table 1B. Myositis syndrome features for which detection of myositis specific autoantibodies should be considered after excluding other, more common, diagnoses.

- Isolated seronegative and non-erosive polyarthritis* Interstitial Lung Disease of unknown cause
- Isolated high CK level on repeated samples
- LGMD-like disease with no known molecular diagnosis nor familial history**

**For interpretation the focus should be only on the anti-synthetase antibodies. Anti-Jo1 detection, most often, is already included in the anti-ENA analysis, but may go unnoticed in case of negative HEp-2 IFA results.*

***For interpretation the focus should be only on the anti-SRP and anti-HMGCR antibodies.*

Next, consensus was defined for the testing algorithm and the way test-results should be reported to the clinician (Fig. 1). Finally, there was agreement on the research agenda in terms of the explicit need for a collaborative multicenter study to better define the test characteristics of the MSA in different immune-assays currently in clinical practice or under development. Optimal interpretation of the test-results, eventually, will benefit the patient in terms of appropriate diagnosis and treatment.

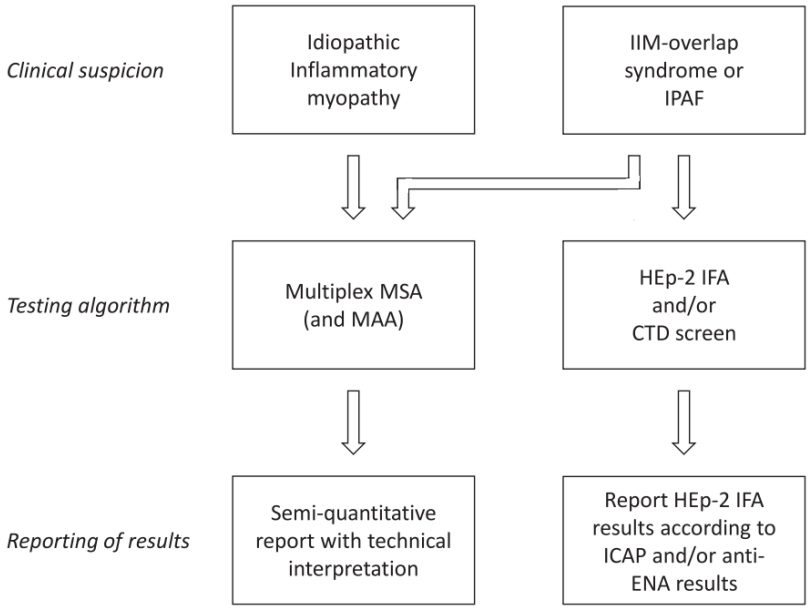

Figure 1. Harmonization of autoantibody detection in IIM: from requesting to reporting

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ANA in autoimmune hepatitis: detection on HEp-2 cells versus rodent tissues

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Abstract

Presence of antinuclear antibodies (ANA) are among other autoantibodies substantial arguments to diagnose autoimmune hepatitis (1). 1993 the term “autoimmune chronic active hepatitis” was replaced by “autoimmune hepatitis” (2) and since then detection of ANA to diagnose AIH was recommended to be determined by indirect immunofluorescence on rodent tissue sections.

Nowadays the recommended and most often used ANA test to detect ANA is the immunofluorescence assay on HEp-2 cells (HEp-2 IFA) (3).

On HEp-2 cells several immunofluorescence patterns can be distinguished by staining the nucleus, the cytoplasm or mitotic figures (4). The international consensus of ANA patterns (ICAP; *www.ANApatterns.org*) describes 29 patterns defined with an AC (anti cellular) number. Up to now 29 patterns (AC-1 to AC-29) are described and AC-0 for a negative sample without any specific immunofluorescence staining. The clinical relevance of different immunofluorescence patterns has been published (5).

The use of HEp-2 cells or ELISAs instead of rodent tissue sections to detect ANA in AIH was discussed and validated based on a restricted number of AIH patients (62 and 113) and controls (72 and 202) (6). The authors conclude that HEp-2 cells are comparable to tissue sections if the cut off titer is adjusted by four-fold increase. We verified these results using a number of consecutive patients´ samples sent for screening on autoantibodies associated with autoimmune liver diseases (7, 8).

In the present study we extended the number of patients to 4249. Out of these 888 samples were tested positive for ANA using rodent kidney-stomach-liver (KSL) sections, 2101 samples were tested positive for autoantibodies on HEp-2 cells using the same cut off (1:40) as for KSL. Excluding isolated cytoplasmic or mitotic patterns, 1812 samples were tested positive for ANA on HEp-2 cells. Thus, the number of positive samples on HEp-2 cells was more than twice compared to the rate on KSL. If the cut off for ANA on HEp-2 cells is adjusted to 1:160 as suggested (6), 842 samples

are considered as ANA positive on HEp-2 cells, 834 if isolated cytoplasmic or mitotic patterns are excluded. 249 samples were tested positive using a cut off of 1:80 on KSL, 340 samples were tested positive on HEp-2 cells using the adapted cut off of 1:320, 336 excluding isolated cytoplasmic and mitotic patterns.

Using adjusted cut offs on HEp-2 cells, the number of ANA positive patients still slightly exceeds the number of positive results on KSL with cut off 1:80 but is comparable to the number of samples with cut off 1:40 (figure 1). But clear description of the HEp-2 cell pattern according to ICAP helps to exclude positive ANAs which are not relevant for AIH (e. g. AC-2 suggestive for anti-DFS70 antibodies) or patterns which add information on clinical relevance (e. g. indication of overlap to PBC in case of AC-6, AC-12 or AC-21 patterns).

Our results confirm the use of the HEp-2 assay with an adapted cut off is suitable for screening on ANA if autoimmune hepatitis is suspected.

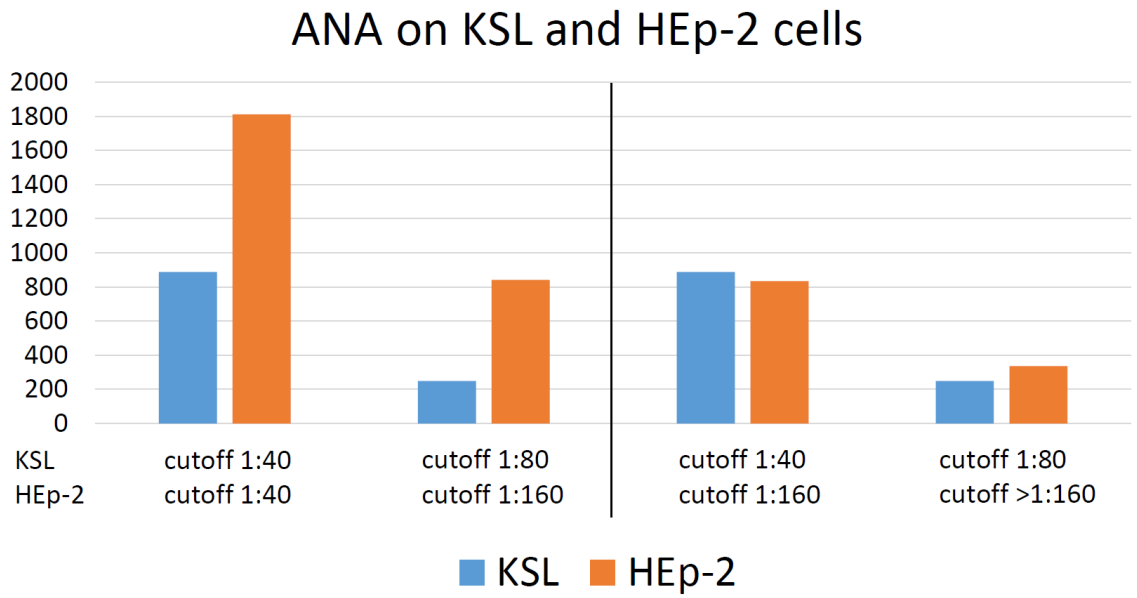

Figure 1: ANA detected on HEp-2 cells and KSL slides

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Challenge of harmonization of antiphospholipid antibody detection

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Abstract

Three assays are commonly used for anti-phospholipid antibodies (aPL): Lupus Anticoagulant (LAC) which is a functional coagulation test and aCL and anti-β2-glycoprotein-I (β2GPI) which are solid phase techniques. aPL laboratory assays are classification but also diagnostic tools; their crucial role in antiphospholipid syndrome (APS) is supported by their predictive value for the clinical manifestations of the disease (1).

The performance of LAC and the interpretation of the results should be carried out according to the International Society of Thrombosis and Hemostasis guidelines.

The solid phase assays (SpA) for aPL detection have been originally represented by RIA and then by ELISA and FEIA. Despite the huge effort in the standardization of both ELISA and FEIA, the harmonization of the aPL SpA is still incomplete. More recently, the Luminex and chemiluminescence (CLIA) techniques offered better feasibility and reproducibility but displayed results with much higher values because of the increase in sensitivity (2). Consistent with this finding, the major issue of the new techniques is represented by the clinical meaning of aPL levels close to the cutoff established for ELISA and FEIA. In particular, this is a critical point since the new ACR/EULAR APS classification criteria include medium/high aPL levels according to the values detectable by ELISA (3). Because of the increasing use of the new techniques among diagnostic laboratories, the correct clinical interpretation of the results based on the comparison between different methods represents an unmet need in the routine setting.

According to the fact that β2GPI-dependent aPL are widely accepted as the clue antibodies in APS, the new classification criteria pointed out the combined positivity for aCL and anti-β2GPI assays. It is not surprising that an effort was made for setting up a reference material for anti-β2GPI test in order to reduce the variability of the methods. The Committee on Harmonization of Autoimmune Testing of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in collaboration with the Joint Research Institute of the European Commission developed a certified reference material (CRM) with an assigned property value (anti-β2GPI IgG

antibodies concentration in a matrix material). The CRM was shown to be commutable and it can serve as a quality control of anti-β2GPI IgG measurements and/or for the calibration of immunoassay (4).

There is growing evidence that the epitope specificity of anti-β2GPI antibodies can offer additional diagnostic and prognostic information. For example, antibodies against D1 display higher diagnostic/prognostic value. While the different SpA for anti-β2GPI antibodies display apparently the same specificity against the whole molecule, preliminary data show that this is not the case for the different domains of the molecule. The reason for such a difference can be due to conformational changes related to the diverse coating systems in the different assays.

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Two decades of external proficiency testing at INSTAND e.V. in Germany - what can we expect, what have we learned?

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Abstract

The INSTAND QA program for autoantibody proficiency testing started as an inter-laboratory comparison. Initially results were evaluated via Excel sheets. Since a few years results are submitted and assessed via an online system.

Ab initio, the question arose as to how it should be possible to compare the results with each other. In general, there are no internationally established units for quantifying autoantibody tests. The reasons are obvious. Antibodies in patient sera and thus in interlaboratory test samples are the product of a patient-specific, individual "evolution" in B cell development. They are polyclonal and each antibody targets very specific 3-D structures. This immune response is constantly changing. Antibody responses and profiles are subject to constant change due to affinity maturation. They are as individual as fingerprints or facial features at a given time.

Despite many highly sophisticated efforts, what is known as international standards are actually not real standards in the literal sense, but are rather reference materials on which various international bodies have agreed. This certainly may be debatable, but as of today this unfortunate situation remains. Test manufacturers can obtain such reference materials from the relevant institutions in order to calibrate their tests against them. The volumes are limited with the obvious result that if these reference materials are no longer available, it is usually not possible to establish new materials that have exactly the same dilution kinetics. Therefore, and despite several attempts and efforts it may never be possible to establish a uniform quantification system for antibody tests. In addition, the cut-off values used by the manufacturers with the help of such reference materials are subsequently expressed as a numerical value, but in reality, they are the result of statistical surveys based on more or less large patient cohorts compared to healthy control populations. Values achieved in this way therefore only formally represent an absolute cut-off value, but in practice should rather be understood as diffuse clouds. Statistical

and technical fluctuations must therefore also be taken into account when assessing measured values. Each laboratory should therefore adjust their cut-off values internally.

Hence, any comparative measurements must rely on auxiliary variables. In the context of the EQAs on autoimmune diseases (RV 251-275), INSTAND e.V. uses evaluation categories in addition to titrations or qualitative results. These evaluation categories range from '0' (negative), '1' (borderline), '2' (weakly positive), '3' (clearly positive), '4' (strongly positive).

Additional complications arise from the incoherent presentation and definition of the target antigen involved, as are exemplified by the ANA proficiency testing. ANA testing usually is done by IIFT (indirect immunofluorescence test). Results have to be submitted as inverse end point titers and staining patterns. Certification is based solely on the reported titers, but not patterns, as HEp-2 cells from various manufacturers show great variation in staining patterns produced by a given sample. Nevertheless, an indication of the observed pattern via a coded descriptor is expected. However, a tendency can be observed that an increasing number of participating laboratories utilize non-IFT methods for the determination of ANA. While the overall performance of the laboratories using ANA-IFT proves to be generally very good with success rates of >90%-100%, the success rate among the laboratories using non-IFT methods varies considerably between 25%-100%. Upon comparison of the success rate with the sample characteristics it becomes evident, that this heterogeneity can be attributed to the specificities of the samples used. Not surprisingly, the success rate among the participants using non-IFT methods for ANA detection is clearly connected to the specific target antigens recognized by the autoantibodies in the sample material. While samples containing either one or several of the “classic” ENAs (U1-snRNP, Sm, Ro/SS-A, La/SS-B, Scl-70, Jo-1) are easily being detected, antibodies against slightly rarer antigens (e.g., PM/Scl, SP-100, nuclear envelope protein gp210 or others) may cause severe problems. Observations like these led to the proposal that the term ANA screening or ANA test should be restricted solely to results obtained by indirect immunofluorescence on Hep-2 cells [1].

Since the introduction of ANA proficiency testing in Germany in 2001 the pattern descriptors used were based on the patterns suggested by the European Consensus Finding Study Group (ECFSG) [2, 3]. This pattern descriptors proved to be useful in the past. However, with the introduction of the International Consensus on ANA Patterns (ICAP) in 2014 an internationally unified language for ANA pattern descriptions with individual codes ranging from AC01 - AC28 became widely accepted [4, 5]. Therefore, as of spring 2017 the ECFSG pattern descriptions were replaced by the new ICAP nomenclature in ANA proficiency testing. The use of similar samples before and after introduction of the ICAP nomenclature showed that participants hardly had any difficulties to adapt to the new pattern descriptions.

The initial paper-bound submission with a possible discrimination oft the submitted results by assay type (Elisa, IIFT, Westernblot. EIDA/Lineassay) was replaced by an online submission system where participants now report the manufacturer of the assays utilized. The effects of this recent change in reporting are interesting in terms of result distribution.

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3.2 Likelihood Ratios in Medical Practice

Results to decision making: Incorporating likelihood ratios (LR) within medical practice

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Abstract

Up to 70% of medical decisions are dependent on laboratory results, and 80% of diagnostic guidelines require laboratory testing.[1] It is not surprising then that laboratory medicine lends to the highest volume of medical activity. Diagnosis is one of the most important aspects of medicine and has critical implications related to patient care and disease burden. Yet, it is estimated that approximately 5% of adults in high income countries are impacted by diagnostic error. This rate is likely higher in low-income countries. [2,3]

General practice (GP) settings in particular pose a high-risk for diagnostic errors, given the high number of patients seen with vague and overlapping symptoms. Some figures demonstrating as much as 58% of diagnostic errors stemming from GP.[4] This challenge has even been categorized as a high priority problem by the World Health Organization.[2,3] Misinterpretation of test results is one aspect of diagnostic testing that adds to diagnostic error rates.

Accurate interpretation of laboratory test results guides decision making and treatment plans for patients. These results are often viewed as either being positive or negative; positive indicating disease and negative indicating no disease. However, in practice there are many tests in which results should be interpreted as more than positive or negative. Rather the test result should be interpreted in the context of pre-test probability, which is dependent on multiple factors (e.g., clinical setting, age, gender, clinical symptoms etc.), as well as the result value.

Likelihood ratios (LR) are the ratio of the probability that a test result is correct to the probability that a test result is incorrect and is calculated using the sensitivity and specificity values of a given test. No diagnostic test is perfect, so a benefit of the LR approach is that context (i.e., pre-test probability) is considered within the calculation and final decision making. This approach allows result interpretation to move beyond a single cutoff and considers disease prevalence and disease severity within an individual patient.

A recent study out of the UK highlighted that nearly 25% of missed diagnostic opportunities within GP were due to result interpretation. Incorporating solutions such as LR within lab results to

support interpretation and increase clinician confidence in diagnosis is an avenue that should be explored by clinicians, researchers, laboratorians and industry.

In this session, our speakers will elaborate on:

- The utility of LR in diagnosis and prediction of disease, using Rheumatoid Arthritis (RA) as an example
- The practical application of LR within RA
- Their personal experiences using LR in practice
- Industry perspectives and considerations on LR

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Current application and interest of likelihood ratio reporting in autoimmune serology: results of a Belgian and EASI forum survey

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Introduction

Likelihood ratio (LR) reporting facilitates correct test result interpretation and harmonizes interpretation across assays. For several, commonly used autoimmune diagnostic tests, test result (interval)-specific LRs have been determined in large, multicenter study cohorts.

Aim

Our study aims to document the current use and interest of test result (interval)-specific LR reporting in routine autoimmune diagnostics.

Materials and methods

On behalf of the Belgian expert committee on non-infectious serology of Sciensano and the Belgian EASI working group, a survey was organized interrogating i) the belief in the added value of LR reporting and ii) the current (intention of) implementation of LR reporting for several autoimmune diagnostic tests. The survey was distributed from May 8th to May 28th 2023 to all Belgian labs certified to perform autoimmunity testing (n=159) and to all EASI forum members (n=56), i.e., the key representatives of the EASI harmonization initiatives in Europe.

Results

Thirty three of the 56 EASI forum members responded to the survey (response rate 58.9%) of which 94% believed in the added value of LR reporting. Currently, 9.1% of the responders had already implemented LR reporting, 78.8% had the intention to implement LR reporting while 12.1% didn't have the intention to implement LR reporting. The immunoassays for which LR reporting was (intended to be) implemented were myeloperoxidase/proteinase-3 (MPO/PR3) ANCA IgG (96.6%), anti-citrullinated peptide IgG (ACPA; 82.8%), rheumatoid factor (RF; 75.9%), anti-nuclear antibody screening (ANA; 72.4%) and to a lesser degree, extractable nuclear antigen IgG (ENA; 48.3%). For >85% of the participants that intended to implement LR reporting, the time frame was within the next 3 years.

Of the 159 Belgian laboratories, 46.2% responded to the survey. Sixty nine percent of the responders believed in the added value of LR reporting, 20.0% had no opinion and 10.9% didn't see the added value. 7.3% of the responders had already implemented LR reporting, 43.6% had the intention to implement LR reporting while 49.1% did not have the intention to implement LR reporting. Of the labs that were interested in LR reporting, 21.4% were serving primary care, 64.3% secondary care and 14.3% tertiary care. The immunoassays for which Belgian labs intended to integrate LR reporting were MPO/PR3-ANCA IgG (71.4%), RF (64.3%), ACPA (60.7%), ANA (60.7%) and to a lesser degree, ENA IgG (35.7%). Eighty five percent planned to implement the LR reporting of these autoimmune tests within the next 3 years.

Conclusion

Although only a minority of the European experts and Belgian labs have currently integrated LR reporting in daily practice, the implementation of test result (interval)-specific LR on autoimmune result reports will, according to the intention of the participants of this study, significantly increase in the coming 3 years, mainly for MPO/PR3-ANCA, RF/ACPA and ANA screening diagnostics.

LR in autoimmune diagnostics and beyond - Personal experience with LR in practice

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Abstract

Correct interpretation of results of diagnostic tests is complex and, generally, we have difficulties to accurately estimate the probability of disease based on sensitivity, specificity, pre-test probability and the test result. Overall, we tend to overestimate the probability of disease in case of a positive test result [1]. An alternative way to express the diagnostic value of a test result is through likelihood ratios. A likelihood ratio is the ratio of the fraction of patients with a particular test result to the fraction of controls with the same test result. For example, a test result with a likelihood ratio of 10 indicates that this particular test result is 10 times more likely to be found in patients than in controls and a likelihood ratio of 0.1 indicates that this test result is 10 times more likely to be found in controls. Likelihood ratios are a better way to convey diagnostic information than sensitivity and specificity [1].

Interpretation of autoantibody test results is usually done in a dichotomous way (positive versus negative). This is an over-simplification as for many autoantibodies the likelihood for disease increases with increasing antibody levels. Such relationship has been recognized by ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition) and EULAR/ACR. The ESPGHAN guidelines for pediatric celiac disease state that a biopsy can be avoided if IgA anti-tissue transglutaminase 2 antibodies exceed 10-times the upper limit of normal, if confirmed by positive endomysial antibodies in a second serum sample [2]. The 2010 EULAR/ACR classification criteria for rheumatoid arthritis assign a higher weight for rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA) levels that exceed three-times the upper limit of normal than for levels that are below three-times upper limit of normal [3].

Even though many clinicians and laboratory professionals appreciate that the likelihood for disease increases with increasing antibody level, such information is not reported by clinical laboratories.

Likelihood ratios can be determined for a positive and a negative test result (single cutoff), for test result intervals and for individual test results. Reporting likelihood ratios for test result

intervals or for single test results allows to give information on how the likelihood for disease increases with increasing antibody level.

For several autoantibody tests (ANCA, RF, ACPA, ANA, IgA tissue transglutaminase), likelihood ratios for test result intervals have been published [4-7]. These likelihood ratios can be reported as a comment to the test results in order to improve test result interpretation.

In the presentation at the 2023 Dresden symposium, experience with reporting likelihood ratios for autoantibodies will be shared.

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Likelihood ratios of anti-MPO and anti-PR3 antibodies for ANCA-associated vasculitis at diagnosis

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Objectives

Antineutrophil cytoplasmic antibodies (ANCA) targeting PR3 and MPO proteins in the primary granules of neutrophils are crucial for the identification and classification of ANCA-associated vasculitis (AAV). The objective of this study was to define thresholds for PR3- and MPO-ANCA QUANTA Flash assays, determine test result intervals based on predefined specificities, and calculate test result interval-specific likelihood ratios (LRs) for AAV at diagnosis. We compared the interval-specific LRs with previously published LRs (1) and determined the diagnostic value of anti-MPO and anti-PR3 detected by either chemiluminescent assays (QUANTA Flash MPO and PR3) or in-house direct ELISAs or capture ELISAs for the diagnosis of AAV.

Methods

Three different ANCA assays (an in-house direct ELISA, a capture ELISA, and a chemiluminescent assay QUANTA Flash on a BIO-FLASH analyzer) were performed to detect anti-MPO and anti-PR3 in sera from 39 patients with AAV, 55 patients with various non-AAV vasculitides, and 66 patients with different connective tissue diseases. The results of all three assays were evaluated and their clinical performance for AAV was determined. In addition, interval-specific LRs for AAV at diagnosis were calculated for QUANTA Flash assays using thresholds corresponding to specificities of 95.0, 97.5, 99.0, and 100%.

Results

The diagnostic sensitivity, specificity, and diagnostic accuracy (area under the ROC curve - AUC) for AAV of tested assays were comparable (in-house ELISA 89.7%, 95.0%, and 0.937; capture ELISA 92.3%, 98.3%, and 0.939; and QUANTA Flash 89.7%, 95.9%, and 0.972, respectively).

For the QUANTA Flash assay, the following interval-specific LRs were established for AAV at diagnosis: results between 0-8 CU had LR 0.08 (published 0-12.5 CU, LR 0.10), results between 8-29 CU had LR 1.03 (published 12.5-23.8 CU, LR 1.22), results between 29-121 CU had LR 7.76 (published 23.8-78.2 CU, LR 10.23), results between 121-191 CU had LR 12.41 (published 78.2-1049.8 CU, LR 64.85), and results >191 CU with LR ∞ (published 1049.8-3500 CU, LR ∞).

Conclusions

The LRs calculated in our study were consistent with previously published LRs for AAV, confirming the utility of LRs for harmonizing ANCA test results. The compared assays showed similar clinical utility in AAV.

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3.3 Novel Developments in Autoantibody Diagnostics

Autoantigenomics: Exploring patterns in the repertoires of autoantibody-targeted proteins

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Abstract

Autoantigenomics is a systematic approach that identifies and characterizes the repertoire of autoantibody-targeted proteins, also known as the "autoantigenome," which is a subcategory of the proteome. This promising subdomain of proteomics enables a systemic understanding of a patient's autoimmune reaction (Moritz et al., 2020). Here, we discuss our recent research examples of using autoantigenomics to study patients with autoimmune-related neuropathies, specifically chronic inflammatory demyelinating polyneuropathy (CIDP) and sensory neuronopathy (SNN).

To obtain the autoantigenome, we utilized protein microarrays containing over 16,000 full-length proteins. We then filtered the lists of significantly targeted and study-group-specific autoantigens, and perform over-representation analyses, cluster analyses, heat maps, and pathway analyses.

We present three examples of using autoantigenomics. In the first example, we found that CIDP patients who responded to intravenous immunoglobulin targeted three times more antigens than non-responders (Moritz et al., 2021). Furthermore, we identified anchoring junction proteins as a major target in CIDP, which is a recently revealed autoimmune target in this disease. In the second example, we identified the P-body and RISC complex as significantly overrepresented in the SNN autoantigenome, which may serve as potential biomarkers for underlying autoimmune context in sensory neuronopathies. In the third example, we found that immune system pathways were significantly targeted by non-paraneoplastic SNN sera.

We conclude that autoantigenomics is a powerful approach that identifies global events in the repertoire of autoantibodies. It provides important insights into the underlying pathophysiology

of autoimmune neuropathies. This may lead to the development of new diagnostic tools and assist treatment decisions.

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Identification of autoantibodies recognizing conformational epitopes by immunoprecipitation – mass spectrometry

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Abstract

Solid phase assays are widely used for autoantibody detection and quantification. Advantages of solid phase assays are the leeway for automation, low imprecision and the use of well-characterized recombinant antigens. Disadvantages of solid phase assays are the possibility of aspecific reactivity, poor standardization and discrepant results between (commercial) assays. Further weaknesses of solid phase assays are related to the fact that the autoantigens are bound to the solid phase and therefore not fully accessible for the autoantibodies and/or not in their native conformation. Moreover, when autoantigens are part of a multi-protein complex, setting up a solid phase assay for autoantibody detection is difficult. Examples of autoantigens that are part of a multi-protein complex include anti-OJ antibodies, anti-RuvBL1/2 antibodies and anti-THO antibodies [1-3].

Immunoprecipitation has the advantage that the interaction between autoantigen and autoantibody occurs in solid phase. As such the native conformation of the autoantigen is preserved and the autoantibody can directly interact with the native autoantigen and/or with the autoantigen as part of a multi-protein complex. The identity of the immunoprecipitated autoantigens can be directly revealed by mass spectrometry.

In the presentation we will illustrate how immunoprecipitation-mass spectrometry can be used to identify new and known rare autoantigens (e.g., anti-Ly, anti-OJ, anti-THO, anti-Ki, anti-EIF2B) [3-6]. Using anti-RuvBL1/2 as example we will also illustrate that immunoprecipitation can be combined with targeted mass spectrometry using a triple quadrupole mass spectrometry instrument (which is widely used in clinical laboratories) [unpublished data].

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Generation and evaluation of conformation-specific antibodies to prothrombin from Antiphospholipid Syndrome (APS) patients

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Background

Antiphospholipid syndrome (APS) represents an immune system disorder that causes an increased risk of blood clots and it's characterized by the presence of antiphospholipid autoantibodies (aPL). The detection of persistently elevated levels of aPL antibodies, targeting cardiolipin (CL), beta2-glycoprotein I (ß2GPI), together with Lupus anticoagulant (LA) activity represent laboratory parameters required for the diagnosis of APS. In the recent years, the presence of anti-phosphatilylserine/prothrombin (aPS/PT) antibodies in APS patients have gained considerably notoriety but their clinical significance remains uncertain. In physiological conditions, and due to the flexibility of the inter-Kringle domain linker, the target antigen prothrombin coexist in equilibrium between its closed (cPT, 70-80%) and open (oPT, 20-30%) conformations. Two main groups of antibodies with different conformation affinity have been described in APS patients. Group A comprised of Type I antibodies with predominant affinity to cPT, and Group B containing Type I antibodies in addition to Type II antibodies with predominant affinity to pPT. It is known the potential pathogenic effect of both group of antibodies and its potential role in thrombotic events, although the molecular mechanism remains unclear. Thus, the correct differentiation of these antibodies, their pathogenic mechanism and use in patients' stratification represents an important tool that will improve the diagnosis and management of patients with coagulation disorders.

Objective

The objective of this study was the characterization of the differential binding of isolated anti-PT antibodies to specific prothrombin conformation.

Methods

For the conformational characterization of human native prothrombin, analytical tools such as size exclusion chromatography (SEC) and thermal protein stability (NanoDsf) were applied to cPT and oPT protein. Native PT protein was utilized, and a complex comprised of PT exposed to the thrombin specific active-site inhibitor Argatroban, known to shift the equilibrium to the open form, as oPT.

Protein G affinity chromatography was used to isolate Total IgG from serum samples from APS patients containing aPS/PT or serum from healthy individuals as controls.

An affinity anti cPT resin was generated by conjugating native PT protein through Cyanogen Bromide chemistry to Sepharose beads. This was used to isolate anti-PT antibodies from the previously mentioned purified Total IgGs from APS PSPT positive patients and healthy individual controls' sera. Subsequently, antibodies targeting the cPT were isolated by affinity chromatography using the generated PT-conjugated resin.

The functional reactivity characterization of the different purified fractions was carried out by ELISA using QUANTA Lite® aPS/PT IgG ELISAs (Inova Diagnostics, San Diego, US) and biolayer interferometry (BLI) using OCTET (Sartorius, Germany).

Results

SEC analysis of the native PT compared to PT incubated with increasing concentrations of the anticoagulant drug Argatroban revealed that native PT is mostly found in its closed conformation. Specifically, thermal stability studies indicate a reduction in the retention time (TR) and increase in the melting temperature for PT while complexed with Argatroban when stabilizes its open form, in comparison to native PT. This was confirmed in a set of affinity purified APS positive patient samples, including Total IgG isolated and enriched anti PT antibodies to cPT form as well as anti cPT abs depleted sample. Differential binding affinity from both populations of antibodies was confirmed by BLI, showing different signal intensity in depleted cPT antibodies sera in comparison to the total IgG and isolated aPT fractions, which showed similar association and dissociation kinetic constants.

Additionally, the successful generation of anti PT antibodies to cPT form was confirmed by anti-PS/PT ELISA, that utilizes oPT form (PT complexed with PS known to open PT). Specifically, results showed that negative samples (FT aPT) for BLI, were positive for this immunoassay. These data confirm the presence different populations of antibodies to both, close and open forms of PT and the validity of these set of tools for its characterization and potential utility in stratification of patient groups in APS.

Conclusions

Overall, we propose a method to isolate and characterize conformation-specific antibodies, a tool that could be used to understand different pathogenic mechanisms which might be relevant as an indicator of risk of thrombotic events, and ultimately for patient stratification.

Isolation and characterization of anti-Prothrombin antibodies from Antiphospholipid Syndrome (APS) patients: a tool to enhance patient stratification studies

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Background

The biological hallmark of the antiphospholipid syndrome (APS) is represented by a thrombotic episode and persistent positivity in plasma (>12 weeks) of antiphospholipid autoantibodies (aPL) in addition to the Lupus Anticoagulant (LA) phenomenon. However, some patients show clinical manifestations highly suggestive of APS diagnosis despite being negative for aPL tests [anti-cardiolipin (aCL) IgG and IgM, and/or anti-Beta-2-glycoprotein-I antibodies (β2GPI) IgG and IgM]. Testing for anti-phosphatidylserine/prothrombin (aPS/PT) antibodies has been proposed as an additional tool both for diagnostic and risk stratification purposes. Even if their clinical significance is still under debate, evidence of pathogenicity comes not only from observational studies, but also from animal models. Additionally, the LA phenomenon, known to be the strongest risk factor for thrombosis development in APS, is thought to be caused mostly by antibodies directed against β2GPI and PS/PT. However, the actual contribution of aPS/PT antibodies to this phenomenon and its potential significance, needs to be further investigated.

Consequently, well characterized biomaterials with the isolated autoantibody specificities represent important tools to shed more light on the contribution of different autoantibodies to the pathogenicity of APS.

Objective

The objective of our study was the isolation by affinity purification of anti-PT antibodies from APS patient sera and its characterization, to help investigate their contribution in the pathogenesis of APS.

Methods

Sera of APS or healthy control individuals were individually clarified by centrifugation and loaded to affinity chromatography with a protein G chromatography column. For each purification process, eluate (total IgG) was collected, and buffer exchanged into phosphate-buffered saline (PBS) through gel filtration using a G-25 Sephadex resin. Next, patients' total IgG was purified using a Cyanogen Bromide-Activated Sepharose resin conjugated to native PT. The eluate (anti-PT IgG) was collected, and buffer exchanged into PBS as stated above.

Monitoring and evaluation of the entire process included techniques such as Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western Blot (WB) and quantification of concentration and aggregation by UV 280/320. Functional characterization of the generated biomaterials was performed by Aptiva® Particle-Based Multi-Analyte Technology (PMAT) (Inova Diagnostics, San Diego, US) for B2GPI and aCL, BIO-FLASH® Chemiluminescent assay (Inova Diagnostics, San Diego, US) for aB2GPI and aCL and QUANTA Lite® aPS/PT IgG ELISAs (Inova Diagnostics, San Diego, US).

Results

Characterization of APS serum samples for the presence of autoantibodies to β2GPI, Cl and/or PS/PT using Aptiva PMAT, Chemiluminescent assay and ELISA served for sample selection. Total IgG of the selected sera was efficiently purified obtaining a high concentration (average 2.7mg/ml), and low aggregation (average concentration 2.5%). Application of total IgG to the generated in-house cyanogen Bromide-Activated Sepharose resin conjugated to a native human PT protein allowed the isolation of highly enriched fractions of anti-PT IgG antibodies (average concentration:85μg/ml). Analytical characterization of the generated biomaterials by SDS/PAGE and WB showed a high purity of the antibodies and specific reactivity to a 72kDa protein corresponding to prothrombin.

Functional characterization of the purified material as performed for the initial sera showed that purified aPT had reactivity to PS/PT, but not to CL or β2GPI while total IgG maintained the reactivity for β2GPI, Cl and/or PS/PT, confirming the successful isolation of aPT autoantibodies.

Conclusions

Here we present the successful generation of well characterized aPT autoantibodies from serum of APS patients through a two-step chromatographic technique and deep characterization biomarker profile.

The generated customized biomaterials by enriching PT antibodies and depleted from other target antibodies as confirmed on Aptiva PMAT, Bioflash and ELISA, with a high titer of specific aPT.

Overall, we have generated specific aPT through a highly monitored process, that can have multiple applications, such as their use experiments to help understand the actual contribution of aPT to APS pathogenesis.

Ex vivo demonstration of Lupus Anticoagulant Phenomenon and Thrombin Generation Activity induced by Anti-Prothrombin Antibodies

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Background

The Lupus Anticoagulant (LA) phenomenon is known for being the strongest risk factor for thrombosis development in the antiphospholipid syndrome (APS). LA phenomenon is thought to be caused mostly by the presence of antibodies directed against β2-Glycoprotein I (β2GPI) and prothrombin (PT). However, aPT actual contribution to this phenomenon and its potential clinical significance, needs to be further investigated.

Objective

To isolate and functionally characterize aPT antibodies by testing their contribution to the LA activity and to the Thrombin Generation Assay (TGA).

Methods

Serum samples from one tetra-positive female APS patient (antibody specificities all positive at high titers: LA, anti-cardiolipin IgG, aβ2GPI IgG, anti-phosphatidylserine/PT – aPS/PT - IgG and IgM) with history of recurrent unprovoked venous thrombotic event (while under anticoagulation) and recurrent extra-criteria APS manifestation (distal ulcers) were collected. Two female aged-matched healthy controls (HCs) with no history of thrombotic events and persistently negative for criteria antiphospholipid antibodies (aPL) and aPS/PT IgG/IgM specificities, were also recruited. Total IgG were purified through gel filtration and subsequently aPT antibodies were isolated employing a resin conjugated to native PT, as previously described.

Purified total IgG and aPT were then spiked into a normal pool plasma (NPP) employing different dilutions [1:3 (purified material: NPP), 1:6, 1:21, 1:51] and functional evaluation was assessed through LA testing (Silica Clotting Time - SCT - HemosIL) and TGA (Technothrombin TGA kit, Techonoclone, Vienna, Austria) for each sample and each dilution, as previously described.

Purified total IgG and aPT were then spiked into a normal pool plasma (NPP) employing different dilutions [1:3 (purified material: NPP), 1:6, 1:21, 1:51] and functional evaluation was assessed through LA testing (Silica Clotting Time - SCT - HemosIL) and TGA (Technothrombin TGA kit, Techonoclone, Vienna, Austria) for each sample and each dilution, as previously described.

Results

When performing TGA testing on HCs enriched with total IgG at different dilutions, we did not observe any effect on TGA curves (Figure 1). Also, when testing the same samples for LA, SCT was not prolonged and LA ratio resulted negative at all dilutions (Figure 2).

When performing TGA testing on the study sample (tetra-positive APS patient) enriched with total IgG, we observed a prolongation of both tLag and tPeak. The effect progressively increased from higher to lower concentrated samples (Figure 1). A similar effect was obtained when employing aPT purified enriched sample (Figure 1). Moreover, when testing the study sample for LA, SCT testing was prolonged for both total IgG and aPT purified enriched samples, with positive LA ratios at 1:3, 1:6 and 1:21 dilutions for total IgG, and 1:3, 1:6 dilutions for anti-PT purified (Table 1).

Conclusions

In our study, we observed that aPT antibodies, isolated from a high-risk APS patient with both thrombotic and extra-criteria manifestations, showed an overt LA activity and TGA effect when compared with healthy aPL negative donors. These results might help clarifying the actual contribution of aPT to the LA phenomenon, with important implications for patients' diagnosis, classification, and overall management.

Pursuing appropriateness in antiphospholipid antibodies testing: feasibility study with a reflex test approach for anti-beta-2-glycoprotein I domain 1

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Background

We evaluate the effectiveness of the reflex approach in testing for anti-β2GPI–D1 IgG antibodies (aβ2GPI-D1) of aβ2GPI positivity in a real world setting (development cohort) and validate it in an external setting (validation cohort).

Methods

The development cohort was constituted by a population screened for aPL from January 2021 to May 2022. Samples were tested for aβ2GPI-D1 only when aβ2GPI testing obtained positive results. In the validation cohort, consecutive patients at high clinical suspicion for antiphospholipid syndrome referred from 2019 to 2022 were tested for aPL, including aβ2GPI-D1 regardless the aβ2GPI status. Sera were tested by CLIA-QUANTA-Flash, Inova Diagnostics, San Diego, CA.

Results

Out of 5250 requests, 283 samples included in the development cohort resulted positive for aβ2GPI (5.4%). Of those, 81 (28.6%) resulted positive for aβ2GPI-D1 (Table 1). In the validation cohort, out of the 489 tested patients, aβ2GPI antibodies resulted positive in 201 (41.1%) cases. aβ2GPI-D1 antibodies were positive in 73 patients (36.3%). In 7 cases, low-borderline titers of aβ2GPI-D1 were observed in the absence of positive aβ2GPI antibodies (mean titer 25.73 CU/mL±2.3CU/mL). None of these 7 patients presented with thrombotic events.

		aβ2GPI-D1+ve	
		Development Cohort (N.283)	Validation Cohort (N.489)
aβ2GPI	Moderate-high titers (>40 CU/mL)	73(25.8%)	61(12.4%)
	Low titers (20-40 CU/mL)	8(2.8%)	12(2.4%)

Conclusion

Up to 28% and 36% of patients with aβ2GPI tested positive also for aβ2GPI-D1 antibodies in the development and validation cohort, respectively. Our study supports the feasibility of aβ2GPI-D1 reflex algorithm to further investigate aPL positive results, especially when aβ2GPI antibodies are detected to moderate-high titers.

Novel Autoantigen Peptide Epitopes as Theranostic Targets in Incurable Autoimmune Blistering Diseases

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Introduction

The major obstacle to develop innovative immune therapies in rare bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) is the identification of continuous peptide epitopes displaying high affinity binding to autoreactive B-cells receptors in the patient's peripheral blood. Using peptide autoantigen-CAR T-cells, one can deliver more precise immune modulation than Rituximab, which gives largely non-specific B-cell depletion and incomplete remission.

Methods

Prospective serum samples of thirty-three BP patients, 16 female 17 male, median age 85 years (range 64-90) having confirmed direct immune fluorescence (DIF) IgG and/or complement C3 deposition clinically associated with blisters, erosion and/or lesions on limbs and trunk were tested in enzyme linked immunoassay (MBL ELISA BP180 IgG and BP230 IgG). Twelve sera with BP180 IgG >60 U/mL or BP230 IgG > 60 U/mL were screened for reactivity in a 600peptide array of 17mer peptides printed in

duplicate derived from overlapping sequences of NC16-BP180, BP180C-terminus and BP230 C-terminus and control peptides of polio and HA influenza viruses.

Results

Positive BP180 IgG in 24 of 33 (73%) and positive BP230 IgG in19 of 33 (58%) BP patients gave ELISA reactivity with mean values of 56 U/mL (95% CI 39-74 U/mL) and mean 48 U/mL (95% CI 30-66 U/mL), respectively. The peptide array fluorescent intensities of 13 BP versus 2 control scleroderma sera reveal high reactivity against continuous epitopes (nine BP180, four BP230). The autoantibody profile of a larger BP cohort by ELISA, and binding of specific fluorescent peptides to patient CD19 B cells by flow cytometry are undergoing evaluation.

Conclusions

Our preliminary findings underscore the potential of BP180-BP230 peptide ELISA to have greater sensitivity than the recombinant auto-antigen based commercial ELISA. Further studies on BP180 and BP230 peptide epitopes which selectively target autoreactive B cells in BP patients are warranted to develop novel therapeutics; CAR-T cells and auto-monoclonal antibodies (AutoMAbs).

4 Serology of Rheumatoid Arthritis – Update and new Autoantibodies

Autoantibodies in Rheumatoid Arthritis – Laboratory and Clinical Perspectives

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Abstract

Measurement of two groups of autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA) have gained increasing significance in the diagnosis and classification of rheumatoid arthritis (RA) over the last 65 years. RA and ACPA can occur independently of each other and are found in about 2/3 of RA patients. Despite rising importance of autoimmune serology in RA, there is a palpable lack of harmonization between different commercial RF and ACPA tests. While a minimal diagnostic specificity has been defined for RF tests, which almost always are related to an international reference preparation, neither of this have hitherto applied to ACPA. Assays with low diagnostic specificity, like most RF tests, are associated with very low positive predictive values or post-test probabilities in real world settings. This is especially palpable in primary care settings where pre-test probability of RF often is low, and where most investigated individuals do not have RA.

In my talk I will focus on issues of practical bearing both for the autoimmunity laboratories measuring RA-associated autoantibodies and for the clinical physicians diagnosing patients who potentially have RA, or treating patients diagnosed with RA. I will discuss ways to align results from different autoantibody tests, and give examples from the Swedish approach aiming at high diagnostic specificity especially in the primary care setting.

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Pre-RA: Can early diagnosis lead to prevention?

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Abstract

Rheumatoid arthritis (RA) is currently diagnosed and treated once an individual has signs and symptoms of joint inflammation that can be termed 'clinical RA'. For decades, the presence of clinical RA has been established based on the presence on physical examination findings of a swollen joint that is indicative of underlying synovitis. However, established and emerging data support that there is a 'pre-RA' period of RA development that can be characterized by abnormalities of biomarkers (e.g., antibodies to citrullinated protein antigens [ACPA] and rheumatoid factor [RF]), joint symptoms and/or imaging abnormalities of the joint in absence of physical examination findings of synovitis; see key studies of pre-RA[1]. Furthermore, there appear to be sub-stages of pre-RA including a 'non-articular' stage during which there is no articular involvement (and that may be driven by mucosal inflammation [2]) and an 'articular stage' during which there is early joint Involvement detectable through symptom assessment and/or imaging, but without clinical RA.

Importantly, the pre-RA period is now being targeted with pharmacologic and other therapies with the goal to prevent, delay or modulate future clinical RA. To date, several agents used in pre-RA including corticosteroids alone, atorvastatin and hydroxychloroquine have not delayed or prevented clinical RA [3-5]. There have been mixed results with other agents; in particular, rituximab delayed but did not prevent the onset of clinical RA [6] and methotrexate did not delay or prevent clinical RA but did appear to improve the severity of clinical RA that developed [7]. In addition, emerging data suggests that abatacept may delay and in some cases prevent clinical RA [8,9]. The current knowledge of prediction, and the growing knowledge of effective preventive interventions represent exciting advances in the field of rheumatology.

However, there are **several key challenges**:

(1) The ability to predict which individual will develop future clinical RA is good (and supports current prevention trials), but it could be improved, specifically with greater ability to identify the likelihood of a specific individual to develop clinical RA, and within a definable time-period. These two factors (i.e., will someone get RA, and if yes when will they get it) are important metrics in the design of clinical prevention trials and in education of individuals who are at-risk for RA. To address this, there are ongoing efforts that will use combinations of symptoms, imaging studies and biomarkers to improve the ability to stratify an individual's risk for future clinical RA and estimate their projected time to developing clinical RA.

(2) While there have been some modest successes in pharmacologic interventions to delay, modulate or prevent RA, additional approaches are needed to effectively target the mechanisms that are relevant at each stage of RA development [10]. To address this, there are a growing number of studies designed to understand the biology of disease during the pre-RA stages, and this should translate into more effective preventive and treatment interventions in the future.

(3) Advancing the prevention of RA, as well as other autoimmune rheumatic diseases, will require the participation of a broad range of stakeholders including individuals at-risk for future RA, diagnostic and therapeutic industries, public health and regulatory agencies.

In summary, results from studies in RA prevention as well as other ongoing natural history studies of RA will help to change the paradigm of how RA is managed, potentially adding prevention to the possibilities for management.

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In early rheumatoid arthritis, anti-citrullinated peptide antibodies associate with low number of affected joints, and rheumatoid factor with systemic inflammation

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Objectives

To investigate how individual autoantibodies associate with different signs and symptoms at the time of RA diagnosis.

Methods

Sixteen individual ACPA reactivities, anti-CCP2, IgA-, IgG- and IgM-RF were analyzed centrally in baseline sera from 1600 RA patients classified according to the 1987 ACR criteria. The results were related to CRP, ESR, number of swollen and tender joints, 28-joint disease activity scores (DAS28 and DAS28CRP), global disease activity evaluated by the patients (visual analogue scale), and health assessment questionnaire obtained at baseline.

Results

Individually, all autoantibodies except IgG RF associated with low counts of swollen and tender joints, and with high ESR. In IgM RF negative patients ACPA associated strictly with low number of swollen and tender joints. This association persisted in multiple regression adjusted for age, sex and inclusion year, where IgM RF instead associated with increased inflammatory markers, especially ESR. The association to low joint counts was stronger to number of individual ACPA reactivities than to anti-CCP2 levels. The ACPA microarray detects residual ACPA positive patients in the anti-CCP2 negative subjects, and among subjects without any ACPA peptide reactivity there was no association between RF isotypes and ESR. The effect of RF on ESR increased with the number of ACPA, the effect being most prominent for IgM RF.

Conclusion

In early RA, ACPA associate with low counts of affected joints and IgM RF associates with elevated ESR in an ACPA-dependent manner. Future studies in early RA may benefit from evaluating the impact of individual autoantibodies in seropositive patients as well as distinct DAS28 components separately.

Leveraging the combination of classification criteria biomarkers and demographic information to improve rheumatoid arthritis diagnosis

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that principally attacks the diarthrodial joints which is characterized by synovial inflammation and hyperplasia, pain, and joint degradation. Currently, RA diagnosis is guided by a combined approach involving patient history, clinical examination, imaging, and measurement of the widely established serological markers rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), as recommended by the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA. The criteria assign a higher score to higher level of antibodies, but it does not consider the combination of multi-marker positivity meaningfully. This pilot study aims to evaluate the established serological markers in a multiple biomarker approach, utilizing RA and non-RA cohorts collected at an Italian rheumatology laboratory.

Methods

Five hundred consecutive sera were recovered from left-over samples of the routine work up. Among them, excluding subjects without available clinical data, serum from 150 RA and 241 non-RA (various rheumatological, connective tissue diseases, other autoimmune disorders, interstitial lung disease [ILD]) patients were tested on chemiluminescence immunoassays for the detection of ACPA (anti-CCP3 IgG), RF IgA, RF IgM (QUANTA Flash, Inova Diagnostics, San Diego, CA). The

clinical performance of the assays was measured using receiver operating characteristic (ROC) curves. Marker prevalence based on the manufacturer's cut-offs, combinations, and antibodies levels were analyzed along with available demographic information.

Results

Among the RA patients, 127 (84.7%) were positive for at least one marker, while 23 (15.3%) were seronegative (Figure 1a). Of the 241 non-RA patients, 78 (32.3%) were positive for at least one marker, while 163 (67.6%) were seronegative (Figure 1b). The clinical performance of anti-CCP3 IgG, RF IgA, RF IgM, and a triple marker cumulative score - calculated as an arbitrary composite sum of unit values from the 3 assays, are summarized in Table 1, and ROC curves are shown in Figure 2.

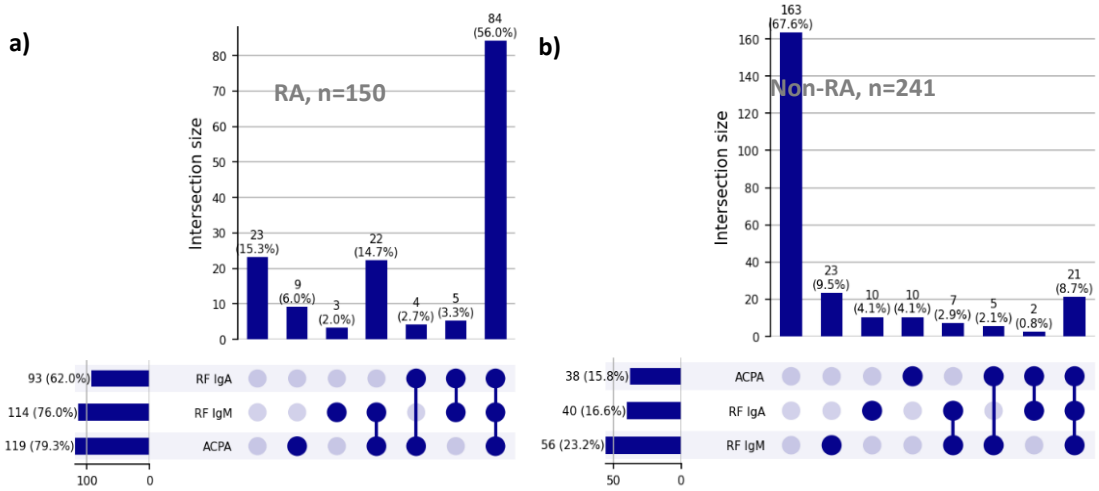

Figure 1. UpSet plots showing the frequency and combinations of RA markers among the RA (n=150), panel a, and non-RA (n=241) patients, panel b, using the manufacturer's recommended cut-offs

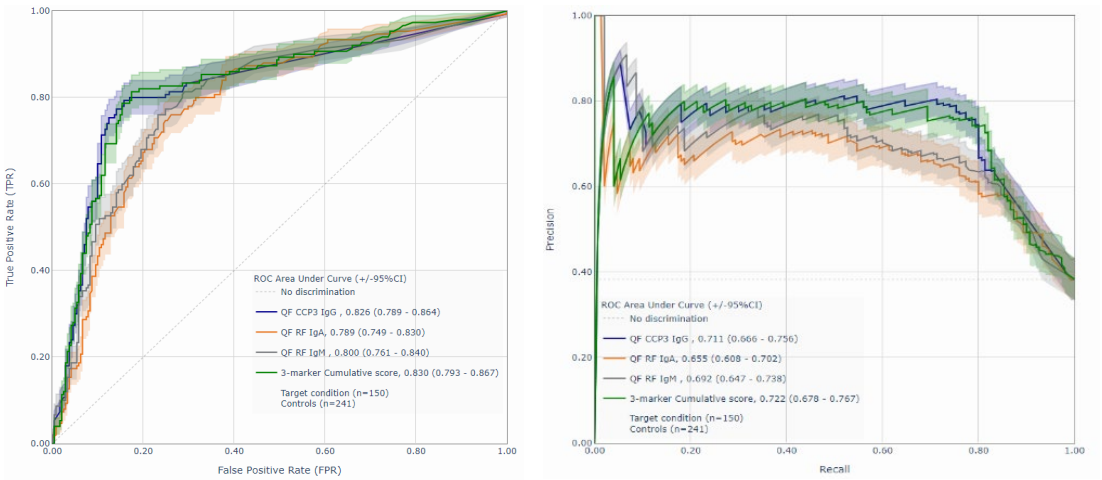

Figure 2. ROC and precision recall curves featuring the performance of individual chemiluminescence immunoassay results and a cumulative score in the Italian cohort (n=341)

Table 1. Performance of the individual chemiluminescence immunoassay results and a cumulative score in the Italian cohort consisting of RA (n=150) and non-RA (n=241) patients.

Assay	Threshold category	Threshold (Units)	Sensitivity (95% CI)	Specificity (95% CI)	LR- (95% CI)	LR+ (95% CI)	Odds Ratio (95% CI)
QF CCP3 IgG	Manufacturer's recommendation	20.0 CU	0.79 (0.72 - 0.85)	0.84 (0.79 - 0.88)	0.2 (0.2 - 0.3)	5.0 (4.9 - 5.0)	20.5 (12.1-34.7)
	Optimal*	21.2 CU	0.79 (0.71 - 0.85)	0.84 (0.79 - 0.89)	0.3 (0.3 - 0.3)	5.0 (4.9 - 5.0)	19.7 (11.7 - 33.2)
QF RF IgA	Manufacturer's recommendation	20.0 CU	0.62 (0.54-0.69)	0.83 (0.78-0.88)	0.46 (0.4-0.4)	3.7 (2.8-5.1)	8.2 (5.1-13.2)
	Optimal*	10.6 CU	0.75 (0.68 - 0.82)	0.74 (0.68 - 0.80)	0.3 (0.3 - 0.3)	2.9 (2.9 - 3.0)	8.8 (5.5 - 14.1)
QF RF IgM	Manufacturer's recommendation AND Optimal*	5.0 IU/mL	0.75 (0.68 - 0.82)	0.77 (0.71 - 0.82)	0.3 (0.3 - 0.3)	3.2 (3.2 - 3.3)	10.1 (6.3 - 16.3)
Triple marker Cumulative score**	Optimal*	67.8 Combined Units	0.81 (0.73 - 0.87)	0.83 (0.77 - 0.87)	0.2 (0.2 - 0.2)	4.6 (4.6 - 4.7)	19.8 (11.7 - 33.4)

Note: *Optimal thresholds were determined by the following software libraries: Python, version 3.8; Numpy, version 1.24.2; Pandas, version 1.5.3; Scikit-learn, version 1.2.2; SciPy, version 1.10.1; Plotly, version 5.13.1. **Triple marker cumulative score is an arbitrary composite sum of unit values from the 3 assays. QF= QUANTA Flash; LR=likelihood ratio

When considering various marker combinations in RA diagnosis in this cohort, anti-CCP3 in addition to any RF positivity yielded the highest odds ratio of 20.7 (95% CI 11.9-37.1) with a *p*-value of <0.0001, followed by anti-CCP3 alone [OR=20.3 (95% CI 11.8-35.9, *p* <0.0001], and triple positivity [OR=13.2 (95% CI 7.5-24.3, *p* <0.0001]. Twenty-one patients in the non-RA group that are triple positive have high anti-CCP3 IgG and moderate to high RF levels, which might be suggestive of RA, and therefore warrants careful monitoring.

The diagnostic performance of the three biomarkers as measured by AUC are notably higher in age group 16-53 (CCP3: 0.867 [0.810-0.925], RF IgA (0.853 [0.792-0.913], RF IgM: 0.850 [0.789-0.911]) versus 53-66 and 66-87 with AUC ranging from 0.732-0.828. The same phenomenon was observed when comparing the markers' performance among females (CCP3: 0.845 [0.804-0.885],

RF IgA 0.825 [0.782-0.867], RF IgM (0.824 [0.781-0.867]) versus males with AUC ranging from 0.735-0.780 (see table 2).

Table 2. Diagnostic performance of the individual markers and a combination as measured by area under the curve (AUC), by age group and gender.

Demographic parameter	Age			Gender	
Grouping	16-53	53-66	66-87	Male	Female
RA vs. non-RA (N)	43 vs. 89	58 *vs*. 75	50 *vs*. 79	39 *vs*.61	112 *vs*. 190
CCP3	0.867 (0.810-0.925)	0.828 (0.764-0.892)	0.784 (0.713-0.855)	0.780 (0.699-0.861)	0.845 (0.804-0.885)
RF IgA	0.853 (0.792-0.913)	0.807 (0.740-0.874)	0.733 (0.656-0.809)	0.735 (0.649-0.822)	0.825 (0.782-0.867)
RF IgM	0.850 (0.789-0.911)	0.807 (0.740-0.874)	0.732 (0.655-0.808)	0.748 (0.663-0.833)	0.824 (0.781-0.867)

Conclusion

The diagnosis of RA may be further improved by the combination of established serological markers while considering demographic information (e.g., age and gender). A scoring system tool consisting of biomarkers and demographic information, where various clinical information can be added, holds continuous promise and may aid clinicians to better diagnose RA.

NORA and ScandRA – Personalised medicine in RA by combining genomics, biomarkers, clinical and patient-data from the Scandinavian countries

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Background

Rheumatoid Arthritis (RA) is a chronic inflammatory disease showing significant variation in clinical picture, response to therapy, and long-term outcomes. Only a few and crude predictive biomarkers and indicators are available for an individualised treatment strategy. A personalised medicine approach would require the identification of new biomarkers and other predictors, and prediction algorithms, to support timely diagnosis and the choice of effective and tolerable treatment. Development of such personalised approaches requires large and harmonised datasets.

Objectives

The ScandRA (Scandinavian RA) and NORA (Nordic RA) collaborative projects aim at developing and tailoring new serological and other biomarkers by testing biobanked samples from existing large-scale RA cohorts from the Scandinavian countries, and to use these results to develop algorithms to bring personalised medicine to clinical rheumatology practice.

Methods and results

Project ScandRA started in 2022 and builds on a successful collaboration between academia, healthcare, patients, the diagnostic industry, data interoperability and e-health, which started in 2019 with project NORA. We included cohorts from Sweden, Norway and Denmark covering over 10,000 samples from patients with early or established RA. Cohorts were observational but also included randomised trials, with treatment naïve, early but also established RA patients treated with standard anti-rheumatic drugs. For some of the patients several follow-up samples over a period of 12 months were included. We measured all available samples (plasma and DNA material) using EliATM technology for CCP IgG (anti-cyclic citrullinated peptides), CCP IgA, RF IgM (rheumatoid factor) and RF IgA, a targeted sequencing panel based on the AmpliSeqTM technology for the genetic dataset and a multiplex research chip to include novel biomarkers. The work we already achieved highlighted the advantages of using and combining different technologies, and the unique dataset we could create.

Expected outcomes

Using this unique dataset in regard to biomarker but also clinical information, we are now looking into several analytic topics e.g., stability of serologic biomarker over time and added value of new serologic biomarker compared to CCP IgG and RF IgM, as well as identification of predictors for therapy response.

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Novel Autoantibodies in “seronegative” RA

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Background

Identification and characterisation of novel autoantibodies and their role in disease pathogenesis of rheumatoid arthritis (RA) is an area of intense ongoing research. Aiming to find novel diagnostic markers particularly for seronegative RA we have used in previous studies a 16k protein array presenting 6,371 human proteins for de-novo discovery of disease-specific autoantibodies using plasma from patients with seropositive and seronegative RA, osteoarthritis and healthy subjects (1).

Methods

Differentially reactive proteins (n=742) from different class comparisons were used to generate a high density 74k peptide array containing 73,160 peptides including 12,951 citrullinated peptide variants, 16 amino acids (aa) long peptides with 4aa overlap. Out of these a set of 318 differentially reactive peptides (thereof n=63 containing at least one citrulline residue) was selected and used for generation of a Luminex bead array and subsequently characterised on a large cohort of clinical samples, comprising seropositive RA (n=155), seronegative RA (n=141), healthy subjects (n=72) and disease controls with other rheumatic disorders including osteoarthritis, psoriatic arthritis and seronegative spondyloarthropathies (n=96).

Results

Among the citrullinated peptides we identified 15 interesting peptides which showed significant reactivity with samples from seropositive RA patients being recognized by at least 10% of the patients. Samples from seronegative RA patients were much less reactive and apart from some

exceptions, disease controls did not recognize these peptides. Overall, the majority of seropositive patients recognized at least one of the citrullinated peptides which were derived from diverse proteins and 72% showed reactivities to ≥ 3 peptides. Interestingly, also approximately 15% of seronegative RA patients showed multiple reactivities whereas such patterns were rarely observed in controls.

Among the unmodified peptides approximately 30 peptides were preferentially recognized by a subset of seronegative patients but less frequently in plasma from healthy subjects or disease controls. Remarkably, again the presence of reactivities against ≥3 peptides proved highly specific for RA. Thus, 13.5% of seronegative and 9% of seropositive patients showed multiple reactivities which were seen in only 2.4% of controls resulting in a positive likelihood ratio of 5.5 for seronegative RA.

Conclusion

This newly developed bead assay may indeed become a useful diagnostic tool for identifying a subset of hitherto seronegative RA patients. The potential clinical relevance remains to be determined in future studies.

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Novel antibodies that predict failure to reach early and sustained remission or low disease activity after first-line therapy in rheumatoid arthritis

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Background/Purpose

One third of rheumatoid arthritis (RA) patients do not respond to first-line treatment with a classical synthetic disease-modifying anti-rheumatic drug (csDMARD), such as methotrexate, and short-term glucocorticoids. Therefore, this study aimed to identify novel antibody biomarkers that can predict the lack of response to first-line treatment in RA.

Methods

Two cDNA phage display libraries from hip and knee tissues representing RA synovial antigens, were screened for antibody reactivity in baseline sera from participants of the CareRA trial, a Flemish multicenter study of different treatment regimens, failing to reach early remission at week (w) 8. Antibody reactivity to identified University Hasselt (UH)-RA antigens was validated using ELISA in 177 baseline samples. To test if antibody reactivity against a panel of three UH-RA antigens can predict failure to reach remission or low disease activity (LDA) according to the DAS28CRP/ESR and CDAI/SDAI, multivariate analyses including age, gender and RF/ACPA status were performed. Immunohistochemical analysis was carried out on synovial knee tissue sections from one RA patient using purified human anti-UH-RA antibodies to determine the tissue expression of the UH-RA antigens.

Results

Screening for antibody reactivity resulted in 41 novel antigens. A panel of 3 of these antigens, UH-RA.305, UH-RA.318 and UH-RA.329, could discriminate between RA patients not reaching DAS28CRP remission at w8 and those that did (31% vs 15% p= 0.015). Baseline anti-UH-RA.305/318/329 antibody reactivity predicted failure to reach w8 DAS28ESR remission in all RA patients (OR: 2.64, p= 0.0116) and failure to reach w8 DAS28CRP remission in RF/ACPA seronegative patients (OR: 13.3, p= 0.0036). Furthermore, baseline antibody reactivity against the UH-RA.305/318/329 antigens was strongly predictive for failure to reach SDAI/CDAI sustained remission (SDAI OR: 9.09, p= 0.0038, CDAI OR: 8.11, p= 0.0053) and sustained LDA according to the DAS28CRP in all RA patients (OR: 2.82, p= 0.008). This baseline antibody reactivity also predicted w8 SDAI LDA (OR: 9.78, p= 0.0028) and SDAI sustained LDA in RF/ACPA seronegative patients (OR: 14.4, p= 0.0033). Notably, human anti-UH-RA.305/318/329 antibodies targeted synovial villus, specifically fibroblasts, surrounding inflammatory infiltrates in synovial knee tissue.

Conclusion

We identified 3 antibody biomarkers that predict the failure to achieve remission or LDA after first-line RA therapy and could contribute to precision medicine decisions.

Anti-PAD antibodies in the diagnosis and management of Rheumatoid Arthritis

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Abstract

The protein-arginine deiminases (PADs) have an important role in the pathogenesis of rheumatoid arthritis (RA). These enzymes generate citrullinated proteins, the hallmark autoantigens of RA. Furthermore, they are targets of the immune response and anti-PAD antibodies can be present in the serum of these patients. Although the knowledge around these autoantibodies has significantly increased over the last few years, there are still many unknowns about their role in RA. In this session, we will review the current knowledge on the PAD enzymes and the anti-PAD antibodies, with focus on the most recent updates in their clinical utility as biomarkers in RA.

Prevalence and role of serum biomarkers in rheumatoid arthritis associated interstitial lung disease

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Background

Interstitial lung disease (ILD) is one of the leading causes of morbidity and premature mortality in rheumatoid arthritis (RA) patients. Autoantibodies are a hallmark of the disease and rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA) are included in the 2010 ACR/EULAR Rheumatoid Arthritis classification criteria. Some studies have evaluated different autoantibody isotypes in some systemic autoimmune diseases. Additionally, some studies have shown that Krebs von den Lungen-6 (KL-6) may be a useful biomarker for diagnosis and stratifying prognosis in patients with RA-ILD.

Objectives

To evaluate the prevalence of IgM, IgG, and IgA isotypes of RF, ACPA, anti-RA33 (RA33), and IgG and IgA isotypes of anti-citrullinated α-enolase-1 (CEP1) autoantibodies as well as to measure KL-6 levels in a RA cohort. To evaluate the diagnostic and prognostic value of these biomarkers in RA-ILD patients.

Patients and Methods

This study includes 175 patients with RA (ACR/EULAR 2010 criteria). ILD was diagnosed by high-resolution computed tomography (HRCT) and confirmed by a multidisciplinary committee. Additionally, 50 healthy controls (HC) were also included.

The IgM, IgG and IgA isotypes of RF, ACPA, and RA33 were measured by the EliA™ platform (Thermo Fisher Scientific, Germany). Cut-off values were selected through ROC curve analysis with 96% specificity between RA patients and HC. The IgG and IgA isotypes of CEP1 were assessed using a commercially available ELISA kit (Euroimmun, Lübeck, Germany). Serum KL-6 levels were measured by Lumipulse® G KL-6 Kit (Fujirebio, Japan), using chemiluminescence enzyme immunoassay (CLEIA). The reference range (118-627 U/mL) for KL-6 was determined with a healthy donor cohort.

Bivariate and multivariate analyses were performed according to the presence of ILD. Mortality was assessed after a 5-year follow-up.

Results

A total of 175 patients were included (37 RA-ILD and 138 RA-non ILD). Patient baseline characteristics were as follows: female gender 76.6%, mean age 53.2 ± 13.4 years, mean disease duration 8.9 ± 8.6 years, RF-IgM positive 86.3%, ACPA-IgG positive 93.1%, erosive disease 54.9%, and mean DAS28 3.0 ± 1.2.

Patients with RA-ILD were older (57.6 ± 11.5 vs 52.0 ± 13.5 years; p=0.025), were more frequently males (35.1% vs 20.3%; p=0.079), had a longer disease duration (11.8 ± 8.1 vs 8.2 ± 8.6 years; p=0.001), a higher disease activity (DAS28 3.78 ± 1.22 vs 2.78 ± 1.13; p<0.001), higher body mass index (29.9 ± 4.8 vs 26.6 ± 4.4; p=0.001), and higher mortality (32.4% vs 8.0%; p<0.001).

There were no differences in the prevalence of autoantibodies between patients with and without ILD, except for RF-IgG (67.6% in RA-ILD vs 43.5% in RA-non ILD; p=0.010). Autoantibody levels were similar in both groups except for RF-IgG (48.54 ± 69.07 in RA-ILD vs 26.47 ± 40.42 µg/L in RA-non ILD; p=0.021).

KL-6 levels were available for 172 patients. Twenty from 36 (55.6%) RA-ILD patients have abnormally elevated KL-6 levels while only 17 from 136 (12.5%) RA-non ILD patients presented with elevated KL-6 (p<0.001). Serum levels of KL-6 in the RA-ILD group were significantly higher than those in the RA-non ILD group (906.97 ± 804.31 vs 412.32 ± 269.21 U/mL; p<0.001).

After correction for possible confounders (p<0.150 in bivariate analysis), age at diagnosis (OR 0.922/year), male sex (OR 3.663), RA duration (OR 1.193/year), body mass index over 25 (OR 4.048), elevated KL-6 (OR 3.883), and RA activity (OR 3.403) were independently associated with ILD. Additionally, only male sex (OR 12.987) and ILD (OR 5.409) were independently associated with mortality in a 5-year follow-up period.

Conclusion

Our results suggest that body mass index, high KL-6 levels, male sex and active disease might be useful biomarkers for diagnosing RA-ILD while only male sex and the presence of ILD seem to be associated with worse prognosis in patients with RA.

Clinical performance and method comparison of Aptiva CCP3 for the detection of anti-citrullinated protein antibodies using a particle-based multi-analyte technology

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Introduction

Anti-citrullinated protein/peptide antibodies (APCA) one of the most significant serological markers of rheumatoid arthritis (RA), demonstrating not only diagnostic utility, but also prognostic and predictive value. Several generations of cyclic citrullinated peptide (CCP) assays used to detect ACPA are widely used in routine testing where measuring for ACPA is now part of the ACR/EULAR classification criteria for RA. This study aims to evaluate the clinical performance of the Aptiva CCP3 assay on the novel particle-based multi-analyte technology (PMAT) in comparison to a well-established CCP3 ELISA.

Methods

Our study cohort included a total 207 patient samples: 71 from patients with RA and 136 from disease controls consisting of non-RA arthritis controls and other autoimmune diseases. All samples were tested on the Aptiva CCP3 PMAT assay (research use only, Inova Diagnostics, USA) and the QUANTA Lite CCP3 IgG ELISA (Inova Diagnostics, USA).

Results

The PMAT assay showed similar clinical performance compared to the ELISA (see Table 1). Receiver operating characteristic (ROC) curve analysis resulted in an area under the curve (AUC) value of 0.872 (95% CI 0.811-0.933) for the PMAT assay and 0.907 (95% CI 0.854-0.960) for the ELISA. Good quantitative and qualitative agreement was found between the PMAT assay and ELISA (Overall agreement = 95.2%, Cohen's kappa = 0.876, 95% CI 0.802-0.951, Spearman's rho =0.683, 95% CI 0.600-0.751, p<0.0001).

Table1. Performance Characteristics of the Aptiva CCP3 PMAT assay and QUANTA Lite CCP3 ELISA.

Performance Characteristic	Aptiva CCP3 PMAT	QUANTA Lite CCP3 ELISA
Sensitivity (95% CI)	73.2% (61.9-82.1%)	74.6% (63.4-83.3%)
Specificity (95% CI)	98.5% (94.8-99.6%)	97.8% (93.7-99.2%)
Likelihood Ratio (LR+)	49.8	33.8
Likelihood Ratio (LR-)	0.27	0.26
Odds Ratio (95% CI)	183.4 (44.9-734.5)	130.5 (38.6-434.6)

Conclusion

The Aptiva CCP3 PMAT assay demonstrated good clinical performance in comparison to the CCP3 ELISA. In addition, high agreement was observed between the PMAT and ELISA. Aptiva CCP3 PMAT demonstrates a promising alternative to screening for CCP3 in high-volume laboratories.

An agglutination assay for ACPA detection in rheumatoid arthritis patients

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Introduction

Anti-citrullinated protein antibodies (ACPA) are the most specific serological marker of rheumatoid arthritis (RA) and are produced by about 75% of RA patients. The presence of ACPA early in the disease, even before onset of clinical symptoms, facilitates early diagnosis and ACPA is among the most prominent classification criteria for RA (Ref. 1). Importantly, early diagnosis and immediate start of treatment is strongly correlated with improved outcomes. Several ACPA-detection assays are available for clinical use, which nearly all are based on the same principle: ELISA or related assays with cyclic citrullinated peptides or citrullinated proteins (Ref. 2). While these methods can be automated in modern diagnostic laboratories, they are ill-suited for low volume laboratories or resource-poor environments.

Objectives

The aim of this study was to develop a rapid and easy to perform assay for ACPA detection. The assay is based on ACPA-dependent agglutination of erythrocytes and can be executed with whole blood.

Methods

An agglutination mediator was developed by protein engineering. Addition of this mediator to (diluted) blood samples results in hemagglutination when ACPA are present. After optimization of the assay with RA serum-spiked blood samples, the applicability was assessed by the analysis of fresh blood samples from 200 RA patients and from 100 psoriatic arthritis (PsA) patients as a control group. Anti-CCP2 and RF levels in these patient samples were determined by standardized ELISAs.

Results

The agglutination mediator that was generated is based on a single-chain antibody fragment that binds to glycophorin A (Ref. 3), one of the major surface proteins of erythrocytes. It is conjugated to a citrullinated peptide that is efficiently recognized by ACPA. In the presence of erythrocytes and ACPA the mediator induces agglutination of the erythrocytes, which can be detected by the naked eye. The addition of the mediator resulted in detectable agglutination in 48-61% percent of the RA patient samples. Agglutination correlated well with the results obtained with a commercial anti-CCP2 ELISA for ACPA detection (63-67%) and dit not show any correlation with RF. Efficient agglutination was observed with only 9% of the PsA samples.

Conclusion

An ACPA-dependent hemagglutination mediator was generated. This agglutination mediator allows the rapid and efficient detection of ACPA by hemagglutination in human blood samples.

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Evaluation of circulating levels of soluble urokinase plasminogen activator receptor (suPAR) in a cohort of patients with chronic arthritis

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Background and Objectives

Because of the lack of specific markers, the measurement of chronic inflammation for clinical or research purposes is primarily carried out by assessing biomarkers of acute inflammation. Recently soluble urokinase plasminogen activator receptor (suPAR), the circulating form of a cell surface receptor which activates intracellular inflammatory pathways destroying extracellular matrix (Smith HW et al, Nat Rev Mol Cell Biol 2010), has been proposed as potential marker of chronic inflammation. SuPAR can be found in different body fluid such as blood, urine, saliva and cerebrospinal fluid and commercially available assay (ELISA an Turbidimetry) are now available in different laboratory.

Recent research has shown that suPAR is more sensitive than acute phase reactants in identifying low-grade inflammation in rheumatoid arthritis (RA) (Manfredi M et al, J Pers Med 2023) and association of suPAR levels with the numbers of swollen joints (Pliyev BK at al., Inflammation 2010) and the presence of erosions (Toldi G at al., Clin Chem Lab Med 2013) in RA was also found. The aim of this study was to analyze the suPAR levels in patients with chronic arthritis under treatment to intercept persistance of a chronic inflammatory state.

Methods

Fourteen RA [male/female=13/1; median age (25°-75° percentile)=60 (52-69) years; 71% seropositive; CRP=5.78 (3.45-9.70) mg/L (normal value <5), CRP-28-joint Disease Activity Score

(CRP-DAS28)=3.33 (2.40-4.23); 43% in a moderate/high disease activity], 12 PsA patients [male/female=9/3; median age=49 (44-54) years; 8% seropositive; CRP=2.25 (1.02-5.27) mg/L; CRP-DAS28=2.08 (1.82-2.47); 8% in a moderate/high disease activity] and 10 healthy controls (HC) [female/male=5/5; median age=49 (38-52)], were enrolled. EULAR criteria were used for the disease activity (≤3.2, remission and low, >3.2 moderate and high). suPAR serum levels (ng/ml) were detected in serum by a commercial kit (suPARnostic AUTO Flex ELISA, ViroGates).

Results

suPAR was higher in RA patients than in HC [2.96 (2.47-4.08) vs 2.28 (2.19-2.43); p=0.030] and in PsA patients [vs 1.94 (1.73-2.83); p=0.046], while no differences were found among PsA and HC (p=0.821). In the whole cohort of patients and in RA, no differences were found in patients according with their seropositivity (p=0.180 and p=0.453, respectively) or with the presence of elevated CRP levels (p=0.097 and p=0.573, respectively). suPAR levels were not different in PsA patients when compared according with the presence of elevated CRP levels (p=0.373) or when compared with ACPA- RA patients' levels (p=0.529). suPAR was positively correlated with CRP, if considering the entire cohort (r=0.41; p=0.039). Dividing patients in two groups according with their disease activity, no differences were found. No correlation was found among its levels and CRP-DAS28 score in the entire cohort or in the sub-cohorts of RA and PsA patients.

Conclusion

There is still an unmet need for robust biomarkers to objectively monitor RA activity and response to therapy and measurement of suPAR could be a good candidate since suPAR levels are higher in RA when compared with those found in HC and PsA patients. In this cohort of RA patients under treatment we demonstrate its potential capability to underline the persistence of a chronic inflammatory state, which may influence their prognosis. On the contrary, we were not able to demonstrate its potential value as biomarker in PsA, where suPAR levels were not different from those found in HC or in seronegative RA.

Circulating calprotectin in rheumatoid arthritis: unravelling the impact of (pre-)analytical confounders on meta-analysis results

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Introduction

Circulating calprotectin (cCLP) is a promising diagnostic and prognostic biomarker in neutrophil-related inflammation, with most evidence in rheumatoid arthritis (RA). However, recent meta-analyses, investigating the clinical evidence of cCLP in RA patients versus healthy individuals (HI), described an important inter-study heterogeneity.

In the current study we aimed to replicate our recent meta-analyses investigating the impact of pre-analytical and analytical confounders on the inter-study heterogeneity of available publications from January 2000-February 2023.

Materials and methods

A systematic literature search was performed in the electronic databases Medline, Embase and Cochrane databases (March 23st, 2023). After removal of duplicates from the different databases, article eligibility was assessed based on following inclusion criteria: articles should be available as i) English ii) full-texts, describing iii) unique study results obtained from iv) clinically defined RA patients and a healthy control cohort, based on v) clearly specified sample types (serum/plasma) and vi) analytical method, and vii) providing quantitative cCLP data (mean +/- SD, or median and interquartile range). When median and interquartile range were reported, previously formulas were used to calculate mean and standard deviation.

MEDCALC statistical software version 19.3 (MedCalc Software Ltd, Ostend, Belgium) was applied for meta-analysis (random effects model).

Results

Our literature search resulted in a total of 646 titles: 44 titles were retrieved for full-text screening and 18 studies including 21 cCLP datasets were eligible for inclusion in the meta-analysis.

Totally, cCLP was measured in 2627 RA and 897 HI and cCLP levels were significantly higher in RA patients (estimated standard mean difference (SMD)= 1.032; 95%CI= 0.767-1.297). Significant ($p<0.0001$) heterogeneity was observed between the different studies (Cochran's Q=171.04; I^2 statistic=88.3%; 95%CI= 83.5-91.7%).

cCLP analyses was tested using 14 different cCLP assays, comprising both in-house and commercial assays. Only in 9 out of the 18 included studies, adherence to pre-analytical conditions (i.e., time to centrifugation and storage temperature) was specified. Subgroup analysis of the datasets (n=12) of these 9 studies, still showed significant heterogeneity (Cochran's Q=96.107; I^2 statistic=88.6%; 95%CI= 81.9-92.8%), mainly caused by the use of Bühlmann cCLP assay (n=5). Excluding the latter datasets: datasets compliant to pre-analytical recommendations (n=8), using 8 different cCLP assays on samples of 715 RA patients and 284 HI, showed absence of heterogeneity (Q=1.31; I^2 statistic=0.0; 95%CI=0.0-0.0), whilst in the other subgroup (n=8) significant inter-study heterogeneity persisted (Q=66.0; I^2 statistic=89.40%; 95%CI: 81.48-93.93). cCLP concentrations remained significantly higher in RA patients compared to HI (estimated SMD= 0.698; 95%CI= 0.551-0.844).

Conclusion

The interstudy variability of cCLP in RA is, besides analytical issues, mainly caused by pre-analytical confounders. Adherence to pre-analytical recommendations, significantly reduced inter-study heterogeneity and provided a reliable SMD, accurately revealing higher cCLP levels in RA versus HI.

Discriminating signal from noise: the biological variation of circulating calprotectin

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Introduction

Circulating calprotectin (cCLP) is a promising systemic biomarker of disease activity in neutrophil-related inflammation and especially in the field of rheumatoid arthritis (RA). Currently, biological variation (BV) data on cCLP in healthy individuals (HI) are lacking. However, BV data are essential for a correct interpretation of cCLP in diagnosing and monitoring of systemic inflammation on the one hand and to define critical analytical performance specifications for cCLP assays on the other hand.

Aim

Our study aimed to define the within-subject (CV_I) and between-subject (CV_G) BV of cCLP in HI. Given the impact of pre-analytics on cCLP levels, BV was determined on different sample matrices.

Materials and methods

Every HI included in the study, voluntarily underwent 10 blood collections, equally spread over 5 consecutive weeks. Each collection comprised a serum, citrate and EDTA sample (Sarstedt Monovette® recipients, Nümbrecht, Germany). Pre-analytical requirements were strictly addressed. Besides C-reactive protein analysis (CRP; Cobas Pro c503, Roche Diagnostics, Mannheim, Germany) at the University Hospital of Leuven, cCLP measurement was performed by EliA Calprotectin 2 assay (Thermo Fisher Scientific, Freiburg, Germany) on the Phadia 2500 instrument (Thermo Fisher Scientific, Uppsala, Sweden) at Thermo Fisher Scientific, Freiburg,

using a serum/plasma protocol for research use only. Outlier detection was performed on the CV-transformed data using the Cochran's method. After correction for trends over time, the estimated geometric mean values and CV_G were calculated using linear mixed models on the log-transformed data, including a random intercept per patient. The standard error (SE) of transformed variables was calculated using the delta method. CV_I was calculated using the same model on the CV-transformed trend-corrected data. Impact of gender and age on estimated geometric mean cCLP values, CV_I and CV_G was evaluated.

Results

In total, 55 HIs were included in the study (58.2% female; median age [range] = 39 [21-62] years). After applying the exclusion criteria, 538 serum samples, 534 EDTA plasma samples and 534 citrate plasma samples were statistically analyzed. The estimated geometric mean values, CV_I and CV_G were calculated for each sample type.

cCLP concentrations measured in serum were more than triple the concentration in EDTA and citrate plasma (1.837 µg/mL versus 0.366 µg/mL and 0.471 µg/mL respectively). Furthermore, cCLP levels obtained from male HI were higher than for female HI ($p<0.05$ on citrate and EDTA plasma).

cCLP CV_I [95%CI] were substantially lower in citrate and EDTA plasma samples (resp. 28.99 [27.25-30.74]% and 23.81 [22.38-25.24]%) than in serum samples (38.60 [36.29-40.91]%). CV_G tended to be lower for cCLP levels in citrate plasma (36.54 [28.60-44.47]%) compared to EDTA plasma (45.77 [35.85-55.70]%) and serum (41.27 [31.76-50.77]%). In contrast to geometric mean values, CV_G was not gender dependent ($p>0.05$ all matrices). Impact of age on cCLP CV_I or CV_G couldn't be revealed either.

Conclusion

cCLP CV_I and CV_G results are essential in accurately interpreting cCLP concentrations in the follow-up of neutrophil-related inflammatory diseases. The highest values for cCLP CV_I were observed in serum samples, whereas the lowest values for cCLP CV_G were observed in citrate plasma samples. Furthermore, despite gender-specific differences in the mean, there was no evidence of gender or age-related impact on cCLP CV_I and CV_G.

Verification of Siemens Latex anti-TNF-alpha nephelometric assay on Atellica Neph630 nephelometer

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Background and aim

Tumor necrosis factor alpha inhibitors (anti-TNF-alpha), are in widespread use for treatment of inflammatory diseases, including autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases. Therapeutic drug monitoring (TDM) of biologics is a tool for individual dose optimization. The aim of the study was to verify the manufacturer performance specifications for the N Latex anti-TNF-alpha (adalimumab-ADA and infliximab-IFX) nephelometric assay on AtellicaNeph630 nephelometer (all Siemens) and to compare with the results of ELISA method.

Materials and methods

Verification procedure included a) the study of precision using manufacturer quality control samples low and high measured in triplicate for five days according to Clinical and Laboratory Standards Institute (CLSI) EP15-A2 protocol, and manufacturer declared specifications were used as the acceptance criteria; b) comparison with the methods currently in use in our laboratory (manual ELISAs, RIDASCREEN® IFX Monitoring, RIDASCREEN® ADM Monitoring, both R-Biopharm AG, Germany) which was performed on residual sera samples (N=33 for IFX and N=33 for ADA); c) limit of quantification (LoQ) using one sample in seven replicates over three days, according to modified CLSI EP17-A2 protocol, both for ADA and IFX and less than 4 samples exceeding the 20% deviation from the target concentration was defined as acceptable.

Results

Repeatability and within-laboratory imprecision was for IFX 7.2% and 6.1% for the high QC and 4.5% and 4.0% for the low QC, while for ADA it was 5.0% and 5.4% for the high QC and 4.6% and 4.7% for the low QC. For repeatability, minimal exceed of defined criteria were obtained for ADA on both levels, and on high level for IFX. Within-laboratory precision were acceptable according to defined criteria for both ADA and IFX. Comparison study confirmed that there is no proportional or constant deviation between methods for IFX, with regression equation y = -0,062 (95%CI -0,381 - 0,439) + 0,958 (95%CI 0,834 - 1,107) x, nor for ADA with equation y = -0,095 (95%CI -0,148 - 0,092) + 0,995 (95%CI 0,881 - 1,053) x. Bland-Altman analysis confirmed there is no proportional nor constant difference between methods for both IFX and ADA. Results for LoQ study was in concordance with manufacturer declared values, both for IFX (0,37 mg/L vs declared 0,30 mg/L) and ADA (0,39 mg/L vs declared 0,30 mg/L).

Conclusion

Results of verification study confirmed manufacturer declared performances of the ADA and IFX nephelometric assay.

Bone mineral density in patients with alkaptonuria

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Introduction

Osteopenia is a common finding in patients with alkaptonuria (AKU); however, prospective data and description of the effect of the drug nitisinone treatment on bone mineral density (BMD) are lacking.

Objective

To determine the effect of nitisinone on the bone mineral density in the group of patients with alkaptonuria.

Methods

AKU patients aged 25 years or older were randomly assigned to receive either oral nitisinone 10mg daily (N=68) or no treatment (N=64). The total hip BMD Z- scores were recorded yearly at baseline, 12, 24, 36 and 48 months.

Results

At baseline, the treatment and non-treatment groups did not differ in total hip BMD Z- scores (-0.86 (0.13) in nitisinone vs -0.83 (0.11) in non-treatment group). There was a significant (F=2.62, p=0.035) interaction between the effects of time and treatment on the total hip Z- scores. The difference in the total hip BMD Z-score at month 48 compared to baseline tended to be higher in patients on nitisinone compared to untreated patients (0.07 (0.06) in nitisinone vs –0.06 (0.06) in non-treatment group, p=0.056. The results of the study demonstrate a modest beneficial effect of 10 mg/day of nitisinone on the BMD in AKU during the relatively limited follow-up time. It is more appropriate to prioritize bone density values expressed as Z-scores instead of T-scores in

the diagnosis of osteoporosis (a diverse group of patients), likewise also to examine the hip joints than the L region of the spine (calcifications in the intervertebral discs and ligaments can cause false negative results).

Bisphosphonates are ineffective in preventing fractures in AKU in the long term.

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5 Serology and Therapy of ANA associated Rheumatic Diseases – Update and new Autoantibodies

New hope? Novel therapies for systemic autoimmune diseases

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The last three decades have brought tremendous progress in the treatment of inflammatory rheumatic and systemic autoimmune diseases. Next year, routine therapy of rheumatoid arthritis (RA) with tumor necrosis factor (TNF) blockers will celebrate its 25th anniversary. TNF blockers found numerous new indications, including psoriatic arthritis and severe psoriasis, ankylosing spondylitis and other forms of axial spondyloarthropathies, most forms of juvenile idiopathic arthritis (JIA), chronic inflammatory bowel disease (IBD), or refractory uveitis. Off label indications, such as sarcoidosis or even SLE, round off the picture.

Other cytokine blockers followed. Interleukin-1 (IL-1) receptor blockade and later IL-1 blockade turned out to be much less useful for RA, but of high potency in various febrile diseases, including most fever syndromes, from familial Mediterranean fever, via TRAPS and CAPS, to systemic JIA and adult onset Still's disease, and gout. IL-6 receptor blockade is established for RA and giant cell arteritis, but also apparently works for polymyalgia rheumatica (PMR) and at least the interstitial lung involvement of diffuse cutaneous systemic sclerosis (dcSSc).

Blockade of IL-12 and IL-23 p40 is effective for psoriatic arthritis and psoriasis, with IL-23 p19 blockade showing even higher efficacy for psoriasis. IL-17 blockade is comparable to TNF blockade in axial spondyloarthropathy and psoriatic arthritis, but with higher efficacy on psoriatic skin lesions. Blockade of the TNF family cytokine B lymphocyte stimulator (BLyS) became the first approved biological therapy for systemic lupus erythematosus. SLE also constitutes the first indication for blockade of the common type I interferon receptor.

The kinases downstream of interferon receptors and of many of the interleukin receptors are likewise therapeutic targets. This is predominantly true for Janus kinase (Jak) 1, with no unequivocal evidence for an importance of Jak 2 and/or Jak3 blockade in the efficacy of the four different Jak1 inhibitors licensed for RA (Table1) and, in part, for other indications. Deucravacitinib, which blocks the fourth Janus kinase Tyk2 downstream of the type I interferon receptor, is approved for psoriasis, and is in phase 3 trials in SLE, after success in phase 2.

A randomized controlled trial of tofacitinib against the TNF blockers etanercept and adalimumab failed to established non-inferiority with regard to cardiovascular safety and has found a small, but significant increase in malignancies, predominantly lung carcinoma in smokers in North and South America. These data have led to a 2023 EMA warning letter regarding all four Jak1 inhibitors, which are otherwise very successful oral drugs.

Table 1. Approved and (some) off-label (in brackets) indications of targeted immunomodulatory approaches (often not approved for all drugs in the group).

Target	Approved drugs	RA	PsA	axSpA	JIA	GCA	AAV	SLE	Others
Cytokines									
TNF	Adalimumab	+	+	+	+		(+)	(+)	Psoriasis
	Certolizumab-pegol								IBD
	Golimumab								Uveitis
	Infliximab								(Sarcoidosis)
	Etanercept							(+)	Psoriasis
BLyS	Belimumab							+	
IL-1R	Anakinra				+				Fever syndromes
IL-1	Canakinumab								Gout
IL-6R	Sarilumab	+			+	+		(+)	(PMR)
	Tocilizumab								(SSc)
IL-12/23	Ustekinumab		+						Psoriasis
IL-23	Guselkumab		+						Psoriasis
	Risankizumab								IBD
	Tildrakizumab								
IL-17A	Ixekizumab		+	+	+				Psoriasis
	Secukinumab								Hidradenitis suppurativa
IL-17A/F	Bimekizumab								Psoriasis
IFNAR*	Anifrolumab							+	
Cytokine signal transduction									
Jak1	Baricitinib	+	+	+	+				IBD
	Filgotinib								Atopic dermatitis
	Tofacitinib								Alopecia areata
	Upadacitinib								
Tyk2	Deucravacitinib							(+)	Psoriasis
Co-stimulation									
CD80/86	Abatacept	+	+		+				IBD
B cells, plasma cells									
CD20	Rituximab	+					+	(+)	(SSc)
	Ocrelizumab								MS
CD19	CAR T cells							(+)	Lymphoma, (SSc), (ASS)
CD38	Daratumumab							(+)	Myeloma

RA rheumatoid arthritis, PsA psoriatic arthritis, axSpA axial spondylarthritis, JIA juvenile idiopathic arthritis (several subforms), GCA giant cell arteritis, AAV ANCA-associated vasculitis, SLE systemic lupus erythematosus, IBD inflammatory bowel disease, PMR polymyalgia rheumatica, SSc systemic sclerosis, MS multiple sclerosis, ASS anti-synthetase-syndrome. *The interferon-alpha receptor is in fact the common receptor for all type I interferons.

Cellular therapies approved so far include co-stimulation blockade with abatacept, a CTLA4-Ig construct tha targets CD80/86 co-stimulation and thus T cells activation, and B cell depletion. The B cell-depleting antibodies rituximab, ocrelizumab and the more novel obinutuzumab target CD20. These antibodies were all primarily developed for B cell lymphoma.

Rituximab is licensed for RA and ANCA-associated vasculitides, but also constitutes a rescue option for connective tissue diseases, such as SLE, SSc, anti-synthetase syndrome (polymyositis), dermatomyositis, or Sjögren's syndrome with severe organ involvement. Ocrelizumab is approved for multiple sclerosis (MS), and obinutuzumab is now the first anti-B cell antibody with at least a positive phase II lupus nephritis study.

When the standard therapies for B cell lymphoma, including depleting anti-CD20 antibodies, fail, chimeric antigen-receptor T (CAR T) cells against CD19 are a rescue option. After rather spectacular successes in Erlangen in refractory patients with SLE, anti-synthetase syndrome, and systemic sclerosis, there is worldwide interest in this very expensive, but highly effective approach to depleting B cells, but also plasmablasts. Indeed, antibodies to double-stranded DNA in the SLE patients disappeared within months, while protective humoral memory remained intact. Long-term remissions without therapy nourish hopes for a cure, at least for some of the systemic autoimmune diseases.

While long-lived plasma cells should not be depleted by anti-CD19 approaches, plasma cells are directly targeted by the anti-CD38 antibody daratumumab. Designed for treating multiple myeloma, daratumumab also had significant impact on SLE disease activity in single patients, albeit not at a level comparable to the anti-CD19 CAR T cells.

To some degree, co-blockade of BLyS and its sister cytokine APRIL by TACI-Ig constructs has similar functions. Atacicept showed some efficacy in SLE, but its development program was terminated due to issues in finding a dose that was effective, but did not increase the risk of severe infections through relevant hypogammaglobulinemia. Telitacicept, a similar molecule developed in China, was approved there and is now being tested in other countries and in various indications.

Thus, for severe systemic autoimmune disease, B cell and plasma cell targeting is on the rise and indeed offers hope of a potential cure for some of the patients.

“Untargeting” Autoantibodies using Genome Editing, A Proof-Of-Concept Study

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Highlights

- Autoantibodies can have a direct pathogenic role in some autoimmune diseases

- Elimination of specific B/plasma-cell clones is not attainable with current therapies

- CRISPR/Cas9 allows targeting of specific DNA sites, such as V(D)J rearrangements

- CRISPR/Cas9 genome-editing was very effective in knocking out the heavy chain of autoantibodies

- Indels introduced at Cas9 cut site interfered with autoantibody-antigen interaction

Abstract

Autoantibodies (AAbs) are useful biomarkers and many have direct pathogenic role. Current standard therapies for elimination of specific B/plasma-cell clones are not fully efficient. We apply CRISPR/Cas9 genome-editing to knockout V(D)J rearrangements that produce pathogenic AAbs in vitro. HEK293T cell-lines were established stably expressing a humanized anti-dsDNA Ab (clone 3H9) and a human-derived anti-nAChR-α1 Ab (clone B12L). For each clone, five CRISPR/Cas9 guided-RNAs (T-gRNAs) were designed to target the heavy-chain`s CDR2/3. Non-Target-gRNA (NT-gRNA) was control. After editing, levels of secreted Abs were evaluated, as well as 3H9 anti-dsDNA and B12L anti-AChR reactivities. T-gRNAs editing decreased expression of heavy-chain genes to ~50-60%, compared to >90% in NT-gRNA, although secreted Abs levels and reactivity to their respective antigens in T-gRNAs decreased ~90% and ~95% compared with NT-gRNA for 3H9 and B12L, respectively. Sequencing indicated indels at Cas9 cut-site, which could lead to codon jam, and consequently, knockout. Additionally, remaining secreted 3H9-Abs presented variable dsDNA reactivity among the five T-gRNA, suggesting the exact Cas9 cut-site and indels further interfere with antibody-antigen interaction. CRISPR/Cas9 genome-editing was very effective to knockout

the Heavy-Chain-IgG genes, considerably affecting AAbs secretion and binding capacity, fostering application of this concept to in vivo models as a potential novel therapeutic approach for AAb-mediated diseases.

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5.1 Methodical Aspect and Clinical Strategies

Autoantibodies as stratification tools for prediction and treatment decisions in ANA associated rheumatic diseases

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Abstract

Many ANA-associated rheumatic diseases (AARD) including systemic lupus erythematosus (SLE) constitute a heterogenous presentation and are multifaceted in pathogenesis. A better approach to diagnosis and understanding of their underlying biology may be achieved by stratifying patients into common endotypes. Autoantibodies are a hallmark of disease and have proven useful as diagnostic, prognostic, and predictive biomarkers for disease development and outcome. A recent study utilizing machine learning identified four unique clusters of SLE patients based on longitudinal ANA and autoantibody profiles alone in the first five years of disease (1). These clusters were associated with specific clinical features and predictive of disease activity, organ involvement, treatment course, and mortality. Similar studies have performed in other AARD suggesting that characterisation of autoantibody profiles may be helpful in reconciling disease heterogeneity and understanding disease pathogenesis. This may guide clinical prognostication and inform design of personalised diagnostic and treatment strategies (2).

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Predicting progression from undifferentiated connective tissue disease to clinically overt disease using particle-based multi-analyte technology

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Abstract

Undifferentiated Connective Tissue Disease (UCTD) is a term that brings together a heterogeneous range of clinical manifestations and laboratory abnormalities suggestive, but not sufficient, to allow a definite connective tissue disease (CTD) diagnosis. Although UCTD is recognized as a separate entity and emerging data support its wide prevalence among patients with symptoms evocative for autoimmune disease, classification criteria, epidemiological data and targeted guidelines are still debated.

We will review the state of the art on diagnostic approach in UCTD and report our experience in a long term longitudinal single-center cohort of patients with UCTD. The role of conventional and upcoming diagnostic approach (e.g., the use of particle-based multi-analyte technology (PMAT) will be discussed.

Our experience highlighted that up to 44.2% of UCTD patients might developed novel clinical and/or laboratory features during the follow-up, leading to an evolution into a definite CTD in 1 out 5 cases. An adequate long term clinical and serological follow-up is therefore mandatory, in order to identify patients at high risk of progression to a definite CTD.

Multi-analyte testing in autoimmunity: From early days to modern technologies

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Abstract

Since the beginning of autoantibody testing as part of the diagnosis of autoimmune conditions, it was recognized that several autoantibodies are needed to correctly diagnose patients. Consequently, a desire to perform multi-analyte detection of those biomarkers led to the development of several methods, including but not limited to counter-electrophoresis, western-blotting, line immunoassays, immunoprecipitation and indirect immunofluorescence. Although these methods allow for the detection for multiple antibodies at the same time, technological aspects make their automation challenging and unsuited for large laboratories. Therefore, more modern particle-based technologies have been developed. Here we summarize the history and future perspectives of multi-analyte detection of autoantibodies.

Does anti-Ro52 antibodies have an impact in diagnosis of systemic autoimmune diseases?

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Objective

Anti-Ro52 antibody is one of the most common autoantibodies found in systemic autoimmune diseases (SAID). However, the clinical significance of anti-Ro52 antibodies remains controversial. The aim of this multicentric retrospective observational study was to investigate the significance of anti-Ro52 antibodies with and without other nuclear antibodies, particularly anti-SSA/Ro60 and anti-SSB/La in various SAID.

Methods

Patients tested positive for anti-Ro52 antibodies by EUROLINE Anti-ENA Profile (Euroimmun, Germany) and diagnosed with a SAID by a rheumatologist at six tertiary care hospitals in Turkey between January 2019 and May 2023, were included in the study. Demographic and related laboratory data were extracted from the medical records and the clinical associations were analyzed.

Results

A total of 1268 patients with anti-Ro52 positive result in EUROLINE blot was analysed. Among those, 995 patients with the diagnosis of SLE, Sjogren syndrome, rheumatoid arthritis, systemic sclerosis, dermatomyositis/polymyositis, mixed connective tissue disease, overlap syndrome and undifferentiated connective tissue disease (UCTD) were taken for further analysis. The mean age was 51.24 ± 15.59 (range: 6-89 years) and 91.16% female, 8.84% male.

Among the patients 44.62% (n=444) had isolated anti-Ro52 (Group 1) whereas 30.75% (n=306) had anti-Ro52+anti-SSA/Ro60, but SSB/La negative (Group 2) and 24.62% (n=245) had anti-Ro52+ anti-SSA/Ro60+ anti-SSB/La (Group 3).

In group 1 UCTD (n:134), Sjogren syndrome (n:121) and SLE (n:74); in group 2 Sjogren syndrome (n:120), SLE (75) and UCTD (67); in group 3 Sjogren syndrome (n:107), UCTD (n:59) and SLE (n:51) were the most frequent three diagnosis. Isolated Ro52 positivity was highest for UCTD patients. The average age of the isolated anti-Ro52 positive patients were higher than the group 2 and 3.

A test of independence was made between the three anti-Ro52 antibody groups and the other antinuclear antibodies. No significant dependence was found between the presence of DFS70, AMA M2, histone, PCNA, Jo-1, Scl-70, Sm, Sm/RNP, Ku and Mi-2 antibodies and the anti-Ro52 antibody groups. However, in group 1, CENP-B, PM-SCl and Scl70 antibodies were detected higher, while ribosomal P protein and nucleosome were more common in Group 2 and anti-dsDNA in Group 3.

Conclusion

The findings of this study further indicate that separate detection and reporting of anti-Ro52 antibodies is important to distinguish between anti-Ro52 and anti-SSA/Ro60 due to their different clinical associations. In case of SSA/SSB negativity, isolated Ro52 positivity may have an additional impact in the diagnosis of Sjogren syndrome. Concurrence of isolated anti-Ro52 with CENP-B, PM-Scl and Scl70 antibodies requires further investigation.

These findings together with the clinical data indicate that anti-Ro52 should be considered as an additional and independent serum marker. Elucidating the diagnostic role of anti-Ro52 in SAID might have prognostic and therapeutic implications.

Our clinical findings were supportive of the potential association between isolated anti-Ro52 antibodies and RA, SSc, MCTD, and inflammatory myopathies. However, the current understanding of the role of anti-Ro52 in autoimmunity is limited, and the precise association of these antibodies with clinical parameters remains obscure.

Anti-p56 in patients with Idiopathic Pulmonary Fibrosis targets Annexin A11

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Background

Autoantibodies are a hallmark of autoimmune connective tissue disease, and thus a potential useful tool in differentiating connective tissue disease associated interstitial lung disease from idiopathic forms. In this study we aimed to identify a novel autoantibody targeting an unknown 56kDa antigen in 7 patients with idiopathic ILD.

Methods

Autoantibody status was determined by immunoprecipitation in 499 patients with idiopathic interstitial lung disease recruited to the UK Biomarkers of Interstitial Lung Disease cohort including 251 with a diagnosis of idiopathic pulmonary fibrosis. An autoantibody targeting unknown 56 kDa protein was identified in 7 patients. The immunoprecipitated protein was identified by liquid chromatography with tandem mass spectrometry and subsequently confirmed by ELISA.

Results

The 56kDa autoantigen detected by immunoprecipitation was demonstrated to be Annexin A11. Six of the patients with anti-annexin A11 had idiopathic pulmonary fibrosis and one had non-

specific interstitial pneumonia. All 7 patients plus an additional patient with non-specific interstitial pneumonia were positive on ELISA. One myositis and one systemic lupus erythematosus (SLE) samples were positive on ELISA out of 133 controls (22 myositis, 26 juvenile dermatomyositis, 16 SLE and 69 healthy controls). There were no obvious clinical associations that separated the anti-Annexin A11 positive patients from other subgroups of interstitial lung disease.

Conclusion

The presence of anti-Annexin A11 in patients with idiopathic interstitial lung disease, notably in a small subset of patients with idiopathic pulmonary fibrosis, may indicate occult underlying connective tissue disease in a condition hitherto not considered to have an autoimmune aetiology. Further work is required to determine the clinical significance of anti-Annexin A11 in interstitial lung disease and whether a differential treatment approach is indicated.

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Comparison of Aptiva and routine methods for the detection of antibodies associated with connective tissue disease in an Australian laboratory

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Background

Detection of antinuclear antibodies (ANA) is important for the diagnosis of connective tissue disease (CTD). The adoption of fully automated solid phase assays to supplement existing testing methods is increasing in various geographies. This study aimed to evaluate the performance of Aptiva® CTD Essential Reagent based on a novel fully automated particle-based multi-analyte technology (PMAT) in comparison to BioPlex® 2200 ANA and other reference methods using characterized samples from an Australian reference laboratory.

Methods

A total of 205 banked serum samples characterized for various CTD-associated autoantibodies were included in the study. All samples were tested blinded on Aptiva® CTD Essential IgG reagents (Inova Diagnostics, USA) and in parallel by BioPlex® 2200 ANA IgG (Bio-Rad, USA). Qualitative and quantitative correlation parameters were calculated for each of the analytes with available results. Historical Euroimmun line immunoassay (LIA Euroimmun, Luebeck, Germany) results, ANA results as measured by indirect immunofluorescence (AESKUSLIDES® ANA-Hep-2 (Aesku, Germany) and other clinical notes were available and assessed.

Results

Out of the 205 total samples, the prevalence of autoantibodies as measured by Aptiva CTD ranged from 0.5% (Jo-1 and Ribo-P) to 16.1% (Ro60 and RNP) (Table 1). For samples where reference method results were available (n=153), the agreement to Aptiva was also determined (Table 1).

In some cases, the assays had too few positive samples to assess agreement. The majority of discrepant samples between Aptiva and BioPlex was for the RNP analyte, where 76 samples were positive for BioPlex RNP and negative for Aptiva RNP, of those only 1/76 (1.3%) was positive for LIA RNP (RNP68+, negative for Sm/RNP full-length or Sm by LIA). Centromere, out of the 8 samples positive by Aptiva, 0/8 were positive by BioPlex, 4/8 were positive by LIA, and 3 were confirmed Centromere pattern by HEp-2 IFA.

Table 1. Prevalence of Aptiva CTD Essential analytes and agreement with other methods

Analyte	Prevalence N (%)	Overall Agreement (*kappa*) vs. BioPlex	Overall Agreement (*kappa*) vs. LIA
Ro60	33(16.1%)	94.5% (0.795)	97.3% (0.879)
SSB	29 (14.1%)	91.8% (0.742)	94.5% (0.828)
Ro52	22 (10.7%)	n/a	97.9% (0.915)
RNP	33 (16.1%)	47.3% (0.156)	93.2% (0.766)
Sm*	2 (1.0%)	93.8% (-0.012)	95.9% (0.241)
Scl-70	14 (6.8%)	88.4% (0.451)	91.1% (0.502)
Jo-1*	1 (0.5%)	99.3% (0.664)	97.3% (0.326)
Centromere	9 (4.4%)	n/a (no positives for BioPlex found) (0.00)	97.3% (0.654)
Ribo-P*	1 (0.5%)	97.3% (-0.010)	99.3% (0.664)
DFS70	8 (3.9%)	n/a	n/a
dsDNA	18 (8.8%)	n/a	n/a

*low number of positive samples

Conclusion

This evaluation is the first to show an analytical comparison between the Aptiva® CTD Essential and BioPlex® 2200 ANA. Our data shows fair to substantial agreement for Aptiva CTD Essential to reference methods among most analytes with a sufficient number of positive samples. Further definition of samples with clinical diagnosis will allow for expanded clinical performance and association analyses.

Retrospective Evaluation of “Rings and Rods” Pattern Detected in the Anti-Nuclear Antibody (ANA) Indirect Immunofluorescence (IIF) Test

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Objective

Anti-rods and rings antibodies have recently been described as a cytoplasmic pattern in IIF-based screening of autoantibodies on HEp-2 cells and ICAP has named it as AC-23. It is most frequently related to drug-induced antibody generation. This study was aimed to investigate the clinical significance of AC-23 positivity and its significance in the diagnosis and/or follow-up of the associated diseases and/or drug use.

Methods

Patients with AC-23 pattern detected in the ANA HEp-2 IIF (Euroimmun, Germany) test between 1 January 2017-31 December 2021 were included. Demographic data, clinical diagnosis with focus on hepatitis C (HCV), drugs used for treatment and concomittent Hep-2 IIF patterns were analysed in AC-23 positive patients.

Results

The study included 600 patients with AC-23 from ten different centers in Turkey. 67% was female, 33% were male. Concomitant ANA positivity was detected in 54% of patients, the most common patterns being AC-4 and AC-5 (57.4%). The distribution of AC-23 positive patients by years indicated a steady increase between 2017-2021. While 10% of the AC-23 patients were detected in 2017, 30% were in 2021. Among the 600 patients included, 66 (11%) were diagnosed as HCV, 28 (4.6%) as HBV and 9 (1.5%) as HCV+HBV. Data about the anti-viral therapy of HCV patients were available for 34 patients; 70.6% had history of interferon alfa+ribavirin, 29.4% NS5B polymerase inhibitor drug use. Besides HCV and HBV, 5.6% of the AC-23 patients had rheumatoid arthritis, 2% had systemic lupus erythematosus and 1% Sjogren's syndrome.

Conclusion

There is a lack of report on the clinical association of AC-23 on HEp-2 IIF tested patients. Although AC-23 was most frequently associated with HCV and ribavirin treatment, we found that it could also be detected in HCV negative cases. Increasing number of AC-23 detection following COVID-19, raised the question of whether immune dysregulation during COVID-19 has led to this increase, however, data was not appropriate for the evaluation of a possible relationship between COVID-19 and AC-23 pattern. For better understanding of the clinical relevance of AC-23, larger scale prospective studies are required.

Comparison of a novel multi-analyte assay for the detection of autoantibodies in the diagnosis of connective tissue diseases with routine laboratory methods

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Background

Early diagnosis with enlarged antibody profiles combined with powerful lab technology platforms can help to deliver more efficient diagnosis in patients affected by connective tissue diseases (CTDs). The aim of the present study was to evaluate the performance of a new fully automated digital system, using particle-based multi-analyte technology (PMAT), with a multiparametric CTD antibody profile. This new system was tested in comparison to the existing lab routine methods.

Methods

Serum samples from 187 patients diagnostically characterized by routine methods (indirect immunofluorescence, IIF, Euroimmun; ELISA, Orgentec by Sebia; Blot, Euroimmun) were analyzed with the Aptiva-PMAT instrument (Werfen) for a panel of 11 autoantibodies. Correlation between different methods has been measured.

Results

Concordance analyses showed a very good overall agreement between existing routine methods and Aptiva CTD Essential, with highest correlation for ELISA vs. Aptiva. Excellent correlation (Cohen´s kappa >0.8) was found for Jo-1, DFS70 and Centromere. RNP, SS-B, Scl-70 and SS-A (ELISA

SS-A compared to Aptiva SS-A Ro 52 and Ro60) showed good correlation results (kappa >0.6) and moderate correlation (kappa >0.4) was measured for dsDNA, and Sm.

Conclusions

New Aptiva-PMAT method may represent a good alternative method for the detection of autoantibodies in diagnosis of patients with autoimmune rheumatic diseases.

Harmonization of ANA testing challenge: quantification strategy to accurately predict end-point titers avoiding serial dilution

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Objectives

Despite the advantages of automated systems for antinuclear antibody (ANA) analysis, the prediction of end-point titers avoiding serial dilutions is still in progress. The aims of this study were to set a conversion table providing discriminant ranges of fluorescence signal intensity values (FI) corresponding to the end-point titers and validate this tool in a real-life laboratory setting.

Methods

894 serum samples were analyzed for ANA using Image Navigator system. In order to classified FI into non-overlapping groups corresponding to conventional end-point titers, statistical discriminant analysis was used. Validation study was performed calculating agreement and error rates between visual readings and conversion table of 1119 routine ANA positive samples.

Results

Setting of FI ranges corresponding to the end-point titers for different staining patterns was computed. For samples showing single pattern, the overall agreement between visual readings and conversion table was 98.4% for all titers ranging from 1:160-1:2560, of which 68.0% had the same titer and 30.4% were within ±one titer difference. Concordance rates according to ANA patterns were as follows: 1) nuclear 98.4%, of which 67.0% had the same titer and 31.4% ±one

titer; 2) cytoplasmic 100%, of which 72.7% had the same titer and 27.3% than ±one titer; 3) mitotic 66.6%, of which 33.3% had more ±one titer.

Conclusions

Our study developed a quantification method for autoantibodies titers assessment based on just one single sample dilution instead of traditional serial dilution approach, providing significant advantages in routine laboratory in terms of reduction in hand-on time and harmonization of results.

Antinuclear Antibody-HEp-2 IIF Competency Assessment Programme of Society for Clinical Microbiologists of Türkiye: Analysis of Five-Year Results

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Introduction and Aims

Due to the diversity of antigens in the HEp-2 cell line, which is used in testing antinuclear antibodies (ANA) with indirect immunofluorescence (IIF), a wide variety of patterns can be seen. Therefore, education and experience are important in ANA assessment. Diagnostic Immunology Study Group of Society for Clinical Microbiologists of Türkiye has prepared a resource guide to ensure national harmonization in the ANA evaluation, and the third version of this guide was published in 2020. In addition, since 2018, Antinuclear Antibody-HEp2 IIF Competency Assessment Program has been developed in order to create a common language at the national level and ensure standardization. This program is carried out in accordance with the International Consensus on Antinuclear Antibody Patterns (ICAP) targeting international harmony.

The aim of this study is to collectively evaluate the performance of participants in 14 assessment cycles performed between 2018-2023, and to determine the current situation for future planning.

Material and Methods

This program, which is carried out free of charge in cooperation with Diagnostic Immunology Study Group and the Quality and Accreditation in Microbiology Laboratories Study Group, was launched in December 2018 and announced to the members via Society for Clinical Microbiologists of Türkiye.

Clinical laboratory specialists who want to be included in the program have registered to the system with the participant link. In the program, which is implemented as three cycles every year, 14 cycles have been completed so far. It was announced that the cycles would start and new participants were allowed to participate. Since laboratory levels were defined as “competent” and “expert” in the ICAP ANA assessment, five (three competent and two expert) HEp-2 IIF images were sent to the participants in each cycle, and the system was kept open for 15 days to provide answers. Participants answered each question by choosing the pattern and fluorescence intensity from the system. Competent level laboratories were expected to answer only the first 3 questions.

Updates in ICAP codes were followed, and the nomenclature and classification were updated in the programme when necessary. Since 2021, reflex test recommendation questions have been added with each image. Statistical analysis of the evaluation results was shared with the participants on the basis of confidentiality.

Results and Conclusions

Between 26 and 52 laboratories participated in the 14 completed cycles. Competent level patterns that can be identified with more than 90% performance were centromere AC-3; discrete nuclear dots AC-6, 7; nucleolar AC-8, 9, 10; nuclear envelope AC-11, 12; cytoplasmic fibrillar AC-15, 16, 17; and cytoplasmic polar (Golgi-like) AC-22. Among the expert level patterns, the highest performance was achieved in intercellular bridge (midbody) AC-27 pattern. Within competent level patterns, those identified with less than 80% accuracy were negative AC-0 and nuclear dense fine speckled AC-2; and the least performances at expert level were detected as anti-topoisomerase-I AC-29; nuclear large/coarse speckled AC-5; multiple discrete nuclear dots AC-6; cytoplasmic linear fibrillar (anti-F-actin like) AC-15 and anti-Jo-1 like AC-20 (Table 1).

It was observed that, in some expert level patterns, the answers were given as competent level, but correct suggestions were made as a reflex test.

This programme is based on image-based reporting, and laboratory performance is determined according to the statistical analysis of the results. Among the participants, the proficiency of the ANA IIF definition was evaluated as being 80% or more successful in the cycle, and the proficiency rate was 86.39% in advanced participants. The proficiency level of the competent level laboratories could not be calculated due to the low number of competent level participants in most cycles and even because all participants were expert level in some cycles. Although competent level ANA IIF reporting is sufficient for routine laboratory service, expert level reporting is useful in terms of recommending reflex testing to the clinician and beneficial for patient management.

Our future goals as working groups are to add different IIF test parameters to the cycles, and to take initiatives to increase competence in expert level reporting.

Table 1. Accurate identification rates of competent-level and expert-level patterns in cycles

Pattern	Rates of correct identification of patterns in cycles, %													
	Cycles													
	2018-1	2019-1	2019-2	2019-3	2020-1	2020-2	2020-3	2021-1	2021-2	2021-3	2022-1	2022-2	2022-3	2023-1
Negative, AC-0	93,75		76,92	92,85		100						79,31		
Nuclear homogeneous, AC-1		82,85			97,05			100				100		
Nuclear dense fine speckled, AC-2	87,5		96,15				79,31	92,85			91,17			
Centromere, AC-3	96,87				97,05				96,29			96,55		100
Nuclear speckled, AC-4, 5		80		100		96,66			100	92,59			97,3	
Discrete nuclear dots, AC-6, 7						96,66							91,89	
Nucleolar, AC-8, 9, 10		97,14			94,11						97,05			100
Nuclear envelope, AC-11, 12			100			95,83				96,29			97,3	
Cytoplasmic fibrillar, AC-15, 16, 17				100										
Cytoplasmic speckled, AC-19, 20										85,18				
Cytoplasmic reticular (anti-mitochondrial antibody), AC-21	100						93,10	92,85			88,23			
Cytoplasmic polar (golgi-like), AC-22			92,30				100		92,59					92,31
Topo I; anti-DNA topoisomerase I; anti-Scl-70-like, AC-29			55				53,84			42,30		42,85		
Nuclear large/coarse speckled, AC-5	62,5													
Multiple discrete nuclear dots, AC-6		28,57									53,12			
PCNA-like, AC-13														80,49
Cytoplasmic linear fibrillar (anti-F-actin like), AC-15		71,42						64,28			65,62			
Cytoplasmic discrete dots/GW body-like, AC-18									96,29			78,57		
Cytoplasmic dense fine speckled (anti-ribosomal P proteins-like), AC-19				95,65									81,81	
Cytoplasmic fine speckled (anti-Jo-1-like), AC-20					51,85									75,61
Centrosome, AC-24						83,33			81,48					
Spindle fibers, AC-25				86,95			92,30			96,15				
Intercellular bridge (midbody), AC-27					100			96,42					90,91	

Yellow: Competent level reporting; Green: Expert level reporting

Reference

(1) https://www.anapatterns.org

Platform comparison and assessment of a 48-plex cytokine panel using a high throughput particle-based multi-analyte technology

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Introduction

Cytokines and chemokines play a central role in the pathogenesis of various autoimmune diseases (AID), including but not limited to rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD)1-5,8. Consequently, cytokines represent valuable biomarkers for disease monitoring especially during biologic therapies in patients with AID. In addition, during the COVID-19 pandemic a significant correlation of cytokine levels to disease severity was reported6,7. Although cytokines are used frequently in research settings, challenges to commercialize them persist, mostly related to accurately measuring cytokine levels in blood primarily due to pre-analytical sample handling inconsistency9. Multi-analyte technology allows measurement of a large number of analytes simultaneously in each biological sample. In this study, we investigated the commutability of running a 48-plex cytokine panel on a new high-throughput fully automated particle-based multi-analyte technology (PMAT) system compared to Luminex multiplex technology (xMAP) system.

Methods

A total of 40 patient serum samples were collected based on testing for 48-plex Milliplex® MAP Cytokine/Chemokine Magnetic Bead Panel Kit (HCYTOMAG-60K, Millipore-Sigma, MA, USA) run on Luminex 100/200TM instruments (Luminex/DiaSorin, Saluggia, Italy), with the Bio-Plex Manager™ (BPM) software (BioRad, Hercules, CA) at Eve Technologies (Calgary, Canada; CLIA certified lab). All samples were also tested on the PMAT system (Aptiva, Inova Diagnostics, San Diego, USA) (research use only) (Figure 1) and the correlation between the 48 cytokine results between sites was established. Median fluorescent intensity (MFI) raw unit values as well as

converted units using the cytokine standards into pg/mL from both systems were used for analysis, and p-values <0.05 was considered significant.

Results

Using Spearman's correlation analysis, significant correlation in signal between platforms was found for 36/48 (75.0%) of the cytokines, with Spearman's rho ranging between 0.0109 (95% CI 0.090-0.637) and 0.868 (95% CI 0.759-0.930) with an average of 0.455. For some cytokines, an insufficient number of positive samples was found for significant correlation among the 40 samples. The top markers with the highest level of linear correlation are IP-10, EGF, CXCL9, MDC, IL-1RA, and IL-17F. Using heatmap and correlogram analysis, overlap reactivity between the different cytokines was found.

Conclusion

The measurement of 48 cytokines showed good commutability between Aptiva and MagPix xMAP. Additional studies are needed to assess the PMAT system for measuring cytokine profiles of patients with known AID and correlation of clinical parameters with cytokine levels (disease severity, disease activity, treatments, etc). Additional pre-analytical studies to improve the accuracy of measuring cytokine levels in blood are also needed. The Aptiva PMAT system represents a promising platform for measuring cytokines with a random-access instrument with quick turnaround with special utility for pharmaceutical or biotechnology companies to identify precision medicine approaches in AID.

Routinely use of BioCLIA® 6500 (HOB Biotech/ Eurobio Scientific), a novel chemiluminescent immunoanalyzer in autoimmune diseases diagnosis

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Introduction

The detection of antibodies was often useful to diagnose and/or to classify autoimmune diseases as connective tissue diseases and antiphospholipid syndromes. BioCLIA® 6500 is a fully-automated chemiluminescent immunoanalyzer described to perform safety diagnostics tests in a short time.

Objective

The aim of this study was to compare the predictive and discriminative performance of BioCLIA® Anticardiolipin (ACL), anti-B2GP1, anti-ENA (extractable nuclear antigen) and anti-dsDNA tests to conventional ELISAs or multiplex assays on clinical well defined groups of patients and to daily evaluate, using multiparametric runs, the determination of these antibodies in a hospital laboratory.

Results

BioCLIA® reagents showed good diagnostic performances, in term of sensitivity and specificity. Global agreements between BioCLIA® and routine-used assays were from 90 to 98% for anti-ENA and 70 to 97% for antiphospholipid antibodies in screening assays as well as specific assays.

Analysis of discrepancies showed a higher sensitivity for the detection of anti-Ro52, anti-RNP and anti-B2GP1 IgM on BioCLIA® and it appeared to be less sensitive for anti-dsDNA and ACL IgG determinations.

Conclusion

BioCLIA® 6500 analyzer is easy to rapidly detect the most common autoantibodies in autoimmune diseases. This system has the potential to provide clinically useful data within a short time. Because of the flexibility of its work modalities (random access and stat position), it is well adapted to determine antigenic specificities in daily practice.

Preliminary comparison between ELISA test routinely used and fully automated ELISA monotest system in solid-phase for the therapeutic drug monitoring

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Introduction

Infliximab (IFX) and Adalimumab (ADL) are biological drugs widely used in clinical practice, as anti-tumor necrosis factor (TNF) agents, in patients with inflammatory bowel diseases and rheumatic diseases. Sometimes these drugs fail to produce a satisfactory clinical response often due to the generation of anti-drug antibodies (ADA). Our study aims to compare and verify differences between enzyme-linked immunosorbent assay (ELISA), LISA TRACKER Duo Drug (LISA-Tracker Duo Adalimumab and LISA-Tracker Duo Infliximab) routinely used in our laboratory, and an automated quantitative method, the DIESSE CHORUS TRIO instrument, for the determination of IFX and ADL drugs and ADA levels.

Materials and Methods

The study was performed on 37 patients affected by selected Rheumatology and Gastroenterology diseases, 21 treated with IFX and 16 treated with ADL, attending Rheumatology and Gastroenterology departments at the University of Modena, Italy.

All sera were analyzed for IFX, ADL, anti-IFX and anti-ADL using LISA-Tracker Duo Adalimumab and Infliximab (Theradiag) and the fully automated assays CHORUS Promonitor (DIESSE Diagnostica Senese). The level of agreement between two methods was evaluated through Spearman correlation coefficient for drugs determination and through Cohen kappa for anti-drug antibody results. Drugs determination results were analyzed by Bland-Altman plots, to evaluate comparability of the two methods and to estimate the differences.

Results

For the detection of drugs levels, the two methods showed a perfect agreement with Spearman coefficient values of 0.98 (p = 3.37e-14) and 0.96 (p = 2.90e-09) for the dosage of IFX and ADL, respectively. Bland-Altman Plots for detection of IFX levels showing a bias ranging from −8,37 to 18,46 and a standard deviation of difference equal to 1.96 with limits of agreement from −11,90 to 9,42. The scatter of differences through Bland-Altman plot for ADL detection levels showing a bias ranging from −6,89 to 5,17 and a standard deviation of difference equal to 1.96 with limits of agreement from -7,01 to 6,73. Concordance between two methods for the detection of anti-drug antibody levels, assessed by Cohen's kappa test, displayed a good agreement with a value of 0.61 for anti-IFX and 1.0 for anti-ADL.

Conclusion

Our results revealed a strong correlation between methods, but the impact of ELISA is more laborious. CHORUS Promonitor being a mono-test, fully automated and faster, offers timing advantages for laboratory and clinical monitoring. The choice of the method must be made according to the equipment and the workflow of the laboratory.

Identification of thresholds for moderate and high positive anticardiolipin and anti-β2-glycoprotein I levels detected by chemiluminescent assays

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Introduction

The antiphospholipid antibodies (aPL) included in the current classification laboratory criteria for antiphospholipid syndrome (APS) are lupus anticoagulant (LA), anticardiolipin (aCL), and anti-β2-glycoprotein I (anti-ß2GPI) antibodies [1]. Test results for aPL remain inconsistent among different detection methods, in part because of the lack of standardization of cut-off values, calibration and quantification methods, choice of solid phase and coating, type and source of antigen, and other analytical problems. Traditionally, enzyme-linked immunosorbent assay (ELISA) has been used because of its relative time and cost efficiency. In recent years, new automated detection systems such as chemiluminescent immunoassay (CLIA) have been introduced. According to the classification criteria, the 99th percentile of healthy blood donors or 40 GPL and MPL have been suggested as the thresholds between low and moderate aCL antibody levels and the 99th percentile for positive anti-β2GPI, both with ELISA. In the new draft ACR/EULAR Classification criteria [2], 40 and 80 GPL/MPL are proposed as thresholds for moderate and high positive aPL levels by ELISA only. Thresholds for CLIA assays have not yet been defined.

Therefore, we aimed to identify thresholds for moderate and high positive aCL and anti-ß2GPI levels using chemiluminescent assays.

Methods

The study included 151 samples collected at the Department of Rheumatology, University Medical Centre Ljubljana. The population comprised patients with primary APS (n=54), secondary APS

(n=3, all SLE), seronegative APS (n=66), and control sera from other CTD (n=28, SLE, Sjögren´s syndrome, rheumatoid arthritis, and systemic sclerosis). All samples were tested with the in-house aCL and anti-β2GPI ELISA for IgG and IgM and additionally with the CLIA QUANTA Flash assays on the analyzer BIO-FLASH. ROC analysis, overall agreement, and Cohen`s Kappa coefficient (κ) were calculated using Analyse-it software.

Results

Moderate and high thresholds were set for in-house aCL [3] and anti-β2GPI ELISAs [4] performed in our laboratory for more than two decades. We used ROC analysis to calculate chemiluminescence units (CU) that provide the same diagnostic specificity and sensitivity for the QUANTA Flash as the in-house ELISA`s threshold. The following thresholds were calculated: aCL IgG moderate 40 CU, high 200 CU; aCL IgM moderate 30 CU, high 90 CU; anti-β2GPI IgG moderate 40 CU, high 100 CU; and anti-β2GPI IgM moderate 30 CU, high 90 CU. The agreement between the results of the aCL assays was 91.6% for IgG with κ 0.749 and 92.6% for IgM with κ 0.719. The agreement between the results of the anti-β2GPI assays was 89.4% for IgG with κ 0.705 and 91.4% for IgM with κ 0.501.

Conclusion

We determined thresholds for moderate and high levels of aCL and anti-β2GPI CLIA QUANTA Flash assays in our population and laboratory setting that achieved the same diagnostic specificity and sensitivity as the in-house ELISAs. The established thresholds showed high agreement and substantial Cohen`s Kappa agreement. Future studies are needed to evaluate the clinical utility of the QUANTA Flash results using new thresholds.

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5.2 Systemic Lupus erythematosus

Comparison of prospective anti-double stranded DNA (dsDNA) assays relative to antinuclear antibodies and *Crithidia luciliae* indirect fluorescent test

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Background

The presence of antibodies to double stranded DNA (dsDNA) is a hallmark immunologic feature of systemic lupus erythematosus (SLE). Assessment of anti-dsDNA autoantibodies, however, can be challenging due to a number of factors not limited to heterogeneity in host immune responses and lack of harmonization of immunoassays for antibody testing. The evaluation of prospective anti-dsDNA assays in parallel with additional relevant testing, including *Crithidia luciliae* indirect fluorescent test (CLIFT) and anti-nuclear antibodies (ANA) is therefore an important part of assay selection for the clinical laboratory. The objective of this study was to evaluate the analytical performance of 4 prospective dsDNA solid phase assays (SPAs) relative to ANA by HEp-2 by indirect immunofluorescent assay (IFA) and CLIFT.

Methods

In total, 497 specimens were collected and analyzed. Sample selection was based on the laboratory's predicate dsDNA ELISA (Innova QUANTA Lite® dsDNA ELISA) and included 172 dsDNA positives, 47 equivocals and 140 dsDNA negatives (analytical cohort: n=359). An additional 65 healthy, and 73 disease controls (n=138) were included to assess specificities of the different assays. All positive dsDNA samples in the analytical cohort were collected in a consecutive manner between the dates of 02/27/2023 and 05/05/2023 from both the local clinic and external reference laboratory testing populations. Healthy and disease control groups were obtained from Mayo Clinic's Biorepository. All samples were evaluated with 4 different dsDNA SPAs (Werfen: QUANTA Flash® dsDNA and QUANTA Lite® dsDNA ELISA, ThermoFisher: Phadia EliA™ dsDNA, and Euroimmun: anti-dsDNA-NcX ELISA), ANA with HEp-2 by IFA (NOVA Lite® Hep-2 ANA) and CLIFT (NOVA Lite® dsDNA *Crithidia luciliae*). The analytical performance of each dsDNA SPA was

assessed by determining negative percent agreement (NPA) relative to ANA and positive percent agreement (PPA) relative to CLIFT.

Results

The median age of all subjects in the dataset was 53 (IQR: 35.8 – 35.0) and the percentage of females was 71.5%. Within the analytical cohort, the NPA relative to ANA ranged from 80.7% – 93.3% (Table 1), while the PPA relative to CLIFT ranged from 58.3% – 82.7%. The number of results falling in the equivocal range was 16 for the Quanta Flash and EliA kits, 47 for the QUANTA Lite kit and 0 for the Anti-dsDNA-NcX kit (which does not have an equivocal range). When considering all equivocal results as negative, the differences in PPA relative to CLIFT were smaller between the kits, ranging from 58.3% - 69.4%. NPA relative to ANA was similar for all kits in the healthy and disease control groups. PPA relative to CLIFT in the healthy control group was 50% for the Phadia EliA kit, and 25% all others, however, there were only 4 total CLIFT positive samples in this group.

An analysis of discrepant results among the kits revealed that each assay had results which were uniquely positive relative to the other kits (Figure 1, A). The number of samples which were uniquely positive on each kit ranged from 11 – 33, corresponding to 2.2% – 6.7% of all results. Clustering analysis illustrated that a subset of the results which were indeterminant or uniquely positive on one kit had high fluorescence intensity by CLIFT and were also positive for ANA (Figure 1B).

Table 1. Analytical performance of each dsDNA solid phase assay (SPA).

		Negative Percent Agreement (Relative to ANA)	Positive Percent Agreement (Relative to CLIFT)	# of Equivocal Results
Analytical Cohort	QUANTA Lite	80.7 (70.6-88.6)	82.7 (76.7-87.7)	47
	QUANTA Flash	85.1 (75.8-91.8)	68.9 (62.4-74.9)	16
	Phadia EliA™	87.5 (78.7-93.6)	61.6 (54.9-68.1)	16
	Anti-dsDNA-NcX	93.3 (85.9-97.5)	58.3 (51.7-64.7)	-
Analytical Cohort (Equv. as Negative)	QUANTA Lite	82.0 (72.5-89.4)	69.4 (63.0-75.2)	-
	QUANTA Flash	85.4 (76.3-92.0)	65.1 (58.6-71.2)	-
	Phadia EliA™	87.6 (79.0-93.7)	57.7 (51.1-64.1)	-
	Anti-dsDNA-NcX	93.3 (85.9-97.5)	58.3 (51.7-64.7)	-
Healthy Controls	QUANTA Lite	100 (93.3-100)	25.0 (0.60-80.6)	1
	QUANTA Flash	100 (93.3-100)	25.0 (0.60-80.6)	0
	Phadia EliA™	100 (93.3-100)	25.0 (6.80-80.6)	2
	Anti-dsDNA-NcX	100 (93.3-100)	25.0 (0.60-80.6)	-
Disease Controls	QUANTA Lite	100 (91.8-100)	25.0 (3.2-65.1)	2
	QUANTA Flash	100 (92.1-100)	25.0 (5.50-57.2)	1
	Phadia EliA™	97.7 (88.0-99.9)	25.0 (5.50-57.2)	0
	Anti-dsDNA-NcX	100 (92.1-100)	23.1 (5.00-53.8)	-

Conclusions

The performance of each dsDNA SPA in this comparison varied with the primary differences being that each kit displayed a unique balance between NPA relative to ANA, and PPA relative to CLIFT. When equivocal results were considered negative, the differences between the kits were reduced. Clustering analysis suggested that each kit is able to detect a subset of dsDNA positive samples which are negative on other assays. Overall, the results of this comparison suggest that these dsDNA SPAs are complementary to each other and offering a combination of SPAs or a SPA and Crithidia test may provide optimal disease evaluation and or management.

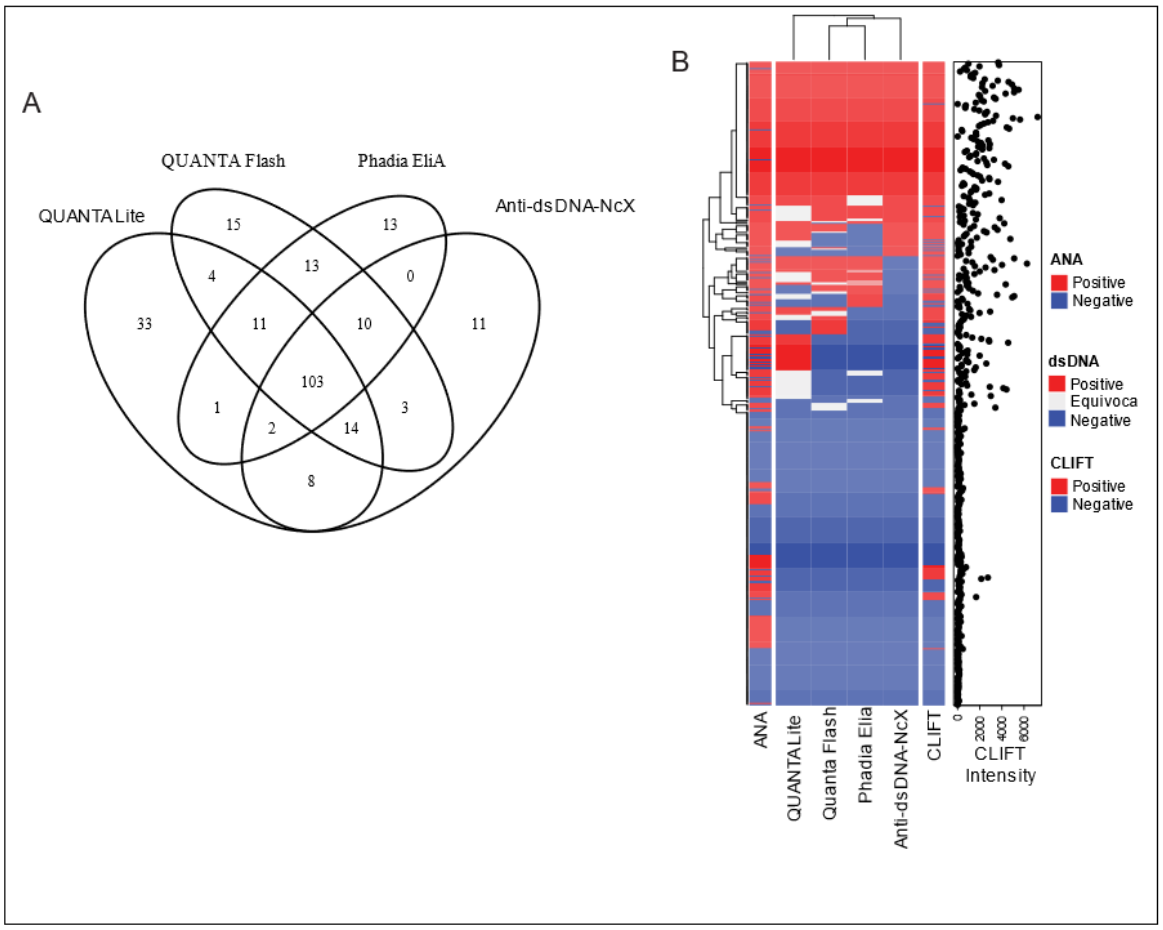

Figure 1: Comparison of dsDNA solid phase assays (SPAs). A) The overlap of dsDNA positivity for each dsDNA SPA is displayed in a Venn diagram. B) The results from each dsDNA SPA are displayed in a heatmap relative to antinuclear antibody (ANA) and Crithidia luciliae indirect fluorescent test (CLIFT) testing results. The fluorescence intensity from CLIFT for each sample is displayed on the right of the heatmap.

Urinary sCD163 performs better than anti-C1q and anti-dsDNA antibodies as biomarker in lupus nephritis for monitoring remission and impending flares

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Objective

Detecting an active lupus nephritis (LN-A) on a background of systemic lupus erythematosus (SLE) is challenging in the absence of kidney biopsy and there is an unmet need to develop serum and/or urinary biomarkers as an alternative.

Material and methods

A monocentric and retrospective study was conducted in 99 SLE patients with biopsy-proven nephritis having LN-A (n=45) or not (n=54), as well as 48 non-renal SLE patients. The panel of biomarkers included: 11 autoantibodies (e.g., anti-C1q Abs, anti-dsDNA Abs), 3 complement parameters, and the urinary soluble CD163 factor normalized to creatinuria (sCD163/Cre).

Results

Four out of the 15 (26.7%) selected biomarkers were effective to discriminate LN-A with an area under the curve (AUC) ranging from 0.96 (sCD163/Cre, threshold = 330 ng/mmol), 0.78 (anti-C1q Abs), 0.74 (anti-dsDNA Abs), to 0.73 (C3). In patients with LN-A, anti-dsDNA/Chromatin/C1q Ab levels were higher in proliferative glomerulonephritis (class III/IV vs V), which was not the case for sCD163/Cre. Finally, and among evaluated biomarkers, urinary sCD163/Cre best predicts disease activity (SLEDAI-2K) when tested in a cross-sectional analysis (r=0.8) or during patient's follow-up.

Conclusion

Among the biomarkers that can be used to follow LN evolution, urinary sCD163/Cre provides the optimal accuracy for monitoring remission and impending flares.

Anti-KIF20B Autoantibodies are associated with Cranial Neuropathy in Systemic Lupus Erythematosus

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Background

Cranial neuropathies (CN) are a rare neuropsychiatric SLE (NPSLE) manifestation associated with significant morbidity and impaired quality of life. Previous studies reported that antibodies to kinesin family member 20B (KIF20B) (anti-KIF20B) were associated with idiopathic ataxia and CN. We assessed anti-KIF20B as a potential biomarker for NPSLE in a large international SLE inception cohort.

Methods

Individuals fulfilling the revised 1997 American College of Rheumatology (ACR) SLE classification criteria were enrolled from 33 centres from 1999-2011 and followed annually. Anti-KIF20B testing was performed at baseline (within 15 months of diagnosis) or first annual assessment samples using an addressable laser bead immunoassay. Univariate and multivariate logistic regression (using penalized maximum likelihood and adjusting for confounding variables) examined the association between anti-KIF20B positivity and NPSLE manifestations (1999 ACR case definitions) occurring over the first 5 years of follow-up.

Results

795 SLE patients were assessed; 88.7% female, and 52.1% White. The frequency of anti-KIF20B antibody positivity was 29.8%, which differed only for those with versus without CN (70.0% vs. 29.3%; odds ratio [OR] 5.16, 95%CI 1.44, 18.54). Compared to patients without CN, patients with CN were more likely to fulfill the ACR hematologic (90.0% vs. 66.1%, difference 23.9%, 95%CI

5.0%, 42.8%) and ANA criteria (100% vs. 95.7%, difference 4.3%, 95%CI 2.9%, 5.8%). In the multivariate analysis adjusting for age at anti-KIF20B testing, female, White race/ethnicity, ACR hematologic and ANA criteria, anti-KIF20B positivity remained associated with CN (OR 5.24, 95%CI 1.44, 19.09).

Conclusion

Anti-KIF20B is a potential biomarker for SLE-related CN. Further studies are needed to examine anti-KIF20B antibodies over the disease course to determine whether anti-KIF20B are predictive of CN development, and how anti-KIF20B, which is differentially expressed in a variety of neurological cells and tissues, contributes to disease pathogenesis. contribute to disease pathogenesis in SLE.

Prevalence of Autoantibodies Directed Against Secondary NEcrotic Cells in SLE Patients

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Background

Systemic lupus erythematosus (SLE) is characterized by autoantibodies against nuclear autoantigens. During apoptosis antigens are congregated and modified increasing the potential of autoimmune reactions. It was shown that a novel diagnostic test which employs apoptotically modified nuclear remnants, Secondary NEcrotic Cells (SNEC), allowed sensitive detection of pathologically relevant autoantibodies in serum of patients with lupus disease.

Within this study, we aimed to validate technical and clinical performance of this assay with an independent patient cohort.

Methods

For the validation study, an indirect enzyme-linked immunosorbent assay (ELISA) detecting autoantibodies directed against SNEC was used to measure serum samples from 115 SLE patients, 50 healthy donors and 286 disease controls. To compare the results with classical, serological biomarkers, the samples were measured using the EliA™ technology (Thermo Fisher Scientific, Phadia AB, Sweden).

Results

The technical evaluation demonstrates a high stability and reproducibility of the ELISA using secondary necrotic cells as immobilized antigens. The measurement of an SLE patient cohort revealed a comparable clinical performance as shown by Biermann et al. with a sensitivity and specificity of 56% and 98%, respectively. When comparing the results with established, serological biomarkers, the measurement of anti-SNEC antibodies results in an added value of approx. 27% sensitivity in the seronegative patient group.

Conclusions

This study validated the previous results regarding technical and clinical performance of the SNEC-ELISA. Comparison to established serological markers reveals an added sensitivity of 27% among seronegative samples indicating that SNEC-ELISA includes to date unidentified additional autoantigens and therefore allows more accurate classification of suspected SLE patients.

Isolated ribosomal P antibodies, are they useful clinically?

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Introduction

Ribosomal P antibodies (RibP) are specific markers of systemic lupus erythematosus (SLE), usually associated with lupus nephritis, psychiatric manifestations or hepatitis. However, their role as isolated antibody is not defined.

Methods

At the Fundación Jiménez Díaz Universitary Hospital (Madrid, Spain) we performed a retrospective search from all RibP processed in our autoimmunity laboratory, between December 28, 2018 and December 28, 2022. We selected all RibP with positive result.

RibPa positive result was defined as the presence AC-19 (ICAP nomenclature) in HEp2 cells, with a positive result in two solid phase assays: one immunoblotting (Euroimmun® /DTEK®) and the other: Biorad-Bioplex®/ ELIA-Thermofisher. Patients with antibody profile associated to other antibodies (anti-SSA/Ro52, anti-SSA/Ro60, anti-SSB/La, anti-Sm, anti-U1RNP, dsDNA, nucleosomes or histones) were excluded.

Results

Of the 296,606 RibP determinations, 137 positive results were obtained for the study inclusion criteria. Of these, 110 were associated with other antibodies. RibPa alone was present in 27 determinations, of which we had access to the medical records of 12 patients (23 determinations). Regarding the clinical profile: 4/12 met SLE criteria, 3/12 in an active inflammatory process, 2/12 in an oncologic process, and the rest without definitive diagnosis. Four of the 12 patients presented analytical follow-up by one or two immunoassay techniques without relapses during this period.

Conclusions

- In our cohort, isolated RibP determination is low, appearing mostly associated with other antibodies.

- In patients with isolated RibP in our series, no cases of hepatitis or psychiatric manifestations were observed, being mostly described in different clinical scenarios and with little association with SLE.

- The sample size does not allow conclusions to be drawn on the usefulness of monitoring RibP levels. However, in 4 patients with follow-up, a decrease in values coinciding with periods of remission was observed.

- Further studies are needed to see its long-term behavior, including episodes of relapse.

Efficacy of intravenous immunoglobulin therapy in a haematological form of systemic lupus erythematosus: a case report

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Abstract

Intravenous immunoglobulin products (IVIg) are obtained from the plasma of healthy subjects by ethanol fractionation. IVIg affects autoimmune conditions through multiple mechanisms. Cellular mechanisms include: immune modulation of T- and B-cell activation; inhibition of anti-inflammatory cytokine production. Humoral mechanisms include: blocking Fc-receptors, effects on autoantibodies and their production via idiotype-antiidiotype interactions, prevention of immune complex formation and neutralization of microbial toxins. The use of IVIg in SLE remains off-label as an option in patients who are refractory to or have contraindications to cyclophosphamide, mycophenolate mofetil and azathioprine therapy. High doses of glucocorticoids and cytotoxic drugs used in some cases even worsen the clinical presentation.

We present a case of a 61-year-old man who was admitted for elective cholecystectomy. During routine preparation thrombocytopenia and significant abnormalities of the coagulation parameters were noted. There was no evidence of bleeding. Therefore, surgical treatment got delayed and the patient was referred to the hematology clinic. He received treatment with methylprednisolone, fresh frozen plasma and got further referred to the rheumatology clinic for diagnostic clarification and treatment. There was also a familial burden of a mixed connective tissue disease: his sister had Lupus Nephritis. After physical examination, anamnesis, laboratory tests and imaging Systemic Lupus Erythematosus (SLE) with cutaneous, articular and hematological involvement with immunological activity was confirmed. So, pulse therapy with methylprednisolone and intravenous Immunoglobulin was started. Although data on the

usefulness of IVIG therapy in SLE are limited, this agent was effective to treat this patients' various manifestations of SLE.

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Impact of imlifidase treatment on immunoglobulins in an HLA-hypersensitized SLE patient with anti-SSA/SSB antibodies after kidney transplantation

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Abstract

Removing pathogenic auto- or allo-antibodies (Ab) is a common therapeutic approach in the context of autoimmune and allograft diseases. Initially performed by plasmapheresis and/or immunoadsorption, pathogenic Ab removal can be alternatively obtained by a single administration of a streptococcus cysteine protease that specifically degrades human IgG1-4. To this end, imlifidase treatment (Ides, Idefirix®, Hansa Biopharma) was evaluated in numbers of autoimmune diseases (notably anti-GBM disease and thrombotic thrombocytopenic purpura), and has been approved by the European Medicines Agency in 2020, for desensitization of highly sensitized adult kidney transplant patients with a positive crossmatch against an available deceased donor.

Here we report the case of a 51 years old woman suffering from a systemic lupus erythematosus (SLE), who has been registered in 2015 in the awaiting list of kidneys for a second allograft. At the time of the second allograft in 2023, imlifidase treatment was selected as she was highly HLA-sensitized due to miscarriage, and a first allograft which has become quickly nonfunctional. The longitudinal monitoring of different immunoglobulin subsets included: (i) IgG, IgA, IgM and IgG1-4 subclasses (i.e., capillary electrophoresis, Sebia®; immunoturbidimetry, The Binding Site® and

Roche®); (ii) IgG autoantibodies (Bio-plex ANA Screen, Bio-Rad®); (iii) IgG anti-vaccine Abs against tetanus toxoid, pneumococcus and haemophilus influenzae (ELISA, Binding Site®); and (iv) IgG anti-HLA (One lambda® Single Antigen and Labscreen kits). The monitoring was initiated at the time of imlifidase initiation and the last time point obtained after 4 months.

Total IgG Abs and related subclasses, but not IgA/IgM Abs, start to decrease only six hours after imlifidase injection, and the lowest detection (IgG 2.41g/L) was obtained after 48 hours, which is compatible with imlifidase half-life (78 hours). IgG depletion was associated with the negativity of the anti-SSA and anti-SSB Abs (< 0.2 AI), and anti-vaccine Abs. Anti-SSA and anti-SSB Abs depletion was transitory: both were detectable again after 1 and 2 months (anti-SSA Abs: 4.6 and 7.5AI, anti-SSB 1.4 and 3.2AI, respectively). Concerning alloimmunization, four donor specific antibodies (DSA), initially detected at 9000, 7000, 6000 and 1000 of MFI were immediately negativized (MFI<500), undetectable at 48h (MFI=0), and still undetectable at the last follow-up (four months after the graft). At this time, the patient presented a good kidney function (creatinine = 103µmol/L, creatinine clearance = 54mL/min, hemoglobin = 11.0g/L, and proteinuria = 0.11g/L four months after transplantation).

This case report underlined the biological follow-up of an SLE patient concerning his auto- and allo-sensitization. From our knowledge, this is the first report of the imlifidase impact on a large spectrum of immunological parameters present in SLE.

Identification of new autoantigens in HEp-2 IIFA-positive usual interstitial pneumonia without known autoantibody

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Introduction and objective

Up to a third of patients with idiopathic usual interstitial pneumonia (UIP) have a positive HEp-2 indirect immunofluorescence assay (HEp-2 IIFA) without known underlying autoantigen. We aimed to identify autoantigens in these patients through immunoprecipitation-mass spectrometry (IP-MS).

Methods

Thirty-seven HEp-2 IIF-positive patients with an UIP pattern on high-resolution computed tomography without known autoantibody specificity after screening with the Farr assay and EliA CTD Screen (Thermo Fisher, Germany) were identified from a database of 641 patients with a tentative diagnosis of idiopathic pulmonary fibrosis. Sera of five HEp-2 IIF-negative patients with idiopathic pulmonary fibrosis were included as controls. HEp-2 IIFA-positivity was defined as a

nuclear speckled, nuclear homogeneous or cytoplasmic pattern at ≥1:160 titer; or nucleolar or centromere pattern at ≥1:80 titer. We performed immunoprecipitation-mass spectrometry with HeLa nuclear (n = 35) or cytoplasmic extract (n = 2), according to HEp-2 IIF pattern.

Results

Three new autoantigens were identified: regulator of chromosome condensation 2 (RCC2, n = 1), protein arginine N-methyltransferase 1 (PRMT1, n = 1) and Superkiller complex protein 8 (SKIC8 or WDR61, n = 1). In addition, the rare DDX21/Gu autoantigen (n = 1), the recently discovered HMBOX1 autoantigen (n = 1), the systemic sclerosis autoantigens Th/To (n = 2, of which only one was positive on a dot blot assay) and Ku (n = 1), and the systemic lupus autoantigen histone 1.3 (n = 5) could be identified.

Conclusion

IP-MS revealed new and rare autoantigens in patients with HEp-2 IIFA-positive UIP. Multiple new autoantigens are functionally linked with established autoantigens in systemic autoimmune diseases. Extended autoantibody profiling with IP-MS could be useful in HEp-2 IIF-positive UIP to assess the risk of evolution towards a definite systemic autoimmune disease.

5.3 Systemic Sclerosis and Idiopathic Inflammatory Myopathies

Rare and new autoantigens in systemic sclerosis and how to detect them

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Abstract

Autoantibody production is a characteristic feature of systemic sclerosis (SSc). However, in up to 20% of patients with SSc no established SSc-associated autoantibody can be identified, even though many of these patients have a high-positive result on the HEp-2 indirect immunofluorescence assay (HEp-2 IIFA).

In the last few years, new autoantigens in the scleromyositis spectrum have been identified, including RuvBL1/2, eIF2B, SMN and TERF1. The technical and clinical features will be discussed with emphasis on characteristics that might hint at these rare autoantigens.

In addition, we recently identified new autoantigens with a new approach based on unlabeled protein immunoprecipitation combined with gel-free liquid chromatography-tandem mass-spectrometry (LC-MS/MS) with sera from individuals with SSc and unidentified HEp-2 IIF staining from the Hospices Civils de Lyon, University hospitals Gent and University Hospitals Leuven [1]. We identified eight telomere- and telomerase-associated autoantigens, including the THO complex, telomeric repeat-binding factor 2 (TERF2), homeobox-containing protein 1 (HMBOX1), regulator of chromosome condensation 1 (RCC1), nucleolar and coiled-body phosphoprotein 1 (NOLC1), dyskerin (DKC1), probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase (NOP2) and nuclear valosin-containing protein-like (NVL) [1]. Furthermore, we identified the NineTeen Complex (NTC) [2] and cyclin-dependent kinase 9 (CDK9) as novel autoantigens in SSc [3]. With IP-MS we were also able to identify anti-U5 RNP and anti-eIF2B autoantibodies [4]. The potential applications of IP-MS as autoantibody discovery and detection method in SSc and other autoimmune diseases will be discussed.

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Novel autoantibodies in systemic sclerosis detected using multi-analyte testing

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Abstract

Systemic sclerosis (SSc) is a severe autoimmune disease characterized by multiple-organ involvement, unpredictable clinical course, and poor prognosis. It has a wide range of manifestations with cutaneous, pulmonary, cardiac, renal and gastrointestinal involvement. SSc is challenging to diagnose in the early phase due to low sensitivity of the classification criteria in patients without skin involvement, however, the presence of Raynaud's phenomenon (RP) and antinuclear antibodies (ANA) can be predictors for early treatment intervention and potentially avoiding irreversible organ damage. More than 95% of patients with SSc present autoantibodies (Abs) against different nuclear proteins of which three have been included in the classification criteria: anti-topoisomerase I (ATA, Scl-70), anti-centromere (ACA), and anti-RNA-Pol III (ARA). Additionally, other specific but less frequent Abs are anti-Th/To (e.g., Rpp25/38) or anti-fibrillarin, as well as Abs that may be present but are not specific to SSc only. New biomarkers are needed to close the seronegative gap and enable patient stratification, and to aid in the establishment of valid disease predictors for early treatment. Additionally, the time gap between disease onset and diagnosis is a "margin of opportunity" for SSc patients, where clinicians can intervene with therapies to impede the progression of the disease. This talk focuses on samples from the VEDOSS project, which is a multicenter, longitudinal registry study done in 42 European Scleroderma Trial and Research group centers (located in 20 countries in Europe, North America, and South America), evaluated on new assays based on particle-based multi-analyte technology (PMAT) for the detection of SSc autoantibodies.

The never-ending story of Antibodies in Systemic Sclerosis

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Aim

In Systemic Sclerosis (SSc), a diagnostically important immunological aspect is the presence of circulating antinuclear antibodies (ANA). There are several ANA specific for SSc. Autoantibodies have been linked to distinct clinical features. ACA are typically detected in lcSSc, Scl70 have been associated to dcSSc. Therefore, detecting a particular antibody type is important in predicting a possible organ involvement and prognosis and may have an impact on monitoring and treatment.

Methods

This study analysed 188 SSc patients with a specific clinical manifestation or SSc autoantibodies.

Patients were recruited by the Rheumatology Unit, AOU, University of Modena, Italy (approval Ethical Committee) during 2021-2022 years. They were patients belonging to the Scleroderma Unit, undergoing routine testing.

We performed Hep 2000 ANA IIF pattern (Immunoconcepts and Image Navigator Automated Microscope).

All patients were tested for Elia CTD screen and single specificity antibodies by ImmunoCap FEIA ThermoFisher Scientific. All patients were tested for 3 PMAT: panel 1. CTD Essential, panel 2 CTD Comprehensive, panel 3, Myopathy RUO, by Aptiva Inova Diagnostics, San Diego, USA.

Results

In this first examination of the results, we considered a first group (1) of 78 ACA positivity and a second group (2) of 65 anti Scl-70 positivity. We investigated within each of these 2 groups to

detect the presence and frequency of other antibodies because the subsets have different clinical features distinguishing predominating types of circulating ANA.

In group 1 we had 23 patients with BICD2, 22 Ro52, 8 Ro60, 5 RNP, 3 PM-Scl, 2 SSB, 2 MDA5, 1 NXP-2, 1 Rpp25, 1 Rpp38, 1 Ku, 1 RNApol3. In group 2 we had 22 patients with Ro60, 8 RNP, 5 SSB, 4 BICD2, 4 Rpp38, 4 Ro52, 4 Ku, 3 PM-scl, 3 Fibrillarin, 2 RibP, 1 HMGCR, 1 SRP54, 1Rpp25, 1 RNApol3.

We further analysed antibody associations and clinical aspects of patients, organ damage and distinct prognosis. The restricting subset of SSc patients to only those based on the two mainly characterizing antibodies, may not capture the complete heterogeneity of the disease.

Conclusion

Subgrouping patients based on the type of autoantibodies can be useful in diagnosing and management.

Despite availability of validated and standardized immunoassays for the detection of classical autoantibody markers of SSc, the search and validation of novel autoantibodies is very important since we keep on having several diagnostic gaps in the diagnosis of SSc, particularly in seronegative SSc patients. Autoantibodies presenting high diagnostic specificity and high predictive values are required for early SSc diagnosis, for a specific follow-up and to define the best therapy for specific SSc subsets. For this purpose, the Autoimmune Laboratory plays a very crucial and fundamental role because it has to organize and make the coexistence in an optimal way of different technology platforms for autoantibodies analysis and make every effort towards the harmonization of results.

Added value of systemic sclerosis and anti-synthetase antibody testing on ILD classification in patients with distinct radiological HRCT pattern

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Background and aims

Multidisciplinary team discussion (MTD) increases the diagnostic confidence in discriminating idiopathic from non-idiopathic interstitial lung disease (ILD). We aim to investigate the added value of systemic sclerosis (SSc) and anti-synthetase syndrome (ASS) antibody (Ab) testing on ILD classification in patients with a distinct HRCT pattern (e.g., usual interstitial pneumonia (UIP), organizing pneumonia (OP) and non-specific interstitial pneumonia (NSIP)).

Methods

ILD patients, diagnosed in our hospital from 02/2018-08/2022, were retrospectively included. Two consecutive MTDs, composed of a radiologist, a pneumologist and a rheumatologist were organized including

1) standard serology only, e.g.:
    - RF IgM and ACPA IgG (Thermo Fisher Scientific)
    - ANA testing (dsDNA IgG and ENA IgG (ANA Profile 3 IgG, Euroimmun) guided by indirect immunofluorescence (IFA) (NOVAView, Werfen)
    - ANCA IgG (myeloperoxidase IgG and proteinase-3 IgG) (BIOFlash, Werfen) guided by IFA (Euroimmun)
2) additional SSc (SCL12DIV-24) and ASS (MYOS12DIV-24) Ab results (D-tek)

Results

181 ILD patients were included (n=110 UIP, n=48 NSIP, n=23 OP). Additional serological testing resulted in a decrease of idiopathic ILD classifications from 58.0% to 53.6% and a significantly (p=0.036) increased ASS/SScl ILD classification from 6.1% to 12.2%, irrespective of the HRCT pattern (n=3 UIP, n=6 NSIP, n=2 OP). The additionally identified ASS/SScl-ILD diagnoses constituted of i)9 ASS/PM-ILD based on PL12 IgG(+++)(3x), PL7 IgG(+++)(2x), Th/To IgG(+++), EJ IgG(++/+++) (2x), SRP IgG (+), ii)1 SSc based on PM-Scl75 IgG(+++) and iii)1 PM-Scl disease based on PM-Scl75 IgG(+). In 5/9 newly identified ASS/PM-ILD, Ro52 IgG co-occurred.

Extending the serological domain of the current IPAF classification, resulted in 5 new IPAF classifications (PM-Scl 100 IgG; Th/To IgG(+++), RNA-PIII(+++), SAE-2 (+), SAE-1 (+)).

Of the newly identified antibodies, 37,5% (n=6/16) couldn't be confirmed by an alternative line-dot technology (Euroimmun SScl/Myositis) (Th/To (+++) (2x), RNA-PIII(+++), PM-Scl 75 (+), SAE-1 (+), SAE-2 (+)). Accuracy of the Ab detection will be verified by immunoprecipitation-mass spectrometry.

Conclusions

Independent of the presenting HRCT pattern, serological testing for SSc and ASS Ab added value in ILD classification. An update of the serological domain of the current IPAF classification criteria is warranted, in line with an optimalization and harmonization of routinely used commercial line-immunoassays.

Identification and characterization of novel functional autoantibodies associated with systemic sclerosis

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Abstract

Systemic sclerosis (SSc) is a chronic autoimmune connective tissue disease with multiple organ involvement and heterogenous clinical manifestations. It is characterized by the presence of high levels of autoantibodies in the serum. Previous studies have shown, that in addition to autoantibodies directed against nuclear antigens, SSc patients also exhibit autoantibodies targeted to cell surface receptors which not only serve as biomarkers but also play a functional role in disease pathogenesis.

Therefore, in this study we aim to identify novel functional autoantibodies contributing to the development of the disease. For that purpose, we collected serum samples from SSc patients (n=30) and age- as well as gender-matched healthy controls (n=37). First, we determined the binding of IgG and IgM autoantibodies to the surface of different cell lines using flow cytometry. Both IgG and IgM autoantibodies against HEK293 cells could be detected in human sera which were both significantly elevated in SSc patients compared with healthy controls.

Interestingly, autoantibodies against HEK293 were associated with the two major SSc subsets, diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc). While dcSSc patients show significantly higher levels of IgG autoantibodies than patients with lcSSc, the latter show significantly higher levels of IgM autoantibodies, suggesting that they likely contribute to the development of the two distinct subtypes of the disease. Although several functional autoantibodies have been reported in SSc before, none of them are specifically associated with dcSSc or lcSSc.

In the next steps, we will investigate the role of the autoantibodies against HEK293 cells in the development of these two subsets of SSc and identify the autoantigen.

IgA Autoantibodies in Systemic Sclerosis Patients

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Background

Systemic Sclerosis (SSc) is an autoimmune disease characterized by excessive fibrosis and vasculopathy. It usually affects the skin and, variably, other organs. Interstitial lung disease (ILD) is the most important cause of mortality.

Different IgG autoantibodies (Abs) against nuclear proteins are present in >95% of patients. Anti-topoisomerase I (ATA) Abs, are associated with diffuse-cutaneous (dcSSc) phenotype and ILD; anti-CENP-B (ACA) Abs, with limited-cutaneous (lcSSc) involvement and lower risk of ILD, and anti-RNA polymerase III (RP3) Abs, with an aggressive diffuse skin involvement, and scleroderma renal crisis. These three Abs are included in the 2013 SSc-ACR/EULAR classification criteria. The detection of additional Abs, like Ro52 among others, is of interest when those are negative. Currently, the study of IgA isotype is limited to a few Abs but we wonder whether it could play a role in SSc and ILD.

Objective

The aim of this study was to analyse the prevalence of IgG and IgA autoantibodies and its associations with clinical manifestations in a SSc cohort of Hospital Clínic de Barcelona.

Methods

Ninety-seven patients, including 56.7% lcSSc, 15.5% dcSSc and 27.8% sine-scleroderma phenotype (sine-SSc), were tested for IgG and IgA isotypes of ATA, ACA, RP3 and Ro52 Abs using the EliATM platform (Thermo Fisher Scientific). Manufacturer reference values for IgG (7 U/mL) were used and 50 healthy individuals were tested to establish the IgA isotype cut-off value that was set at 1.65 μg/L.

Results

Prevalence of isolated IgG Abs was 3/97 (3.1%), 17/97 (17.5%), 1/97 (1.0%) and 7/97 (7.2%) for ATA, ACA, RP3 and Ro52, respectively. Moreover, double positive prevalence, IgG+IgA for the same specificity, was 19/97 (19.6%), 30/97 (30.9%), 5/97 (5.2%) and 7/97 (7.2%) for ATA, ACA, RP3 and Ro52, respectively. There were only 2 patients with isolated IgA isotype for SSc criteria Abs (1 for ATA; 1 for ACA). Additionally, 24/97 (24.7%) patients were seronegative for SSc criteria Abs.

Regarding clinical manifestations, ACA IgG+IgA was associated with lcSSc, ACA IgG with sine-SSc, ATA-IgG with dcSSc and Ro52 IgG+IgA with sine-SSc. Moreover, ILD was associated with ATA IgG and ATA IgG+IgA. Telangiectasias were associated with ACA IgG+IgA and arthritis with ACA-IgA. We did not find associations with RP3.

Conclusions

This study is one of the first evaluations of IgA isotype against ATA, ACA, RP3 and Ro52. Many of the SSc patients are actually double positive IgG+IgA for the same specificity except for Ro52.

Interestingly, the addition of ACA IgA isotype to the presence of ACA IgG, is associated with a distinct cutaneous phenotype (ACA IgG with sine and ACA IgG+IgA with lcSSc). Ro52 IgG+IgA positivity was also associated with sine-SSc but it was independent from ACA.

More prospective studies are necessary to explore the role of IgA isotype in the diagnosis and pathogenesis of SSc.

Assessment of systemic sclerosis markers utilizing a particle-based multi-analyte technology in an Italian cohort for the very early diagnosis of systemic sclerosis (VEDOSS)

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Introduction

Systemic sclerosis (SSc) is a severe autoimmune disease characterized by multiple-organ involvement, unpredictable clinical course, and poor prognosis1. It has a wide range of manifestations with cutaneous, pulmonary, cardiac, renal and gastrointestinal involvement. SSc is challenging to diagnose in the early phase due to low sensitivity of the classification criteria in patients without skin involvement, however, the presence of Raynaud's phenomenon (RP) and antinuclear antibodies (ANA) can be predictors for early treatment intervention and potentially avoiding irreversible organ damage2. More than 95% of patients with SSc present autoantibodies (Abs) against different nuclear proteins of which three have been included in the classification criteria: anti-topoisomerase I (ATA, Scl-70), anti-centromere (ACA), and anti-RNA-Pol III (ARA). Additionally, other specific but less frequent Abs are anti-Th/To (e.g., Rpp25/38) or anti-fibrillarin, as well as Abs that may be present but are not specific to SSc only. New biomarkers are needed to close the seronegative gap and to enable patient stratification. The goal of this study was to evaluate a novel particle-based multi-analyte technology (PMAT) for the detection of autoantibodies in an Italian cohort of SSc patients part of the Very Early Diagnosis of Systemic Sclerosis (VEDOSS) initiative, as well as investigating the correlation of markers to the clinical manifestations of the disease.

Methods

A cohort of 188 patient samples collected at University Modena (Italy) comprising of early SSc patients (126 limited cutaneous lcSSc, 45 diffuse cutaneous dcSSc, 6 VEDOSS, 7 RP, 1 sine scleroderma, and 3 unconfirmed diagnosis) were tested by three PMAT panels: (1) CTD (connective tissue disease) Essential, (2) CTD Comprehensive and, (3) Myopathy (research use only, Inova Diagnostics, San Diego, USA). SSc-associated Abs included in these panels were directed against Scl-70, centromere, Ku, RNA-Pol III, Th/To subunit Rpp25, Th/To subunit Rpp38, PM/Scl, BICD2, and Fibrillarin. Analysis of all markers in the panels was conducted and p values less than 0.05 were considered significant.

Results

As expected, for SSc patients, ACA and ATA were the most sensitive markers (41.5% and 34.0%, respectively) where ACA occurred more frequently in lcSSc (56.3% vs. 13.3%, $p<0.0001$) and ATA occurred more frequently in dcSSc (71.1% vs.23.0%, $p<0.0001$). ARA had a sensitivity of 4.8% (11.1% in dcSSc and 3.2% in lcSSc, $p=0.0549$). For the non-criteria Abs, the prevalence was as follows: Fibrillarin (5.9%), PM/Scl (5.3%), Th/To Rpp25/38 (5.3%), Ku (3.7%), and BICD2 (13.3%). In addition, 45/188 (23.9%) of the overall cohort were negative for the criteria Abs and 12/45 (26.7%) were positive for at least one of the non-criteria SSc markers (Th/To, PM/Scl, Ku, BICD-2, and Fibrillarin). When looking at lab result findings compared to PMAT, the SSc markers typically associated with nucleolar pattern by HEp-2 IFA (Th/To, Fibrillarin, PM/Scl, RNA Pol III) were primarily found in patient samples with nucleolar pattern (53.8%, $p=0.0039$). All patients (64/64) that are ACA positive by PMAT were found for 100.0% of the patient samples identified as centromere pattern by HEp-2 IFA. For the correlation with clinical parameters, most notably there were several antibodies significantly associated with manifestations. ATA levels and prevalence were significantly higher in patients with interstitial lung disease (ILD) ($p<0.0001$).

Conclusions

In addition to the expected prevalence of SSc classification criteria antibodies, non-criteria markers reduced the serological gap significantly. Additional markers included in the diagnostic workup of patients especially for the VEDOSS population may be beneficial for early intervention. Additional studies assessing autoantibody levels and involving more patients are warranted to evaluate novel biomarkers.

Performance of a novel, fully automated planar microarray immunoassay for the detection of autoantibodies against Centromere Protein B and correlations with autoimmune disease diagnosis

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Background

Autoantibody presence is a hallmark in autoimmune diseases (AID). Its testing, including against centromere proteins (CENP) is important in the identification of these conditions; however, some current test methods may be manual, time-consuming, and/or fragmented. Development of highly automated sensitive and specific diagnostic tools is needed.

Objective

Evaluate the performance of the novel, fully automated planar microarray immunoassay, MosaiQ CENP-B (CENP-B-MA), designed for use with the MosaiQ System, for qualitative detection of anti-CENP-B autoantibodies (ACA-B) and to describe the clinical diagnosis of ACA-B reactive samples.

Methods

A comparator study was conducted at Hôpital Pitié-Salpêtrière (Paris, France) using anonymized serum samples, characterized as ACA-B non-reactive or reactive with a composite of CE-marked devices. Chart review was performed to correlate CENP-B-MA results with clinical diagnosis.

Results

99 ACA-B reactive samples (median age 57 years, range 8–88 years, 94 females) and 199 non-reactive samples were included in the analysis. CENP-B-MA correctly identified 96/99 samples characterized as CENP-B reactive and all 199 non-reactive samples: positive percent agreement 97% (95% CI, 91.4% – 99.4%), negative percent agreement 100% (95% CI, 98.2% – 100%), overall percent agreement 99% (95% CI, 97.1% – 99.8%). Clinical diagnosis was available for all 99 ACA-B reactive samples. 37 (37.37%, 35 female) were directly related to systemic sclerosis; 5 (5.05%, all female) had Raynaud's phenomenon/disease. Sjögren's syndrome was recorded in 11 (11.11%, 10 female) samples; 7 (7.07%, all female) had mixed connective tissue disease; 7 (7.07%, all female) had lupus; and 6 (6.06%, all female) idiopathic inflammatory myopathies. Diagnosis in the remaining 26 (26.26%) ACA-B reactive samples varied from other AID to cancer. The centromere ANA-immunofluorescence pattern was the most frequent, observed in 92 (92.93%) of ACA-B reactive samples.

Conclusions

CENP-B-MA showed high performance concordance with other CE-marked assays for detecting ACA-B. These autoantibodies were associated with a range of AID and other conditions. Additionally, the microarray can be printed with multiple other antigens, allowing simultaneous detection of various autoantibodies on one microarray, which may help to reduce time to diagnosis by providing comprehensive results in a single test. The fully automated, continuous random access, high-throughput MosaiQ system has the potential to improve AID testing, increasing laboratory efficiency and productivity.

Autoantibodies in Idiopathic Inflammatory Myopathies

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Abstract

The discovery of novel autoantibodies and other proteomic biomarkers in idiopathic inflammatory myopathies (IIM) continues to expand thereby decreasing the seronegative gap while increasing the diagnostic, prognostic, and predictive value of serology testing. Newer autoantibodies of note include those directed against Cytoplasmic Cysteinyl-tRNA-Synthetase (CARS1/anti-Ly), Varyl-tRNA Synthetase (VARS), Survival of Motor Neuron (SMN) Complex, Cell division Cycle and Apoptosis Regulator 1 (CCAR1), transcription factor Sp4, cortactin and unique anti-mitochondrial antibodies (reviewed in [1]). Antibodies to CARS1 and Sp4 are of particular interest because they are reported to be *negatively* associated with contemporaneous cancer (reviewed in [1]). In addition, there is emerging evidence that anti-SMN antibodies are associated with a poorer prognosis. Elucidation of older and descriptions of newer IIM autoantibody markers provides increasing clinical value by revealing novel clinical features including IIM subsets and overlap syndromes (e.g., scleromyositis) [2, 3]. It is important to understand the gaps and pitfalls in using contemporaneous immunoassays (i.e., ELISA, line immunoassays/dot blots, immunoprecipitation) to detect IIM autoantibodies. The widening spectrum of autoantibodies In IIM has increased the value of multi-analyte diagnostic testing. An appreciation of these limitations and opportunities has prompted the development of promising particle based solid phase multianalyte technology (PMAT) [1, 4, 5]. There continues to be interest in sporadic inclusion body myositis (sIBM) with description of biomarkers and appreciation of its occasional overlap with Sjögren syndrome (reviewed in [1]).

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A comparison of methods for the detection of anti-cN1A autoantibodies in patient sera

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Abstract

The discovery of serum autoantibodies targeting a 44 kDa skeletal muscle protein in the serum of many sporadic inclusion body myositis (IBM) patients has aroused new interest in the role of autoimmunity in the pathogenesis of IBM. The identification of the target autoantigen for these autoantibodies as the cytosolic 5'-nucleotidase 1A (cN1A, also indicated with cN-1A, cN-IA, NT5C1A and Mup44) facilitated the development of various tests to detect these autoantibodies [1]. ELISA studies consistently demonstrated that anti-cN1A autoantibodies are more prevalent in IBM compared with other forms of myositis, other neuromuscular disorders and most other autoimmune diseases. In light of these findings, anti-cN1A autoantibodies provide utility in distinguishing IBM from other myopathies [2], an important distinction when determining therapy regimes, considering that other inflammatory myopathies, but not IBM, are typically responsive to immunosuppressive medication.

Methods that have been applied to analyse the presence of anti-cN1A autoantibodies in patient sera include (i) immunoblotting with lysates from human skeletal muscle tissue or lysates from transfected HEK293 cells expressing cN1A, and a line blot with bacterially expressed recombinant cN1A, (ii) immunoprecipitation of in vitro translated recombinant cN1A, (iii) ELISA with synthetic cN1A-derived peptides or with recombinant cN1A expressed in bacterial or eukaryotic cells, (iv) addressable laser bead-based assays with recombinant cN1A or fragments thereof, and (v) immunofluorescence with transfected, cN1A-expressing COS and HEK293 cells. Most of these are non-standardized tests developed in research laboratories. The only standardized commercial anti-cN1A tests that are currently available are the 'ELISA cN-1A (Mup44, NT5C1A)', the 'EUROLINE Autoimmune Inflammatory Myopathies 16 Ag et cN-1A', and the 'EUROLINE Profile Autoimmune Inflammatory Myopathies 20 Ag (IgG)' tests, all developed by Euroimmun [3].

Although the available tests for anti-cN1A detection in serum samples have not been systematically compared, a number of observations indicate that discordant results can be obtained with different test formats, with full-length cN1A expressed in different systems, and

with different parts of cN1A. IBM sera display heterogeneity in the reactivity with full-length cN1A in different assays and in the recognition of different linear epitopes. At least in part this will be due to the denaturation/renaturation status of full-length cN1A and differences in the accessibility of distinct epitopes. Also, the influence of differences in the isotype specificity of secondary antibodies is not clear yet and needs to be explored in more detail.

Irrespective of the current lack of a gold standard for anti-cN1A testing and although anti-cN1A autoantibodies are rare in other forms of myositis and in other rheumatic and neuromuscular diseases, it has been demonstrated that they are not specific for IBM. For example, in several studies anti-cN1A autoantibodies have been detected in patients with Sjögren's syndrome and systemic lupus erythematosus [2, 4].

Although the availability of the standardized ELISA and Lineblot tests (Euroimmun) has made an important contribution to anti-cN1A detection in clinical practice, the relatively large variation in the results obtained in different assays for anti-cN1A autoantibody detection suggests that there is room for further improvement. To establish an international gold standard for anti-cN1A autoantibody testing a collaborative effort of multiple researchers is required, in which not only the same samples are tested in various assays, but in which also the only commercially available standardized ELISA test is used as a reference test.

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Anti-Mi2 assays - Are they interchangeable?

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Introduction

Anti-Mi2 are specific markers of dermatomyositis (DM). Several commercial assays allow its detection.

Aim

We aimed to compare the main anti-Mi2 commercial assays available.

Methods

Part A.

We retrospectively reviewed anti-Mi2 ELiA consecutive positive samples (Thermo. Positive >7 U/mL) from 01/2013 to 09/2022.

ELiA results were compared to two immunoblots: Immunoblot-Euroimmun® (BlotA) (MYO4G-automatic reading by EuroblotOne) and immunoblot-Dtek® (BlotB) (PMS12D-24, subjective interpretation).

Immunofluorescence patterns on Hep2 (ICAP nomenclature), other autoantibodies associated to Systemic autoimmune diseases (SARD) and clinical records were reviewed.

Part B.

BlotA specificity was tested on a further analysis: samples routinely tested and positive for either anti-Mi2 alfa and/or beta by BlotA from 01/2020 to 05/2022 were retrospectively analyzed.

Results

Part A results:

Out of 2020 samples analyzed by ELiA, 5.6% were positive [114 samples/46 patients; 76% woman, mean age=43 years (5-88). Mean anti-Mi2 ELiA levels=219.6 U/mL (7.4-3520). 46% patients had at least other autoantibody (50% anti-CENPB).

41/46 samples were tested simultaneously by at least one immunoblot.

In 30 samples both anti-Mi2 blot A and B were tested. 24/30 were concordant: 19 positives (13/19 α+β in BlotA) and 5 negatives. In 6/30 a discordant result was observed: 3/5 BlotA+/BlotB- (mean anti-Mi2 levels=28 U/mL) and 3/5 BlotA-/BlotB+ (mean anti-Mi2 levels=59 U/mL).

DM diagnosis gathered among BlotA+/BlotB+ and BlotA-/BlotB+ (Table1).

Table 1. Clinical data of anti-Mi2 ELiA positive processed by both immunoblots

CLINICAL DATA	DM	OTHER SARD
Blot A+/Blot B+	5/19	4/5	0/5
Blot A-/Blot B-	1/5	0/1	0/1
Blot A+/Blot B-	1/3	0/1	0/1
Blot A-/**Blot B+**	2/3	1/2	0/2

For those in which only one blot was studied: BlotB Negative were not DM and had lower anti-Mi2 levels (p=0.0325) compared to BlotB positive (Table2).

For those processed exclusively by BlotA, BlotA negative results included DM patients with anti-Mi2 ELiA at high levels (Table2).

Table 2. Clinical data of anti-Mi2 ELiA positive processed by either blotA or blotB

	CLINICAL DATA	DM	Anti-Mi2 levels (ELiA, mean U/mL)	OTHER SARD
Blot A+	3/8	2/3	199	1/3
Blot A-	4/5	2/4	136	1/4
Blot B+	7/9	3/7	133	2/7
Blot B-	6/9	0/6	31	3/6

BlotA positive samples were frequently isolated profiles for either alfa or beta (4/8 beta, 1/8 alfa).

Part B results:

1259 samples routinely tested by BlotA. 93% were negative for anti-Mi2 alfa and anti-Mi2 beta.

Among the 88 positive samples, the most frequent profile was isolated positivity for anti-Mi2 beta (75%), at weak intensity (+, 68%). In this profile we did not find DM patients (without other DM-specific antibody) and this anti-Mi2 beta was anti-Mi2 ELiA negative in 95% samples.

Conclusions

In our cohort, anti-Mi2 in DM usually appears isolated, at high levels by ELiA and positive by both BlotA (alfa+beta+) and BlotB.

We describe limited sensitivity and specificity for BlotA, with profile alfa+beta+ being the most clinically specific.

We describe a cohort of anti-Mi2 at low levels by ELiA, associated to other autoantibodies and without DM.

Anti-Th/To: Clues for facing puzzling results

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Introduction

Anti-Th/To are markers of Systemic Sclerosis (SSc) with pulmonary involvement. Discrepancies between commercially available assays have been described, mainly related to the different epitopes [hPop (Euroimmun) and Rpp25 (DTEK)].

Aim

We aim to analyze our anti-ThTo cohort, describing its clinical association, as well as its behaviour in the two commercial assays.

Methods

We retrospectively reviewed anti-ThTo positive patients (described as positivity by at least one commercial assay -Euroimmun and/or Dtek-) from January/2020 to April/2022. Dtek (SCL10D24) results were subjectively interpreted, Euroimmun line-immunoassays (EurolineSSc) were automatically read by EuroblotOne.

Clinical records, IFA pattern (ICAP nomenclature) and positivity for other autoantibodies (subclassified as: SSc criteria, SSc non-criteria and related to other systemic autoimmune diseases) were recorded.

Results

We obtained 71 anti-ThTo positive samples by at least one assay (65 patients). Mean age 57 years (range 21-86), 66% women. 27 samples with anti-ThTo results ≥++ by at least one immunoassay.

In 34/65 patients both assays were tested simultaneously. 76% were discordant (Table 1).

Table 1. Comparisson of anti-Th/To positive samples according to intensity.

		DTEK				
		NA	NEG	+	++	+++
Euroimmun	NA					
	NEG			3	2	7
	+	25	10			2
	++	4	2		1	
	+++	2	2			5

Regarding association to other SSc autoantibodies, coexistence of other SSc non-criteria autoantibodies was the most frequent finding (18/28). When anti-ThTo was associated, it was frequently at low titres among all groups (SSc criteria, SSc non-criteria and other: 9/10, 12/18 and 4/4, respectively).

69% Isolated anti-ThTo were AC-8 IFA pattern, 36% among associated anti-ThTo.

Clinical records were available in 38/65 patients and 15/38 were diagnosed of SSc. 60% SSc were monospecific for anti-ThTo, without other SSc markers, of which 89% were discordant between both commercial assays (Table2).

Table 2. Comparisson of anti-Th/To positivity among SSc without other SSc markers.
*Orange highlights anti-ThTo with AC-8 pattern.
**Patient with anti-ThTo+anti-Ro and AC-1 pattern.

		DTEK				
		NA	NEG	+	++	+++
Euroimmun	NA					
	NEG			1*	1*	3* 1**
	+	1				
	++		1*			
	+++					1*

Conclusions

Positivity for both hPop and Rpp25 are rare. In our cohort we find SSc among both groups, highlighting the importance of searching for anti-ThTo by both commercial immunoassays.

Anti-Mi2 and anti-CENPB, An odd finding?

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Abstract

In a previous study performed in a cohort of 46 patients with anti-Mi2 by ELiA® (>7 U/mL-Thermo), we found that 22% of patients also had anti-CENPB (>0.8 AI Bioplex2200®-BioRad). Mean levels in this cohort for anti-Mi2 and anti-CENPB were 47±46 U/mL and 3.1±1.8 AI, respectively.

Considering this preliminary finding, we undertook a prospective active search for the presence of anti-Mi2 by EliA® in patients with anti-CENPB values between 0.5 and 7.9 AI (Bioplex220®) from June/2022 to January/2023.

In patients that resulted positive for anti-Mi2, it was further studied using two immunoblots (Euroimmun® and Dtek®). The immunofluorescence pattern in HEp2, other autoantibodies associated with Systemic Autoimmune Rheumatic Diseases (SARD), and clinical data were recorded.

We found concomitant presence of anti-Mi2 and anti-CENPB in 18 patients (17 females). Table 1. Mean age was 30 years [5-74], and 44% were under 16 years old. This finding was confirmed in at least one follow-up sample in 5 patients, with a mean follow-up time of 68 months [23-164]. Additionally, other antibodies were detected in 13 patients (2/13 anti-Ro52). Clinical information was available for 15 patients, with 2 having a diagnosis of SARD and 5 of Celiac disease.

Mean anti-Mi2 EliA® levels were 55±40 U/mL, and at least one immunoblot confirmed the positivity in 14 out of 18 patients. Mean values for anti-CENPB were 2.7±1.6 AI. Anti-CENPB was confirmed by another technique in 16 patients [2/15 by immunoblot (Euroimmun/Dtek), 9/15 by ELiA->7 U/mL, and 5/15 by both].

Regarding Hep2 indirect immunofluorescence, 6/18 patients showed a mixed AC-3+AC-4 pattern, 2/18 AC-3+Other AC, 8/18 AC-4±Other AC, and 2/18 no AC-3/AC-4.

We describe a cohort with an atypical association of antibodies that is related to a low prevalence of SARD diagnosis. The high percentage of paediatric population, patients with Celiac disease, and

association with other antibodies in this series are noteworthy. Further studies are needed to determine the implications of this finding.

Table 1. Anti-Mi2+ anti-CENPB cohort.
– not available, POS: positive, NEG: negative, *Patients diagnosed with Celiac disease

Patient	Age	Mi2Ac ELiA (U/mL)	Blot Mi2Ac	CENPB Bp (AI)	CENPB ELiA (U/mL)	Blot CENPB	ICAP	Other antibodies	Specificity	SARD
1*	16	55	POS	6,3	116	-	AC-1+AC-3	NO	-	NO
2*	35	34	NEG	0,9	6,7	POS	AC-4+AC-XX	YES	DFS70	NO
3	16	102	POS	4,2	-	-	AC-4+AC-3	YES	PM-Scl	NO
4	14	49	POS	1,1	7,8	-	AC-4 + AC-7	YES	PML	NO
5*	52	35	POS	3,7	18	POS	AC-1	YES	CN1A	NO
6	16	11	POS	2,7	11	POS	AC-1	YES	CN1A	NO
7	71	34	POS	1,6	6,2	POS	AC-4	YES	PM-Scl; Th/To	SSc
8*	52	118	POS	0,8	3,7	INDETERMINATE	AC-4	YES	NXP2; DFS70	SLE
9*	12	33	POS	1,7	31	POS	AC-4	YES	DFS70	NO
10	5	150	POS	4,4	121	POS	AC-1+AC-3	YES	DFS70; U1RNP	NO
11	74	12	NEG	2,1	12	-	AC-4+AC-3	NO	-	NO
12	9	23	NEG	5,2	68	-	AC-4+AC-3	YES	Scl-70	-
13	51	89	POS	4,4	40	-	AC-4+AC-3	NO	-	NO
14	31	42	POS	0,9	5,1	POS	AC-4+AC-3	YES	Ro52KDa	NO
15	24	27	POS	0,9	ND	POS	AC-4+AC-6	YES	RNA-Polimerase III	-
16	38	82	-	3,9	43	-	AC-4	NO	-	NO
17	22	21	POS	1,2	14	-	AC-4+AC-3	YES	CN1A	NO
18	6	25	POS	4,5	27	-	AC-4	NO	-	NO

Adjustment of the band intensity value for myositis specific and associated autoantibodies using line-blot techniques

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Introduction

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of systemic autoimmune disorders in which muscle weakness is usually the classical clinical manifestation. Other organs can be affected, including the skin, joints, lungs, heart, and the gastrointestinal tract. Different myositis-specific and associated autoantibodies (MSA and MAA, respectively) have been identified. On the basis of clinical, histopathological, and serological features, IIM can be classified into several subgroups: dermatomyositis (DM) (including amyopathic dermatomyositis), antisynthetase syndrome (ASS), immune-mediated necrotizing myopathy (IMNM), inclusion body myositis, polymyositis (PM), and overlap myositis.

Detection of MSA and MAA was originally restricted to research laboratories using immunoprecipitation (IP) techniques, but nowadays, several commercial assays have become available, enabling widespread introduction of these assays in clinical laboratories. Currently, line-blot assays (LBA) are widely used because they represent a faster and semi-quantitative option to detect autoantibodies in a single run. However, problems with sensitivity and specificity were detected depending on the antigen. A recent study postulated that the diagnostic performance of Euroline LBA could be improved using an adjusted band intensity (BI) of each antigen in relation to the intensity of the positive control band (PCB) included in each strip (1).

Aim

This study aimed to analyze whether applying adjusted band intensity can improve the specificity of LBA.

Patients and Methods

From January 2022 to April 2023, 1001 serum samples from Hospital Clínic de Barcelona were requested to study MSA and MAA.

All sera were tested in a commercial LBA (EUROLINE Myositis Antigen Profile 3 IgG, Autoimmune Inflammatory Myopathies 16 Ag IgG and Autoimmune Inflammatory Myopathies 16 Ag et cN-1A IgG; EUROIMMUN, Lübeck, Germany). The band intensity of each antigen was scanned using EUROLineScan (EUROIMMUN, Lübeck, Germany). The PCB, composed of anti-human IgG, served as an indicator of correct colorimetric reaction on the strips. The PCB-adjusted BI (aBI) was calculated as: (BI of each MSA or MAA)/(BI of the corresponding PCB) × 100. The cutoff value for the aBI was set at 20 instead of 15 (cutoff value recommended by the manufacturer).

Results

From the 1001 samples received, 325/1001 (32.5%) had, at least, one positive result according to BI and cutoff value >15.

In relation to MSA, prevalence of each antibody was: 8.0% for anti-Jo1, 2.2% for anti-EJ, 0.9% for anti-OJ, 15.1% for anti-PL7, 3.1% for anti-PL12, 6.6% for anti-MDA5, 8.3% for anti-TIF1γ, 3.4% for anti-NXP2, 4.8% for anti-SAE1, 5.7% for anti-Mi2, 6.2% for anti-Mi2α, 7.2% for anti-Mi2β, and 4.3% for anti-SRP. Regarding MAA, prevalence was: 33.5% for anti-Ro52, 13.9% for anti-PM/Scl75, 12.9% for anti-PM/Scl100, and 6.5% for anti-Ku.

After calculating the aBI, 174/325 (53.5%) samples remain positive. These 174 samples account for 158 different patients. The new prevalence of each antibody was: 4.9% for anti-Jo1, 1.2% for anti-EJ, 0.3% for anti-OJ, 4.6% for anti-PL7, 0.6% for anti-PL12, 2.1% for anti-MDA5, 3.8% for anti-TIF1γ, 1.4% for anti-NXP2, 0.4% for anti-SAE1, 0.0% for anti-Mi2, 2.4% for anti-Mi2α, 2.4% for anti-Mi2β, and 2.2% for anti-SRP, in case of MSA and 25.2% for anti-Ro52, 5.9% for anti-PM/Scl75, 3.4% for anti-PM/Scl100, and 4.3% for anti-Ku, in case of MAA.

With the use of aBI, >40% of results became negative, and the most affected antigens were OJ, PL7, PL12, MDA5, NXP2, SAE1, Mi2, and PM/Scl100.

Of the 158 patients with a positive aBI result, 16 (10.1%) were excluded because, with the data collected in the medical history, the diagnosis of IIM could not be confirmed or ruled out. From the rest, 59/142 (41.6%) had an IIM diagnosis being 22/59 (37.3%) DM, 19/59 (32.2%) ASS, 4/59 (6.8%) MDA5 associated DM, 4/59 (6.8%) SRP associated MNIM, and 10/59 (17.0%) other form of IIM.

Conclusion

The application of the adjustment in relation to the intensity of the positive control band (aBI) allows the elimination of weakly positive results with low specificity in an objective and reproducible manner. The positive predictive value of the aBI was 41.6%. A more in-depth review of this data is necessary to calculate the sensitivity and specificity of this LBA and to determine optimal cutoff values for distinct autoantibodies. More studies are necessary to compare the test characteristics between LBA and other methods based on the same cohort of patients and controls.

References

(1) Chang SH, Choi SR, Choi YS, et al. Performance of cut-offs adjusted with positive control band intensity in line-blot assays for myositis-specific antibodies. Rheumatol Int. 2023 Aug;43(8):1507-1513. doi: 10.1007/s00296-023-05287-5. Epub 2023 Feb 21. PMID: 36809556.

6 Serology of Organ Specific Autoimmunity – Update and new Autoantibodies

6.1 Autoantibody Associated Neurological Diseases

The antibody-mediated encephalitis – From discovery to new clinical insights and mechanisms

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Abstract

Investigations over the last 18 years have revealed that many neurologic and psychiatric disorders are due to antibody-mediated mechanisms against neuronal proteins and neurotransmitter receptors. The discovery of these remarkable disorders has changed the landscape of how physicians approach the diagnosis and treatment of these patients. Indeed, cases of rapidly progressive memory loss, psychosis, seizures, abnormal movements or impaired level of consciousness previously considered idiopathic are now known to be mediated by antibodies and curable with immunotherapy. In my presentation I will show how the most frequent of these diseases, anti-NMDA receptor encephalitis, was discovered and what we have learned since, including the main clinical manifestations and triggers of the disease. On a more basic level, I will describe the underlying pathogenic mechanisms and show how an antibody can lead to memory deficits or psychosis through a reduction of the levels of NMDA receptors in neurons, resulting in changes in synaptic transmission and plasticity. The experience gained from these investigations has led us to discover 11 additional diseases mediated by antibodies against other brain receptors or proteins, each with specific patterns of symptoms and distinct mechanisms. Overall, these studies have resulted in novel treatment strategies that have improved patient outcomes and help us to understand other diseases in which the same receptors are affected by other mechanisms.

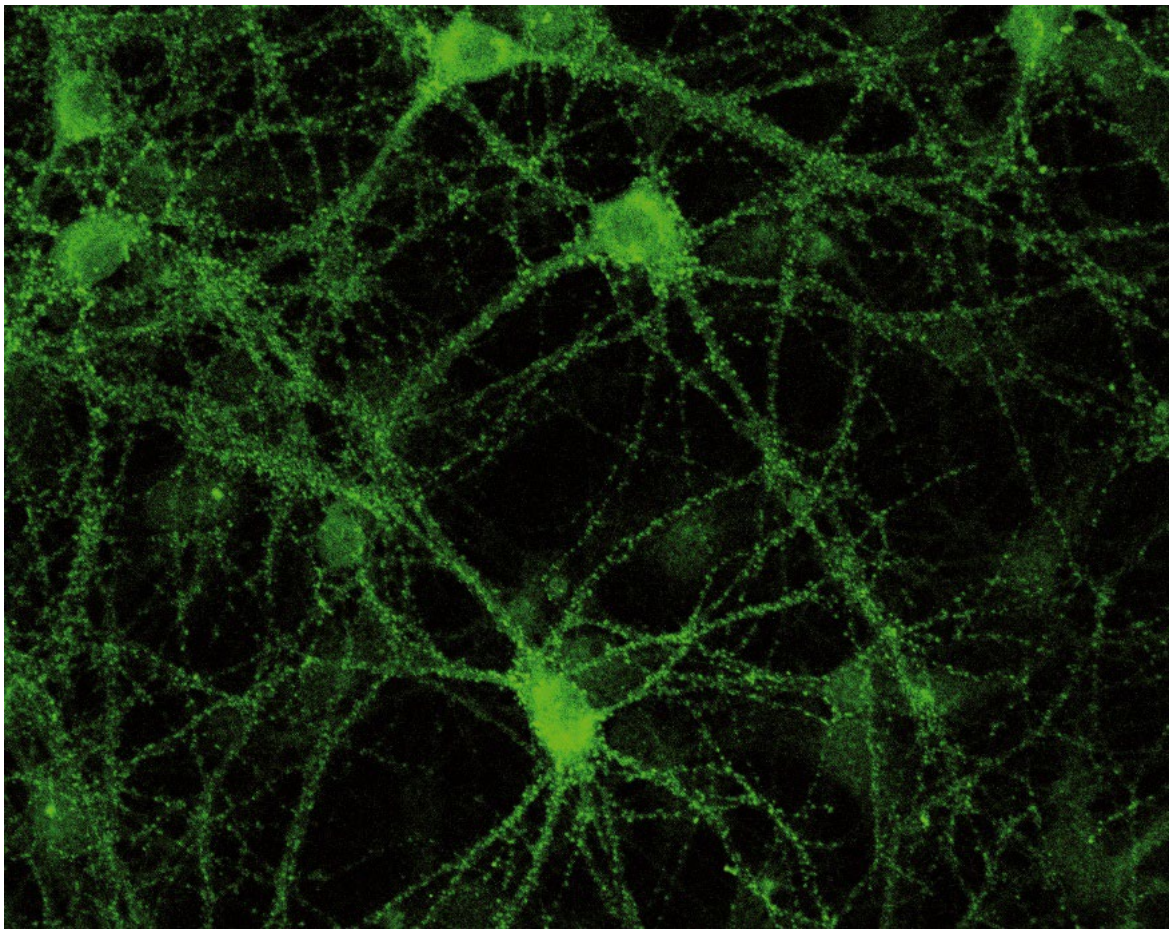

Figure 1: Cultured rat hippocampal neurons incubated with CSF from a patient with anti-NMDA receptor encephalitis, showing immunolabeling of NMDA receptors located on the cell surface.

The clinical impact of antibody testing in neurology

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Abstract

A plethora of novel antibodies directed against glial and neuronal antigens have been described in the last two decades. Routine testing of these antibodies has led to new diagnostic strategies and therapeutic approaches in the field of clinical neurology. Antibodies against aquaporin-4 have become biomarkers of the demyelinating disorder neuromyelitis optica (NMO), thereby clearly discriminating NMO from multiple sclerosis, with important implications on treatment decisions. In addition, antibodies against aqaporin-4 mark a subset of patients with systemic lupus erythematosus and Sjögren's syndrome suffering from myelitis and optic neuritis. In a subgroup of anti-aquaporin-4 negative NMO patients, antibodies against myelin oligodendrocyte glycoprotein (MOG) have become diagnostic markers. A hallmark has been the subsequent identification of antibodies against distinct receptors and synaptic proteins in patients with encephalitis, leading to a reclassification of neurological and psychiatric syndromes. Based on these classifications, national registries, e.g. the German Network for Research on Autoimmune Encephalitis, have been established. In collaborative projects, new insights have been gained into the interplay between infection, autoimmunity and neurodegeneration.

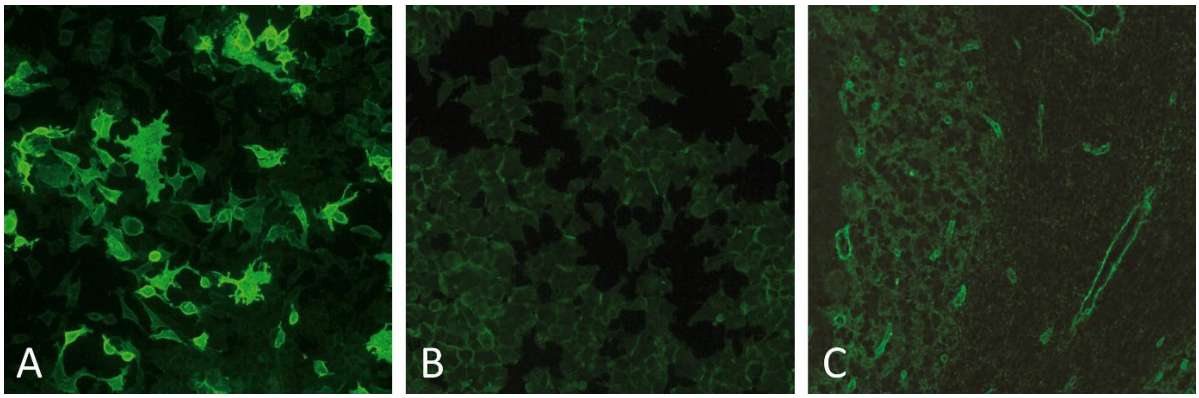

Figure 1: Autoantibodies against aquaporin-4 detected in serum from an NMO patient by indirect immunofluorescence testing on (A) aquaporin-4-transfected HEK cells, (B) control-transfected HEK cells and (C) cerebellum sections.

Paranodopathies: Antibody-mediated diseases of the peripheral nervous system

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Abstract

Paranodopathies are an evolving subgroup of acquired immune-mediated demyelinating syndromes. They are clinically similar to chronic inflammatory demyelinating neuropathy, although often more acute in onset and prone to relapse. They can also cause generalized polyradiculitis, including the cranial nerves. The target antigens are located on the Schwann cell and axonal plasma membrane and constitute an essential cell adhesion complex that is also found in the central nervous system. The autoantibodies are often not yet exclusively of the IgG4 isotype and cause demyelination and conduction blocks in animal models. This talk will focus on the clinical picture, pathophysiology and therapy of these rare but instructive autoimmunopathies.

Autoimmune encephalitis: Connecting clinical clues and immunological finger prints

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Abstract

Autoimmune encephalitis has evolved as a new entity over the last 15 years, including over 10 different antibody-mediated diseases. Despite the antibodies being pathogenic, the clinical phenotype and outcome can vary widely, and additional mechanisms have to play a role explaining this. Clinical characteristics, abnormalities in ancillary testing as well as relevant markers for specific immunological pathways or compartments are targets to explain the clinical phenotype and assist in predicting long-term outcome. This talk will focus on the progress made in predicting long-term outcome of anti-NMDAR encephalitis and defining the extent of clinical phenotype of anti-IgLON5 disease.

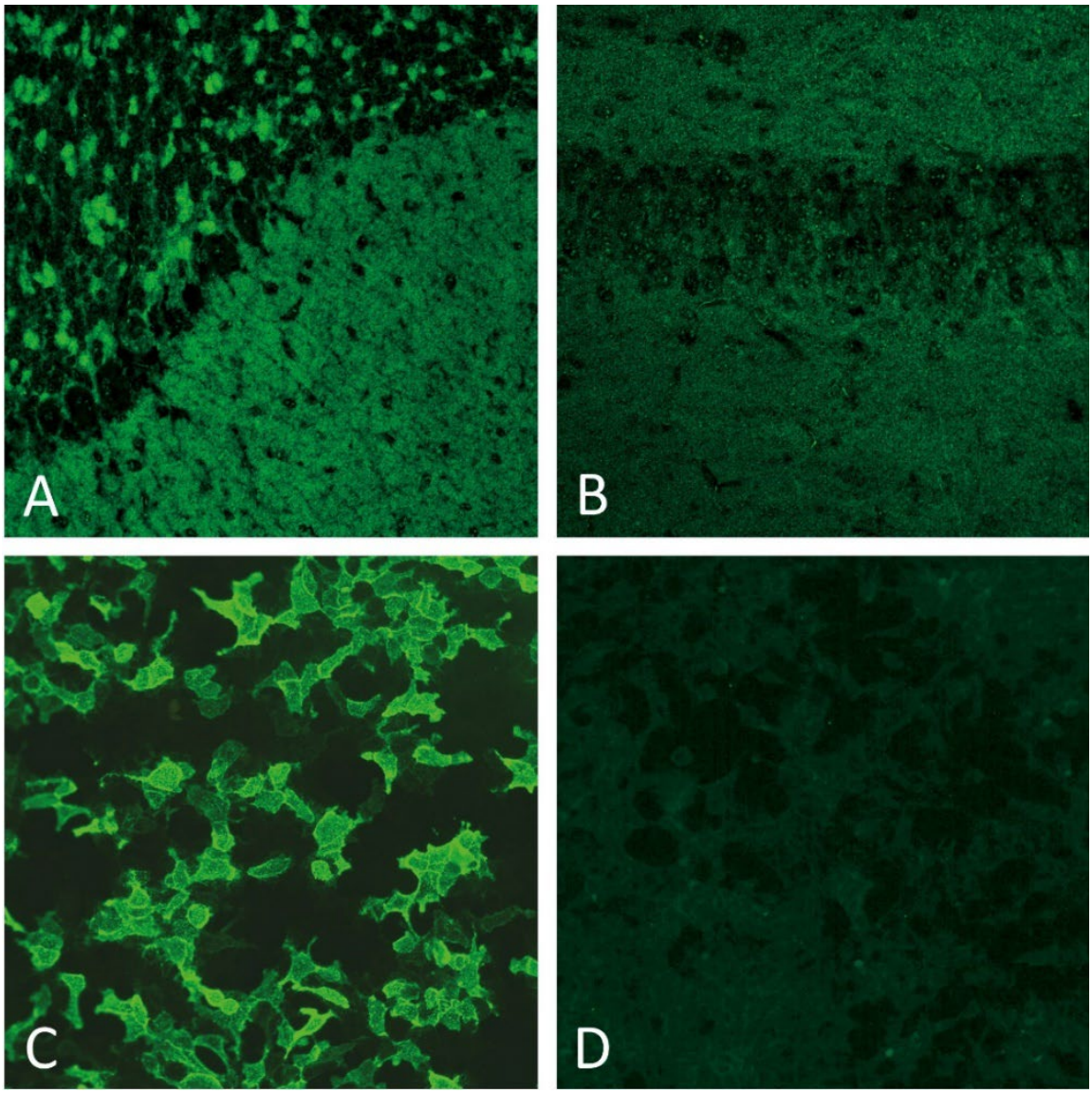

Figure 1: Anti-IgLON5 autoantibodies detected in patient serum by indirect immunofluorescence assay on (A) rat cerebellum, (B) rat hippocampus, (C) IgLON5- and (D) control-transfected HEK cells.

Discovery of autoantibodies in autoimmune neurological syndromes

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Introduction

Since the discovery of the first autoantigens in neurological autoimmune diseases, the number of identified autoantibody targets has continuously increased. Despite the growing number of discovered autoantigens, there is still a diagnostic gap when patient samples show autoantibody binding in a tissue-based immunofluorescence assay (IFA) but no reaction with known autoantigens in a recombinant cell-based IFA (CBA). Here, we compare three different antigen identification approaches and show examples of recently identified autoantigens.

Methods

Tissue IFAs and CBAs with known neuronal target antigens were used for sample characterization. Identification of the target antigen was achieved by immunoprecipitation (IP) and mass spectrometry analysis of 1) unique bands of IP eluates separated by SDS-PAGE or 2) whole IP eluate fractions and determination of relevant antigens by comparison of hit lists with control samples. In a third approach, a commercial Phage Display Immunoprecipitation Sequencing (PhIP-Seq) service was used.

Results

Using IP and mass spectrometry, we identified over 50 neuronal autoantibody targets over the last 10 years. Using the mass spectrometry approach with whole eluates and CBA-positive samples, 11/17 antigens were clearly identified as first hits, 4/17 antigens were identified as lower hits and 2/11 antigens were not detected. PhIP-Seq identified the target antigen of 3/6 CBA-positive samples. For 5/16 samples targeting unknown neuronal targets, the PhIP-Seq identification was confirmed using recombinant antigens, including the zinc finger and SCAN domain-containing protein 5B (ZSCAN5B).

Conclusion

The continuous development of biochemical tools for autoantigen identification offers great potential to unravel previously unknown biomarkers. For all the methods, a well-characterized group of samples is mandatory for the identification of the target antigen. Future challenges will include the implementation of raising numbers of known but often rare neuronal autoantibodies in the diagnostic workup.

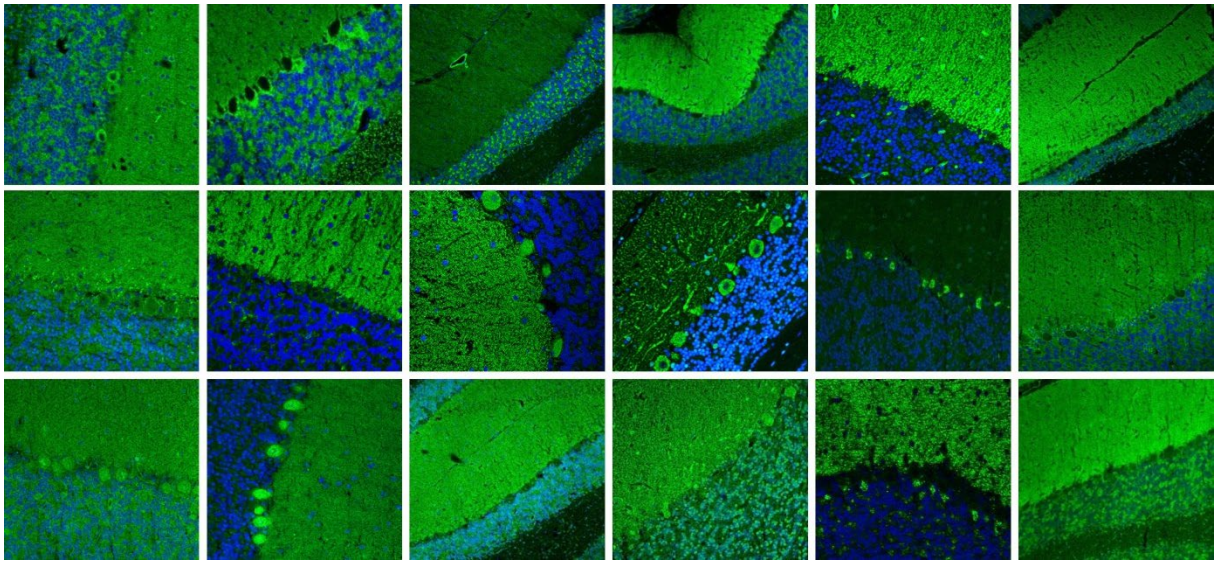

Figure 1: Immunofluorescence staining of rat cerebellum using serum samples from patients with idiopathic neurological syndromes. For all depicted fluorescence patterns, the autoantibody-targeted antigens have recently been identified.

Identification of neural autoantigens by combining indirect immunofluorescence of tissue sections and phage immunoprecipitation sequencing (PhIP-seq)

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Abstract

Neurological syndromes are frequently associated with autoantibodies to neural antigens. Identification of the underlying autoantigen target is critical for the development of specific immunoassays. Here, we combined an indirect immunofluorescence assay (IFA) using patient sera with whole human proteome phage immunoprecipitation sequencing (PhIP-Seq) for autoantigen identification. First, sera from patients suffering from neurological autoimmune syndrome and unknown autoantibody signatures were sorted into groups according to specific indirect immunofluorescence patterns on brain tissue cryosections (rat, monkey). These sera were then subjected to PhIP-Seq to identify the neural autoantigen underlying the indirect immunofluorescence pattern. Potential autoantigen hits were selected from the PhIP-Seq data if an antigen was present in sera of the same group but not in others. Autoantigens were confirmed by recombinant cell-based assays and by neutralizing the patients' autoantibodies with the corresponding recombinantly expressed antigens in a tissue-based immunofluorescence assay. Combination of those two approaches led to the identification of the transcription factor ZSCAN5 as the underlying autoantigen of a distinct immunofluorescence pattern only found in the granular layer of the cerebellum in monkey but not in rat. This result is consistent with the lack of a homolog of ZSCAN5 in rodents and underscores the need for human-based approaches to identify human autoantigens. The clinical relevance of ZSCAN5 autoantibodies in patients with neurological syndromes is currently under investigation.

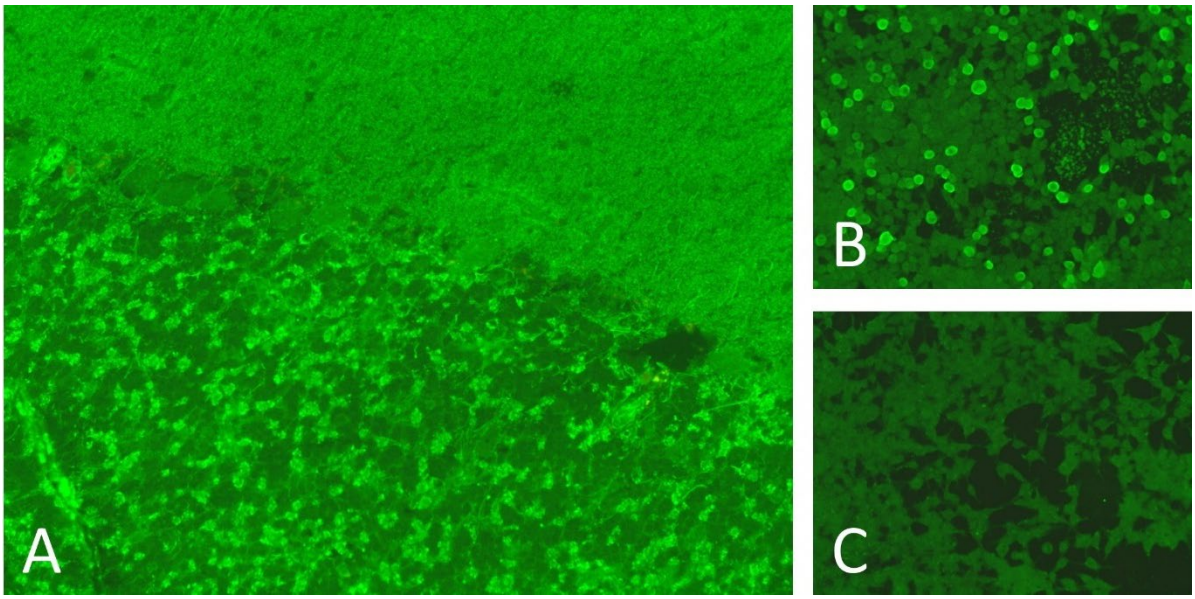

Figure 1: Detection of autoantibodies against ZSCAN5B by indirect immunofluorescence assay using (A) primate cerebellum, (B) ZSCAN5B- and (C) control-transfected HEK cells.

Autoantibodies in peripheral neuropathies – What are the news?

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Abstract

Autoantibodies play a crucial role in the diagnostics of various peripheral neuropathies, leading to a wide range of clinical manifestations. In this presentation, we aim to provide an overview of autoantibody-related diseases affecting the peripheral nervous system, focusing on recent developments in diagnosis and treatment. The presentation is structured around four major themes: Paraneoplastic neuropathies, diseases with autoantibodies against glycosylated epitopes, autoimmune nodopathies, and idiopathic sensory neuronopathies.

Paraneoplastic neuropathies involve the immune system's response to neuronal proteins expressed by tumors. The activated immune cells produce antibodies against these proteins, which can cross-react with neuronal tissues. Traditionally classified as onconeural intracellular antibodies, recent research has revealed a broader range of surface antibodies, even playing a potentially pathogenic role. Examples include VGKC-complex antibodies and anti-CASPR2.

Diseases with autoantibodies against glycosylated epitopes, particularly monoclonal gammopathies, can be associated with another class of peripheral neuropathies. Polyneuropathy associated with immunoglobulin M (IgM) monoclonal gammopathy is the most common paraproteinemic neuropathy, with a diverse clinical spectrum. Neuropathies with monoclonal anti-MAG IgM are particularly notable, presenting with a chronic course and distal sensory ataxic neuropathy. The identification of the immunoglobulin isotype can be critical in defining the disease and determining the appropriate treatment.

Autoimmune nodopathies, characterized by autoantibodies against proteins at the node of Ranvier, represent a distinct subset of autoimmune neuropathies. Antibodies such as anti-NF186 IgG4, anti-NF155 IgG4, and anti-CNTN IgG4 have emerged as important serological diagnostic markers, contributing to the definition of the disease. These conditions exhibit unique clinical features, limited inflammation, and poor response to conventional CIDP treatments, with rituximab showing promise as an alternative therapy.

Idiopathic sensory neuronopathies, often immune-mediated, present challenges in diagnosis and treatment decisions. Approximately 50% of sensory neuronopathies are associated with the immune system, and novel markers are needed to identify underlying autoimmune contexts. Anti-FGFR3 and anti-AGO autoantibodies have shown potential as biomarkers for autoimmune sensory neuronopathies, which may guide treatment decisions.

Table 1. Autoantibodies associated with neuropathies

Category	Targeted antigen	Associated Neuropathy
Paraneoplastic neuropathies	Hu (ANNA-1)	Sensory neuropathy, SNN
	Yo (PCA-1)	Peripheral neuropathy, myeloneuropathy
	CV2/CRMP5	SNN
	SOX1	LEMS
	PCA2/MAP1B	Sensorimotor/ autonomic neuropathy
	MA1 (PNMA1/2)	Peripheral neuropathy
	ANNA-3	Sensory/sensorimotor/ autonomic neuropathy
	Amphiphysin	Polyradiculoneuropathy, SNN
	P/Q VGCC	LEMS
	ITPR1/Sj	Peripheral neuropathy (sensory & motor)
	CASPR2	Morvan syndrome, Isaac syndrome
	KLHL 11	Myeloneuropathy
	LZUP4 (Leucin-Zipper 4)	Motor neuropathy, polyradiculoneuropathy
	Neurofilament light chain (Nfl)	Peripheral/cranial neuropathy
Diseases with Aabs against glycosylated epitopes	MAG	anti-MAG neuropathy
	SGPG	Neuropathies
	Monosyalilated/ disyalilated gangliosides	CANOMAD/Guillain-Barré & Fisher syndrome
Autoimmune nodopathies	NF155	Subacute/chronic nodopathy
	NF186	
	Pan-NF	Acute/subacute nodopathy
	CNTN1	Subacute aggressive motor nodopathy
	Caspr1	Acute/subacute nodopathy
	LGI4	Juxtaparanodal neuropathy/CIDP
Idiopathic sensory neuronopathies	FGFR3	Autoimmune neuropathy, SNN, SFN
	AGO 1/AGO2	**Autoimmune neuropathy, SNN, SFN**

Neuropathy	Antigen	Frequency	Isotype	Causal	Diagnostic value
Guillain-Barré syndrome and variants: - Classical - AMAN - Miller-Fisher syndrome	none Monosialylated gangliosides Disialylated gangliosides	80-90% 80-90%	IgG 1-3 IgG 1-3	yes yes	No supportive
Chronic demyelinating neuropathy and variants: - Classical CIDP - autoimmune nodopathies - MMN with conduction blocs	none Node of Ranvier proteins GM1 ganglioside	5-10% 50-70%	IgG 4 IgM	yes prob	Yes No
Neuropathy with M-IgM: -Anti-MAG neuropathies -CANOMAD	MAG Disialylated gangliosides	100% 100%	M-IgM k M-IgM k>l	Yes ?	Yes Yes
Sensory Neuronopathy: -Paraneoplastic -Non-paraneoplastic	HuD, CRMP5 FGFR3, AGO	90% 15-30%	IgG 1-3 IgG 1-3	No ?	Yes supportive
Small fiber neuropathy	FGFR3, S-HDS Plaxin D1	?	IgG 1-3	? pos	supportive
Neuromyotonia (Isaak syndrome)	Caspr2	?	IgG/IgG 4	?	No
Lambert Eaton myasthenic syndrome	Voltage gated calcium channel	90%	IgG 1-3	yes	No

Bold antigen names: antigen/association with neuropathy discovered in the last 5 years.
Abbreviations: SNN = sensory neuronopathy; LEMS: Lambert-Eaton myasthenic syndrome; CANOMAD: chronic ataxic neuropathy, ophthalmoplegia, immunoglobulin M [IgM] paraprotein, cold agglutinins, and disialosyl antibodies; CIDP = chronic inflammatory demyelinating polyneuropathy; SFN = small-fiber neuropathy

This presentation aims to provide a comprehensive update on the role of autoantibodies in peripheral neuropathies and their clinical implications. Novel treatment options and emerging diagnostic criteria hold promise for improving patient outcomes in these challenging neurological disorders.

Autoimmune encephalitis: the specific cell based assays are not enough and it is essential to analyze neuronal tissue reactivity!

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Introduction

Mosaic cell-based assays (CBA) are now widely used in autoimmunology to test the most known and frequent antibodies to neuronal surface antigens. However, the guidelines for the diagnosis of autoimmune encephalitis indicate to always carry out a test on neuronal tissue (cerebellum or hippocampus) and to analyze the cerebrospinal fluid (CSF) primarily. The indirect immunofluorescence (IIF) test on cerebellar tissue, although limited in its performance, allows not only to confirm a positivity found by CBA or blot methods, but also to suggest the presence of other important specificities, not yet present in the commercial panels. The purpose of this report is to demonstrate how the introduction of the IIF test on primate cerebellum can allow the identification of some specificities, such as anti-IgLON5 and anti-GFAP antibodies, in cases that would otherwise have been defined as negative.

Methods

Antibodies associated with autoimmune encephalitis were evaluated by mosaic CBA comprising NMDAR, CASPR2, LGl1, GABA-B, DPPX, AMPAR1/2 (Euroimmun, Germany). Immunofluorescence testing on primate cerebellar tissue was performed using Inova slides (Werfen, USA). Anti-IgLON5 antibodies were confirmed by Euroimmun CBA. Anti-GFAP and anti-GABA-A by live-CBA in house test at third-level centres.

Results

Our Laboratory is the Regional Neuroimmunology Hub Center and receives now around 200 requests/year for antibodies to surface neuronal antigens. The review of cases with confirmed positivity for antibodies related to surface antigens of the last 5 years (June 2018 - June 2023) highlighted a total of 15 cases, including 5 CASPR2 (33.3%), 3 Lgl1 (20%), 2 IgLON5 (13.3%), 2 GFAP (13.3%), 1 GABA-B (6.7%), 1 GABA-A (6.7%), 1 DPPX (6.7%). The IIF test on primate cerebellum was positive in all cases. In the IgLON5, GFAP and GABA-A positive cases, the positivity of the IIF test on the cerebellum and the clinical information led to search for these antibodies as well. All specificities have been confirmed on CSF and also at the Reference Centers.

Conclusions

Ascertained positivities for antibodies associated with autoimmune encephalitis are rare, but if we stop to analyze only the best-known antibodies with the basic CBA mosaic, we risk losing important positivities. In our reality 5/15 (33.3%) of the positive cases would not have been identified without the detection of a suggestive fluoroscopic pattern in IIF on the cerebellum and the subsequent referral to third-level centers for confirmation, in case the commercial test is not available.

Neuronal autoantibodies: Evaluation of a seven years' experience as a reference clinical laboratory

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Background and aims

The discovery of novel antibodies in autoimmune neurological syndromes has expanded substantially in the last two decades. Innovative screening techniques like multiplex indirect immunofluorescence mosaics using transfected cells substrates and line blots of monospecific neuronal antigens added value to screening and diagnostic methods like the traditional autoantibody detection using neuronal antigen tissue substrates like cerebellum, nerve and intestine.

Recent identification of autoantibodies against cell surface antigens, like glutamate receptor of type NMDA or AMPA, GABA receptors, the voltage-gated potassium channel-associated proteins, LG1, CASPR 2 or DPPX alongside with classical anti-neuronal antibodies against intracellular targets like Yo, Hu and Ri.

The authors present a 7-year revised casuistic as a reference clinical laboratory centre in autoimmune diseases diagnosis, focusing on the prevalence of anti-neuronal antibodies and associated autoimmune neurological syndromes.

Methods

IIFT Autoimmune Encephalitis Mosaics, Euroimmun™: Monospecific detection of autoantibodies against NMDAR, AMPAR, GABAR, LGI1, CASPR2, DPPX and IgLON5 using transfected cell substrates; IIFT NMOSD Mosaic, Euroimmun™: Monospecific detection of autoantibodies against AQP-4 and MOG using transfected cell substrates; IIFT Neurology Mosaics, Euroimmun™: Detection of autoantibodies against e.g., Hu, Yo, Ri, CV2, Ma, amphiphysin, MAG, myelin, Tr (DNER) using tissue substrates (cerebellum, nerve, intestine); EUROLINE Paraneoplastic

Neurological Syndromes 12 Ag Profile, Euroimmun™: Monospecific detection of autoantibodies against amphiphysin, CV2 (CRMP5), PNMA2

(Ma2/Ta), Ri, Yo, Hu, recoverin, SOX1, Titin, Zic4, GAD65 and Tr (DNER) by line blot.

Results

The authors present a 7-years revised casuistic from October 2015 to October 2022 as a reference laboratory center in autoimmune diseases diagnosis, focusing on autoimmune neurological syndromes, specific autoantibodies and clinical manifestations.

Conclusions

The expanding portfolio of neuronal autoantibodies is an increasingly important tool for early diagnosis of the multiple autoimmune neurological syndromes. Moreover, autoantibodies associated with paraneoplastic syndromes enhances early diagnosis and chances of a favorable outcome.

Anti-pyruvate dehydrogenase autoantibodies may be of functional relevance in patients with suspected autoimmune encephalitis

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Objective

The aim of the present study was to evaluate the presence and functional relevance of specific anti-mitochondrial autoantibodies (AMA) targeting the pyruvate dehydrogenase (PDH) complex in a cohort of 565 patients with a differential diagnosis of autoimmune encephalitis presenting with psychiatric symptoms and/or seizures.

Methods

We screened for anti-PDH autoantibodies in sera samples from 565 patients who presented to the University Hospital of Bonn, Germany, which were clinically suspected of having an autoimmune encephalitis, manifested either by psychiatric symptoms and/or epileptic seizures. This also included patients with temporal lobe epilepsy (TLE) of unclear aetiology. We have combined a wide range of approaches from immunoblotting, immunoprecipitation, mass spectrometry, immunohistochemistry, and in vitro assays of PDH enzyme activity, neuronal uptake and viability.

Results

Different subunits of the intramitochondrial PDH complex were identified as target proteins by mass spectrometry in serum samples from three different patients with psychiatric symptoms or seizures with suspected autoimmune encephalitis. Subsequently, a total of 17 patients in our

cohort were identified with immunoblotting as positive for anti-PDH complex autoantibodies. Localisation of the target structures in the brain revealed that the PDH complex is widely distributed in the cortex, cerebellum and hippocampus as well as at pre- and postsynapses of inhibitory and excitatory neurons. Finally, exposure of specific anti-PDH antibodies leads to neuronal uptake and altered enzyme activity in vitro.

Conclusion

Anti-PDH complex autoantibodies appear to have a functional role in patients with suspected autoimmune encephalitis.

This work was supported by the German Research Foundation (SFB1089, FOR2715), Else Kröner-Fresenius-Stiftung, and the BONFOR programme of the University Hospital Bonn.

Identification of novel antigenic targets using antibody profiling in spinal cord injury patients

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Introduction

Traumatic spinal cord injury (SCI) is a devastating condition that is caused by damage to the spinal cord, often leading to paralysis and loss of sensory function. Current neurological scoring systems and imaging techniques are insufficient to predict disease progression and therapy response due to high patient heterogeneity. During SCI, the blood-spinal cord barrier is disrupted, resulting in the release of central nervous system proteins into the blood. These proteins elicit an immune response involving the production of autoantibodies, which can aggravate the damage during the secondary injury phase. The goal of this project was to identify and characterise novel SCI-induced autoantibodies that can be used as biomarkers for disease course or severity.

Methods

A human spinal cord (hSC) cDNA phage display library made from RNA of healthy spinal cords (n=18) was screened for novel antibodies in plasma samples of SCI patients (n=12). Additionally,

a new human SCI cDNA phage display library was constructed from spinal cord lesion tissue of a traumatic SCI patient collected during surgery. This novel SCI cDNA phage display library was also screened for antibodies in plasma samples of SCI patients (n=11). Antibody reactivity against their selected antigens was validated in 303 individual plasma and serum samples of 190 SCI patients collected at baseline and follow-up (0-54 days post-injury) and 119 age- and gender-matched HC samples using phage enzyme-linked immunosorbent assay (ELISA).

Results

We identified antibodies to 42 novel antigens using the hSC cDNA phage display library. Furthermore, the newly constructed SCI cDNA phage display library had a diversity of 1.04 million antigens. Screening for antibodies using this SCI cDNA phage display library resulted in identification of 66 novel antigens. Antibodies to six antigens were further validated in individual SCI and HC plasma samples. Currently, analysis of these results is ongoing. Preliminary data showed an increased antibody reactivity towards four antigens in SCI patients compared to healthy controls.

Conclusion

Increased antibody reactivity to four novel antigens was indicated in SCI patients. Future research will focus on the biomarker potential of these novel autoantibodies.

Paraneoplastic ataxia-ophthalmoplegia syndrome associated with anti-Ri antibodies: a case report

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Background

Paraneoplastic neurological syndromes (PNSs) are rare autoimmune disorders caused by the remote immune-mediated effects of tumor expression. PNSs are caused by a variety of autoantibodies that associate with specific tumors and different symptoms. Anti-Ri antibodies (or ANNA-2), usually associated with breast and small cell lung cancer, often manifest with clinical symptoms such as opsoclonus-myoclonus.

Case presentation

A 60-year-old woman, with a history of infiltrating ductal breast carcinoma, was admitted to the hospital for the occurrence of subacute symptoms arising in the preceding nine months. The patient showed a severe oculomotion impairment and ataxia, as well as a passive limitation in the mandibular opening, and mild hypophonia. Postural tremor with mild diffuse upper limbs mixed hyper tone was noted, being prevalent on the right side, associated with cervical dystonia.

Laboratory analyses showed strong positivity for anti-Ri antibodies both in serum and in cerebrospinal fluid (CSF) samples: being confirmed with both immunofluorescence assay (IFA) and line blot assay (LBA). CSF analysis also showed mild protein increase with normal cellular count and positivity of blood-brain barrier damage indices (Link index=0.74; Kappa-index=10.30; BEL index=0.9). Imaging tests performed in suspicion of a paraneoplastic genesis of the disorder were normal, ruling out a new tumor diagnosis and possible recurrence of breast cancer. Treatment with intravenous immunoglobulins (IVIg) and high-dose intravenous corticosteroid therapy was performed, reporting limited clinical benefit.

Conclusion

The PNSs diagnosis often occurs late, but treatment at an early stage may provide a good prognosis for these patients. PNSs may affect any level of the central and peripheral nervous system. We report a case of anti-Ri not associated with opsoclonus-myoclonus, but an ophthalmoplegia-ataxia syndrome. Although anti-Ri antibodies are frequently associated with ataxia, these are rarely found in association with oculomotion impairment. However, there are other cases in the literature similar to the patient described in our work in which, instead, anti-Ri identification preceded the tumor diagnosis.

PNSs are still difficult to diagnose due to several factors, including the variety of autoantibodies involved and the plethora of related symptoms. The laboratory plays a pivotal role as the identification of these autoantibodies is diagnostic. However, these assays are of recent introduction and require the remarkable expertise of both the laboratory technician and the senior registrar. In addition, close collaboration between the clinician and laboratory technician is essential to increase prescriptive appropriateness and evaluate possible follow-up tests based on diagnostic suspicion.

Advancing Autoimmune Encephalopathy Diagnosis: Leveraging Cell-Free Protein Synthesis for Enhanced Detection of Autoantibodies

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Abstract

Autoantibodies play a crucial role in various clinical pictures. Accurate diagnosis is essential for effective treatment and improved patient outcomes. As the field of autoimmune-mediated diseases is rapidly emerging, a diagnostical technique that is modular is urgently needed to close the diagnostic gap. However, current diagnostic methods in the field of neurological disorders, such as anti-neuronal encephalopathies, face challenges in attaining the required flexibility, specificity, and objective assessment, which can weaken their effectiveness.

Traditional laboratory techniques for autoimmune encephalitis detection are based on neuronal tissue sections and recombinant or cultured primary neuronal cells. However, these methods require complex cloning procedures for protein production, are limited in protein yield and the necessary truncations alter protein stability, which negatively affect the binding of conformation-specific autoantibodies. Cell-free protein synthesis (CFPS) attracts as an alternative with open and modular character based on translationally active eukaryotic Spodoptera frugiperda 21 cell lysate. Due to the mild disruption procedures, microsomal vesicles derived from the endoplasmic

reticulum are preserved, enabling efficient synthesis of the post-translationally modified full-length proteins. By supplementing DNA-coding plasmids, proteins can be synthesized within 3 h and directly applied to immunochemical validation without the need for further downstream processing. Therefore, low protein accessibility on the cell surface, laborious cloning and purification procedures, that disrupt the conformation of the proteins can be tackled and enable the production of autoantigens in a highly scalable and parallelized manner.

In the presented study, we demonstrate the on-demand synthesis of autoantigens, including anti-N-methyl-D-aspartate receptor (NMDA receptor, subunit 1), anti-contactin-associated protein-like 2 (Caspr2), anti-metabotropic glutamate receptor type 1 and 5 (GRM1, GRM5) in a eukaryotic CFPS system. Subsequently, the suitability of these synthesized membrane proteins as autoimmune biomarkers is furthermore highlighted by immunochemical analysis using patient-derived autoantibodies. By employing a parallel panel of autoantigens, we underscore the versatility and suitability of the CFPS system for the development of an efficient and high-throughput capable system for autoantibody detection.

In conclusion, our study highlights the potential of leveraging the modular CFPS system as a valuable tool for assay development to detect autoimmune encephalopathies. This on-demand technology will help improve the diagnostic and enable the development of multiparameter assays targeting autoantibodies.

Clinical and laboratory re-evaluation of anti-GFAP antibody positive patients

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Introduction

Anti-GFAP (glial fibrillary acidic protein) antibody-associated astrocytopathy was first described in 2016. The main clinical syndromes reported are meningitis, meningoencephalitis and myelitis, however several case reports suggest a broader clinical presentation. The pathomechanisms of this astrocytopathy are not fully understood, but an autoimmune etiology is suspected due to frequent CSF inflammatory signs and rapid response to immunotherapy. As the target antigen is located intracellular, the direct pathogenic effect of antibodies is questioned and T-cell mediated mechanisms are being discussed. There are also several reports of secondary anti-GFAP associated astrocytopathy after viral VZV and HSV encephalitis.

Aims

(1) evaluate the clinical presentation of anti-GFAP positive patients from our routine laboratory,

(2) quantify astrocytic and neuroaxonal damage in patients with positive anti-GFAP antibody findings, (3) screen for secondary anti-GFAP antibody development after viral and bacterial meningitis/ meningoencephalitis.

Methods

Clinical characterisation of anti-GFAP positive patients tested in our laboratory between 2016 and 2023. Measurement of GFAP and NfL (neurofilament light) serum concentrations using a highly sensitive platform based on single-molecule array analysis (SiMoA, Quanterix) in patients tested positive for anti-GFAP antibodies and correlation with clinical and laboratory findings. Screening for anti-GFAP antibodies in CSF in a cohort of patients with viral and bacterial meningitis/ meningoencephalitis using cell-based assays.

Results

24 patients were positive for anti-GFAP antibodies by fixed cell assay and confirmed by tissue based assay. 8 patients were serum positive only. The main clinical syndromes observed in our cohort were myelitis (N=10) and meningoencephalitis (N=4). All patients with meningo-encephalitis were positive for anti-GFAP antibodies in CSF and serum. 4/10 patients with myelitis were positive in serum only. Serum GFAP and NfL levels were elevated compared to healthy controls, with the highest GFAP and NfL levels measured in patients with encephalitis syndrome. We also observed pathological MRI findings (17/19), increased CSF cell counts (13/24) and intrathecal B-cell expansion (3/3) during early stages of the disease. In the screening of 78 patients with herpes simplex encephalitis, viral and bacterial meningitis, no positive results for anti-GFAP antibodies were found, neither in the clinical manifestation nor in the short follow-up (up to 10 weeks).

Conclusions/Discussion

Our patients with anti-GFAP antibodies showed similar clinical manifestations as described in the literature. The most severe astrocytic and neuroaxonal damage was observed in patients with meningoencephalitis. In our cohort of patients with viral and bacterial CNS infections, we could not detect secondary anti-GFAP autoimmunity, but this may be partly due to the small sample size.

Clonally expanded CSF plasma cells produce highly specific autoantibodies in patients with LGI1/CASPR2 autoimmune encephalitis

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Abstract

Autoimmune encephalitis (AE) are inflammatory diseases of the central nervous system (CNS) characterized by adaptive autoimmunity against synaptic proteins and receptors. Autoantibodies targeting the neuronal proteins contactin-associated protein-like 2 (CASPR2) and leucine-rich-glioma-inactivated-1 (LGI1) define common subtypes of AE associated with cognitive dysfunction, epileptic seizures, and psychiatric syndromes. Inflammatory changes of in cerebrospinal fluid (CSF) are mostly absent and serologic findings (serum>CSF) have suggested primarily systemic autoantibody production. However, we recently identified the presence of plasma cells in CSF and a strong HLAII restriction as hallmarks of these diseases. Therefore, we hypothesized that clonally expanded plasma cells in CSF (1) produce antibodies targeting the respective autoantigen and (2) show a high rate of expansion in CSF, affinity, and somatic hypermutation (SHM).

We analyzed CSF using scRNA (10x, 5' and VDJ) of untreated patients with AE (3 LGI1, 2 CASPR2), and performed a comprehensive in silico analysis of their B cell repertoire. We synthesized recombinant antibodies (rHumAB) of the top 5-7 clonally expanded antibody-producing cells per patient and analyzed these rHumAbs for specificity, epitopes, affinity and SHM. We could show, that 10/10 rHumAbs from CASPR2 and 15/17 rHumAbs from LGI1 patients' CSF were indeed autoantigen reactive, with high affinity and somatic hypermutation.

We conclude that patients with LGI1/CASPR2-AE harbor a high rate of intrathecally expanded, autoantigen-specific plasma cells in their CSF compartment. This finding needs to be considered for monitoring response to therapy and future therapy development.

GanglioCombi, a unique standardized multiparametric ELISA for the specific detection of anti-neural antibodies compliant with new regulatory IVDR standards

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Objective

In Vitro Diagnostic Device Regulation (EU) 2017/746 (IVDR) is the new harmonized EU regulatory framework to ensure safety and performance of *in vitro* diagnostics (IVD). We present validation data according to stringent IVDR guidelines of the BÜHLMANN GanglioCombi® MAG ELISA (aka "GanglioCombi"), a multiparametric IVD test to detect clinically relevant anti-neural antibodies. The IVDR version of GanglioCombi uniquely features a MAG glyco-epitope (modified HNK-1) and 5 gangliosides (GM1, GD1a, GD1b, GT1a, and GQ1b), which can be screened for IgG and/or IgM isotypes.

Methods

We have determined the **reference interval** with 120 sera from random donors with unknown medical history in 2 kit lots. **Cross-reactivity** with other neurological or autoimmune disorders was investigated with 85 sera. A systematic literature review has been performed to evaluate clinical performance of GanglioCombi. A series of study schemes has established repeatability (within-laboratory precision), reproducibility (between lots/operators), and analytical sensitivity (limit of blank, limit of detection). Finally, we have compared the current GanglioCombi against the renovated IVDR version using a case series of healthy donors, diseased controls, and seropositive samples.

Results

> 90% of random donor sera and diseased control samples are negative. Between 1.3 and 6.3% are measured within the grey zone (where the test result is inconclusive), and < 3% are positive. The combined clinical performance analysis of six published studies reveals a sensitivity of 68.1% and a specificity of 88.0%. Within-laboratory precision for anti-ganglioside antibodies is between 5.7 and 13.2% (14.4 – 36.5% for MAG). Reproducibility is between 7.7 and 19.1% (23.5 – 33.2% for MAG). The IVDR-compatible version of GanglioCombi, which includes a traceable and standardized calibrator is highly comparable with the current IVDD version (agreement > 98%).

Conclusions

GanglioCombi is a robust, standardized, multiparametric ELISA test that has been renovated and validated according to the high IVDR standards of the EU. Both reference interval and cross-reactivity study justify a distinct grey zone in addition to seronegative and/or seropositive titer categories. The two regulatory versions (current IVDD and renovated IVDR-compatible) are in perfect agreement. The new version is a reliable tool for supporting the diagnosis of autoimmune-mediated neuropathies. IVDR-clearance of GanglioCombi by regulatory bodies is expected soon.

Detection of anti-AMPA receptor autoantibodies using a new substrate for recombinant cell-based testing

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Introduction

Human autoantibodies against the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) are associated with a rare subtype of autoimmune encephalitis (AE) that predominantly affects the limbic system. Early recognition and treatment can improve the disease outcome. This study evaluated the performance of a newly developed cell-based assay (CBA) for the detection of AMPAR-specific autoantibodies in patients with suspected anti-AMPAR encephalitis.

Methods

The commercial EUROIMMUN Anti-Glutamate receptor (type AMPAR1/2) indirect immunofluorescence assay (IFA), a CBA based on formalin-fixed HEK293 cells recombinantly expressing the AMPAR1 and AMPAR2 subunits, was compared to a prototype CBA in which the same transfected cell substrate with acetone fixation is used. Clinical performance was analyzed using two sample groups: Panel A (non-problematic samples) comprised 21 samples (11 sera, 10 CSF) from 14 clinically characterized AE patients with concordant anti-AMPAR results obtained by

pre-characterization using the IDIBAPS in-house and EUROIMMUN commercial CBA. Panel B (problematic samples) consisted of 18 samples (11 sera, 7 CSF) from 15 clinically characterized AE patients with discordant anti-AMPAR pre-characterization results.

Results

In panel A, anti-AMPAR autoantibodies were detected in all samples (100%) irrespective of the fixation type. In panel B, 3/18 (14.3%) samples were anti-AMPAR-positive using the formalin-fixed substrate compared to 18/18 (100%) using the acetone-fixed substrate. This corresponds to an increase in the detection rate of 83.3% by applying the prototype CBA and confirms an autoimmune etiology of encephalitis in all patients. Overall, acetone fixation produced stronger signal intensities than formalin fixation for most samples (33/39, 84.6%).

Conclusion

The new acetone-fixed substrate increases the efficiency of anti-AMPAR autoantibody detection, thus improving the serological diagnostics of patients with anti-AMPAR encephalitis.

Conflict of interest: ADC, DJ, SM and SS are employed by EUROIMMUN, a manufacturer of diagnostic assays. JD holds patents and receives royalties for antibody studies (NMDAR, AMPAR, GABAbR, IgLON5, and GABAaR).

Evaluation of EUROIMMUN anti-LGI1 cell-based assays: novel prototype versus commercial substrate

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Introduction

Detection of autoantibodies against leucine-rich glioma-inactivated 1 (LGI1) is important for diagnostic confirmation and prompt immunotherapeutic treatment of patients with suspected autoimmune encephalitis (AE). This study evaluated the performance of newly developed prototypes of the EUROIMMUN anti-LGI1 cell-based assay (CBA) to find out whether co-expression of LGI1 with the synaptic anchor proteins ADAM23/ADAM22 can improve the sensitivity of antibody detection.

Methods

The commercial EUROIMMUN anti-LGI1 indirect immunofluorescence CBA (based on LGI1 expressed in HEK293 cells) was compared to two EUROIMMUN prototype anti-LGI1 CBAs (LGI1+ADAM23 or LGI1+ADAM22 expressed in HEK293 cells). Clinical performance was analyzed using three sample groups: Panel A (non-problematic samples) comprised 27 samples (14 sera, 13 CSF) from 20 clinically characterized AE patients with concordant anti-LGI1 results obtained by pre-characterization using the IDIBAPS in-house and EUROIMMUN commercial CBA. Panel B

(problematic samples) consisted of 11 samples (2 sera, 9 CSF) from 10 clinically characterized AE patients with discordant anti-LGI1 pre-characterization results. Panel C contained sera from 99 healthy blood donors (HBD).

Results

In panel A, the anti-LGI1-positivity rate was increased from 85.2% using the commercial CBA (LGI1 alone) to 100% and 88.9% using the LGI1+ADAM23 and LGI1+ADAM22 prototype, respectively. In panel B, all samples were non-reactive in the commercial CBA, while 63.3% were positive using the LGI1+ADAM23 prototype and 18.2% using the LGI1+ADAM22 prototype. The substrate LGI1+ADAM23 produced stronger signal intensities compared to LGI1 alone in 78.9% (30/38) of all samples, and compared to LGI1+ADAM22 in 60.5% (23/38). All HBD samples were negative using the prototype assays (100 % specificity).

Conclusion

The newly developed prototype CBA substrate, co-expressing LGI1 with ADAM23, improves the detection of anti-LGI1 autoantibodies in serum and CSF of AE patients.

Conflict of interest: ADC, DJ, SM and SS are employed by EUROIMMUN, a manufacturer of diagnostic assays. JD holds patents and receives royalties for antibody studies (NMDAR, AMPAR, GABAbR, IgLON5, and GABAaR).

Performance assessment of the new EUROLINE Neurologic Syndrome 15 Ag (IgG) for the determination of autoantibodies associated with neurological disorders

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Introduction

Many paraneoplastic as well as non-paraneoplastic autoantibodies (Aabs) have been described in neurological disorders in the last decade. By integrating the associated antigens into existing assays, the diagnostic work-up of patients is being improved and diagnostic gaps reduced. Here, we assess the performance of the new EUROLINE Neurologic Syndrome 15 Ag (IgG) which expands the EUROLINE Paraneoplastic Neurologic Syndrome 12 Ag by adding CDR2L (together with CDR2 targeted by anti-Yo[1,2]), AK5[3], and Neurochondrin (NCDN)[4,5].

Methods

Sensitivity of each Aab was analyzed using a total of 194 clinically and diagnostically pre-characterized samples (table 1). Specificity of each Aab was investigated using a minimum of 100 sera from healthy blood donors.

Results

Using the EUROLINE Neurologic Syndrome 15 Ag, autoantibody positivity was confirmed in 89-100% of samples. In particular, all samples for which clinical and tissue-based indirect immunofluorescence assay pre-characterization indicated anti-Yo positivity were anti-CDR2 and -CDR2L double positive. Anti-AK5 was determined in serum and cerebrospinal fluid (CSF) with a sensitivity of 90 and 100%, respectively, and anti-NCDN with a sensitivity of 100%. The individual specificities were ≥99%.

Conclusion

The EUROLINE test kit provides a tool for the qualitative *in vitro* determination of Aabs against a large panel of 15 different neuronal autoantigens to support the diagnosis of neurologic syndromes. The parallel detection of anti-CDR2 and anti-CDR2L (both anti-Yo) increases the diagnostic significance, as double positivity is strongly related to paraneoplastic cerebellar degeneration.

Table 1: Autoantibodies detectable using the EUROLINE Neurologic Syndrome 15 Ag (IgG) and their respective sensitivity (determined using pre-characterized samples) and specificity (determined using sera from blood donors).

Antibodies	Number of pre-characterized samples	Sensitivity (%)	Specificity (%)
Anti-amphiphysin	2	100	99-100
Anti-CV2	4	100	
Anti-PMNA2 (Ma2/Ta)	9	89	
Anti-Ri (Nova1)	8	100	
Anti-CDR2 (anti-Yo)	20	100	
Anti-CDR2L (anti-Yo)	14	100	
Anti-Hu (HuD)	27	100	
Anti-Recoverin	3	100	
Anti-SOX1	8	100	
Anti-Titin	14	100	
Anti-Zic4	6	100	
Anti-GAD65	51	96	
Anti-Tr (DNER)	14	93	
Anti-AK5	10	90 (serum) 100 (liquor)	
Anti-Neurochondrin	4	100	

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Conflict of interest: All authors are employed by EUROIMMUN, a manufacturer of diagnostic assays. EUROIMMUN holds patents such as EP3086120 directed to the detection of autoantibodies to NCDN.

Evaluation of the performance of the EUROIMMUN anti-$GABA_A$ Receptor IFA in patients with suspected autoimmune anti-$GABA_A$R encephalitis

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Introduction

Autoantibodies (Aabs) against Gamma-aminobutyric acid receptors A and B ($GABA_AR$, $GABA_BR$) are found in patients with autoimmune anti-GABAR encephalitis. Because of the rareness of anti-$GABA_AR$ encephalitis the full spectrum of symptoms is still unknown. Most patients suffer from seizures accompanied by status epilepticus. Treatment of epileptic seizures might be ineffective without simultaneous autoimmune therapy, demonstrating the importance to diagnose this rare form of encephalitis. This study analyzes the performance of a cell-based anti-$GABA_AR$ indirect immunofluorescence assay (IFA; for research use only, EUROIMMUN) using sera from patients with suspected anti-$GABA_AR$ encephalitis.

Methods

Clinically pre-characterized sera from 30 patients with suspected anti-$GABA_AR$ encephalitis were tested for presence of anti-$GABA_AR$ and anti-$GABA_BR$ Aabs with specific cell-based IFAs using $GABA_AR$- or $GABA_BR$-transfected cells. Anti-GABAR-positive sera were further investigated with tissue-based IFAs using cryosections of rat hippocampus as well as rat and monkey cerebellum. Serologically pre-characterized samples from 21 patients positive for encephalitis-specific Aabs other than anti-$GABA_AR$ served as controls.

Results

Of the 30 patient sera, one serum was positive for anti-$GABA_AR$ and one for anti-$GABA_BR$ Aabs. Aab positivity was confirmed using tissue-based IFA. All control samples were anti-$GABA_AR$-negative in the cell-based anti-$GABA_AR$ IFA, indicating an analytical specificity of 100%.

Conclusion

This cell-based anti-$GABA_AR$ IFA shows excellent analytical specificity for the detection of anti-$GABA_AR$ Aabs in human serum. Thus, the test provides a diagnostic tool to support recognition of rare anti-$GABA_AR$ encephalitis.

Conflict of interest: RK, LZ, EL, ADC, DJ, and AS are employed by EUROIMMUN, a manufacturer of diagnostic assays.

Simultaneous detection of multiple Purkinje cell antigens using the EUROLINE Purkinje Cell Profile (IgG)

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Introduction

In several neurologic syndromes, autoantibodies (Aabs) are directed against antigens in the cerebellum, including those expressed exclusively in Purkinje cells. These Aabs can be associated with inflammation, altered neurotransmission and cerebellar degeneration. They are commonly detected by means of indirect immunofluorescence assays (IFA) using tissue cryosections and confirmed by blot assay. Here, we report the assessment of the new EUROLINE Purkinje Cell Profile (IgG) (EUROIMMUN) line blot which enables simultaneous detection of multiple autoantibodies against Purkinje cell antigens.

Methods

Sensitivity of the line blot was determined using pre-characterized (tissue-based IFA, cell-based IFA, or immunoprecipitation) patient sera that showed a biomarker-specific immunofluorescence signal on cryosections. Sera were positive for anti-Yo (indicating positivity for anti-CDR2 and anti-CDR2L[1,2], n=14), anti-Tr (DNER) (n=14), anti-PRKCG (n=3), anti-ARHGAP26 (n=3), anti-Homer-3 (n=5), anti-RGS8 (n=3), anti-RYR2 (n=3) and anti-AP3B2 (n=3). Specificity was tested using 150 control sera from healthy blood donors.

Results

Presence of Aabs against Yo (CDR2 and CDR2L[1]), PRKCG, ARHGAP26, Homer-3, RGS8, RYR2 and AP3B2 was confirmed in all sera. Anti-Tr (DNER) positivity was detected in all but one serum. A specificity of 100% was found for anti-Tr (DNER), anti-PRKCG, anti-Homer-3, and anti-RGS8, of 99.3% for anti-ARHGAP26 and anti-AP3B2, and of 98.7% for anti-RYR2.

Conclusion

The EUROLINE Purkinje Cell Profile (IgG) provides excellent sensitivity and specificity for the determination of Aabs against nine Purkinje cell antigens, including the seven newly developed substrates CDR2L, PRKCG, ARHGAP26, Homer-3, RGS8, RYR2, and AP3B2. The simultaneous detection of relevant Aabs supports fast serodiagnosis of autoimmune neurologic syndromes.

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Conflict of interest: All authors are employed by EUROIMMUN, a manufacturer of diagnostic assays. EUROIMMUN holds patents such as US10466239 and patent application EP18168251 directed to means and methods for the detection of Aabs to RGS8.

Detection of autoantibodies against the acetylcholine receptor, evaluation of commercially available methodologies: fixed Cell-Based Assay, Radioimmunoprecipitation Assay and Enzyme-Linked Immunosorbent Assay

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Introduction

Myasthenia Gravis (MG) is an autoimmune disease resulting from the action of pathogenic autoantibodies (AAbs) directed against nicotinic acetylcholine receptors (AChR), which interfere with communication between the neurotransmitter acetylcholine and its receptor on the muscle fiber. The detection of anti-AChR has 100% specificity for the diagnosis of MG, but the most reliable methods to detect this AAb, such as RadioImmuno Precipitation Assay (RIPA), have high execution complexity due to radioactive materials and are therefore restricted to some laboratories.

Objective

We evaluated the performance of different commercial assays to detect anti-AChR AAbs: the gold standard RIPA, a newly marketed cell-based assay (CBA), as well as some solid-phase ELISAs.

Results

145 samples were included with medical indication for anti-AChR testing. By the RIPA method, 63 were negative (RIPA-Neg <0.02 nmol/L), 17 were classified as Borderline (≥0.02 – 1 nmol/L), and 65 were positive (RIPA-Pos >1 nmol/L). Samples were also tested by two ELISAs: a competitive ELISA, which yield a poor performance with only a fair Kappa agreement with RIPA (0.210); and an indirect ELISA which yields a substantial Kappa agreement (0.652), with a ~70% sensitivity and ~96% specificity, compared to RIPA. Best performance was observed with the CBA that uses as substrate fixed cells expressing clustered AChR. The Kappa showed an almost perfect agreement with RIPA (0.969), with ~97% sensitivity and 100% specificity. However, in the Borderline group, only 5 (~30%) were positive by the CBA, suggesting CBA performance was weak in samples with low Ab titers.

Conclusion

For detection of anti-AChR reactivity, the fixed CBA showed the best performance, almost in perfect agreement with RIPA, with potential to replace the RIPA in clinical applications. However, since we observed a good Spearman correlation between indirect ELISA and RIPA levels (r=0.845), this could be an option to estimate anti-AChR AAb levels after confirming positivity by the CBA.

Detection of KLHL11 autoantibodies using a new cell-based indirect immunofluorescence assay

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Introduction

Autoantibodies against kelch-like protein 11 (KLHL11) were first described in 2019 as markers of paraneoplastic encephalitis [1]. Early diagnosis and treatment are imperative to improve prognosis and outcome of affected patients. Rat brain immunohistochemistry was reported to be not useful in routine screening for KLHL11 antibodies [2]. This study evaluates the performance of a new recombinant cell-based assay for the standardized detection of KLHL11-specific IgG and reports the characteristics of the examined patients.

Methods

Serum (n=9), CSF (n=5) and/or plasmapheresis (n=1) samples from 10 patients with clinical presentations compatible with anti-KLHL11 encephalitis as well as sera from 100 healthy blood

donors were analyzed using a prototype indirect immunofluorescence assay (IFA; EUROIMMUN) based on recombinant HEK293 cells expressing human KLHL11.

Results

KLHL11 autoantibodies were detected at high titers in all samples (serum >1:1,000; CSF >1:320; plasma >1:100,000) from all (10/10) patients but in none (0/100) of the blood donors, indicating 100% sensitivity and specificity. Anti-KLHL11-positive patients had a median age of 52 years (range 27-71) and 80% were male. The most common reported clinical presentations were cerebellar syndrome (ataxia), brainstem diencephalic encephalitis, rhombencephalitis and limbic encephalitis. Testicular or ovarian tumors were found in 87.5% (7/8) of the cases with available results from malignancy screening.

Conclusion

The new cell-based IFA enables the sensitive and specific detection of KLHL11 autoantibodies, thus supporting the diagnosis of patients with predominantly paraneoplastic brainstem cerebellar syndrome. Future studies will evaluate assay performance in larger patient cohorts to address the reported association of KLHL11 autoimmunity with a wider spectrum of syndromes and tumors [2-4].

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Conflict of interest: SM, DJ, SS and LK are employed by EUROIMMUN, a manufacturer of diagnostic assays. JD holds patents and receives royalties for antibody studies (NMDAR, AMPAR, GABAbR, IgLON5, and GABAaR).

Identification of DAGLA as an autoantibody target in cerebellar ataxia

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Introduction

We report the identification of diacylglycerol lipase alpha (DAGLA) as target autoantigen in severe cerebellitis.

Methods

Serum and CSF samples from four index patients (P1-4) were subjected to comprehensive autoantibody screening by indirect immunofluorescence assay (IFA), showing an unclassified pattern on tissue substrates. Immunoprecipitation followed by mass spectrometry was used to identify the autoantibody target, which was verified by recombinant protein assays. Sera from patients with various neurological symptoms showing a similar tissue IFA pattern as the index patient samples (disease cohort, DC, n=101), and healthy control sera (HC, n=102) were analyzed

in recombinant cell-based IFA (RC-IFA) using HEK293 cells expressing the identified protein. Epitope characterization was performed using different immunoassays.

Results

All four index patients suffered from rapidly progressive midline-accentuated cerebellar syndrome characterized by gait ataxia, dysarthria, and visual impairments. Paraclinical hallmarks in early-stage disease were inflammatory CSF changes (WBC 132-406 cells/µl, ASI>2) and MRI revealing cerebellar cortex hyperintensity and swelling. The overall response to immunotherapy was limited. Severe cerebellar atrophy developed in 3/4 patients within six months.

In IFA on cerebellar tissue sections, all patient samples revealed a consistent IgG reactivity with the molecular layer. DAGLA was identified as the target antigen and confirmed by competitive inhibition experiments and RC-IFA with recombinant DAGLA (IgG titer serum 1:32-1:3200, CSF 1:320-1:3200). Among disease and healthy control sera, additional anti-DAGLA positive samples were detected by RC-IFA (DC: n=17, IgG titer 1:100-1:3200, HC: n=1, IgG titer 1:1000), including patients with different clinical phenotypes compared to the index patients, but only one with tissue IFA-positive CSF (DC_9, cerebellitis). In ELISA using six DAGLA fragments, none of the cerebellitis patient samples (P1-4, DC_9) displayed a positive reaction, while 16/17 DC sera and the anti-DAGLA positive HC serum reacted with the C-terminal, intracellular DAGLA aa 583-1042 fragment. In immunoprecipitations or RC-IFA with C-terminally truncated DAGLA variants lacking the C-terminal intracellular domain, only the sera or CSFs of the cerebellitis patients reacted positively.

Conclusion

Our data indicate the existence of at least two subtypes of anti-DAGLA autoantibodies targeting different epitopes and having distinguishable clinical associations. We propose that anti-DAGLA autoantibodies detected in CSF showing a characteristic tissue IFA pattern, represent novel biomarkers for a severe form of autoimmune cerebellitis. In these cases, rapid disease diagnosis, followed by aggressive and prolonged immunotherapy might effectively attenuate the dramatic disease progression.

Conflict of interest: RM, MS, NR, CR, YD, CP and LK are employed by EUROIMMUN, a manufacturer of diagnostic assays. EUROIMMUN holds patent US10989712 and patent applications, such as EP3629021, concerning the detection of an autoantibody against DAGLA.

Septin-3 as a novel autoimmune target antigen in patients with paraneoplastic cerebellar ataxia

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Introduction

Septins are cytoskeletal proteins with filament forming capabilities, which have multiple roles during cell division, cellular polarization, morphogenesis, and membrane trafficking. Autoantibodies against septin-5 are associated with non-paraneoplastic cerebellar ataxia, and autoantibodies against septin-7 with encephalopathy with prominent neuropsychiatric features. Here, we report on newly identified autoantibodies against septin-3 in patients with paraneoplastic cerebellar ataxia. We also propose a strategy for anti-septin autoantibody determination.

Methods

Sera from three patients producing similar immunofluorescence staining patterns on cerebellar and hippocampal sections were subjected to immunoprecipitation followed by mass

spectrometry. The identified candidate antigens, all of which were septins, were expressed recombinantly in HEK293 cells either individually, as complexes, or combinations missing individual septins, for use in recombinant cell-based indirect immunofluorescence assays (RC-IFA). Specificity for septin-3 was further confirmed by tissue IFA neutralization experiments. Finally, tumor tissue sections were analyzed immunohistochemically for septin-3 expression.

Results

Immunoprecipitation with rat cerebellum lysate revealed septin-3, -5, -6, -7, and -11 as candidate target antigens. Sera of all three patients reacted with recombinant cells co-expressing septin-3/5/6/7/11, while none of 149 healthy control sera was similarly reactive. In RC-IFAs the patient sera recognized only cells expressing septin-3, individually and in complexes. Incubation of patient sera with five different septin combinations, each missing one of the five septins, confirmed the autoantibodies' specificity for septin-3. The tissue IFA reactivity of patient serum was abolished by pre-incubation with HEK293 cell lysates overexpressing the septin-3/5/6/7/11 complex or septin-3 alone, but not with HEK293 cell lysates overexpressing septin-5 as control. All three patients had cancers (2x melanoma, 1x small cell lung cancer), presented with progressive cerebellar syndromes, and responded poorly to immunotherapy. Expression of septin-3 was demonstrated in resected tumor tissue available from one patient.

Conclusion

Septin-3 is a novel autoantibody target in patients with paraneoplastic cerebellar syndromes. Based on our findings, RC-IFA with HEK293 cells expressing the septin-3/5/6/7/11 complex may serve as a screening tool to investigate anti-septin autoantibodies in serological samples with a characteristic staining pattern on neuronal tissue sections. Autoantibodies against individual septins can then be confirmed by RC-IFA expressing single septins.

Conflict of interest: RM, MS, NR, YD, CP and LK are employed by EUROIMMUN, a manufacturer of diagnostic assays.

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Immune checkpoint inhibitor-related myotoxicity: musculoskeletal and/or neuromuscular junction disorder?

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Importance

Immune checkpoint inhibitors (ICIs) may induce myositis with myasthenia gravis (MG) features, sparking current debate about pathophysiology, therefore management, of this emerging and lethal disease entity.

Objective

To clarify whether ICI-induced (ir-) myositis and ir-MG represent distinct diseases or exist concurrently. Retrospective multicentric study at tertiary centers for myositis/MG cohort study from 2012 to 2021. In the Greater Paris University Hospitals database (n=2.910.417), we screened all patients with International Classification of Diseases codes related to ICI and myositis/MG signs (n=620).

Main outcomes and measures

We investigated incidence of ir-MG signs (fatigability, repetitive nerve stimulation (RNS) decrement, acetylcholine receptor antibodies (AchR Abs)) in patients with and without ir-myositis (inflammatory infiltrates, CK >5x upper reference limit). We also assessed pre- and post-ICI AchR Abs positivity (>0.5nmol/l) and presence of myositis in diaphragm and extraocular muscles necropsies.

Results

Ir-MG signs were never observed in absence of myositis. Among ir-myositis patients, fatigability (2%; n=1/62) and electromyographic evidence of myasthenia (2%; n=1/41) were demonstrated only in one patient with pre-existing MG. An additional patient exhibited an atypical RNS decrement. AchR Abs were present at diagnosis in 26% of cases (n=14/53). Pre-ICI, AchR Abs test results were positive in all but one of these patients (n=8/9), and borderline negative (0.39nmol/L) in the remaining case. Another patient displayed AchR Abs pre-, but not post-ICI. Among ir-myositis patients, we observed oculomotor disease (60%; n=18/30), bulbar dysfunction affecting speech (40%; n=12/30) and swallowing (47%; n=14/30) and respiratory disorders (67%; n=20/30). Diaphragm and extraocular muscles necropsies disclosed intense muscle inflammation (100%; n=5/5).

Conclusion and relevance

In our extensive database, we found no evidence of isolated ir-MG, nor of neuromuscular junction disorder in ir-myositis. These findings lead to reconsider the entity of ir-MG. “MG-like” symptoms may stem from ir-myositis -specific predilection for oculo-bulbo-respiratory musculature. Indeed, we revealed florid inflammatory infiltration of the oculomotor and respiratory muscles. Additional studies are needed to confirm these results and to elucidate the role of pre-existing AchR Abs in ir-myositis.

The enigma of sclera-specific autoimmunity in scleritis

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Abstract

Scleritis is a severe and painful ophthalmic disorder, in which a pathogenic role for autoimmunity, including collagen type II-specific, was previously suggested.

We evaluated the presence of sclera-reactive autoantibodies and potential human leucocyte antigen (HLA) associations in a large cohort of patients with non-infectious scleritis. Therefore, we prospectively collected serum samples from 121 patients with non-infectious scleritis in a multicenter cohort study in the Netherlands. In addition, healthy (n=39) and uveitis disease controls (n=19) were included. Serum samples were tested for anti-native human type II collagen antibodies using a validated enzyme-linked immunosorbent assay (ELISA). Further, the presence of sclera-reactive antibodies was investigated using indirect immunofluorescence (IIF) test on primate retinal/scleral cryosections. Lastly, HLA typing was performed in 111 patients with scleritis. Anti-type II collagen antibodies were found in 13% of scleritis patients, in 10% of healthy controls and in 11% of uveitis controls ($p = 0.91$). A specific immunoreaction to scleral nerve tissue on IIF was observed in 33% of patients with scleritis, which was higher than healthy controls (11%; $p = 0.01$), but similar to uveitis controls (26%; $p = 0.38$). Reactivity to the scleral nerve tissue was

significantly associated with earlier onset of scleritis (48 versus 56 years; $p < 0.001$), bilateral involvement (65% versus 42%; $p = 0.01$), and less frequent development of scleral necrosis (5% versus 22%; $p = 0.02$). HLA-B27 was found to be more prevalent in patients with scleritis compared to a healthy population.

In conclusion, our results provide some evidence for autoimmunity in scleritis, although not collagen type II-specific. The observed scleral nerve autoantibody reactivity is potentially relevant for scleritis development and prognosis and warrants further investigation.

6.2 Autoantibodies in Autoimmune Liver Diseases

Autoimmune Hepatitis Societies and awareness of difficulties in autoantibody detection

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Abstract

Most diseases or group of diseases stimulate the gathering of scientists, clinicians as well as researchers who promote efforts for the better understanding of the pathogenesis and management of the diseases. In the case of specialists, over the years societies have created societies to promote the professional rights, to accelerate efforts for better education and to represent the community of given specialists and relevant stakeholders to the outer world. Concerning autoimmune hepatitis (AIH), an autoimmune liver disease, national or international societies which relate to the relevant specialists, namely hepatologists are those of the European Association for the Study of the Liver (EASL) as well as the American Association for the Study of Liver Diseases (AASLD) in the outskirt of such national and international associations, specialized groups of scientists and clinicians focusing specifically on AIH have been created. The most prominent of those is the International Autoimmune Hepatitis Group (IAIHG). IAIHG is an international panel of experts, created in 1992 in order to establish criteria for AIH. Over the years this group has been evolved and involved in several projects including the proper testing of autoantibodies related to autoimmune liver diseases. IAIG currently aims to "establish a web-based data registry for international, prospective, registration of AIH patients and follow-up data, according to the standardised directives as well as to develop resources and centres of excellence for the management of AIH accessible to clinicians, researchers, and patients". Amongst the most recent influential publications of the IAIHG are those concerning the expert clinical management of AIH in the real world, the consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group and the simplified criteria for the diagnosis of AIH, to name a few. Attempts must be made the efforts of IAIHG to coordinate with efforts made by other international bodies in order to facilitate the aims and goals related to the proper testing of autoantibodies for AIH, and the treatment and management of this troubling disease. Fine tuning of such efforts is not going to be an easy task as the relevant stakeholders must place a tremendous work force to deliver a fruitful completion of unfinished projects. For example, the

latest consensus statement for the proper testing of autoimmune serology has been published in 2004 and has not been renewed to facilitate current needs and expectations.

Autoimmune hepatitis (AIH): Implications of discrepancies between guidelines and routine labs

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Abstract

AIH is a highly heterogenous, immune-mediated acute or more frequently chronic hepatitis of uncertain cause characterized by a). hypergammaglobulinemia with distinct elevation of IgG, b). circulating autoantibodies, c). absence of other markers (viral hepatitis, alcohol, genetic, etc.) and d). lymphoplasmacytic interface hepatitis on liver biopsy. The disease is characterized by high mortality (56% after a follow-up of 30-72 months) if undiagnosed and untreated. According to the autoantibodies detected, AIH is subclassified is AIH-type 1 (ANA, SMA and/or anti-SLA/LP antibodies detection; 90% of cases) and AIH-type 2 (LKM-1/3 and/or LC1 autoantibodies detection; 10% of cases usually in children and young adulthood).

The diagnosis of the disease is based on the simplified criteria published in 2008 (Table 1).

Table 1: Simplified criteria for AIH

Variable	Cutoff	Points
ANA or SMA	≥1:40	1
ANA or SMA	≥1:80	
or LKM	≥1:40	2*
or SLA	Positive	
IgG	>Upper normal limit	1
	>1.10 times upper normal limit	2
Liver histology (evidence of hepatitis is a necessary condition)	Compatible with AIH	1
	Typical AIH	2
Absence of viral hepatitis	Yes	2
		≥6: probable AIH ≥7: definite AIH

*Addition of points achieved for all autoantibodies (maximum, 2 points).

Accordingly, the detection of non-organ specific autoantibodies (NOSA) is one of the major hallmarks to establish a prompt and timely AIH diagnosis. However, NOSA are detected in ≥ 95% of suspected cases if only tested according to the clinical practice guidelines (CPG) which suggest indirect immunofluorescence (IIF) on fresh frozen multi-organ rodent tissue substrates (liver, kidney, stomach) and anti-SLA/LP by ELISA and/or western blot as the preferable screening methods. Awareness of the significance and rules of liver autoimmune serology is a prerequisite for physicians both for requesting testing and for the interpretation of the results, while labs should adhere to CPG on serology methods used and reporting.

However, these international recommendations are rarely followed by routine clinical labs which usually use IIF on HEp2 cells or ELISAs for ANA and/or SMA detection even though, the simplified score for AIH diagnosis does not account for ANA/SMA detected by IIF on HEp2 cells or ELISAs.

Therefore, a recent multicenter study was design (Galaski et al J Hepatol 2021) in an attempt to assess the diagnostic validity of IIF on HEp2 and ELISAs testing for AIH diagnosis and make the simplified score usable all over the world. The results showed that ELISA-based and IIF on HEp2 testing can be used in the diagnostic work-up of patients with liver diseases, although assays vary considerably in performance. Most importantly, the cut-offs (specifically for ELISAs) need to be validated and established locally (not by the manufacturer). As a result, an adaptation of the simplified score for AIH diagnosis for everyday use in different labs has been proposed:

Table 2: Simplified criteria for AIH – Update of serological criteria

Variable	Cutoff	Points[1]
ANA or SMA/F-Actin	Positive[2]	1
ANA or SMA/F-Actin	Strongly positive[3]	
or LKM	≥1:40	2
or SLA	Positive	
IgG	>Upper normal limit	1
	>1.1 times upper normal limit	2
Liver histology (with evidence of	Compatible with AIH	1
hepatitis)	Typical AIH	2
Absence of viral hepatitis	Yes	2
		≥6: probable AIH
		≥7: definite AIH

[1]Addition of points achieved (maximum 2 points for autoantibodies); [2]IIF: ≥1:40 when assessed on tissue sections; ≥ 1:80 or 1:160 for ANA when assessed on HEp-2 cells, depending on local standards. ELISA with locally established cut-offs; [3]IIF: ≥1:80 when assessed on tissue sections; ≥ 1:160 or 1:320 for ANA when assessed on HEp-2 cells. ELISA with cut-offs established locally; Note: If ELISA-based autoantibody assessment is negative despite high clinical suspicion of AIH, IIF should be performed in addition.

Conclusions

IIF assay on HEp-2 cells seems valid alternative for AIH diagnosis when cut-off titers are increased. ANA detection by ELISAs and F-actin ELISA represent potential alternatives to IIF for AIH diagnosis, but cut-offs need to be established locally to be predictive in diagnosing AIH. ANA ELISA kits should include HEp-2 nuclear extracts to account for unrecognized autoantigens. Adaptation of the simplified score is needed in all above cases. Labs should adhere to CPG on serology methods & reporting.

Autoantibodies after liver transplantation

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Abstract

Organ transplantation involves a mandatory degree of interference with the native immune system. This is achieved through indefinite use of immune suppressant drugs, typically calcineurin inhibitors (CNI) tacrolimus and cyclosporin A. Their effect cannot be limited solely to prevention of rejection and therefore common post-transplant side effects include increased susceptibility to infections and higher risk of lymphoid neoplasms. In addition, other immune-mediated phenomena may be observed, not necessarily with the clinical effects. Such example is production of donor-specific antibodies and organ-specific and non-specific autoantibodies. After liver transplantation a unique condition termed *de novo* autoimmune hepatitis (dn-AIH)) has been described, initially in paediatric recipients, triggered by autoantibody production and subsequent auto aggression aimed at the liver graft. The proposed pathogenic mechanism involves impaired production and control of autoreactive T cells in the thymus and at periphery, presumably caused by CNIs. However, between 40 and 70% of organ transplant recipients develop autoantibodies with no obvious clinical ill effects. It remains unclear why only a minority of them – estimated at 5-7% - develop clinical manifestations of dn-AIH. One study suggested possession of certain HLA types shared with classical autoimmune hepatitis in the liver grafts, could be one of the contributory pathogenic explanations. History of suboptimal adherence to CNIs has also been noted in dn-AIH.

Antibody-mediated rejection is relatively common in ABO-incompatible liver transplantation, but in ABO-compatible setting is much less common. It could be acutely graft-threatening and should be aggressively treated with combination of intravenous immune globulins, rituximab and eculizumab.

Donor-specific antibodies are increasingly detected after organ transplant, but their significance in liver transplantation remains unclear. Preformed donor specific antibodies bind to endothelial cells and could initiate a complement activation cascade. Some studies have linked presence of elevated class II HLA antibody titres with late cellular of chronic rejection, but this still remains controversial.

Primary biliary cholangitis: the disease of 100 autoantibodies

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Abstract

Primary biliary cholangitis (PBC, formerly known as primary biliary cirrhosis) is an autoimmune liver disease affect the small intrahepatic ducts. The pathogenesis of the disease is not clear, but an immune-mediated destruction of the biliary epithelial cells is witnessed in the early phases of the diseases. The diagnostic criteria of the disease are currently in place and include a) the presence of cholestasis in the form of elevated cholestatic enzymes (ALP), b) the existence of histopathological features compatible with the disease and c0 the detection of disease-specific autoantibodies. Those autoantibodies are largely divided in two types, namely, the anti-mitochondrial antibodies (AMA) and the PBC-specific anti-nuclear antibodies which are directed against various subunits of the 2-oxoacid complex and are present in more than 90% of the patients with this disease and less than 2% of the healthy and disease controls. AMA largely recognize the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) and to a lesser extent other E2 and non-E2 subunits of the 2-oxo-acid complex. The universal presence of AMA has led to the appreciation of a distinct entity termed AMA-negative PBC. This phenotype shares the same clinical and histological features of AMA-positive PBC, but the affected patients do not have AMA in their serum. In AMA negative PBC, ANAs specific for the disease are more frequent compared to AMA-positive PBC. PBC-specific ANA are directed against two organelles, namely the nuclear pore complex and the nuclear body. The most frequent antigens recognized by PBC-specific ANA are gp210, a nuclear pore complex and sp100 a nuclear body antigen. Original testing of AMA and PBC-specific ANA was performed by indirect immunofluorescence using liver, kidney stomach triple tissue and HEp-2. The identification of the major autoantigens has led to the development of molecularly based assays which use the corresponding antigens for the testing of both AMA and disease-specific ANA. This study attempts to provide a meticulous assessment of the plethora of autoantibodies seen in patients with PBC. We focus on the additional 2-oxo-acid complex antigens which are recognized by AMA. We also wish to bring the attention to the excess of nuclear pore complex and nuclear body antigens which are detected (into a lesser extent compared to gp210 and sp100) by PBC sera. During the last decade, antibodies against kelch-like

protein (KLHL12) and hexokinase 1 (HK-1) were proposed as novel biomarkers in PBC patients, especially those lacking AMA.

Finally, we wish to refer to the dozens of other autoantibodies such as ANA and their respective targets, which are not disease specific but can be disease-related. Those ANA come into two forms. Those which related to co-existent/concurrent autoimmune diseases (usually autoimmune rheumatic) such as Sjogren's disease and systemic sclerosis etc. and those which do not reveal the presence of an underlying autoimmune disease. In addition, we make reference to numerous other autoantigens and their respective autoantibodies which have been identified over the years and their presence has been documented in PBC. If we wish to sum up all these autoantibody-autoantigen pairs, we can easily notice that over exceed the number of 100, making PBC an enigmatic disease resembling the autoantibody continuum of other autoimmune diseases such as systemic lupus erythematosus. The relevant questions, in part unsolved, are those which refer to the diagnostic and clinical relevance of all those non-conventional autoantibodies detectable in patients with PBC. Can they also assist efforts to better understand the pathogenesis of this puzzling disease or not?

PR3-ANCA detected by third-generation ELISA predicts severe disease and poor survival in primary sclerosing cholangitis

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Objective

Highly sensitive detection of anti-neutrophil cytoplasmic antibodies to serine proteinase 3 (PR3-ANCA) aids in the serological diagnosis of autoimmune liver disorders and the prediction of severity in primary sclerosing cholangitis (PSC). Here, we evaluate a novel third-generation ELISA for the detection of PR3-ANCA.

Methods

In total, 309 patients with PSC, 51 with primary biliary cholangitis (PBC), and 120 healthy blood donors (BD) were analyzed. For the survival analysis in PSC, the outcome was defined as liver transplantation-free survival during the follow-up.

Results

Positive PR3-ANCA levels were found in 74/309 (24.0%) of patients with PSC. No BD and one patient with PBC demonstrated PR3-ANCA positivity. PR3-ANCA was revealed as independent predictor for poor PSC outcome (study endpoint: liver transplantation/death, log-rank test, $p=0.02$). PR3-ANCA positivity, lower albumin levels and higher bilirubin concentrations were independent risks of a poor survival (Cox proportional-hazards regression analysis, $p<0.05$, respectively). The Mayo risk score for PSC was associated with PR3-ANCA positivity ($p=0.01$) and disease severity assessed by model of end-stage liver disease (MELD) and extended MELD-Na ($p<0.05$, respectively).

Conclusion

PR3-ANCA detected by third-generation ELISA is a diagnostic and prognostic marker in PSC. Its wider use could help to identify patients who are at-risk of a more severe disease.

Antimicrobial glycoprotein 2 (GP2) detected in gallstones and in bile fluid and peribiliary glands of patients with primary sclerosing cholangitis

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Background & Aims

IgA autoantibodies against secreted antimicrobial glycoprotein 2 (GP2) are predictive biomarker of disease progression and cholangiocarcinogenesis in primary sclerosing cholangitis (PSC). To date, GP2 has not been detected in the biliary tract. To test the hypothesis that biliary GP2 is a target of autoantibodies in cholangiopathies, we examined its occurrence and source in bile and gallstones.

Methods

GP2 was analyzed in 20 bile/30 serum samples from patients with PSC, 23/11 with gallstone disease (GD), 15 bile samples from healthy individuals undergoing liver-donation surgery (HILD), 20 gallstone extracts (GE) from patients undergoing cholecystectomy, and 101 blood donor (BD) sera by ELISA (enzyme linked immunosorbent assay) and immunoblotting. GP2 presence in bile and biliary tissues was confirmed by mass spectrometry and immunohistochemistry, respectively.

Results

Bile GP2 concentrations were significantly higher in patients with PSC and GD than in those with HILD ($p<0.0001$). Serum GP2 levels were similar in PSC and GD patients and controls ($p>0.05$) but lower than biliary levels in the respective cohorts ($p<0.0001$). GP2 was detected in 20 (ELISA) and 13 (immunoblotting) GE samples. Mass spectrometry identified GP2 in the bile of 2/2 randomly selected GD and 2/2 PSC patients and 0/2 HILD. Immunochemistry showed that GP2 was localized to the peribiliary glands in 8/12 PSC patients, with concomitant morphological changes in acinus cells.

Conclusion

GP2 is present in the bile of patients with PSC and GD but not in healthy individuals and in the peribiliary glands of individuals with PSC. These data support a role for biliary GP2 in the pathogenesis of autoimmune cholangiopathies.

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Author contributions to the manuscript: Planning and concept of study: PM, MK, FL, CS and DR. Acquisition of data: SL, PS, JK, MP, RK, RS, ŁK, BK, FG, MG, WP and KZ. Statistical analysis: SL and DR. Data interpretation and manuscript revision: SL, DR, JK, PS and PM. Writing committee: SL, MK, CS and DR.

Molecular mimicry in primary biliary cholangitis accounts for disease-specific anti-nuclear antibodies

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Abstract

Various ANA specificities have been reported in up to 60% of patients with primary biliary cholangitis (PBC). These autoantibodies are detected either by IIF using HEp-2 cells or molecular based assays since their identity is well known. PBC-specific ANA are mainly directed against the nuclear pore complex antigen gp210 and the nuclear body antigen sp100. Antibodies against gp210 and sp100 are present in almost one third of patients with PBC. They can be found in isolation (i.e., without AMA being present) or in combination with AMA, since the former autoantibodies are practically universal found in approximately 90-95% of the patients. How gp210 and sp100 autoantibodies arise is not clear. The mechanism of molecular mimicry and immunological cross-reactivity has been initiated to explain their presence. The first hypothesis is that molecular mimicry involving mitochondrial autoantigens and either gp210 or sp100 could explain their co-occurrence in patients with PBC. The second, more provocative, hypothesis is that microbial or viral antigens are sharing antigenic mimicry with gp210 or sp100 or both and such mimicry may be the apparent explanation of their presence in patients with PBC. The involved pathogens may be the same of those currently formulated for the explanation of the induction of AMA, the typical example being that of *E. coli* which has been heavily involved in the induction of AMA and AMA PDC-E2-specific T cells. Our group has studied in great detail the induction of antigen-specific AMAs by molecular mimicry and identified various pathogens implicated in their likely induction. *E. coli* is the dominant mimic, while others such as *H. pylori* appear not to be involved. In the current study, we present data relating molecular mimicry in the induction of PBC-specific ANA and in particular PBC-specific sp100. Those data, linked with epidemiological data suggest recurrent urinary tract infections being associated with PBC, suggest that infection with *E. coli* may indeed be a trigger of PBC-specific AMA and ANA. Other pathogens have also been identified and those must be the focus of ongoing research.

Loss of mucosal tolerance to glycoprotein 2 isoform 1 is a potential diagnostic biomarker in cholangiocarcinoma without concomitant primary sclerosing cholangitis

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Objective

Anti-Glycoprotein 2 (anti-GP2) IgA and antineutrophil-cytoplasmic antibodies to proteinase 3 (PR3-ANCA) are predictive markers of cholangiocarcinoma (CCA) in patients with primary sclerosing cholangitis (PSC) but their occurrence in CCA without PSC is yet elusive.

Methods

Discovery (118 Chinese patients) and validation (38 Polish patients) cohorts with CCA without PSC were compared with 49 patients with pancreatic ductal adenocarcinoma (PDAC), 21 patients with benign pancreatic neoplasms (BPN), 45 patients with hepatocellular carcinoma (HCC), and 75 healthy controls (HC) for the occurrence of PR3-ANCA, IgA and IgG to large (anti-$GP2_1$) and short (anti-$GP2_4$) GP2 isoforms, and carbohydrate antigen 19-9 (CA19-9).

Results

Anti-$GP2_1$ IgA was the most prevalent autoantibody in both CCA cohorts (discovery: 65/118, 52.6%; validation: 17/38, 44.7%) and corresponding levels were significantly elevated compared to other controls except PDAC patients. Anti-$GP2_4$ IgA levels were significantly increased in CCA patients compared with PDAC patients and HC. Both IgA autoantibody levels were not significantly correlated with CA19-9 levels or CCA differentiation status. Of all autoantibodies, anti-$GP2_1$ IgA demonstrated the best diagnostic performance for the differentiation of CCA from disease controls and HC by receiver-operating characteristic-curve analysis. Compared with tumor markers, anti-$GP2_1$ IgA demonstrated also the highest performance although not significantly different from that of CA19-9. Anti-$GP2_1$ IgA was an independent predictor of CCA with old age and gamma-glutamyl aminotransferase and anti-$GP2_4$ IgG as confounders by logistic regression.

Conclusion

Mucosal loss of tolerance in the form of anti-$GP2_1$ IgA distinguishes CCA from BPN and HCC and is an independent risk predictor for the occurrence of CCA.

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Human antimicrobial glycoprotein-2 expressed in Brunner glands – a putative autoimmune target and link between Crohn's and celiac disease

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Introduction

Crohn's disease (CD and celiac disease (CeD) are immunologically-mediated chronic inflammatory diseases primarily of the gastrointestinal tract but are both associated with many extra-intestinal manifestations. Although many individual factors, conditions, mechanisms, and processes have been deciphered in recent years, the pathogenesis of these diseases is not yet fully understood. CD and CeD arise in individuals with a genetic predisposition and have at least autoimmunological features, even though they are not classic autoimmune diseases and their classification as autoimmune diseases is subject of controversy[1, 2]. A significant part of the pathogenesis takes place at the intestinal interfaces, resulting in a disrupted intestinal barrier with increased permeability. Both entities also share an altered gut microbiome[3, 4]. The incidence and prevalence of both inflammatory bowel disease and celiac disease have increased worldwide in industrialized countries, particularly in North America and Europe, in recent decades[5–7]. The prevalence of IBD is estimated to be 0.5-0.7% in Germany, and that of celiac disease is to be

approximately 1%[8, 9]. There is a bidirectional (causal?) relationship between inflammatory bowel disease (IBD) and CeD: patients with IBD have a statistically significant increased risk of CeD and vice versa[10, 11].

Antibodies against Saccharomyces cerevisiae (ASCA), along with other antimicrobial antibodies, have long been known as specific markers for CD. It was only after the identification of pancreatic major zymogen membrane granule glycoprotein 2 (GP2) as the autoantigen of antibodies against exocrine pancreatic tissue that an autoantibody for Crohn's disease has been discovered[12]. An association with a more complicated course of CD could also be shown[13]. Interestingly loss of tolerance to GP2 was also described in patients with active CeD, which disappeared on a gluten free diet[14].

Four different isoforms of GP2, differing in the length of their amino acid chain, have been identified to date: two larger isoforms referred to as isoform 1 ($GP2_1$) and 2 ($GP2_2$) and two smaller ones termed isoform 3 ($GP2_3$) and 4 ($GP2_4$)[15, 16]. GP2 is predominantly produced in the acinar cells of the exocrine pancreas and forms a major component of pancreatic juice. It apparently plays no role in the exocrine function of the pancreas, i.e., in digestion, and therefore its function was unclear for a long time[17]. GP2-deficient mice show severe intestinal inflammation, increased mucosal colonization with E. coli, and shortened survival in induced colitis compared with control mice. This indicates that GP2 binds *Escherichia coli* and other adhesive and invasive commensal bacteria and prevents adhesion and invasion into the mucosa[18]. However, GP2 is also expressed outside the pancreas, including in the antigen-presenting M cells (microfold cells) of Peyer's plaques of the small intestine, a component of mucosa-associated lymphoid tissue (MALT)[19]. GP2 appears to be a specific receptor for certain bacterial components (e.g. of FimH-bearing *Enterobacteriaceae*) at the apical membrane of M cells and thus a modulator of both innate and adaptive immune responses[20, 21]. Evidence is accumulating that an imbalance in the interaction of GP2 with the gut microbiota is involved in the induction and/or maintenance of intestinal inflammation with autoimmunity to GP2[22].

Since GP2 has been identified in mucosal glands of the intestinal tract in mice and particularly in duodenal glands, GP2 synthesis in human duodenal glands (Brunner glands) was investigated by immunohistochemistry[23]. Brunner glands are mainly confined to the submucosa of the proximal duodenum and secrete an alkaline fluid composed of mucin glycoproteins that exert a physiological anti-acid function by coating the duodenal epithelium.

In our study we have investigated reactivity of human GP2 isoforms to CD- and CeD-related antigens and the level of transcription of different GP2 isoform mRNAs along the entire intestine in controls and patients with CeD and IBD and the expression of GP2 in the duodenum and jejunum with focus on Brunner glands.

Patients and methods

Patients and controls

We obtained biopsy specimens from the duodenal bulb, 2nd part of the duodenum, jejunum from 13 patients with CeD. Furthermore, specimens along the whole intestine were taken from 8 paediatric patients with CD, 4 with ulcerative colitis (UC) and from 13 healthy children (HC). During the endoscopies, 3–5 biopsies were additionally taken, snap-frozen in liquid nitrogen and stored at – 80° Celsius. All of the following inclusion criteria had to be met by the participants: 1) age ≥ 2

and < 18 years, 2) informed consent signed by at least one parent or legal guardian, 3) one of the following diagnoses IBD (CD or UC), active CeD on a gluten-containing diet or exclusion of any chronic bowel disease (HC). The study was approved by the local ethics committee of the faculty of medicine of the Technical University Dresden.

Reverse transcription quantitative PCR

For details see the paper by Roggenbuck et al. 2023[24]. For evaluation of the results, the standard curve method was applied. Therefore, a prediluted standard curve consisting of known copy numbers of the respective GP2-isoform or β-actin was used. Relative mRNA transcription was calculated using β-actin as reference: 1000 x GP2 copy number/β-actin copy number.

Protein expression of GP2 by immunohistochemistry

Analysis of GP2 protein expression by immunohistochemistry was performed on formalin-fixed paraffin-embedded intestinal biopsies from HC and patients with CD and CeD. The tissue was deparaffinised and stained with a rabbit polyclonal antibody against all GP2 isoforms (HPA015739) and a second rabbit antibody against only the larger isoforms $GP2_{1/2}$ (HPA 016668, both antibodies from Sigma-Aldrich, St. Louis, US). For the quantification of epithelial cells stained by the antibodies, two different areas of each section were counted and positive cells were referred to 10 crypts, respectively. The occurrence of GP2 positive cells was classified into 3 groups: low (1–5 positive cells in 10 crypts), medium (6–10 positive cells in 10 crypts), and high GP2-positive cell count (>10 positive cells in 10 crypts).

Analysis of GP2 isoform reactivity by ELISA

For testing the reactivity of GP2 isoforms to CD- and CeD-specific antigens an ELISA technique employing gliadin and enzymatically (pepsin and trypsin) digested variants thereof, deamidated gliadin, as well as phosphopeptidomannan (PPM) of Saccharomyces cerevisiae were used. Recombinantly expressed GP2 isoforms were used at different concentrations ranging from 0.15 mg/mL to 10.0 mg/mL). For further details on the methods used, see our paper Roggenbuck *et al.* 2023[24].

Results

GP2-isoform-expression (mRNA transcription) in the intestine of CD, CeD and UC patients and HC

In patients with CD and UC and in the HC, we examined samples from the small and large intestine (from duodenal bulb to the rectum), whereas in the patients with CeD, we were only able to take biopsies from the upper small intestine (duodenum and jejunum) for relative mRNA transcription. For most sections of the intestine of all patient groups the relative mRNA levels of isoforms $GP2_2$ and $GP2_4$ were higher than those of isoforms $GP2_1$ and $GP2_3$. Thus, also in the biopsies from the duodenal bulb, duodenum, and jejunum of CeD patients, the relative $GP2_2$ and $GP2_4$ mRNA-transcription rates were significantly higher than $GP2_1$ and $GP2_3$ mRNA-transcription rates. In CeD and CD patients, transcription of $GP2_2$ and $GP2_4$ isoforms was highest in the duodenal bulb and decreased distally. The relative transcription of these isoforms in CeD and CD was also significantly increased compared with HC and UC. $GP2_2$ and $GP2_4$ isoforms were expressed more in the small intestine than in the large bowel in CD, UC, and HC patients.

Reactivity of recombinant GP2 isoforms to CD- and CeD-specific antigens (PPM, native and digested gliadin by pepsin and trypsin)

The four GP2 isotypes showed partially significantly different binding behavior to PPM and native gliadin with the two largest isoforms $GP2_1$ and $GP2_2$ demonstrating the highest binding in a concentration dependent manner. Proteolytic cleavage of native gliadin by pepsin and trypsin increased binding to all GP2 isoforms in ELISA in contrast to undigested gliadin, whereas cleavage of gliadin by pepsin only improved the binding of $GP2_1$ and $GP2_4$ only. In contrast, deamidation of gliadin significantly reduced its binding to all GP2 isoforms.

Intestinal identification of GP2 by immunochemistry

To further investigate and confirm the increased transcription of GP2 isoforms in the upper small intestine in patients with CeD and CD, we examined the expression of GP2 by immunohistochemical staining in duodenal biopsies from 25 CeD patients, 23 CD patients, and 24 HC. By using two different polyclonal antibodies, one recognizing all GP2 isoforms (HPA 15739) and one recognizing only the larger isoforms $GP2_1$ and $GP2_2$ (HPA 016668), we were able to detect different expression patterns of the small and large GP2 isoforms in the upper small intestine of patients and HC.

The antibody against the large GP2 isoforms stained preferentially Brunner glands (figure 1). The acinar cells of Brunner glands showed staining with the antibody against all GP2 isoforms in the cytoplasm and membrane. The antibody against all GP2 isoforms additionally interacted with duodenal epithelial cells of triangular appearance and without a distinct brush border, which therefore might probably be M cells. Significantly different was the apical staining of all GP2 isoforms of Brunner glands in CeD (19/25) and CD 11/20) patients compared to HC (0/22) ($p < 0.001$). CD patients had a higher proportion of GP2-positive epithelial cells compared with HC.

Discussion

This is the first study investigating transcription of mRNA of different GP2 isoforms along the entire intestine in controls and patients with CeD and IBD as well as the first study of reactivity of human GP2 isoforms with antigens of CD and CeD. We have shown that GP2 synthesis also occurs in the Brunner glands of the duodenum and that the pancreas is not the only source of fecal GP2. Thus, exocrine glands outside the pancreas are also autoantigenic targets and presumably involved in the pathophysiology of CD and CeD. Our study supports the suggestion that increased transcription of different GP2 isoforms is a feature of inflammatory processes in patients with CeD and CD and a common feature of the etiopathogenesis of these two clinical entities. Although the inflammation of CD and CeD are histologically distinct, overlapping patterns of inflammation have also been found by other investigators in the Brunner glands in patients with CD and CeD[25].

The demonstration that GP2 primarily binds digested gliadin as well as PPM in a concentration-sensitive manner and possibly translocates antigens is further evidence for an involvement of GP2 in the etiopathogenesis of CeD and CD.

Abbreviations

CD, Crohn's disease; GP2, glycoprotein 2; IBD, inflammatory bowel disease; CeD, celiac disease; HC. Healthy children; M cell, microfold cell; MALT, mucosa-associated lymphoid tissue; PAB, pancreatic autoantibodies; PPM, phosphopeptidomannan; UC, ulcerative colitis

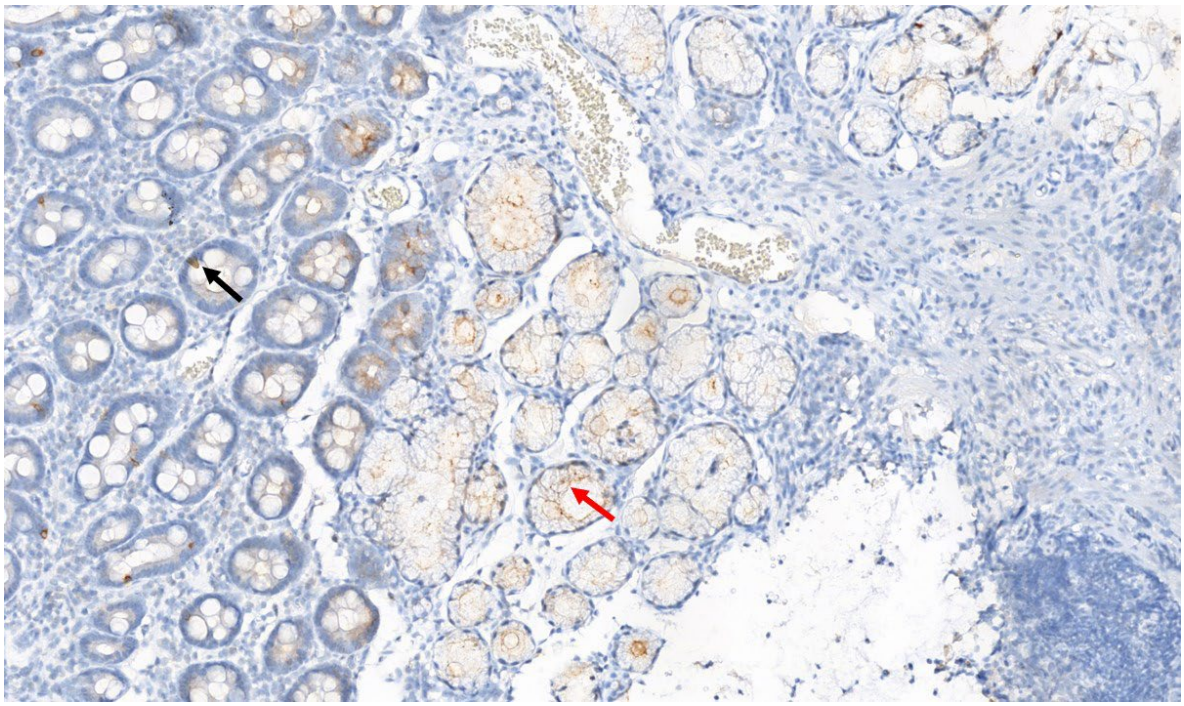

Figure 1. Staining of deparaffinised tissue with rabbit polyclonal antibody against all glycoprotein 2 (GP2) isoforms (HPA 015739). Predominant apical staining in Brunner glands of duodenal tissue sections from a patient with celiac disease (red arrow). In addition, small triangular cells in the epithelium of the crypts showed a positive staining by the same antibody (black arrow).

Conclusion

In our study, we have found evidence that GP2 isoforms in the gut originate from a source other than the pancreas and that they may react with disease-related antigens. GP2 appears to play an important role as part of the innate and acquired immune system: in modulating the microbiota, in antigen presentation in CD and CeD and as an antigenic target in both conditions.

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F-actin as a new marker antigen on an established multiparametric immunoassay profile for liver diseases

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Introduction

Autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC) are severe diseases with heterogenous manifestations, which affect people of all populations and age groups. Diagnosis and differentiation from other liver diseases such as primary sclerosing cholangitis (PSC) remain a substantial challenge for hepatologists. Since AIH and PBC are caused by different sets of autoantibodies, we previously developed a line blot for the parallel detection of all relevant parameters. We now added the AIH-specific antigen filamentous actin (F-actin) to this established liver immunoassay profile.

Methods

Sera from 15 patients with AIH, 15 patients with overlap AIH/PBC or AIH/PSC, 32 patients with PSC, 53 patients with PBC, 4 patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH), 30 patients with hepatitis B infection and 79 patients with hepatitis C infection as well as sera from 150 blood donors were examined using the EUROLINE line immunoassay system (EUROIMMUN autoimmune liver diseases 9 Ag plus F-actin (IgG); contains the PBC-relevant antigens AMA-M2, M2-3E (BPO), Sp100, PML and gp210, the AIH-associated antigens LKM-1, LC-1 and SLA/LP and F-actin as well as Ro52). The newly included F-actin antigen was polymerized from human globular actin (G-actin) and assessed by sedimentation analysis and dynamic light scattering. The scan software EUROLineScan (EUROIMMUN) was used to automatically determine presence and intensity of the resulting bands on the blot.

Results

Quality assessment revealed successful polymerization of F-actin. Autoantibodies against this newly developed F-actin antigen were detected in 53.3% of sera from AIH patients and 40% of AIH+PBC/AIH+PSC overlap patients. 93% of these positive sera did not contain other autoantibodies associated with AIH. Only one PSC patient (3.1%), two patients with hepatitis C (2.5%) and two blood donors (1.3%) also showed anti-F-actin positivity. All tested sera of PBC, NAFLD/NASH and hepatitis B patients were evaluated as anti-F-actin negative.

Conclusion

The addition of F-actin to the established multiparametric EUROLINE profile for the simultaneous and monospecific detection of AIH- and PBC-relevant autoantibodies is a great advance for routine diagnostics of patients with symptoms associated with various liver diseases.

Conflict of interest: All authors are employed by EUROIMMUN, a manufacturer of diagnostic assays.

Concordance of the results of antimitochondrial antibodies (AMA) testing with immunofluorescence on rodent tissue sections and line immunoassay

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Background and aim

Antimitochondrial antibodies (AMA) are a serological hallmark of primary biliary cholangitis (PBC), with 90 - 95% positivity. The main AMA targets in PBC are proteins of 2-oxo-acid dehydrogenase complexes known as the M2 antigen. Common laboratory practice for the detection of AMA include screening with indirect immunofluorescence (IIF) on rat liver-kidney-stomach (L/K/S) tissue sections followed by M2-specific immunoassay in case of a positive result. The aim of the study was to evaluate the concordance of AMA detection with IIF and Line immunoassay (LIA).

Materials and methods

The study included sera from 471 consecutive patients referred for AMA testing from October 2022 to June 2023. All samples were tested for AMA with IIF on rat L/K/S sections (Inova Diagnostics, USA) and LIA which encompasses 9 antigens associated with autoimmune liver diseases, including native AMA-M2 and AMA-M2-3E(BPO) fusion protein from the E2 subunits of the three enzyme complexes (branched-chain 2-oxoacid dehydrogenase (BCOADH), pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) (Euroimmun, Germany). Results of the LIA test were classified according to the manufacturer from weak positive (1+) to high positive (3+). The results of ANA IIF (performed with ANA IIFT HEp-2, Euroimmun, Germany) were available for 398 patients. Twelve samples with AMA discordant results were tested with AMA IIF reagent kit from another manufacturer (Substrates: rat L/K/S, and vascular smooth muscle cell line (VSM47) (Liver Mosaic 9, Euroimmun, Germany).

Results

Median age of 471 patients was 56 years (min 6 and max 86) and 59,4% were female (P<0,0001). AMA positivity was found in 56/471 (11,9%) samples: 24/56 (0,43) IIF pos/LIA pos, 1/56 (0,018) IIF pos/LIA neg, and 31/56 (0,55) IIF neg/LIA pos. Out of 31 samples positive with LIA only, 10 had weak positivity for only one antigen (M2 or M2-3E) and were neglected due to the low specificity. Therefore, 21/56 (0,38) samples were considered IIF neg/LIA pos. Out of these 21 samples, 14 were positive for both M2 and M2-3E (9 M2 3+/M2-3E 3+; 2 M2 3+/M2-3E 2+; 2 M2 3+/M2-3E 1+; 1 M2 1+/ M2-3E 3+). Seven out of 21 were positive for only one antigen: 4 M2 only (all 2+), 3 M2-3E only (1 sample 3+, 2 samples 2+). In 12 out of the total 22 samples with IIF/LIA discordant results (21 IIF neg/LIA pos and 1 IIF pos/LIA neg), retesting for AMA with different IIF reagent kit yielded 8 patients positive for AMA. These 8 patients were M2 3+/M2-3E 3+ with AC-21 on HEp-2 substrate in 5 cases; M2 1+/M2-3E 3+ with AC-21 in 1 case; and 2 M2-3E positive only, 2+ and 3+ with AC-21 and AC-0, respectively.

Conclusion

Results indicate a significant number of inconsistent results for AMA between the two methods. The combination of different methods could improve the sensitivity of AMA detection and reduce the number of AMA-negative symptomatic PBC, as well as asymptomatic patients at risk for PBC development in the future.

6.3 Inflammatory Bowel Diseases and Coeliac Disease

Utility of PR3-ANCA in patients with inflammatory bowel disease: a systematic review and meta-analysis

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Background/purpose

Anti-neutrophil cytoplasmic antibodies (ANCA) directed to proteinase 3 (PR3) represent a highly established marker for patients with ANCA-associated vasculitis (AAV). PR3-ANCA have also demonstrated utility in the management of inflammatory bowel disease (IBD) with certain immunoassays. More specifically, PR3-ANCA discriminated patients with ulcerative colitis (UC) from Crohn`s disease (CrD) patients and were associated with disease severity and activity. Lastly, evidence is mounting that PR3-ANCA helps to stratify patients according to treatment response. Here, we aimed to summarize the current data on the diagnostic utility of PR3-ANCA in IBD.

Methods

A structured systematic literature review including three electronic databases (Pubmed, Scopes, and Web Of Science) was conducted on June 6th 2023 to identify studies assessing the diagnostic accuracy of the QUANTA Flash PR3 assay (RUO for IBD) in UC *vs.* CrD patients. Electronic searches were supplemented by hand searching. A hierarchical, bivariate, mixed-effect meta-analysis was conducted using metandi function in STATA MP v17.0, as per the Cochrane collaboration recommendations for a review of diagnostic test accuracy studies. Study quality was assessed using QUADAS-2 tool which considers the risk of bias and applicability.

Results

Out of 111 citations retrieved for titles and abstracts screen, a total of 6 studies met the inclusion criteria and reported QUANTA Flash PR3 diagnostic accuracy in UC *vs.* CrD. The six studies included

667 UC patients and 682 individuals with CrD. Of these, 2 articles (Horn et al. and Laass et al) were case-control studies in children. Sensitivity point estimate for UC was 34.9% [95% confidence interval (CI): 26.2-44.6%]. Specificity point estimate for CrD was 95.9% (95% CI: 92.0-98.0%). This results in a Diagnostic Odds Ratio (DOR) of 12.6 (95% CI: 5.7-27.9%), a positive likelihood ratio of 8.5 (95% CI: 4.2-17.5), and a negative likelihood ratio of 0.68 (95% CI: 0.59-0.78).

The risk of bias was low in the index test and reference standard domains. Four studies (67%) showed an unclear risk of bias in the patient selection and flow and timing domains. All studies had low concerns of applicability in all the domains.

Table 1. Sensitivity and specificity of PR3-ANCA for diagnosis of UC

	UC pos	**UC total**	**Sensitivity (95% CI)**	**CrD pos**	**CrD total**	**Specificity (95% CI)**
Laass et al. 2022	49	121	40.5% (31.7-49.8%)	20	187	89.3% (84.0-93.3%)
Xu et al. 2022	35	84	41.7% (31.0-52.9%)	1	91	98.9% (94.0-100%)
Horn et al. 2018	19	33	57.6% (39.2-74.5%)	2	28	92.9% (76.5-99.1%)
Mahler et al. 2017	18	98	18.4% (11.3-27.5%)	6	94	93.6% (86.6-97.6%)
Mahler et al. 2013	88	283	31.1% (25.9-36.8%)	4	208	98.1% (95.2-99.7%)
Arias-Loste et al. 2013	14	48	29.2% (17.0-44.1%)	2	74	97.3% (90.6-99.7%
Estimates	**223**	**667**	**34.9% (26.2-44.6%)**	**35**	**682**	**95.9% (92.0-98.0%)**

Conclusion

In this meta-analysis QUANTA Flash PR3 assay has demonstrated to be able to discriminate between UC from CrD with a sensitivity of 34.9% and a specificity of 95.9%. Due to the limited number of studies published, clinical trials should consider the inclusion of PR3-ANCA as an exploratory biomarker for patient stratification.

Method comparison of two calprotectin imunoassays: the EliA™ Calprotectin 2 test (Thermo Fisher Scientific) and CALiaGold® (Sentinel Diagnostics)

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Introduction

Calprotectin is an important biomarker in the diagnosis and follow-up of IBD patients. Calprotectin immunoassays are not harmonised - despite the common cut-off of 50 mg/kg. Thight control of IBD patients also requires a good knowledge of assay performance in a higher range, especially 100-300 mg/kg[1,2].

Aim

Our aim was to evaluate the performance of the CALiaGold® (Sentinel Diagnostics) calprotectin immunoassay, compared to the established method in our laboratory, the EliA™ Calprotectin 2 test (Thermo Fisher Scientific) including the respective calprotectin extraction methods.

Methods

Study population and design

At the clinical laboratory of Imelda Hospital, which performs the calprotectin assays for four secondary-care hospitals and which has a large IBD-department, routine samples were prospectively included for 2 weeks, resulting in 113 samples.

Preanalytical aspects and extraction methods

Stool samples were stored at -20°C until batch extraction (3 batch extractions/week). Extracts for the reference method were prepared with a weighing-based method (Fecal sample preparation kit®; Roche Diagnostics). Extracts for the CALiaGold® assay were prepared with the CALiaGold® Tube extraction tool (a volume-based dipstick method) as prescribed by the manufacturer. The extract for the study method was prepared at the same time as the standard method, on residual stool material.

Analytical methods

Both the extracts per sample were analyzed with the reference method and the study method on the same day, to minimize the effect of differences in storage conditions on the analytical result. The reference method is the EliA™ Calprotectin 2 test (Thermo Fisher Scientific), performed on the Phadia 250 analyzer (Thermo Fisher Scientific). The study method is CALiaGold® (Sentinel Diagnostics) on the SENTiFIT®270 analyzer (Sentinel Diagnostics). Samples with CALiaGold® were measured in duplicate.

Results

Analytical performance

The duplicate CALiaGold® calprotectin measurements (performed on one CALiaGold® extract) have a good coefficient of variation of 3.96% (calculated for 78 samples with a result in the measuring range of 20-2200 mg/kg).

Concordance at the cut-off of 50 mg/kg

From 113 included samples, 51 samples were considered negative with the cut-off of 50 mg/kg with both the CALiaGold® method and the EliA™ Calprotectin 2 test. 41 samples were considered positive (>50 mg/kg) with both methods. However 16/113 samples (14%) had a different qualitative interpretation (for both methods, 8 samples positive classified negative with the alternative method).

Method comparison

When calculated for the subset of samples with a result in the measuring range for both methods (n=77), the passing bablok fit is y = 20.05 + 0,5549x (x is the reference method). The Pearson correlation coefficient is 0.80 (95%CI 0.70-0.87).

The subset of samples with a result in the clinical relevant range for follow-up (defined as 100-300 mg/kg with the reference method) consists of 19 samples. For this subset, with the EliA™ Calprotectin 2 result (Thermo Fisher Scientific) considered as the reference method, only 10/19 samples measured with CALiaGold® have a bias between -50% and 50% and bias is ranging from -89% up to +363%. The mean total analytical error (TEA%) is 75.1%.

Preanalytical aspects

When the CALiaGold® extracts of a challenging selection of 10 discordant samples are analyzed with Calprotectin 2 test (Thermo Fisher Scientific), performed on the Phadia 250 analyzer, better

agreement is obtained, suggesting that the extraction is probably not the most critical factor in the differences obtained, but the analytical method itself.

Conclusions

The concordance in qualitative interpretation of calprotectin at the cut-off of 50 mg/kg is acceptable for CALiaGold® (Sentinel Diagnostics) compared to the EliA™ Calprotectin 2 test (Thermo Fisher Scientific). However, large differences are observed in the quantitative results: methods are not interchangeable in the follow-up of IBD patients. Method validation data communicated to clinicians should not only focus on qualitative interpretation at the cut-off of 50 mg/kg.

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The comparison and optimization of calprotectin extraction in routine IBD care

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Introduction

The preanalytical phase contributes to variability in calprotectin measurements between laboratories

while tight control of patients with IBD relies on accurate and precise calprotectin results. The 'weighing-based' extraction method is considered to be more accurate for calprotectin than 'volume-based' methods. However, weighing-based methods are challenging in routine practice. This procedure is laborintensive and poses a risk for identification errors and other preanalytical concerns in a batch extraction setting - such as prolonged sample storage and freeze-thaw cycles. The consolidation of calprotectin samples from four hospitals in our laboratory network urged us to re-evaluate the preanalytical phase of calprotectin measurement.

Aim

Our aim was to optimize the preanalytical phase in calprotectin analysis, by comparison of three different calprotectin extraction methods combined with a single analytical method, the EliA™ Calprotectin 2 test (Thermo Fisher Scientific).

Methods

From February 2022 onwards, 50 prospective calprotectin samples from the gastroenterology department in our hospital underwent simultaneous calprotectin extraction with 3 methods. The reference method (the current extraction method in our lab), a weighing-based method (Fecal sample preparation kit®; Roche Diagnostics) was compared with two volume-based (dipstick) methods (CALiaGold® pierceTube; Sentinel diagnostics, and Elia™ Stool Extraction Kit plus; Thermo Fischer Scientific). The extracts were measured simultaneously to eliminate difference in sample or extract storage and to minimize analytical (interrun) variability.

Results

Both the volume-based extraction methods correlate well with the reference method (Pearson's R>0.98). At the diagnostic cut-off of 50 mg/kg, there is a good concordance in terms of positive or negative results, between the extraction methods. Of 26 negative (calprotectin <50 mg/kg) samples obtained with the reference method (Roche), 25/26 samples were also negative with the alternative extraction methods. The discordant samples had low positive values: 54 mg/kg for the discordant Caliagold® extract, compared to 35 mg/kg with the reference method and 42 mg/kg with Elia™ Stool Extraction Kit plus; the discordant Elia™ Stool Extraction Kit plus extract measured 72 mg/kg compared to 41 mg/kg with the reference method and 34 mg/kg with Caliagold extraction.

In 24 positive samples with a calprotectin result >50 mg/kg with the reference method, the concordance for positivity is 23/24 for Elia™ Stool Extraction Kit plus and 21/24 for the Caliagold extraction. All discordances – gaining a negative result with the alternative extraction methods - were borderline results with the reference method (ranging 56-65 mg/kg).

In positive samples >50 mg/kg, the Caliagold extraction gained lower results with a median negative bias of -28.4% (95%CI -45.7 to 0.0), even ranging to -74.3% (267 mg/kg compared to 1040 mg/kg with the reference method) in one sample. The Elia™ Stool Extraction Kit plus showed a median bias of 11.1% (95%CI: -24% to 40%) and less variability – especially in the clinically important range of 100-300 mg/kg, with the exception of some distinct discordant results. However, when these discordant samples were repeated with a good homogenisation of the samples before repeating both extractions, the Elia™ Stool Extraction Kit plus (Thermo Fisher Scientific) and the Fecal sample preparation kit® (Roche) gained comparable results and even confirmed the result obtained with the Elia™ Stool Extraction Kit plus.

Conclusions

The 'volume-based' (or 'dipstick') calprotectin extraction method Elia™ Stool Extraction Kit plus (Thermo Fischer Scientific) was judged clinically equivalent to the 'weighing-based' gold standard Fecal sample preparation kit® (Roche Diagnostics) in combination with the EliA™ Calprotectin 2 test, while another 'volume-based' calprotectin extraction method (CALiaGold® pierceTube; Sentinel diagnostics) was not. Since the Elia™ Stool Extraction Kit plus (Thermo Fischer Scientific) can be performed daily - excluding the need to freeze samples, has a lower risk for identification errors and facilitates a good homogenization of the faecal sample, it might be the preferred technique in a large-volume clinical lab.

Results of a novel multiparametric test for gluten related diseases in a pediatric cohort

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Background

The AESKUBLOTS® Gluten Related Diseases (GRD) is a novel membrane-bound multiparametric enzyme immunoassay for the overall quantitative determination of total IgA and IgA antibodies against gliadin, DGP (deamidated gliadin peptides), tTG (tissue transglutaminase), tTG neo-epitope (tTG crosslinked to gliadin peptides), TG 3 (tissue transglutaminase 3), mTG neo-epitope (microbial transglutaminase crosslinked to gliadin peptides), mTG (microbial transglutaminase) and PT-gliadin (digested gliadin by Frazer et al. in 1959) in human serum or plasma.

Aims

To evaluate the performance of a novel test system in a study cohort of pediatric GRDs and the correlation of the results to well-established methods in GRD diagnostics.

Methods: Antibody titers of AESKUBLOTS® GRD IgA were evaluated in a cohort of 50 CD, 5 Morbus Chron, and 15 Ulcerative Colitis pediatric patients. Results were correlated to AESKUSLIDES ® EMA IgA IFA read out, and AESKUBLOT® Gastro Pro IgA / IgG, Receiver-Operating Characteristic (ROC) curves, method agreement, and Pearson correlation were calculated.

Results

Using cut-offs estimated from Receiver-Operating Characteristic (ROC) curves, the highest area under the curve (AUC) of antigens was 0.955 tTG IgA, followed by tTG-neo IgA and mTG-neo IgA (0.955 and 0.907 respectively) on the AESKUBLOTS® GRD IgA. tTG-neo IgA showed the highest correlation with EMA read out (r^2=0.7453, $p<0.001$) followed by tTG-IgA and mTG-neo IgA (0.7035, $p<0.001$; 0.5504, $p<0.001$ respectively). IgA deficiency was detected in 1 patient, and the relevant antibodies were determined with respective IgG tests.

Conclusion

This novel test system is a unique and powerful tool to support diagnosing and monitoring of GRDs, such as celiac disease or non-celiac-wheat sensitivity. Excellent correlation with the highly specific EMA IFA and comparability to well-established markers show its superior performance as a screening assay.

Tales of the unexpected. sIgAd unravelled by Gluten Free Diet

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Introduction

Patients with total, selective IgA deficiency (sIgAd) are generally asymptomatic. Nevertheless, they are 10 to 15 times more likely to develop Celiac Disease (CD). Classically, the absence of IgA isotype antibodies in this cohort makes CD diagnosis more difficult.

Aim

Here we present a series of patients diagnosed of CD with normal basal IgA serum levels and IgA antibodies at the time of diagnosis. Surprisingly, after starting a Gluten Free Diet (GFD) they diminish not only their IgA antibodies, but also their IgA total levels, evolving to an IgA deficiency status.

Methods

We retrospectively reviewed patients with normal total IgA levels and positive IgA anti-tTG (±anti-deaminated gliadin peptides -DGP- antibodies) at CD diagnosis who after introducing a GFD lost IgA, paralleling autoantibodies' clearance.

Anti-tTG and anti-DGP IgA/ IgG were measured with Bioplex2200 (BioRad). Bioplex2200 contains an IgA verification bead that identifies samples with total IgA <7mg/dL. IgA levels were also measured by chemoluminescence (Cobas, Roche) and confirmed by turbidimetry (Binding Site) when IgA levels by chemoluminescence were <5 mg/dL. Clinical records were reviewed.

Results

7 patients, 2 adults and 5 paediatric (epidemiological data are shown in Table 1) met our criteria. Median age at diagnosis was 2 years old (range1-47 years). All paediatric patients were diagnosed with clinical and serological criteria as described in ESPGHAN 2012 guidelines. Biopsy was performed in adults.

All patients were HLA-DQ 2 positive. Anti-tTG IgA levels at diagnosis were above 10 times the Upper Limit of Normal (xULN) in all but one patient. Total IgA levels at diagnosis were reported in 4 patients and only one of them had a partial IgA deficit (defined as IgA levels 2 SD below the normal levels for its age).

Median time since starting GFD to the first avb error was 7 months (range 4-40 months). In Figure 1 we can see the progression of IgA anti-tTG after starting GFD and every time that the avb error appeared. As expected, anti-tTG levels decreased in every patient after starting GFD. In 4 patients we observed that the avb error appeared in the first anti-tTG measurement after starting GFD. Patient 3 maintained anti-tTG levels despite GFD, due to known gluten transgressions. This patient also had her first avb error in her second and third anti-tTG IgA measurement but the avb did not appear again in the following measurements. The other patient that report transgressions in GFD (patient 4) did not show the avb error until 40 months after GFD.

Discussion

As long as we know, this is the largest cohort of CD patients that share a “functional IgA deficiency” unravelled by GFD. We hypothesize that, after starting GFD, the gluten related antigenic stimulation stops and reveals an unknown cause that leads to the IgA deficiency. The early, high affinity and specific development of anti-TG antibodies in CD patients, the long life of the intestinal plasmatic cells in lamina propria and its role as antigen presenting cells in the intestinal mucosa could explain the development of a gluten-restricted IgA response.

Our results emphasize also the importance of careful follow-up in these patients, since both IgA and IgG antibodies, as well as total IgA quantification should be implemented.

Table 1. sIgAd GFD-induced cohort.

Patient	Sex	DoB	Age (Dx)	Dx anti-tTg IgA (UI/mL)	HLA DQ	Total IgA levels (mg/dL)	Months in GFD until first AVB error	Reported transgressions to GFD
1	M	17/05/2015	2	>250	2,5/2,2	NA	7	NO
2	F	21/06/2014	2	>250	2,5/8	52	6	NO
3	F	20/05/1969	47	>250	DQ 2*	30	6	YES
4	F	23/12/2014	1	>250	2,2/7,5	81	40	YES
5	F	23/04/2016	2	>250	2,5/2,2	83	25	NO
6	M	08/03/1999	20	76,7	2,2/8	NA	4	NO
7	F	15/05/2018	1	>250	2,5/2,2	110	7	NO

*lack of additional information.
NA: not available. DoB: Date of birth. Dx: Diagnostic. AVB: IgA Verification Bead.

Temperature, Ph dependency and activity of microbial transglutaminase and its gliadin cross-linked neo-complexes

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Background

Microbial transglutaminase (mTG) is a survival factor for bacteria and is heavily used as a food additive in the processed food industry. Being an enzyme, its temperature and pH range of activity are sensitive.

Aims

Study the mTG temperature and pH operating ranges by exploring its capacity to cross-link gliadin peptides.

Methods

After optimizing the conditions to cross-link gliadin peptides by mTG (Zedira, Germany), temperature and pH dose-response curves were explored. Gliadin peptides, mTG, and cross-linked products were analyzed on SDS gels.

Results

mTG showed activity at 60°C by cross-link gliadin peptides. Also, various processed food products are not boiled during production processes. On the other hand, the mTG-gliadin docked complexes turn more immunogenic when heated to 90°C. Most probably, more epitopes are exposed to the immune system during denaturation. Concerning the pH impact on mTG activity, the enzyme is active at pH 4.0 and above.

Conclusions

Generally, during processed food preparation, the mTG cross-linked complexes are created before heating or boiling. The resulting covalent isopeptide bonds are incredibly resistant to the luminal proteases. During meal intake, gastric acidity is neutralized, and the pH can reach 4.5. Many children and adults consume acid-suppressive medications, infants and the elderly have a higher gastric pH, and alkaline reflux is not rare. Temperature and pH do not jeopardize the mTG induced cross-linking of gliadin peptides during food preparation. The stomach pH allows those cross-linked complexes to pass and reach the gut lumen.

Generation of mAbs against TTG and MTG neo epitopes

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Background and Aims

Gluten-related diseases occur in 5% of the population. An increase in diagnosis seems to be due to a real increase in the incidence rather than the increased use of food additives, such as microbial transglutaminase (mTG).

Gliadins are cross-linked by tissue transglutaminase (tTG) and/or mTG to form complexes, exposing immunogenic neo-epitopes, triggering the production of anti-neo-epitope antibodies. Detection of these antibodies is a powerful tool in early detection of enteric damage in pediatric CD.

Anti-neo-epitope transglutaminase antibodies represent a new generation of markers offering several advantages like better diagnostic performance, a higher reflection of intestinal damage, better predictability at an early age, more diverse epitopes, and less false positivity.

Recently, we generated monoclonal antibodies specifically recognizing tTG/mTG neo-epitopes.

Method

In a first-of-its-kind attempt to generate mAbs against tTG/mTG neo epitopes, we injected mice with the tTG-gliadin and mTG-gliadin complexes. The resulting antibodies were tested for specificity using tTG-Neo, tTG, mTG, mTG-Neo, and gliadin ELISAs. Cell lines generating specific mAbs against tTG neo, tTG, mTG, mTG neo, and gliadin were identified and cultured to produce large quantities of the mAbs. These mAbs were purified and stored until further use.

Result

IFA EMA slides using anti-tTG- and mTG-neo mAbs revealed new patterns, previously not observed, different from the well-known tTG honey-comb pattern, as well as the gliadin pattern.

Conclusion

The purified mAbs are specific to the neo-epitopes and can be used for various research applications.

First multiparametric point of care test for gluten related disorders

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Background

Increasing incidence of Gluten Related Disorders (GRD) and their vast diversity of symptoms still need novel techniques and fast support for help in diagnosis.

Aims: Develop a novel near patient Point-Of-Care (POC) test system for evaluating and direct comparing of all responsible markers.

Methods

AESKUCARE® GRD IgA is a near-patient in-vitro-immunoassay for the simultaneous measurement of human-specific IgA antibodies against human tissue transglutaminase (tTG), neo-epitopes of human tTG (tTG neo), microbial transglutaminase (mTG), neo-epitopes of mTG (mTG neo), deamidated gliadin-specific peptides (DGP), gliadin, peptic-tryptic digests of gliadin (Frazer's fraction), human epidermal transglutaminase (TG3) and total-IgA in heparinized or Na-EDTA venous or capillary whole blood, plasma or serum. Just 2 to 3 drops of blood from the fingertip or earlobe are needed and evaluated within only 30 minutes.

Results

The weighted average sensitivity is 91.5% and the weighted average specificity is 97.4% compared to the reference test system AESKUBLOTS® GRD IgA (AESKU.DIAGNOSTICS). Performance evaluation shows an average lot-to-lot dependent reproducibility of > 96% and the correlation coefficient R2 of the linear straight line for each positive antigen was > 0.95.

Conclusion

AESKUCARE® GRD IgA is a unique tool. In conjunction with the patient's medical history and other findings, test results can be used by professionals to make a fast nutritional recommendation or support the diagnosis of GRDs.

Retrospective analysis of different diagnostic procedures for diagnosis of pediatric coeliac disease in a local German study

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Objectives

The diagnosis of pediatric coeliac disease (CD) can be established without biopsies when IgA antibodies against tissue transglutaminase (IgA-TTG) values show at least 10-fold the upper limit of normal (xULN), and further criteria are met. However, the rate of patients not demanding gastroscopy strongly depends on the analytic sensitivity of the antibody test applied. However, a prospective antibody study by Werkstetter et al. 2017 showed that a reliable positive predictive value (PPV) was even exceeded if IgA-TTG reached 2xULN. Therefore, we speculated that IgA-TTG ≥1xULN in addition to a positive test result of IgG antibodies against deamidated gliadins (IgG-DGP) would comply with a PPV comparable to that of the current no biopsy approach.

Methods

We performed a monocentric, retrospective study by analyzing the dataset of 620 children and adolescents undergoing endoscopy between 2010-2017 to confirm or challenge diagnosis of CD at Hospital St. Georg Leipzig, Germany. Subjects without serology results, with IgA deficiency, or patients with unclear final diagnosis were excluded. Blinded measurement of IgA-TTG and IgG-DGP in sera samples was performed with tests from EUROIMMUN (Manufacturer A, Lübeck, Germany) and Thermo Scientific/Phadia (Manufacturer B, Freiburg/Breisgau, Germany) with thresholds of ≥20 U/ml and ≥25 U/ml as well as ≥7 U/ml and ≥7 U/ml, respectively. Performance and predictive values with 95% confidence intervals were calculated for IgA-TTG ≥1xULN, IgA-TTG ≥10xULN (no-biopsy approach/diagnostic procedure 1), and IgA-TTG and IgG-DGP, both with results ≥1xULN (diagnostic procedure 2).

Results

In total, 319 datasets of children and adolescents (124 with CD, 59.2% females, aged between 1 to 17.7 years) were included. The proportion of subjects with autoimmune diabetes type 1 and inflammatory bowel disease in the study cohort was 3.1% and 1.9%, respectively. The diagnostic procedures 1 and 2 identified CD patients at a prevalence of 38.9% with a PPV of 0.989 ($CI_{95\%}$ 0.934-0.999) and 0.989 ($CI_{95\%}$ 0.933-0.999), or 0.986 ($CI_{95\%}$ 0.915-0.999) and 0.990 ($CI_{95\%}$ 0.940-0.999), respectively. Despite comparable PPV, the rate of CD patients not demanding gastroscopy to establish CD diagnosis differed significantly between both manufacturers using the current no-biopsy approach (73.4% versus 57.3%, $p<0.01$). However, by combining diagnostic procedures 1 and 2, the none-biopsy rate was comparable (83.9% versus 85.5%).

Conclusions

In a monocentric study, we approved that IgA-TTG results ≥10xULN can considered as a reliable antibody level for CD diagnosis. Furthermore, we confirmed that the proportion of CD patients requiring biopsy depends on factors such as the antibody test applied. Additionally, our data suggest that biopsies can be omitted in case of IgA-TTG ≥1xULN and IgG-DGP ≥1xULN when measured together and requested by a pediatric gastroenterologist. Further prospective studies should be endorsed to optimize diagnostic procedures for pediatric patients.

Novel fully automated anti-GAD65 autoantibodies immunoassay: technical comparison to ELISA and CLIA reveals different sensitivities

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Objective

The majority of patients suffering from Type 1 Diabetes (T1D) can be characterized by autoantibodies against 65kDa glutamic acid decarboxylase (anti-GAD65 AAb). The serological diagnosis of anti-GAD65 AAb needs a special test to ensure the best possible assay performance. So far only bridging Immunoassays have proven sensitive enough. Here we report a technical comparison of the new Phadia EliA GAD 65 to a commercial particle-based Immunoassay.

Methods

For technical evaluation, a total of 113 serum samples were tested, consisting of n=68 GAD65 parameter positive remnant samples, selected for positive test results on GAD65 ELISA by RSR Ltd. (Kronus Inc.) and 45 samples from apparently healthy blood donors. All sera were analyzed by a commercial particle-based Immunoassay (CLIA, SNIBE Diagnostic, Shenzen, China) and also by the novel GAD65 EliA assay (Research use only, Thermo Fisher Scientific, Freiburg, Germany) for the presence of anti-GAD65 AAb. Data analysis was performed using Analyse-it for MS Excel.

Results

A good correlation of the results could be observed between the CLIA and the novel GAD65 EliA technology on a Phadia instrument. Comparison of test results revealed a moderate positive percent agreement between CLIA and ELiA of 88,4% (95% CI 0.788 -0.940). Negative percent agreement in the technical control group was observed at 100% (95% CI 0.920 -1.000). Comparison to RSR ELISA as predicate Assay revealed a technical sensitivity of 100% for the EliA assay, but only 88.4 % for the CLIA assay.

Conclusions

We compared a fully automated EliA GAD65 assay for serological identification of autoimmune type 1 diabetes against another fully automated assay, using CLIA technology. We found a high technical agreement of the novel automated EliA GAD65 assay with the established ELISA. The GAD65 EliA outperformed the CLIA based anti GAD65 assay by displaying higher sensitivity.

Serum levels of anti-elastin and anti-fibrillin 1 autoantibodies in recurrent pregnancy loss (RPL) patients

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Abstract

A basic component of the microfibrils that make up the extracellular matrix is fibrillin-1, a glycoprotein, alone or in association with elastic fibrils containing elastin. Both elastin and fibrillin make up structures that undergo intense remodelling during the menstrual cycle and pregnancy, such as the endometrium and decidua. The present study aimed to determine the serum levels of anti-fibrillin-1 and anti-elastin autoantibodies in women with a history of recurrent pregnancy loss (RPL) and to compare those in normal ongoing pregnancy. Anti-fibrillin-1 and anti-elastin IgG and IgM antibodies were measured by homemade ELISA in serum samples from 26 women during normal first-trimester pregnancies, 15 non-pregnant women with a history of previous first-trimester pregnancy losses, and 25 healthy non-pregnant women with a history of successful one or more pregnancies. Anti-fibrillin-1 IgM levels were significantly lower in the group of women with normal ongoing pregnancies compared to non-pregnant controls. The group of women with history of RPL had significantly elevated IgM anti-fibrillin-1 antibody levels compared to healthy non-pregnant controls. There were no significant differences in IgG anti-fibrillin-1 levels between the study groups. Comparing the RPL non-pregnant patients with the healthy non-pregnant controls showed a significantly increased anti-elastin antibody level in RPL from both classes.

Variations in serum levels of anti-fibrillin-1 IgM anti-elastin IgM and IgG autoantibodies were found in normal pregnancy as well as in RPL patients compared with healthy non-pregnant women. Elevated autoantibodies to fibrillin-1 and elastin may contribute to the pathogenesis of immune-mediated pregnancy loss but may also be a consequence of abnormal degradation during the remodelling of pregnancy-related structures.

7 Infection, Vaccination and Therapy associated Autoimmunity

Rheumatic symptoms after COVID infection or vaccination

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Summary

Rheumatic symptoms may begin after COVID infection as well as after one or more COVID vaccinations. The mechanism seems to be similar in both cases and related to the coronavirus or its specific consequences. In most publications it is named reactive arthritis although the term COVID-19 associated arthritis seems more likely to fit to the symptoms. The number of patients with COVID-related symptoms is extremely low compared to the number of COVID-infected and COVID-vaccinated people. The prognosis seems to be rather good.

Background

Soon after the beginning of the SARS-COV19 pandemic at the end of 2019 an increasing number of reports described symptoms similar to chronic inflammatory rheumatic diseases which may happen after COVID-19 infection or vaccination.

Rheumatic symptoms associated with viral or bacterial infections are not uncommon, may persist for a few days after infection, and are usually self-limiting. Rheumatic complaints can also begin days to weeks after an infection, last for weeks to months and sometimes may develop into a chronic inflammatory rheumatic disease summarized as reactive arthritis (ReA).

In contrast to common infections SARS-COV19 disease seems to be different. After an acute infection with SARS-Cov2 symptoms of the disease can persist and are summarized under the term long COVID. The most common complaints are persistent tiredness (fatigue), feeling of illness (post-exertional malaise, PEM) with limited physical and mental performance, shortness of breath, smell and taste disorders and other symptoms. It is estimated that around 10 % of COVID-19 survivors suffer from long COVID [Garg M et al. 2021, Calabrese C et al. 2022, Davis HN et al. 2023].

Irrespective of patients with long COVID, however, there are increasing reports of patients in whom symptoms of a chronic inflammatory rheumatic disease begin after a COVID-19 infection

[Kocivnik N et al. 2022, Pires RE et al. 2022, Roseti L et al. 2022]. These include symptoms of arthritis, myositis, autoinflammatory symptoms, complaints related to collagenosis or chronic inflammatory rheumatic diseases that are not associated with autoantibodies such as psoriasis with psoriatic arthritis or other spondyloarthropathies [Zhou Q et al. 2021, Essien F et al. 2022].

Arthritis that occurs days to weeks after a documented infection is commonly referred to as reactive arthritis (ReA). The term ReA was introduced in 1963, provisional diagnostic criteria were formulated in 1983, and ReA was assigned as a subgroup of spondyloarthritis in 1991 [Selmi C & Gershwin ME 2014, Zeidler H 2022].

Arthritides after known infections can be divided into 3 groups depending on the evidence in the joint aspirate. Infective arthritis is present if a pathogen can be detected in the joint aspirate, post-infective arthritis if microbial components are detected but an intact pathogen is missing, and ReA is present if there is a clear medical history but pathogens or pathogen components cannot be found (Table 1).

Table 1. Type of arthritis with or after an infection (modified from Zeidler 2022)

group	infection known	causative organism in a joint	parts of causative organism in a joint	type of arthritis
1	yes	+	+	infective
2	yes	-	+	postinfective
3	yes	-	-	reactive

There is still no consensus on the designation of arthritis after COVID-19 infection. Most scientific papers report ReA after COVID-19 infection [Ono K et al. 2020, Bekaryssova D et al. 2022, Dombret S et al. 2022, Ruiz-Del-Valle V et al. 2022 Slouma M et al. 2022, Migliorini F et al. 2023]. ReA currently belongs to the group of spondyloarthritis, but there are no classification or diagnostic criteria formulated in an international consensus.

However, the correctness of the designation ReA in connection with SARS-Cov-2 is repeatedly questioned and discussed whether the designation viral arthritis, SARS-Cov-2 associated or post-COVID arthritis instead of ReA would be more appropriate [Kobayashi S et al. 2021, Yokogawa N et al. 2021, Baimukhamedov C et al. 2022, Farisogullari B et al. 2022, Pal A et al. 2022]. From the numerous case reports on the occurrence of acute arthritis after COVID infection the clinical picture emerges differences to classic ReA in a few points (Table 2)

	reactive arthritis	**post-COVID Arthritis**
age (years)	predominantly below 40	predominantly over 45
gender	men dominate (m:f ≈6:1)	roughly equally distributed
precipitating factor	gut or urogenital infection	respiratory tract infection
triggering agent	bacteria	virus
phenotype	spondylarthritis like	various
HLA-B27	mostly positive (up to ~80 %)	negative
preferred joints	large joints	small joints
chronicity	≈30 % chronic (>3 months)	most resolve within 3 months
treatment	like spondyloarthritis	low dose steroid ± NSAIDs

Table 2. Differences between reactive arthritis as subclass of spondyloarthritis und post-COVID Arthritis (modified from Bekaryssova D et al. 2022)

ReA typically presents as an asymmetric oligoarthritis with large-joint dominance. Post-COVID arthritis tends to be more diffuse in distribution with involvement of hand and ankle joints, finger and toe joints. ReA is more likely to be found in younger patients, a post- COVID arthritis increases in patients in the second half of life. Typical triggers of ReA are bacteria, in post-COVID viruses. ReA is seen more frequently in men, post-COVID arthritis appears to be roughly equally distributed in both sexes. Patients with ReA are predominantly HLA-B27 positive. In patients with post-COVID arthritis, susceptibility of HLA-B27 positive patients has not yet been observed after COVID infection occurred [Dombret S et al. 2022, Baimukhamedov C et al. 2022]. Some studies also tried to include the HLA status as far as it was present. Of 26 patients listed, 3 were HLA-B27 positive, 12 negative and in 11 patients the HLA status was unknown [Farisogullari B et al. 2022]. Similar results are described in a review of 20 patients, 10 of whom had known HLA-B27 status. 2 patients were positive, 8 negative [Bekaryssova D et al. 2022]. In an additional study, out of 13 patients with post-COVID arthritis 5 were HLA-B27 positive [Slouma M et al. 2022]. Even in individual case reports in which the HLA status is mentioned, the HLA-B27 negative finding predominates [Ono K et al. 2020, Dombret S et al. 2022].

The clinical picture of ReA has also been described in other viral diseases [Bekaryssova D et al. 2022] such as HIV (human immunodeficiency virus), hepatitis B and C [Kemmer NM et al. 2010, Chen Y-L et al. 2018], Eppstein-Barr (EBV), parvovirus, dengue, chicungunya [Tritsch SR et al. 2020]. Whether the virus itself or an accompanying bacterial infection triggers ReA has not been clarified. In the cases of post-COVID arthritis a recent COVID infection was detected by an antigen test.

Arthritis after COVID infection

The occurrence of arthritis after a COVID infection is described in numerous publications and case reports. A search of PubMed using the terms “arthritis” and “COVID” returned more than 30000 hits.

A summary of case reports described 25 patients (11 female, 14 male) with a mean age of 45 years. The time from infection to onset of arthritis was between 6 and 48 days. Peripheral joint involvement was described in 20 cases. Knees (n=11), feet (n=2), ankles (n=9), hips (n=3), wrists (n=6), hands (n=3), elbows (n=3) and shoulders (n=4). There have been isolated reports of tendinitis and dactylitis [Slouma M et al. 2022].

Similar results were seen in a review of papers until July 2021. The time from diagnosis of COVID to diagnosis of ReA was 7-90 days (median 18). The most affected joints were knees, ankles, and interphalangeal joints [Kocyigit BF et al. 2021].

The number of reports about post-acute COVID-19 musculoskeletal manifestations after COVID-19 is rapidly rising [Ciaffi J et al. 2023, Yaday S et al. 2023]. Arthritis is one of the most common post-COVID-19 rheumatological manifestation.

The prognosis of post-COVID arthritis appears good. In most cases, treatment was symptomatic with local or systemic NSAIDs and steroids. Basic therapeutics such as salazopyrin, hydroxychloroquine, methotrexate or biologics were rarely administered [Migliorini F et al. 2023].

Other rheumatic complaints after COVID infection

COVID infection also appears to cause autoimmune diseases. Guillain-Barré syndrome (GBS), autoimmune hemolytic anemia, antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE), multiple sclerosis (MS), acute disseminated encephalomyelitis, multisystem inflammatory syndrome in children (MIS-C) and rheumatoid arthritis (RA) have been reported [Kocivnik N et al.2022]. A COVID-related disturbance of the immunological balance with the promotion of autoimmune reactions including autoantibody formation is suspected. All cases are described individually. So far, no center has reported a larger accumulation of autoimmune diseases after COVID infection [Sher EK et al. 2023].

Some studies suggest that patients with autoimmune diseases are more prone to hospitalization, have more severe complications, and higher mortality from COVID. This assumption is based on the idea that patients with autoimmune diseases are more susceptible to a SARS-Cov-2 infection compared to the healthy normal population as a result of a weakened immune system, the use of immunomodulating drugs and the organ damage that has already occurred. In contrast, other studies suggest that regular medication intake protects patients with autoimmune diseases from severe COVID complications [Kocivnik N et al. 2022].

In addition to the most frequently described COVID-associated arthritis, other chronic inflammatory rheumatic diseases are suspected to be caused by COVID. The onset of different forms of vasculitis after COVID infection has been reported [Batu ED et al. 2022, Bryant MC et al. 2022, Gracia-Ramos AE et al. 2021, McGonagle D et al. 2021].

Not only isolated chronic inflammatory rheumatic diseases may begin after COVID but also autoinflammatory syndromes such as systemic juvenile idiopathic arthritis in children [Alexandri et al. 2022, Waheed N et al 2022], as well adult onset still syndrome (AOSD) [Alshablan A et al. 2021, Bamidis AD et al. 2021] or Behcet's disease [Espinosa G et al. 2021].

Since the beginning of the pandemic, there has been concern that patients with autoimmune diseases are at increased risk of COVID with more serious complications. However, previous experience has not really confirmed these suggestions [Alzahrani ZA et al. 2021, Rorat M et al. 2022, Figueroa-Parra G et al. 2022].

Rheumatic complaints after COVID-19 vaccination

Vaccination skepticism is high among the general population and among patients with chronic inflammatory rheumatic diseases. From the very beginning of COVID pandemic, the international rheumatological societies have recommended to accept the vaccination against SARS-Cov-2 as vaccination offers the best protection against severe or fatal courses [Landewé RBM et al. 2022, Jeffrey R et al. 2022, Mease PJ et al. 2022]. So far there is no indication that patients with chronic inflammatory rheumatic diseases are more prone to vaccination reactions or on increased risk that the vaccination could trigger a reactivation of their underlying disease [Spinelli FR et al. 2022]. Nevertheless, in individual cases, a chronic inflammatory or autoinflammatory disease seems to be induced by a COVID vaccination [van Laar JA 2022].

Rheumatic diseases similar to those after COVID infection can occur after COVID-19 vaccination [Chen Y et al. 2022, Al-Allaf AW et al. 2022, Chen CC et al. 2022, Jara LJ et al. 2022, Rodríguez Y et al. 2022, Ursini F et al. 2022]. After SARS-Cov-2 vaccination, the onset was described of diffuse cutaneous sclerosis, ANCA-associated vasculitis [Ma Y et al. 2022], RS3PE (remitting seronegative symmetric synovitis with pitting edema) syndrome [Arino H et al. 2022], adult Still syndrome (AOSD) [Matsuda M et al. 2023], inflammatory muscle diseases such as polymyalgia rheumatica (PMR) or PMR-like symptoms [Manzo C et al. 2022], myositis [Ding Y et al. 2022], and myocarditis [Lai YW et al. 2022].

Despite the increasing reports of autoimmune syndromes after COVID vaccination, the total number of affected people in relation to the number of people vaccinated is very small and does not argue against the benefits of vaccination.

Discussion

After COVID infection or vaccination patients sometimes react with symptoms similar to chronic inflammatory diseases. The connection between COVID and rheumatic or musculoskeletal complaints is not clear. SARS-Cov-2 and vaccination appear to induce autoimmunity and autoinflammation via similar mechanisms, the diseases observed are similar after infection and vaccination.

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Functional implications of promiscuous autoantibodies in COVID-19

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Abstract

COVID-19 was previously shown to precipitate the autoimmunity in severe patients. However, the functional implications of the generated autoantibodies remain unclear. Autoantibodies were characterized to bind multiple targets, including ACE2 and interferons. ACE2, the viral receptor, was demonstrated to be elevated in blood of the patients, which raises the question of whether spike-ACE2 complexes might induce the ACE2 reactivity. In our work, we screened COVID-19 patients and observed that an increase in soluble ACE2 level is poorly correlated with anti-ACE2 IgG titer. We probed the sera for reactivity against various self-targets and found that the levels of antibodies against ACE2, IFNα2, and CD26 strongly correlate in severe cases of COVID-19, with most of the sera displaying polyreactivity rather than narrow autospecificity. In contrast to specific autoantibodies, promiscuous autoreactive sera failed to inhibit the enzymatic activity of ACE2 and IFNα2 signaling. Our study suggests that the functional impact of the autoantibodies in COVID-19 might have been misinterpreted in earlier studies.

Evidence of the presence of antinuclear antibodies (ANA) in healthcare workers after mRNA based anti-SARS-CoV-2 vaccines: two years follow-up study

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Background and aim

The BNT162b2 and mRNA-1273 vaccines were the first mRNA-based vaccines to be approved. However, their effects and potential to induce autoimmune reactions are still not well understood. This two-year follow-up study aimed to assess the development and persistence of autoantibodies, particularly antinuclear antibodies (ANA), in healthcare workers (HCWs) who received either the BNT162b2 mRNA or mRNA-1273 vaccines.

Methods

A total of 155 HCWs were initially enrolled in the study. However, only 77 participants (60 females and 17 males, age range 26-67 years, median age 48) completed all scheduled blood draws and received a minimum of 3 doses of either the BNT162b2 mRNA or mRNA-1273 vaccines. Blood samples were collected at multiple time points: before vaccine administration (T0), at 3 months

(T1), 12 months (T2), and 24 months (T3) after the first dose. ANA levels were assessed using indirect immunofluorescence on Hep-2 cells, with dilutions of 1:80, 1:160, 1:320, and 1:640.

Results

Out of the initial population, 52 subjects who tested negative for ANA at T0 were included in the final analysis. Among them, 35 participants completed sample collection until the last follow-up (T3). After excluding subjects who contracted COVID-19 between T2 and T3, the final population for analysis comprised 17 subjects. Among the initially ANA-negative subjects at T0 (n=17), 2 individuals tested positive at T1 and maintained positivity until T3. At T2, 6 subjects became ANA positive, resulting in a total of 8 ANA-positive subjects at T2. Among the 6 individuals who tested positive at T2, 3 remained positive at T3, while the remaining 3 reverted to ANA-negative status. At T3, two additional subjects tested positive, bringing the total number of positive subjects to 7 at that time point. All participants who were negative for ANA at T0 were surveyed to determine if they had undergone specialized rheumatology examinations. Additionally, two participants were monitored for arthralgia.

Conclusion

This two-year follow-up study of HCWs vaccinated with BNT162b2 mRNA and mRNA-1273 vaccines observed the onset of ANA in a subset of individuals who were initially ANA-negative. These findings provide preliminary insights into the potential immune responses associated with mRNA vaccines. However, it is important to interpret these results with caution and consider several factors. First, the sample size reduced over time, limiting the statistical power of the analysis. Second, the study showed that only two participants displayed clinical manifestation of arthralgia. Further investigations would be necessary to understand the clinical implications of ANA development in vaccinated individuals.

Humoral response to SARS-Cov-2: a perspective study in vaccinated subjects and COVID19 patients

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Abstract

In December 2020, a massive vaccination campaign against anti-SARS-CoV-2 started worldwide. In Italy, 4 vaccines were authorized: Pfizer; Moderna; Astrazeneca (AZ); Johnson & Johnson (J&J). This study aimed to investigate the quantity and persistence of humoral response in vaccinated subjects and COVID-19 patients.

We were able to enrol 179 individuals, although only 169 sticked until the end of the project. In total, the subjects were divided in 114 vaccinated subjects and 55 COVID-19 patients (age: 19-95 years). Blood samples were collected through a dried blood spot at specific time points during an entire year and the antibodies levels were checked by ELISA assay.

We found that subjects receiving Moderna and Pfizer vaccines had a similar kinetic, reaching the antibodies peak concentration at 7 and 6 weeks after the first dose. AZ vaccine was administered in homologous and heterologous manner giving both the same trend and showing a peak at 15 and 13 weeks after the first dose respectively. However, subjects receiving AZ heterologous had antibodies concentration more than ten times higher compared to AZ homologous. J&J revealed the worst performance in terms of antibodies concentration and maintained a constant trend just above the cut off.

COVID-19 patients underwent vaccination too. They respond similarly after 1 or 2 doses of vaccine, reaching the antibodies peak at 2 and 5 weeks respectively. In addition, the IgG levels in COVID-19 patients that got two shots was almost 2 times higher and remained longer.

Our study lasted one year thus, 132 volunteers got mRNA-based vaccine booster: 91 vaccinated subjects and 41 COVID-19 patients. Subjects that previously had Pfizer and Moderna vaccine, they achieved antibodies levels like the one reached after the second dose; instead, the ones who got AZ homologous, AZ heterologous and J&J, after the booster, they achieved antibodies level higher then after the second dose. Independently of the vaccine received at the beginning, in all the

subjects, after the booster, the antibodies concentration decreased slower than after two doses. In the COVID-19 patients the humoral response was quick and already at 2 weeks after the booster the IgG levels were the same obtained after the first dose. The IgG levels had a slow decrease during the following 74 weeks.

In conclusion, the humoral response in the general population is influenced by vaccine type. Moderna vaccine determines the highest antibodies concentrations followed by Astrazeneca heterologous vaccination. The number of doses in COVID-19 infected subjects does not affect the kinetics, however IgG levels remained longer in the two doses patients compared to the one who received only one dose. Therefore, the number of doses is fundamental to maintain/recall a humoral response and have an antibody persistence over time. The heterologous vaccination seems to be more effective to achieve a good humoral response in term of IgG concentration.

Antiphospholipid antibodies are enriched post acute COVID-19 but do not modulate the thrombotic risk

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Background and objectives

COVID-19-assocatiated coagulopathy, shown to increase the risk for the occurrence of thromboses and microthromboses, displays phenotypic features of the antiphospholipid syndrome (APS), a prototype antibody-mediated autoimmune disease. Several groups have reported elevated levels of criteria and non-criteria antiphospholipid antibodies (aPL), assumed to cause APS, during acute or post-acute COVID-19. However, disease heterogeneity of COVID-19 is accompanied by heterogeneity in molecular signatures, including aberrant cytokine profiles and an increased occurrence of autoantibodies. Moreover, little is known about the association between autoantibodies and the clinical events. Here, we first aim to characterise the

antiphospholipid antibody, anti-SARS-CoV-2 antibody, and the cytokine profiles in a diverse collective of COVID-19 patients (spanning the whole range of disease severity from asymptomatic to intensive care), using vaccinated individuals and influenza patients as comparisons. We then aim to assess whether the presence of aPL in COVID-19 is associated with an increased incidence of thrombotic events in COVID-19.

Methods and results

We conducted anti-SARS-CoV-2 IgG and IgA microELISA and IgG, IgA, and IgM antiphospholipid line immunoassay (LIA) against 10 criteria and non-criteria antigens in 155 plasma samples of 124 individuals, and we measured 16 cytokines and chemokines in 112 plasma samples. We additionally employed clinical and demographic parameters to conduct multivariable regression analyses within multiple paradigms. In line with recent results, we find that IgM autoantibodies against annexin V (AnV), β2-glycoprotein I (β2GPI), and prothrombin (PT) are enriched upon infection with SARS-CoV-2. There was no evidence for seroconversion from IgM to IgG or IgA. PT, β2GPI, and AnV IgM as well as cardiolipin (CL) IgG antiphospholipid levels, significantly elevated in the COVID-19 but not in the influenza or control groups. They were associated predominantly with the strength of the anti-SARS-CoV-2 antibody titres and the major correlate for thromboses was SARS-CoV-2 disease severity.

Conclusion

While we have recapitulated previous findings, we conclude that the presence of the aPL, most notably PT, β2GPI, AnV IgM, and CL IgG in COVID-19 are not associated with a higher incidence of thrombotic events.

Severe acute respiratory syndrome coronavirus-2 variants and antibody responses in Bangladesh

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Abstract

Novel SARS-CoV-2 variants are emerging at an alarming rate. The delta variant and other variants of concern (VoC) carry spike (S)-protein mutations, which have the potential to evade protective immunity, to trigger break-through infections after COVID-19 vaccination, and to propagate future waves of COVID-19 pandemic.

To identify SARS CoV-2 variants in Bangladesh, patients who are RT-PCR-positive for COVID-19 infections in Dhaka and Chittogram were screened by a RT-PCR melting curve analysis for spike protein mutations. To assess the anti-SARS CoV-2 antibody responses, the levels of the anti-S -proteins IgA and IgG and the anti-N-protein IgG were measured by ELISA. Of a total of 36 RT-PCR positive samples (75%), 27 were identified as delta variants, with one carrying an additional Q677H mutation and two with single nucleotide substitutions at position 23029 (compared to Wuhan-Hu-1 reference NC 045512) in the genome sequence. Three (8.3%) were identified as beta variants, two (5.5%) were identified as alpha variants, three (8.3%) were identified as having a B.1.1.318 lineage, and one sample was identified as an eta variant (B.1.525) carrying an additional V687L mutation. The trend of higher viral load (lower Cp values) among delta variants than in the alpha and beta variants was of borderline statistical significance ($p = 0.045$).

Prospective studies with larger Bangladeshi cohorts are warranted to confirm the emergence of S-protein mutations and their association with antibody response in natural infection and potential breakthrough in vaccinated subjects.

Prediction of the occurrence of neutralizing antibodies for SARS-CoV-2 according to the titer of anti-Spike IgG antibodies after vaccination

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Background

Neutralizing antibodies (NAbs) inhibit the binding of the receptor-binding domain (RBD) of the SARS-CoV-2 virus surface spike (S) protein to the human angiotensin-converting enzyme 2 (ACE2) receptor. This interaction disables entrance of the virus to the host cells and may prevent infection and reduce disease severity. Neutralizing antibodies assay measures the real protective immunity for SARS-CoV-2 in comparison with assays which measure all antibodies that can recognize the virus S protein (anti-S-Abs).

Aim

The aim was to assess the possibility to predict the existence of NAbs according to anti-S IgG SARS CoV-2 antibody titer after vaccination.

Materials and Methods

TECO SARS-CoV-2 Neutralization Antibody competition ELISA Assay (TECO medical, Switzerland, TE1076) and S-antigen-specific anti-SARS-CoV-2-IgG CMIA assay (Abbott, North Chicago, IL, USA, I) were used to determine the NAbs and anti-S-IgG antibody titer in 174 persons (133 females, 41 males), median age 40 years (minimum 22, maximum 78), after period of at least 15 days after vaccination with 1st and/or 2nd dose against SARS-CoV-2. According to the manufacturer

declaration NAbs >50 IU/mL was considered positive. Anti-S-IgG-Abs ≥50 BAU/mL has been established as protective by the World Health Organization. ROC curve analysis were performed to determine cut-off, sensitivity (Se) and specificity (Sp) for anti-S-IgG-Ab titer in cohorts classified according to the presence/absence of NAbs and multivariate logistic regression analysis to find which parameter among anti-S IgG Abs, age, height, weight, body mass index (BMI) could be valuable for prediction of NAbs presence.

Results

With NAbs cut-off >50 IU/mL defined as positive, ROC analysis revealed anti-S-IgG-Abs cut off at >451 BAU/mL with sensitivity 94% (95% 88.1 - 97.0) and specificity 94 % (95CI 80.3 - 99.3%), positive and negative likelihood ratio +LR 15,9 and -LR 0,068, respectively, for discrimination between presence and absence of protective immunity. Multivariate logistic regression analysis revealed only anti-S-IgG-Abs as significant for NAbs protective status prediction ($p < 0.0001$) while other variables were insignificant.

Conclusion

Our analysis revealed that anti-S-IgG Abs values above 451 BAU/mL indicate the presence of Nabs with high probability and therefore could be considered as protective for SARS-CoV-2 infection.

Detection and Clinical Relevance of Non-HLA Autoantibodies in Kidney Transplantation

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Abstract

Kidney transplantation is the best option for end stage kidney failure. Despite improvement of kidney transplant results in the last decade, chronic antibody-mediated rejection (ABMR) is still one of the main causes of long-term graft loss. Identification of patients at risk for ABMR remains a challenge. Although the clinical significance and pathogenic role of donor-specific HLA antibodies (DSA) in this process has been well established, the occurrence of ABMR in the absence of DSA is a well-known phenomenon in kidney transplantation. Multiple studies have shown an association between the presence of non-HLA auto-antibodies and kidney graft loss. In a recent multicenter study, the presence of 14 different pretransplant non-HLA antibodies was correlated to renal allograft survival in a nationwide cohort of 4770 recipients transplanted between 1995 and 2006, ensuring an adequate period of follow-up. This showed that auto-antibodies against ARHGDIB/RhoGDI2 were significantly associated with graft loss in recipients transplanted with a deceased-donor kidney. All non-HLA autoantibodies investigated differ completely from the classic autoantibodies known in diagnostics used for connective tissue diseases or other autoimmune disorders. At present, the analysis for prevalence and clinical relevance of non-HLA autoantibodies is hampered due to the broad diversity of autoantigens recognized, and lack of standardized testing methods fulfilling requirements such as reproducibility and definition of antibody concentrations. This has prevented a wide adoption in the clinical routine diagnostics. As the non-HLA field is currently in progress, this highlights the ongoing need for experts in the field of autoimmunity, transplant immunologists and nephrologists to collaborate in order to improve diagnostics and define the role of different non-HLA autoantibodies in kidney transplantation.

Preexisting autoantibodies as predictor of immune related adverse events (irAEs) for advanced solid tumors treated with immune checkpoint inhibitors (ICIs)

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Importance

Immune checkpoint inhibitors (ICIs), used alone or as a combination are standard of care in many cancers. Although generally well tolerated, they can generate immune related adverse events (irAEs). No biomarkers are available to identify patients who are more likely to develop irAEs.

Objective and Design

To assess the association between preexisting autoantibodies and occurrence of irAEs. Prospective single center study

Setting and participants

All consecutive patients receiving ICIs for advanced solid tumors between May 2015 and July 2021 and for which circulating autoantibodies (Anti-Neutrophil Cytoplasmic Antibodies, Antinuclear Antibodies, Rheumatoid Factor anti-Thyroid Peroxidase and anti-Thyroglobulin) have been tested.

Main outcomes and measures

Associations of pre-existing autoantibodies with onset, severity and time to irAEs; associations of pre-existing autoantibodies and irAEs with progression-free survival (PFS) and overall survival (OS).

Results

221 patients were included. Most had renal cell carcinoma (n=99; 45%) or lung carcinoma (n=90; 41%). Treatments received were PD-(L)1 monotherapy (n=162; 73%) and PD(L)1-combinations (n=59; 27%). Using the recommended positivity thresholds, 129 patients (58%) had preexisting autoantibodies (positive group). Grade >1 irAEs were more frequent among patients with preexisting autoantibodies: 64 pts (50%) vs. 20 (22%), (Odds-Ratio= 3.5, [95%CI=1.8-6.8]; $p=2.4^{10-5}$) in the positive vs negative group respectively. IrAEs occurred earlier in the positive group with a median time interval between ICI initiation and irAE of 13 weeks (IQR= 8.8-21.6) vs. 28.5 weeks (IQR=10.6-55.1) in the negative group (p=0.01). Twelve patients (9.4%) experienced multiple (≥2) irAEs in the positive group vs. 2 (2%) in the negative group (OR=4.5, [95%CI:0.98-36], p=0.04). Positive and negative groups did not differ in terms of ICIs exposure (p=0.95). After a median follow-up of 25 months, median PFS and OS were significantly longer among patients experiencing irAE (p=0.00034 and p=0.016, respectively), but did not differ according the presence or absence of preexisting autoantibodies (p=0.09 and p=0.66, respectively).

Conclusion and relevance

The presence of preexisting autoantibodies is significantly associated with the occurrence of grade >1 irAEs in patients treated with ICIs. Earlier and multiple irAEs were observed in the presence of preexisting autoantibodies. Thus, autoantibody detection should be part of the work-up prior to ICI initiation in order to identify patients most at risk of developing irAEs.

Bullous Pemphigoid (BP) induced by Anti-PD1 medication

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Introduction

In recent years, new cancer immune checkpoint inhibitors emerged and anti-programmed cell death protein 1 (anti-PD1) treatment have raised concerns about immune-related adverse events. It has been reported that 1% of treated patients will develop iatrogenic bullous pemphigoid (BP) with atypical clinical and biological presentation. BP is an autoimmune, antibody mediated disease characterized by the formation of large, tense blisters on the skin and mucosal membranes. In classical BP, almost 95% of patients have anti-BP180 directed against NC16A antigen and/or anti-BP230 antibodies (Ab). In literature, only 60% of patients with anti-PD1 induced BP will be positive for anti-NC16A Ab. LAD-1, a cleavage product of the BP180 protein, have been characterized as a targeted antigen in BP induced by anti-PD1 treatment. IgG auto-Ab directed against LAD-1 have been described in 60% of BP including anti-PD1 induced BP, and should regain interest in order to help diagnosis in seronegative BP suspicions.

Methods

A retrospective study in the immunology laboratory department of Toulouse University Hospital was conducted on a panel of 39 patients under anti-PD1 treatment with dermatologic manifestations to evaluate IgG anti-LAD1 Ab prevalence. Autoreactivity against the basement membrane zone (BMZ) has been explored by direct immunofluorescence on skin/mucosa biopsy, when available, to evaluate the local deposit, and in sera with indirect immunofluorescence on monkey esophagus (Werfen) and/or on NaCl-split skin sections (EuroImmun). This was completed with IgG anti-NC16A and BP230 Ab identification by ELISA (Euroimmun). Additionally, IgG anti-LAD 1 Ab were searched by Western blot on human keratinocytes plasmid's treated supernatant. Anti-LAD1 Ab were also searched in 11 BP patients without anti PD1 treatment.

Results

In our cohort, only 23% (9/39) presented anti-NC16A Ab and 33% (13/39) of them were positive at least on indirect immunofluorescence on monkey esophagus and/or NaCl-split skin sections and/or direct immunofluorescence on skin biopsy. 19 patients were tested on Western Blot: regardless of their positivity on anti-BP180 antibodies, 12 (63%) showed positive antibodies directed against LAD-1 antigen. Moreover, 50% of anti-PD1 treated patients have antibodies directed against LAD-1 antigen without anti-NC16A Ab. Only 18% (2/11) of BP patients without anti PD1 treatment had anti-LAD1 Ab.

Conclusion

BP induced by anti-PD1 medication represents an important immune-related adverse event in cancer patients undergoing immunotherapy. An important number of patients under anti-PD1 medication are seronegative to BP180 and BP230 antibodies despite strong clinical presentation and positive indirect immunofluorescence. Our study showed on a cohort of 39 patients under anti-PD1 treatment and with dermatological manifestations that anti-LAD1 Ab detection could be helpful for BP diagnosis. It is now necessary to study if anti-LAD1 Ab precede anti-PD1 treatment or if it precedes clinical BP expression.

8 Update on Anti-DFS70 Autoantibodies

Recent advances on the biology of the DFS70/LEDGFp75 autoantigen: clues for understanding the significance of the anti-DFS autoantibody response

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Abstract

The monospecific dense fine speckled (DFS) immunofluorescence assay (IFA) pattern (ICAP AC-2) is considered a potential marker to aid in the exclusion of antinuclear antibody associated rheumatic diseases (AARD). This pattern is typically produced by autoantibodies against the stress transcription co-activator and active chromatin reader DFS70, also known as LEDGFp75 and PSIP1. Monospecific anti-DFS70/LEDGFp75 autoantibodies are frequently found in healthy individuals and patients with miscellaneous inflammatory conditions. These antibodies also circulate in some patients with AARD, albeit generally mixed with other disease-associated antibodies. The biological and clinical significance of the DFS IFA pattern remains elusive, which warrants more detailed studies on the anti-DFS70/LEDGFp75 antibody-antigen system. Our recent studies showed that the DFS IFA pattern is not exclusively produced by anti-DFS70/LEDGFp75 antibodies since a very similar pattern, called "DFS-like" or "pseudo-DFS", does not appear to target DFS70/LEDGFp75. High resolution confocal microscopy analysis showed that the target antigen(s) recognized by the "pseudo-DFS" antibodies co-localize with DFS70/LEDGF/p75 and its interacting partners H3K36me2, a marker of active chromatin, and MLL, an oncogenic transcription factor. This suggested that these antibodies may be also directed against active chromatin-associated antigen(s), possibly including interacting partners of DFS70/LEDGF/p75. However, immunoblotting studies by our group and others using "pseudo-DFS" sera failed to show immunoreactivity to any specific proteins, suggesting that these sera may recognize diverse proteins or conformational epitopes within DFS70/LEDGFp75. Recent efforts from our group and others have also focused on understanding DFS70/LEDGFp75 regulation, biological functions, protein-protein interactions, and gene targets in the context of resistance to environmental

stress. We have developed a cellular model that endogenously overexpresses this protein and its interacting partners, consisting of cancer cells gradually selected to develop resistance to chemotherapeutic drugs. Using this model, we have shown that the expression of DFS70/LEDGFp75 is upregulated by glucocorticoids via the glucocorticoid receptor (GR), and that DFS70/LEDGFp75 interacts in active chromatin with GR and other oncogenic factors to promote cancer cell survival against drug-induced stress. RNAseq analysis of drug-resistant cells depleted of DFS70/LEDGFp75 indicated a role for this autoantigen in the regulation of gene pathways associated with cell death and survival under stress as well as immune function. An in-depth understanding of the biology of DFS70/LEDGFp75 is critical to elucidate the clinical significance and diagnostic utility of the DFS and "pseudo-DFS" autoantibodies, and develop novel therapeutic strategies for cancer and autoimmune diseases.

Anti-DFS70 autoantibodies: is there any evidence in support of underlying molecular mimicry?

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Abstract

Anti-nuclear antibodies (ANA) are frequently found in patients with autoimmune rheumatic diseases, as well as in other autoimmune disorders. Their targets are heterogenous. By in large, ANAs can be divided in three broad categories: disease-specific, disease-related and disease-irrelevant or unspecific. It must be noted that various reports have demonstrated the presence of ANAs in apparently healthy individuals, mostly adults at a frequency ranging in between 12-35%.

The presence of these autoantibodies in autoimmune diseases has been attributed amongst others to infectious-induced polyclonal activation or an antigen-driven loss of immunological tolerance triggered by pathogens or other environmental factors. Epstein-Barr virus is a viral trigger of autoimmunity which induces polyclonal activation and development of ANAs by various mechanisms including that of B- and T-cell molecular mimicry between viral and nuclear mimics. So far, molecular mimicry and immunological mimicry involving EBV and nuclear autoantigens has been considered a likely mechanism which could explain the presence of ANA targeting RNP, centromere A and B, scleroderma 70 (DNA topoisomerase-I),

Another virus which has frequently linked with autoimmunity is that of human cytomegalovirus (HCMV), arguably reported as a probable inducer of various antigen-specific ANA. Amongst bacteria, several have been considered inducers of autoantibodies and indeed autoimmune phenomena, the focus of intense interest being that of Helicobacter pylori (Hp). We and others provided data in support (but also against) of the role of this bacterium in bridging infection and autoimmunity, and highlighted the complex nature of host-microbial interactions, and their relation to the loss of immunological tolerance to disease-specific autoantigens.

The recent demonstration of ANAs against an antigen giving a dense fine speckled pattern (anti-DFS70), being present in a significant proportion of patients with autoimmune and non-autoimmune rheumatic diseases, as well as in apparently healthy individuals has raised the

expectation that a common virus or a bacterium may indeed be responsible for their induction. If this holds true, detection of anti-DFS70 could be considered an epiphenomenon i.e a results of 'false positivity' due to a cross-reaction implicating one or more foreign pathogens.

On the basis of the above and in view of the enormous potential of EBV, CMV and Hp infection to induce disease-specific or disease-related ANA, as well as the fact that infection with these pathogens is very frequent in adulthood, we considered that any of those could be the likely candidates that may trigger anti-DFS70 antibodies.

To address our hypothesis, we followed two approaches. The first was to consider whether DSF70 specific antibodies are able to cross-react with EBV- or CMV- or Hp-specific antigens. The second was to attempt to identify a specific association between anti-DFS70 and EBV or CMV or Hp-specific antigens. We comprehensively defined pathogen-related antigen-specific antibody reactivity in an anti-DFS70 positive cohort of serum samples from healthy as well as disease individuals (affected with various diseases) and compared it with that noted in a cohort of anti-DFS70 antibody negative sera. In the present study, we present the data of our experimental study.

Over-expression of LEDGF/p75 / DFS70 in HEp-2 cells enhances autoimmune IgG response in patients with benign prostatic hyperplasia - a novel diagnostic approach with therapeutic consequence?

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Abstract

Benign prostate hyperplasia (BPH) is an enlargement of the prostate with a prevalence of 50% to 75% in men 50 years of age and older. The pathophysiology of BPH is not yet fully elucidated, but for almost 100 years, the possibility of autoimmune disease has always played a role. Therefore, autoantibody (autoAb) testing could aid in the diagnosis of BPH patients profiting from a corresponding immunosuppressive therapy by TNFalpha antagonists.

Lens epithelium-derived growth factor-splice variant of 75kDa (LEDGF/p75), also known as DFS70, is an autoantigen which is over-expressed in solid tumors and acts as a stress-related transcriptional co-activator. Thus, we generated CRISPR/Cas9 modified HEp-2 LEDGF knock-out (KO) and HEp-2 LEDGF/p75 over-expressing (OE) cells and examined IgG autoantibody reactivity to LEDGF/p75 in patients with prostate cancer (PCa, n=89), bladder cancer (BCa, n=116), benign prostatic hyperplasia (BPH, n=103), and blood donors (BD, n=60) by indirect immunofluorescence assay (IFA).

Surprisingly, we could not detect elevated binding of autoAbs against LEDGF/p75 in cancer patients, but an unexpectedly significantly increased autoAb reactivity to LEDGF/p75 OE cells in about 50% of patients with BPH. Furthermore, a line immunoassay enabling the detection of 18 different autoAbs revealed a significantly increased occurrence of anti-dsDNA autoAbs in 34% of BPH patients in contrast to tumor patients and BD. We could confirm this finding by anti-mitochondrial (mDNA) autoAb detection with the Crithidia luciliae immunofluorescence test that also showed a significantly higher prevalence (34%) of anti-mDNA autoAbs in BPH.

In summary, our study provided further evidence for the occurrence of autoimmune responses in BPH. Furthermore, LEDGF/p75 over-expression renders HEp-2 cells more autoantigenic and a perfect target for autoAb analysis in BPH with a potential therapy consequence.

Silencing the DFS70/LEDGFp75 autoantigen reveals potential roles in lymphocyte function and immune evasion

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Abstract

The dense fine speckled (DFS) antinuclear autoantibody (ANA) pattern is generated by autoantibodies targeting the DFS70 autoantigen, which is also known as lens epithelium derived growth factor p75 (LEDGFp75). Patients with ANA-associated autoimmune rheumatic diseases (AARD) generally have low frequencies of monospecific anti-DFS antibodies, compared to healthy individuals and patients with certain non-AARD inflammatory diseases.

Elucidating the biological functions of DFS70/LEDGFp75 in different disease contexts is crucial to enhance our understanding of the clinical and biological significance of these autoantibodies. DFS70/LEDGFp75 is a cellular survival protein that is naturally upregulated in cancer cells exposed to environmental stressors, including cytotoxic drugs, and that plays an important role in cellular protection against stress-induced cell death. The C-terminus of DFS70/LEDGFp75 contains the Integrase Binding Domain (IBD), which binds to HIV-integrase complex. Its N-terminal PWWP domain tethers this complex to active chromatin sites to promote viral integration and replication. The IBD also corresponds to the autoepitope recognized by the anti-DFS autoantibodies. In cancer cells, the IBD serves as the binding site for multiple oncogenic transcription factors, such as JPO2, PogZ, Menin, and MLL, that co-localize with DFS70/LEDGFp75 in the nucleus and regulate the transcription of cancer-related genes. Since cancer cells, especially those that develop resistance to specific chemotherapeutic drugs, upregulate the expression of DFS70/LEDGFp75, they serve as powerful models to define the functions of this autoantigen.

This study was designed to gain mechanistic insights on the biological functions of DFS70/LEDGFp75 in docetaxel (DTX) resistant prostate cancer (PCa) cells, which express high levels of this protein. To evaluate the functions of DFS70/LEDGFp75 in this cellular model, we silenced its expression via RNA interference, followed by RNA-sequencing analysis. The silencing

of DFS70/LEDGFp75 in chemoresistant PCa cells led to the identification of 970 differentially expressed genes (DEGs), and pathway analysis was conducted using gene set enrichment analysis (GSEA). We confirmed the efficiency of the DFS70/LEDGFp75 silencing using immunoblotting and by evaluating the expression of previously established genes regulated by this autoantigen. GSEA analysis revealed a role for DFS70/LEDGFp75 in B cell and T cell pathways. We also identified DEGs involved in immune evasion in cancer cells, thus implicating DFS70/LEDGFp75 as a potential target for cancer treatment. In addition, we assessed the cytotoxicity of candidate DFS70/LEDGFp75 inhibitors in DTX-resistant PCa cell lines. Our initial results showed increased cytotoxicity upon treatment with these inhibitors in the presence or absence of DTX.

Knowledge of the role of DFS70/LEDGFp75 in B-cell and T-cell immunity may provide important insights on the biological relevance of the anti-DFS70 autoantibodies. It may also reveal the potential of this autoantigen as a therapeutic target for the treatment of patients with cancer and, possibly, certain inflammatory diseases.

The presence of antibodies against DFS70 antigen are not correlated with antibodies against infectious antigens

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Introduction

How antibodies against dense fine speckled-70 antigen (anti-DFS70) arise remains obscure. Human Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Helicobacter pylori (Hp) infection are considered common triggers of autoantibody production but whether they can induce anti-DFS70 antibodies remains obscure.

Aim

To assess antigen-specific antibody responses against specific, CMV, EBV and Hp in anti-DFS70 positive and anti-DFS70 negative patients with various autoimmune diseases.

Material and Methods: Fifteen anti-DFS70 positive patients (12 females; median age 42yrs, range 11-70yrs) with various autoimmune and non autoimmune diseases were studied. Serum samples from 22 demographically matched (age, sex, ethnicity) anti-DFS70 negative patients (13 females; median age 57 yrs, range 17-77yrs) (either with psoriasis or with psoriatic arthritis) were used as controls. Reactivity to CMV EBV, Hp-specific antigens was tested by western immunoblotting, (Euroimmun).

Results

CMV: Magnitude of antibodies against pp28 CMV antigen were found higher in anti-DFS70 negative patients than positive patients (51.5 ± 11.2 vs 33 ± 7.2, p=0.04) EBV: Abs against p79

EBNA, p42-VCA and p33-VCA were found more frequently in anti-DFS70 negative than positive patients (22/22,100% vs 8/15,53.5%; p=0.001, 10/22,45.5% vs 0/15,0%; p=0.002, 13/22,59.1% vs 2/15, 13.3%; p=0.007 respectively). The magnitude of antibody response against p33-VCA antigen were stronger in anti-DFS70 negative compared to anti-DFS70 positive patients (57.1 ± 22.7 vs 36 ± 5.6; p=0.022) while on the contrary response against p22-VCA antigen were stronger in anti-DFS70 positive patients (117.5 ± 334.5 vs 95 ± 20.6; p=0.048). Hp: Only a trend of association was found of the presence of anti-p26 Hp in anti-DFS70 positive patients (100% vs 44.4%, p=0.089)

Conclusion

Our data do not support any significant association between antibody responses to CMV. EBV and Hp antigens and DFS70, raising concerns as to whether these infectious agents are a likely trigger of these autoantibodies.