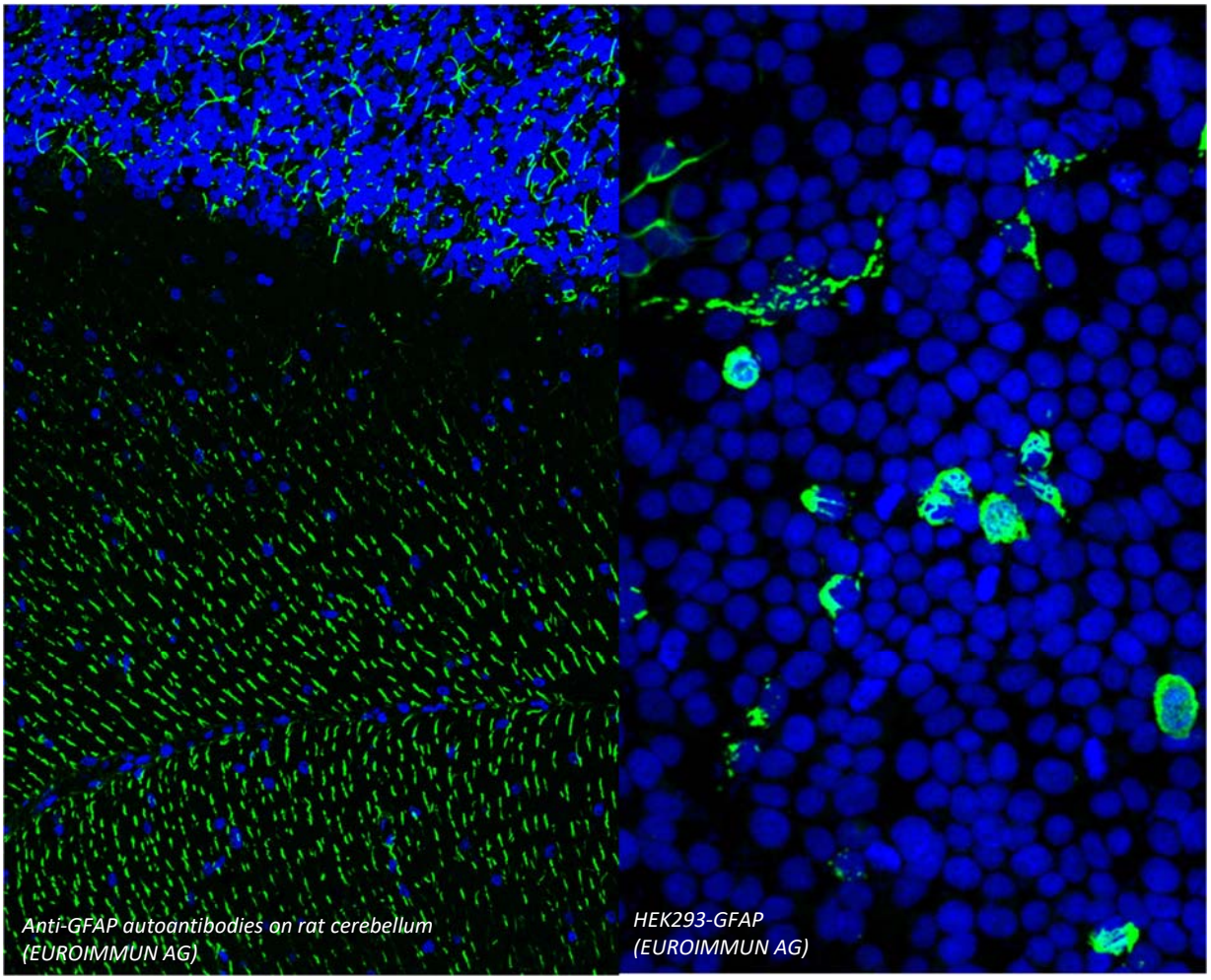
Karsten Conrad, Jan Damoiseaux, Christian Moritz, Luis E. C. Andrade (Eds.)

The Autoantibody Spectrum: Bridging Health, Diagnostics, and Disease

Abstracts of the 17th Dresden Symposium on Autoantibodies, September 08/09-12, 2025



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Gesellschaft zur Förderung der Immundiagnostik e.V.
Dresden

http://www.GFID-eV.de

Manufacturer: EUROIMMUN AG, Lübeck, Germany
Proof reading: Dr. Silke Zwjatkow, Dresden, Germany
Typesetting: Dr. Rico Hiemann, Thiendorf, Germany
Cover: Dr. Rico Hiemann, Thiendorf, Germany
Cover figure: EUROIMMUN AG Lübeck, Germany

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Preface

A plethora of autoantibodies has been discovered since the concept of horror autotoxicus was overruled by studies of Noel Rose in animal models of thyroid autoimmunity. Although multiple relevant biomarkers, not being autoantibodies, have been described over the years, the high specificity of autoantibodies has made them important and superior diagnostic tools in a wide spectrum of autoimmune diseases. Beyond diagnosis, autoantibodies have increasingly been shown to be pathogenic, predictive and prognostic. The ongoing quest for discovering novel autoantibodies and additional applications warrants the organization of the 17th Dresden Symposium on Autoantibodies, entitled "The Autoantibody Spectrum: Bridging Health, Diagnostics, and Disease". This symposium is organized in appreciation of Prof. Eng M. Tan's outstanding merits in the field of autoimmunity.

The current symposium covers the role of autoantibodies in multiple autoimmune diseases varying from traditional autoantibodies in the systemic autoimmune rheumatic diseases to the novel spectrum of autoantibodies more recently discovered in neurological autoimmune diseases. With the continuous discovery of new autoantibodies, novel detections methods and distinct commercial assays, the challenge is to find the best way to interpret the results obtained either in the clinical context of the patient or for classification of patients to be included in clinical studies. As such, the concept of likelihood ratios of test-result intervals will be further elaborated upon. In addition, creating awareness that for monitoring patients by autoantibody levels distinct interpretation modes are required, will be addressed.

Special attention is paid to the concept of natural autoantibodies, including those against the physiological network of G protein-coupled receptors, the pathophysiology of IgG4 auto-antibodies in IgG4-mediated autoimmune diseases, and the generation of autoimmune phenomena upon treatment of patients with malignancies with immune checkpoint inhibitors.

Hopefully, the data, viewpoints and discussions in this volume will further stimulate collaborative initiatives to improve diagnosis and management of the wide spectrum of autoimmune diseases.

The editors

1 Introduction and Keynotes

Autoantibodies - Increasing importance in diagnostics and research

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Disease specific autoantibodies (AABs) play an important role in the diagnosis of most of the known autoimmune diseases (AIDs) as well as in the research of their pathogenesis. More and more of those AABs are or will be part of classification criteria of defined AIDs. But also non-disease specific AABs may become more relevance in monitoring of disease course (activity, severity, manifestations) and therapy response.

Autoimmune disorders recently defined by AABs

The search for novel AABs may identify novel autoimmune systemic (e.g., subtypes of idiopathic inflammatory myopathies) or organ specific (e.g., neurologic, nephrologic, dermatologic, hematologic, and cardiovascular autoimmune manifestations) entities or clinical subtypes. The majority of novel entities where AABs are definitely or probably pathogenic and are of diagnostic and therapeutic relevance were found in the last two decades in the broad spectrum of neurologic diseases (COLLET et al., 2023; GILLIGAN et al., 2024). Among the group of neuronal surface antibody syndromes (NSAS) different kinds of autoimmune encephalopathies have been described so far (BOKHARI et al., 2024; DALMAU & GRAUS, 2023). Furthermore, immune-mediated diseases responsible for symptomatic combined central/peripheral nervous system involvement (ICCPs), that means syndromes of peripheral neuropathies combined with CNS features, are an important group of autoimmune pathogenesis (LEBOYAN et. al., 2022). More and more neurologic entities with characteristic AABs were identified at short intervals using modern methods of AAB discovery like cell-based assays using neurons and astrocytes derived from human-induced pluripotent stem cells (Mathias et al., 2024). Because of the variety and complexity of these disorders, and the resemblance of symptoms to those of infectious and other inflammatory neuronal disorders, the neural (neuronal and glial) AAB determination becomes a very important diagnostic approach (DALMAU & GRAUS, 2023). The differentiation from non-autoimmune entities can lead to the improvement of the management of AAB mediated neuronal diseases by the early introduction of the appropriate and effective immune therapy (e.g., plasma exchange, IVIg, B cell depletion, CAR-T cell therapy). Examples of recently defined autoimmune neurologic disorders and their AAB specificities are shown in table 1.

Besides autoimmune neurologic diseases, novel autoimmune entities have been identified in nearly all organ systems. A rare autonomic cardiovascular autoimmune manifestation is the **postural orthostatic tachycardia syndrome (POTS)** with ganglionic A3 acetylcholine receptor and α1-and β1/2 adrenergic receptor antibodies (DAHAN et al., 2016). Human **podocytopathies**

include focal and segmental glomerulosclerosis, minimal change disease, membranous nephropathy, collapsing glomerulopathy and diabetic nephropathy. The early identification of autoimmune podocytopathy subtypes with pathologic AABs targeting nephrin, phospholipase A2 receptor (PLA2R), or thrombospondin type 1 domain–containing 7A (THSD7A) can prevent the progression to end-stage renal disease by early effective immune therapy such as B cell depletion (HENGEL et al., 2024). Further examples of other entities whose clinical course and prognosis can be improved by an early diagnosis through defined AABs followed by an effective immune therapy are listened below:

- **Acquired Generalized Lipodystrophy (AGL):** A recent study shows that AABs targeting perilipin 1(anti-PLINl) are useful biomarkers for diagnosis of AGL. The association anti-PLINl antibodies with metabolic worsening suggest that they may also be a prognostic biomarker. The capacity anti-PLINl AABs to block PLIN1/ABHD5 interactions provides a mechanistic explanation to the pathophysiology of the disease and opens the possibility for novel therapeutic strategies (CORVILLO et al., 2023).
- **Autoimmune chronic spontaneous urticaria (type IIb aiCSU)**: This endotype is associated with mast cell-activating IgG AABs against high-affinity IgE receptor (FcεRI) or FcεRI-bound IgE. Type II aiCSU is characterized by high disease activity, autoimmune comorbidities such as Hashimoto's thyroiditis and vitiligo, low total IgE levels, and poor response to antihistamines and omalizumab. Novel immune therapies like dupilimumab (inhibition of IL4Ra), barzolvolimab (inhibition of KIT activation by stem cell factor that leads to depletion of skin mast cells) or remibrutinib (inhibition of the Bruton tyrosinkinase) seem to be effective (KOLKHIR et al., 2025; LANG et al., 2025).
- **Immune thrombocytopenia (ITP)**, an acquired autoimmune disorder characterized by low platelets and an increased risk of bleeding caused by platelet AABs that target major platelet glycoproteins (e.g., GPIIbIIIa and anti-GPIbIX) and cause Fc-mediated platelet destruction in the spleen and reticuloendothelial systems. Because platelet AABs are postulated to be the principal mechanism of ITP, targeting this pathway (e.g., FcRn inhibitors and new BTK inhibitors) as novel treatment approaches may improve efficacy and limit short and long term toxicities (DALMIA et al., 2024).
- **Immune thrombotic thrombocytopenic purpura (iTTP)** with AABs against the metalloprotease ADAMTS13 leading to catastrophic microvascular thrombosis. A case report showed that the administration of recombinant human ADAMTS13 may be a novel adjunctive therapy in iTTP (BENDAPUDI et al., 2024).
- **Atypical hemolytic uremic syndrome (aHUS**), a devastating form of thrombotic microangiopathy (TMA) predominantly involving the kidney, is a rare but life-threatening systemic disorder caused by the dysregulation of the complement alternative C3 convertase of the complement pathway probably by AABs targeting Factor H of the complement system. The binding of C5 by eculizumab leading to the blockade of the terminal complement activation has become the cornerstone of the treatment of aHUS. However, this therapeutic breakthrough has been dulled by persistent difficulties in the positive diagnosis of aHUS (Fakhouri et al., 2023).
- **Vaccine-induced immune thrombotic thrombocytopenia (VITT)** is a rare but dangerous side effect of adenoviral vectored COVID-19 vaccines linked to production of AABs recognizing platelet factor 4 (PF4). In case of suspicion of VITT, patients should be tested for anti-PF4 antibodies and treatment targeting autoimmune processes with intravenous immunoglobulin and prothrombotic processes with non-heparin anticoagulation should be initiated (KLOK et al., 2021).

In some of disease conditions, AABs against mediators and effectors of the immune system may play an important role, e.g., AABs targeting interleukins, interferons, cytokine and chemokine receptors, complement factors as well as components of the innate immune system.

Autoantibodies against immune system components

AAB against pentraxins (PTX), the soluble innate immunity receptors involved in sensing danger molecules, have been reported in various autoimmune diseases, especially in systemic lupus erythematosus and ANCA-associated vasculitis. The major antigenic targets are the short pentraxins C reactive protein (CRP/PTX1) and serum amyloid P component (SAP/PTX2) as well as the long pentraxin PTX3. In SLE, anti-CRP and anti-SAP antibodies reflect disease activity rather than a specific pattern of SLE nephritis. On the other side, anti-PTX3 autoantibodies might provide protection from renal involvement as they represent an independent factor negatively associated with kidney injury (BRILLAND et al., 2021). In RA patients, elevated anti-PTX3 antibodies indicate residual active disease despite controlled inflammation. They may serve as a biomarker for true active disease, especially in seronegative RA patients who might be undertreated (Salvato et al., 2025). Because of the multifactorial role of pentraxins, the interplay of PTX and anti PTX antibodies may be an interesting model to elucidate the complexity of the potential pathogenic and/or protective role of anti-PTX antibodies. Based on the role of pentraxins in efferocytosis, it is tempting to speculate that these AABs could alter the efferocytosis process and, consequently, the outcome of immune responses against self-antigens. Furthermore, these AABs could re-confer immunogenicity to apoptotic cells by promoting the presentation of apoptotic cell-associated antigens by professional APCs and, consequently, the priming and/or expansion of self-reactive T cells (BRILLAND et al., 2021). However, the complexity regarding cross-reactivity, differences in epitope recognition, the isotypes and the sides of production of anti-PTX antibodies as well as the immune status of the tissue at the time of efferocytosis (such as chronic inflammation) may have important consequences on the regulatory versus activating potential of these autoantibodies and could lead to misinterpretation of the roles of anti-PTX Abs in the pathogenesis of autoimmune diseases.

Neutralizing AABs targeting mediators of the immune system may elucidate their physiologic or pathologic role in different kind of diseases as well as in the spectrum of biologic functions in defence against infections as shown by the following examples:

- Recently, the potential role of **AABs neutralizing the interleukin-1 (IL-1)-receptor antagonist (IL1-Ra)** in the pathogenesis of a subgroup of adult-onset Still's disease (AOSD) and systemic juvenile idiopathic arthritis (sJIA) has been described. Anti-IL1-Ra antibody positive Still's disease patients were either new-onset active disease or unresponsive to IL-1 blocking drugs and showed decreased IL1Ra plasma/serum levels. The in vitro impaired IL-1Ra bioactivity caused by these AABs could be reversed by therapy with recombinant human IL -1 receptor antagonist (anakinra) or a fully human interleukin-1b inhibiting monoclonal antibody (canakinumab) (Hoffmann et al., 2024).
- **Naturally occurring autoantibodies targeting chemokines** were found in tumors and autoimmune diseases. In few reports, the role and functions of anti-chemokine AABs have been described: They can promote or prolong chemokine activities. On the other hand, thanks to their neutralizing properties, they may be beneficial to the host, through

dampening excessive inflammation (Cecchinato, et al., 2023). AABs targeting the chemokine **CXCL8** are shown to be associated with adverse outcome of acute respiratory distress syndrome and shows proinflammatory activity in patients with asthma and RA. A beneficial role of anti-chemokine antibodies with neutralizing properties has been described for **anti-CCL3** antibodies in type 1 diabetes mellitus and atopic dermatitis. **Anti-CCL2** antibodies limited tumor growth in patients with prostate cancer. In COVID-19 patients, the neutralizing AABs targeting several chemokines, including **CCL19, CCL22 and CXCL17** could be beneficial in dampening the inflammatory response, preventing hyperactivation of the immune system and tissue damage associated to severe COVID-19. Furthermore, it was shown that ABBs against **CCL21, CXCL13 and CXCL16** are present in individuals that do not develop long COVID. In conclusion, AABs targeting chemokines sustain pathology or prevent adverse outcome by either neutralizing their activities or promoting Fc-mediated functions. The authors believe that all these features should be taken into account for future drug development studies and novel approaches into personalized medicine (Cecchinato, et al., 2023).

- AABs against the the CXC-motif-chemokine receptor 3 (**CXCR3**), a G protein-coupled receptor with a key role in atherosclerosis, may predict cardiovascular risk. It was shown that anti-CXCR3 AAbs in individuals free of autoimmune disease were related to cardiovascular end-organ damage and predicted cardiac morbidity and mortality in conjunction with the acceleration of experimental atherosclerosis (MÜLLER, et al. 2023).
- In patients suffering from **late-onset primary immune deficiency** with unusual susceptibility to mycobacteria, fungi, and viruses, AABs against different cytokines, including so far **IL-6, IL-17/22, CSFR, GM-CSF**, and interferons were identified (NETEA e al, 2024). Recently, also AABs targeting **IL-23** were described to be associated with opportunistic mycobacterial, bacterial, and fungal infections in patients with thymoma (CHENG et al., 2024). The authors suggest that these results should lead to the design of new diagnostic tests and therapeutic approaches based on anti-IL-23 AABs in patients with rare and severe infections of unknown cause.
- High titers of **AABs neutralizing GM-CSF** have been reported in patients with idiopathic pulmonary alveolar proteinosis (PAP). They were also discovered in patients with cerebral nocardiosis or cryptococcosis.
- In autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (**APECED**, or autoimmune polyendocrine syndrome 1), the presence of neutralizing AABs against type-17 cytokines (**IL-17A, IL-17F, IL-22**) and **type-I interferons** (IFNω and the majority of the 13 subtypes of IFN-α) is associated with chronic mucocutaneous candidiasis (CMC), the "signature" infectious disease in APECED patients (FERRE et al., 2021).
- The **adult-onset immunodeficiency (AOID)** syndrome (anti-interferon-gamma autoantibody-associated immunodeficiency syndrome) was described as an immune-deficiency syndrome related to the presence of AABs targeting **interferon-γ** (**IFN-γ**) in adults with multiple opportunistic infections similar to that observed in patients with advanced human immunodeficiency virus (HIV) infection. Disseminated nontuberculous mycobacterial (NTM) infection is the most common infectious disease, but Talaromyces marneffei, Cryptococcus neoformans, Histoplasma capsulatum, Burkholderia pseudomallei, Salmonella species, and Varicella–zoster virus are also frequently identified as opportunistic pathogens in this patient group. The potential pathogenicity may be concluded by the difference in the neutralizing capacity of anti-IFN-γ AABs found in patients and non-neutralizing antibodies in healthy populations. This is further supported by the observation that patients with anti-IFN-γ AABs without neutralizing capacity did

not have opportunistic infections. Cyclophosphamide or B cell depletion therapy has been reported to reduce anti-IFN-γ AAB titers effectively and might be used as adjuvant therapies combined with antimicrobial therapy for AAB positive patients with a refractory opportunistic infection (CHAWANSUNTATI et al, 2021).

- AABs targeting **type I Interferons** (**IFN-α, IFN-β, IFN-ω**) increase the susceptibility to severe diseases caused by certain viruses, including SARS-CoV-2, herpes zoster, and varicella pneumonia and represent a form of **acquired immunodeficiency.** Many studies have demonstrated that AABs to type I IFN were detected in plasma or serum from patients with **severe or critical COVID-19**. Most AABs detected in COVID-19 patients were directed against IFN-α2 or IFN-ω, and AABs to IFN-β were very rare. It has been shown that AABs targeting type I IFN in the plasma from COVID-19 patients suppressed IFN-α2-induced STAT1 activation, suggesting the possible neutralizing activity of these AABs. An autoimmune phenocopy of inborn errors of type I IFN immunity **accounts for life-threatening COVID-19 pneumonia.** Removal of IFN-I AABs by plasma exchange or plasmablast depletion may rescue life-threatening cases of COVID-19. Therefore, the detection of AABs to type I IFNs might be an appropriate treatment to patients with COVID-19. Considering the possibility that other emerging viral infectious diseases may appear in the near future, large-scale screening of AAB carriers to type I IFNs and the other cytokines would be beneficial (AOKI et al., 2024).
- IFN-I AABs were further identified in patients with chronic graft-versus-host disease following allogeneic bone marrow transplantation, pemphigus foliaceus and **SLE**. They are less prevalent in other AIDs like Sjøgren's disease and rheumatoid arthritis, where reported frequencies depend on the cohorts, subtypes of disease, IFN-I AAB assays and "threshold level". In SLE patients, a high titre of anti-IFNα may protect against lupus flare by inhibiting IFNα signalling but predispose to COVID-19. A strong IFNα production induced by a virus like SARS-Cov-2 may overpass the protective role of anti-IFNα in SLE and thus contribute to lupus flare.
- AABs against IFN-α might be associated with the less aggressive autoimmunity in latent autoimmune diabetes in adults **(LADA)** compared to early-onset type 1 diabetes (T1D). Autoimmunity against IFN-α is peculiar to autoimmune diabetes and appears to be distinctive to its slowly progressive forms. Understanding the underlying molecular mechanisms and clinical significance of this novel autoimmunity could lead to the development of new therapeutic strategies in autoimmune diseases, advancing personalized medicine (Amendolara et al., 2025).
- AABs to type I IFNs can also be observed in patients with **autoimmune polyglandular syndrome (APECED**). The likelihood of developing IFN-I autoimmunity follows a spectrum from ~100% in AIRE deficiency, to various, but lower, frequencies in other genetic disorders that affect the development and function of the thymus and is also reflected by dominant AIRE mutations. Patients with systemic autoimmune disorders probably need additional environmental triggers, such as infections, drugs or vaccines, to develop IFN-I autoimmunity. (OFTEDAL et al., 2024). High titre of neutralizing AABs to most IFN-α subtypes and especially IFN-ω (60% homologous to IFN-α) were found - mostly in the earliest samples. Lower titres were detected against IFN-β (30% homologous to IFN-α) (BASTARD et al., 2024). Anti IFN-I AABs may have practical value in the clinic, e.g., in diagnosing unusual or prodromal AIRE-mutant patients with only single components of APS1, and possibly in prognosis if they prove to predict its onset (MEAGER et al., 2006). Interestingly, patients with APECED and acquired thymoma-associated myasthenia gravis (MG) share several common features, including defective expression of the transcription

factor AIRE and AABs targeting against type I interferons. More than 70% of MG patients with thymoma were found to have high-titer neutralizing AABs to IFN-α. Titers increase strikingly if the thymomas recur, indicating a closer tumor relationship than for anti-AChR. AABs against IFN-ω, -α2, and -α8 occurred more often in MG patients with thymoma (73%) than in late-onset MG (39%) and early-onset MG (5%). These AABs showed preferences for IFN-ω in APS I and for the IFN-α in MG, hinting at thymic aberrations in both groups. The exact profile of type I IFN antibodies may indicate MG subtype and may hint at thymoma recurrence (HAPNES et al. 2012; SHIONO et al., 2003).

- Patient with **Inborn Errors of the Immune System** (IEI) should be screened for AABs targeting type I IFNs, particularly those with known genetic etiologies of AABs against type I IFNs. Children with a history of unusually severe viral infection should also be tested. **Children with AABs neutralizing type I IFNs** should be followed prospectively and should be vaccinated against SARS-CoV-2 and influenza, but not with live-attenuated vaccines. Finally, it would be of interest to conduct pilot studies of the screening of selected populations, such as children with autoimmune conditions (e.g., SLE, which is associated with these AABs in adults) (BASTARD et al., 2024)

Autoimmune phenomena in a variety of disease manifestations

Novel as well as known AABs may be also of help in undercovering of autoimmune phenomena in a variety of disease manifestations:

- **IgG4-associated autoimmunity:** AABs of the IgG4 subtype are found in a range of different diseases (autoimmune and allergic diseases, helminth infection, cancer). The most clinical relevant disease groups are IgG4-related and IgG4 mediated autoimmune diseases:

  IgG4-related diseases (IgG4RD), recognized as a novel clinical entity, is a rare, chronic, immune-mediated systemic fibroinflammatory disorder of unknown origin characterized by organ enlargement and fibrosis leading to functional impairment. These manifestations include “autoimmune” pancreatitis, retroperitoneal fibrosis, fibrosing mediastinitis, Riedel thyroiditis, orbital pseudotumor, and hypertrophic pachymeningitis, among others (Kamisawa et al., 2015). An autoimmune mechanism is strongly suspected in the pathogenesis of IgG4-RD: IgG4 antibodies may directly cause the disease by targeting self-antigens or may modulate an immune response based on different pathways. As potential autoantigens galectin-3, IL-1Ra, prohibitin 1, annexin A 11, and laminin 511-E8 (42) were described so far (MOTTA & CULVER, 2024). Although the pathogenic mechanism remains unclear, possible multipathogenic factors such as genetic backgrounds, disease-specific or related antigens, and abnormal innate or adaptive immunity may be involved. Many immunocytes, including B and T-cells (Th2-$CD4^+$T, follicular helper T cells, and $CD4^+SLAMF7^+$cytotoxic T cells) play important roles in the pathogenesis. Conventional therapies with glucocorticoid or rituximab in combination with or without immunomodulators are recommended in all symptomatic patients with active IgG4-RD. Because of the few of randomized clinical trials, the comprehensive management for IgG4-RD has not been established yet. Targeted treatment approaches against the B cells and the $CD4^+SLAMF7^+$cytotoxic T cell seem to be promising for the future-directed treatment (OKAZAKI et al., 2025).

IgG4 mediated autoimmune diseases are characterized by the presence of disease specific AABs of the IgG4 subclass and contain well-characterized diseases such as myasthenia gravis with MuSK antibodies, pemphigus with dsg1 and dsg3 antibodies, thrombotic thrombocytopenic purpura with ADAMTS3 antibodies, membranous nephropathy with PLA2R and THSDA7A antibodies, anti-IgLON5 disease, and further IgG4 mediated autoimmune neurologic diseases (HUIJBERS et al., 2015, KONECZNY et al., 2021). The list of IgG4 autoantigens is rapidly growing and contains more than 29 candidate antigens. So far, IgG4 autoimmune diseases are restricted to four distinct organs: the central and peripheral nervous system, the kidney, the skin and mucous membranes and the vascular system. The AABs may be protective or pathogenic. The pathogenicity of IgG4 is usually excerted by functional blocking of protein-protein interaction (KONECZNY 2020).

- **IgE-associated autoimmunity** is generally termed "**autoallergy**" and the target molecules of the response are called "autoallergens", IgE reactivity against autoantigens has been found in "classical" AIDs along with the typical IgG (and IgM or IgA) AABs: in SLE patients targeting nucleosomes dsDNA, SS-A/Ro, SS-B/La, Sm, and RNP; in RA patients targeting nuclear antigens; in patients with Graves disease targeting thyreoperoxidases and muscle antigens; and in patients with autoimmune blistering diseases targeting BP180, BP320, dsg1, dsg3, and laminin-332. The pathogenic and/or clinical relevance of these IgE reactivities are not clear yet. In SLE, dsDNA-specific IgE may be a reasonable clinical indicator of increased disease activity; in RA patients IgE-ANA is associated with neutropenia; in pemphigus vulgaris a strong correlation between dsg3-reactive IgE has been observed in patients with acute disease onset, indicating a role for auto-IgE in pemphigus (MAURER et al, 2018). In another group of disease, IgE-mediated autoimmunity plays a more important pathogenetic and diagnostic role: autoimmune uveitis (IgE against retinal S antigen and galectin-1 associated with progressive disease and poor prognosis); allergic rhinoconjunc-tivitis (IgE against profilin); atopic dermatitis (IgE directed to more than 100 self antigens may contribute to a chronification of the disease). The prototype of autoallergic diseases is the **type I autoimmune chronic spontaneous urticaria** (**type I aiCSU or autoallergic CSU)**: In this endotype complexes of IgE antibodies and autoallergens (e.g., FcεRI, dsDNA, TPO, Thyreoglobulin, IL-24) cross-link and activate FcεRI leading to activation and degranulation of mast cells and basophils. IgE-targeted therapies, such as anti-IgE, must have shown to be of benefit to CSU patients. The efficacy of anti-IgE treatment with omalizumab or ligelizumab supports both type I and type IIb autoimmune pathomechanisms in CSU. Omalizumab reduces the levels of IgE, the driver of type I autoimmune CSU, and of its high-affinity receptor FcεRI, the target of type IIb autoantibodies. However, type I and type IIb autoimmune CSU patients differ in their rates of response and in their speed of onset of improvement. The co-occurrence of anti-FcεRI IgG and IgE autoantibodies was documented only in late- and non-responders, but not in early ones, crediting the co-existence of autoimmune and autoallergic mechanisms as a driver of late/poor response to omalizumab (LANG et al., 2025; MAURER et al., 2020).

- **IgA associated autoimmunity:** AABs of the IgA isotype targeting the same antigen as the typical IgG antibodies are present in many of the classical AIDs but are only rarely used for diagnostic or prognostic purposes with the exception - although still in discussion - of IgA RF in RA and IgA anti-phospholipid (aPL) and b_2GPI antibodies in APS and SLE patients.

Recently, IgA anti-β2GPI antibodies were found as the most frequently detected aPL in COVID-19 patients and an association with thrombosis and severe COVID-19 was shown suggesting the use of these AABs as a possible marker to identify high-risk patients (MELLOR-PITA al., 2024a e). IgA anti-β2GPI seems to be the most prevalent isotype in patients with SLE and is significantly associated with thrombotic events. Such a clinical relevance has been recognized by the inclusion of these AABs among the aPL tests in the SLICC classification criteria for SLE (ANDREOLI et al., 2013). Furthermore, the combined positivity of IgA, IgG and IgM AABs may increase the diagnostic specificity for classical AIDs as has been shown for RA (IgA-RF/IgM-RF/IgG-ACPA) and systemic sclerosis (IgA and IgG anti-CENP-B antibodies) (CONRAD et al., 2010; van HOOVELS et al, 2022). In the search for early diagnosis of classical AIDs, IgA AABs may be helpful if they are the first detectable AABs. As an example, about one third of ACPA IgG and/or RF IgM seronegative patients had IgA AAB levels in their sera (RF, ACPA or anti-CarP) which are therefore helpful in diagnosing early RA (SIEGHART et al., 2018; van DELFT et al., 2017).

IgA mediated diseases: On the other side, IgA AABs are definitely or probably pathogenic and important diagnostic markers in intestinal diseases (celiac disease with tissue transglutaminase antibodies), skin disease (IgA-related autoimmune blistering diseases, e.g., IgA pemphigus with dsg1, dsg3, and desmocollin-1 antibodies; linear IgA bullous dermatosis (LABD), and dermatitis herpetiformis (DH); characterized by IgA AABs targeting adhesion components, leading to blister formation), autoimmune vasculitis and kidney diseases (IgA vasculitis (IgAV) or Henoch-Schönlein purpura (HSP), IgA vasculitis with nephritis (IgAVN), IgA nephropathy (IgAN). The later diseases are characterized by deposition of immune complexes containing abnormally *O*-glycosylated IgA1 in the vessel walls and glomeruli (FENG et al., 2025; NOVAK et al., 2024). A further example of a probable role of IgA AABs was shown in the pathogenesis of osteoporosis in patients with RA:

- **Bone loss in patients with rheumatoid arthritis:** AIDs are often accompanied by bone loss and AABs are discussed to play a probable role. In RA patients, an association of ACPA with the loss of bone mineral density (BMD) has been described in several studies. Moreover, it was shown that ACPA positive RA patients had a higher 10-year probability of major or hip fracture, independent of ACPA levels, and a lower BMD of the femoral neck than ACPA negative patients (CHEN et al., 2018). Interestingly, blocking of the IL-6R by tocilizumab potentially prevents bone loss in patients with ACPA positive RA. This is important because ACPA are associated with ongoing BMD loss at the spine despite suppression of inflammation and adoption of prophylactic measures. The association between ACPA and BMD loss was shown to be independent of other variables including age, sex, disease activity, cumulative dose of glucocorticoids and duration of therapy with bisphosphonates. ACPA-positive RA patients should be therefore strictly monitored for the development of osteoporosis (BUGATTI et al., 2021). It has been described that IgA AABs induce the release of IL-6 and IL-8 by immune cells as well as osteoclasts, which enhances bone resorption by osteoclasts. Therefore, it was anticipated that this will result in more severe disease activity in RA patients. Targeting IgA-FcaRI interactions therefore represents a promising novel therapeutic strategy for RA patients with IgA autoantibodies (BREEDVELD et al., 2021).

- **Transplant associated autoimmunity (TAA) following solid organ transplantation:** Extending the graft survival is one of the major goals in the modern era of organ transplantation. However, long-term graft survival has not significantly improved in recent years despite the improvement of patient management and advancement of immunosuppression regimen. Antibody-mediated rejection is a major obstacle for long-term graft survival. Donor human leucocyte antigen (HLA)-specific antibodies were initially identified as a major cause for antibody-mediated rejection. However, 40% of patients with biopsy-proven acute antibody-mediated rejection do not have donor HLA-specific antibodies. Several studies (e.g., long term follow-up of large numbers of human transplant patients) provided strong evidence that **non-HLA AABs** contributes to allograft rejection (ZHANG & REINSMOEN 2020). As targets for the TAA response the angiotensin II type 1 receptor (AT1R, a G-protein-coupled receptor expressed on endothelial cells) and the endothelin-1 type A receptor (ET1AR, in conjunction with AT1R) following renal transplantation; vimentin intermediate filament, myosin motor protein, and skeletal muscle glycolipid following heart transplantation; glutamic acid decarboxylase (GAD) enzyme and islet antigens following pancreas transplantation; endothelial cell antigens, the glomerular basement membrane protein agrin, and the perlecan proteolytic released laminin G-like 3 (LG3) fragment in renal transplant recipients; fibrillar collagen IV and V (ColV, often in association with AAB responses against Kα1 tubulin) and the matrix glycoprotein fibronectin followed lung transplant recipients, have been described so far (SIU et al., 2020). These non-HLA AABs may lead to early graft dysfunction, reduced allograft survival through acute or chronic transplant rejection, transplant glomerulopathy, and severe bronchiolitis obliterans syndrome. Non-HLA AABs may injury the graft via the classical or the alternative pathway of complements activation, microvascular injury, or via activation of endogenous AT1R signaling regulating vasoconstriction. The TAAs could be treated with plasmapheresis, B cell depletion (rituximab) and/or IVIG, and complement blockade (ZHANG & REINSMOEN 2020).

Further interesting aspects of scientific activities are investigations of

- the role of **natural autoantibodies (NAA)** in the regulation of the immune response and maintenance of immune homeostasis as well as in the pathogenesis of Alzheimer disease, systemic inflammatory responses syndrome (SIRS), ischemia, cryoglobulinemia, RA and SLE. Furthermore, NAA (mainly of IgM isotype) may be protective regarding disease progression through enhancing phagocytic clearance in apoptotic cells, blocking pathogenic IgG-immune complex-mediated inflammatory responses, elimination of cell debris during inflammation and protection against chronic inflammation by AABs targeting inflammatory cytokines (MADDUR, et al., 2020; SILOSI et al., 2016). These understandings may harness NAAs as future tool in the combat of disease (MADDUR et al., 2020).
- the **protective role of AABs** (NNAs as well as induced AABs) was found, e.g., in RA (subset of ACPAs), and atherosclerosis (AABs targeting ALDH4A1, a mitochondrial dehydrogenase involved in proline metabolism) (HE et al., 2023; LORENCO et al., 2021). For example, IgM autoantibodies were present at significantly higher levels in SLE patients with lower disease activity and with less severe organ damage with specificity for apoptotic cells. In lupus patients, higher levels of IgM targeting b_2-glycoprotein I (b_2-GPI) and cardiolipin were correlated with less frequent renal disease manifestation). The probable use of

protective AABs in the treatment of AIDs is under discussion: e.g., IgM anti-ds DNA might serve as useful treatments for SLE patients and anti-OxLDL antibodies for patients with atherosclerosis (SILOSI et al., 2016).

- the **special role of functional autoantibodies against G-protein coupled receptors** (GPCRs): AABs directed to GPCR, their levels and correlations to other AABs, serve as biomarkers for various diseases. They also could reflect the individual interplay between the environment and the immune system. Thus, GPCR AABs could display pathogenic chronic conditions and could help to identify disease-related pathways. Moreover, by acting as ligands to their corresponding receptors, GPCR AABs modulate autoimmune as well as non-autoimmune diseases (RIEMEKASTEN et al., 2020). Functional autoantibodies targeting GPCRs have been associated with multiple disease manifestations: High levels of autoantibodies targeting the angiotensin II type 1 receptor (ATlR) and endothelin-1 type A receptor (ETAR) promote severe disease complications in systemic sclerosis (SSc); AABs targeting the ß2 and α2a adrenergic receptors (ADRB2 and ADRA2A) are among the strongest predictors of post-COVID-19 outcomes; and an autoimmune autonomic syndrome (dysautonomia) was characterized by the presence of AABs directed against adrenergic and muscarinic acetylcholine receptors. Beyond their association with disease phenotypes, intense research related to the mechanistic action of these AABs on immune regulation and pathogenesis has been developed, underscoring the role of autoantibodies targeting GPCRs on disease outcomes and etiopathogenesis (Cabral-Marques et al., 2023).
- the role of AABs in **immune-related adverse events (irAEs)** of immune checkpoint inhibition (ICI) as well as in other side effects of immune therapies.
- the role of AABs in **post-infection and post-vaccination syndromes** (e.g., post-acute infection syndromes (PAIS), long COVID syndrome, multisystem inflammatory syndrome in children (MIS-C) associated with COVID-19).

Concluding remarks

The importance of AAB determinations, especially for the early diagnosis of AIDs, increases continuously. To fulfill the requirements of the growing relevance of AAB diagnostics, harmonized procedures must be established including disease-targeted stepwise multiparametric testing. The challenges for optimized AAB diagnostics require cooperation and coordination between manufactures, clinical laboratories and physicians as well as expert committees (e.g., ICAP) and networks (e.g., EASI).

The identification of novel AABs may lead to the definition of novel autoimmune entities, to a better differentiation of clinical subgroups, to earlier diagnosis and therapy of AIDs, and to the improvement of disease monitoring (e.g., prognosis, organ manifestations, therapy response). Along with the exploration of the pathogenetic or protective role of AABs this may help in the development of optimized and personalized management of AIDs and, as the main task, the prediction and prevention of AIDs. However, economic restrictions, the lack of enough highly specialized laboratories, and sometimes also problems in accepting the relevance of novel AABs, may often lead to a longtime delay of the introduction of novel AABs in routine practice or even will not be integrated into a probable personalized management of diseases.

Table 1: Examples of defined autoimmune neurologic disorders and their AAB specificities

disease entity	**description**	**target antigens**
Autoimmune nodo- and para-nodopathies	immune-mediated subtype of polyneuropathy involving AABs against cell adhesion molecules located in nodes of Ranvier and paranodal regions	Neurofascin 155 & 186, Contactin-1, Contactin-associated protein-1 (Caspr1),
Neuromyelitis optica spectrum disorders (NMOSD) & MOG antibody-associated disease (MOGAD)	rare, yet severe, autoimmune diseases of the CNS, which predominantly affect the spinal cord and optic nerves leading to recurrent optic neuritis (ON) and transverse myelitis (TM) attacks, that may lead to vision loss, motor and sensory impairment, and permanent neurological disability; different response to B cell depletion therapy	Aquaporin-4 Myelin oligodendrocyte glycoprotein (MOG)
Autoimmune encephalitis (several subtypes, among them anti-NMDA receptor encephalitis is the most frequent type; other autoimmune entities are, for example, the LGI1-antibody encephalitis, the anti-CASPR2 encephalitis, the anti-AMPAR encephalitis, the anti-DPPX encephalitis, the anti-mGluR5 antibody-associated encephalitis	an ever-growing group of rare but potentially devastating neurological disorders characterized by inflammation of the brain due to an aberrant idiopathic, paraneoplastic or therapy induced (immune checkpoint inhibition) immune response	N-methyl-D-aspartate receptor (NMDAR) leucine-rich glioma-inactivated 1 (LGI1) contactin-associated protein-2 (Caspr2) alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) Dipeptidyl-peptidase-like protein-6 (DPPX) metabotropic glutamate receptor 5 (mGluR5)
Anti-IgLON5 disease	a newly defined clinical entity with progressive course, high disability and mortality rate, characterized by a distinctive sleep disorder, associated with a heterogeneous spectrum of neurological symptoms, including characteristic daily sleep attacks, progressive brainstem syndromes, gait abnormalities and neuropsychiatric symptoms	Neuronal cell adhesion molecule IgLON5

Paraneoplastic neurological syndromes	relatively rare paraneoplastic immune-mediated peripheral (e.g., subacute sensory neuronopathy and central nervous manifestations (e.g. cerebellar ataxia, limbic encephalitis) in patients with malignant tumors caused by an aberrant expression of neuronal antigens in carcinomas or by a disrupted immune regulation in hematological tumors	intra-cellular onco-neuronal (e.g. Hu, Ri, Yo, Ma, Sox1, ITPR1, KLH11) or neuronal surface antigens (e.g., Caspr2, Lgi1, GluR, GABAR)
Autoimmune narcolepsy	chronic sleep disorder linked with disturbances in the hypocretin system	tribbles homolog 2 (TRIB2) of hypocretin neurons
GFAP astrocytopathy,	novel autoimmune inflammatory disease of the central nervous system (CNS) characte-rized by involvement of the meninges, brain, spinal cord, and optic nerve	glial fibrillary acidic protein (GFAP) α-isoform
Autoimmune autonomic ganglionopathy (AAG),	disease of widespread autonomic dysfunction (orthostatic intolerance, lower and upper GI dysfunction, bladder dysfunction, sicca symptoms) caused by ganglionic acetylcholine receptor (gAChR) AABs	α3 subunit of ganglionic nicotinic AchR (α3 nAchR)
subgroup of autism spectrum disorder (ASD)	autoimmune disorder involving social communication impairments and repetitive behaviors or interests	Folate receptor α (FRα)
anti-GlyR antibody-related disease	multiple clinical phenotypes in anti-GlyR antibody-related disease: progressive encephalomyelitis with rigidity and myoclonus (PERM), variant of stiff-person syndrome (SPS), epileptic seizure, and paraneoplastic disease	Glycine receptor (GlyR) α1-3 and β subunits (major target GlyR α1)

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In Memory: Eng M. Tan (1926 - 2024) – A pioneer in autoantibody research

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In Memory

Professor Eng Meng Tan who was Professor Emeritus, Department of Molecular Medicine at The Scripps Research Institute in La Jolla, California, passed away on March 9th, 2024, at the age of 97. Dr. Tan was an internationally renowned clinical research scientist whose life and mentorship influenced the careers of numerous researchers, clinicians and their patients due to his seminal studies on the identity and diagnostic importance of autoantibodies in systemic rheumatic diseases and other conditions.

He was the eldest of a Malaysian family of eight and he migrated to the United States of America on a full scholarship to attend Johns Hopkins University where he received a BA in 1952 and an MD in 1956. He then undertook an internship at Duke University Medical Center in North Carolina followed by a residency in Internal Medicine at Case Western University School of Medicine in Cleveland, Ohio. In 1962, a week after his marriage to Lisa, he joined the laboratory of Professor Henry Kunkel at The Rockefeller University (New York, USA).

His ground-breaking studies included elucidation of anti-dsDNA antibodies, the discovery of the Sm (Smith) and proliferating cell nuclear antigens (PCNA), the importance of anti-histone antibodies in drug-induced lupus, and innovative studies in systemic sclerosis led to the reports on anti-Scl-70 (topoisomerase and antibodies directed to the kinetochore/centromere. Many other studies included a focus on rheumatoid arthritis, Sjögren disease, juvenile arthritis, chronic fatigue syndrome and cancer (reviewed in (1-3).

In addition, to his prowess in discovery research, he was also a leader of several organizations, including President of the American College of Rheumatology. He was co-founder of several international meetings that thrive today. These include the International Congress on SLE, International Workshop on Autoantibodies, and The Henry Kunkel Society meeting. Last but not least, Dr. Karsten Conrad asked Dr Tan along with other leading scientists about the notion of turning the Dresden Autoantibody meeting into one of international scope. Enthusiastic feedback was given and this year at the Seventeenth International Meeting we are benefactors of one of the very best meetings on autoantibodies.

The world of autoantibodies has lost one of the true ‘exemplars’ of medical research. His insights, no-nonsense approach to research, unique sense of humor, and friendship are sorely missed by his family, friends, and scores of collegues around the world.

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Historical perspective of autoantibody detection: from discovery to interpretation

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Abstract

At the turn of the 19th to 20th century the concept of antibodies was coined by Paul Ehrlich in his side chain theory. He hypothesized that side chains are antigen specific receptors on white blood. These side chains are generated independent from antigen exposure and are eventually shed into the circulation upon antigen exposure. This concept appeared to have a bright future as it resulted in seven Nobel prizes ranging from understanding the complexity of the antibody repertoire, the generation of monoclonal antibodies, and most recently the clinical introduction of check-point inhibitors. The use of antibodies as analytical tool was introduced by Coombs in the so-called Coombs test, which detects antibodies to red blood cells.

Paul Ehrlich did not only introduce the concept of antibodies, but also the term “horror autotoxicus”. Although this was initially interpreted as the unwillingness of the organism to endanger itself by the formation of toxic autoantibodies, the intended meaning of “horror autotoxicus” was not that antibodies against self cannot be formed, but that they are prevented from exerting any destructive action. This concept was first challenged by the Donath – Landsteiner Test for paroxysmal cold hemoglobinuria (PCH). In this test antibodies in the patient serum bind to autologous red blood cells when incubated at 4°C, eventually resulting in hemolysis if subsequently incubated at 37°C in the presence of functional complement activation. Hans Sachs, Ehrlich’s assistant, considered this example of PCH as an exception to the rule. Ernest Witebsky, Sachs’s student, declared the existence of autoimmune disease impossible. This was again challenged by his own student, Noel Rose, by the discovery of thyroid autoantibodies in experimental thyroidits. Although Witebsky first delayed publication of these results, he next changed his opinion and formulated the so-called Witebsky’s postulates for proving that a disease is of autoimmune origin. These postulates, in parallel with Koch’s postulates for infectious diseases, entail evidence that autoantibodies are causative in the respective disease. This opened the way for acceptance of a multitude of autoimmune diseases in the early sixties of the previous century as precipitated in the landmark books of Ian Mackay and Noel Rose.

When describing the history of autoantibodies, it seems obvious to enlist all the many autoantibodies that have been discovered over the following years. Here, only a selected number of key autoantibodies will be mentioned. Evidently, this starts with the discovery of anti-thyreoglobulin antibodies in Hashimoto's disease by Witebsky (1957). This was an important game changer at that time. Next, antibodies to neuromuscular endplates were described by Simpson (1960) in patients with myasthenia gravis (MG) and the development of a radio-immuno assay (RIA) for antibodies to the acetylcholine receptor (AChR) by Lindstrom (1976). This enabled quantification of undisputed pathogenic autoantibodies. The discovery of antinuclear antibodies by Svec (1967) and subsequent identification of recognized antigens by Tan paved the way to autoimmune serology in the systemic autoimmune diseases. At the end of the millennium autoantibodies in celiac disease and rheumatoid arthritis appeared to be reactive to tissue transglutaminase (Dieterich, 1997) and citrullinated proteins (Schellekens, 1998), respectively. These discoveries not only introduced recombinant proteins (tTG) or even artificial antigens (cyclic citrullinated peptides) in autoantibody assays, but also raised discussion on the use of these assays in population screening for identifying individuals at risk for the respective diseases. Finally, the more recent discoveries of antibodies to the NMDA-receptor in limbic encephalitis by Dalmau (2007) and to the PLA2-receptor in membranous nephropathy by Beck (2009) broadened the spectrum of autoimmune diseases affecting the central nervous system and kidney, respectively. Obviously, this also enabled adequate therapeutic intervention for these diseases.

Over the years the methods of detection for autoantibodies have changed enormously. At first, technologies like immuno-diffusion and immunoprecipitation were used and they were based on immune complex formation in liquid phase. Next to these two methods also Western blotting was widely applied. All these technologies have in common that they do not unequivocally identify the antigen recognized and the results are only qualitative. More importantly, performing the assays and reading the results required substantial expertise. Next, RIA and indirect immunofluorescence assays (IFA) were introduced. The first required purification of the antigen, or in case of the RIA for anti-AChR antibodies a very specific ligand of the receptor, while the second was based on tissues or cells as substrate revealing a particular staining pattern. These assays were hampered either by the need for special laboratory facilities enabling to work with radio-active reagents or by the need for expertise to read the microscope slides and adequately recognize the relevant IFA patterns. The latter has been partially solved by the introduction of software supported microscopes for pattern recognition. In addition, the technology for IFA has been expanded with the introduction of cell-based assays in which cells are transfected in order to express membrane bound receptors that heavily depend on the integration within the cell membrane for appropriate exposition of relevant epitopes. In the next phase solid-phase immuno-assays have been introduced for either single-plex (ELISA, FEIA, CLIA) or multiplex (ALBIA, LIA/DIA, micro-arrays) autoantibody detection. This transition was accompanied by automation and widespread availability in routine clinical laboratories. The easy access to these assays has increasingly resulted in testing of patients with low pre-test probability. Furthermore, the introduction of multiplex testing as well as testing algorithms share the risk of incidental findings out of the clinical context of the patient for which the test is requested. The transition also enabled reporting of results in quantitative results, uncovering the need for standardization and, eventually, harmonization. Very recently, it has been argued that for adequate conservation of the three-dimensional structure of the antigen liquid phase interactions with the autoantibody are to be preferred above solid-phase coupling of the antigen. The introduction of combined immunoprecipitation with mass spectrometry offers the advantage of optimal antigen-antibody

interactions and antigen identification. This approach currently awaits widespread introduction in clinical practice, but also entails the risk of finding autoantibodies of undefined significance.

The clinical value of autoantibody detection initially was restricted to supporting or excluding a diagnosis. This required a good balance between sensitivity and specificity. Over the years the presence of autoantibodies has been included in both diagnostic as well as classification criteria. In particular in case of pathogenic autoantibodies they appeared also to have added value in follow-up. Indeed, to monitor efficacy of plasmapheresis in anti-GBM disease the antibodies should disappear in the peripheral blood. On the other hand, anti-AChR antibodies in MG should be reduced to about 50% of the initial concentration to achieve clinical remission. Besides monitoring therapeutic efficacy also prediction of clinical relapses has been promoted, for instance for anti-dsDNA antibodies and anti-neutrophil cytoplasmic antibodies (ANCA). Initial studies, however, were based on small patient cohorts and with methods that are currently less available in routine clinical practice. It may be questioned, therefor, if the predictive value still holds for current methods. Due to the lack of standardization, interpretation may require alternative cut-off settings and/or clear definitions for clinically relevant increases in autoantibody levels. Moreover, most autoantibody assays are marketed by diagnostic companies for diagnostic purposes only. Hence, the follow-up application is beyond the recently installed IVD-R 2017/746 within Europe. The observation that autoantibodies may also precede clinical manifestations by many years has started the discussion that this might be used for a more early diagnosis of diseases like rheumatoid arthritis (RA) and other systemic autoimmune rheumatic diseases. It should be noted, though, that the first observations were based on case-control studies. This implies that one cannot conclude that the presence of autoantibodies inevitably results in development of the respective disease. In case of RA, however, the predictive value of the autoantibodies increases if multiple RA-related autoantibodies are detected and if the Fab fragment is heavily glycosylated during T-cell dependent affinity maturation.

As already mentioned, the introduction of novel quantitative methods in many routine clinical laboratories revealed that the results were not aligned between distinct methods. Multiple initiatives (WHO, ASC/IUIS, EASI, IRMM, ….) were taken to generate international standard preparations for distinct autoantibodies. Although these preparations could very well be used for calibration of methods, they have not resulted in real standardization of autoimmune serology because it has become apparent that each patient produces his/her own repertoire of autoantibodies, being a unique fingerprint in terms of epitope recognition, affinity, isotype/subclass distribution, glycosylation, etc. Harmonization seems to be the best alternative to optimize interpretation of autoantibody results. In particular Alan Wiik pioneered in such harmonization initiatives with attention for gating strategies, testing algorithms, and adequate reporting of test results. This was further elaborated upon by Xavier Bossuyt by advocating the added value of likelihood ratio’s and by Luis Andrade by starting the international consensus on ANA patterns (ICAP).

In summary, the concept of autoantibodies, coined by Paul Ehrlich, opened the road to the discovery of autoantibodies, but was in the first decades hampered by the idea of “horror autotoxicus”. From the acceptance of autoantibodies as a real phenomenon the plethora on autoantibodies has, and still is, expanding. Increased clinical awareness of the multitude on autoimmune diseases has increased the number of requests, has propelled forward the development of new methods that could be automated and incorporated in routine clinical practice. Eventually, this uncovered the need for harmonization, in particular for test result interpretation. The organization of the many Dresden Symposia on Autoantibodies, now number

17, by Karsten Conrad has been of utmost importance to facilitate exchange of ideas on how to push autoimmune serology to a bright future.

The ouroboros of autoimmunity

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Abstract

Human autoimmunity against elements conferring protective immunity can be symbolized by the 'ouroboros', a snake eating its own tail. Autoantibodies neutralizing granulocyte-macrophage colony-stimulating factor impair alveolar macrophages, thereby underlying pulmonary proteinosis and airborne infections, type I interferon viral diseases, type II interferon intra-macrophagic infections, interleukin-6 pyogenic bacterial diseases and interleukin-17A/F mucocutaneous candidiasis. Each of these five cytokine autoantibodies underlies a specific range of infectious diseases, phenocopying infections that occur in patients with the corresponding inborn errors. Immunity against immunity should be considered as a factor in patients with unexplained infection.

The potential role of the likelihood ratio approach to support diagnosis of autoimmune diseases

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Abstract

Classically autoantibody test results are interpreted in a dichotomous way in which a single cutoff is applied and results are considered positive or negative. This overlooks the fact that for many autoantibodies the likelihood for disease increases with increasing antibody level.

The use of likelihood ratio allows to enhance interpretation of autoantibody test results. The likelihood ratio indicates how much more (or less) likely a particular test result is in patients compared to controls. It can not only be applied for a single cutoff but also for test result intervals and even for individual test results. Additional advantages of applying likelihood ratios in autoimmune serology are that likelihood ratios are unit independent and that likelihood ratios allow harmonization of antibody interpretation.

During the presentation, the concept of likelihood ratio will be explained and illustrated. Detailed background on the use of likelihood ratios in autoimmune serology can be found in the following papers:

1. Bossuyt X, Van Hoovels L. Reporting Likelihood Ratio in Autoimmune Serology: Just a Theory or Feasible in Practice? Clin Chem. 2024 Mar 2;70(3):482-485.

2. Deane KD, Van Hoovels L, Joy VE, Olschowka N, Bossuyt X. From autoantibody test results to decision making: Incorporating likelihood ratios within medical practice. Autoimmun Rev. 2024 May;23(5):103537.

3. Broeders S, Vercammen M, Bonroy C, Goletti S, Lutteri L, Schouwers S, Van Hoovels L, Bossuyt X; Belgian Expert Committee on Non-Infectious Serology of Sciensano. Applying the likelihood ratio concept in external quality assessment for ANCA. Clin Chem Lab Med. 2024 Nov 27;63(5):e137-e138.

4. Fierz W, Bossuyt X. Likelihood Ratio Approach and Clinical Interpretation of Laboratory Tests. Front Immunol. 2021 Apr 16;12:655262.

5. Fierz W, Bossuyt X. Likelihood Ratios as Value Proposition for Diagnostic Laboratory Tests. J Appl Lab Med. 2020 Sep 1;5(5):1061-1069.

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2 The Complex Autoimmune Response in Rheumatoid Arthritis

Autoantibodies in Rheumatoid Arthritis – Rheumatoid Factor, Antimodified Protein Antibodies and Beyond

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Overview

The presence of autoantibodies in blood and joint fluid is a characteristic feature of RA that distinguishes this disease from other inflammatory joint disorders [1, 2]. The hallmark antibodies of RA are rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPA) which are detectable in 60-70% of RA patients already in the earliest stages of the disease and may precede onset by several years. Autoantibody positive patients are clinically distinct from seronegative patients showing a more severe disease course and extra-articular manifestations that are less frequently observed in seronegative patients. Remarkably and contrary to autoantibodies present in other systemic autoimmune diseases, autoantibodies of patients with RA are typically directed to epitopes contained in post-translationally modified proteins. These antibodies are now collectively termed anti-modified protein antibodies (AMPA) (Figure 1).

The first AMPA species described were anti-citrullinated protein antibodies (ACPA) which are directed to epitopes containing deiminated arginine (i.e., citrulline). They show high disease specificity >90% making them the most valuable serologic markers of RA. Subsequently, it was found that antibodies may be also directed to other post-translationally modified epitopes containing carbamylated or acetylated lysine (anti-CarP and AAPA, respectively) [3]. Specificity of these antibodies is significantly lower than ACPA and comparable to rheumatoid factor (RF). Another “family” of AMPAs are antibodies against malondialdehyde-acetaldehyde adducts (anti-MAA) [4]. However, these antibodies are not specific for RA but may be associated with inflammation and clinical outcome [5].

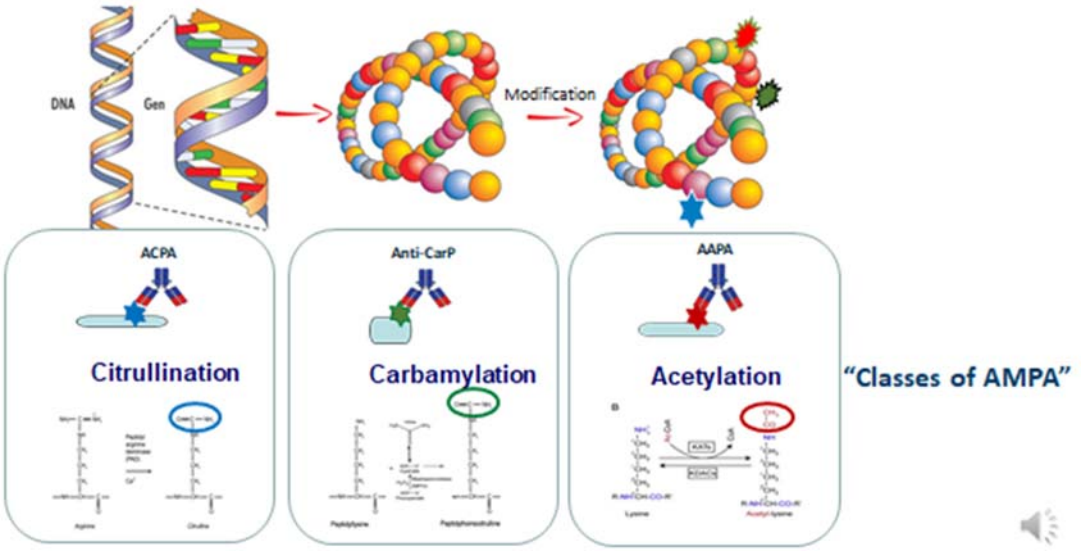

Figure 1. Posttranslational protein modifications connected to RA autoimmunity [2]. Proteins are synthesized in the cytosol by translating the genomic information in a protein. After translation proteins can be modified enzymatically or chemically. Three Post Translational Modifications (PTMs) are intimately connected to RA as 50-70% of RA patients harbor autoantibodies collectively termed Anti-Modified Protein Antibodies (AMPAs) to proteins carrying citrulline, homo-citrulline or acetyl-lysine.

AMPAs are typically detected in RF positive patients and in a low percentage of seronegative patients but their added diagnostic value is limited because they may be detected also in other seronegative rheumatic diseases [6]. Therefore, only ACPA and RF are routinely used for RA sero-diagnostics and part of the ACR/EULAR classification criteria for RA where they have been given the same scoring weight despite the fact that ACPA are considerably more specific than RF [7]. Apart from their usefulness for RA diagnostics AMPAs and RF may substantially contribute to the pathogenesis of RA, e.g., by immune complex formation and subsequent induction of proinflammatory immune responses aggravating destructive processes in the joint of RA such as activation of synovial fibroblasts and bone resorbing osteoclasts [8-10].

Rheumatoid Factors

RFs are a family of autoantibodies that recognize diverse antigenic determinants on the Fc portion of IgG. In contrast to most other autoantibodies, the major RF species is the IgM isotype, whereas IgG-RF and IgA-RF occur less frequently. Low-titer IgM-RF is not specific for RA and can be found in several rheumatic and infectious diseases and also in up to 10% of healthy individuals. However, chronic persistence of IgM-RF at elevated titer, as well as the presence of IgG and IgA subtypes, is a characteristic feature of RA. IgM-RF can be detected in 50-60% of RA patients at disease onset usually co-occurring with ACPA (and related AMPAs). The co-occurrence of IgM-RF and ACPA is a characteristic serological feature of RA that is rarely observed in other rheumatic diseases [7, 11].

RFs of all subtypes may be present already in the earliest stages of the disease and can precede the onset of RA by several years [12]. Among RF subtypes IgA-RF shows the highest specificity but is less sensitive than IgM-RF whereas diagnostic performance of IgG-RF is inferior to the other isotypes. However, also the added diagnostic value of IgA-RF is limited because it occurs almost

exclusively together with IgM-RF [13]. Nevertheless, its determination might be helpful in ACPA negative patients because double positivity for IgM- and IgA-RF has a higher specificity than either isotype alone [14]. Importantly, IgA-RF also has some prognostic value because, similar to high-titer IgM-RF, it is associated with the severity of RA, such as erosiveness, disease progression and extra-articular manifestations. Moreover, the presence of IgA-RF was found associated with a blunted response to TNF-blocking biologicals [15]. Remarkably, a similar finding was recently reported for IgA-ACPA [16].

The fine specificity of RF epitope recognition revealed different binding patterns in RA patients, arthralgia patients at risk for RA, patients with primary Sjögren´s syndrome (pSS) and healthy subjects [17]. An epitope was identified that appeared to be strongly associated with RA being targeted by both IgM-RF and IgA-RF. Moreover, distinct differences in RF binding patterns between RA-derived and pSS-derived RFs were seen but also some similar reactivities. Interestingly, the IgA-RF repertoire appeared to be largely restricted to pathology-associated specificities which is corroborated by recent findings showing different effector functions of IgA subclasses being associated with distinct glycosylation profiles [18] and the capacity of IgA complexes to induce neutrophil extracellular trap formation more potently than IgG complexes [19]. In line with these findings, total IgA1 and IgA2 levels were found to be significantly elevated in RA patients, especially in the seropositive population further supporting the mucosal origin hypothesis [20]. Furthermore, in patients with early RA the presence of IgM-RF and IgA-RF was found associated with the degree of systemic inflammation, but this was dependent on the concomitant presence of ACPA pointing to a role of ACPA-RF immune complexes in driving and/or enhancing inflammation [21].

Anti-Modified Protein Antibodies

ACPA are the prototype member among the family of AMPAs and recognize proteins that have undergone a PTM wherein an arginine residue is transformed into a citrulline by peptidyl arginine deiminases (Fig. 1). While ACPA inherently require citrulline for antigen binding, their recognition of citrullinated proteins is diverse. On the monoclonal antibody level, some ACPA “cross-react” to a broad range of citrullinated antigens [22, 23, 24] while others exhibit a more restricted pattern of citrullinated antigen-recognition, likely due to interactions with amino acids neighboring the citrulline residue [25].

Citrullination is not the exclusive PTM of significance in RA as RA patients can also harbor autoantibodies interacting with proteins altered through carbamylation [26]. This non-enzymatic process transforms a lysine into homocitrulline (Fig. 1). While often considered a distinct “family” of autoantibodies, the reactivity pattern of anti-carbamylated protein (anti-CarP) antibodies, although not identically, resemble the reactivity pattern of ACPA. This is explained by the promiscuous nature of ACPA as evidenced by the observation that many monoclonal ACPA also react to carbamylated antigens [22, 23].

A third member of the AMPA family that is related to ACPA includes antibodies to acetylated proteins [27]. These proteins form when acetyltransferases modify lysines present in proteins into acetylated lysines (Fig. 1). Anti-Acetylated Proteins Antibodies (AAPA) also occur in RA, and, like ACPA, can cross-react with various acetylated proteins as well as other PTMs (i.e.: homocitrulline and citrulline) [22, 23]. Although ACPA and anti-CarP antibody reactivity often align, AAPA reactivity can be absent in ACPA-positive subjects. These observations suggest that AAPA belong

to a different category of AMPA [28].Taken together, these observations indicate that AMPA constitute a group of antibodies that is not only promiscuous toward the same PTM expressed by different proteins, but also to various PTMs. This promiscuity underscores that AMPA can recognize many modified autoantigens, as well as modified foreign antigens.

Seronegative RA - beyond RF and AMPA

Seropositive RA is defined by the presence of RF and/or ACPA and is now generally considered a disease subtype that is distinct from its seronegative counterpart [6]. Nevertheless, it is obvious that this definition is partially dependent on the diagnostic performance of the assay systems used for their determination. This may have some impact on diagnosis and also classification of RA [29]. Therefore, efforts are ongoing to standardize ACPA [30] and to harmonize routinely used assay systems in order to reduce discrepancies between them [31]. Furthermore, also autoantibodies to native proteins may be detectable in sera of RA patients including also a subset of seronegative patients. In recent years several array-based studies have revealed novel candidate antigens for seronegative disease including peptides derived from joint specific antigens RA [32-35]. Although some candidates seem promising confirmation in larger multicenter cohorts is required to confirm their potential value for RA diagnostics. It is nevertheless interesting to note that in most studies only 20-30% of seronegative patients were found to show serological features of autoimmunity targeting not only native proteins or unmodified peptides but also citrullinated, carbamylated and acetylated peptides [36]. This suggests the existence of at least three subsets of seronegative patients: One subset is characterized by the presence of ACPA fine specificities not covered by commonly used assays which are based on synthetic cyclic citrullinated peptides that despite their high sensitivity cannot detect all ACPA specificities [16]. Therefore, these patients may be considered truly ACPA positive, thus actually belonging to the seropositive population. In fact, with respect to clinical features, genetic background and environmental factors this subgroup of patients appears to be very similar to seropositive patients [37]. The second subgroup is characterized by antibodies to unmodified native epitopes such as anti-RA33 or the recently described anti-PTX3 and anti-DUSP11 antibodies [33, 38]. This group appears to be clinically more related to completely seronegative patients which would then form the third subgroup. Of note, while single reactivities are generally not very specific the presence of multiple reactivities appears to be a feature highly characteristic of RA [7, 11, 37, 38]. Therefore, assays allowing concomitant detection of a large number of autoantibodies might indeed increase the sensitivity and specificity of RA serodiagnostics and moreover allow further stratification of patients into clinically distinct subgroups.

Concluding considerations

The progress in recent years obtained in the area of autoantibodies in RA has led to several intriguing findings. At present it is not clear how these findings relate to each other, if/how they are involved in a breach of tolerance or are linked to the microbiome found at mucosal surfaces. It can be expected that in forthcoming years the characteristics of the AMPA response will be further elucidated and put in clinical context. Moreover, the presence and high specificity of IgA autoantibodies hints towards a pivotal role of IgA-RF and IgA-AMPAs in the inflammatory and destructive processes of RA which may support the mucosal origin hypothesis of RA pathogenesis. The issue of seronegative RA is still not fully clarified but according to most studies a subset of

patients defined as seronegative by current routine diagnostics shows features of autoimmunity including reactivities to modified peptides as well as antibodies to native proteins which may allow further subclassification of seronegative patients. Overall, these novel findings will not only further our understanding of the pathogenetic processes of RA but likely open new avenues relevant for diagnosis, prognosis and design of tailored therapeutic interventions allowing personalized treatment in the earliest stages of the disease.

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It is time to refine the serological scores in rheumatoid arthritis classification criteria by changing RF and ACPA scores

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Background

Key autoimmune features of rheumatoid arthritis (RA) include the presence of autoantibodies. In the 2010 ACR/EULAR classification criteria for RA, an equal scoring weight is attributed to the presence of rheumatoid factor (RF) and anti-cyclic citrullinated protein/peptide antibodies (ACPA), although the diagnostic performance characteristics differ significantly between RF and ACPA.

Materials and methods

Diagnostic samples from 398 RA patients and from 448 diseased controls were evaluated with five RF and ACPA assays from five different companies (1). LRs for RA were calculated by means of a statistical grid search. Different serological weight scores were allocated to the absence, low or high levels of RF and ACPA and combinations of those.

The same model was applied for three confirmatory cohorts (2,3,4) comprising, respectively 67, 135 and 132 RA patients and 441, 175 and 193 diseased controls.

Results

In the investigative cohort, LR for RA was (i) higher for ACPA than for RF, (ii) increased with increasing antibody levels, and (iii) higher for double antibody positivity than for single antibody positivity. RA classification according to the 2010 ACR/EULAR depended on the RF and ACPA assays used, with 7.8-10.5% and 67.8-74.0% of control and RA patients, respectively, fulfilling the RA classification criteria. Refining of the weighing scores (RSS), resulting in an optimal diagnostic specificity, decreased RA misclassification of controls (6.3-6.9%), without affecting diagnostic sensitivity (67.1-74.0%). Moreover, applying the RSS reduced the inter-manufacturer variation in specificity (from 2.7% to 0.4%).

In the confirmatory cohorts, application of the refined classification criteria (using optimized serological weights) revealed a specificity and sensitivity for RA diagnosis of 82.4-92.3% and 82.1-87.4% respectively, compared to 78.2-91.4% and 82.1-88.9% using the 2010 RA classification criteria. In addition, in specifically seropositive patients, application of the refined classification criteria improved specificity and sensitivity for RA diagnosis respectively from 21.0-80.0% to 63.2-91.4% 86.7-97.3% to 86.7-100%.

Conclusion

Serological weight factors for RA classification can be improved by taking into account the antibody type (RF versus ACPA), the antibody levels, and single or combined positivity. The application of such refined serological weights reduced the number of RA misclassifications without affecting the diagnostic sensitivity.

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IgA ACPA as prognostic factor for development of RA

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Abstract

Antibodies against citrullinated proteins (ACPA) are one of the strongest risk factors for the development of rheumatoid arthritis (RA) and are associated with more severe disease. The main immunoglobulin class of ACPA is IgG. However, a considerable proportion of RA patients in addition display IgA ACPA. The presence of IgA ACPA has been associated with increased disease severity, joint erosion, and extra-articular manifestations. Similar to IgG ACPA, IgA ACPA precede the development of RA and can be found at similar levels in RA at-risk individuals as in patients with established RA. Since IgA ACPA rarely occur in the absence of IgG ACPA, the value of IgA ACPA in RA diagnosis is somewhat limited. Nevertheless, our findings suggest that IgA ACPA might serve as a prognostic marker for RA development. In individuals at-risk for RA, positivity for IgA ACPA was associated with an increased risk of progression to RA within the following 14 to 18 months. Conversely, at-risk individuals positive for IgA ACPA showed a much better response to abatacept than at-risk individuals negative for IgA ACPA. Together, these data suggest that IgA ACPA could be used to assess an individual's risk of developing RA and to stratify at-risk individuals for preventive therapeutic interventions.

Shift in perspective: autoimmunity protecting against rheumatoid arthritis

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Abstract

More recently the importance of B cells has been highlighted for the therapy of several autoimmune diseases including RA. Still, the functional role of B cells and antibodies in the disease process are unclear. Using animal models, antibodies specifically binding cartilage are pathogenic, but it has also recently been shown that both B cells and antibodies could be protective (1-4). These have specificities also carried by autoantibodies detected in humans, including antibodies to citrullinated proteins and collagen type II, and may play an important role hindering an inflammatory attack. The inflammatory attack is likely due to that pathogenic lymphocytes and antibodies start to dominate and these will initiate and drive the clinically observable disease. I will further discuss the possibility to detect protective and pathogenic antibodies in RA (5, 6).

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Anti-type II collagen antibodies associate with high levels of inflammation at RA diagnosis but with good clinical outcome

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Abstract

Collagen type II (CII) is the major protein in hyaline cartilage. IgG autoantibodies against native human CII (anti-CII) are found in a subgroup of rheumatoid arthritis (RA) patients at the time of diagnosis, and are associated with an early inflammatory phenotype but good long-term prognosis (Mullazehi M et al. Ann Rheum Dis 2007;66:537-41; Manivel VA et al. Ann Rheum Dis 2017;76(9):1529-1536). Anti-CII forms immune complexes with surface-bound CII *in vitro* and induces cytokine and chemokine production via FcgRIIa- and TLR4-dependent mechanisms (Mullazehi M et al. Arthritis Rheum 2006; 54(6):1759-71; Manivel VA et al. Eur J Immunol 2016; 46(12):2822-2834). Levels of anti-CII antibodies drop during the first year post-diagnosis, in parallel with decline in cytokine induction *in vitro* and reduced levels of CRP and ESR *in vivo*. These parallel *in vitro* and *in vivo* changes argue that anti-CII may be functionally active *in vivo*, and that declining anti-CII levels in initially anti-CII positive patients may be associated with reduced disease activity over time.

We have previously described the anti-CII-associated acute onset RA phenotype with good prognosis in two Swedish cohorts; one from the Stockholm region (Mullazehi M et al. Ann Rheum Dis 2007;66:537-41) and in the National Epidemiological Investigations in Rheumatoid Arthritis

(EIRA) study (Manivel VA et al. Ann Rheum Dis 2017;76(9):1529-1536). In the current talk, we will present unpublished confirmatory data from two independent cohorts: the Norwegian ARCTIC study including 221 DMARD-naïve early RA patients treated with the same DMARD escalation strategy for two years (Haavardsholm EA et al. BMJ 2016; 354; i4205), and 224 early RA patients recruited via consecutive enrolment in the city of Malmö, Sweden, followed in an observational study during five years (Eberhard A et al. Arthritis Res Ther 2021; 23: 169).

Collectively our previously published and present data argue that measurement of anti-CII may be a clinically useful biomarker to distinguish early RA patients with a good long-term prognosis.

Acknowledgment

The ARCTIC study was supported by Vinnova, Innovationsfonden and The Research Council of Norway, under the frame of Nordforsk (Grant agreement no. 90825, Project NORA) and by The Swedish Research Council, Innovationsfonden, the Federal Ministry of Education and Research in Germany and The Research Council of Norway, under the frame of ERA PerMed (Project ScandRA). ERA PerMed is supported by funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 779282. The Malmö early RA study was supported by the Swedish Research Council and the Swedish Rheumatism Association.

Prediction and prevention of rheumatoid arthritis: Where are we in 2025?

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by systemic inflammation and progressive joint damage. Early prediction and prevention strategies are critical to mitigating long-term disability and improving patient outcomes. Due to the lack of clear clinical symptoms biomarkers play a pivotal role in identifying individuals at risk before disease onset. Anti-cyclic citrullinated peptide (anti-CCP) antibodies are highly specific for RA and often precede symptoms by years. Other emerging biomarkers include anti-Ra33, anti-PAD4, and anti-CarP antibodies (among others), which reflect distinct immunological pathways and may enhance risk stratification in seronegative populations. In addition, combinations of such biomarkers using classical decision trees or artificial intelligence can further enhance disease prediction. Lifestyle modifications also contribute to RA prevention. Smoking cessation is paramount, as tobacco use is a well-established risk factor for seropositive RA. A Mediterranean-style diet rich in omega-3 fatty acids, antioxidants, and fiber may reduce systemic inflammation. Regular physical activity and maintaining a healthy weight further support immune regulation and joint health. Several prevention trials have demonstrated that immunomodulatory interventions in anti-CCP-positive individuals prior to diagnosis can delay or prevent the onset of RA. Ongoing trials continue to explore the efficacy of B-cell depletion, T-cell modulation, and cytokine blockade in early or even preclinical RA. These efforts underscore the importance of identifying at-risk individuals through biomarker screening and monitoring. Recently, a study revealed a clear link between *H. pylori* infection and the upregulation of PAD4 which is a key enzyme in the application of RA. These findings have the potential to help manage patients with RA and potentially play a role in disease prevention.

Optimizing the hemagglutination-mediator for rapid detection of anti-citrullinated protein antibodies in rheumatoid arthritis patients

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Abstract

Anti-citrullinated protein antibodies (ACPA) are the most specific serological marker of rheumatoid arthritis (RA). Several ACPA-detection assays are available for clinical use, which are almost all based on ELISA(-like) assays with citrullinated peptides (CCP2; CCP3) or proteins (MCV). To facilitate ACPA-detection in low-volume laboratories and resource-poor environments, we aimed to develop a rapid and easy to perform test.

An agglutination mediator was generated by protein engineering. This mediator consists of an anti-glycophorin A, one of the major surface proteins of erythrocytes, single-chain antibody fragment (scFv) and a citrullinated synthetic peptide, which is linked to the scFv by sortase A-mediated conjugation. Addition of this mediator to (diluted) whole blood samples results in hemagglutination when ACPA are present, which can be detected by the naked eye. The applicability was assessed by the analysis of fresh blood samples from 204 RA patients, 77 psoriatic arthritis (PsA) patients and 100 healthy individuals (Kruis et al., 2025).

ACPA-dependent erythrocyte agglutination was observed in up to 61% of the RA samples, which correlated well with the results obtained with a standardized anti-CCP2 ELISA (63-67%). Depending on the minimal agglutination score – the level of agglutination was scored 1 (very low) to 4 (very high) – agglutination was observed with only 3-21% of the PsA samples and with 1% of the healthy controls.

Because scFv's have a slight tendency to dimerize, which is for obvious reasons not desired for an agglutination mediator, may be relatively unstable, and the production of the anti-glycophorin A scFv is not very efficient, we generated an alternative agglutination mediator using an anti-glycophorin A nanobody. This nanobody can be very efficiently produced in HEK293 cell cultures.

The synthetic citrullinated peptide was conjugated to the nanobody by sortase A-mediated conjugation. The resulting nanobody-based mediator binds to erythrocytes and is recognized by ACPA in RA patient sera. Its capacity to mediate ACPA-dependent agglutination is currently being explored.

We conclude that ACPA detection by erythrocyte agglutination represents a rapid and efficient ACPA detection method using human whole blood samples. The nanobody-based agglutination mediator may be an attractive alternative for the scFv-based mediator, because of production and stability issues.

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Inflammation rather than anti-citrullinated protein antibodies associates with cardiovascular mortality in RA: Insights from rheumatoid arthritis and coronary artery disease cohorts

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Objectives

The presence of anti-citrullinated protein antibodies (ACPA) is independently associated with increased (cardiovascular) mortality in rheumatoid arthritis (RA) patients. Increased prevalence of ACPA (around 10-11%) is also described in two non-RA cohorts of patient with coronary artery disease (CAD) (1,2), where ACPA positivity was associated with worse long-term survival (2). However, the observed association between ACPA and increased mortality in RA and non-RA patients might (partly) reflect underlying inflammatory burden as systemic inflammation is known

to contribute to accelerated atherosclerosis. Therefore, we investigated not only ACPA prevalences in larger cardiovascular non-RA cohorts and the association between seropositivity and mortality in these patients, but also the role of systemic inflammation in the association between ACPA and mortality in RA patients.

Methods

The prevalence of ACPA was investigated in two large CAD cohorts using commercial ELISAs, after excluding participants with reported comorbidity of RA. CLARICOR (n=959) is a Danish randomised trial treating patients with a previous diagnosis of myocardial infarction or angina pectoris with clarithromycin or placebo for two weeks. The LURIC study (n=2189 patients and 656 controls) is a German prospective cohort study that included patients with CAD defined as luminal narrowing on coronary angiography. Kaplan-Meier curves, log-rank tests and multivariate Cox models adjusted for age and sex were performed to investigate the association between ACPA and all-cause mortality.

Two prospective cohorts of early RA patients, the Dutch EAC (n=764) and the Swedish BARFOT (n=794), were used to investigate the role of CRP over time, a proxy for systemic inflammation, in the association between ACPA and mortality in RA. In EAC CRP levels were measured annually for up to 10 years, while in BARFOT CRP was measured at 0, 6, 12, 24, 60 and 96 months after inclusion. Joint models, incorporating both a linear-mixed model and a Cox regression model adjusted for age, sex, smoking status and year of inclusion, were employed and the indirect effect of (logarithmically transformed) CRP on mortality was calculated. Hazard ratios (HR) and 95% confidence intervals (CI) are given.

Results

The average follow-up time was 8.2 to 11.8 years. ACPA prevalence was low in both CAD cohorts (4.6% in CLARICOR; 0.9% in LURIC patients and 0.3% in controls (non-significant)). Seropositivity was not associated with increased all-cause mortality (Figure 1) (CLARICOR clarithromycin group: HR 0.94, 95%CI (0.52-1.68); CLARICOR placebo group: HR 0.63, 95%CI (0.26 -1.53); LURIC: HR 0.87, 95%CI (0.39-1.94)).

In both RA cohorts, multivariate Cox models showed a significant association between ACPA and all-cause mortality (EAC: HR 1.66, 95%CI (1.17–2.37); BARFOT: HR 1.50, 95%CI (1.13–1.99)). However, when CRP was added to the joint model, the association between ACPA and all-cause mortality was attenuated and became non-significant (EAC: HR 1.22, 95%CI (0.70–2.17); BARFOT: HR 1.14, 95%CI (0.73–1.75)). In contrast, a significant indirect effect of log(CRP) on all-cause mortality was observed in RA patients (EAC: HR 1.24, 95%CI (1.14–1.34); BARFOT: HR 1.33, 95%CI (1.24–1.42)). Similar analyses using cardiovascular mortality as outcome showed comparable results.

Conclusions

ACPA prevalence was not increased in non-RA CAD cohorts and seropositivity was not associated with mortality in these patients. In RA, the association between ACPA positivity and increased all-cause and cardiovascular mortality was primarily explained by CRP. Thus, rather than the

presence of ACPA as such, the chronic inflammatory process seems to play a pivotal role in the development of cardiovascular disease in RA patients and is therefore an important therapeutic target.

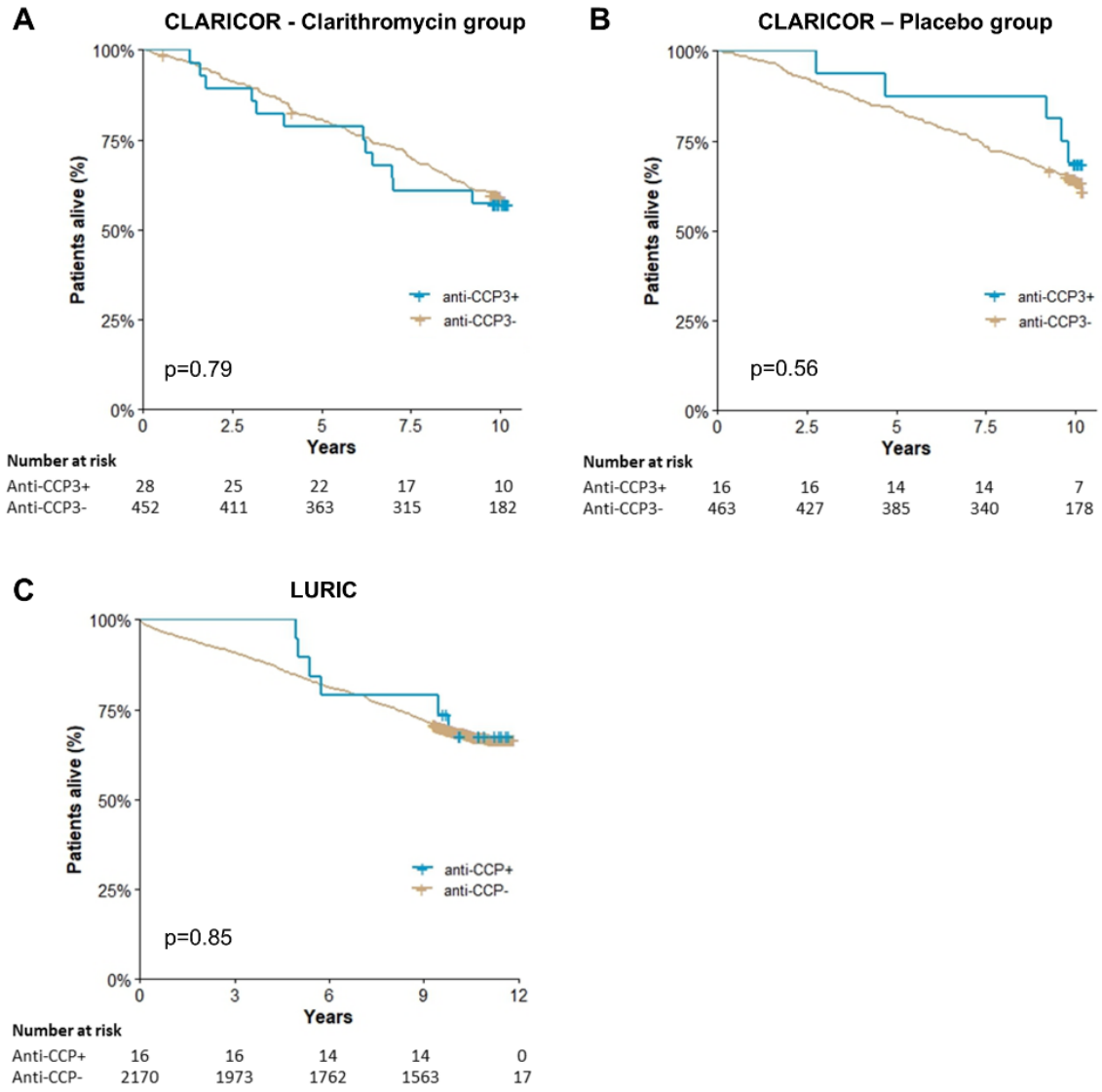

Figure 1. Kaplan-Meier curves for ACPA positivity on all-cause survival in CAD non-RA patients. P-values of log-rank test are given. Number of patients at risk over time is stated.

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The interplay between novel markers for rheumatoid arthritis: Experience from a large cohort of 60,046 patients

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Background/Purpose

The diagnosis of rheumatoid arthritis (RA) strongly depends on the detection of anti-citrullinated peptide antibodies (ACPA) as well as rheumatoid factor (RF). However, a significant portion of RA patients are negative for those markers, which poses a diagnostic challenge. Therefore, biomarkers that can help to close the serological gap are desired. Over the last decades, several potential markers have been proposed including anti-carbamylated protein (anti-CarP) and anti-RA33 antibodies. Until recently, no routine diagnostic test was available for the novel antibodies. The objective of this study is to explore the interplay between anti-CarP, and anti-RA33 alongside the traditionally assessed RF and ACPA.

Methods

During a 6-month observation period, a total of 60,046 samples were available with results for all RA biomarkers (except anti-CarP, n=15,267). Anti-CCP2 and RF were measured on the Phadia 2500 instrument (ThermoFisher). Anti-CarP IgG and anti-RA33 IgG, IgA and IgM were detected by ELISA and fluorescence enzyme immunoassay (FEIA) (both laboratory developed tests, LDT), respectively. Correlation between the different methods was studied using Spearman's correlation. Un-supervised cluster-analysis was performed to explore relationships between the different markers. IC-10 codes as proxy for clinical status were used to assess clinical associations.

Results

The positivity rate among all samples ranged from 3.58% for CC2 IgG to 16.72% for anti-CarP. In the seronegative population, defined as anti-CCP2 and RF negative, the positivity rates were as follows: anti-RA33 IgA (4.36%), anti-RA33 IgM (5.20%), anti-RA33 IgG (9.68%) and anti-CarP IgG (16.72%). While some biomarkers showed strong age and gender associations, others were expressed equally in the different populations. When the association between the different

biomarkers was assessed, the correlation ranged from Rho=0.059, p<0.001 (RF IgA vs. RA33 IgM) to Rho=0.550, p<0.001 (RF IgA vs. RA33 IgA). Lastly, using un-supervised cluster analysis identified defined groups of biomarkers.

Conclusions

In this largest series of patients studying the intersection between anti-CarP and anti-RA33 antibodies, we detected the novel autoantibodies anti-CarP and anti-RA33 in seropositive and seronegative samples. Age and gender dependent expression of some of the novel antibodies might shed more light on different subgroups of RA patients. Further studies using well characterized cohorts are required to further validate the utility of the markers.

Generation of a sialic acid–binding conjugate for high-throughput detection of RA biomarkers using particle-based multi-analyte technology

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Background

Anti-Citrullinated Protein Antibodies (ACPA) serve as specific marker for the diagnosis of rheumatoid arthritis (RA) as they are present in a significant proportion of patients, also in the early stages of the disease. Although detected years before the onset of the disease, not all patients with ACPA develop autoimmune diseases (AID).

Glycan structures on antibodies play a critical role in immune function and disease progression, especially in autoimmune conditions. Recent evidence implicates antibody glycosylation, particularly Fab-linked N-glycans of ACPA, as a contributor to RA pathogenesis. These glycans, frequently galactosylated, bisected, and disialylated, are present in over 90% of ACPA and can influence antigen affinity and inflammatory responses.

ACPA Fab linked glycans may play a role in preventing binding to self-antigens while potentially maintaining cross-reactivity to foreign antigens, allowing B cell receptors (BCRs) to avoid autoreactivity. For example, Sialylation on Fab could modulate binding to specific antigens by steric repulsion (negatively charged) or by competition between antigen and Glycan to binding pocket. Thus, the study of sialylation of ACPA has the potential to proactively identify which ACPA-positive patients will progress to clinically overt disease.

Objective

To develop and characterize a glycan-specific conjugate for detecting sialic acid on ACPA using the Particle-Based Multi-Analyte Technology (PMAT) platform, enabling high-throughput, automated assessment of RA-associated glycosylation patterns.

Methods

Sialic acid binding protein (SBP) was conjugated with a reporter fluorophore molecule using heterobifunctional PEG-crosslinker SM(PEG)2. SBP was conjugated using a molar excess of N-succinimidyl S-acetylthioacetate (SATA) for introducing protected sulfhydryl into amines protein groups. Fluorophore protein was conjugated with SM(PEG)2, a heterobifunctional crosslinker with N-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine- and sulfhydryl-containing molecules. Previous to reaction, sulfhydryl SBP was deprotected (deacylated) to generate sulfhydryl for use in cross-linking with maleimide- fluorophore protein using a molar ratio 1:1. To remove unconjugated SBP and Fluorophore protein, a size exclusion chromatography (SEC) using Cytiva HiLoad™ 26/600 Superdex™ 200 pg column was used. High molecular weight species were purified from unconjugated molecules and were quantified by spectrophotometry using Absorbance at 565 nm. The final purified conjugate was functionally tested in combination with anti-IgG tracer on CCP3 coated particles using the PMAT platform. Performance metrics, scale-up feasibility, and conjugate stability were evaluated.

Results

The fluorescent conjugate generated via the SATA-maleimide strategy showed superior performance in detecting sialylation on ACPA compared to commercially available alternatives. Among 36 tested samples, the maleimide-derived conjugate produced higher signal-to-noise ratios and more specific detection. This conjugate strategy allows a controlled and scalable strategy for better process control.

A successful scale-up produced 20 mg of conjugate though variability in conjugation efficiency between batches was observed. The conjugate demonstrated stable performance after three weeks at 2–8 °C (Spearman's rs = 0.81), whereas onboard stability declined significantly over the same period, indicating the need for more frequent monitoring.

Conclusions

The SATA-maleimide approach enabled the development of a robust and scalable conjugate for high-throughput detection of sialylated ACPA using PMAT. This method bested commercial alternatives in sensitivity and specificity while supporting efficient scale-up. Although batch-to-batch conjugation consistency and stability remain areas for further studies, this conjugate provides a valuable tool for identifying disease-relevant glycosylation features and holds promise for advancing early diagnosis and risk stratification in RA.

Refining the serological scores in rheumatoid arthritis classification criteria: an international study

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Background

Rheumatoid factor (RF) and anti-cyclic citrullinated protein/peptide antibodies (ACPA) are included in the ACR/EULAR 2010 classification criteria for rheumatoid arthritis (RA). The presence (yes/no) and the level (high/low) of both markers is given the same weight in the criteria although the diagnostic performance characteristics differ significantly between RF and ACPA. To address this issue, we set out to refine the serological weight factors for RA classification.

Materials and methods

Diagnostic samples from 398 RA patients and from 1073 diseased controls were evaluated with five RF assays (two RF IgM isotype-specific and three total RF assays) and five ACPA IgG assays from five different companies. Based on this investigative cohort, different serological weight scores were allocated to the absence, low or high levels of RF and ACPA and combinations of those. We performed a grid search across all integer score pairs (0-5) for RF and ACPA and retained the serological weight score combination that optimized the specificity of the RA classification criteria.

The same model was applied for Belgian and Spanish confirmatory cohorts comprising, respectively, 133 and 135 RA patients and 651 and 1099 diseased controls.

Results

In the investigative cohort, the optimal combination of weighting scores for RF, ACPA and combinations thereof (out of >23000 possible combinations) was characterized by higher weighting scores (i) for ACPA than for RF, (ii) for high antibody levels than for low antibody levels and (iii) for double positivity than for single positivity (Table 1).

Table 1. Combination of serological weight scores optimizing the specificity of the RA classification criteria in the investigative study cohort

ACPA	**RF**		
	Negative	**Low positive**	**High positive**
Negative	0	0	1
Low positive	1	3	4
High positive	3	4	5

Applying the optimal combination of weighting scores in the refined classification criteria resulted in improved specificity (93.4%) compared to the specificity of the current 2010 ACR/EULAR RA serological classification (90.5%), without affecting the sensitivity (71.2%). Besides, with the refined criteria, the manufacturer-dependent variability in classification was reduced.

In the confirmatory cohorts, application of the refined classification criteria (using optimized serological weights) revealed a specificity and sensitivity for RA diagnosis of 89.7-92.3% and 82.1-

87.4% respectively, compared to 89.1-91.4% and 82.1-88.9% using the 2010 RA classification criteria.

Conclusion

Serological weight factors for RA classification can be improved by taking into account the antibody type (RF versus ACPA), the antibody levels, and single or combined positivity. The application of such refined serological weights reduced the number of RA misclassifications without affecting the diagnostic sensitivity.

Mass spectrometry-based analysis of rheumatoid factor

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Abstract

Rheumatoid factor (RF) autoantibodies are detected in approximately two-thirds of patients with rheumatoid arthritis (RA), a chronic autoimmune disease characterized by, potentially destructive, symmetrical joint inflammation. RF consists of polyclonal antibodies targeting the Fc region of immunoglobulin G (IgG). Despite its clinical relevance, RF is not specific for RA, and conventional assays for RF detection, predominantly solid-phase tests detecting IgM RF, suffer from poor harmonization and the disability to test more than one RF isotype.

In this study, we employed a mass spectrometry-based proteomic approach to characterize RF autoantibodies at the peptide level in RF(+) and RF(–) RA patients, as well as in disease control patients. Our methodology involved capturing RF on IgG Fc-coated microwell plates, followed by enzymatic digestion into peptides, and subsequent analysis by liquid chromatography-tandem mass spectrometry.

Multivariate statistical analyses, including principal component analysis and sparse partial least squares discriminant analysis demonstrated that peptide profiles derived from RF(+) RA patients clustered away from peptide profiles derived from disease controls. Peptides originating from the framework and variable regions of RF, as well as de novo sequenced peptides absent from the reference human proteome, were significantly enriched in RF(+) sera compared to disease control sera. These de novo sequenced peptides have not previously been reported in literature and might be situated on the hypervariable regions of RF. Furthermore, mass spectrometry enabled

the identification of multiple RF isotypes beyond the conventionally measured IgM, including IgA and IgG subclasses. RF IgG2 subclass was detected in RF(+) and RF(–) RA cohorts in lower titers.

In summary, our findings highlight that mass spectrometry provides a platform for elucidating the heterogeneity and isotypic diversity of RF autoantibodies in RA, overcoming limitations inherent to current solid-phase RF assays.

From antigen expression to autoantibody detection: engineering, generation, and assay integration of the human recombinant protein-arginine deiminase 1 (PAD1) antigen for a Particle-Based Multi-Analyte Technology (PMAT) autoimmune diagnostic platform

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Background/Purpose

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation and the presence of autoantibodies. While current serological markers serve as tools for diagnosis and patient stratification, identifying additional autoantigens may support uncovering novel immunological subsets and help reduce the serological gap. The protein arginine deiminase 1 (PAD1) is an enzyme involved in protein citrullination and recently emerged as a novel autoantigen in RA.

The aim of this study was to generate a high-quality recombinant human PAD1 protein and evaluate its performance for the detection of anti-PAD1 IgG, both individually and in combination with other serological RA biomarkers in a multi-analyte panel. PAD1 was evaluated alongside the third generation cyclic citrullinated peptide (CCP3), a well-established RA biomarker, and anti-PAD4, an emerging biomarker with potential utility in the early diagnosis and prognosis of RA.

Methods

Several lots of N- or C-terminal His-tagged variants of the recombinant human PAD1 antigen were generated using a vector-based bacterial expression system and purified via a two-step chromatography process. Purity, identity and thermal stability were evaluated using biochemical and biophysical characterization techniques.

The purified PAD1 antigens were subsequently covalently conjugated onto paramagnetic microparticles compatible with the particle-based multi-analyte technology (PMAT) and the Aptiva platform. Preliminary assay performance was evaluated by assessing the discrimination between RA patients and healthy controls as well as by comparing the signal-to-noise ratio, defined as the signal intensity in positive samples relative to negative controls. Anti-PAD1 IgG assay performance was evaluated both individually and in a multi-analyte panel together with anti-CCP3 and anti-PAD4 IgG. Preliminary cut-off determination was performed by maximizing specificity. The sample panel used for this study included a cohort of RA patients (n=40) and healthy individuals (n=38).

Results

Both N- and C-terminal His-tagged PAD1 antigens were expressed and purified with high purity (>90%) and yield. Multiple lots of each variant were generated, with reproducibility and lot-to-lot consistency supported by comparable protein characterization profiles and assay performance data. Biophysical characterization confirmed the monomeric state of PAD1 and showed slight differences in thermal stability between variants. Variants uniquely detected subsets of patient samples, indicating that His-tag location may influence epitope accessibility and autoantibody recognition. Both in single-analyte format, or when assessed together with anti-CCP3 and anti-PAD4 IgG in a multi-analyte format, the anti-PAD1 IgG assay demonstrated strong signal-to-noise ratios, with minimal background in healthy control samples. Based on preliminary cut-offs, a subset of anti-CCP3- and anti-PAD4- positive samples also exhibited anti-PAD1 reactivity (50% and 77% overlap, respectively). More importantly, the anti-PAD1 IgG assay detected monospecific samples within the cohort (n=2), indicating that anti-PAD1 may identify a partially overlapping but distinct autoantibody profile.

Conclusion

The anti-PAD1 IgG assay demonstrated a good performance in both single- and multi-analyte formats, successfully detecting anti-PAD1 autoantibodies in RA patient samples. These findings support the potential utility of anti-PAD1 as a valuable complementary biomarker for RA. Tag orientation influenced antigen reactivity, underlining the importance of antigen engineering in assay design. Ongoing investigations in large and well-characterized cohorts aim to further evaluate the clinical relevance and potential added value of anti-PAD1 IgG in RA diagnostics.

Combined testing of anti-CD74 IgA and anti-UH-axSpA antibodies increases the diagnostic potential for axSpA

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Background

Diagnosis of axial spondyloarthritis (axSpA) is challenging and a specific laboratory diagnostic test is lacking. Previously, we identified novel immunoglobulin G (IgG) and IgA antibodies to 4 Hasselt University (UH)-axSpA antigens (UH-axSpA-IgG 4, 8 and UH-axSpA-IgA 1,10), corresponding to non-physiological peptides and to a novel axSpA autoantigen, Double homeobox protein 4 (DUX4) (1). Validation of antibody reactivity in plasma samples of axSpA patients from the Leuven spondyloarthritis biologics cohort (BIOSPAR) cohort revealed antibody reactivity against at least one of these 4 peptide targets in 15.9 % of axSpA patients (26/164) (2). In addition, IgA antibodies against CD74, involved in the assembly of and the prevention of premature peptide-binding to major histocompatibility complex (MHC) class II has been shown in patients with axSpA.

Objectives

Here we aim to determine the diagnostic potential of the anti-CD74 IgA antibodies in combination with previously determined IgG and IgA antibodies against the 4 UH-axSpA antigens.

Methods

Anti-CD74 IgA antibodies were measured using the AESKULISA SpA Detect Kit (AESKU Diagnostics, Wendelsheim, Germany) in axSpA patients from the BIOSPAR cohort and patients with chronic low back pain (CLBP) served as the control group. The cut-off of the ELISA was 20 U/ml.

Results

In the BIOSPAR cohort, anti-CD74 IgA antibodies were present in 29.3% of axSpA patients (48/164) versus no presence in the CLBP patients (0/58) ($p<0.0001$). Additional testing for the presence of antibodies against the 4 UH-axSpA peptides further increased the antibody reactivity in 40.2 % of the patients (66/164) and only in 3.4% of the CLBP patients (2/58) ($p<0.0001$). Interestingly, we found a significant increase in age, disease duration and Bath Ankylosing Spondylitis Functional Index (BASFI) in axSpA patients who tested positive for antibodies against at least one of the 4 UH-axSpA antigens or CD74 compared to seronegative patients.

Conclusions

Combined testing for antibody reactivity against the 4 novel UH-axSpA peptides and anti-CD74 results in improved diagnosis of axSpA.

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Antinuclear Antibodies in Rheumatoid Arthritis: Sign or Disease?

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Background

Rheumatoid arthritis (RA) is a chronic progressive inflammatory disease with irreversible joint destruction if inadequately treated. Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) are the most specific antibodies found in patients with RA. But also antinuclear antibodies (ANAs) are often found in patients with RA which has been described in several publications [1, 2, 3].

Methods

To estimate the amount of ANA positive patients we used a different method compared to previous papers. We did a chart view of all patients tested on ANA and anti-citrullinated peptide antibodies (ACPAs) from January 2020 until March 2025 and calculated the frequency of ANA positive among ACPA positive patients. We screened 43052 files of 31829 patients. ACPA was tested at least once in 11270 patients. In our laboratory the upper limit of ACPA's normal range for is 10 U/ml. Statistical analyses were done by chi-squared tests.

Objectives

ACPAs are specific antibodies for RA. We suppose that ACPA positive patients have high probability for RA. We tested the number of ANA positive patients in ACPA positive versus ACPA negative patients.

Results

The frequency of ANA in patients with ACPA < 10 U/ml (N = 7903) compared to patients in whom ACPA was not measured (n = 23472) revealed no statistical difference (p = 0,18; 25,25 % versus 24,60 %). All comparison with patients in whom ACPA was measured showed highly significant

differences regarding the proportion of ANA. We compared patients with ACPA < 10 U/ml (n = 10573) versus ACPA ≧ 10 U/ml (n = 697), ACPA < 10 U/ml (n = 10573) versus ACPA ≧ 30 U/ml (n = 583), ACPA < 10 U/ml (n = 10573) versus ACPA ≧ 100 U/ml (n = 458). All comparisons ANA positive versus negative resulted in highly significant differences (p = 0,00001). The amount of ANA positive patients was 48 % in ACPA positive (ACPA > 10 U/ml) patients, 51 % in ACPA high positive (ACPA > 30 U/ml) and 51 % in ACPA very high positive (ACPA > 100 U/ml) patients. Most often we found a titer of 1:160 (n = 6020), but also very high (> 1:2500) ANA-titers (n = 872)

Conclusion

About half of our ACPA-positive patients have ANA. This is more than described in most previous papers with a selected number of patients with definite RA (17 - 41 %). Our data suggest that ANA correlates with the presence of ACPA.

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3 The Complexity of Anti-Phospholipide Syndrome and Systemic Lupus Erythematosus

2023 ACR/EULAR APS classification criteria: the starting point to move on

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Abstract

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by arterial, venous, and/or pregnancy morbidities in patients persistently carrying antiphospholipid antibodies (aPL). The presence of aPL is a hallmark of both the classification and diagnosis of APS. Moreover, the aPL titer and number of positive assays are important factors for risk stratification by individual aPL profile (1).

Since 2006, the revised Sapporo APS classification criteria (2) have required at least one clinical feature (i.e., a thrombotic or obstetric manifestation) together with a minimum of one positive laboratory test for aPL, among lupus anticoagulant (LA), anti-cardiolipin (aCL) and anti-beta 2 glycoprotein 1 (β_2GPI) antibodies. The positivity has had to be confirmed in two occasions at least 12 weeks apart (2).

The new 2023 ACR/EULAR classification criteria (3) have been designed primarily for their application in clinical studies and trials. They strictly define the clinical and laboratory features, which are differently weighted and organized into 8 domains (6 clinical and two laboratory domains), and include an entry criterion of at least one documented clinical domain (domains 1-6) plus one aPL positive test [LA (domain 7), or aCL or anti-β_2GPI antibodies IgG or IgM (domain 8) within three years of the clinical criterion], followed by additive weighted criteria. Classification as APS requires at least three points from both clinical and laboratory domains.

Persistent LA, aCL and anti-β_2GPI remain the landmarks of APS laboratory classification criteria, as in the revised Sapporo criteria (2). LA should be detected and interpreted according to the last International Society of Thrombosis and Haemostasis guidelines (4). Measurement of aCL and anti-β_2GPI antibodies is restricted to standardized ELISA methods with moderate and high titers, as defined by the tresholds of 40 and 80 Units, respectively. The antibody quantification, which overcomes the dichothomus positive/negative results, is in line with the evidence that aPL titer has a diagnostic/prognostic value for both vascular and obstetric APS (1).

The aPL profile plays a pivotal role not only in the classification but also in the diagnosis of APS, and clinicians tend to use the classification criteria as surrogates of diagnostic criteria. Solid-phase (automated) assays other than ELISA are currently widely employed to detect aCL and anti-β_2GPI antibodies in the diagnostic practice owing to their feasibility and reproducibility. Fluorescence enzyme immunoassay (FEIA), multiplex flow immunoassay (MFI), and chemiluminescent immunoassay (CLIA) are the most frequently used non-ELISA techniques. However, based on the test's analytical characteristics, there is variability between the results from ELISA and non-ELISA methods, and in the daily routine, a sample positive in one assay may be negative in a different assay, or in the same assay performed by another laboratory (5). Moreover, the moderate or high titers of aCL and anti-β_2GPI in non-ELISA platforms have not yet been definitely established. So, the distinction between the detection and interpretation of aPL for classification purposes or for their use in the clinical care is of utmost importance.

Classification criteria aim to identify "definite" APS for clinical and basic research, allowing the recruitment of relatively homogeneous cohorts of patients. To this end, highly comparable laboratory results are mandatory, and specificity has to be privileged over sensitivity (3,6). In the clinical setting, APS diagnosis is provided by healthcare professionals who evaluate signs, symptoms, clinical history, physical examinations, and laboratory results of one individual patient, in order to guide therapeutic recommendations (7,8). Based on this crucial difference, classification criteria can be used to diagnose APS, but should not restrict the evaluation of each patient's conditions by clinicians.

From a laboratory perspective, though ELISA is required for aCL/anti-β_2GPI antibody detection to classify APS patients, this item should not affect the diagnostic approach of laboratories employing non-ELISA systems. Establishing thresholds to define "moderate" and "high" aCL and anti-β_2GPI antibody levels for non-ELISA methods is a big challenge (9), and a great deal of effort is needed to harmonize the interpretation of the results of the different aPL assays in daily clinical practice and research (10). Initiatives from different research groups and international societies (e.g., APS Action and ISTH Scientific Committee Subcommittee on LA/aPL collaboration) have been promoted to achieve this goal, and the use of interval-specific likelihood ratios (11) or internationally validated reference materials (12) has been proposed to improve the appropriateness of aCL/anti-β_2GPI results interpretation to support APS diagnosis.

In conclusion, aCL and anti-β_2GPI IgG/IgM antibody low and medium/high levels have different clinical significance. The definition of low, moderate, and high antibody ranges comparable across the available platforms may enhance a harmonized interpretation of the results. When managing a suspected APS patient, clinicians should be aware of the classification or diagnostic context and of the detection method for an accurate interpretation of the results. Further external quality control studies involving large cohorts of patients and appropriate controls should be designed to validate the different harmonization approaches and best aid the clinical diagnosis of APS.

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The probable role of the likelihood ratio approach in diagnosing/classification of APS

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Background

A key laboratory finding for diagnosis and classification of antiphospholipid syndrome (APS) includes the presence of anticardiolipin (aCL) and anti-b_2-glycoprotein I (ab_2GPI) antibodies. However, an important interassay variation is described among currently available commercial aCL and ab_2GPI assays, impacting the diagnostic performance of aPL assays.

Materials and methods

Commercial aCL IgG/M and ab_2GPI IgG/M assays of three different diagnostic companies (Thermo Fisher Scientific, Orgentec, Werfen) were evaluated using (i) 176 diagnostic samples from patients with APS and 433 disease controls (DCG), (ii) international APS reference materials (Harris/Louisville, Koike/Sapporo, NIBSC 21/266), and (iii) samples of 120 healthy controls (HC).

Results

Analytically, the imprecision ranged from 3.0% to 23.0% and was the highest for the ELISA assays and for the double-layered aCL assays. Between the different aPL methods only a low-to-moderate correlation was obtained among test results obtained with patient samples and with reference materials. Except for the Orgentec ELISAs, the 99% upper reference limit (99%URL) differed from the cutoff proposed by the manufacturer.

The area under the curve of the ROC curves of the different aPL assays were comparable, indicating comparable diagnostic performance between ELISA, CLIA and FEIA aPL methods. Independent of the assay technology, the diagnostic performance of ab_2GPI IgG tended to be higher than aCL IgG and aCL IgM; the diagnostic performance of aCL IgM outperformed ab_2GPI IgM.

Using manufacturer's proposed cut-offs, there was large variability in diagnostic sensitivity and specificity among assays. The difference in diagnostic performance and corresponding LRs for APS between assays, was lower when thresholds corresponding to a predefined specificity were used. LRs for APS increased with increasing Ab levels for aCL IgG, aCL IgM, ab_2GPI IgG but less so for ab_2GPI IgM. For all aPL tests expect EliA aCL IgM, a LR>10 was obtained for Ab levels > 97.5% specificity threshold. In addition, for almost all manufacturers and assays the 99.0% URL determined in the HC cohort aligned with the 97.5% specific threshold determined in the DCG.

Thresholds corresponding to 97.5% and 99.5% specificity in diseased controls were used to delimit test result intervals [negative (<97.5% specificity threshold), weak positive and high positive (>99.5% specificity threshold)]. Test result interval-specific LRs were concordant across the different aCL and ab_2GPI assays. LRs for APS significantly increased by combining aCL with ab_2GPI and were higher for IgG than for IgM and for double and triple antibody positivity. The added diagnostic value of ab_2GPI IgM was limited.

Conclusion

Defining thresholds for antibody levels and assigning test result interval-specific LRs allows alignment of clinical interpretation for aCL and ab_2GPI assays. To confirm our specificity-based thresholds, additional studies mimicking routine APS diagnostics are warrented.

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Appropriateness and sustainability of aPL testing

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Abstract

Efficient utilization of healthcare resources, including laboratory testing, is fundamental for environmental sustainability and cost-effectiveness. Antiphospholipid syndrome (APS) is a rare autoimmune disorder requiring specific diagnostic investigations. However, inappropriate requests for antiphospholipid antibodies (aPL) tests contribute to unnecessary healthcare expenses and environmental impact. This study evaluates the appropriateness of aPL testing in a clinical setting.

A retrospective analysis was conducted on 642 patients attending the San Giovanni Di Dio Hospital, Florence (11/2023–02/2024). Diagnostic suspicion underlying aPL test requests were classified as appropriate, inappropriate, or unevaluable using a scoring system based on clinical recommendations. Appropriateness assessment was performed independently by two researchers and reconciled with a third expert. Patient demographics, test results, and the specialty of the physicians ordering aPL were recorded and analysed.

Of the 642 queries, 36% were deemed appropriate, 42% inappropriate, and 22% unevaluable. Family physicians accounted for 53% of all test requests but exhibited the highest rate of inappropriate prescriptions (44%). Rheumatologists, internal medicine physicians, and gynecologists demonstrated better adherence to recommendations. Only 4.9% of patients underwent comprehensive aPL testing according to international guidelines. Among the 115 aPL-

positive cases, multiple antibody positivity was more frequently observed in appropriately requested tests. In contrast, inappropriate test requests often originated from clinical scenarios with no established association with APS, such as alopecia, hypercholesterolemia, and dysmenorrhea. A substantial proportion of aPL testing in routine practice lacks clinical justification, highlighting inconsistent adherence to diagnostic guidelines across medical specialties. Unwarranted testing not only increases healthcare costs and unnecessary specialist referrals but also contributes to environmental burden. Enhancing clinician education, promoting adherence to established diagnostic criteria, and fostering more sustainable testing practices are essential steps toward optimizing APS diagnostics and resource use.

Anti-PS/PT antibodies: results of a very long follow-up in large series of unselected patients

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Abstract

Among all the candidates for inclusion in the diagnostic criteria for APS syndrome, anti-PS/PT antibodies appear to be the most promising, given the extensive literature attesting to their significant and independent pro-thrombotic activity.

In tetrapositive patients, their presence contributes to the majority of the lupus anticoagulant (LA) phenomenon. In fact, they showed the most significant correlation with LA activity, so much so that they can substitute LA when a correct interpretation is not possible.

LA is associated with activated protein C (APC) resistance, which might contribute to thrombotic risk in patients with antiphospholipid syndrome (APS). Recently, it was demonstrated that while anti-β2GPI antibodies with LA-activity contribute to a procoagulant state by causing APC resistance via interference with the cofactor function of Factor V during activated Factor VIII inactivation, anti-PS/PT antibodies interfere with the anticoagulant function of APC by preventing activated Factor V cleavage (Noordermeer T et al., 2023).

Numerous studies have demonstrated that anti-PS/PT positivity is an independent risk factor for venous vascular thrombosis and obstetric complications. There is also an association with thrombocytopenia, suggesting that the presence of anti-PS/PT antibodies may be associated with more severe clinical APS. Among seronegative APS patients, 6-17% of patients are positive for anti-PS/PT antibodies. However, the relevance of the sole aPS/PT positivity for APS diagnosis is still a matter of debate.

Of note, we recently studied the procoagulant effect in vitro of anti-PS/PT antibodies versus the anti-β2GPI antibodies, finding comparable ability to enhance TF expression, both in monocytes and in endothelial cells (Cifù et al., 2019).

Based on our preliminary positive experience (Fabris et al., 2014), we introduced anti-PS/PT testing by ELISA in our diagnostic Laboratory in Udine in 2013. Initially, according to many Authors, we suggested anti-PS/PT testing in case of strong APS suspicion and absence of criterial aPL. While the number of patients tested per year for aCL, aB2 GpI and LA has remained stable

(approximately 4500, 2600 and 3900 respectively, from 2013 to today), patients tested for aPS/PT have increased from approximately 850 in 2013 to an estimated 1200 in 2025 (+ 33%). Nowadays, in many patients, anti-PS/PT are requested together with the aPL criterion in cases with greater clinical suspicion, since they contribute to estimating the overall risk (GAPPS score, Sciascia et al., 2013).

An unresolved limitation in the analysis of these antibodies is represented by the fact that, while aPL can be performed daily with an automated method, anti-PS/PT remain in ELISA with a TAT of approximately 10 days.

Some emblematic cases taken from the case history of our centre will be presented.

Even low levels of Anticardiolipin antibodies are associated with pregnancy-related complications: A monocentric cohort study

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Specific objectives

Moderate and high levels of anticardiolipin antibodies (aCL), especially in the setting of the antiphospholipid syndrome, are associated with adverse obstetric outcomes. However, the clinical relevance of low aCL levels (<40 MPL/GPL units) is still a matter of debate. The aim of the study was to evaluate obstetric outcomes in pregnancies with low immunoglobulin M (IgM) and/or immunoglobulin G (IgG) aCL positivity. The association between low aCL positivity and maternal baseline characteristics was also studied.

Methods

The retrospective monocentric cohort study of prospectively collected data involved a total 3047 singleton pregnancies that underwent the first-trimester screening involving an aCL test and delivered on site. Obstetric outcomes were compared between the low-titer aCL group (IgM ≥7 MPL units and <40 MPL units and/or IgG ≥10 GPL units and <40 GPL units) and the aCL negative group (IgM <7 MPL units and IgG <10 GPL units, reference group). In addition, obstetric outcomes were evaluated with regard to the antibody isotype: IgM-positive group (IgM <40 MPL units, IgG negative) and IgG-positive group (IgG <40 GPL units, IgM negative or <40 MPL units).

Results

Overall, the occurrence of pregnancy-related complications was significantly higher (27.91% vs. 19.32%, p = 0.034) in the low-titer aCL group. Concerning the antibody isotype, a higher rate of pregnancy-related complications was observed in the IgG-positive group (54.55% vs. 19.32%, p =

0.001), but not in the IgM-positive group (22.43% vs. 19.32%, p = 0.454). The stillbirth rate did not reach statistical significance. Low-titer aCL pregnancies were more frequently of advanced maternal age (p < 0.001), suffered from autoimmune diseases (p < 0.001), chronic hypertension (p = 0.040), and hereditary thrombophilia (p = 0.040). In addition, they had more often a positive history of stillbirth (p < 0.001), underwent conception via assisted reproductive technologies (p < 0.001), were administered low-dose aspirin (p < 0.001), low-molecular-weight heparin (p = 0.018) and immunomodulatory drugs (p < 0.001), and delivered earlier (p = 0.018).

Conclusions

Even low aCL levels are associated with a higher incidence of pregnancy-related complications, but only in the case of IgG antibody isotype presence. Screening for aCL in the first trimester has some prognostic value, but further studies are needed to determine whether its potential implementation into routine clinical practice would improve antenatal care.

Recent advances in the serological domain of antiphospholipid syndrome diagnosis

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Abstract

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder for which diagnosis remains challenging due to its broad clinical spectrum and the diverse, often heterogeneous, serological profiles associated with the disease. This session will provide an in-depth examination of both criteria and non-criteria antiphospholipid-associated autoantibodies, including anti-cardiolipin (aCL), anti-β2 glycoprotein I (anti-β2GPI) across three isotypes (IgG, IgM, IgA), β2GPI Domain 1 IgG, and phosphatidylserine/prothrombin (PS/PT) antibodies of three isotypes (IgG, IgM, IgA). A major emphasis will be placed on the molecular underpinnings of these biomarkers, highlighting recent advances in understanding their immunologic specificity, pathogenic roles, and analytical detection. The discussion will explore how serological profiles can support risk stratification, improve prognostic assessment, and guide clinical decision-making in APS. Finally, the session will address the practical and conceptual challenges introduced by the recently updated APS classification criteria.

Reporting of Reference Intervals for Anti-Cardiolipin Antibody (IgG and IgM) Immunoassay: Normal Reference Ranges in Singapore

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Background

The detection of IgG and IgM isotypes of anticardiolipin (aCL) antibodies is a laboratory criterion for antiphospholipid syndrome (APS) [1]. However, the testing and reporting of aCL antibodies is not well standardized due to the use of different assay methodologies and reporting cut-offs. The use of locally derived 99th centile cut-off values for both aCL IgG and IgM has been recommended by some groups, but published studies demonstrate substantial variation of these values [2,3,4,5]. There is also a lack of clear evidence from manufacturers on the establishment of their recommended cut-off values [6,7]. Hence, there is a need for each laboratory to evaluate assay and population-specific cut-offs values.

Objectives

To establish and compare local in-house cutoff values for the Phadia EliA aCL IgG and IgM fluoroenzyme immunoassay, the immunoassay used by our laboratory for aCL antibodies testing, to the manufacturer cutoff values.

In addition, we also examine the impact that the established local in-house cutoff values will have on our College of American Pathologist (CAP) External Quality Assessment (EQA) aCL survey performance, as compared to responses using the manufacturer cutoff values.

Methods

A total of 154 volunteers were recruited from the Division of Pathology, Singapore General Hospital, from September 2024 to March 2025. Information on basic demographic data and medical information including pregnancy status, history of autoimmune disease and prior medical illness were collected using an online questionnaire. aCL IgG and IgM levels were measured from serum samples using EliA Cardiolipin IgG and IgM test wells and reagents on the Thermo Fisher Scientific Phadia250 platform. Volunteers with the presence of systemic autoimmune disease; current pregnancy; and history of thrombotic events were excluded from downstream analysis. The non-parametric percentile method was used to calculate the 95th, 97.5th and 99th percentiles (CLSI standard C28-A3c) [8]. Potential outliers were identified and excluded by the Dixon/Reed method. Stratification analysis by gender and age were performed with differences between groups determined using Wilcoxon test.

Subsequently, results from the laboratory submissions to CAP aCL EQA program from the period 2021 to 2025 were retrieved from records. CAP EQA program provides intended positive samples that are derived from patients clinically defined to have antiphospholipid syndrome and intended negative samples comprising pooled normal serum. Comparative analysis was carried out with the locally derived 99th centile cutoffs against manufacturer recommended cut-offs, using intended results provided by CAP as reference results.

Results

The in-house 99th percentile cut-off values were 23.04 GPL-U/mL and 13.04 MPL-U/mL for aCL IgG and IgM, respectively. There were no statistically significant differences in aCL IgG and IgM antibodies levels between men and women (aCL IgG p-value = 0.37, aCL IgM p-value = 0.63) or between the different age groups (aCL IgG p-value = 0.51, aCL IgM p-value = 0.89). Compared to other published 99th percentile cut-offs for aCL using Phadia / EliA, our 99th percentile cut-off for IgG aCL was fairly similar to other studies, but our cut-off for IgM aCL was lower than most other studies.

Using CAP aCL EQA proficiency testing survey values previously submitted by the laboratory, the use of the in-house derived 99th percentile cut-off would result in 2 of 27 aCL IgG results being misclassified (with false negative results), while all 27 aCL IgM results would be accurate. In comparison, using the manufacturer recommended cut-off values [negative: <10 GPL-U/mL or MPL-U/mL, weak positive: 10-40 GPL-U/mL or MPL-U/mL, positive: >40 GPL-U/mL or MPL-U/mL], all 27 aCL IgG results would be classified correctly but 1 of 27 aCL IgM result would be misclassified (with false positive result).

Conclusion

We present the reference intervals for Phadia EliA aCL IgG and IgM determined in accordance to CLSI guidelines for our study population, which to our knowledge is the first such study in a Southeast Asian population. Given that variations in autoantibodies exist among different populations, we show how the use of a locally derived 99th percentile cut-off may affect the classification of aCL status in patients with antiphospholipid syndrome.

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Evaluation of criteria and non-criteria anti-phospholipid antibodies in pregnant women with systemic lupus erythematosus or antiphospholipid syndrome: a longitudinal study and method comparison using a novel particle-based multianalyte technology

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Introduction

Pregnant women with antiphospholipid syndrome (APS) or systemic lupus erythematosus (SLE), particularly those with antiphospholipid antibodies (aPL), are at increased risk of obstetric complications. Although advances in clinical management have significantly improved maternal and fetal prognosis, the ability to predict adverse perinatal outcomes (APO) remain limited. Therefore, the identification of novel biomarkers with high prognostic value would help to individualize the follow-up and optimize the best treatment for these pregnant women, with the consequent improvement of perinatal results. Thus, we conducted a prospective study of pregnant women with SLE, APS and non-criteria APS (NC-APS).

This study aimed: first, to assess the prevalence of criteria aPL using chemiluminescence immunoassay (CIA) and particle-based multi-analyte technology (PMAT); second, to evaluate the variation in aPL levels throughout the three trimesters of pregnancy; and third, to evaluate the potential role of non-criteria aPL in predicting APO in our cohort.

Patients & Methods

From 2021 to 2023, 49 pregnant patients were enrolled (30.6% SLE, 18.4% SLE with aPL, 14.3% obstetric APS, 2.0% thrombotic APS, and 34.7% NC-APS). In all three trimesters of pregnancy, following biomarkers were studied:

• Criteria aPL: anti-Cardiolipin (aCL) IgG and IgM and anti-β2 glycoprotein I (aβ2GPI) IgG and IgM by CIA (QUANTA Flash) and PMAT technologies (Aptiva).

• Non-criteria aPL: aCL IgA and aβ2GPI IgA by enzyme-linked immunosorbent assay (ELISA) (QUANTA Lite) and CIA (QUANTA Flash), aβ2 GPI-domain I (aD1) by CIA (QUANTA Flash) and anti-Phosphatidilserine/prothrombine (aPS/PT) IgG and IgM by ELISA (QUANTA Lite).

All assays were performed using Inova Diagnostics (San Diego, USA) kits, with manufacturer-recommended cut-offs.

We evaluated both clinical and histopathological APO and conducted comparative analyses based on the presence or absence of aPL. Clinical APOs were defined as the occurrence of at least one of the following events: preterm birth before 37 weeks of gestation, preeclampsia at any gestational age, a 5-minute Apgar score <7, neonatal acidosis (umbilical cord arterial pH <7.0), or fetal/neonatal death. Histopathological APOs were categorized according to the Amsterdam Placental Workshop Group Consensus Statement, and encompassed placental or fetal vasculo-stromal lesions, immunological lesions, and other histopathological abnormalities. Additionally, reduced placental size, defined as placental weight below the 10th percentile for gestational age, was considered an adverse histological finding.

Results

All autoantibodies were analyzed at various time points corresponding to first, second and third trimester of pregnancy. No differences in prevalence of different aPL were observed throughout the three trimesters.

In the first trimester of pregnancy, CIA detected aCL IgG/IgM in 28.6%/7.1% and aB2GPI IgG/IgM in 16.7%/4.8%. PMAT and CIA methods showed strong total and negative agreement for aCL and aβ2GPI, both isotypes (IgG and IgM). Furthermore, aCL and aβ2GPI IgM demonstrated a strong positive agreement between PMAT and CIA. In contrast, aCL IgG and aβ2GPI IgG presented weak and moderate positive agreement, respectively. Among the discrepant aCL IgG results, 12 out of 16 data points from seven patients were classified as low positive (<50 CU) by CIA but negative by PMAT. Additionally, three patients (five data points) presented aB2GPI IgG results near the CIA cutoff value, while remaining negative for PMAT. Finally, total agreement was observed between aD1 by CIA and aβ2GPI IgG by PMAT or CIA.

Among seronegative patients for aPL criteria by PMAT and CIA in the first trimester, 14.7% and 16.7% were positive for aPS/PT IgM, respectively. IgA isotypes were rarely positive, though CIA and ELISA showed strong total agreement.

In our cohort, the overall prevalence of clinical APOs was 22.4%, while histopathological APOs were identified in 65.5% of cases. The association between different aPL and APO is under study.

Conclusion

Our findings suggest that aPL levels remain stable throughout gestation, suggesting that repeat testing during pregnancy may be unnecessary. Notably, aPS/PT antibodies IgM emerged as a promising biomarker, especially in seronegative patients for aPL criteria. Although no statistically significant differences in APO were observed according to aPL status, the overall prevalence of both clinical and histopathological complications in this cohort was substantial. Importantly, certain histopathological features appeared more frequently in aPL-positive patients, suggesting possible subclinical placental involvement in the absence of overt clinical manifestations. Due to the limited sample size and low number of aPL positivity, larger studies are warranted to confirm these findings and to enhance risk stratification and clinical management in this high-risk population.

Isolated anti-cardiolipin reactivity in Syphilis sera highlights diagnostic utility of CL/β_2GPI complex in APS testing

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Objective

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of β_2-glycoprotein I (β_2GPI) antibodies and antiphospholipid antibodies (aPL), particularly directed against cardiolipin (CL). These autoantibodies target phospholipid-binding proteins and play a key role in promoting abnormal blood clot formation. Patients with APS, especially those with lupus, can have a false-positive non-treponemal screening test for syphilis due to cross-reaction between antiphospholipid antibodies and cardiolipin. In this case, accurate identification of specific antibody profiles is essential for guiding treatment decisions and assessing long-term risks. This study aimed to evaluate a spot immunoassay for the quantitative detection of IgG, IgM, and IgA antibodies against an extended panel of APS-relevant antigens.

Methods

A novel spot immunoassay prototype was developed for the quantitative detection of IgG, IgM, and IgA autoantibodies against APS-relevant antigens in human serum and plasma. This new assay includes the following core antigen panel: CL, β_2GPI, CL/β_2GPI complex, Phosphatidylserine/Prothrombin complex and Prothrombin. Furthermore, Annexin V, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine were integrated into the prototype analysis as additional antigens. To evaluate the assays performance, isotype-specific antigen reactivities were analyzed using 16 external quality assessment samples from syphilis patients, 45 samples from APS patients, and 32 from healthy blood donors.

Results

The prototype demonstrated high sensitivity for the detection of CL/β_2GPI complex-specific antibodies: 93.3% for IgG, 100% for IgM, and 60% for IgA, with specificities of 100% (IgG, IgM) and

96.9% (IgA) in blood donor samples. To further investigate potential cross-reactivity, a panel of 16 syphilis sera was analyzed. These samples showed no reactivity to β_2GPI in the IgM isotype. Notably, isolated anti-CL reactivity was observed exclusively in the syphilis group: 3 sera were positive for IgG and 9 for IgM. In contrast, within the APS panel, isolated CL reactivity was rare, observed in only two samples for IgG. Most APS sera demonstrated antibody responses against either β_2GPI alone or the CL/β_2GPI complex, underscoring the diagnostic relevance of these antigens.

Conclusion

This study presents a novel multiparameter spot immunoassay capable of detecting IgG, IgM, and IgA autoantibodies against an extended panel of APS-associated antigens with high sensitivity and specificity. The inclusion of the CL/β_2GPI complex significantly improved diagnostic accuracy, distinguishing APS from potential cross-reactive conditions such as syphilis. These results emphasize the clinical relevance of comprehensive antigen profiling and highlight the value of advanced multiparameter assays such as the SeraSpot® APS spot immunoassay for enhancing the serological diagnosis of APS.

Autoantibodies and biomarkers in follow-up of systemic lupus erythematosus

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Objectives

The autoimmune response targeting nuclear antigens, in conjunction with complement activation, is commonly implicated in the pathogenesis of systemic lupus erythematosus (SLE). Nevertheless, these biomarkers individually exhibit limited efficacy in monitoring SLE. Consequently, this study aimed to redefine biomarker combinations based on SLE clusters and evaluate their utility for longitudinal follow-up.

Methods

This single-center retrospective study, conducted from 2009 to 2024, included 380 SLE patients with available follow-up data. Bio-clinical parameters were assessed at multiple time points through medical record review to investigate fluctuations of 30 SLE-associated biomarkers in relation to disease activity measured by the clinical SLEDAI-2K score (using repeated measures correlation analysis or RmCorr), flare events necessitating specific treatment (analyzed via mixed models), and therapeutic response evaluated according to revised DORIS criteria using Kaplan-Meier analysis.

Results

Disease activity during follow-up demonstrated significant associations with anti-double-stranded DNA antibodies (anti-dsDNA Ab; RmCorr=0.412, p<10-4), anti-chromatin antibodies (anti-Chr Ab; RmCorr=0.349, p<10-4), and complement component C3 levels (RmCorr= -0.348, p<10-4). These biomarkers exhibited intercorrelations allowing the classification of patients experiencing disease flares into serological clusters: double-positive dsDNA/Chr with complement activation (DP-C+), double-positive dsDNA/Chr without complement activation (DP+), single positive chromatin antibody group (SP-C), single positive dsDNA antibody group (SP-D), and seronegative dsDNA/Chr group (SN). Notably, the DP-C+ subgroup showed a significantly increased risk of developing lupus nephritis (49% versus 23%; p<10-7). Variations in anti-dsDNA/Chr antibodies and complement levels were predominantly observed within this serological profile and correlated with severe flares as well as therapeutic outcomes. Furthermore, stratification based on serological groups proved valuable for predicting treatment responsiveness: poor responders were primarily identified within the DP-C+ cluster (median response time of five years), intermediate responders corresponded to the DP+ group, whereas other groups demonstrated favorable responses.

Conclusion

The combined assessment of anti-dsDNA antibodies, anti-chromatin antibodies, and complement activation enables the delineation of distinct endotypes during disease flares. This approach is particularly informative within the DP-C+ subgroup for monitoring disease activity, forecasting lupus nephritis development, and anticipating therapeutic response.

This study is part of the European Autoimmunity Standardization Initiative (EASI)-network related to autoantibodies and biomarkers in the follow-up of autoimmune diseases: Carlé C et al. The added value of coupling anti-dsDNA and anti-chromatin antibodies in follow-up monitoring of systemic lupus erythematosus patients. J Transl Autoimmun. 2025;10:100274.

Comparing the performance of a newly developed anti-dsDNA antibody 6-plex assay with other anti-dsDNA antibody detection methods: Farr-RIA, CLIA and EliA

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Abstract

Anti-dsDNA antibodies are a key biomarker to aid in the diagnosis of systemic lupus erythematosus (SLE). A wide range of assays are currently implemented in the clinical laboratories, each exhibiting their own strengths and drawbacks. Anti-dsDNA antibodies are a mix of anti-dsDNA antibody subpopulations each targeting a specific epitope on the complex dsDNA molecule and the composition of the anti-dsDNA antibody subpopulations may differ between patients with SLE. As each anti-dsDNA antibody test presents one dsDNA molecule with a specific size, sequence and origin, the detected subpopulation(s) can differ between anti-dsDNA antibody assays and thus can give different test results. Aiming to detect as many anti-dsDNA antibody subpopulations as possible, we developed an anti-dsDNA antibody multiplex assay on the Luminex platform (Diasorin) that presents 6 different dsDNA molecules with different sizes (ranging from 17 - 150 base pairs) and sequences, in contrast to one dsDNA molecule in other available anti-dsDNA antibody assays. The WHO 151174 reference serum was used to construct a 5-PL calibration curve to assess anti-dsDNA antibody concentrations.

A retrospective patient cohort was collected al UZ Leuven consisting of 1296 serum samples from 134 patients with SLE obtained at time of diagnosis, from 962 disease controls and from 200

healthy controls. The cohort was tested with the newly developed anti-dsDNA antibody multiplex assay as well as with commercially available anti-dsDNA antibody assays: Farr-RIA (Trinity and EUROIMMUN), CLIA (Werfen, BioFlash) and EliA (ThermoFisher, Phadia 250). Test result interval-specific likelihood ratios (LR) were determined for test result intervals delimited by thresholds corresponding to specificities of 95o/o,97o/o and 99%. lnterval-specific LR increased with anti-dsDNA antibody concentration and were comparable between CLIA, Farr-RIA and EliA. With the multiplexed Luminex assay, the LR ratio increased with increasing combined positivity with the highest LR found for samples that tested positive for antibodies to all six dsDNA antigens.

To conclude, combined positivity in a multiplexed dsDNA assay was associated with a higher LR for SLE. For commercially available anti-dsDNA assays, interpretation of test results can be improved by test result-interval-specific LRs.

Anti-dsDNA antibody isotypes in systemic lupus erythematosus: the neglected diagnostic parameters

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Introduction

In systemic lupus erythematosus (SLE) many different organs are affected by an immune response including the skin, blood, muscles, heart, lung or kidneys making the range of symptoms vary widely. SLE occurs in about 0.1% of the general population and is predominant in females, especially in the age between 20 and 40 years, and may be linked to the hormone estrogen. Anti-double stranded DNA (dsDNA) antibodies are highly specific for the disease and can be found in 30-40% of patients.

Aim

Perform a comprehensive literature search to identify all relevant available published data for demonstration of the State-of-the-Art (medicine and technology, SOTA), the Scientific Validity (SV) of the analyte and the Clinical Performance (CP) of anti-dsDNA autoantibodies.

Method

Systematic literature search, evaluation and documentation was done by applying PRISMA method. The search strings are assembled with the use of Boolean operators and search was restricted to peer-reviewed literature and systematic reviews.

Results

In total 22 publications have been identified as significant for SOTA, SV and CP of anti-dsDNA antibodies. The reviewed literature concludes that anti-dsDNA antibodies are specific and pathogenic biomarkers for monitoring SLE. IgG anti-dsDNA antibodies are the gold standard for diagnosing and monitoring SLE, especially in patients with kidney involvement (Lupus Nephritis) being the most common and severe organ manifestation. These antibodies can bind to self-

antigens or immune complexes and accumulate in the glomerular and tubular basement membranes. Defective clearance of apoptotic cells may trigger the production of anti-dsDNA antibodies.

Anti-dsDNA IgA and IgG show a strong association with disease activity, and the IgA isotype is additionally associated with several symptoms of skin involvement. However, the IgA isotype has no association with nephritis and arthritis and may therefore define a distinct subset of SLE patients. The presence of IgM anti-dsDNA antibodies shows a negative correlation with various parameters indicating Lupus Nephritis. Due to the contrary roles of IgG and IgM anti-dsDNA Isotypes in the pathogenesis of Lupus Nephritis, there is strong scientific evidence to use the IgG/IgM Isotype ratio for prediction of nephritis (IgG/IgM >0.8 nephritis; IgG/IgM <0.8 no nephritis) also considered as replacement for kidney biopsy. Anti-dsDNA isotype evaluation in ELISA might indeed improve diagnostic accuracy, and multiple isotype detection (IgG, IgA, IgM) could enhance sensitivity in detecting the disease.

Discussion

Almost all patients with renal problems show anti-dsDNA antibodies and they are also suitable for disease monitoring, since anti-dsDNA antibody concentration increases before disease flares but there is still some controversy. But even though anti-dsDNA antibodies have been established as one of the American College of Rheumatology (ACR) and Systemic Lupus International Collaborating Clinics' criteria for diagnosing SLE, IgA and IgM anti-dsDNA isotypes are not included in follow-up routine of the patients. The combination of analysis of different anti-dsDNA isotypes (IgG, IgA, IgM) provides a more nuanced perspective on SLE disease. It not only enables more precise diagnosis, but also better monitoring of disease activity and progression, especially when distinguishing between organ involvement and tracking treatment courses. Overall, the analysis of anti-dsDNA antibody isotypes could provide a tailored and more precise diagnostic strategy in clinical practice, which may be particularly important in the monitoring of Lupus Nephritis and the specific treatment of SLE patients.

Broad autoantibody profiles reveal novel diagnostic markers and HLA-linked self-reactivities in systemic lupus erythematosus

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Abstract

In systemic lupus erythematosus (SLE), addressing diagnostic delays and disease heterogeneity remains critical. To tackle this, we employed high-throughput microarray technology (i-Ome Discovery; Sengenics) to profile IgG and IgA autoantibodies against 1609 protein antigens in plasma from two independent cohorts. These included SLE patients (n=199 and n=30), healthy controls (n=111 and n=84), and individuals with primary Sjögren's disease (n=115 and n=31) or systemic sclerosis (n=115 and n=24). We validated novel autoreactivities, such as anti-LIN28A, anti-TGIF1, anti-HNRNPA2B1, anti-HMG20B, anti-HMGB2, anti-SUB1, and anti-TFCP2, which exhibited high SLE specificity (0.91–0.94) and substantial sensitivity (0.22–0.69). Notably, anti-LIN28A outperformed conventional markers like anti-double-stranded (ds)DNA in certain diagnostic metrics, yielding positivity (defined as levels exceeding the median plus two interquartile ranges in the healthy control population) in 53% of patients with active central nervous disease and 47% of patients with active kidney involvement. Moreover, LIN28A appeared to be part of enriched pathways linked to neurological manifestations. IgA anti-FOSL2 was

uniquely elevated in patients with active musculoskeletal involvement. Levels of these autoantibodies were positively associated with high disease activity (SLE disease activity index 2000 score ≥10) and inversely with Lupus Low Disease Activity State (LLDAS). Over 14 months, longitudinal tracking in newly diagnosed SLE patients revealed evolving autoantibody patterns, with IgA seroreactivity pointing to mucosal tissue roles. Autoantibody-based clustering identified diverse SLE subgroups, ranging from highly reactive to non-reactive, supporting refined molecular classification. HLA association analysis revealed a cluster of variants in the HLA locus, which was significantly associated with reactivity to CBX5 (top SNP rs2182658: $\beta = -1.34$, $p = 3.0\times10^{-6}$), as well as to other nuclear antigens including HMG20B (rs6937545: $\beta = 0.69$, $p = 1.1\times10^{-5}$) and CCNB1 (rs116464870: $\beta = 1.01$, $p = 7.0\times10^{-5}$). Analysis of the associations between autoantibody profiles and HLA haplotypes identified novel HLA allele associations, such as HLA-B*50 with anti-PSIP1 and HLA-A*25 with anti-CCNB1, indicating currently unknown genetic influences on autoantibody specificity in SLE and other systemic autoimmune diseases, warranting further exploration.

Serological inconsistency in SLE: How assay choice influences anti-dsDNA antibody detection and monitoring

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Objectives

The detection of anti-double-stranded DNA (anti-dsDNA) antibodies is important for the diagnosis, assessment of disease activity and follow-up of patients with systemic lupus erythematosus (SLE). Despite its clinical importance, there remains a significant lack of standardization across anti-dsDNA antibody assays. Commercially available tests vary widely in antigen source, antigen presentation, and detection methodology, leading to marked differences in sensitivity, specificity, and inter-assay agreement. This study compared the performance characteristics of five commercial assays to detect anti-dsDNA antibodies: a recently launched chemiluminescent immunoassay (CLIA; dsDNA IgG assay, IDS), a fluorescence enzyme immunoassay (FEIA; EliA™ dsDNA, Thermo Fisher), an ELISA assay (anti-dsDNA-NcX ELISA (IgG), Euroimmun) and two Crithidia luciliae indirect immunofluorescence tests (CLIFT; NOVA Lite® dsDNA Crithidia luciliae Kit with DAPI, Werfen & dsDNA EUROPattern, Euroimmun).

Material & Methods

A multi-part single-center retrospective study was conducted at AZ Sint-Jan AV, Bruges, Belgium. (a) To evaluate the diagnostic test accuracy, 506 stored serum samples from unique patients with

a positive ANA nuclear pattern (titer ≥1:80) were analyzed using the five anti-dsDNA antibodies assays. All samples had been submitted for ANA testing by internal medicine specialists (rheumatologists, nephrologists, gastroenterologists, or pulmonologists). (b) To evaluate the performance in disease monitoring, 136 longitudinal samples from 17 SLE patients (3–14 samples per patient collected over a 6.5-year period, diagnostic sample available for 2/17 patients) were assessed using the same five assays. For both study parts, relevant medical information was collected in the patient's medical file with approval of the local ethical committee.

Results

(a) Among the 506 patients, 50 (10%) were diagnosed with any form of lupus (including SLE, cutaneous lupus, drug-induced lupus), regardless of disease state (including patients in remission or in lupus flare). Anti-dsDNA antibody results were discordant between assays in 11/50 cases (22%). In 2/50 (4%) samples, anti-dsDNA antibodies were detected by all five assays, whereas 37/50 (74%) samples tested negative in all assays. In patients without any clinical suspicion of lupus (n=456), 418 (92%) samples were concordantly negative by all assays. Assay-specificity among non-lupus cases was high: 98.9% (CLIA), 97.1% (FEIA), 99.1% (ELISA), 97.4% (CLIFT/Werfen), 98.5% (CLIFT/Euroimmun).

(b) When using the manufacturer's recommended diagnostic cut-off to interpret anti-dsDNA antibody kinetics in follow-up samples (n=136) of patients with SLE (n=17), 61 (45%) discordant results between the five assays were detected. Three (18%) patients were concordantly in serological remission by all five assays (in 2/3 patients, anti-dsDNA antibodies were previously objectified). Two (12%) patients exhibited identical patterns of serological disease activity between all five assays. In the majority of patients (71%), serological assessment of disease activity depended heavily on the assay used.

Conclusions

The recently launched CLIA assay (dsDNA IgG assay, IDS) fulfills the international recommended assay specificity level of ≥90% to detect anti-dsDNA antibodies against relevant disease controls. However, this study clearly illustrates the ongoing lack of assay standardization, evidenced by the substantial inter-assay variability in both diagnostic and longitudinal assessments of anti-dsDNA antibodies. These findings underscore the need for harmonized testing strategies in the serological management of SLE.

An unfrequent typical case of PCNA autoantibodies in SLE

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Background and aim

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by aberrant production of an heterogenous group of autoantibodies (Abs). PCNA-Abs are present in 2-10% of SLE patients, especially those with arthritis and hypocomplementemia, and are frequently (>70%) associated with negative anti-dsDNA autoantibodies. The aim of this work is to emphasize the role in renal involvement of these unfrequent autoantibodies, by the description of a clinical case.

Material and methods

Here we describe a case of a 34-years-old Asian woman, with persistent arthralgias and low-grade fever, who was hospitalized in Nephrology department for these findings: anemia (Hb 7.8 g/dL), renal failure (sCr 2.3 mg/dL), proteinuria (uProt 1,5 g/die) and microhematuria. Further laboratory evaluations revealed hypocomplementemia (C3 and C4 reduced) and ANA-IFI positive (titer 1:1280) for a double fluoroscopic pattern: AC-4 and AC-13. These findings were confirmed with a line-immunoblot method, which revealed a strong positivity to PCNA-Abs and with a CLIA method, which showed a positivity for SSA-52 and SSA-60, both above the limit of quantification. Negative was the detection of anti-dsDNA instead. The patient finally underwent a renal biopsy with histological report of class IV+V lupus nephritis.

Results

The diagnosis of SLE was clear. The patient underwent a steroid therapy with prednisone at 1 mg/kg/die (with progressive tapering down to 5 mg/die), and subsequently mycophenolate mofetil was added at 1.5 g twice daily for 3 months, then reduced to 1 g twice daily. After rheumatological evaluation, hydroxychloroquine, ACEi and SGLT2i and native vitamin D were introduced.

One year after diagnosis, the patient showed significant clinical improvement, with reduced renal function markers (sCr 1.4 mg/dL) and no pathological proteinuria (140 mg/die), despite persistently high ANA titers (>1:1280). A repeat renal biopsy is currently pending.

Conclusions

The key points of this clinical report are: the diagnosis of lupus nephritis with anti-dsDNA negativity and positivity for the rare anti-PCNA fluoroscopic pattern, associated with severe histopathological involvement; the stabilization of renal function and the normalization of proteinuria after one year of corticosteroids and mycophenolate therapy, despite persistent high titers of anti-PCNA antibodies.

Although the rare presence of anti-PCNA antibodies has been associated with increased severity of organ involvement, very few data exist on the clinical-serological correlations of anti-PCNA Abs in lupus nephritis.

Biomarker-based forecasting of SLE flares: Toward a new era of precision and proactive disease management

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Background

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease characterized by unpredictable disease activity, posing significant clinical and scientific challenges. This study aims to explore dynamic autoantibody profiling as a biomarker-driven strategy to predict SLE flares, aiming to shift disease management toward a more proactive and precision-guided approach.

Methods

In a prospective cohort study, patients with SLE were monitored longitudinally at the University Medical Center Utrecht, the Netherlands. Standardized study visits were conducted every three months, including comprehensive clinical assessments and flare evaluations using the SELENA-SLEDAI Flare Index. Concurrently, blood samples were collected for biomarker profiling. Samples were processed using a highly standardized biobanking protocol to ensure maximal analyte stability and reproducibility. Blood was collected in citrate tubes, centrifuged within two hours of venipuncture, and plasma was aliquoted under sterile conditions. Aliquots were immediately stored at –80°C and were never subjected to freeze–thaw cycles, preserving protein and antibody integrity. Levels of SLE-associated autoantibodies and calprotectin - a marker of neutrophil activation - were semi-quantitatively measured in citrate plasma using the EliA™ platform (Phadia AB, Sweden). Binary logistic regression was used to assess whether baseline biomarker positivity could predict flare occurrence within the study period.

Results

A total of 100 patients with SLE were screened, with 98 meeting the eligibility criteria. These patients were followed up every three months, resulting in a total of 844 study visits with stored blood samples. At baseline, disease activity was generally low, with a median SLEDAI score of 4 (IQR 2–6). Additional baseline characteristics are presented in Table 1. During the follow-up period, 193 disease flares were documented, comprising 180 moderate and 13 severe episodes. At baseline, anti-double-stranded DNA (dsDNA) antibody positivity was the most frequently observed at 41%, followed by positivity for Ro60, Ro52, and RA33 IgM antibodies (33%, 20%, and 20%, respectively). The associations between baseline antibody positivity and the occurrence of disease flares over the subsequent two years are presented in Table 2. Initial analysis showed that higher dsDNA antibody levels were associated with flares (Figure 1). Calprotectin levels also showed associations with flares. More in-depth analysis is ongoing to investigate the associations between clinical parameters, disease flares, and specific autoantibody patterns. Additionally, cytokine patterns will be evaluated in both plasma and urine samples collected within the study.

Conclusion

This study highlights the potential of dynamic autoantibody profiling, particularly changes in anti-dsDNA and calprotectin levels, as a promising strategy for predicting disease flares in patients with systemic lupus erythematosus. The use of rigorously collected, never thawed biobanked plasma samples ensures high data fidelity, thereby strengthening the reliability of our findings. While preliminary, these results support a shift toward more proactive, biomarker-informed disease monitoring, laying the groundwork for precision-guided management in SLE. The planned analysis of cytokine profiles in these patients will provide further insight into the disease's pathogenicity and enhance the tools available for precision medicine. Ongoing analyses will further elucidate the temporal dynamics of specific antibody and cytokine profiles and their predictive value for the occurrence of flares.

Table 1. Baseline characteristics

	N = 98
Age; median (IQR)	50 (39 - 57)
Etnic distribution; n (%)	
- White	76 (77.6)
- Asian	9 (9.2)
- Black	4 (4.1)
- Unknown/other	9 (9.2)
Female sex; n (%)	86 (87.8)
Disease duration, months?; median (IQR)	18 (8 – 28)
SLEDAI-2K score; median (IQR)	4 (2 – 6)
SDI-score; median (IQR)	1 (0 – 2)

IQR: interquartile range; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index 2000; SDI: SLICC/ACR Damage Index

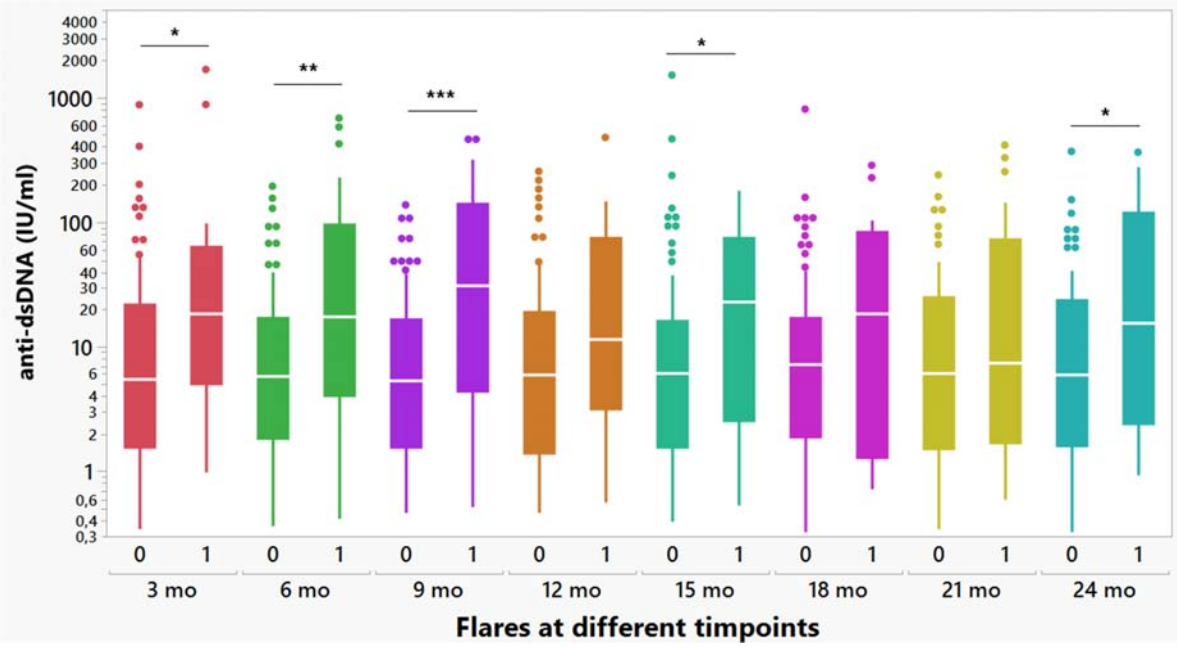

Figure 1. Differences in dsDNA levels at different timepoints split by flares 0 = no flare; 1 = flare

Table 2. Prediction of flares based on antibody positivity at baseline

Antibody	% positive at baseline	OR	p-value	95% CI	
				Lower	Upper
dsDNA	41	2.41	0.084	0.89	6.55
aβ2 IgG	11	1.64	0.543	0.33	8.10
aβ2 IgM	7	1.27	0.771	0.25	6.47
aCL IgG	5	0.92	0.911	0.23	3.74
aCL IgM	2	0.81	0.768	0.20	3.34
RA33 IgM	20	1.84	0.371	0.49	6.98
RF IgM	4	0.76	0.754	0.14	4.21
Calprotectin	5	1.02	0.975	0.38	2.71
SmDP-S	12	3.84	0.210	0.47	31.49
La/SSB	16	0.70	0.544	0.22	2.24
RibP	3	1.26	0.842	0.13	11.85
RNP70	7	2.30	0.448	0.27	19.73
Ro52	20	1.21	0.761	0.36	4.08
Ro60	33	1.39	0.519	0.51	3.80

Results of the binary logistic regression analysis assessing whether antibody positivity at baseline predict the occurrence of a flare during the study follow-up. Odds ratios (OR) with 95% confidence intervals (CI) are reported. OR > 1 indicates an increased risk of flare. $p < 0.05$ was considered statistically significant. aB2: anti-beta 2 glycoprotein 1; aCL: anti-cardiolipin

Lupus and nutrition – The first step to control your flares?

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease, characterized by the presence of autoantibodies and diverse clinical manifestations, including one or even more organs. Still, the exact cause of Lupus is unknown, and symptoms include also fevers, rashes, swelling and pain. Some gastrointestinal symptoms in lupus patients, such as bloating or abdominal pain, could be related to food allergies or intolerance. Gluten intolerance or celiac disease may occur more frequently in lupus patients, which can increase gastrointestinal symptoms. Research even from 1993 shows that people with lupus are at a much higher risk of developing allergies to drugs, skin, and insects. Moreover, family members of individuals with systemic lupus erythematosus are also more likely to experience at least one type of allergy.

Aim

To have a closer look on the antibody and autoantibody profile related to lifestyle and nutrition of SLE patients to improve disease management and quality of live.

Method

A total of 17 patients with a clinical diagnosis of SLE were tested using AESKUBLOTS® Allergy and AESKUBLOTS® Gluten-Related Disorders (GRD) IgA. AESKUBLOTS® Allergy is a membrane-based enzyme immunoassay for quantitative detection of allergen-specific IgE antibodies against allergens/allergen mixtures and total IgE in human plasma or serum. The AESKUBLOTS® GRD IgA is a membrane-based enzyme immunoassay for quantitative detection of IgA subclass antibodies against gliadin, DGP, tTG (tissue Transglutaminase), tTG neo (cross-linking of tTG with gliadin-specific peptides induces the formation of tTG neo-epitopes), TG3 (epidermal Transglutaminase), mTG (microbial Transglutaminase), mTG neo (cross-linking of mTG with gliadin-specific peptides induces the formation of mTG neo-epitopes), Frazer's Fraction in and total IgA in human serum or plasma. The antigens are positioned as parallel lines at precisely defined locations on a nitrocellulose membrane.

Results

A total of 52% of patients exhibited high IgE levels (class 5-6) against allergens such as wheat, spelt, egg white, casein, and various nuts including almond, hazelnut, peanut, pistachio, and cashew. Additionally, 5 out of 17 (30%) patients showed elevated antibodies against gliadin (>3-5x ULN (Upper Limit of Normal)), DGP (>3-5x ULN), mTG-neo (>2-3x ULN) and autoantibodies against tTG (>2x ULN) and tTG-neo (>3-5x ULN) antigens, which are highly associated with gastrointestinal disorders like celiac disease and non-celiac gluten sensitivity. Three SLE patients demonstrated significantly elevated food allergy-specific IgE levels (≥class 4) along with high levels of autoantibodies related to GRDs.

Discussion

Even within this small cohort, antibodies and autoantibodies associated with food allergies and GRD are significantly elevated compared to the general population. A comprehensive understanding of SLE epidemiology is urgently needed to gain deeper insights into the disease and better manage healthcare resources. The close interaction between autoimmunity, inflammation, and allergies means that during a lupus flare, both gastrointestinal symptoms and allergic reactions can be exacerbated, which further complicates the treatment and management of SLE patients. Accurate identification, antibody monitoring via multiplex and treatment of gastrointestinal complaints and allergies during a lupus flare are crucial to improving the quality of life for affected patients and preventing complications.

4 Advances in Research and Diagnostics of Autoantibody in Systemic Sclerosis and Idiopathic Inflammatory Myopathies

Immunoprecipitation–mass spectrometry: an emerging reference method for autoantibody detection in myositis and systemic sclerosis

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Abstract

Immunoprecipitation is regarded as the golden standard for identification of antinuclear antibodies. The technique has been used to identify many novel autoantigens. However, it is only performed in a handful of laboratories worldwide as it relies on the use of radiolabeled cell extract and the availability of reference sera. Moreover, interpretation is hampered when different autoantigens have a similar molecular weight.

An alternative immunoprecipitation approach has been proposed in which the proteins in the immunoprecipitate are directly analyzed by mass spectrometry. The advantage of the immunoprecipitation-mass spectrometry approach is that it does not rely on radioactivity and that identification of the proteins is not affected by the molecular weight. Moreover, as mass spectrometry is increasingly implemented in clinical laboratories, it can be offered in a clinical context.

The immunoprecipitation-mass spectrometry approach is powerful for identification of antibodies to complex autoantigens. A typical example is the detection of anti-OJ antibodies Anti-OJ antibodies are directed against the multi-enzyme synthetase complex which consists of several synthetases. With immunoprecipitation-mass spectrometry the whole complex is precipitated and identified. Immunoprecipitation mass spectrometry has also proven superior to line or dot blots for detection of anti-Zo antibodies.

Immunoprecipitation-mass spectrometry is also powerful for detection of antibodies to complex autoantigens in systemic sclerosis, such anti-Th/To, anti-PM-Sc and anti-fibrillarin. Finally, immunoprecipitation-mass spectrometry allows to identify novel autoantibodies, such as anti-Ly antibodies and antibodies to the TFIID complex.

During the presentation, the technology will be explained and illustrated for detection of antibodies in myositis and systemic sclerosis.

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4.1 Autoantibodies in SARD-Associated Interstitial Lung Diseases (ILD)

Autoantibodies in myositis associated ILD

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Abstract

Interstitial lung disease (ILD) is reported in 40-50% of patients with Idiopathic Inflammatory Myopathies (IIM) and it is the primary cause of morbidity and mortality in this patient group. So far, autoantibodies are the most reliable biomarkers predicting occurrence and severity of ILD. Among myositis-specific autoantibodies (MSAs), anti-synthetase autoantibodies, of which anti-Jo1 is the most frequent, identify a subgroup of IIM, called anti-synthetase syndrome (ASSD) with a prevalence of ILD up to 60-100%. In addition to anti-synthetase autoantibodies, antibodies targeting the melanoma differentiation-associated protein 5 (MDA5) are strongly associated with dermatomyositis and ILD that, especially in Asian cohorts, often develops as rapidly progressive leading to premature death. In the presence of ILD, patients with IIM are treated with a combination of high doses of glucocorticoids and immunosuppressive drugs but response to treatment varies.

The potential role of the myositis specific autoantibodies in the disease mechanisms of ILD in myositis have not been clarified but indirect support for a role in disease development will be discussed.

Phenotypic clusters of anti-MDA5 antibody-positive dermatomyositis: Prediction of rapid progressive ILD

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Abstract

Anti-melanoma differentiation-associated gene 5 (anti-MDA5) dermatomyositis (DM) represents one of the most clinically severe and distinct subsets of autoimmune inflammatory myopathies. This subtype can be associated with rapidly progressive interstitial lung disease (RPILD), a complication that carries a high risk of mortality. However, lung involvement is not universally present among all patients with anti-MDA5 DM, underscoring the heterogeneity of the disease presentation and clinical course.

The mechanisms underlying the development of RPILD in anti-MDA5 DM remain incompletely understood, and there is a critical need to elucidate the pathogenic pathways that drive this aggressive phenotype. Better identification of patients at risk for RPILD is needed, as early diagnosis and intervention may improve outcomes. Recent studies have begun to reveal distinct clinical and serological phenotypic clusters within the anti-MDA5 DM population, offering potential insight into disease stratification and prognosis.

This presentation will review the emerging evidence around the anti-MDA5 DM phenotypes as well as current gaps in knowledge. We will discuss future directions for research, including the development of targeted therapeutic strategies aimed at improving outcomes for patients affected by this life-threatening complication.

Epitope mapping of Anti Ro52 antibodies in antisynthetase syndrome and inflammatory myositis

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Introduction

Idiopathic inflammatory myositis (IIM) is a heterogeneous group of autoimmune diseases characterized by muscle inflammation, weakness, and the presence of specific autoantibodies [1]. Among the various forms of IIM, polymyositis and dermatomyositis are the most recognized, each with distinct clinical features and associated autoantibody profiles [1,2]. We and others have shown that myositis-specific autoantibodies (MSAs), including anti-Jo1 and other anti-synthetase antibodies, play a crucial role in the diagnosis and classification of IIM, as they are often associated with specific clinical manifestations and disease outcomes [1-7].

Notably, a significant proportion of patients who test positive for anti-Jo1 and other anti-synthetase antibodies also exhibit reactivity against Ro52, an autoantigen that has received attention in the context of rheumatic and other autoimmune diseases (8-10). Despite the frequent occurrence of anti-Ro52 antibodies in this subset of patients, and particularly their co-occurrence with anti-synthetase antibodies, the underlying mechanisms driving this association remain unclear, raising essential questions about the antigen-driven processes that may contribute to the induction of anti-Ro52 antibodies in this group of heterogeneous diseases. The epitopic regions of Ro52 have been mapped in Sjogren's disease and systemic lupus erythematosus, primarily targeting conformational (and linear) epitopes within the coiled-coil domain (aa 125–268).

Considering the complexities surrounding the immunological landscape of IIM, this study aims to assess the epitope specificity of anti-Ro52 antibodies in patients with IIM and look for differences or similarities posed by the concurrent anti-synthetase antibodies, and in particular anti-Jo1, anti-PL7 and anti-PL12 which are the most frequent. Understanding the nuances of Ro52 epitope

recognition may illuminate the factors contributing to the development of anti-Ro52 antibodies and enhance our comprehension of the antigen-driven impetus in these multifaceted diseases.

Material and Methods

Patients

Serum samples from a total of 35 anti-Ro52 antibody positive patients with IIM were studied. The status of myositits-specific (MSA) and myositis-associated antibodies (MAA) was pre-determined in all patients using a myositis profile [7]. Main demographic characteristics and frequencies of MSA reactivities are shown in Table 1.

Table 1. Main demographic characteristics and frequencies of MSA reactivities.

	N=35, n (%)
Sex (F/M)	26 (74.3) / 9 (25.7)
Age (Mean ± 2sd)	61.76 ± 26.26
Autoantibodies	
Ro52	35 (100)
Jo1	17 (48.6)
PL7	6 (17.1)
PL12	9 (25.7)
NXP2	2 (5.7)
OJ	2 (5.7)
EJ	2 (5.7)
FARSA1	4 (11.4)
FARSB1	4 (11.4)
MDA5	1 (2.9)

Line Immunoassay testing

Epitope mapping was conducted with a custom line immunoassay incorporating five recombinant Ro52 fragments expressed in *Escherichia coli*: Ro52-1 (aa 1–127), Ro52-2 (aa 125–268), Ro52-3 (aa 268–475), Ro52-4 (aa 57–180), and Ro52-5 (aa 181–320) (Figure 1), in line with prior published protocols of out research group [11-13].

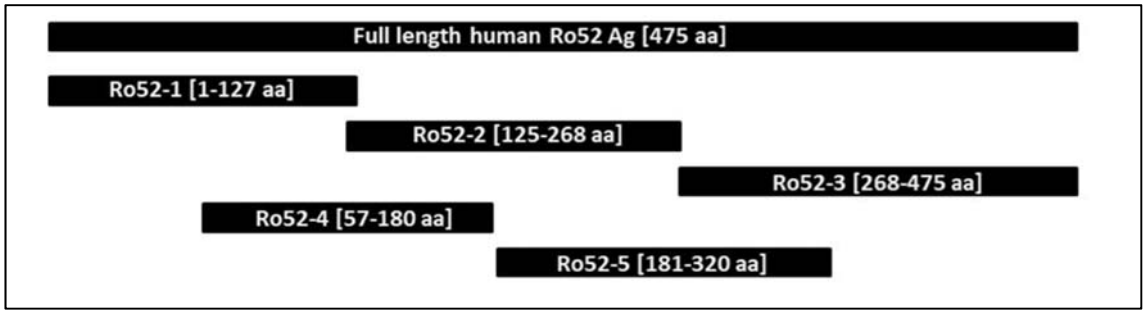

Figure 1. Amino acid sequences of the 5 Ro-52 fragments

The full-length Ro-52 antigen, produced via a baculovirus/insect cell system, served as the positive control. Titration experiments established optimal assay conditions. Fragment concentrations were chosen based on ROC analysis from four tested concentrations (1, 5, 25, 100 µg/mL) using sera from 20 anti–Ro52–negative healthy individuals as previously described (11-13]. The concentrations providing up to 94% specificity were 100 µg/mL for Ro52-1 and 25 µg/mL for Ro52-2 through Ro52-5. The specially designed line strips were incubated with sera at a 1:100 dilution on a rocking platform at room temperature for 30 minutes [11-13]. Following aspiration, strips were washed three times with 1.5 mL wash buffer (Euroimmun) for 5 minutes each. Strips were then incubated with an alkaline phosphatase–labeled anti-human IgG conjugate (Euroimmun) for 30 minutes, followed by three 5-minute washes [11-13]. Development was performed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate (Euroimmun) for 10 minutes, after which strips were rinsed with distilled water and air-dried. Strips were evaluated using EUROLineScan software (Euroimmun) and results were expressed in arbitrary units (AU/mL), as described previously [11-13].

Results

Representative results of the analyses of antibody reactivities against separate Ro52 fragments based on their autoantibody status are shown in Tables 2 & 3.

In the analysis of Ro52 fragment reactivities among patients with IIM, statistically significant differences were observed exclusively for the Ro52-1 fragment. No difference was observed regarding fragments Ro52-2 (34/35 pos, 97.1%, panepitope universally recognized), Ro52-3 (all negative), Ro52-4 and Ro52-5 (no difference amongst groups). As it is noted, anti-Jo1 negative patients tended to react more frequently against Ro52-1 fragment compared to Jo1 positive patients (12/18, 66.7% vs 6/17, 35.3% p=0.063).

Those anti-Jo1 positive patients who were also positive for one or more of the other MSAs tested were totally unreactive against Ro52-1 compared to anti-Jo1 negative (12/18, 66.7%, p=0.014). Notably, IIM patients with anti-PL7 or anti-PL12 positivity were more frequently reactive against Ro52-1 fragment compared to patients being anti-PL7 and anti-PL12 negative ($p<0.001$).

Table 2. Reactivity against full Ro52 antigen and its separate fragments in patients with Inflammatory Myopathies (IM) **(Jo-1+ vs Jo-1-).**

	Total patients (n=35), N (%)	**Ro52+/Jo-1+ (n=17), N (%)**	**Ro52+/Jo-1- (n=18), N (%)**	**P-value**
Ro52	35 (100)	17 (100)	18 (100)	NS
Ro52-1	18 (51.4)	6 (35.3)	12 (66.7)	0.063
Ro52-2	34 (97.1)	17 (100)	17 (94.4)	NS
Ro52-3	0 (0)	0 (0)	0 (0)	NS
Ro52-4	8 (22.9)	4 (23.5)	4 (22.2)	NS
Ro52-5	14 (40)	7 (41.2)	7 (38.9)	NS

Table 3. Reactivity against full Ro52 antigen and its separate fragments in patients with Inflammatory Myopathies (IM) **(Jo-1 only+ VS Jo-1- VS Jo-1+ plus other MSA+).**

	Total patients (n=35), N (%)	Ro52+ Jo-1 only+ (n=12), N (%)	Ro52+ Jo-1- (n=18), N (%)	Ro52+ Jo-1+ plus other MSA+ (n=5), N (%)	P-value Jo-1 only+ VS Jo-1-	P-value Jo-1 only+ VS Jo-1+ plus other MSA+	P-value Jo-1- VS Jo-1+ plus other MSA+
Ro52	35 (100)	12 (100)	18 (100)	5 (100)	NS	NS	NS
Ro52-1	18 (51.4)	6 (50)	12 (66.7)	0 (0)	NS	0.102 - NS	0.014
Ro52-2	34 (97.1)	12 (100)	17 (94.4)	5 (100)	NS	NS	NS
Ro52-3	0 (0)	0 (0)	0 (0)	0 (0)	NS	NS	NS
Ro52-4	8 (22.9)	2 (16.7)	4 (22.2)	2 (40)	NS	NS	NS
Ro52-5	14 (40)	6 (50)	7 (38.9)	1 (20)	NS	NS	NS

Discussion

The complicated serological setting of IIM, characterised by the frequent simultaneous presence of anti-Ro52 and MSAs, has long been regarded as puzzling, pointing towards a nexus of interconnected autoimmune responses [1,2]. Our present study attempts to dissect the humoral response to Ro52 at the epitope level, in a continuum to reveal clues regarding the origin of the striking anti-Ro52/MSA relationship [7]. We have found an appealing MSA-dependent hierarchy of epitope recognition, challenging the notion of an uninterrupted anti-Ro52 response and highlighting the complexity of the immune response in IIM. The central astonishment of our work is the conditional immunogenicity of the N-terminal domain of Ro52 (Ro52-1, aa 1–127). While the coiled-coil domain (Ro52-2) is the most immunodominant pan-epitope, recognized almost universally across our cohort, the N-terminus emerged as a key site of distinct epitope recognition. Strikingly, reactivity against Ro52-1 was not only lessened in the presence of anti-Jo1 but was completely extinguished in patients holding both anti-Jo1 and additional MSAs (p=0.014). This potent, MSA-associated suppression of an epitope response specifically limited to one of the many epitopes of Ro52 suggests a profound level of immunological governance.

Among the anti-Jo-1 antibody negative, anti-Ro-52 positive patients (n=18), 12 (66.7%) reacted with Ro-52-1, whereas none of the 5 anti-Ro-52 positive patients with anti-Jo-1 and additional MSA showed reactivity to this polypetidyl peptide. Previously, we have mapped the epitope profile of anti-Ro-52 antibodies in various diseases, including systemic sclerosis, Sjögren's disease, systemic lupus erythematosus, and even in patients with cancer who also possess anti-Ro52 antibodies. We have shown that Ro-52-1 in an autoantibody target in almost half of the patients with SLE or SjS, but in just 12% of patients with systemic sclerosis. However, SSc patients who had an "SjS"-like profile, meaning they had both anti-Ro52 and anti-Ro60 antibodies, more frequently reacted with Ro-52-1 (33%) compared to anti-Ro52+/anti-Ro60-patients, who do not respond at all with Ro-52-1 [12]. Considering that autoantibodies against Ro-52 co-exist with Jo-1 (as with Ro60 in SjS and SLE), we expected to find in patients with the former co-occurrence what was found with those with the latter autoantibody status, the same seroprevalence in terms of Ro-52-1 epitopic recognition [12], but we failed. In fact, in the presence of anti-Jo-1 and any other MSA, Ro-52-1 is totally unreactive. The reasons behind this observation are beyond the scope of this study. It compels us to consider mechanisms beyond mere epitope spreading, that of intramolecular epitope masking mediated by the binding of MSAs to a putative synthetase-Ro52 complex being the most likely. Another possibility is that of distinct antigen processing pathways dictated by the primary autoantigen, namely Ro52. Notably, patients with additional MSAs, such as **anti-PL7 or anti-PL12,** showed even greater reactivity to Ro52-1 (p<0.001). In practice, anti-PL12/PL7 presence is analogous to the presence of anti-Ro60 in making the R052-1 sequence accessible to Ro52 autoantibodies. As for other disease cohorts, like SjS, SLE and SSc, **Ro52-3 fragment (aa 268–475)** is entirely unreactive also in IMM, while **Ro52-4 (aa 57–180)** and **Ro52-5 (aa 181–320)** showed no significant differences in reactivity across IIM patient groups.

From a diagnostic perspective, the distinct epitope pattern recognition surrounding Ro52-1 may have a practical utility and may serve as a biomarker for **anti-Jo1-negative patients** or those with anti-PL12/PL7 antibodies. The fact that the coiled-coil domain (Ro52-2) is a panepitope underscores its potential use as a fragment for anti-Ro52 testing across anti-Ro52 seropositive cohorts, irrespective of their disease state (autoimmune or non-autoimmune). Comprehensive autoantibody profiling, including epitope-specific analysis at short peptides spanning the whole Ro52, may enhance the diagnostic and prognostic accuracy in IIM and related conditions and

provide additional clues regarding the peculiar epitope recognition. Additionally, **mechanistic studies** focusing on the most likely Ro52/MSA antibody binding are needed to elucidate the immunological processes underlying the observed epitope recognition patterns. They will be crucial for advancing our understanding of the immunology behind IIM.

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Importance of biomarkers in the diagnosis and management of interstitial lung disease

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Abstract

Interstitial lung disease (ILD) is a common and serious complication across a range of systemic autoimmune rheumatic diseases (SARD), including systemic sclerosis, rheumatoid arthritis, and autoimmune inflammatory myopathies. ILD contributes substantially to morbidity and is a leading cause of mortality in several SARD subtypes. Notably, a significant proportion of patients present with ILD either at or soon after their initial SARD diagnosis, underscoring the importance of early detection and intervention.

At present, the primary modality for identifying and monitoring ILD is high-resolution computed tomography (HRCT), often in conjunction with pulmonary function testing. There is a growing interest in the use of biomarkers to complement current diagnostic tools. Biomarkers hold the promise of enabling earlier diagnosis, stratifying risk, predicting disease trajectory, and evaluating treatment response.

This presentation will explore the evolving landscape of biomarker research in SARD-associated ILD, highlighting currently available biomarkers, their utility in clinical practice, and the limitations that hinder their widespread use. We will also discuss promising novel biomarkers under investigation to better understand disease mechanisms and personalize patient care. Ultimately, this session will address how biomarker-driven strategies may transform the future diagnosis, monitoring, and management of ILD in SARD.

4.2 Autoantibodies in Idiopathic Inflammatory Myopathies

Predictive value of myositis antibodies: role of semiquantitative classification and positivity for more than one autoantibody

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Objectives

We assessed the positive predictive value (PPV) of 17 myositis antibodies for having a diagnosis of myositis and other myositis-spectrum conditions (interstitial lung disease (ILD), connective tissue diseases (CTD), malignancy) and evaluated the impact of semiquantitative classification and antibody overlap on the PPVs.

Materials and methods

We retrospectively identified 1068 individuals ≥18 years who tested positive for ≥1 antibody in the EUROLINE myositis line blot assay or positive for anti-3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in an ELISA-based test between 2015 and 2020 in 15 out of the 20 hospital districts in Finland. We extracted clinical diagnoses from the Care Register for Health Care between January 2013 and June 2022.

Results

The PPV for a myositis diagnosis (ever during data collection) was highest for anti-HMGCR antibodies (94%), followed by anti-MDA5, anti-Jo-1 and anti-TIF1-γ (49–54%). Regarding other myositis antibodies, 18–42% of cases had myositis. Anti-synthetase antibodies, anti-MDA5, anti-PM-Scl100, anti-SAE1 and anti-Ro52 had a PPV for ILD of 25–47%. A PPV for CTD was highest for

anti-Ro52 (57%). The PPV for malignancy was highest for anti-TIF1-γ (38%), followed by anti-PL-7 (32%). Stronger antibody band intensity was associated with higher PPVs for myositis and CTD but not for ILD or malignancies. Simultaneous positivity for ≥2 antibodies compared with single antibody was associated with higher PPVs for myositis, CTD and ILD.

Conclusion

The PPV of myositis antibodies for diagnoses of myositis or other myositis spectrum diseases vary considerably between individual autoantibodies. Higher PPVs can be expected with stronger band intensities and with the presence of ≥2 overlapping myositis antibodies.

RMD Open, published on 20 January 2025

Frequencies of myositis-specific antibodies (MSAs) and myositis-associated antibodies (MAAs): Our experience

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Background

Myositis-specific autoantibodies (MSAs) or myositis-associated autoantibodies (MAAs) play a crucial role in the diagnosis, classification, and prognosis of idiopathic inflammatory myopathies (IIMs) and are generally considered mutually exclusive.

Aim

To assess the frequencies of MSAs and MAAs positivity, with special emphasis on the occurrence of multiple concurrent MSAs positivity.

Patients and methods

We retrospectively analyzed data from 412 patients referred to our laboratory for IIM antibodies testing between December 2023 and May 2025. Autoantibody testing was performed using line immunoassay (LIA) (EUROLINE Autoimmune Inflammatory Myopathies 16 Ag (IgG), EuroImmun, Lübeck, Germany). When requested, antinuclear antibody (ANA) testing by indirect immunofluorescence (IIF) was performed on HEp-2 cells (EuroImmun, Lübeck, Germany).

Results

Out of 412 samples, all autoantibodies were negative in 245 (59.5%) cases. The frequencies of individual antibodies positivity were as follows: anti-Mi-2alfa 18 (4,4%), anti-Mi-2beta 16 (3,9%), anti-TIF1y 9 (2,2%), anti-MDA5 5 (1,2%), anti-NXP2 14 (3,4%), anti-SAE1 4 (1,0%), anti-Ku 17 (4,1%), anti-PMScl100 20 (4,9 %), anti-PMScl75 31 (7,5%), anti-Jo-1 6 (1,5%), anti-SRP 8 (1,9%), anti-PL-12 15 (3,6%), anti-PL-7 10 (2,4%), anti-EJ 4 (1,0%), anti-OJ 4 (1,0%), anti-Ro-52 54 (13,1%). Monospecific reactivity was found in 120 (29.1%), and multiple antibody reactivity in 47 (11.4%)

cases, including double positivity in 33 (8,0%), triple in 8 (1,9%) and four or more antibodies in 6 (1,5%). After exclusion of weakly positive results (1+), the number of cases with multiple antibody positivity decreased to 19 (4.6%): double positivity in 15 (3,6%), triple in 3 (0,7%) and four antibodies in 1 (0,2%). Multiple MSAs positivity was observed in 5/19 (26.3%). The antibody profiles (including MAAs) were: 1. anti-PL-12 (2+) and anti-OJ (3+); 2. anti-TIF1γ (2+) and anti-SAE1 (2+); 3. anti-Mi-2beta (2+) and anti-PL-12 (2+); 4. anti-NXP2 (2+), anti-OJ (2+) and anti-Ku (3+); 5. anti-Mi-2beta (2+), anti-TIF1γ (2+) and anti-PL-12 (2+). In 3/5 MSAs multi-positive cases, ANA IIF results were available. Concordance with LIA findings was observed only in Case 2, where anti-TIF1γ (2+) corresponded with AC-4 pattern (1:320) and negative ENA7. Regarding clinical correlation, in Case 1 symptoms and MSAs were consistent with anti-synthetase syndrome; in Case 2 clinically amyopathic dermatomyositis and breast carcinoma were confirmed, aligning with detected MSAs; in Cases 3–5 the MSAs identified did not correlate with the clinical presentation.

Conclusion

Our findings confirm the limited specificity of weakly positive LIA results and highlight the need to reconsider the thesis of mutual exclusivity among MSAs. Consistency with expected ANA pattern can be useful for interpretation of multipositive reactivity.

Detection of anti-synthetase antibodies by immunoprecipitation-mass spectrometry

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Abstract

The presence of anti-synthetase autoantibodies is important for the diagnosis of anti-synthetase syndrome (ASyS) and has the highest weight in the recent development of candidate classification criteria [1]. However, commercial solid phase immunoassays (including line/dot blot) lack reliability for the identification of non-anti-Jo1 autoantibodies. Immunoprecipitation (IP) using radiolabeled cell extract is considered the golden standard for the detection of anti-synthetase autoantibodies. We here present the added value and validation of IP (using HeLa cellular extract as antigen source) with mass spectrometry-based identification of the precipitated proteins (IP-MS) [2] for detection of anti-synthetase autoantibodies. Furthermore, we identified anti-synthetase autoantibodies in cytoplasmic ANA-positive samples (n=4), that were not reported by commercially available assays.

Anti-synthetase antibodies were not detected by IP-MS in 23 samples obtained from healthy individuals. Method comparison of IP-MS against the golden standard, radiolabeled IP (performed at the University of Bath, UK), showed concordant results for 16/17 anti-synthetase antibodies [Jo1 (n=3), PL-7 (n=2), PL-12 (n=3), EJ (n=3), OJ (n=3), Ha (n=2)]. In one sample, IP-MS revealed anti-Jo1 antibodies whereas radiolabeled IP revealed anti-PL-7 antibodies. The added value of IP-MS compared to commercial assays was further demonstrated by the IP-MS based identification of anti-synthetase antibodies [OJ (n=3) and KS (n=1)] in myositis patients with a cytoplasmic fine speckled ANA pattern but unidentified autoantibodies from two different medical centres [Centre Hospitalier Lyon Sud (France) (n=3) and University Medical Centre Ljubljana (Slovenia) (n=1)].

In summary, IP-MS offers complementary benefits to the currently used commercial assays in the detection of rare anti-synthetase antibodies. Given the clinical relevance and importance of these antibodies for diagnosing ASyS, we validated the IP-MS method for use in a clinical laboratory.

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Is the PL-7 index a useful immunological biomarker in antisynthetase syndrome?

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Purpose

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of systemic autoimmune diseases that primarily affect skeletal muscle. Among them, antisynthetase syndrome (ASS) represents a well-defined clinical subset, associated with autoantibodies directed against aminoacyl-tRNA synthetases, such as anti-PL-7. Although immunoblotting is the most widely used method for their detection, its performance may be influenced by technical variability. To minimize these inaccuracies and improve diagnostic performance, the PL-7 index is employed.

Aims

Evaluate the diagnostic utility of the PL-7 index (adjusted band intensity) as a semi-quantitative measure of anti-PL-7 antibodies, and to assess its association with immunological and clinical variables in patients with suspected ASS.

Methods

A retrospective observational study was conducted on a cohort of patients from a tertiary hospital (2019–2025) who tested positive for anti-PL-7 antibodies using the Euroline immunoblot assay (EUROIMMUN), with a manufacturer-recommended cut-off of ≥15.

Clinical data, including the diagnosis of ASS or interstitial lung disease (ILD) were recorded, along with immunological findings such as ANA patterns and semi-quantitative densitometric measurements of the internal control band and the PL-7 antigen band.

The PL-7 index was calculated as the ratio of PL-7 band to internal control multiplied by 100. Statistical analysis included Kruskal–Wallis, Mann–Whitney U, ROC curves, and McNemar's test.

Results

A cohort of 55 patients with anti-PL-7 antibodies (70.9% female) was analyzed. 10 (18.2%) met ASS criteria, 7 ILD (12.7%), 20 (36.4%) were ANA-positive and 2 (3.6%) with AC-19/AC-20 patterns.

The PL-7 index showed no global differences among ASS, ILD, and patients with other diagnoses ($p = 0.0568$). Pairwise analysis of the PL-7 index showed statistical significance in patients with ASS compared to other diagnoses ($p = 0.038$) and in ANA AC-19/AC-20 positive patients compared to ANA-negative patients or those with other patterns ($p = 0.045$).

For PL-7 band densitometry in ASS, the optimal cut-off was ≥38 (AUC = 0.698), with sensitivity 90%, specificity 51%, positive predictive value (PPV) 29%, negative predictive value (NPV) 96%, positive likelihood ratio (LR+) 1.84 (95% CI 1.28–2.65), and negative likelihood ratio (LR–) 0.20 (95% CI 0.03–1.28). For the PL-7 index in suspected ASS, the optimal cut-off was ≥27.0 (AUC = 0.712), with sensitivity 70%, specificity 71%, PPV 35%, NPV 91%, LR+ 2.42 (95% CI 1.31–4.47), and LR– 0.42 (95% CI 0.16–1.11).

At the optimal cut-offs, there were no differences in sensitivity (McNemar $p = 0.50$), whereas specificity was higher for the PL-7 index (McNemar $p = 0.0039$), indicating fewer false positives with the PL-7 index.

Conclusions

The results indicate that a high proportion of patients with anti-PL-7 antibodies detected by immunoblot do not exhibit a confirmed ANA AC19-20 pattern, underscoring the methodological limitations of the immunoblot strip, likely due to antigenic characteristics or nonspecific reactivity.

It is also observed that the PL-7 index provides greater diagnostic utility than the raw PL-7 densitometric value in the context of ASS. The index enables better discrimination between patients by yielding fewer false-positive results, thereby facilitating patient management while also reducing the influence of technical variability.

Further studies in larger, independent cohorts are recommended to validate and generalize these findings.

Becoming familiar with the unusual. Anti-Mi2 and anti-CENPB, not that weird!

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Introduction & Objectives

In a previous study at our center H.U. Fundación Jiménez Díaz (C1), we identified a cohort of 18 patients with concomitant anti-Mi2 and anti-CENPB antibodies. We described an atypical association of antibodies that was related to a low prevalence of SARD (systemic autoimmune rheumatic diseases) diagnosis, with high percentage of pediatric population, celiac disease diagnosis and association to other antibodies.

Given the unusual nature of this association, we aimed to confirm this finding by conducting an active search for these patients at C1 and at another centre, H.U. Son Espases (C2).

Methods

Between January 2013 and December 2024, both centers performed a retrospective and prospective search for the simultaneous presence of these antibodies to finally compare two profiles: anti-Mi2 positivity with or without anti-CENPB.

Anti-Mi2 was tested by EliA® Thermo in all cases and by Euroimmun line immunoassay/ Dtek dot immunoassay in 87% (132/152). Anti-CENPB was tested by Bioplex 2200®BioRad or by Euroimmun line immunoassay/Dtek dot immunoassay in 147/152 patients.

Immunofluorescence pattern in HEp2 (INOVA-Werfen C1/ Euroimmun C2), results obtained for anti-Mi2 and anti-CENPB by further antigen-specific immunoassays, presence of other antibodies associated with SARD, and patient clinical histories were reviewed.

Results

Out of 152 patients with positive anti-Mi2 antibodies (96 C-1 + 56 C-2), we found concomitant anti-Mi2 and anti-CENPB antibodies in 63 patients –41%.

In the anti-CENP-B +anti-Mi2 cohort the mean levels of anti-Mi2 EliA® were 37.8±31.6 [7.5-150] U/mL. Anti-Mi2 was further studied by immunoblot in 46, with a positive result in 35. Anti-CENPB positivity was confirmed by at least two alternative CENPB specific techniques in 33/63 patients. Regarding indirect immunofluorescence, 28/63 patients had a mixed AC-3+AC-4 pattern (Image 1), 14/63 had AC-3±others, 17/63 had AC-4±others, and 4/63 were negative for both AC-3 and AC-4. In 33/63 patients, another SARD antibody was also detected (7/63 anti-Ro). Among the 53/63 patients with available clinical information, none had dermatomyositis (DM), none had systemic sclerosis, 2 had other connective tissue disease (CTD) and 7 had celiac disease.

In the anti-Mi2 cohort without anti-CENPB, the mean levels of anti-Mi2 EliA® were 244.8±4656 U/mL. Anti-Mi2 was further studied by immunoblot in 86 samples, with a positive result in 70. Anti-CENPB was tested in 84/89 patients. As for the indirect immunofluorescence, 63 patients had AC-4 pattern, 8 had AC-4±others, 14 had others ACs and 2 were negative by immunofluorescence in HEp2. Other SARD antibodies were detected in 28/89 patients (3/28 anti-Ro). Among the 62/89 patients with available clinical information, 20 had DM, 3 had connective tissue disease, and 1 had celiac disease.

Conclusion

Anti-Mi2 is frequently described in association to anti-CENPB in our cohorts. Nevertheless, striking differences in clinical behavior must be highlighted. Anti-Mi2 without anti-CENPB displays higher levels of anti-Mi2 and higher association with dermatomyositis.

Expanding our initial cohort, extending the follow-up period, and adding a new center to the study has allowed us to reaffirm our initial conclusions and extrapolate them to other settings (different techniques, algorithms, and reference populations). These results support that this is not a random finding. The high proportion of pediatric patients and celiac disease remains consistent. Despite the referral bias, the low prevalence of connective tissue disease in this unexpected antibody association warrants the need to report these results with caution.

Case report of HMG-CoA reductase necrotizing myopathy: novel approach to antibody confirmation

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Abstract

We present a case of statin-induced myopathy, demonstrating serological confirmation of anti-3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (anti-HMGCR).

Radioimmunoprecipitation, the gold standard method for detection of anti-HMGCR, is impractical in routine diagnostic immunology laboratories and has been superseded by alternative immunoassays.

This case illustrates practice of triangulation of results from three different analytical methods to confirm the presence of this antibody.

First line testing for anti-HMGCR was performed via commercial line immunoassay (LIA, Euroimmun Autoimmune Inflammatory Myopathies), a multiplex of 18 antigens including HMGCR.

Use of manufacturers cut-off is known for high diagnostic false positive rates. Sera with signal intensity >21 for anti-HMGCR on LIA are routinely reflexed for confirmation by counter-current immunoelectrophoresis (CIEP). This agarose gel-based method utilises commercial recombinant HMGCR antigen (Cayman Chemical). Following electrophoretic counter-current application the patient serum showed a characteristic precipitin band that formed a line of identity and positive reference sera, visualised by addition of Coomassie blue stain. (1)

In addition, routine autoantibody indirect immunofluorescence (IFA) screening tests showed characteristic staining patterns for anti-HMGCR. Cell-cycle dependent cytoplasmic staining was observed on HEp2000® substrate and distinctive distal tubule kidney staining on rodent kidney tissue respectively, supporting the presence of anti-HMGCR. (2, 3)

LIA requires second line confirmation for anti-HMGCR to minimise false positives. In the absence of highly specific confirmatory tests (CIEP or ELISA) IFA tests which are readily available in routine clinical Immunology labs could form useful diagnostic strategies to support findings from LIA.

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Anti-HMGCR tests, reliable enough? When avoiding biopsies is at stake

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Introduction

Anti-HMGCR antibodies identify a subset of immune-mediated necrotizing myopathy (IMNM) that typically shows high CK and proximal weakness. Antibodies allow diagnosis without muscle biopsy. Several commercial assays are available.

Objectives

Test the performance of anti-HMGCR by a novel quantitative chemiluminescence assay.

Methods

Anti-HMGCR were quantified by chemiluminescence assay (CLIA-QUANTA-Flash®HMGCR, INOVA-Werfen; Positive>19CU) in a cohort of frozen samples (n=25) with a known positivity for anti-HMGCR by dotblot (BlueDOT-Myositis-IgG®, D-tek) and/or HALIP (HMGCR Associated Liver Immunofluorescence Pattern) on rat liver (NOVA-lite®, INOVA-Werfen).

23 samples were HALIP+: 12 dotblot+, 9 dotblot-, 2 anti-HMGCoAr confirmed at a reference laboratory by in-house test.

2 samples were HALIP-: 2 dotblot+.

Results

Samples displaying an HALIP pattern and confirmed by dotblot or by in-house assay at a reference laboratory were positive by CLIA in 100% of cases (n=14):

- 2 moderate positive dotblot (++): median of CLIA=82CU (77-88).
- 10 strong positive dotblot (+++): 4 samples had CLIA results above upper limit of quantification (>550CU), 6 had a median of CLIA=185CU (66-543).

None of the HALIP+ samples with a negative dotblot were positive by CLIA (89% <1,5CU).

Among the 2 HALIP-, the 2 samples positive by dotblot had a negative CLIA result:

- Weak positive dotblot (+) vs CLIA=13,4CU
- Moderate positive dotblot (++) vs CLIA <1,5CU.

Conclusion

In general, we identify a good concordance between CLIA and dotblot.

There is a group of patients displaying an HALIP pattern with consistent negative results for anti-HMGCR in both tested assays.

The absence of antibodies detection reinforces the nonspecificity of the HALIP pattern and the need of a confirmation test.

In our cohort, the profile HALIP+ and dotblot+++ is associated with high levels of anti-HMGCR by CLIA (median>10xUpper Limit of Normality).

Clinical profile and outcomes in anti-TIF1γ positive idiopathic inflammatory myositis patients: a Greek cohort study

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Background

Anti-transcription intermediary factor 1-gamma (anti-TIF1γ) antibodies are closely associated with Inflammatory myositis (IIM) and cancer-associated myositis (CAM).

Objective

Description of clinical characteristics of anti-TIF1γ(+) IIM patients in a Greek population.

Material & Methods

Retrospective analysis with 113 IIM cases between 2001 and 2024 was performed and clinical and laboratory data were collected. Disease manifestations and outcomes were compared between anti-TIF1γ-positive and -negative groups.

Results

Twenty patients (17.7%) were anti-TIF1γ(+), of which 70% were women. The mean age was 64.8 ± 12.5 years vs 59.61 ± 12.81 of anti-TIF1γ(-) patients ($p>0.05$). Anti-TIF1γ was strongly associated with Dermatomyositis (DM) (95%, $p < 0.001$) and more severe cutaneous involvement (mean CDASI=27.35 ± 15.01 vs 14 ± 12.25 $p =0.0015$). Malignancy was significantly more frequent in the anti-TIF1γ(+) group (60% vs. 20.4%, $p = 0.001$), with an odds ratio of 5.84 (95% CI 2.09–16.31). Logistic regression identified anti-TIF1γ positivity as independent predictor of malignancy. Interstitial Lung Disease (ILD) was uncommon among anti-TIF1γ(+) cases (15%, $p = 0.004$), while dysphagia was far more prevalent (55% vs. 22.6%, $p = 0.001$). Muscle power (MMT-8score) and

CPK levels did not differ significantly, and survival was lower in anti-TIF1γ(+) patients (55.7% vs. 82.6% $p<0.001$), associated with malignancy.

Conclusions

In our cohort, anti-TIF1γ antibodies define a distinct IIM subset marked by severe skin disease, high malignancy risk, and poorer survival, supporting comprehensive cancer screening and tailored immunosuppressive treatment. This study describes this phenotype in a Greek cohort, aligning with international evidence and highlighting the need for collaborative studies.

A new immunofluorescence assay allows the sensitive detection of anti-cytosolic 5'-nucleotidase 1A autoantibodies

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Abstract

Inclusion body myositis (IBM) is a progressive idiopathic inflammatory myopathy, mostly occurring in men over 50 years old. IBM is characterized by immune cell infiltration in skeletal muscles, rimmed vacuoles in patients' muscle fibers, muscle and mitochondrial degeneration, and the accumulation of proteins such as TDP-43 and p62. In the serum of 33-80% of IBM patients, anti-cN1A autoantibodies are found. These autoantibodies are also observed in patients with dermatomyositis, polymyositis, systemic lupus erythematosus, and Sjögren's syndrome, albeit at lower frequencies (Herbert et al., 2016). However, the new ENMC criteria for the diagnosis of IBM have recently included anti-cN1A autoantibodies as a supportive criterion (Lilleker et al., 2024).

We have observed that the ectopic expression of cN1A in cultured human cells results in the accumulation of cN1A in specific subcellular structures. These accumulations are formed in the perinuclear region and, in most cases, appear as filaments. These cN1A accumulations may be related to the accumulations of cN1A previously found in muscle fibers of IBM patients, which were associated with rimmed vacuoles or located in the perinuclear region. We hypothesized that these cN1A accumulations may display conformational epitopes that do not exist in currently applied assays for anti-cN1A autoantibody detection. Therefore, we investigated whether these cN1A-containing filamentous structures in cultured cells can be utilized to develop a specific and sensitive assay for detecting anti-cN1A autoantibodies.

HEp-2 cells were transiently transfected with a construct encoding the enhanced green fluorescent protein (EGFP)-cN1A fusion protein. After 48 hours of culturing, the cells were fixed, permeabilized, and stained with 50-fold diluted patient sera and a secondary antibody reactive with the heavy and light chains of human IgA, IgG, and IgM. For every patient, two images were made of non-overlapping regions. Three reviewers independently assessed the images. Patient sera were considered anti-cN1A positive when there was an extensive overlap of the EGFP and patient antibody signals.

Forty-eight of 82 IBM patients (59%) appeared to be positive in the newly developed anti-cN1A-immunofluorescence assay. Ten of the 141 disease controls (DM, PM, SLE, and SjS; 7%) were positive, while none of the sera of 35 healthy individuals showed anti-cN1A autoreactivity in this assay. The same serum samples were previously tested for the presence of anti-cN1A in an ELISA with three synthetic peptides of cN1A (Herbert et al., 2016). In this ELISA, 31 of the 82 IBM sera (38%) were reactive. Moreover, for SLE and SjS patients, two diseases in which relatively high frequencies of anti-cN1A were detected by ELISA (Herbert et al., 2016; Rietveld et al., 2018), 14 of the 47 patients (30%) were positive in the ELISA, while only two patients (4%) were positive in the immunofluorescence assay. Although the total number of sera analyzed is still limited, our data strongly suggest that the new immunofluorescence assay for anti-cN1A autoantibodies represents a highly sensitive and specific test.

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Association of PTPN22 and STAT4 polymorphisms with anti-Jo-1 positive antisynthetase and myositis-scleroderma overlap syndromes

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Introduction

PTPN22 encodes a tyrosine phosphatase expressed by hematopoietic cells. The 1858C>T (rs2476601) polymorphism causes an arginine-tryptophan substitution at amino acid position 620, altering an interaction motif of the protein. This polymorphism has been associated with the development of several autoimmune diseases (myositis, rheumatoid arthritis, type 1 IDDM, SLE, alopecia areata, etc.). The STAT4 gene encodes a transcription factor that plays a role in the differentiation and proliferation of T helper 1 and 17 cells. These cells are important effectors in chronic inflammatory disorders, and consequently the STAT4 gene may play an important role in the pathogenesis of autoimmune diseases.

Aims

To investigate the PTPN22 rs2476601 and STAT4 rs7574865 polymorphisms and assess them as risk factors in the groups of patients with myositis-scleroderma overlap and anti-Jo-1 positive antisynthetase syndrome.

Methods

19 (13 women and 6 men) patients with myositis-scleroderma overlap and 27 (13 women and 14 men) with anti-Jo-1 positive antisynthetase syndrome were included in the study. 18 healthy individuals (13 women, 5 men) were selected to form the control group. DNA isolated from peripheral samples was analyzed for the mentioned polymorphisms by Sanger sequencing

Results

Using the chi-square test to evaluate the PTPN22 and STAT4 genes, no statistically significant differences were found when comparing patients with controls for PTPN22 ($p = 0.112$) or STAT4 ($p = 0.544$). Furthermore, the comparison between the subgroups of patients with myositis-scleroderma overlap and anti-Jo-1 positive antisynthetase syndrome revealed no significant differences for PTPN22 ($p = 0.748$) or STAT4 ($p = 0.752$).

Conclusion

Based on our results, the presence of the PTPN22 rs2476601 and STAT4 rs7574865 polymorphisms in either homozygous or heterozygous form is not a risk factor in the myositis-scleroderma overlap and anti-Jo-1 positive antisynthetase syndrome groups of patients.

4.3 Autoantibodies in Systemic Sclerosis

RP11 and RP155 autoantibodies in patients with systemic sclerosis phenotype

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Background

Anti-RNA polymerase III (Pol III) antibodies, particularly those targeting the RP11 and RP155 epitopes, are clinically significant in systemic sclerosis (SSc). They are often associated with diffuse cutaneous disease and scleroderma renal crisis (1). Despite their diagnostic value, the distribution and overlap of autoantibodies to RP11 and RP155 in the SSc patient population remain under characterized (2).

Methods

We evaluated the prevalence of RP11 and RP155 antibodies in a cohort of 30 consecutive and unselected patients with a systemic sclerosis phenotype using line immunoassay (LIA) provided by Quest Diagnostics (San Juan Capistrano, CA, USA) as part of their standard SSc antibody panel, which includes centromere protein (CENP)-A, CENP-B, fibrillarin (U3-RNP), PM/Scl-75, PM/Scl-100, RP11, RP155, Scl-70 (topo I), Th/To, U1-snRNP RNP-70kd, U1-snRNP RNP A, and U1-snRNP RNP C IgG antibodies. At the time of testing, patients had at least bi-phasic Raynaud's, puffy fingers, and positive ANA by IIF.

Results

Seven patients out of 30 tested were positive for anti-RNA Pol III antibodies. Of these, three exhibited both RP11 and RP155 reactivity, three were positive for RP11 only, and one was positive for RP155 alone. Notably, one double-positive patient also tested positive for topo-I antibodies.

Conclusion

RP11 and/or RP155 antibodies were present in 23% (7/30) of patients with systemic sclerosis phenotype, with notable co-positivity in the cases of diffuse (n=2) and limited cutaneous systemic sclerosis (n=1). Interestingly, single reactivity to specific regions of the RNA Pol III complex - RP11 or RP155, showed diverse clinical presentations that are described in the table. These findings highlight the importance of testing for both epitopes in routine SSc panels to enhance diagnostic sensitivity and better characterize serologic subgroups. In 2 of our cases, the rare simultaneous positivity for several SSc autoantibodies was observed (RP11 and RP155 with topo-I, and RP155 with PM-Scl and NXP2). This result confirms previous reports of multiple autoantibody positivity in SSc sera when analyzed by LIA (3,4). Double positive RP11/RP155 cases may represent epitope spreading within the RNA Pol III complex (5). Careful clinical and serological follow-up, especially in the early stages of the disease, could answer questions on prognosis and need for more precisely targeted early therapy for RP11/ RP155 positive patients.

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Frequency of autoantibodies in Systemic Sclerosis

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Background

Systemic sclerosis (SSc), also known as scleroderma, is an immune-mediated rheumatic disease of unknown etiology characterized by vascular abnormalities and fibrosis involving the skin and internal organs. Disease-specific autoantibodies are considered the most important biomarkers for SSc, due to their ability to stratify patients with different severity and prognosis into more homogeneous subsets. Serum autoantibodies directed against multiple intracellular are detectable in most patients and are characterized by SSc-specific autoantibodies directed against nuclear or nucleolar autoantigens (topoisomerase I (Scl-70); centromere (CENP); RNA polymerase III (RP11, RP155); Th/To; fibrillarin and NOR90). Autoantibodies targeting PM/Scl proteins, Ro52, or Ku are not specific for SSc and are also found in other systemic autoimmune diseases.

Aim

To determine the prevalence and coexistence of SSc-specific and SSc-associated autoantibodies in a large cohort of patients suspected of having SSc.

Methods

In our study conducted in the period from 02/2020 to 06/2024, a total of 583 serum samples from patients suspected of having SSc were analyzed for the presence of one of the specific or associated SSc autoantibodies. Determination of autoantibodies was performed with the EUROLINE Systemic sclerosis (Nucleoli) profile (lgG) line –blot assay (Euroimmun, Lübeck, Germany). With line-blot assay detection of 13 systemic sclerosis specific and associated antibodies against Scl-70, CENP A, CENP B, RP11, RP155, fibrillarin, NOR90, Th/To, PM-Scl100, PM-Scl75, Ku, PDGFR and Ro-52, was possible.

Results

A total of 225 (38.6%) serum samples were negative for the presence of autoantibodies. Of the 358 positive samples, in the 170 samples only one SSc antibody was detected, 95 samples were presented with two antibodies, 63 samples were positive for three antibodies while 21 samples were positive for 4 antibodies. Only 6 samples showed positivity for 5 antibodies and three samples for 6 antibodies.

Antibodies to Ro52 were most frequently detected and were found in the serum of 103/358 (28,8%) positive samples. Among SSc specific antibodies, the most common were antibodies to the Scl70 antigen (74/358, 20.6%), most of which were strongly positive. 72 samples showed positivity for Th/To antigen (20,1%), and 64 for fibrillarin antigen (17.9%), but most were borderline positive. For CENP A, CENP B, RP155, NOR90, PM-Scl100, PM-Scl75 and Ku antigen, autoantibodies showed similar frequency (16.7; 15.4; 14.2; 14; 12.8; 12.3 and 10.6 % respectively). Most antibodies against CENP A, CENP B and Ro 52 were strongly positive, while antibodies to RP155, NOR90, PM-Scl100, PM-Sc75 and Ku showed weak positivity.

Conclusion

SSc-specific and SSc-associated autoantibodies were found in most serum samples from patients suspected of having SSc. The most common SSc-specific antibodies were against Scl-70 and Th/To antigens. Among SSc-specific antibodies, anti-Scl-70, CENP A and CENP B showed strong positivity.

Evaluation of anti-CENP reactivity in samples exhibiting the centromere HEp-2 pattern, which is associated with a better prognosis within the limited cutaneous systemic sclerosis spectrum

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Introduction/Objectives

Anti-centromere antibodies are associated with limited cutaneous systemic sclerosis (lcSSc) and a more favorable prognosis. The centromere HEp-2 pattern (AC-3) suggests the presence of antibodies against CENP antigens, mainly CENP-B and A. This study analyzed the clinical and demographic associations of anti-centromere antibodies in a cohort of patients exclusively with the lcSSc form of SSc. The frequency of CENP-B and CENP-A reactivity in samples with the AC-3 pattern was also evaluated.

Method

Samples from 38 lcSSc patients with AC-3 were evaluated for reactivity to CENP-B and CENP-A using line-blot and ELISA. Clinical data from 68 lcSSc patients (20 AC-3 and 48 Non-AC-3) were analyzed.

Results

Of the AC-3 samples, 84.2% and 81.6% were reactive against CENP-B and CENP-A, respectively, by line-blot, and 92.1% were positive for CENP-B by ELISA. Concordance for CENP-B reactivity between ELISA and line-blot was 78.9%. Reactivity to both CENP-B and CENP-A was found in 68.4% of AC-3 samples, while one sample was positive only for CENP-A. Overall, 97.3% of AC-3 samples were reactive to CENP-B, and all were reactive to either CENP-B or CENP-A. Clinically, interstitial lung disease (ILD) was less frequent in AC-3 patients compared to Non-AC-3 (10.5% vs. 54.2%; p=0.001). Other organ involvement frequencies were similar.

Conclusions

Among lcSSc patients, anti-CENP reactivity is associated with a less severe prognosis, with ILD being less frequent in AC-3-positive patients. In addition, anti-CENP-B was the predominant autoantibody in samples yielding the AC-3 pattern, but anti-CENP-A reactivity was also prevalent and exclusive anti-CENP-A reactivity was also observed.

Key-points

- In a cohort composed exclusively of limited cutaneous Systemic Sclerosis patients, those with the AC-3 centromere HEp-2 pattern showed significantly lower prevalence of interstitial lung disease compared to those without AC-3.
- CENP-B is the dominant autoantigen in AC-3 samples, but anti-CENP-A reactivity was also prevalent and exclusive anti-CENP-A reactivity was observed in one sample.
- HEp-2 IFA was 100% sensitive for detecting anti-CENP antibodies, meaning all AC-3-positive samples reacted with CENP-B and/or CENP-A in at least one solid-phase immunoassay, reinforcing HEp-2 IFA as a highly reliable screening method.

Prevalence of systemic sclerosis-associated antibodies in patients with positive nucleolar or centromere anti-nuclear antibody patterns

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Background

Anti-nuclear antibodies (ANA) are positive in over 90% patients with systemic sclerosis (SSc). Among these, anti-Topoisomerase I (Scl-70), anti-centromere (CENP) and anti-RNA polymerase III (RP) are included in the American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) classification criteria for SSc. Additional SSc-associated autoantibodies can be detected using the EUROLINE Systemic Sclerosis (Nucleoli) profile immunoblot assay. This study aimed to determine the prevalence of SSc-associated antibodies in patients with nucleolar or centromere ANA patterns at titers >1:320.

Methods

A retrospective analysis was conducted using data from the laboratory information system of Pula General Hospital over a two-year period. The study included patients who had both a positive ANA indirect immunofluorescence test (titer >1:320) and results from the SSc immunoblot profile test. ANA patterns were categorized as nucleolar (homogeneous, clumpy, punctate — ICAP AC-8, AC-9, AC-10) or centromere (AC-3).

ANA was determined using the Euroimmun Hep-2 kit, and SSc antibody profile using the Euroimmun SSc (Nucleoli) profile kit, detecting 13 specific autoantibodies: Scl-70, CENP A, CENP B, RNA Polymerase III subunits RP11 and RP155, fibrillarin, NOR90, Th/To, PM-Scl100, PM-Scl75, Ku, PDGFR and Ro-52.

Results

A total of 62 patients met inclusion criteria: 59 (95%) were females and 3 (5%) males. Of these, 28 patients (median age 62, range 33–79) had nucleolar ANA patterns, and 34 patients (median age 64, range 36–84) had centromere patterns.

Among patients with nucleolar patterns, six had no detectable SSc-specific antibodies, and one was positive only for Ro-52. Th/To antibodies were most prevalent in patients with the AC-8 pattern (8/14), either alone or in combination. NOR90 antibodies were detected in 5/7 patients with AC-10 patterns, and fibrillarin antibodies in 2/5 patients with AC-9 patterns.

Among patients with AC-3 (centromere) patterns, both CENP-A and CENP-B antibodies were positive in 32/34 cases. One patient was positive for CENP-B and Ku, while another showed CENP-B and RP155 positivity.

Conclusion

Distinct ANA patterns are associated with specific systemic sclerosis-related autoantibody profiles. Patients with centromere patterns (AC-3) showed a high prevalence of CENP-A and CENP-B antibodies, consistent with limited cutaneous SSc. Nucleolar patterns, particularly AC-8, AC-9, and AC-10, were more heterogeneous and associated with less common SSc-specific antibodies such as Th/To, NOR90, and fibrillarin. Combining ANA pattern recognition with extended autoantibody profiling can enhance diagnostic precision and support early identification of SSc subtypes.

Anti-fibrillarin autoantibodies induced by viral molecular mimicry in a paediatric patient

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Background

Anti-fibrillarin autoantibodies (AFA) as serological hallmarks of systemic sclerosis, mainly react with epitopes arranged in the NH2-(aa-1-80) and COOH-terminal-(aa-276-321)-domains of fibrillarin. Interestingly, the fibrillarin NH2-hexapeptide sequence is shared with an Epstein-Barr-virus (EBV)-encoded nuclear antigen.

Case presentation

We herein report a case of a 14-year-old girl presenting with a history of vomiting, sore throat, arthralgias and fever. Laboratory tests revealed leukocytosis, an increased level of CRP, transaminases and total/direct bilirubin. On further investigation, a positivity of ANA testing showing a clumpy nucleolar indirect immunofluorescence (AC-9) pattern on HEp-2000 substrate, due to anti-fibrillarin antibodies, was found. Concomitantly, high concentrations of EBV-VCA-IgM and a slight increase of EBV-VCA-IgG were detected, helping establish a diagnosis of ongoing EBV infection. After a follow-up of six months, all autoimmunity tests were repeated, and together with infection resolution, the negativity of ANA was determined, confirming the transient nature of the autoimmune phenomenon.

Conclusions

Our findings confirm how molecular mimicry plays an important role in the viral-induced autoimmunity. Given the significant homology between fibrillarin and EBV protein sequences, caution in interpreting AFA positivity is suggested, especially in pediatric patients without clinical evidences of an autoimmune condition, and a simultaneous screening for EBV infections is recommended.

Immunoprecipitation – mass spectrometry allows to reduce the seronegative gap in systemic sclerosis

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Abstract

The identification of anti-nuclear autoantibodies can aid in the diagnosis and prognosis of various systemic rheumatic diseases, including systemic sclerosis (SSc). Currently, several SSc-specific antibodies are known, the most established being anti-DNA topoisomerase I (Scl-70) and anti-centromere antibodies. Other, less prevalent SSc-specific/associated antibodies include (among others) anti-RNA polymerase III, anti-fibrillarin, anti-Th/To, anti-PM/Scl, anti-Ku and anti-RuvBL1/2 antibodies. Commercial solid phase immunoassays (including line/dot blots) are widely used (in clinical laboratories) for detection of SSc-specific/associated antibodies, but may be limited by insufficient sensitivity and/or specificity. Moreover, not all SSc-specific/associated antibodies can be detected by commercial assays, leaving a seronegative gap of ~10-15%. We aimed to characterize the antibodies that remained unidentified by commercial solid phase assays using immunoprecipitation combined with mass spectrometry (IP-MS). With IP-MS, the interaction of the antibodies with the antigen occurs in fluid phase, which allows identification of antibodies targeting complex autoantigens.

We analyzed samples from 127, mainly anti-nuclear antibody positive, patients diagnosed with SSc or SSc-myositis overlap syndrome with unknown autoantibodies from six cohorts [Cliniques Universitaires Saint-Luc (Belgium) (n=45), University of Calgary (Canada) (n=33), Chris Hani Baragwanath Hospital (South Africa) (n=18), Centre Hospitalier Universitaire d'Angers (France) (n=15), Centre Hospitalier Lyon Sud (France) (n=13) and University Medical Centre Ljubljana (Slovenia) (n=3)] using the IP-MS technique (with HeLa cellular extract as antigen source) [1]. SSc-specific/associated autoantibodies targeting Th/To (n=19), RNA polymerase III (n=7), Scl-70 (n=5), U3-RNP/fibrillarin (n=6) and THO complex (n=2) were identified in 39 patients. In 17 other patients SSc-myositis overlap syndrome-associated autoantibodies targeting PM/Scl (n=7), Ku (n=1), U1-RNP (n=4) and RuvBL (n=5, 1 anti-RuvBL antibody-positive patient also had anti-Th/To antibodies) were detected. In addition, several rare SSc-associated antibodies (NVL, SMN, RCC1, NOLC1 and NOP2) were identified and two samples had myositis-specific autoantibodies (Mi-2 and Jo1). In other patients, anti-mitochondrial autoantibodies (against pyruvate dehydrogenase E1 and a component of the branched-chain alpha-keto acid dehydrogenase complex) and possible novel autoantibodies were identified. In most of the IP-MS-positive patients autoantibodies against one single antigen/antigen complex were identified.

In conclusion, IP-MS can be used to fill the seronegative gap in a substantial proportion of individuals with SSc and SSc-myositis overlap.

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5 IgG4-Mediated Autoimmune Diseases

The diagnostic and pathogenic role of IgG4 autoantibodies

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Abstract

Our understanding of IgG4 autoantibodies, previously considered mostly tolerogenic due to their relative immunological inertness, has significantly broadened in the past decade with the recognition of two IgG4-centric disease groups: 1) IgG4-related diseases, a group of inflammatory conditions with multi-organ involvement that present with elevated serum IgG4 levels and IgG4-positive plasma cell infiltrates in affected tissues, despite limited evidence for direct IgG4 pathogenicity. This inflammatory condition must be distinguished from 2) IgG4 autoimmune diseases (IgG4-AIDs), an expanding group of rare, severe autoimmunopathies defined by the presence of antigen-specific IgG4 autoantibodies. These include diseases such as MuSK myasthenia gravis, pemphigus vulgaris, and thrombotic thrombocytopenic purpura. In recent years, new diseases have been identified, and now over thirty antigens are associated with IgG4 subclass antibodies. In these diseases, IgG4 has been observed to be pathogenic, primarily through functional blocking of protein-protein interactions. To establish IgG4 pathogenicity in individual diseases, we developed a classification system based on Witebsky's postulates. In some cases, the pathogenic role of these antibodies has been demonstrated by passive transfer of patient IgG4 or patient IgG4+ B-cell-derived monoclonal antibodies to experimental animals, solidifying their pathogenic role. Furthermore, lower concentrations of IgG1-3 autoantibodies are often present in IgG4-AIDs and may contribute to pathogenicity, although this requires further study. We will highlight IgG4 autoantibody diagnostics and the commonalities of IgG4-AIDs.

IgG4 anti-GBM antibodies - laboratory challenges and solutions

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Abstract

Autoantibodies against the α3 chain of type IV collagen within the glomerular and alveolar basement membranes can cause both rapidly progressive glomerulonephritis and alveolar hemorrhage, known as anti-glomerular basement membrane (anti-GBM) disease or Goodpasture syndrome. In literature patients have been described who exclusively produce polyclonal IgG4 anti-GBM, often characterized by less rapidly progressive disease. Anti-GBM test-results in these patients are often false-negative since the currently available immunoassays poorly detect antibodies of the IgG4 subclass. We report a young adult male with a nephrotic syndrome and deteriorating kidney function and negative ANCA/GBM serology. Kidney biopsy, which was delayed due to personal circumstances of the patient, showed a diffuse segmental and partial extracapillar proliferative glomerulonephritis and linear IgG staining like anti-GBM disease. IgG-subtyping showed IgG4 dominance. Classification of this patient was difficult due to the chronic histopathology result and the absence of anti-GBM antibodies using Phadia250 ELiA and an equivocal result for anti-GBM using the ANCA-GBM Euroimmun immunoblot. Due to high suspect of possible IgG4 predominance of anti-GBM antibodies, a modified anti-GBM ELiA assay was performed in which the regular anti-GBM ELiA on the Phadia250 was combined with an IgG4 conjugate.

The results showed a positive response (3499 signal intensity) for IgG4 anti-GBM antibodies using this experimental setup which was not detected in 5 healthy controls (all < 80 signal intensity). Subsequent treatment in this patient with oral cyclophosphamide, (methyl)-prednisolone and plasmapheresis resulted in a gradual decrease of the IgG4 anti-GBM antibodies towards the levels measured in the healthy controls. The patient's kidney function showed partial recovery over time because of treatment, with a decrease in serum creatinine from 422 µmol/L to 286 µmol/L by the

end of plasmapheresis. Further recovery was observed in the outpatient setting, with serum creatinine improving to 189 µmol/L (eGFR-CKD-Epi 25 ml/min/1,73m^2) after 5 months. During follow-up, the IgG4 anti-GBM antibodies remained not detectable with the modified anti-GBM ELiA test. However, microscopic hematuria and severe proteinuria persisted during the first three months after plasmapheresis. Afterwards, a marked decrease is seen, however patient is still nephrotic 5 months after stop plasmapheresis. An additional urethrocystoscopy was normal.

To investigate whether other anti-GBM assays also report false-negative results, we have included this sample in the Dutch ANCA/GBM-External Quality Assessment (EQA) program. None of the 36 participants reported a positive test result in any of the five different anti-GBM immunoassays that were performed and only one participant reported an equivocal result.

We conclude that a modified IgG4 anti-GBM ELiA is able to detect IgG4 anti-GBM antibodies aiding in the diagnosis and follow up of a patient who has exclusively IgG4 anti-GBM antibodies. To prevent delays in diagnosing IgG4 anti-GBM disease, laboratory specialists and clinicians should be aware of false-negative test results in these patients and the possibility to detect these IgG4 anti-GBM antibodies with a modified anti-GBM ELiA assay.

Fab glycosylation in IgG4 autoimmune diseases: A clue to pathogenicity?

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Rationale

IgG4 antibodies are typically anti-inflammatory and functionally monovalent, yet paradoxically, they are central to a group of rare but severe autoimmune diseases termed IgG4 autoimmune diseases (IgG4-AIDs). This contradiction raises the important question of what transforms IgG4 from a normally tolerogenic molecule into a pathogenic autoantibody. IgG4 generally has increased Fab glycosylation compared to the other IgG subtypes. Additionally, recent studies have highlighted increased Fab glycosylation in chronic B cell mediated autoimmune diseases, including two IgG4-AIDs. Fab glycans are often introduced near antigen-binding sites, such as the complementarity-determining regions and DE loop, through somatic hypermutation during antigen-driven B cell responses and could thus affect antigen affinity. Hence, our research hypothesizes that post translational modifications such as Fab glycosylation contribute to pathogenicity in IgG4-AIDs. We specifically focus on IgG4-AIDs of the nervous system, including anti-IgLON5-disease, LG1I and CASPR2 autoimmune encephalitis and NF155 and CASPR1 paranodopathies.

Methods

Serum and CSF samples from patients with IgG4-AIDs (targeting antigens IgLON5, LGI1, Caspr1/2 and NF155) as well as NMDAR encephalitis patients (IgG1 control) were fractionated using SNA lectin chromatography. SNA specifically binds to terminal α2,6-linked sialic acid residues, allowing the separation of Fab glycan-enriched (sialylated) and glycan-depleted fractions. These fractions are analyzed using total IgG and subclass-specific ELISAs, as well as antigen-specific assays, to determine the percentage of sialylated antibodies within total IgG and IgG4 and withing antigen-specific IgG and IgG4.

Results

We will show Fab glycosylation levels in IgG4-AIDs with preliminary data suggesting differential Fab glycosylation of antigen specific IgG compared to total IgG.

Future Plans

We will perform functional assays to investigate the role of Fab glycans in pathogenicity. Finally, we aim to explore potential differences in Fab glycosylation between serum and CSF-derived antibodies to better understand their contribution to disease mechanisms.

Are there differences in the structural lipids of the different B-cells types that influence the pathogenesis of IgG4-autoimmune disorders?

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Abstract

IgG4 autoimmune diseases (IgG4-AID) are an emerging group of autoimmune diseases caused by IgG4 autoantibodies. These antibodies mainly target antigens present in: 1) the central and peripheral nervous system with diseases such as MuSK myasthenia gravis (MG), different autoimmune encephalitis (anti-LGI1 and anti-Caspr2), anti-IgLON5 disease or chronic inflammatory demyelinating polyneuropathy (CIDP), 2) the skin and mucosa with skin blistering diseases such as pemphigus vulgaris (PV) and pemphigus foliaceus (PF), 3) the kidneys with antibody positive membranous glomerulonephritis and 4) the haematological system with diseases such as thrombotic thrombocytopenic purpura (TTP, ADAMTS13) or GPIHBP1 autoantibody syndrome. Despite significant advances in understanding the immunological mechanisms underlying IgG4-AID, the role of structural lipids of the B-cells involved in the pathogenesis and progression of these disorders remain poorly explored. Structural lipids are crucial components of cellular membranes and play a pivotal role in modulating immune responses, inflammation, and tissue remodeling. Investigating the structural lipid profiles associated with IgG4-AID using LC-MS could provide novel insights into disease mechanisms and identify potential biomarkers or therapeutic targets. For that, in a first pilot study, healthy individuals derived B cells at different developmental stages (naïve B cells, memory B cells, transitional B cells, regulatory B cells) and different antibody isotype production (IgG1-4) will be studied. Later, and if significant differences are identified, specific cell sorting will be performed to isolate IgG4-specific antibody-producing B and plasma cells in patients with IgG4-AID. These will be compared with healthy controls.

Leukotraps from three healthy controls within the same age and sex group will be used. Initially, PBMCs will be isolated and subsequently sorted into different subpopulations by FACS sorting. We aim to isolate naïve, memory, regulatory, and transitional B cells, as well as isotype-specific B cells (IgG1-2 and IgG4). To this end, a specific FACS sorting panel has been established. After sorting, lipids will be extracted from each cell group using a biphasic solvent system comprising

cold methanol, methyl tert-butyl ether (MTBE), and water. Subsequently, the lipids will be analyzed by LC-MS, and relevant statistical comparisons will be performed to observe differences in the structural lipids of each cell group.

If significant differences are found, the next step of the study will focus on analyzing the structural lipids of IgG4-specific B cells in IgG4-AID conditions, such as CASPR2+ or LGI1+ autoimmune encephalitis, and comparing them to IgG4-producing B cells from healthy controls. Comparisons will also be made between IgG1, IgG2, and IgG3 positive B cells.

This study will enhance our understanding of the role of structural lipids in immune cells, particularly their involvement in the pathogenic mechanisms underlying the production of disease-specific antibodies in IgG4-AID. Additionally, identifying significant differences in the structural composition of membrane lipids in these specific cells could lay the foundation for the search for biomarkers that predict disease progression.

6 Autoimmune Neurological Diseases

“Ouroboros" in neurology? Anti-immune-system antibodies in sensory neuronopathies

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Abstract

Sensory neuronopathies (SNN) encompass diverse etiologies, with autoimmunity playing a major role through both cellular and humoral responses. To investigate the humoral autoantibody repertoire in autoimmune SNN, we conducted a retrospective cohort study using large Human Proteome-wide protein microarrays (HuProt 3.1, HuProt 4.0, ProtoArrays). We specifically analyzed immune system pathways targeted within the autoantigen repertoire (the autoantigenome). We included 131 participants: 44 patients with non-paraneoplastic autoimmune SNN (12 with anti-FGFR3 and/or anti-AGO antibodies), 8 with paraneoplastic SNN, and 79 controls. Findings were validated in an independent cohort of 16 SNN patients. Overrepresentation of immune-system-related proteins was assessed using the Reactome database, and serum levels of IFN-γ and IL-6 were measured with the Bio-Plex Pro™ Reagent Kit. Autoimmune SNN sera interact with significantly more immune system proteins than healthy controls (ProtoArrays: 271/863 vs. 14/863, HuProt: 112/1694 vs. 39/1694, both $p < 0.0001$). Overrepresentation was observed across all major immune sub-pathways, including innate and adaptive immune responses as well as cytokine signaling. Anti-FGFR3-positive SNN patients showed more frequent reactivity to immune system proteins than anti-FGFR3-negative ones. The independent SNN cohort validated the overrepresentation of targeted immune system pathways. Validation with dot blot and ELISA confirmed reactivity to TRIM21 and IL-6 and identified anti-IFN-

γ-positive SNN patients. IFN-γ levels correlated weakly with levels of anti-IFN-γ antibodies (Pearson's r = 0.22, p = 0.03). We conclude that the antibody repertoire of autoimmune SNN targets pathways of the innate and adaptive immune system, potentially reflecting key disease-related immune pathways and highlighting the systemic role of immune dysregulation in SNN.

Autoantibodies in neuropsychiatric diseases – from mechanisms to novel treatments

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Abstract

Autoimmune mechanisms causing dysfunction of the brain are increasingly recognized and brought about a paradigm shift in neurology and psychiatry. The identification of numerous pathogenic autoantibodies against neuronal tissue resulted in unprecedented diagnostic and therapeutic opportunities. Current clinical and experimental data show that diverse neuropsychiatric abnormalities may be the sole symptoms of brain autoimmunity. Affected patients are at risk that such treatable etiologies are overlooked as rheumatic or psychiatric disorders. In some patients, the diagnosis can be made by detection of specific autoantibodies directed against neuronal or glial surface proteins. These epitopes include ion channels or receptors, but also novel antigens not yet tested for autoimmunity, such as cell adhesion molecules or enzymes. The identification and recombinant production of disease-defining human monoclonal autoantibodies from these patients now allow detailed analyses of the pathogenic effects, of signaling cascades leading to neuropsychiatric symptoms, and potential triggers of autoimmunity. Most importantly, they allowed to develop the first autoantibody-selective treatments in patients with autoimmune encephalitis. Using NMDAR encephalitis as an example, preclinical data generated with chimeric autoantibody receptor (CAAR) T cells demonstrated that autoantibody-selective treatment is possible and could potentially avoid side effects of immunosuppression. Ongoing work, including clinical trials, is needed to demonstrate whether CAAR T cells have the potential to cure patients with antibody-mediated neuropsychiatric diseases, and whether the concept of selective immunotherapy can be expanded to other autoimmune neurological diseases in the future.

6.1 Challenges and Advances in Autoantibody Analyses of Autoimmune Neurologic Diseases

Serological analysis of gluten-related antibodies in idiopathic neuropathies and cerebellar ataxia

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Abstract

Immune reactivity to gluten in the development of peripheral neuropathies and cerebellar ataxia has been suggested for decades, but evidence is scarce. The aim of the current study was to test the prevalence of tissue transglutaminase 2 (anti-TG2), tissue transglutaminase 6 (anti-TG6) and gliadin antibodies (anti-gliadin) in a large cross-sectional study.

Sera of patients with idiopathic cerebellar ataxia, idiopathic small fiber neuropathy (SFN) and chronic idiopathic axonal polyneuropathy (CIAP), and controls with a comparable age distribution and men/women ratio were collected. Sera were analyzed for anti-gliadin IgA/IgG (manufacturer's and lower cut-off), anti-TG2 IgA and anti-TG6 IgA/IgG.

In total 683 samples were analyzed: 476 patients (249 SFN, 161 CIAP and 66 idiopathic cerebellar ataxia) and 195 controls. There were no differences between disease and control group in the prevalence of elevated anti-TG6, anti-TG2 and anti-gliadin using the manufacturer's cut-off. Using a lower cut-off of 3 U/mL, previously used by others for gluten-related neurological disorders, anti-gliadin IgA was positive in 20.8% patients vs. 12.8% controls (p=0.017) and anti-gliadin IgG in 7.6% vs. 2.6% (p=0.013), respectively. In subgroup analyses, significant differences were only observed in SFN for anti-gliadin IgA and in idiopathic cerebellar ataxia for anti-gliadin IgG using this lower cut-off after adjusting for sex and age.

In conclusion, no difference in anti-TG2, anti-TG6 and anti-gliadin levels were observed between patients and controls. Only when using the lower cut-off (3 U/mL), patients with SFN and idiopathic cerebellar ataxia were more often positive for anti-gliadin than controls. Whether these low-titer antibodies are gluten related, have any pathophysiological relevance, or reflect an epiphenomenon of neurodegeneration or gut inflammation is unknown.

Controlling background effects in anti-ganglioside antibody testing

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Abstract

Anti-ganglioside antibodies play an important role in many inflammatory peripheral neuropathies, such as Guillain-Barré Syndrome (GBS), Multifocal Motor Neuropathy (MMN), or CANOMAD/CANDA (chronic ataxic neuropathy with ophthalmoplegia, monoclonal gammopathy, cold agglutinins, and disialosyl antibodies / chronic ataxic neuropathies with disialosyl antibodies). Correct identification of autoantibodies remains challenging, since each individual carries a unique set of antibodies with different isotypes, specificities, affinities, and titers. Thus, even small variations in assay production or trace contaminations of raw materials can lead to increased background signals, hampering the specificity of antibody detection.

We measured 59 sera of normal donors and diseased controls (e.g., Myasthenia Gravis, Rheumatoid Arthritis, etc.) across 10 lots of a commercially available anti-ganglioside antibodies ELISA (BÜHLMANN GanglioCombi (MAG) ELISA). Antibodies against GA1, GM1, GM2, GD1a, GD1b, GT1a, GQ1b, and the MAG-epitope HNK-1 were analyzed with an IgG/IgM-Mix detection antibody. In total, 2856 data points were acquired to develop a statistical workflow for the evaluation of background signal.

Six samples (10.2%) reproducibly showed elevated antibody titers against at least one antigen and were excluded from the control set. With the remaining 2575 data points, a statistical workflow to identify increased background signals was developed, which relies on 36 serum samples. With alpha = 0.1, the fraction of positive and negative values in this setup can robustly identify lot-dependent elevated background levels.

Controlling background effects in anti-ganglioside antibody testing is of vital importance to ensure assay specificity. Using a set of 36 control serum samples, elevated background can be robustly detected, based on two criteria: the fraction of positive values, and the fraction of negative values. Applying these criteria during quality control - e.g., when evaluating raw materials - maximizes assay robustness and reliability.

Detection of anti-MAG antibodies with ELISA – The impact of different detergents on performance and stability

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Abstract

ELISA is common and reliable method for the detection of autoantibodies in inflammatory neuropathies. The use of detergents is of particular importance for removal of non-specific binding events, ensuring the specificity of the assay. In Anti-MAG Neuropathy, the BÜHLMANN Anti-MAG Antibodies ELISA is the accepted gold standard and the only IVDR-compliant assay in the market. However, in its traditional formulation, it contained the detergent Triton X-100, which has recently been identified as an environmental toxin, particularly affecting aquatic life forms.

We tested different ecofriendly detergents and their impact on performance and stability of the Anti-MAG Antibodies ELISA. Tween-20 was identified as the most suitable replacement for Triton X-100. The reference limit of 350 BTU of the renovated “green” anti-MAG Antibodies ELISA was determined using sera from 120 healthy controls. A method comparison between the traditional and the new assay revealed no bias by Bland-Altman analysis: 0.5% bias (95%-CI: -5.4% to 6.3%) observed with 44 samples across the measuring range (2’762 – 58’462 BTU). Cross-reactivity was tested with 96 samples of patients with different pathologies, e.g., Myasthenia Gravis, Amyotrophic Lateral Sclerosis, or Rheumatoid Arthritis. 95 out of 96 samples (99%) were measured negative for Anti-MAG-antibodies, i.e. < 1000 BTU. Accelerated stability studies at elevated temperatures showed that all reagents will be stable for at least 12 months at 2-8 °C

In conclusion, our results demonstrate that Triton X-100 can be fully replaced by more ecofriendly detergents in the Anti-MAG Antibodies ELISA, without affecting assay performance, reagent stability, or IVDR-compliance. Importantly, the high specificity of the assay remains intact, ensuring reliable diagnosis of anti-MAG neuropathy.

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

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Background

Glial fibrillary acidic protein (GFAP) is a key intermediate filament protein predominantly expressed in astrocytes, where it plays a crucial role in the structural integrity and function of the central nervous system. Elevated levels of GFAP-specific IgG antibodies in cerebrospinal fluid (CSF) are associated with astrocytopathy, a condition characterized by inflammation and dysfunction of the astrocytes. Autoantibodies targeting GFAP are a biomarker of subacute and progressive autoimmune meningitis, encephalitis and myelitis, which can mimic multiple sclerosis and other idiopathic inflammatory central nervous system (CNS) disorders such as sarcoidosis. A paraneoplastic context is common, with reported neoplasms including adenocarcinomas, myeloma, melanoma, colonic carcinoid, parotid pleomorphic adenomas, and teratomas. Therefore, we also focused on CSF samples, since serum samples often revealed non-specific and clinically irrelevant findings. The goal of this study was to develop a high-sensitive, specific and reproducible cell-based assay (CBA) capable of reliably detecting these anti-GFAP antibodies in clinical samples. Accurate detection of these antibodies is important as they identify patients with an immune-mediated, steroid-responsive disorder who should be screened for malignancy.

Methods

CSF samples from 27 clinically characterized patients with astrocytopathy meningoencephalomyelitis who tested positive in both CBA and tissue-based assay (TBA) (based on in-house tests) were analysed, alongside 149 additional CSF samples from control patients with clinical presentations but no prior positive characterization. The analysis was performed using a

prototype indirect immunofluorescence assay (IFA, EUROIMMUN) based on recombinant HEK293T cells transfected with a GFAP isoform to capture IgG autoantibodies.

Results

GFAP-specific IgG autoantibodies were detected at high titers in CSF samples (1:10 – 1:1000) of all patients (27/27), while no reactivity was observed in CSF samples from controls (149/149). These findings demonstrate that the test is highly specific for GFAP-specific IgG antibodies in CSF samples, with no reactivity in the control group.

Conclusion

The recombinant GFAP prototype CBA is a highly sensitive and specific diagnostic tool for detecting GFAP-specific IgG autoantibodies in CSF, providing a reliable method for supporting the diagnosis of autoimmune astrocytopathy. The detection of anti-GFAP IgG is crucial for identifying patients with immune-mediated, steroid-responsive disorders, and its use can significantly promote early diagnosis and guide the search for paraneoplastic malignancies. Future studies with larger cohorts are needed to further validate the test's performance and evaluate its role in clinical practice for the differentiation of neuroinflammatory disorders, especially in cases mimicking multiple sclerosis or other CNS conditions.

Conflict of interest: SM, MK, and DJ are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of the work.

Evaluation of a cell-based assay for the standardized determination of autoantibodies against IgLON5

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Introduction

Anti-IgLON5 disease is a rare neurological disorder with a heterogeneous spectrum of clinical manifestations, such as sleep disturbances, bulbar dysfunction, gait abnormalities or cognitive deterioration, but also tau accumulation in the brainstem or cerebellum in the late course. Due to the variety of symptoms, it may mimic several other autoimmune or neurodegenerative disorders, requiring thorough differential diagnostics. The presence of autoantibodies against the IgLON family member 5, a neuronal cell adhesion protein, in serum or cerebrospinal fluid (CSF) is crucial to confirm anti-IgLON5 disease. In this study, we evaluated the performance of a commercial cell-based assay (CBA) for the standardized detection of anti-IgLON5 autoantibodies.

Methods

The EUROIMMUN anti-IgLON5 indirect immunofluorescence test, a CBA based on HEK293 cells expressing recombinant IgLON5, was compared to an in-house rat brain immunohistochemistry (IHC) assay and an in-house CBA, both established at the EMC Rotterdam for the detection of anti-IgLON5 antibodies. Clinical performance was evaluated using two panels: [i] 26 samples (17 sera, 9 CSF) collected serially from 12 patients with clinically confirmed anti-IgLON5-positive autoimmune encephalitis (age range: 64–77 years, 5 female, 7 male); [ii] 67 samples (33 sera, 34 CSF) from 33 patients with other autoimmune neurological diseases. Anti-IgLON5 IgG reactivity was assessed qualitatively, with samples being considered positive if specific fluorescence was

detected in serum/CSF at a dilution of 1:200 / 1:2 by IHC, 1:40 / 1:4 by in-house CBA and 1:10 / 1:1 by EUROIMMUN CBA.

Results

IHC and both CBAs demonstrated a sensitivity of approximately 92% and a specificity of 100%. Overall qualitative agreement between the assays was almost perfect (97.2–100%, $\kappa \geq 0.939$). Discrepancies were found in two samples from anti-IgLON5 disease patients: [i] one serum was identified to be anti-IgLON5 positive only by in-house CBA, but not by EUROIMMUN CBA and IHC; a serum sample from the same patient tested negative in all three assays; [ii] one CSF was only seropositive by tissue staining, but negative by in-house CBA and not evaluable by EUROIMMUN CBA; a serum sample from the same patient tested positive in all three assays.

Conclusion

The EUROIMMUN CBA allows the sensitive and specific detection of anti-IgLON5 autoantibodies in serum and CSF and can therefore help to differentiate anti-IgLON5 disease from other neurological disorders. This is particularly important as early diagnosis and initiation of immunotherapy may prevent disease progression and irreversible neuronal damage.

Conflict of interest: ADC, SM and SS are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

Sensitive and specific detection of anti-IgLON5 autoantibodies using a standardized cell-based assay

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Introduction

Anti-IgLON5 disease is a rare disorder of the central nervous system (CNS) that usually presents with a chronic progressive course including manifestations such as bulbar dysfunction, gait instability, abnormal movements, sleep disturbances, neuromuscular symptoms, and cognitive decline, but also tau deposition in the brain in the late course. Due to the variety of symptoms, it may mimic several other autoimmune or neurodegenerative disorders, requiring thorough differential diagnostics. The presence of autoantibodies against IgLON5, an adhesion protein widely expressed in the CNS, is crucial to confirm anti-IgLON5 disease. In this study, we evaluated the performance of a commercial cell-based assay (CBA) for the standardized detection of anti-IgLON5 autoantibodies.

Methods

Serum samples from anti-IgLON5-positive patients (n=43) as well as control sera from patients with multiple system atrophy (n=17), fatal familial insomnia (n=3), progressive supranuclear palsy (n=20), frontotemporal dementia (n=6), multiple sclerosis (n=11) and from healthy blood donors (n=7) were analyzed (dilution 1:10) using the EUROIMMUN anti-IgLON5 indirect immunofluorescence assay, a CBA based on transfected HEK293 cells expressing recombinant IgLON5. Results were compared to those obtained using an in-house rat brain immunohistochemistry (IHC) assay and an in-house CBA, both established at the IDIBAPS Barcelona for anti-IgLON5 testing.

Results

All 43 sera from patients with anti-IgLON5 disease showed the IgLON5-IgG-characteristic tissue staining pattern by IHC and were also positive by both CBAs, indicating a sensitivity of 100% for all three assays. Using the in-house CBA, strong positive specific fluorescence was detected throughout this patient group, whereas the EUROIMMUN CBA showed weak, moderate and strong positive signals in one, seven and 35 cases, respectively. None of the control patients or healthy donors were anti-IgLON5 positive by IHC or CBA. Thus, the specificity of all assays was consistently 100%.

Conclusion

The EUROIMMUN CBA is an easy-to-interpret, highly sensitive and specific tool for the determination of autoantibodies against IgLON5. It is therefore suitable for the differentiation of anti-IgLON5 disease from other neurological disorders. This is particularly important because early diagnosis of anti-IgLON5 disease improves patient management and because the initiation of immunotherapy may prevent disease progression and irreversible neuronal damage.

Conflict of interest: JD is inventor or co-inventor of patents related to antibodies to NMDAR, AMPAR, GABAAR, GABABR, and IgLON5. ADC, SM and SS are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

Antibodies against P-body proteins as potential autoimmunity biomarkers in sensory neuronopathy

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Introduction

Idiopathic sensory neuronopathy (SNN) often presents with suspected autoimmune etiology. Understanding the humoral immune response in SNN could reveal insights into its pathogenesis. This study aims to identify autoantibody signatures in autoimmune-suspected SNN patients, potentially uncovering novel biomarker candidates.

Methods

Serum samples from 12 autoimmune-suspected SNN patients, 22 patients with other autoimmune neuropathies (OAN), and 9 healthy controls (HC) underwent autoantigenome screening using Human Proteome Microarrays containing approximately 16,000 human proteins. A second cohort of 16 idiopathic SNN patients without identifiable autoimmune context and 21 controls was used for validation. Three candidate autoantibodies were examined through ELISA and HEp-2 cell immunostaining with independent patient cohorts.

Results

Within the 219 proteins specifically targeted by SNN patients, 9 (4.1%) were p-body proteins, representing a significant overrepresentation compared to a random distribution ($p<0.0001$, Fisher's exact test). Likewise, the proportion p-body proteins across the SNN autoantigenome was significantly higher than that of both OAN and HC ($p=0.001$ and $p=0.013$, respectively, Fisher's exact test; $p=0.0002$, χ^2 among all groups). In the validation cohort, we confirmed the overrepresentation of p-body proteins in the SNN autoantigenome ($p=0.001$ against the array, 0.009 against OAD, Fisher's exact test; $p=0.0066$ among all study groups, χ^2). In total, 16 p-body proteins were specifically targeted by SNN patients. ELISA confirmed elevated levels of antibodies against all three selected candidate p-body proteins in their corresponding index patients, but also in subgroups (10.5-17.1%) of independent SNN cohorts. Subsets of Sjögren Syndrome patients displayed reactivity, too. High-titer anti-p-body-positive SNN sera demonstrated characteristic staining patterns on HEp-2 cells.

Conclusion

P-body proteins are autoantibody targets in autoimmune-suspected SNN. Degrees of specificity towards SNN or Sjögren Syndrome patients vary among the target proteins. Some of them may represent markers of autoimmune context. Further investigation is warranted to delineate clinical correlations and assess diagnostic utility in SNN.

Autoantibodies against glutamate receptors (type NMDA) of immunoglobulin classes IgA and IgM are associated with cognitive impairment

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Introduction

Of the target autoantigens in the field of neuroimmunology, glutamate receptors (type NMDA, N-Methyl-D-Aspartate) are of high diagnostic relevance (Dalmau J et al., Lancet Neurology 7 (2008):1091-1098). Up to now, only antibodies of immunoglobulin class IgG against NMDA receptors were regarded as diagnostically relevant. We investigated if immunoglobulin classes IgA and IgM of anti-NMDA receptors have at least clinical significance.

Patients and methods

In 1055 patients with various tumors, autoantibodies of the IgA, IgG and IgM classes against 32 different neural antigens including glutamate receptors (NMDA type) were tested separately by indirect immunofluorescence.

A group of 56 tumor patients exhibited anti-NMDA autoantibodies only of immunoglobulin classes IgA and IgM. Among them, the most frequent tumors were prostate carcinoma, malignant melanoma, bronchial carcinoma and breast carcinoma. Cognitive performance tests were carried out on all patients. Tumor patients with and without antibodies against neural antigens were divided into two maximally similar groups. Group 1: One antibody-negative patient was manually selected for each antibody-positive patient. Group 2: For each antibody-positive patient, five antibody-negative patients were selected with software support. Gender, age and tumor were used as comparison data (group 1) and additionally the tumor stage (group 2).

In a VLMT (**Verbal Learning and Memory Test**), the same list of 15 semantically similar words was read out to the patients five times in sequence, which they were asked to recite from memory immediately and after 30 minutes. Each memorized word was scored with one point. The **ROCF test** evaluated the ability of spatial visual construction and visual memory performance. Patients

were asked to draw a geometric figure as accurately as possible. The figure had to be drawn from memory after three minutes and again after 30 minutes. Points were given for the position and accuracy of the individual components. The **Digit span test** was used to test short-term and working memory by asking the respondent to memorize series of numbers and reproduce them forwards and backwards in a certain time.

Results

In the VLMT and ROCF tests, the IgA or IgG antibody-positive group showed significantly weaker memory performance compared to the control groups 1 and 2 (p-value of the t-test < 0.05). Memory performance was also reduced for forward and backward number repetition in the Digit span test, but with low significance.

Conclusion: IgA and IgM autoantibodies against glutamate receptors (NMDA type) may be associated with cognitive deficits. The results are independent of tumor type and suggest that these antibody classes are potentially pathophysiologically relevant. Mild immunosuppression could be an option to treat IgA and IgM positive patients.

Animal model of anti-NMDAR encephalitis by active immunization: Insights into the neuro-immunobiology and therapeutic interventions

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Objective

To develop a mouse model of anti-N-methyl-D-aspartate receptor encephalitis (NMDARe) that allows comprehensive neuro-immunobiological investigations and the assessment of potential therapies.

Background

NMDARe occurs with severe neuropsychiatric symptoms and often improves with immunotherapy. Current animal models do not provide comprehensive physiopathological insights or a clinical course long enough to assess new therapies.

Design/Methods

Eight-week-old female C57BL/6J mice were immunized with GluN1356-385 peptide (or saline) along with AddaVax (adjuvant that favors B-cell autoimmunity) and pertussis toxin, followed by systematically examinations for behavioral and neuro-immunobiological changes. Treatment groups received an anti-CD20, a positive allosteric modulator of NMDAR (NMDAR-PAM, SGE-301), or both. GluN1-antibody synthesis, epitope spreading, antibody effects on NMDAR density and function, brain immunological infiltrates, microglial activation, and antibody synthesis by cultured inguinal (ILN) and deep cervical lymph nodes (DCLN) were assessed by immunohistochemistry, calcium imaging, confocal and super-resolution microscopy, electrophysiology, and flow cytometry. Changes in memory and behavior were assessed with a panel of behavioral tests, and clinical/subclinical seizures with brain-implanted electrodes.

Results

Immunized mice, but not controls, developed serum and CSF NMDAR-antibodies, showing epitope spreading and reduced synaptic NMDAR clusters and hippocampal plasticity. Additionally, they had brain-bound antibodies, inflammatory infiltrates (mainly B- and plasma cells), microglia activation, and presence of NMDAR/IgG complexes in microglial endosomes. Cultured DCLN showed NMDAR-antibody synthesis. These findings were associated with psychotic-like behavior, memory deficits, increased seizure susceptibility, and abnormal movements. Treatment with anti-CD20, NMDAR-PAM or both, reversed most neurobiological and behavioral abnormalities. Repopulation of B cells was associated with re-emergence of clinical-neurobiological alterations, which were abrogated by the NMDAR-PAM.

Conclusions

This model offers an all-inclusive neuro-immunobiology of the disease, allowing testing novel treatments, supporting the therapeutic potential of NMDAR-PAM, and suggesting an immunological paradigm of brain NMDAR-epitope spreading, which along the DCLN might contribute to fine-tuning the immune response.

Comparison of anti-NMDAR antibody titers in cerebrospinal fluid and serum measured by cell-based assay and immunohistochemistry

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Introduction

The diagnosis of anti-N-methyl-D-aspartate receptor (anti-NMDAR) encephalitis depends on clinical criteria in combination with anti-NMDAR antibody detection. The internationally most accepted techniques for the detection of anti-NMDAR antibodies are cell-based assays (CBA) and immunohistochemistry (IHC). Here, these two methods were compared by correlating anti-NMDAR antibody titers determined in cerebrospinal fluid (CSF) and serum.

Methods

The study included 29 CSF and 22 serum samples from 40 patients with clinically and serologically confirmed anti-NMDAR encephalitis (mean age at diagnosis 30±17 years, range 4–64 years; 75% female; 38% with an underlying tumor). Anti-NMDAR IgG titers were determined using an in-house rat brain IHC assay [1] and a CBA (EUROIMMUN) based on transfected HEK293 cells expressing recombinant NMDAR subunits [2]. IHC titrations were performed using 2-fold serial dilutions (1:2, 1:4, 1:8, 1:16, etc.), whereas CBA titrations followed a √10-fold scheme (1:3.2, 1:10, 1:32, 1:100, etc.). Endpoint titers established with both techniques were analyzed using the Spearman's rank correlation method.

Results

For 29/29 (100%) CSF samples, both methods yielded positive anti-NMDAR antibody titers (IHC 1:2 to 1:2048, CBA 1:3.2 to 1:1000). Of the 22 serum samples, 22 (100%) were positive by IHC (1:200 to 1:12800) and 20 (91%) by CBA (1:10 to 1:10000). CSF titers determined using CBA versus IHC showed a very good correlation (r_S=0.92, p<0.0001). Discrepancies in the established CSF titers were mostly attributable to small differences in dilution steps between the titration schemes for IHC (1:16, 1:32, 1:64) and CBA (1:10, 1:32, 1:100). Serum titers showed a moderate correlation (r_S=0.67, p=0.0007). Removing the two CBA-negative sera increased the correlation (r_S=0.71, p=0.0004), suggesting that the only moderate correlation can be partly explained by the lower CBA sensitivity for serum testing.

Conclusion

Equal sensitivity of 100% and high correlation between CBA and IHC support the suitability of both techniques for CSF testing, which is considered the gold standard for establishing the diagnosis of NMDAR encephalitis in symptomatic patients. Serum CBA may require confirmatory CSF analysis due to lower sensitivity and poorer correlation with IHC.

Conflict of interest: ADC, AD and SS are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

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Live versus fixed cell-based assay for anti-MOG antibodies: discrepancies and usefulness of titration

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Abstract

Myelin oligodendrocyte glycoprotein (MOG) antibodies-associated disease (MOGAD) is an inflammatory demyelinating disease for which diagnostic criteria have recently been published (Banwell et al., 2023). In addition to the core clinical phenotypes, the presence of anti-MOG antibodies is a key component of these criteria. However, their significance varies depending on the assay (live cell-based assay [LCBA] versus fixed cell-based assay [FCBA]) and the antibody titer. The cut-off between low and clear positive has been established at 1:100 for FCBA. These criteria still need to be validated in clinical practice.

The aim of this study was to evaluate the relevance of the biological criterion by comparing FCBA and LCBA in our routine practice.

Between August 2024 and January 2025, we conducted a prospective study in which we assessed the presence of anti-MOG in the serum of patients at diagnosis using FCBA (Euroimmun, 1:2 dilution for the screening). Samples with negative results using FCBA were excluded. Positive samples at a dilution of 1:2 were then tested at a dilution of 1:10 and 1:100 with FCBA, and LCBA was also performed.

We identified 155 positive samples for anti-MOG antibodies using FCBA with a titer ≥ 1:2. Clear positivity (i.e., FCBA titer ≥ 1:100) was observed in 49/155 (32%) samples and 39/49 (80%) were also positive using LCBA. Low positivity (i.e., FCBA titer between 1:10 and 1:100) was observed in 42/155 (27%) samples and 14/42 (33%) were also positive using LCBA. Surprisingly, 10/64 (16%) samples that were very low positive using FCBA (titer < 1:10) were positive using LCBA.

This study underscores significant discrepancies between LCBA and FCBA for the detection of anti-MOG antibodies. Further analysis correlating these results with clinical data is needed to refine diagnostic strategies in MOGAD.

6.2 The Improvement of the Serologic Diagnosis of Myasthenia gravis

Laboratory diagnostic testing for myasthenia gravis in the era of cell-based assays

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Abstract

Myasthenia gravis (MG) is an autoimmune neuromuscular junction disorder that is typically mediated by antibodies targeting the muscle-type nicotinic acetylcholine receptor (AChR) or muscle-specific tyrosine kinase (MuSK). Given their central role in immunopathogenesis, testing for antibodies against AChR and MuSK is essential to the diagnostic evaluation of patients with suspected MG. Testing for these antibodies in routine practice has historically been performed by radioimmunoprecipitation assay (RIPA); however, fixed and live cell-based assays (CBAs) have increasingly become clinically available that appear to have higher overall sensitivity and specificity than RIPA, without the need for radioactive reagents. This presentation reviews laboratory diagnostic tests that are currently available for autoantibody detection in patients with suspected MG, examines recent literature investigating their comparative diagnostic performance, and discusses antibody testing algorithms that have been proposed in the era of CBAs.

Detection of myasthenia gravis autoantibodies using an innovative cell-based immunofluorescence assay

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Introduction

Myasthenia gravis (MG) is an immune-mediated neuromuscular junction disorder and one of the most prevalent neurological autoimmune diseases. Serological markers of MG are autoantibodies against nicotinic acetylcholine receptors (AChR) and autoantibodies against muscle-specific kinase (MuSK). Autoantibodies against AChR and MuSK are detected in approximately 80% and 3% of MG patients, respectively, while 15% of MG cases are seronegative [1,2].

The current gold standard for the detection of anti-AChR and anti-MuSK autoantibodies is radioimmunoprecipitation assay (RIA), despite its disadvantage of requiring radioactive reagents. An innovative technology for the detection of autoantibodies is the cell-based indirect immunofluorescence assay (CBA).

Here we studied trueness and diagnostic performance of the Myasthenia Gravis Mosaic 2 IIFT (indirect immunofluorescence test, EUROIMMUN), a newly developed CBA based on transfected human cell lines separately expressing the corresponding target antigens AChR and MuSK for parallel detection of IgG autoantibodies against adult acetylcholine receptor (AChR-E), fetal acetylcholine receptor (AChR-G), and MuSK.

Methods

The trueness was determined by measuring samples with analyte concentrations covering the relevant measurement range of the CBA, i.e. 150 serum samples from MG patients (50 anti-AChR positive, 50 anti-MuSK positive, 50 anti-AChR and anti-MuSK negative), with the CBA and RIAs serving as the reference test system.

To determine the CBA's clinical sensitivity, 395 serum samples from MG patients (144 ocular MG, 251 generalised MG) were analysed. The clinical specificity was determined by analysing 323 serum samples from patients with diagnoses relevant for MG differential diagnostics (223 patients without a neuromuscular disorder, 20 rheumatoid arthritis, 20 systemic lupus erythematosus, 20 Hashimoto's thyroiditis, 20 myositis, and 20 multiple sclerosis patients).

Results

The Myasthenia Gravis Mosaic 2 IIFT had a positive agreement of 96% and a negative agreement of 100% with an Anti-AChR RIA as well as a positive agreement of 98% and a negative agreement of 100% with an Anti-MuSK RIA.

The detection of anti-AChR autoantibodies resulted in a clinical sensitivity of 76.7% and a clinical specificity of 99.4%, while testing for anti-MuSK autoantibodies resulted in a specificity of 100%. The sensitivity for detection of anti-MuSK autoantibodies was 2.8% in the whole patient cohort (N=718), but 39.3% in 351 anti-AChR negative samples (cases of generalized MG).

Conclusion

The Myasthenia Gravis Mosaic 2 IIFT (EUROIMMUN) had excellent agreement with reference RIAs. Due to its high diagnostic sensitivity and specificity, the CBA has gold standard potential as a first-line test for MG serology. The CE-IVDR marked CBA is easy to implement in the laboratory routine; its processing can be either manually or fully automatically with faster incubations compared to ELISA or RIA.

Previous work

Parts of these results were published in: Mirian A et al. Comparison of fixed cell-based assay to radioimmunoprecipitation assay for acetylcholine receptor antibody detection in myasthenia gravis. J Neurol Sci 432:120084 (2022).

Conflict of interest: DJ, LZ, RK, VBL, and EL are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

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Novel recombinant human monoclonal autoantibody IgG1 and IgG4 targeting the human nicotinic acetylcholine receptor

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Introduction

Myasthenia gravis (MG) is a rare chronic autoimmune neuromuscular degeneration affecting breathing, swallowing, movement and vision. The IgG autoantibodies targeting the alpha subunit of nicotinic acid acetyl choline receptor (nAchR), disrupt post-synaptic communication between nerves and muscles causing debilitating and potentially life-threatening muscle weakness in 85% of MG.

Objective

A first-in-kind fully human anti-nAcHR monoclonal autoantibody (nAchR autoHuMAb) was developed to calibrate patient autoantibody levels in the non-radioactive ELISA and cell-based assay to the standard activity measured by radio-receptor immune precipitation assay (RIPA).

Methods

The expression of IgG1 and IgG4 nAChR AutoHuMAbs in transient transfected HEK-293T cell culture and their protein A-purification were under ISO 9001 certification (Absolute Antibodies). Six concentrations of protein A-purified IgG1, range 2-500 ng/mL, were prepared in human serum and measured by RIPA nmole/L activity, immune fluorescence titer Euroimmun Biochip (IIFT-CBA) and ELISA RSR Ltd.

Results

Coomassie stain of SDSPAGE reveals the intact 120 Kd Immunoglobulin in non-reducing (NR) and heavy (H) and light (L) chains in reducing (R) conditions. Indirect immune fluorescence testing

(IIFT) of nAChR AutoHuMAbs on Biochip Mosaic-2 CBA (Euroimmum) shows positive reactivity with the adult nAChR and fetal nAChR versus null reactivity with muscarinic acid receptor (MuSK). The binding activity of seven concentrations of IgG1 and IgG4, range 6-1000 ng/mL, in human serum was measured by RIPA (Tecan GmbH) and by ELISA (RSR Ltd).

Summary/Conclusion

Anti-nAchR AutoHuMAb ng/mL reactivity is robust and stable in immunoassays after repeated freeze-thaw cylces, offering a reference calibrator for quantitative reporting of autoantibody levels. Cost savings and greater accessibility of non-radioactive myasthenia gravis blood diagnostics are anticipated by use of nAchR autoHuMAb. These results underscore diagnostic applications for the nAChR HuMAb with possible therapeutic implications. Investigation of functionality in live cell-based assays of complement activation and receptor clustering and the induction of neuromuscular junction pathology, in a mouse model of Myasthenia Gravis are warranted.

Glycosylation in the Variable Regions of MuSK and AChR Autoantibodies: Effects on Binding and Pathogenicity in Myasthenia Gravis

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Introduction

In autoimmune diseases like myasthenia gravis (MG), autoantibodies targeting acetylcholine receptors (AChR) and muscle-specific kinase (MuSK) disrupt neuromuscular signaling. N-linked glycosylation in the variable (V) regions of antibodies can affect their structure and function, though its role in MG autoantibodies remains unclear. Glycosylation influences antibody binding and pathogenicity, and antibody valency - especially in IgG4 antibodies - also plays a critical role. MuSK antibodies are mainly IgG4, while AChR antibodies are predominantly IgG1 and IgG3.

In this study, we identified glycosylation sites in three new patient-derived monoclonal antibodies (mAbs) targeting MuSK and AChR, along with the previously studied mAb MuSK1A, and investigated how glycosylation removal affects binding and pathogenicity, independent of Fab arm exchange (FAE).

Results

Glycosylation was successfully removed from the V regions of all mAbs, as confirmed by SDS-PAGE. For AChR mAbs (01b and 09), glycosylation removal had no significant effect on binding. For MuSK mAbs (6C6 and MuSK1A), glycosylation removal did not affect pathogenicity in the monovalent Fab form. However, in the divalent IgG4 format, pathogenicity increased, suggesting that glycosylation affects MuSK mAb pathogenicity in a valency-dependent manner. FAE, which contributes to the diversity of IgG4 antibodies, independently influences the pathogenicity of MuSK mAbs.

Conclusion

In summary, glycosylation in the V regions of MuSK-specific antibodies alters their pathogenicity, with the effect varying depending on antibody valency. In contrast, glycosylation removal had no impact on AChR mAbs. These results provide new insights into the molecular mechanisms driving MG and suggest that targeting glycosylation and antibody valency could offer valuable strategies for therapeutic intervention in autoimmune diseases.

Improving diagnostics in seronegative Myasthenia Gravis in the D-A-Ch region through cell-based assays

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Background

Myasthenia gravis (MG) is a rare autoimmune disease. In most cases, pathogenic autoantibodies targeting the acetylcholine receptor (AChR, ~85%) or muscle-specific kinase (MuSK, ~5%) are detectable using assays such as radioimmunoassay or ELISA. However, a subset of patients (10–15%) lack detectable antibodies against these established targets and are classified as seronegative (SNMG), presenting diagnostic and therapeutic challenges. Previous studies suggest that SNMG is likewise antibody-mediated, as SNMG patients often respond to immunotherapies and complement deposition has been observed at their NMJs. We hypothesize that SNMG is driven by unidentified autoantibodies targeting novel NMJ antigens. Our research aims to improve diagnostic sensitivity in the SNMG cohort using live cell-based assays (L-CBA) and to discover novel antigenic targets contributing to the SNMG phenotype.

Methods

The inclusion criteria for the study are MG patients (tested with ELISA or RIA), who are seronegative and from the D-A-Ch region. Using a highly sensitive established L-CBA, we assess

the prevalence of autoantibodies against clustered AChR and MuSK. We employ an NMJ model of agrin-stimulated human myotubes to determine autoimmunity against NMJ proteins. Lastly, we aim to identify potential antigens bound by SNMG patient sera through immunoprecipitation and mass spectrometry.

Results

So far, we received 342 SNMG patient sera, of which 279 patients are from Germany, 57 are from Austria and 6 from Switzerland. Around 70% of the patients are female. 5.26% of the SNMG sera were positive for antibodies against AChR in the L-CBA. Only 2 patients (0.72%) tested positive for anti-MuSK antibodies. We found that approximately 7.29% (24/329) of the remaining SNMG sera show antibody-binding in proximity to the NMJ. We have optimized immunoprecipitation and mass spectrometry workflows to investigate antibody-antigen interactions in SNMG. Using this antigen discovery strategy, we successfully isolated AChR and MuSK from antibody-positive sera, confirming the capability of our protocol to capture and identify antibody-bound NMJ antigens.

Conclusion

In conclusion, a subset of seronegative MG patients has antibodies against AChR or MuSK when detected with L-CBA. We also observe antibody binding near the NMJ in the remaining SNMG sera, suggesting the presence of yet unidentified autoantibodies.

6.3 Paraneoplastic Neurologic Syndromes

Neuropathological findings and implications for the diagnosis of paraneoplastic syndromes

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Abstract

Paraneoplastic neurologic syndromes (PNS) represent a group of immune-mediated complications associated with an underlying tumor. Ectopic protein expression in neoplastic cells or an aberrant immune regulation associated with haemato-oncologic diseases or thymomas trigger an autoimmune response that may affect any part of the central and/or peripheral nervous system. Neuropathologic hallmarks of PNS associated with antibodies directed against intracellular epitopes are characterized by T cell-dominated inflammation, reactive gliosis including microglial nodules, and neuronal degeneration. Moreover, lesions present a prominent neuronal pSTAT1 expression that drives acute phagocytic elimination of synapses followed by CD8+ T cell-mediated attack. Appropriate detection of antibodies as well as understanding of the underlying pathomechanisms in PNS is important because its strongly corresponds with therapy response and prognosis, and should guide treatment decisions.

Towards harmonization of autoantibody detection in relation to Paraneoplastic Neurological Syndromes (PNS): a European Survey on laboratory practices

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Background

The detection of paraneoplastic anti-neuronal antibodies (PNS autoantibodies) can help in diagnosing the particular syndrome and can guide the search for an underlying tumor. In order to identify options for which harmonization might improve its diagnostic contribution, we aimed to document the variability in the current autoimmune serological laboratory work-up of PNS between laboratories.

Materials and methods

A questionnaire was developed by the European Autoimmunity Standardisation Initiative (EASI) to collect information on testing methodology and algorithm, digital methods, test interpretation and clinical context, and quality assurance approaches. The questionnaire was distributed with the support of 2 external quality control (EQC) program providers (UK NEQAS and IfQ Lübeck) amongst clinical laboratories currently performing the assays in daily practice.

Results

A total of 139 laboratory representatives participated in the survey. The vast majority of labs perform both cerebellum IIF and dot/line blot (78%) either consecutive or in parallel, and test both on serum and cerebrospinal fluid (CSF) (76%). In contrast, the sample dilution and the conjugate type are only aligned in 61% and 70% of labs, respectively. Variability in line/dot blot testing is introduced by differences in antigen composition and reporting strategy. Digitization of line blot data is common (87%), while for cerebellum indirect immunofluorescence (IIF) digitization is still limited (31%). Half of labs (53%) use testing algorithms, that differ amongst labs based on the sample type availability, the lab request and the specific PNS autoantibodies that are searched for. Eighty-nine percent and 48% of the laboratories perform internal quality control (IQC) for cerebellum IIF and for line/dot blot, respectively. The origin of the IQC samples is mostly only commercial. Nearly half of the labs (43%) participate in more than one EQA scheme. Nearly half of the participating labs have ISO15189 certification for these assays (46% for IIF, 42% for line/dot blot), and approximately 20% have plans to obtain it. Positivity rates for PNS autoantibodies are low (73%, <6% positivity), indicating that pre-test probability is often low. Limiting the request to certain disciplines is installed in only a minority (46%) of labs. The majority of the lab have access to the clinical context (84%), and many also discuss the results with the requesting clinician (63%).

Conclusion

Although many laboratories pursue high quality for the detection of PNS autoantibodies, significant variation exists in how the assays are conducted, reported and controlled. The introduction of laboratory specific recommendations on the test methodology and reporting may contribute to harmonization and the improvement of the overall quality of these analyses, further optimizing its diagnostic contribution.

Evaluation of a tissue-based assay for KLHL11 autoantibodies in paraneoplastic neurological syndromes

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Background

Anti-Kelch-like protein 11 (KLHL11) autoantibodies were first identified in 2019 as important markers for paraneoplastic neurological syndromes (PNS), a group of disorders in which the immune system attacks the nervous system in response to an underlying malignancy, such as seminomas (testicular cancer) and ovarian tumors. These autoantibodies are specifically associated with paraneoplastic encephalitis (PNE), a severe form of PNS affecting the brain. Early diagnosis and treatment are crucial for improving the prognosis of affected patients. Unlike other neurological markers, where tissue is screened first and a cell-based assay (CBA) serves as a confirmatory test, PNS involving KLHL11 autoantibodies is diagnosed by first screening with CBA, followed by tissue-based assay (TBA) confirmation. This study evaluated whether rat hippocampus and other neuronal tissues can serve as substrates to reliably confirm KLHL11 autoantibody positivity.

Methods

Indirect immunofluorescence was performed on sections from various neuronal tissues to detect KLHL11-specific binding patterns. Clinically characterized samples positive for KLHL11 antibodies [serum (n=15), cerebrospinal fluid (CSF, n=7), and 1 plasmapheresis sample] were tested alongside 24 control samples from healthy blood donors.

Results

KLHL11-specific punctuate fluorescence patterns were detected in the rat hippocampus tissue of all patient samples, confirming the presence of KLHL11-specific autoantibodies. No antibodies were found in the control samples, demonstrating the high specificity of the assay.

Conclusion

Our study demonstrates that rat hippocampus is a suitable and effective substrate for confirming KLHL11 autoantibody positivity following CBA screening. This confirms the potential of the TBA in supporting diagnosis of paraneoplastic brainstem cerebellar syndrome, offering high sensitivity and specificity. Importantly, this approach helps minimize unnecessary cancer screenings, as it provides reliable confirmation of KLHL11 autoantibodies following the initial CBA screening. Further studies are needed to validate its performance in larger cohorts and explore its application in other clinical syndromes and associated tumors.

Conflict of interest: SM, MK, and DJ are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

7 Immune-Related Adverse Events (IRAE) due to Immune Checkpoint Inhibitors (ICI)

Immunogenicity of checkpoint inhibitors: The Italian multicenter study 'RE-ONCHECK'

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Background

Rheumatic and musculoskeletal immune-related adverse events (irAEs) refer to a set of side-effects in patients with cancer receiving immune-checkpoint inhibitors (ICI) treatment. The onset of irAEs has several implication for patient treatment and may affect cancer outcomes. In this context, the investigation of reliable biomarkers to predict which patients are prone to develop toxicities from ICI therapy represents an area of great interest. The RE-ONCHECK study was designed to define the predictive factors of irAEs and their correlation with clinical manifestations associated to systemic autoimmune rheumatic diseases in ICI-treated patients.

Methods

A multicenter, prospective, observational, cohort study has been designed to be conducted in patients diagnosed with different types and stages of solid organ malignant tumors, eligible to ICI therapy. Considering the estimated percentage of ICI-induced irAEs, it has been calculated that a sample size of 250 patients is required, 50 for each participating Italian hospital. In the setting of outpatient clinics, patients will be recruited by the medical oncologists and tested for ordinary blood test as required by protocol; additional blood test (C3, C4, TSH, FT3, FT4, PRC, ESR, amylase, lipase) and a comprehensive autoantibody panel under study will be performed (anti-nuclear antibodies (ANA), anti-ENA, anti-dsDNA, Rheumatoid factor (RF), anti-citrullinated protein antibodies, anti- neutrophil cytoplasmic antibodies (ANCA), anti-DFS70 and myositis-specific antibodies (jo-1, PL-7, PL-12, EJ, SRP, Mi-2, MDA-5, TIF1-γ, SSA/Ro52kD, SAE1, SAE2, NXP-2)) by each laboratory using standardized methods. At timeline of 3, 6, and 12 months after treatment, patients will be evaluated for treatment response and all laboratory test repeated. A telephone interview will be performed by rheumatologists at baseline and at 3 defined longitudinal time, to assess the onset of any rheumatologic or musculoskeletal irAEs. In person rheumatology visits will be scheduled for patients presenting with clinical conditions related to rheumatic diseases. The demographic feature of the patients, type and cycle of ICI, clinical features, biochemical

parameters and autoantibody profile will be included in the analysis. This protocol was reviewed and approved by the Ethical Committee of the Basilicata Region. Informed consent will be obtained from all participants before their enrollment.

Results

To date, 17 patients (14 M, 3 F, average age 77 years) eligible for the ICI treatment (either anti-PD-1 or anti-PD-L1 therapy alone or in combination with anti CTLA-4) were included in this study. At baseline, the autoimmune assessment revealed the positivity for ANA (>1:160) in 5 out 17 patients (29.4%); RF was observed in 2 out of 17 (11.7%), anti-ENA in 4 out 17 (23.5%) and anti-dsDNA antibodies in 1 out 17 (5.88%) patients.

Discussion and conclusion

The protocol of RE-ONCHECK study was designed to prospectively assess the autoantibody status at baseline and over time, the timing of antibody emergence correlated with any rheumatic and musculoskeletal irAEs for several type of cancers and ICI treatments, allowing for a close collaboration across multiple disciplines. Preliminary data showed as the baseline evaluation of a comprehensive panel of autoantibodies is mandatory, to avoid an overestimation of treatment induced-autoimmunity. Indeed, a higher rate of cancer-related ANA positivity was found in patients before treatment, when compared to the general population. This data highlighted the role of cancer cells in eliciting immune responses leading to autoantibodies production. The better characterization of cancer-related and ICI treatment-induced autoimmunity is crucial in order to identify the optimal prevention and precision medicine strategies.

Checkpoint inhibition and autoimmune diseases

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Abstract

Therapy with immune checkpoint inhibitors (ICI) has revolutionised oncological treatment. Patients with various tumours have achieved prolonged remission and even long-term survival as a result of treatment. In addition to tumour defence, ICI-induced immune stimulation is associated with a high rate of immune-mediated side effects (Eisemann N et al.. Dtsch Arztebl Int 2024; Tison A et al., Nat Rev Rheumatol 2022). Particularly in patients with pre-existing autoimmune diseases, there are more and earlier exacerbations of pre-existing autoimmune diseases or new immune-related adverse events. Despite or partly because of this increased immune response, antitumour therapy is very effective in these patients and in some cases even more effective than in patients without pre-existing autoimmune diseases and autoantibodies (Daban A et al., Oncoimmunology2023). Therefore, pre-existing autoimmune diseases are not a contraindication for ICI therapy. However, the autoimmune disease must be very well controlled and new or exacerbated autoimmune reactions should be treated as specifically as possible (Haanen J et al., Annals of Oncology 2020).

Neurological Immune-related Adverse Events (NirAE) due to immune checkpoint inhibitors: a laboratory approach

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Abstract

Immune checkpoint inhibitors (ICIs) are a type of immunotherapy that can cause a range of adverse effects, known as immune-related adverse events (irAEs), as the immune system is activated to attack cancer cells, it may also attack other healthy tissues. These side effects can vary in severity and may affect different parts of the body.

Among the less common, but potentially most serious adverse effects, are the neurological irAE (NirAE). When considering all types of ICI treatments, the incidence of any grade NirAE ranges from 10.2 to 18.9% of treated patients, but high grade NirAEs are below 1%. The most frequent NirAEs involve muscles and neuro-muscular junctions, such as myositis (necrotizing or not) and myasthenic syndromes, which are 3 times more frequent than central nervous system (CNS) disorders and can overlap with myocarditis. Among the CNS disorders, affecting the brain and the spinal cord, encephalitis, meningitis, cerebellitis, demyelinating disorders of the CNS and myelitis were reported. Finally, also events involving peripheral and cranial nerves, such as GBS-like polyneuropathies, were often described.

Many alternative diagnoses need to be considered in patients with cancer experiencing neurological dysfunctions, including neoplastic, infectious, metabolic and iatrogenic complications. The type of ICI and the histological origin of the neoplastic proliferation may influence the neurological phenotypes of NirAE.

All patients developing a neurological disorder under ICI therapies should undergo neurological evaluation, ideally by a Neurologist with experience in autoimmune neurology and/or neuro-oncology. As regards myositis, a strict collaboration with rheumatologists is needed.

Moreover, a multidisciplinary discussion of complex cases in dedicated meetings should be encouraged. In these meetings, also a Clinical Pathologist with experience in autoimmunity and neurologic immune related diseases can be of great help.

Patients affected by ICI-related myositis manifested a spectrum of disease ranging from oligosymptomatic hyperCKemia and myalgias to severe myopathies. Myositis-specific antibodies were found in about 36% of cases. In these cases, the Laboratory has an important role, since

methods available to identify these Abs suffer from both low sensitivity and high false positive results. ANA by IIF is not sufficient, but also the common connective tissue disease screening may be negative. Only dedicated profile may reveal rare specificities, such as SRP, PL7 and PL12 that were often reported in such patients.

Severity was a remarkable characteristic of ICI-induced Myasthenic syndromes, as bulbar involvement and respiratory failure were frequently reported, while isolated ocular involvement was less common. In about 60% of cases, antibodies against AchR were reported and in some cases, these antibodies were present before ICI treatment.

Patients affected by CNS disorders, presented with altered mental status, cognitive impairment, seizures, psychiatric disturbances, ataxia or movement disorders. In many cases patients had an unremarkable brain MRI, but quite all demonstrated remarkable inflammatory alterations in the CSF analyses (moderate increase of leukocytes and protein content). Usually, when the workup for infectious diseases appears negative, the clinicians raised the suspicion for NirAE. Frequently, an associated antibody can be found: the most commonly reported specificities are Ma2-Abs (about 41% of the positive cases), followed by Hu (18%). In some cases, Ma2-Abs tested positive in serum samples before ICI administration.

We recently published the results of a multicenter, retrospective, cohort study of patients developing new-onset, immune-mediated, isolated/predominant cerebellar dysfunction after ICI administration. Neuronal antibodies were detected in 15/31 patients tested (48%). When compared with a series of paraneoplastic cerebellar ataxia (n = 15), the cerebellar irAE group was significantly more associated with male sex, lung cancer (rather than gynecological/breast cancers), isolated ataxia, and a better outcome, thus only partially reflects the associations described in the classic paraneoplastic disorders.

In very few patients with demyelinating disorders or myelitis, positive AQP4 antibodies were found, but in some cases a tissue-based assay suggested the presence of antibodies against similar antigens.

Similarly, in cases with peripheral neuropathies, only few cases demonstrated well defined antibodies, such as anti-gangliosides. But in many cases, when using a tissue-based assay (TBA), we can identify antibodies against neurofilaments (Nf) or GFAP, for which commercial confirmatory assays are not yet available.

In our experience, we found 2 cases with neuroendocrine tumors developing a GBS-like polyneuropathy post combo ICI (Nivolumab+Ipilimumab). In both cases, the TBA on primate cerebellum identified a fluoroscopic pattern suggestive for Nf antibodies. The samples were sent to a third level Centre, where they confirmed positive antibodies against Nf light, medium and high chain and alfa internexin.

An early recognition of potential symptoms and the identification of positive antibodies may lead to early prevention of severe outcomes.

In some cases, antibodies were described even before ICI treatment. The opportunity to comprehensively test at least some autoantibodies before starting ICI therapy is still debated. There is not yet sufficient evidence from randomized, controlled studies. Our neuroimmunology group is carrying out a project on the pre-treatment assessment of the presence of autoantibodies

potentially associated with NirAE, with the aim of understanding whether and how screening may be justified.

But autoantibodies are only part of the story. Recently, we published a review providing a comprehensive overview of the latest findings on NirAEs associated with ICIs, with a focus on their prediction, prevention, as well as precision treatment using autoantibodies but also cytokines, neuronal biomarkers and microbiota. In particular cytokines, such as IL-6, CXCL10 and CXCL13, may have an important role in guiding selection of second line therapies.

In the meantime, with increasing experience, oncologists' sensitivity also grows, enabling them to detect even subtle signs of potential neurological complications and therefore request further investigation from the autoimmunity laboratory. The laboratory must be prepared to analyze these patients, recognizing that it cannot always identify known specificities. Using a TBA test, even a commercial one, can enable the identification of fluoroscopic patterns associated with unknown antibodies or for which a commercial confirmatory test is not yet available. The increased reporting of cases involving these new specificities will allow us to better understand their role and perhaps improve both the prevention and treatment of these toxicities.

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Prediction of immune-mediated side effects in skin cancer patients under immune checkpoint inhibitor therapy using liquid biopsies

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Objectives

Immune checkpoint inhibitors (ICIs) have transformed cancer treatment, particularly in malignant melanoma (MM), yielding remarkable therapeutic outcomes. However, despite their effectiveness, ICIs can cause severe immune-related adverse events (irAEs). The underlying mechanisms remain poorly understood, and currently, there is no reliable method to predict an individual patient's risk. Consequently, there is a critical need for diagnostic approaches to stratify patients according to their likelihood of experiencing irAEs.

Methods

A total of 35 autoantibodies (AABs), each associated with specific autoimmune diseases, were selected to establish a detection panel. Blood samples were collected from each patient before, during, and after ICI therapy. Subsequently, clinical data were incorporated and analyzed in conjunction with AAB test results to correlate their presence with clinical manifestations in the corresponding patient.

Results

From March 2020 to March 2023, 101 patients (30 women, 71 men, average age 69) were included. Of these, 93.1% had MM and 6.9% had squamous cell carcinoma. 49.5% received ICI monotherapy, and 50.4% ICI combination therapy. 66.3% of patients experienced irAEs, with colitis being the most common at 23.8%.

Preliminary analyses revealed 30 patients with elevated AAB levels; 9 had multiple elevated AABs (= >1 AAB), and 21 only one. One aim of this study was to explore a potential association between

elevated AAB levels and the occurrence of irAEs, with a slight correlation observed between the development of irAEs and the presence of more than one elevated AAB. Additionally, we examined whether organ-specific irAEs were associated with specific AABs, but no clear associations were found.

We further investigated the association between elevated AAB levels and treatment response to ICIs. Fisher's exact test comparing disease control rates and irAEs revealed a significant correlation between the number of AABs and partial responses.

Statement

Our findings indicate that elevated AAB levels are modestly associated with irAEs and partial responses in patients receiving ICI therapy. However, no organ-specific AAB patterns could be identified. These results highlight the potential and limitations of AAB panels for risk stratification in ICI-treated patients.

8 Natural, Protective and Pathogenic Autoantibodies

Physiological and pathophysiological functions of autoantibodies against G protein-coupled receptors

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Abstract

G protein-coupled receptors (GPCRs), such as angiotensin receptor type 1 (AT1R) and the chemokine receptor CXCR3, are constitutively expressed in small resistance vessels and large arteries, respectively. Our group has identified autoantibodies (Abs) targeting AT1R and CXCR3 in healthy individuals, with elevated levels observed in patients with inflammatory disorders. Specifically, AT1R Abs are associated with microvascular diseases, whereas CXCR3 Abs are linked to inflammation of large vessels and cardiovascular events. These epidemiological findings suggest that such Abs may serve as biomarkers for diseases affecting distinct vascular beds.

In vitro assays demonstrated that both AT1R and CXCR3 Abs promote immune cell migration. For AT1R Abs, antibody levels in both healthy donors and patients correlated with the number of migrated cells, indicating a physiological role in cell trafficking. Moreover, AT1R Abs activated endothelial cells to upregulate adhesion molecules and reduced the glycocalyx, a surrogate marker of vascular barrier function. In vivo, immunization of mice with CXCR3 increased CXCR3 Ab levels and aggravated atherosclerosis, while immunization with AT1R induced perivascular inflammation of small vessels in the lung and skin. These findings indicate that elevated Ab levels are associated with inflammation in distinct vascular compartments, reflecting the constitutive expression patterns of their target receptors.

Receptor expression of AT1R and CXCR3 also varied among immune cell subsets and across different diseases. In addition to promoting migration, AT1R Abs activated immune cells such as monocytes, further contributing to inflammatory responses. Taken together, our data suggest that these autoantibodies bind specifically to endothelial cells of distinct vascular beds, leading to endothelial activation, immune cell recruitment, and immune cell activation. We propose that GPCR Abs may regulate the site of inflammation (via receptor specificity), the intensity of inflammation (via Ab levels), and the composition of infiltrating immune cells (via receptor specificity). This represents a potential novel mechanism for regulating inflammation in both

physiological and pathological contexts. This hypothesis, however, requires confirmation through further rigorous investigations.

Emerging immunoregulatory functions of DFS70/LEDGF: implications for understanding the possible protective nature of DFS (AC-2) autoantibodies

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Abstract

The dense fine speckled autoantigen of 70 kD (DFS70), also known as lens epithelium derived growth factor p75 (LEDGF/p75), is encoded by the *PSIP1* gene and targeted by IgG antinuclear autoantibodies in subsets of healthy individuals and patients with mild inflammatory conditions, as well as some patients with benign prostatic hyperplasia (BPH) or prostate cancer (PCa). When monospecific in human sera, these autoantibodies produce the ICAP-defined AC-2 immunofluorescence pattern and are considered as biomarkers to aid in the exclusion of a systemic autoimmune rheumatic disease (SARD) diagnosis. In addition, they have been reported to be associated with lower circulating levels of oxidative stress and inflammatory markers, and have been suggested to have protective functions. Functionally, DFS70/LEDGF is a stress response transcriptional co-activator that facilitates HIV integration into T cell chromatin and contributes to cancer cell chemoresistance through its ability to tether transcription factors to active chromatin, promote RNA-loop resolution at transcriptionally active sites, enhance DNA repair, regulate mRNA splicing, and maintain genomic integrity. Its depletion in cancer cells induces genomic instability, likely resulting in upregulation of inflammatory gene pathways. Our recent studies have demonstrated that DFS70/LEDGF is naturally upregulated in chemoresistant prostate cancer (PCa) cells and contributes to their survival by influencing gene pathways associated with regulation of oxidative stress responses, DNA repair, cell cycle progression, and apoptosis. We hypothesized that its silencing in these cells may also influence immune-related gene pathways. Knockdown of DFS70/LEDGF in chemoresistant PCa cells followed by RNA-seq analysis led to the identification of 970 differentially expressed genes (DEGs). Gene set enrichment analysis (GSEA) revealed a role for this autoantigen in modulating gene pathways associated with T and B cell

responses. Interestingly, its silencing led to upregulation of several inflammation-related genes including *IL7R, IL18, SWAP70, BMI-1, ULBPI/2, RAET1E/L, TRPM4, FADD*, as well as several MHC class I genes. Protein expression of selected genes was validated by Western blotting in PCa cells with DFS70/LEDGF knockdown. Elevated expression of several of these inflammatory genes also correlated with favorable overall survival of cancer patients receiving immune checkpoint inhibitor therapy, as revealed by KM Plotter-Immunotherapy analysis. In addition, publicly available RNA-seq data for various immune cells revealed potential roles of DFS70/LEDGF in promoting CD4 and CD8 T cell activation. Moreover, public scRNA-seq data pointed to DFS70/LEDGF expression in T cell clusters in cancer tissues. We conclude that as a guardian of genome integrity, DFS70/LEDGF may contribute to the negative regulation of inflammatory gene pathways when expressed at high levels. This would be consistent with the previous observation that anti-DFS autoantibodies, which are likely triggered by high DFS70/LEDGF tissue expression, are associated with lower circulating levels of markers of oxidative stress and inflammation. While these autoantibodies may not be intrinsically protective, their presence may reflect a protective, anti-inflammatory tissue microenvironment characterized by increased DFS70/LEDGF expression.

Pathogenic autoantibodies in neurologic diseases

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Abstract

Pathogenic autoantibodies have transformed the understanding of autoimmune neurological diseases. Beyond simple biomarkers, they actively cause dysfunction by disrupting receptors, activating complement, or interfering with cell adhesion. Several discoveries have redefined syndromes entirely—anti-AQP4 antibodies separated neuromyelitis optica from classical multiple sclerosis, while anti-NMDAR antibodies introduced a novel, treatable form of autoimmune encephalitis. These examples marked paradigm shifts, altering both diagnostic frameworks and treatment strategies.

This presentation provides a focused overview of key autoantibodies in central and peripheral nervous system diseases. Their classification by target location, immunoglobulin subclass, and effector mechanism offers a clearer understanding of disease biology. Attention is given to IgG4-mediated conditions, which often lack inflammation and respond poorly to standard treatments like IVIg. These differences reflect distinct mechanisms of action and call for tailored therapies, such as B-cell–directed CAR T cells or monovalent antibody blockers.

Rather than catalogue every known antibody, the talk aims to clarify underlying mechanisms and therapeutic implications. The presence of an antibody is no longer the end of the story—understanding how and why it causes disease is becoming central to personalized treatment in neuroimmunology.

Airway autoimmunity: evidence, mechanisms and clinical relevance in complex airways disease

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Abstract

There is increasing evidence of localised airway autoimmune responses in several pulmonary diseases that play a critical role in the persistence of symptoms, steroid dependence, and persistent inflammation, often leading to inadequate responses to novel biologics. Autoantibodies targeting eosinophil degranulation products, anti-nuclear and extractable nuclear antigens (PMID: 28751233, PMID: 35777765), macrophage scavenger receptors (PMID: 36287613, PMID: 35236724), and neutrophil cytoplasmic antigens (PMID: 30179583) have been identified in the sputum of patients with severe asthma and eosinophilic granulomatosis with polyangiitis (eGPA). Notably, in eGPA, a systemic autoimmune disease with pulmonary complications, sputum ANCA was detected in 75% of the population, regardless of their serum ANCA status. Sputum ANCA was associated with severe pulmonary complications, severe asthma, and airway eosinophilia (PMID: 30179583). Again, ~55% of moderate-severe asthmatic patients exhibit airway autoreactivities against two or more pathogenic targets that persist despite guideline-based anti-inflammatory treatment and is associated with exacerbations (PMID: 35777765). The cytokine microenvironment of these patients is primarily T2 high, characterized by elevated IL-13 and eotaxin-3, along with markers of B cell activity such as BAFF and CXCL13 in their sputa (PMID: 28751233). These autoantibodies are pathogenic, inducing extracellular trap formations from granulocytes (neutrophils and eosinophils), which perpetuate inflammation unmitigated by corticosteroids. Additionally, danger-associated molecular patterns (DAMPs) such as HMGB1 and galectin-10, released during eosinophil cytolysis, further activate inflammasome signalling (evident from elevated IL-18) and perpetuate autoimmune responses. Recent findings indicate that these autoantibodies and DAMPs contribute to a complex inflammatory milieu, exacerbating tissue damage, mucus plugging and hindering effective treatment responses to anti-IL-5 biologics (PMID: 32444405, PMID: 37824744). Our research program underscores the importance of local airway autoimmune responses in the pathogenesis of complex airways diseases, highlighting the need for targeted therapeutic strategies to address these pathogenic autoimmune responses and improve patient outcomes.

Compensatory role of natural autoantibodies in systemic autoimmune disease-related autoantibody profiles

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Background

Systemic autoimmune diseases (SARDs) arise from immunological dysregulation and loss of self-tolerance, leading to the production of pathology-associated autoantibodies (pAAbs). The role of natural autoantibodies (nAAbs)—present without prior antigen exposure—remains poorly understood. Recent findings support their functions in immune homeostasis with their plasticity in autoimmune disease-related immune perturbations.

Methods

We analyzed 206 anonymized residual serum samples stratified into serological groups based on elevated pAAb titers (≥3× diagnostic cutoff): (I) anti-dsDNA (n = 32), (II) anti-SSA (n = 31), (III) anti-β2-GP-I/anti-cardiolipin (n = 24), (IV) anti-CCP (n = 49), and (V) seronegative controls (n = 70). Pathological autoantibodies were measured using commercial ELISA kits (Orgentec). Anti-SARS-CoV-2 IgG titers were assessed with QuantiVac ELISA (Euroimmune), while nAAb profiles (anti-CS, anti-HSP60/70 IgG/IgM) were determined by in-house ELISAs. Data were analyzed using JASP.

Results

Compared to controls: (I) anti-dsDNA–positive samples showed elevated IgG nAAb titers against CS, HSP60, and HSP70 ($p = 0.04, 0.034, 0.044$); (II) anti-SSA samples had lower anti-HSP60 IgG/IgM levels ($p = 0.42$); (III) anti-β2-GP-I samples showed reduced anti-CS IgM ($p = 0.042$); (IV) anti-CCP–positive samples exhibited significantly increased anti-CS IgM ($p = 0.004$). Comparing the anti-SARS-CoV-2 IgG titers in the serogroups, we found diminished immune response in CCP-positive samples with higher diagnostic thresholds, alongside an inverse correlation with rheumatoid factor levels ($\rho \approx -0.3$).

Conclusions

nAAb profiles varied across serogroups, reflecting their distinct immunoregulatory roles in SARD. Elevated IgG nAAbs in anti-dsDNA–positive samples may indicate their transition in autoantibody-mediated disease (SLE). Increased IgM nAAbs in anti-CCP–positive samples (RA) may suggest a compensatory role of the nAAbs due to neo-antigen exposure. These findings underscore the immunological heterogeneity of SARDs and highlight the potential relevance of nAAb-pAAb interplay in disease modulation.

Funding: TKP-2021-EGA10 and EGA13

Plasticity of natural autoantibody network in autoantibody positive Hashimoto's Thyroiditis

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Background

Autoantibodies (AAbs) against thyroid antigens—mainly thyroglobulin (TG) and thyroid peroxidase (TPO)—are more common in rheumatic patients and often indicate autoimmune thyroid disease, particularly Hashimoto's thyroiditis (HT). HT features progressive thyroid dysfunction and elevated thyroid-specific AAbs. Natural autoantibodies (nAAbs), a distinct subset of AAbs, help maintain immune balance by clearing apoptotic cells and supporting self-tolerance. Our previous work showed that the nAAb repertoire, once considered stable, may shift in response to immune activation.

Materials and Methods

We first examined whether hormone replacement therapy affects nAAb levels in 11 pregnant HT patients. Subsequently, we analyzed anonymized serum samples from patients with elevated anti-TPO and/or anti-TG levels grouped by AAb titers: 'strong positive' (≥3× diagnostic cutoff; n = 34), 'very strong positive' (≥5× diagnostic cutoff; n = 41), and seronegative controls (n = 70). Natural AAbs—including anti-citrate synthase (a-CS IgG/IgM) and anti–heat shock protein 60/70 (a-HSP60/70 IgG/IgM)—were measured using in-house ELISAs. Anonymized clinical AAb results (commercial ELISAs; Orgentec by Sebia) were retrospectively retrieved from routine diagnostics using GLIMS queries. Data were analyzed with JASP software.

Results

Levothyroxine therapy did not significantly alter nAAb levels. In samples characterized by HT specific pathological AAbs, anti-CS IgG levels showed a positive connection with anti-TG and anti-TPO titers ($p = 0.029$ and $p = 0.072$, respectively). In contrast, IgM isotype levels of anti-CS and anti–HSP70 were inversely associated with elevated thyroid autoantibodies (anti-CS IgM: $p = 0.060$ and $p = 0.027$; anti-HSP70 IgM: $p = 0.040$ and $p = 0.021$).

Conclusion

Stratified analysis reinforced the association between thyroid-specific AAbs and nAAb profiles. The findings suggest that IgG nAAbs may adapt to reflect pathogenic antibody changes, while declining IgM nAAb levels could reflect on compromised immune regulation which facilitate pathological IgG autoantibody overproduction in HT.

Funding: RRF-2.3.1-21-2022-00012 and TKP-2021-EGA10

Autoantibodies to dense fine speckled 70 (DFS70) antigen limit neutrophil extracellular trap formations

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Introduction

Autoantibodies to dense fine speckled 70 (DFS70) are absent or detected at low titers in patients with systemic autoimmune rheumatic diseases. Multivariate regression analysis showed serum DFS70 autoantibodies were differentially higher in survivors and predicted 60-day mortality (unpublished). Given that DFS70 is a chromatin-associated nuclear antigen, we sought to investigate its direct impact on NETosis -a process that contributes to both severe infection outcomes and the development of autoimmunity.

Methodology

Anti-DFS70 autoantibodies in sera (healthy donors) were quantified using a line-immunoassay (Human Diagnostics, Germany) and isolated by magnetic co-immunoprecipitation with recombinant protein (R&D, Minneapolis, USA). An in vitro NETosis model was developed to assess the ability of various stimuli (immunoglobulins, immune complexes, calcium ionophore, and PMA) to induce NETs in neutrophils from healthy donors. Platelet-rich plasma was added to evaluate platelet activation, and NET formation with platelet aggregation to the NET scaffold was imaged via scanning electron microscopy and quantified using MATLAB software. Activation states of

neutrophils (resting vs NETs) were immunostained to colocalize DFS70 antigen with histones and DNA, and cytosolic proteins using confocal microscopy. Western blot quantified DFS70 antigen in neutrophils using the Abby Automated Western system (BioTechne, USA). Neutrophils were stained for Annexin-V and 7AAD to evaluate cell viability (BD BioSciences, USA).

Results

Serum isolated anti-DFS70 IgG significantly attenuated autoantibody-mediated NETosis and platelet aggregation in vitro ($p<0.01$) in a manner comparable to pharmacological inhibitors (dexamethasone and JAK2-inhibitor). Serum isolated DFS70 antibodies demonstrated this effect in a dose-dependent manner. Anti-DFS70 IgG inhibits NETosis downstream in the NET formation cascade, as it also attenuates NETosis induced by non-FcR triggers such as PMA and calcium ionophore. Culture supernatants in conditions with DFS70 autoantibodies demonstrate a decrease in beta-thrombomodulin, indicating reduced platelet activation (USBiological, Massachusetts, USA.) Anti-DFS70 IgG does not reduce platelet activation independent of NETs. Immunostaining of neutrophils with a commercial anti-DFS70 antibody (Abcam, Cambridge, UK) demonstrates nuclear localization of the DFS70 antigen in resting neutrophils and cytoplasmic re-location post-stimulation of NETs. Anti-DFS70 IgG and immunoglobulin-stimulated neutrophils show diffused nuclei instead of the characteristic multi-lobes. Although NETs are not formed, these neutrophils stain with 7AAD and Annexin-V indicating cell death.

Conclusions

Our data suggests endogenous DFS70 autoantibodies are protective by limiting NETosis and platelet aggregation. This antibody-mediated inhibition of NETosis may have implications in infectious disease, sepsis and other conditions where uncontrolled inflammation mediates pathology. However, further investigation is required to fully elucidate the mechanisms involved and the potential clinical relevance of these findings.

Antibodies directed to the angiotensin II type 1 receptor induce endothelial dysfunction in systemic sclerosis

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Objectives

Systemic sclerosis (SSc) is a chronic autoimmune disorder marked by the presence of functional autoantibodies targeting G protein-coupled receptors (GPCRs), including the angiotensin II type 1 receptor (AT1R). These autoantibodies are implicated in endothelial activation and dysfunction, facilitating the infiltration of inflammatory immune cells into tissues and contributing to vasculopathy and fibrosis. This study aims to investigate the impact of AT1R-targeting antibodies on the integrity of the endothelial glycocalyx (eGC), a key regulator of vascular homeostasis whose degradation is associated with endothelial dysfunction and increased monocyte adhesion.

Methods

To evaluate the effects of the AT1R natural ligand angiotensin II (Ang II) and AT1R-specific antibodies on eGC structure, human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured to confluence. Cells were treated for 24 hours with either Ang II or a recombinant monoclonal antibody (mAb) targeting AT1R at a concentration of 50 µg/mL. The eGC height was measured using atomic force microscopy (AFM) with the nanoindentation technique using a Nanowizard V AFM. To assess whether eGC alterations influence monocyte adhesion, U937 monocyte-like cells were fluorescently labeled with Calcein AM and incubated with endothelial cells pretreated with Ang II or AT1R-mAb. Monocyte adhesion was quantified by fluorescence microscopy after 30 minutes.

Results

Treatment of HUVECs with either Ang II or AT1R-mAb significantly reduced eGC height, with median decreases of -17.18% ($p = 0.0014$) and -25.09% ($p < 0.0001$), respectively, compared to untreated controls. The application of losartan, an AT1R antagonist, abrogated these effects, confirming the specificity of the response through AT1R signaling. Despite these structural alterations in the mechanical properties of eGC, static adhesion assays revealed no significant increase in U937 monocyte adhesion to endothelial cells treated with AT1R-mAb.

Conclusion

These findings suggest that, in addition to Ang II, AT1R-specific antibodies contribute to endothelial dysfunction in SSc by promoting degradation of the eGC. However, the absence of enhanced monocyte adhesion under static conditions indicates that AT1R-Ab-mediated eGC disruption alone may be insufficient to drive immune cell recruitment. This highlights the necessity for additional inflammatory cues or biomechanical stimuli in vivo to facilitate leukocyte-endothelial interactions.

9 Challenges and Advances in Autoantibody Detection

9.1 Advance in Autoantibody Discovery and Detection Technologies

Mapping the autoantibody response: towards a systemic understanding via autoantigenomics

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Abstract

Autoantigenomics is an emerging, holistic approach that systematically profiles the full repertoire of autoantibody-targeted proteins—known as the autoantigenome—in autoimmune diseases. Unlike traditional methods focusing on individual autoantigens or small panels, autoantigenomics captures the complexity and diversity of humoral immune responses, providing a more systemic view of autoimmunity.

This presentation highlights recent advances applying autoantigenomics to neurological autoimmune disorders, with a focus on chronic inflammatory demyelinating polyneuropathy (CIDP) and sensory neuronopathies (SNN). Utilizing high-density protein microarrays with over 16,000 full-length human proteins, comprehensive autoantibody repertoires are mapped and analyzed using advanced bioinformatics tools including cluster analysis, pathway enrichment, and overrepresentation statistics.

Key findings demonstrate that CIDP patients responding to intravenous immunoglobulin therapy have broader and distinct autoantigen profiles compared to non-responders, with anchoring junction proteins prominently targeted. In SNN, autoantibody repertoires reveal enrichment of target proteins in immune system pathways and P-bodies, suggesting novel biomarkers and potential mechanisms underlying disease heterogeneity.

By expanding the scope beyond single targets, autoantigenomics shows options for improved patient stratification, biomarker discovery, and mechanistic insights into autoimmune neuropathies. This systemic perspective has the potential to refine diagnosis, guide personalized therapy, and ultimately enhance understanding of autoimmune diseases.

The wider spectrum of autoimmune analyses using orphan autoantibodies

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Abstract

Over 200 autoantibodies in SLE have been described in the literature to date. Despite this abundance, only a limited subset of these autoantibodies has made its way into routine clinical use. This is not unique to SLE, as a similar trend is observed across other systemic autoimmune rheumatic diseases (SARD). In fact, the vast majority of autoantibodies remain in the background, often referred to as “orphan autoantibodies.” These are reactivities that, while detectable, lack a clearly defined clinical association. As such, their role in the broader landscape of autoimmunity remains ambiguous and underexplored.

This presentation will explore the path that autoantibodies must travel to reach clinical relevance, from discovery and mechanistic understanding to validation and diagnostic integration. We will highlight the emerging value of orphan antibodies in potentially closing the “seronegative gap” and serving as predictive indicators of disease onset or prognostic markers of severity, organ involvement, or therapeutic response. Moreover, there is a growing shift toward the use of multianalyte arrays and analytical algorithms integrated with artificial intelligence and advanced omics technologies, aiming to leverage the collective power of multiple biomarkers rather than relying on any single marker. This approach enables a more comprehensive understanding of autoimmunity.

Advancing early diagnosis and prediction in autoimmunity: innovative strategies and the role of biomarkers

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Abstract

Systemic autoimmune diseases including, but not limited to systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc) and primary biliary cholangitis (PBC) often present with complex, overlapping symptoms that delay diagnosis and treatment. Notably, autoantibodies such as anti-dsDNA (in SLE), anti-CCP (in RA), and anti-mitochondrial antibodies (in PBC) can precede clinical onset by months or years, offering a valuable window for intervention.

On the other hand, the growing shortage of rheumatologists poses a significant challenge for patients with autoimmune diseases, often leading to delayed diagnosis and treatment. As demand outpaces specialist availability in many countries, triage becomes critical to prioritize care for those at highest risk of disease progression. Efficient triage systems - leveraging biomarker screening, symptom severity, and comorbidity profiles - can help identify patients who require urgent evaluation versus those suitable for monitoring or primary care management. Without such strategies, patients may experience worsening symptoms, irreversible joint damage, or systemic complications. Addressing this gap through innovative diagnostics and streamlined referral pathways is essential to improving outcomes in autoimmune care.

Consequently, comprehensive biomarker testing to avoid misdiagnosis and ensure appropriate therapeutic pathways is paramount for managing autoimmune diseases. By combining immunological insights with innovative sampling and analytical technologies, the aim is to redefine the paradigm of autoimmune disease prediction and prevention.

Lastly, the landscape of autoimmune diagnostics is rapidly evolving with the introduction of innovative in vitro diagnostic (IVD) platforms such as Aptiva®, a particle-based multi-analyte technology (PMAT) system designed to enhance sensitivity, throughput, and operational efficiency. Aptiva enables simultaneous detection of multiple autoantibodies from a single patient

sample, delivering up to 720 results per hour using a 12-analyte test cartridge - allowing laboratories to complete workflows within a single shift.

This talk explores cutting-edge strategies for early detection and risk prediction, emphasizing the critical role of autoantibodies and emerging biomarkers. We will discuss the utility of diverse sample types - including saliva and dried blood spots as part of the diagnostic workflow.

Use of AI-based algorithms for pattern interpretation of tissue-based indirect immunofluorescence tests

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Background

Indirect immunofluorescence (IIF) on single and multi-organ tissue substrates—e.g. primate esophagus and rodent liver-kidney-stomach (LKS) triple sections—is a powerful diagnostic approach for detecting a comprehensive spectrum of autoantibodies in autoimmune diseases like autoimmune liver diseases, vasculitides, and rheumatological disorders. However, the manual interpretation of diverse and complex staining patterns across these different tissues is challenging.

Methods

This study presents a fully automated deep learning-based pipeline for the comprehensive analysis of IIF tests using the automated interpretation system akiron® NEO (Medipan, Germany). We developed convolutional neural network (CNN) system trained on a large, substrate datasets (~5,000 images) of esophagus and LKS sections, annotated by expert consensus.

The pipeline was designed for three consecutive tasks:

1) Localization of staining to specific anatomical structures across tissues

2) Classification of staining patterns

3) Semantic segmentation and regression for precise semi-quantitative intensity scoring (0 to 4+)

4) Algorithmic prediction of the antibody titer based on the intensity decay across a serial dilution series.

Results

The AI system demonstrated high proficiency across all evaluation steps. It achieved high pattern classification accuracy and a strong agreement with manual intensity scoring. The system provided results in a fraction of the time required for full manual analysis, showcasing a high degree of reproducibility and objectivity.

Conclusion

Our results demonstrate that single AI-based algorithms can fully automate the entire interpretive process of tissue-based IIF, from pattern recognition to final titer reporting. This end-to-end solution has the potential to standardize serological diagnostics, eliminate subjective variability, and dramatically increase laboratory efficiency. The integration of intensity and titer analysis represents a significant step towards fully automated, high-throughput IIF testing, ensuring result consistency.

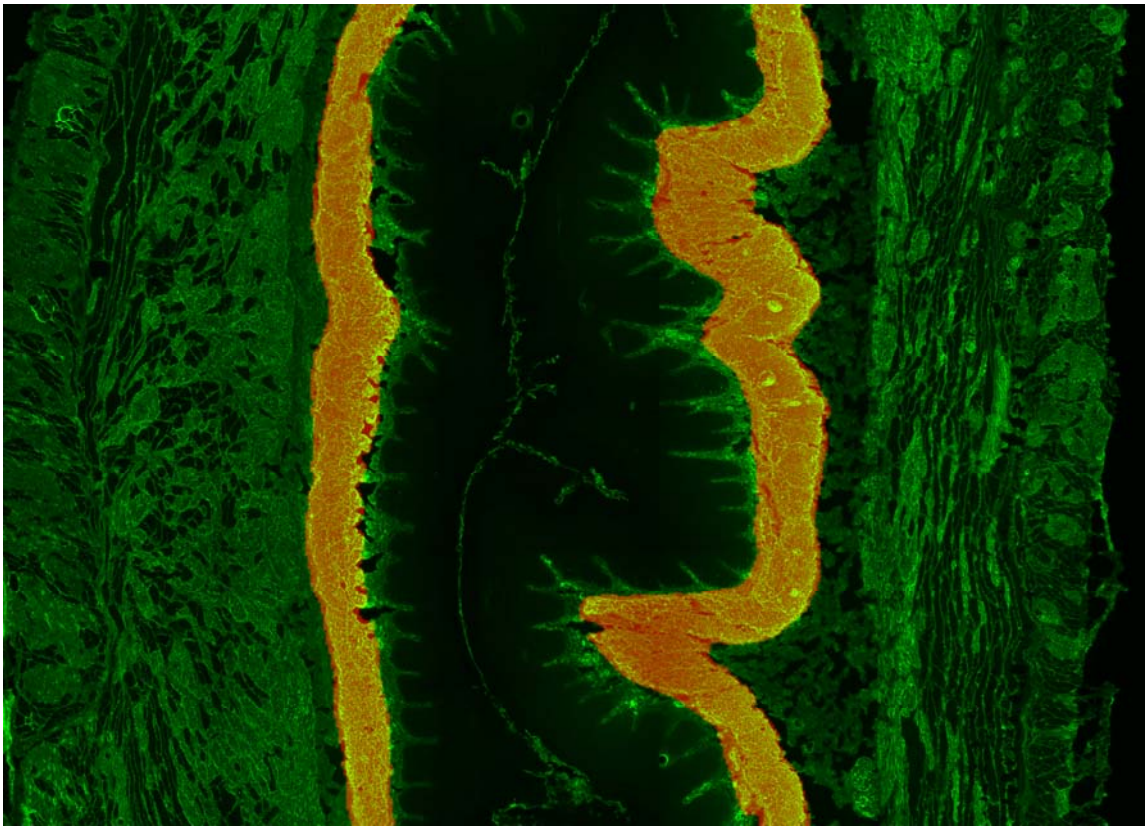

Figure 1. Visualization of detected muscolaris mucosae (endomysium) by convolutional neural network in fluorescence stained cryostat sections of monkey oesophagus

Glycan signature characterization using innovative particle-based multi-analyte technology platform

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Background

Glycan structures on antibodies play a critical role in immune function and disease progression, especially in autoimmune conditions. Recent evidence implicates antibody glycosylation, particularly Fab-linked N-glycans of anti-cyclic citrullinated peptide antibodies (ACPA), as a contributor to rheumatoid arthritis (RA) pathogenesis. These glycans, frequently galactosylated, bisected, and disialylated, are present in over 90% of ACPA and can influence antigen affinity and inflammatory responses.

A range of analytical platforms is available for characterizing glycans on intact proteins, including lectin microarrays, reversed-phase or ion-exchange HPLC, and mass spectrometry (MALDI or ESI). However, many of these methods suffer from key limitations such as low throughput, complex and expensive sample preparation, time-consuming and labor-intensive workflows. Lectin microarrays offer a more streamlined alternative, enabling high-throughput profiling with minimal sample preparation. Nevertheless, conventional lectin microarrays can still face challenges in reproducibility, sensitivity, and quantification.

In this context, the Particle-Based Multi-Analyte Technology (PMAT) platform represents a promising solution. By leveraging particle-based multiplexing, PMAT enables simultaneous, high-sensitivity analysis of multiple analytes using small sample volumes and streamlined protocols.

Objective

The objective of this study was to use PMAT to detect glycans structures on antibodies associated with immune function and disease progression.

Methods

To assess total IgG glycosylation, paramagnetic particles were coated with 14 different glycan-binding proteins (GBP). These beads were incubated with serum samples from healthy controls and autoimmune disease patients. Glycan profiles were detected using an anti-hIgG fluorescent reporter to detect glycosylated IgG from serum samples.

To evaluate biomarker-specific glycosylation, separate sets of particles were coated with RA-related biomarkers; Cyclic Citrullinated Peptides (CCP3), Protein Arginine Deaminases (PADs) and Rheumatoid Factor (RF). These were also tested with serum samples. A dual-reporter system using fluorescent anti-human IgG and a fluorescent sialic acid-binding protein was applied to detect both biomarker presence and sialylation.

Results

The automated PMAT platform successfully detected total IgG glycosylation patterns across disease and control samples using GBP-coated beads. Consistent glycan-binding signatures were observed, with several GBP showing distinct signal profiles in autoimmune patients compared to controls, suggesting differential glycosylation profiles.

For biomarker-specific analysis, the dual-reporter strategy enabled the detection of RA biomarkers and their glycosylation features. Sialylation levels varied across patients, biomarkers and patient groups, with higher sialic acid content observed in certain RA-related targets.

Overall, PMAT demonstrated high sensitivity, scalability, automation compatibility, and robust reproducibility, making it particularly well-suited for high-throughput glycomics in clinical research. This is especially relevant in autoimmune diseases such as RA, where detailed glycan profiling of antibodies can yield critical mechanistic and diagnostic insights.

Conclusions

This study introduces a novel and automated approach for glycan characterization that offers both flexibility and scalability. The PMAT technology enables simultaneous assessment of total and biomarker-specific glycosylation.

While the platform shows potential for applications in autoimmune diagnostics, patient stratification, and therapeutic monitoring, further validation with established methodologies such as HPLC is essential. Additional studies will also be necessary to investigate clinical correlations and optimize biomarker selection for disease-specific glycan profiling.

Development of a multi-disease antigen panel for autoimmune and inflammatory conditions

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Introduction

Autoantibodies serve as important biomarkers for diagnosing autoimmune diseases. However, current clinical assays for their detection often face challenges, such as uncertainty about the specific autoantigen or a narrow focus on well-known autoantigens, which typically results in low sensitivity and specificity. To address these limitations, we developed a multiplex antigen bead-array that enables the parallel detection of both known and novel autoantibodies. Ultimately, we aim to improve the classification and serological characterization of autoimmune diseases.

Methods

Different representations of known autoantigens (full-length proteins and protein fragments) have been used to generate bead-arrays and tested on samples from patients with autoimmune conditions previously classified as seropositive and seronegative for known autoantibodies. Moreover, we performed proteome-wide autoantibody screenings on plasma samples from 1000 vasculitis patients, 180 patients with systemic sclerosis, as well as healthy and inflammatory controls, in order to identify novel candidate autoantigens specific for diseases and subgroups. These broad screenings utilized custom antigen-arrays featuring 42,000 protein fragments (representing 17,000 unique proteins) and 2,000 full-length secreted proteins from the Human Protein Atlas. The Samples were sourced from well-characterised cohorts through collaborations with expert clinicians.

Result

We generated a bead-array for accurate and parallel detection of autoantibodies targeting the well-known autoantigens Scl70/TOPO-1, Ro52/SSA/TRIM21, CENPs, MPO and PR3. Moreover, our screening allowed the identification of novel autoantibodies with relevance in patients with specific diseases or clinical features.

Conclusions

Our multiplex antigen-array allows detection of well-known clinically relevant autoantibodies. Combining clinical expertise with our high-throughput approach enabled the discovery of novel candidate biomarkers, which could improve patient diagnosis and subclassification. These findings underscore the potential of multiplex autoantibody assays for enhancing diagnostic precision across a range of autoimmune conditions.

From Promise to Practice: Assessing Middleware in Autoimmune Labs through an Italian Survey

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Abstract

Autoimmune diagnostics is no longer just a niche discipline—it's a dynamic frontier undergoing profound transformation. In recent years, technological progress has reshaped immunodiagnostics, merging automation, consolidation, and connectivity into a smarter, faster, and more precise laboratory ecosystem. At the heart of this evolution lies a once-overlooked component: middleware. Formerly a simple digital bridge, middleware has emerged as a strategic decision-making platform, revolutionizing how autoimmunity laboratories handle data, trace samples, validate results, and generate clinical reports.

Yet, despite its widespread use in sectors like clinical chemistry and hematology, middleware's penetration into autoimmunology has only recently gained traction. To evaluate its true impact, the Study Group on Autoimmunology of the Italian Society of Clinical Pathology and Laboratory Medicine (GdS-AI SIPMeL) launched a nationwide survey targeting expert laboratories. The questionnaire explored seven core themes: laboratory characteristics, middleware management and traceability, operational flexibility, contribution to results reporting, data archiving and statistical capabilities, technical support, and user satisfaction.

Key findings were revealing: over 90% of participating labs use middleware, often with significant benefits to diagnostic quality and operational efficiency. However, the survey also uncovered disparities in regional access, uneven feature utilization, and knowledge gaps among professionals. Middleware systems were found to excel in reporting functionalities, such as tracking real-time sample status and enabling historical data comparison, while lagging in areas like reagent management and reflex testing protocols. Perhaps most striking was the discovery that 1 in 5 professionals lacked sufficient understanding of their middleware tools—raising urgent questions about training, accessibility, and technological literacy in the field.

Middleware has the potential to reshape autoimmunity diagnostics, but only if laboratories embrace not just its presence—but its full capabilities and if manufacturers commit to enhancing

middleware functionalities in autoimmunity with the same intensity and depth as has already been achieved in hematology and clinical chemistry—fields where the volume of test requests is undeniably higher.

The path forward demands bold solutions: cross-vendor interoperability, standardized interfaces, smarter automation rules, and targeted educational initiatives. Autoimmune diagnostics stands at a critical junction, and middleware—when empowered and optimized—could be its compass toward a more agile, data-driven, and patient-responsive future.

In-depth analysis of the autoantibody associations of the main nuclear patterns in HEp-2 IFA: *What a general laboratory cohort has to tell us about real life pattern-autoantibody association*

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Introduction

Autoantibodies serve as valuable biomarkers for diagnosing and monitoring systemic autoimmune diseases (SAID)[1], and they can appear in the bloodstream either prior to or following the onset of clinical symptoms[2]. In clinical laboratories, a variety of methods are employed to detect these autoantibodies, however, the indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2 IFA) remains the most widely used first-line screening technique worldwide[3,4]. This method can identify more than 30 distinct morphological patterns, each indicative of various autoantibodies pertinent to diagnosing a broad range of SAID[5], and is currently recognized as the gold standard for autoantibody screeining[6].

While the pattern indicates as to which autoantibody might be present the titer reflects their concentration, and with increased titer there is an increased likelihood of SAID[7]. In recognition of the need for harmonization on HEp-2 IFA pattern interpretation and reporting, ICAP (www.anapatterns.org) has classified 32 patterns and caracterized them using an alpha-numerical code (AC) with autoantigen associations and clinical relevance attached to each pattern[3].

Some autoantibodies exhibit strong associations to SAID, to the extent that they have been included in diagnostic criteria[8–11]. Some patterns have no known association, but are distict enough to have been included in the ICAP classification. It is important to note that the observed patterns should not be considered definite indicators of specific autoantibodies. Discrepancies between the expected and actual autoantibody profiles are not uncommon in routine laboratory practice, which is why confirmation with autoantigen-specific immunoassays (such as immunoblot, immunoprecipitation, ELISA, CLIA, or immunodiffusion) is essential[3,12], especially in the context of low pre test probability[13].

A critical review of the HEp-2 IFA pattern highlights several key points: (1) it remains an integral component of the laboratory algorithm for autoantibody testing; (2) there is significant variability in the strength of association between each IFA pattern and its corresponding autoantibodies; and (3) routine practice occasionally reveals deviations from the expected correlations. There is evidence of misinterpretations regarding the immunological and clinical significance of HEp-2 IFA patterns, which may lead to diagnostic and therapeutic delays. Although most studies have been limited by small sample sizes and potential referral bias from specialized autoimmunity centers, there has been no formal investigation into the consistency of pattern/autoantibody associations. In this abstract, we present our findings on the relationships between the principal nuclear HEp-2 IFA patterns observed in a general laboratory setting and the most commonly tested 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, CENP-B, 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. The autoantibody routine is subject to an internal quality control process and two external quality assessment programs, the College of American Pathologists (CAP) and the Brazilian Program of Excellence in Laboratory Medicine (PELM). These characteristics contribute to the consistency of the data over the years. The HEp-2 IFA results were registered according to ICAP recommendations including the AC-30 and 31 patterns, recently classified by ICAP.

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 was 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 nuclear patterns of interest (AC-1, AC-2, AC-30, AC-4 and AC-31).
- Tier 2 analysis (Autoantibody ➔ pattern analysis): the reference parameter was 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 included double immunodiffusion, indirect immunofluorescence on different substrates, ELISA, immunoblot, and CLIA, all of which have been 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 was 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.

Results

Between January 2012 and December 2019, there were 404,134 records of HEp-2 IFA reports and 141,294 had a positive result with a simple immunofluorescence pattern. Altogether, the nuclear patterns predominated in frequency and among them, AC-4 was the most frequent (46,865, 33.2%), followed by AC-30 (27,523, 19.5%), AC-2 (22,636, 16.0%), AC-1 (8,925, 6.3%), AC-5 (6,939, 4.9%), and AC-31 (6,267, 4.4%). As shown in Figure 1, these patterns predominated over all other HEp-2 IFA patterns.

Figure 1. Relative frequency of all HEp-2 IFA patterns in 141,294 records with single-pattern reports.

In the first-tier pattern-driven analysis, several traditional associations were confirmed, although at variable degrees. In addition, some interesting associations emerged. Notably, when selecting AC-1 (the homogeneous pattern), anti-nucleosome was positive in 76.3% of the 333 cases in which this autoantibody was requested, whereas anti-dsDNA was positive in 35.7% of the 4,767 cases with request for this autoantibody. Interestingly, some unexpected autoantibodies were detected in cases with pure AC-1 pattern: anti-DFS70 was present in 14.3%, anti-CENP-B in 5.9%, and anti-RNP in 4.8% of pure 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 98.3% of 233 AC-2 samples in which this autoantibody was requested. Interestingly, other autoantibodies, such as anti-histone and anti-RNA pol III, were present in occasional AC-2 samples.

AC-30 was not predominantly associated with any single autoantibody, and the most frequently observed autoantibodies were anti-DFS70 (34.0% of 50 samples), anti-histone (24.7% of 190 samples), and anti-nucleosome (22.9% of 481 samples). Antibodies against dsDNA and SS-A/Ro lagged behind with less than 4% in over 10,000 samples, respectively.

The AC-1, AC-2, and AC-30 patterns are morphologically related in that these exhibit a diffuse nuclear staining with prominent fluorescence of the metaphase plate. Thus, we directly compared the associations of these three patterns with the four main autoantibodies expected to produce diffuse fluorescence in the interphase nucleus and metaphase plate (anti-dsDNA, anti-nucleosome, anti-histone, and anti-DFS70) (Figure 2). This analysis showed that the AC-1 and AC-2 patterns have opposite associations, with the former strongly associated with antibodies to dsDNA, nucleosome, and histones, and low frequency of anti-DFS70 antibodies, whereas AC-2 was strongly associated with anti-DFS70 and rarely with the other three autoantibodies. Importantly, the recently classified AC-30 pattern showed an intermediate behavior between AC-1 and AC-2 and, therefore, this pattern conveys less assertive information in terms of autoantibody association, compared to AC-1 and AC-2.

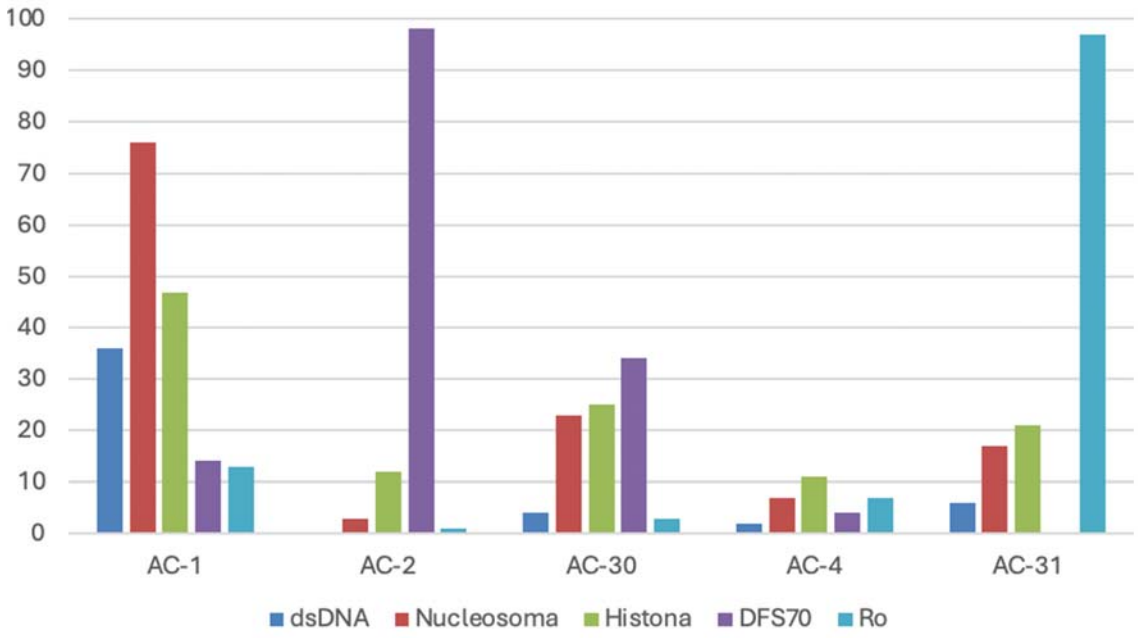

Figure 2. Comparison between the most frequent autoantibodies present in AC-1, AC-2 and AC-30 single pattern samples

In an analogous approach, we compared the autoantibody associations of the three nuclear speckled patterns with no staining of the metaphase plate, namely AC-4, AC-5, and AC-31. Accordingly, we explored the associations with autoantibodies to SS-A/Ro, SS-B/La, and Mi-2. The AC-4 pattern was very frequent but had a low rate of association with known autoantibodies, including those against SS-A/Ro (7.4% of 16,939 requests) and SS-B/La (0.18% of 15,702 requests). There were only 15 requests for Mi-2 in AC-4 samples and all were negative. Occasional AC-4 samples had autoantibodies to RNA pol III, nucleosome, and histone. These results suggest that a variety of uncharacterized autoantibodies are prevalent among the samples yielding the AC-4 pattern.

In contrast, the AC-31 pattern had a very high association with anti-SS-A/Ro autoantibodies (97.1% out of 3,581 requests). Other autoantibodies were observed in lower frequencies, such as anti-histone (20.6% of 34 samples), anti-nucleosome (16.8% of 101 samples), anti-dsDNA (5.5% of 3,072 samples), and anti-SS-B/La (4.2%, of 3,314 samples. The only AC-31 sample tested for anti-Mi-2 antibodies was negative.

Lastly the AC-5 pattern samples showed a high frequency of antibodies against U1-RNP (68.7% of 2,081 samples) and a lower association with anti-Sm antibodies (12.3% of 2,399 samples). Interestingly, AC-5 single-pattern samples had a considerable frequency of antibodies against histones (33.3% of 27 requests), nucleosome (22.9% of 105), and SS-A/Ro (12.5% out of 3,543).

The second-tier autoantibody-driven analysis, presented a myriad of intriguing findings, as outlined below.

Antibodies against dsDNA were found in 4,462 samples (2.6% of all anti-dsDNA requests), almost half of them (45.7%) presented an AC-1 pattern, 13.0% presented the AC-30 pattern, 11.1% presented the AC-5 pattern while 2.1% of anti-dsDNA-positive samples were negative for HEp-2 IFA (AC-0) and eight samples (0.2%) presented the AC-2 pattern. In the case of anti-nucleosome autoantibodies there were 699 positive samples (13.6% of all anti-nucleosome requests) and 41.5% presented the AC-1 pattern while 18.3% presented the AC-30 pattern, 11.8% were negative (AC-0), and 2.0% presented the AC-2 pattern. Of interest the intensity of anti-nucleosome reactivity was higher in the AC-1 samples (76.0 ± 164.0) than in samples presenting the AC-0 (25.0 ± 98.0) ($p<0.001$ by Mann-Whitney U test). The nuclear speckled patterns were also observed in the 699 anti-nucleosome-positive samples: AC-4 in 15.0%, AC-31 in 3.7%, and AC-5 in 4.6%. Lastly for anti-histone autoantibodies there were 299 positive samples (14.5% of 299 requests), out of which no single pattern had a frequency above 20%. Frequencies were as follows: AC-1 17.1%, AC-2 5.0%, AC-30 15.7%, AC-4 13.4%, AC-31 2.3%, and AC-5 3.0%. Interestingly, 121 anti-histone-positive samples (40.5%) had a negative HEp-2 IFA result (AC-0) which was the most frequent HEp-2 IFA result associated with anti-histone-positive samples. Notably, the frequency of the AC-1 pattern increased in samples with two or more autoantibodies against chromatin antigens: 62.9% AC-1 frequency in samples with antibodies to dsDNA and nucleosome, and 53.6% in samples with antibodies to dsDNA and histones.

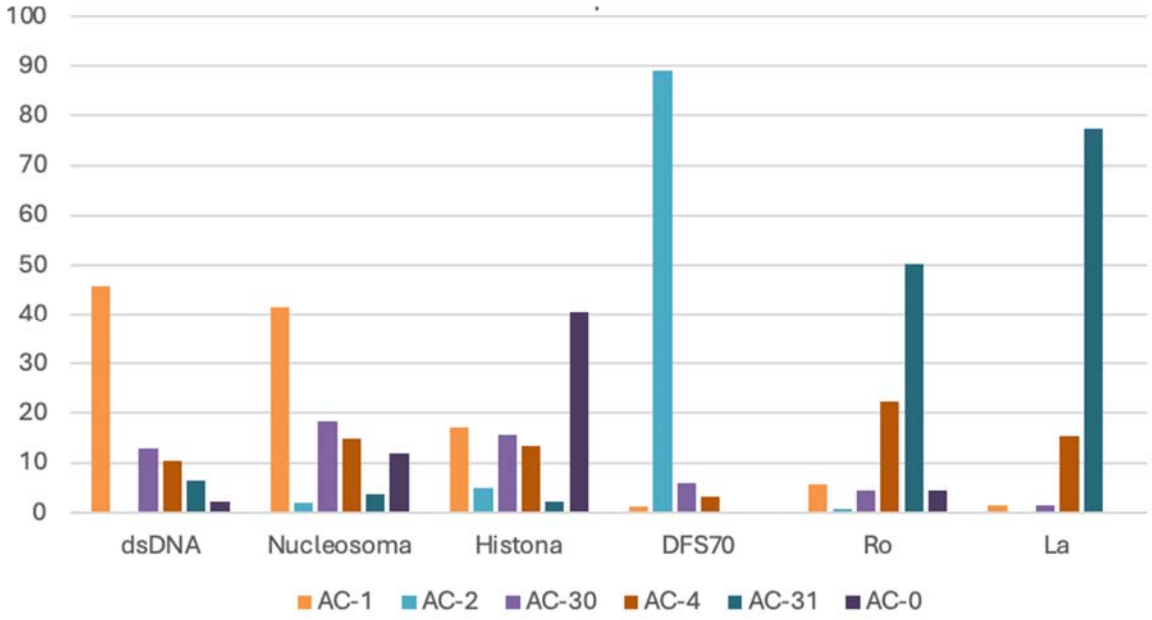

Figure 3. Frequency of ICAP patterns according to specific autoantibodies in the samples

The majority of anti-DFS70-positive samples (284 out of 467 requests, 60.8%) presented the AC-2 pattern (253 samples, 89.1%), with occasional samples presenting other patterns: AC-30 (6.0%), AC-4 (3.2%), AC-1 (1.1%), and AC-5 (0.4%).

Fifty percent of the 7.683 anti-SS-A/Ro-positive samples presented the newly described AC-31 pattern, while 22.3% presented the AC-4 pattern. The AC-1, AC-2, AC-30, AC-5, and AC-0 patterns had a frequency between 5.6% and 0.7%. In the case of anti-SS-B/La antibodies, 203 of them were positive (0.15%). Interestingly only two of these were negative for anti-SS-A/Ro (99.0% were positive for both autoantibodies). Accordingly, 77.3% of anti-SS-B/La positive samples presented the AC-31 pattern, while 15.3% of them presented the AC-4 pattern. Other patterns were positive in less than 2% of cases. This contrasts with the observation from first tier pattern-driven analysis, in which a minority of AC-4 samples had reactivity to SS-A/Ro and SS-B/La highlighting the low specificity of the AC-4 pattern as an indicator of anti-SS-A/Ro autoantibody. In an unbiased scenario of a general clinical laboratory, the most frequent pattern, i.e., AC-4, is mostly not associated with any clinically relevant autoantibody.

The mutual influence of antibodies against dsDNA and SS-A/Ro was analyzed regarding the frequency of patterns AC-1, AC-30, AC-31, and AC-4 (Table 1). The AC-1 pattern was dominant in samples with anti-dsDNA antibodies irrespective of the presence (31.4%) or absence (40.6%) of anti-SS-A/Ro antibodies. In contrast, the strong association with the AC-31 pattern observed in anti-SS-A/Ro-positive samples (48.4%) was strongly affected by the coexistence of anti-dsDNA antibodies, yielding a decrease in frequency to 11.4%.

Table 1. Mutual influence of anti-dsDNA and anti-SS-A/Ro antibodies on the pattern observed in HEp-2 IFA

Pattern	Anti-dsDNA positive Anti-SS-A/Ro positive	Anti-dsDNA positive Anti-SS-A/Ro negative	Anti-dsDNA negative Anti-SS-A/Ro positive
AC-1	31.4 %	40.6 %	3.0 %
AC-30	5.8 %	10.6 %	4.2 %
AC-31	11.4 %	0.1 %	48.4 %
AC-4	7.8 %	8.3 %	1.7 %

Very few samples were positive for anti-Sm antibodies (508 positive samples in 79.474 requests (0.6%)) and AC-5 was the prevailing pattern (74.6%). The rest of the patterns had a very low frequency (lower than 8%) with only one sample (0.2%) presenting a negative HEp-2 IFA pattern. Among 59.596 requests for anti-U1-RNP-positive, 2.200 were positive and 65.0% presented the AC-5 pattern, while other patterns were present in less than 10% of samples. Interestingly for both anti-Sm and anti-U1-RNP, there were very few samples that had a negative HEp-2 IFA (0.2% and 0.5% respectively) compared to the 2.1%, 11.8%, and 40.5% in the case of anti-dsDNA, anti-nucleosome, and anti-histone, respectively. There were 116 samples with antibodies against RNA pol III and none presented the AC-5 pattern, half (50%) had a negative HEp-2 IFA (AC-0), and 41.7% presented the AC-4 pattern.

The pattern-driven and autoantibody-driven analyses provided valuable insights into the intriguing interplay between autoantibodies in the assembly of the corresponding HEp-2 IFA patterns. The several unexpected autoantibody/pattern associations observed in this large cohort highlight the complexity and diversity of the autoimmune responses and how the coexistence of multiple autoantibodies in a given sample may affect the canonical associations of autoantibody specificities and HEp-2 IFA patterns.

Conclusions

The HEp-2 IFA method is a useful tool in the first step laboratory investigation of systemic autoimmune diseases. In particular, the immunofluorescence patterns observed in the HEp-2 IFA test yield valuable information on the possible autoantibody specificities in the sample. ICAP has catalogued the most frequent and relevant patterns into a classification tree and indicated the possible autoantibody specificities associated with each pattern. This study investigated the power of these associations in real-life by exploring the data bank of a large general clinical laboratory. The results showed that while the various pattern/autoantibody associations prevail, they are not absolute. In particular, the coexistence of two or more autoantibody specificities in the sample may cause diversion from the expected pattern association. Two main conclusions can be derived from this study: 1) the canonical pattern/autoantibody associations were confirmed in a large real-life cohort of samples from a general clinical laboratory; 2) the pattern/autoantibody associations are useful as a general guide; however, they are not absolute, especially when more than one antinuclear antibody coexist in the sample under analysis; 3) the relative strength of HEp-2 IFA patterns and autoantibody specificities corroborates the recommendation that further antigen-specific immunoassays are needed to supplement and harmonize the autoimmunity laboratory investigation.

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GAD autoantibody detection comparison between multiple immune-based methods with the focus on avoiding hook-effect occurrence

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Background

Chemiluminescence (CLIA) and immunoblotting (IB) are standard methods for detecting GAD autoantibodies (GAD65-Abs) in type 1 diabetes diagnosis. However, their results frequently disagree. Low CLIA values often fail IB confirmation due to lower IB sensitivity, while IB-positive/CLIA-negative samples typically result from the "hook effect" in CLIA. Notably, elevated GAD65-Abs also associate with neuropsychiatric disorders, where neuronal autoantibody screening commonly employs cell-based (CBA) or tissue-based assays (TBA). The CLIA hook effect for GAD65-Abs remains understudied, and the potential of CBA/TBA to mitigate this effect warrants evaluation.

Objectives

To study the incidence of hook-effect of GAD-Abs detection by chemiluminescence with the comparison of immunoblotting, and to evaluate the performance of CBA and TBA in detection of samples with hook-effect by chemiluminescence method, with the overall aim to provide warning and reference for daily detection results report and clinical interpretation.

Methods

36 out of 7082 serum samples screened for GAD-Abs and yield positive results by immunoblotting from August 15 to September 25, 2023 in KingMed Diagnostic Laboratory, Nanjing were collected. These samples were further measured by chemiluminescence with undiluted, 1:100 diluted and 1:10,000 diluted solutions to determine the hook-effect occurrence and explore the possible samples dilution strategies for avoiding hook-effect. Meanwhile, 5 samples with typical positive

results by immunoblotting and 5 samples with high levels of GAD-Abs by chemiluminescence but negative by immunoblotting were further tested by CBA according to the introductions from the manufacture and a in-house established TBA to roughly evaluate the accuracy of these 2 methods as well as their capability to avoiding hook-effect in GAD-Abs detection.

Results

GAD-Abs levels of 35 samples become obviously higher when diluted to 100 times by chemiluminescence compared to the GAD-Abs levels teste with undiluted samples, so the hook-like effect was confirmed, counting for 97.22% of the positive samples by immunoblotting and 0.49% of the total samples tested during this period. 9 out of the 36 undiluted samples were negative (lower than 10 IU/ml, under the cut-off value) by chemiluminescence, showing false negative results occurred because of hook-effect, which counts for 0.13% of the total samples tested during this period. Notably, all 10 extra samples tested were positive by CBA, but only half of the samples (the 5 samples positive by immunoblotting) were positive by TBA, indicating that these methods could avoid hook-effect compared to chemiluminescence while CBA was more sensitive than TBA and immunoblotting.

Conclusions

The hook-effect of chemiluminescence method leads to inconsistency between the detection results of some samples in GAD-Abs detection. This effect could be effectively reduced by gradient dilution of samples and then detection, or employ immunoblotting, CBA or TBA methods in parallel so as to provide reliable detection results for clinical practice.

Interference of cold agglutinins and/or cryoglobulins in antibody diagnostics for kidney transplantation: a case study

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Abstract

A 59-years old female with anti-glomerular basement membrane induced renal failure was admitted to the kidney transplant waitlist. Despite few immunizing events, no class I and II anti-human leukocyte antigen (HLA) immunoglobulins (IgG and IgM) were detected in EDTA-treated, prozone effect-diminishing, sera from this patient (LABScreen Mixed and Single Antigens). Surprisingly, EDTA-untreated sera induced strong lytic reactions in the complement-dependent-cytotoxicity-screening assay (CDC-screening) using a variable panel of HLA-typed healthy donor peripheral blood mononuclear cells. The percentage of panel reactive antibodies were 100% without Dithiothreitol (DTT; IgM and IgG) and 95% with DTT (IgG only). To investigate the potential interference of autoantibodies, we performed autologous CDC-crossmatch assays (CDC-XMs), which were positive without DTT, but negative with DTT. In allogenic IgG-detecting flow cytometry-based crossmatch assays (FC-XMs) with different peripheral blood mononuclear cells and patient sera, positive scores (>3) were obtained for B cells. Allogenic IgM-detecting FC-XMs were positive for B and T cells. In the patients' medical records, a history of cold agglutinins (CAs) and monoclonal IgM-kappa cryoglobulins (<0.04 g/L; type I) were observed. CAs are mostly IgM molecules that bind to antigens present on red blood cells (RBCs) under hypothermic conditions and cause hemolytic anemia. Cryoglobulins are antibodies that precipitate under hypothermia and can, amongst other things, cause aggregates that plug the small blood vessels or cause vasculitis. Therefore, we speculated that these cold-sensitive antibodies may contribute to our laboratory findings, for which allogenic CDC-XMs were repeated at 20 °C (standard) and 37 °C. In favor of this hypothesis, CDC-XM results remained positive at 20 °C and turned negative at 37 °C

(DTT-independent). When performing these tests using cryoglobulin-only sera from other patients, no consistent interference was observed. We conclude that transplant immunologists should be alert when dealing with a patient that has CAs, as these autoantibodies may interfere with the interpretation of CDC- and FC-XMs. This interference can possibly be overcome by performing these assays at 37 °C.

9.2. New Diagnostic and/or Pathogenic Relevant Biomarkers

Pathogenic autoantibodies in Podocytopathies

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Abstract

Major advances have occurred in the diagnosis and monitoring of immune glomerular diseases with the identification in 2009 of anti-PLA2R1 antibodies which have induced a paradigm shift in the care of patients with membranous nephropathy (MN). My task today will be to discuss recent advances in the mechanisms and diagnosis of other glomerular diseases referred to as ***podocytopathies*** including minimal change disease (MCD) and some forms of nephrotic focal and segmental glomerulosclerosis (FSGS) called podocytopathies. The recent re-discovery of anti-nephrin antibodies associated with those diseases leads to the question: are anti-nephrin the new anti-PLA2R?

Definitions are important. MCD is classically defined by the absence of alterations by light microscopy and immunofluorescence although sometime IgM is present, and by diffuse effacement of foot processes by electron microscopy. MCD represents 10-15% of patients with primary NS in adults but up to 90% in children in whom % decreases around puberty. In children, a biopsy is usually not performed as the diagnosis mainly relies on response to prednisone which defines steroid-sensitive nephrotic syndrome (SSNS).

Primary FSGS is also nephrotic and characterized by the presence of segmental sclerotic lesions associated with diffuse effacement of the foot processes. Its relation with MCD remains controversial, notably in adults.

For those diseases, biomarkers are desperately needed.

For 50 years, MCD was considered a disease of T-cell regulation as suggested by absence of immune deposits in glomeruli, occurrence of remission after measles infection which causes cell-mediated immunosuppression, response to treatments that suppress T cell-mediated immunity such as glucocorticoids and calcineurin inhibitors, association with Hodgkin and lymphoma, and lack of relapse of NS when a graft is removed from a relapsing patient and placed in a patient with another nephropathy.

The lead hypothesis was that the disease was partly due to the presence of soluble mediators released by the dysregulated T cells. But a major breakthrough was the observation of the efficacy of Rituximab, an anti-CD20 antibody, which suggested that this mediator could be an antibody

responsible for alteration of GBM integrity, so that albumin and other serum proteins filter out of into the urinary space.

Actually, it was known for 30 years that anti-nephrin antibodies were pathogenic after infusion in the rat. Fab'2 bivalent antibodies directed against an unknown antigen of the glomerulus (later identified as nephrin), not Fab fragments, induce massive and transient proteinuria with a linear staining of the glomerular basement membrane, while a scattered, fine granular staining is observed at 5 days most likely the consequence of internalization. In sharp contrast, staining was not affected in rats injected with the monovalent Fab. In patients' biopsies also, nephrin staining pattern was shown to be deeply altered in MCD and FSGS but not in IgA nephropathy which led to the suggestion more than 20 years ago, that nephrin redistribution is a potential mechanism for proteinuria also in patients. Another argument favoring the pathogenic role of anti-nephrin is the recurrence of Finnish-type NS after transplantation in patients with biallelic Fyn major mutations as reported more than 20 years ago. In those patients without nephrin, the immune system recognizes the nephrin brought by the graft as an allo-antigen which induces the production of likely pathogenic allo-antibodies that are detected in the circulation.

In 2022, auto-antibodies targeting nephrin were discovered in children with MCD **(1).** Anti-nephrin antibodies were assessed by ELISA: about a third (18/62) of patients had anti-nephrin antibodies correlated with disease activity as they disappeared at remission, but most patients had previously received immunosuppressive (IS) treatment which retrospectively suggests that the rate of anti-nephrin should be higher. By immunofluorescence (IF), dusty staining of nephrin co-localized with IgG was seen, very much alike the nephrin staining observed in the rat. There was a perfect correlation between IgG deposits in the biopsy and circulating anti-nephrin antibodies. However, these findings raised some controversies as per definition IF should be negative in MCD biopsies, but we could confirm these findings by staining our own biopsies for nephrin and IgG after adequate retrieval of the FFPE-fixed paraffin-embedded biopsies. Figure 1 shows co-staining of IgG and nephrin.

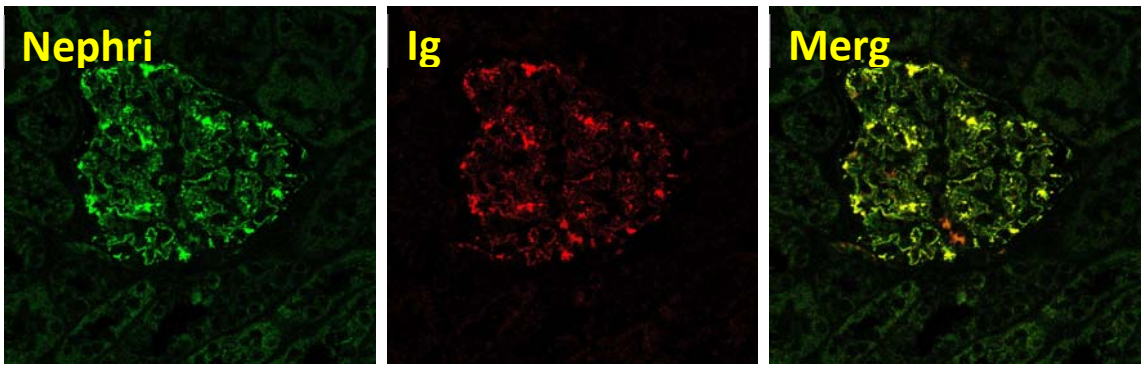

Figure 1. Dual fluorescence staining of IgG and nephrin.

What is the sensitivity and specificity of anti-nephrin antibodies in pediatric and adult patients with primary NS during active disease?

To address this question, we joined efforts with the Hamburg group led by Tobi Huber who had established a novel assay where antibodies were first immunoprecipitated, then measured with

an ELISA. Four cohorts were analysed together: 2 adult cohorts from Hamburg and Bari, and 2 pediatric cohorts from Paris and Rome **(2).**

Figure 2 shows the prevalence of anti-nephrin antibodies among adults and children. The prevalence was 42% in patients with MCD and 10% in those with pFSGS while they were exceptionally detected in other conditions; it increased to 60% in nephrotic MCD and 70% in those treatment naïve. In children, the prevalence was higher from 50% in the whole population rising to 80 % in those who were nephrotic and up to 90% in the children that had not received IS (Figure from Hengel et al, New England Journal of Medicine, 2024) Clinically, in patients that were antibody positive, proteinuria was higher, serum albumin lower, cholesterol higher while GFR was unchanged.We took advantage of this large population of patients, to confirm correlation of anti-nephrin antibodies with remission and relapse both in children and in adults.

Similar results were observed in a large cohort from China (3).

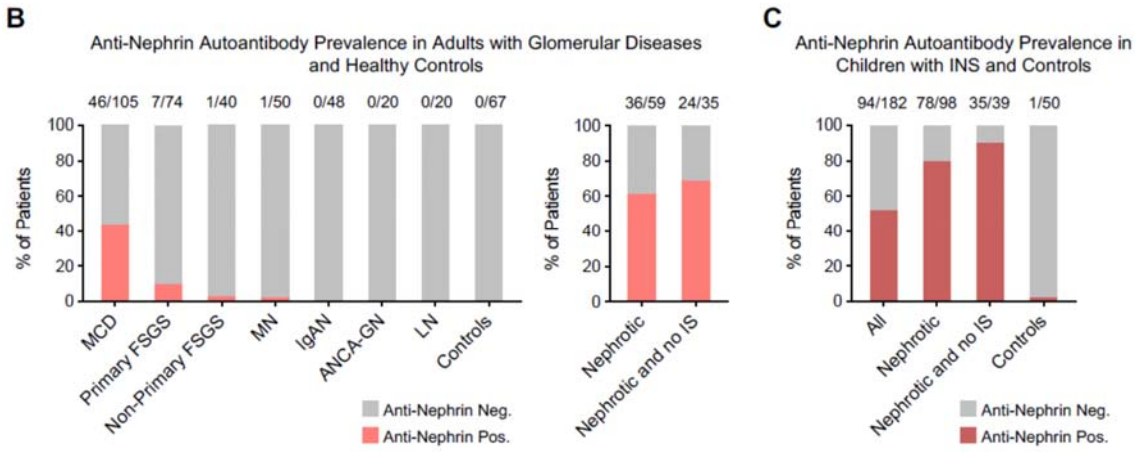

Figure 2. Prevalence of anti-nephrin antibodies among adults and children. (Hengel et al, New England Journal of Medicine, 2024)

Can anti-nephrin antibodies predict response to a second line IS therapy in patients with steroid-resistant nephrotic syndrome (SRNS)?

This question was addressed by an Italian group **(4)** and by our consortium **(5).**

Sixty-two pediatric and 40 adult biopsies were defined as slit positive or slit negative by high resolution microscopy according to the presence of IgG at the slit diaphragm which was found in 30% of pediatric patients with MCD and 25 % of those with primary FSGS, and in 68% and 19% of the adults, respectively but not in other lesion patterns. Then the biopsies were analyzed by STED microscopy for co-localization with nephrin. In pediatric patients, 78% were double positive and in adults 55%, which suggest that other antigens may be involved later identified as podocin and Neph1. Circulating anti-nephrin antibodies were only detected in double positive cases. Sub analysis in patients with SRNS found that about 50 % of non-genetic cases whereas only 8 % of the genetic forms were slit positive. Importantly, slit positive patients were most often responsive to IS and had a better prognosis, thus defining a category of auto-immune SRNS where intensification of therapy could be advised.

Our consortium recently reviewed the prevalence of anti-nephrin antibodies by IP in 333 cases according to response to treatment with emphasis on SRNS **(5**). When patients were grouped by response to treatment, the prevalence was higher in SSNS than in SDNS followed by non genetic SRNS. The prevalence was higher in patients with active disease. In the group of SRNS grouped by response to intensive IS treatment, prevalence was about 18% in responding SDNS whereas they were never detected in MDRS. Very intriguingly, 72 % patients are responsive without having anti-nephrin antibodies.

Overall, these results suggest that response to therapy is more likely in anti-nephrin positive patients.

Can anti-nephrin antibodies predict relapse of refractory forms of primary FSGS after transplantation?

This question was addressed in 2 retrospective studies from Japan (**6**) and from the US **(7).** This Japanese multi-institutional showed that all 11 patients who recurred had high titers of antibodies which were not found in the non recurrent patients. Biopsies at one day showed dusty fluorescence of nephrin co-localized with Ig deposits as in native kidneys and this was associated with increased nephrin tyrosine phosphorylation. These alterations were not observed in the 3 patients with non-recurrent FSGS and in those with genetic FSGS. High level of anti-nephrin antibodies were detected by ELISA in the 11 patients with rFSGS but in none of the nr and genetic FSGS, of the healthy individuals and disease controls.

In the US, the authors performed a retrospective multicenter study gathering 22 recurrent and 17 non recurrent cases of refractory podocytopathy. The main finding was that pretransplant circulating anti-nephrin antibodies was strongly associated with recurrent disease. Punctate staining was only observed in biopsies of patients with recurrent diseases, all with anti-nephrin antibodies.

Recently, we monitored successfully the graft of an adolescent with rapidly progressing FSGS and high level of anti-nephrin antibodies who received a kidney from her mother **(8).** Anti-nephrin were eliminated owing to the use of rituximab and intensive plasma exchanges. No recurrence occurred 20 months after transplantation.

Overall, these findings do suggest a pathogenic role for anti-nephrin antibodies which was confirmed by transfer experiments in the rabbit (**9).**

Conclusion

Anti-nephrin antibodies correlated with disease activity have been definitely confirmed, in keeping with the beneficial effects of anti-CD20, and most likely play an important role in GCW alterations. Those antibodies represent the first biomarker available for diagnosis and monitoring in children and in adults, and may help prevent relapses after transplantation. Development and commercialization of user friendly ELISAs are much awaited.

Many questions remain

Can anti-nephrin antibodies predict steroid dependence and steroid resistance? Is there a correlation between level of antibodies and outcome? Can they predict occurrence of relapse? What are the other antigenic targets in anti-nephrin negative patients, particularly those with primary FSGS? Are there other « permeability factors »?

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Development of a prototype Bridge ELISA for detection of anti-nephrin autoantibodies

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Introduction

Anti-nephrin autoantibodies bind at the slit diaphragm of podocytes in the kidney, leading to failure of the glomerular filtration barrier and inducing podocyte dysfunction leading to nephrotic syndrome [1]. Circulating anti-nephrin autoantibodies are common in patients with podocytopathies such as minimal change disease (MCD), primary focal segmental glomerulosclerosis (FSGS), and childhood idiopathic nephrotic syndrome, and levels correlate with disease activity [2]. The current gold standard for anti-nephrin antibody detection is immunoprecipitation (IP) [2,3]. Conventional ELISAs have been ineffective in detecting these antibodies, possibly because of the low abundance or affinity of these autoantibodies, showing the need for a highly sensitive assay [2, 3]. Here we report on the development of a new prototype Bridge ELISA for the detection of anti-nephrin autoantibodies. The study evaluates the performance of the prototype assay.

Methods

The detection principle of the prototype Anti-Nephrin Bridge ELISA (Euroimmun) involves capturing bivalent anti-nephrin antibodies between two antigens, one capture and one detection antigen, forming a "bridge". The utilization of both antibody valences helps to increase the specificity, which allows to apply serum samples at a significantly lower dilution. This in turn finally is the key to enable the detection of the low-abundance antibodies while still ensuring proper discrimination between anti-nephrin-positive and anti-nephrin-negative samples.

The performance of the prototype Anti-Nephrin Bridge ELISA was evaluated using a panel (n=40) comprising 8 anti-nephrin-positive serum samples from adult patients with MCD precharacterized

by IP (using the recombinant human nephrin ectodomain [2]), 29 sera from healthy adult blood donors, and 3 anti-nephrin IgG positive control substances. Results were compared with those obtained with an in-house Anti-Nephrin ELISA with a conventional detection principle.

From the blood donors, 9 samples were additionally tested for anti-nephrin autoantibodies using IP. Agreement of qualitative results (n=17, 8 patients + 9 blood donors) between ELISAs and IP was calculated using Cohen's kappa (κ).

Results

The prototype Anti-Nephrin Bridge ELISA detected all 40 samples correctly (Table 1), which corresponds to an accuracy of 100%. The agreement between qualitative results of IP and the Bridge ELISA was perfect (κ=1), but slight (κ=0) between IP and the conventional in-house ELISA. All patient sera had higher optical densities when analyzed with the prototype Anti-Nephrin Bridge ELISA than with the conventional Anti-Nephrin ELISA (Figure 2), providing tentative initial evidence for a very high sensitivity of the prototype Anti-Nephrin Bridge ELISA.

Conclusion

The prototype Anti-Nephrin Bridge ELISA could be a valuable tool for supporting precise and sensitive detection of anti-nephrin autoantibodies. Autoantibody quantification can support treatment monitoring of glomerular diseases, enhance treatment evaluation, and improve kidney-transplantation strategies in FSGS patients [4].

Conflict of interest: AD, SR, CR, SM, and VBL are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

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What about Biomarkers in Autoimmune Disease Cardiovascular Risk Assessement?

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Introduction & Objective

Autoimmune Diseases (AID) are chronic inflammatory disorders characterized by immune system dysregulation and the production of autoantibodies. These pathophysiological mechanisms significantly increase the risk of cardiovascular diseases (CVD), including atherosclerosis, myocardial infarction, and stroke.

Biomarkers such as high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and antiphospholipid antibodies (aPL) play a pivotal role in detecting cardiovascular risk early in patients with autoimmune conditions such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Other biomarkers like homocysteine, lipoprotein a (Lpa) and specific lipid profiles are key in cardiovascular prevention strategies. Understanding these biomarkers provides insight into the shared inflammatory pathways driving both autoimmunity and cardiovascular pathology.

This study aims to analyse the casuistry of our laboratory, emphasizing the clinical significance and potential applications of lipid profiles and specific biomarkers in cardiovascular risk assessment in AID.

Methods

ANA screening with IIF in Hep-2 cells (Euroimmun™); ds-DNA by FEIA (Thermofisher ™) with confirmatory IIF with Crithidia luciliae (Euroimmun™). Immunoblotting for SARDS (Euroimmun™). Liver autoimmune diseases study by IIF in Liver mosaic 9 (Euroimmun™) and liver immunoblotting profile by Euroimmun™. Systemic sclerosis and Myositis autoantibody profile by immunoblotting (Euroimmun™). High-sensitivity C-reactive protein (hs-CRP), Lipid profile screening tests by chemiluminescent technology in Attelica CI by Siemens Healthineers TM. Lipid agarose gel electrophoresis by Sebia TM Hydrasys 2 scan focusing and LDL subfractions in polyacrylamide gel electrophoresis by Lipoprint Lipoprotein Subfractions Testing System from Quantimetrix TM.

Results

The authors present a 5 -year revised casuistic from January 2020 to December 2024 as a reference clinical laboratory center in autoimmune diseases and as a reference clinical laboratory center in Dyslipidemia diagnosis.

Conclusions

Autoimmune diseases significantly elevate cardiovascular risk through systemic inflammation and immune-mediated vascular damage. Biomarkers serve as essential tools for early detection and management. Integrating biomarker assessment into clinical practice can guide personalized therapy and reduce the cardiovascular burden in autoimmune populations

Cellular immunophenotypes are associated with heart-reactive antibody profiles in patients hospitalized for acute heart failure

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Abstract

In a retrospective pilot study, we have shown that the induction of heart-reactive autoantibodies (HRA) in the wake of acutely decompensated heart failure predicts worse outcomes. To gain deeper insights into the immunological mechanisms causing induction of HRA after heart failure decompensation we initiated the prospective 'Acute Heart Failure-Immunomonitoring Cohort Study' (AHF-ImmunoCS). This study follows 381 patients for a period of 18 months after an index hospitalisation for AHF and serially collects biomaterials. Analysis of AHF-ImmunoCS samples obtained at baseline and at 6-month follow-up from the first 145 patients enrolled, indirect immunofluorescence (IFT) showed de novo induction of HRA in 19% of patients (previously published percentage: 32%). To understand, what drives HRA induction in these patients we longitudinally immunophenotyped peripheral blood leukocytes in samples obtained at baseline, 6-week and 6-month follow-up by high-resolution spectral flow cytometry. We established 30+ parameter panels to identify lymphocytic and myeloid subsets of peripheral blood leukocytes. Among lymphocytes, induction of HRA in the wake of acute decompensation of heart failure was associated with a higher proportion of CD45RA+ CCR7+ phenotypically naive and, conversely, a lower proportion of memory cells among conventional, i.e., non-regulatory, CD4+ T cells at baseline and 6-week follow-up. Among myeloid cells, the picture was less clear compared to lymphocytes, but HRA induction appeared to be associated with low frequencies/ the absence of plasmacytoid dendritic cells and a sub-cluster of neutrophils marked by high expression of CD16 and CD15 in samples obtained at all three timepoints. Our data, thus, suggest that HRA induction is associated with a skewed composition of both the lymphoid and the myeloid compartment.

This might underly induction of HRA and/ or might in itself constitute a decisive modulatory factor with an impact on disease progression and prognosis in AHF.

This study was supported by a grant from the DFG (SFB1525 - project C05: 453989101).

Diagnostic advancements in Dermatitis herpetiformis: The role of neo-epitopes and multiparametric antibody profiling

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Background

Dermatitis herpetiformis (DH) is a skin manifestation of gluten-related disorders (GRD), strongly linked to celiac disease. Neo-epitopes generated by enzymatic processes are central to its pathogenesis. The AESKUBLOTS® GRD IgA assay offers a novel approach for detecting autoantibodies, including IgA against tissue transglutaminase (tTG), epidermal transglutaminase (TG3), and microbial transglutaminase (mTG).

Aims

This study evaluated the diagnostic accuracy of AESKUBLOTS® GRD IgA in distinguishing DH from other autoimmune blistering diseases and explored the significance of neo-epitope-targeting antibodies in DH.

Methods

Serum samples from 45 DH patients and 27 controls (bullous pemphigoid and pemphigus vulgaris) were analyzed for antibody titers. Receiver operating characteristic (ROC) curves assessed the performance of assay antigens, and antibody profiles were correlated with clinical history.

Results

The assay demonstrated excellent diagnostic performance, particularly for anti-tTG neo IgA (AUC: 0.961, CI: 0.885–0.992), anti-TG3 IgA (AUC: 0.947, CI: 0.867–0.986), and anti-mTG-neo IgA (AUC: 0.961, CI: 0.885–0.992). Specificity reached 100% (CI: 89.424–100%), reliably differentiating DH patients from controls.

Conclusion

Antibodies against gliadin-mTG complexes were identified as potential biomarkers, emphasizing their role in disease pathogenesis. The AESKUBLOTS® GRD IgA assay proves to be a robust diagnostic tool, offering rapid and comprehensive antibody profiling. By targeting neo-epitopes like gliadin-mTG complexes, it provides new insights into DH pathogenesis and could refine diagnostic criteria. These findings support dietary interventions, such as excluding mTG-containing processed foods, to improve DH management, while reducing reliance on invasive biopsies.

Novel peptide epitopes of NC16A BP180 in the diagnostics of Bullous Pemphigoid

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Introduction

Bullous pemphigoid (BP) is a severe autoimmune blistering skin disorder causing significant morbidity and mortality among the elderly. Improved diagnostic strategies rely on the identification of autoantigen epitopes recognized by pathogenic autoantibodies. Continuous (linear) peptide epitopes, unlike conformational epitopes in recombinant proteins, offer advantages such as chemical stability, reproducibility and lower production costs, making them attractive for diagnostic assay development.

Objective

To identify linear epitopes within peptides derived from the NC16A domain of BP180, which are unequivocally the immunodominant epitopes recognized by BP patient's pathogenic anti-BP180 autoantibodies.

Methods

A customized microarray of overlapping peptides spanning the non-collagenous (NC)16A domain of BP180 and the C-terminus of BP230 autoantigens (PEPperPRINT GmbH) was screened with sera

of thirteen BP patients and two control sera. A 25-mer peptide of NC16A, termed Pep7-NC16A, was evaluated by ELISA in two independent patient cohorts (n=35 and 26 BP cases; n=48 and 53 controls).

Results

Pep7-NC16A ELISA showed sensitivity of 71% and 62%, and specificity of 92% and 98%, with AUC values of 0.92 and 0.754 ($p < 0.001$). Reactivity strongly correlated with commercial BP180 NC16A ELISA ($r=0.72$, $p < 0.0001$).

Summary/Conclusion

This is the first study to demonstrate the diagnostic potential of a synthetic peptide in BP. The identified epitope could also serve as a potential biomarker for BP180 associated conditions or future peptide-based immunotherapies.

9.3 Harmonization and Relevance of the Determination of Antinuclear Antibodies

The culture of autoimmune testing: Overtesting and undertesting in times of rising autoimmunity and polyautoimmunity

Thermo Fisher Scientfic Company sponsored symposium

Speakers: Raul Castellanos, Jan Damoiseaux, Marvin J. Fritzler, M. Luisa Mearin, Nina Olschowka

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Abstract

In the era of precision medicine, it is crucial to test the right patient at the right time with the right test and ensure that the results are reported accurately for successful diagnosis. This is particularly relevant in the context of autoimmune diseases and polyautoimmunity, which are steadily increasing in prevalence and scope due to a number of factors including environmental exposures. Autoantibody tests support diagnosis but, in general, only provide a likelihood and/or risk of disease, not a definitive answer. Healthy patients can show positive results, while patients, particularly with early disease, can show negative results. On the other hand, frequently underdiagnosed conditions, like coeliac disease, can be identified by determination of specific antibodies, reducing diagnostic delays and improving health-related outcomes.

Early diagnosis is key in autoimmune diseases, but it brings a dilemma: should we use strategies like massive screening, active case finding, or extensive testing panels to ensure early diagnosis, or should we opt for selective, but early testing based on the disease and marker to avoid misidentifying healthy individuals as autoimmune patients? Is there a single correct approach, or does it depend on the specific disease? Do we need more tests, or do we need to make the most of the results of the relevant ones? Are we over-testing or undertesting? Some of the gaps that need to be addressed are shown in the Table.

Our speakers will delve into these critical questions and discuss who should be tested, when testing should occur, and which tests to use.

Actions Needed to Address Earlier and Accurate Diagnosis of Autoimmune Diseases.

- Develop a standardized definition of autoimmune diseases.
- Establish world-wide registries and repositories for all autoimmune diseases.
- Understand gene-environment interactions. Remediate where possible.
- Define approaches to measure direct and indirect health care costs and benefits, including those in usually underrepresented and underdiagnosed populations.
- Coordinate the use of artificial intelligence and machine learning to identify the matrix of clinical features, risk factors, high risk groups & biomarkers.
- Define best practices for diagnostics, therapies, medical “care” and quality of life.
- Convene stakeholders to identify research opportunities and priorities.

The 8th International Consensus on ANA Patterns (ICAP) Workshop

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Abstract

The International Consensus on ANA Patterns (ICAP) is a global initiative aimed at harmonizing the technical and interpretive aspects of HEp-2 IFA testing (1,2). For over a decade, ICAP has promoted accurate reading and reporting of HEp-2 IFA images across clinical labs, research, industry, and patient care (3). It fosters international collaboration and actively encourages participation from emerging experts worldwide.

The 8th ICAP workshop, held during the day before the 17th Dresden Symposium on Autoantibodies, has addressed several aspects that originated from the interaction with the international community and has presented the initiatives, goals, and tasks to be delivered over the next two years. This article outlines some of the issues addressed at the 8th ICAP Workshop, including the presentation of the new highlights of the AC Patterns Classification Tree, the presentation of some unusual HEp-IFA patterns and Frequently Asked Questions of the www.anapatterns.org website, the novel educational module on unclassified patterns (AC-XX Patterns), new HEp-2 IFA patterns, various aspects and challenges in the quality control of the HEp-2 IFA test, the interplay between the COVID-19 pandemic and HEp-2 IFA results, the judicious use of the “competent-level” and “expert-level” patterns, and the use of artificial intelligence in HEp-2 IFA.

1. Highlights of the AC Patterns Classification Tree (by Edward K. L. Chan)

Edward Chan presented the latest updates to the ICAP classification tree, officially approved during the 7th ICAP Workshop in 2023. Two new nuclear patterns—AC-30 and AC-31—were added, enhancing the recognition of fine-speckled nuclear patterns.

AC-30 is a newly defined variant of the dense fine-speckled nuclear pattern (AC-2), characterized by a more homogeneous distribution of speckles and consistent staining of the metaphase plate. Unlike AC-2, which is strongly associated with anti-DFS70 antibodies and typically not linked to systemic autoimmune diseases, AC-30 is generally not associated with the most common autoantibody specificities (4-6). However, in up to 20% of cases, clinically relevant autoantibodies—such as anti-dsDNA, nucleosome, and TROVE2 (SS-A/Ro60)—may be present. Due to their subtle morphological differences, both AC-2 and AC-30 are classified as Expert-level patterns under the broader Competent-level umbrella of nuclear speckled patterns with mitotic plate staining (AC-2/AC-30) (3).

AC-31 is a newly recognized variant of the fine speckled nuclear pattern (AC-4), distinguished by myriad discrete tiny dots and the absence of metaphase plate staining (7). This pattern has a strong and specific association with anti-TROVE2 antibodies, confirmed through monoclonal antibody studies (8). Initially designated as AC-4a, it has now been formally classified as AC-31, also an Expert-level pattern, with a dedicated page on the ICAP website (3).

Since 2018, ICAP has established the AC-XX category to accommodate unusual HEp-2 IFA patterns not yet included in the official classification tree (9). These unusual patterns may lack fully established clinical significance but are important to recognize to avoid misclassification. Examples include pleomorphic nuclear patterns that resemble—but differ from—known pleomorphic patterns such as AC-13 (PCNA-like) or AC-14 (CENP-F-like). To support analysts, ICAP has recently launched a new Educational Module focused on AC-XX patterns and added a new AC-XX section to the classification tree. In the near future, the AC-XX box will include a hyperlink with representative images and descriptions of these rare patterns.

These additions reflect ICAP's ongoing commitment to refining pattern classification based on morphological and immunological distinctions, ultimately improving diagnostic accuracy and clinical relevance in HEp-2 IFA interpretation.

2. Unusual HEp-IFA patterns and Frequently Asked Questions (presented by Lindsay Brooke Boling and Maria Infantino)

An unusual and complex HEp-2 IFA pattern that resembles—but differs from—the nuclear pleomorphic CENP-F-like pattern (AC-14) was presented. Their presentation focused on the fine morphological details that allow analysts to distinguish between these two patterns.

Infantino used this case as an opportunity to highlight the Frequently Asked Questions (FAQ) section of the ICAP website. As is often the case, the image in question had been submitted by a registered user of the ICAP platform, demonstrating the practical value of the FAQ section. The international community regularly sends questions and HEp-2 IFA images to ICAP, which are carefully reviewed and discussed by the ICAP Executive Board. These discussions often lead to clarifications, updates, and educational resources that are posted at the FAQ section for further benefit of the broader community of analysts and clinicians.

3. The novel educational module on unclassified patterns (AC-XX Patterns) (presented by Werner Klotz)

Since its inception, ICAP has progressively defined 32 distinct fluorescence patterns, labeled AC-1 through AC-31, with AC-0 representing the absence of specific staining on HEp-2 cells. However, during routine HEp-2 IFA testing, laboratories occasionally encounter unusual staining patterns

that do not match any of the established ICAP classified patterns. To address this issue, the AC-XX category was established as an alternative for reporting unusual and unclassified patterns (9). In order to increase the knowledge about HEp-2 IFA unusual patterns, a new educational module has been introduced on the ICAP website. This module is designed to help laboratory professionals distinguish undefined patterns from recognized single, multiple or composite patterns. The primary objective of the module is to clarify the rationale behind using the generic code AC-XX for unusual and unclassified HEp-2 IFA staining patterns, as well as publicizing images and available information for several of these unclassified patterns. The knowledge about several AC-XX patterns is of utmost importance, as it contributes to preventing misclassification of AC-XX patterns as one of the 32 classified AC patterns.

AC-XX pattern category functions as a provisional code for reporting patterns that cannot be classified under existing ICAP definitions. This temporary designation allows such patterns to be flagged for further evaluation and potential classification by ICAP. Although the clinical relevance of these unclassified patterns is not yet fully understood, they may be indicative of autoimmune conditions and should therefore be included in diagnostic reports. The training module presents eight examples of AC-XX patterns—four nuclear and four cytoplasmic—and offers guidance on how to differentiate them from ICAP-classified patterns. While providing detailed descriptions of AC-XX patterns is optional, it is recommended to accompany the AC-XX code with a brief description of the observed pattern and its titer.

4. New HEp-2 IFA patterns (presented by Lucile Musset and Marvin J. Fritzler)

Melanoma Differentiation Associated gene-5 (MDA-5) is a cytoplasmic protein crucial for antiviral immune responses, primarily through the induction of type I interferons. Anti-MDA-5 antibodies were first identified in patients with clinically amyopathic dermatomyositis (CADM) and rapidly progressive interstitial lung disease (RP-ILD), and are now classified as a myositis-specific autoantibody (MSA), helping define clinical subgroups in dermatomyositis (DM). Anti-MDA-5 DM presents with distinct clinical phenotypes—vascular, skin-articular, or pulmonary—and is rare, especially in Europe. Detection of anti-MDA-5 antibodies can be suspected via the HEp-2 IFA test, that shows a fine speckled cytoplasmic pattern in a few clustered cells (10-11). Confirmatory tests include ELISA, line blot, and particle-based assays.

This unique HEp-2 IFA pattern, currently labeled AC-XX, was proposed as a candidate for a new ICAP AC pattern due to its clinical relevance, reproducibility, and distinct morphology. However, limitations include its low detection rate (seen in ~15% of anti-MDA-5 DM cases), possibly because the clusters of stained cells are very few and scattered, thus amenable to be overlooked in the routine HEp-2 IFA analysis. In addition, there is variability in the staining intensity according to the slide brand. Despite these challenges, recognizing this pattern can enhance diagnostic accuracy, especially when supporting a positive result in an MDA5-specific immunoassay. In conclusion, while awaiting ICAP consensus, laboratories should be aware of the MDA5-like pattern and actively search for it in suspected inflammatory myopathy cases, as its identification can guide further testing and improve diagnostic confidence.

Another interesting pattern discussed in the Workshop was the SG2NA pattern, currently an unclassified HEp-2 IFA pattern (AC-XX), sometimes referred to as “pseudo-PCNA”. The SG2NA pattern shows variable nuclear staining depending on the cell cycle phase. This pattern was first described in 1994 and linked to a novel autoantigen named SG2NA (S-G2 phase nuclear antigen), which shows nuclear speckled staining most prominent during the S and G2 phases. Unlike true

pleomorphic patterns like PCNA or CENP-F, SG2NA displays consistent and uniform staining texture but variable intensity (12-14).

Despite studies suggesting that SG2NA was identical to striatin-3 isoform 2, part of the striatin family of WD-40 repeat proteins, which includes striatin and zinedin, only SG2NA produces the characteristic cell cycle-dependent IFA pattern. Human anti-striatin antibodies and commercial anti-striatin monoclonals do not reproduce the SG2NA IFA pattern. Therfore, despite molecular overlap, there appears to be differences in epitope expression, functional, and localization between SG2NA and striatin-3. In conclusion, SG2NA represents a distinct nuclear autoantigen with a unique IFA pattern that remains unclassified in the ICAP system. Its recognition could enhance understanding of cell cycle-related autoantibodies and their clinical relevance.

5. Challenges in the quality control of the HEp-2 IFA test (presented by Jan Damoiseaux, Carolien Bonroy, Maria Infantino, Teresa Carbone, and Dina Patel)

External Quality Assurance (EQA) programs are essential for monitoring and improving laboratory performance, especially in autoimmune diagnostics. The UK NEQAS EQA is a well-established initiative that consistently contributes to quality control in clinical laboratories in several countries. At the 8th ICAP Workshop, Infantino M., Carbone T., and Patel D. presented a structured analysis on the reports from 2013 to 2023 for HEp-2 IFA testing, focusing on harmonization in pattern and titer recognition. The key findings included: a) Improved consensus on positive/negative results over time (from 64.0% in 2013 to 90.9% in 2023); b) Existence of considerable heterogeneity in pattern recognition, where some patterns (e.g., nuclear homogeneous, AC-1) had lower consensus than others, e.g., nuclear speckled (AC-4/AC-5), centromere (AC-3), and negative (AC-0); c) Titer consensus was higher for lower titers (1:80–1:320) than for higher ones; d) Manual vs. digital reading showed no significant difference. ICAP nomenclature adoption significantly improved harmonization (from 82.6% to 93.8%). Despite progress, full harmonization across laboratories and methods remains a challenge.

6. COVID-19 pandemic and HEp-2 IFA (presented by Alessandra Dellavance)

Viral infections, including SARS-CoV-2, are known to trigger autoimmune responses through mechanisms like molecular mimicry and epitope spreading. While early studies reported high HEp-2 IFA positivity in COVID-19 patients, inconsistencies in methods limit their comparability. Importantly, autoantibodies typically linked to systemic autoimmune diseases were not significantly elevated, suggesting a nonspecific immune activation rather than true autoimmunity. Some large studies did find a modest rise in new autoimmune diagnoses and persistent broad-spectrum autoantibodies post-infection, possibly linked to molecular mimicry with SARS-CoV-2 proteins (15-17).

This study analyzed over 1.26 million HEp-2 IFA tests performed at Fleury Laboratory, Brazil, between 2019 and 2024 to investigate whether the COVID-19 pandemic influenced HEp-2 IFA test results—specifically positivity rates, fluorescence patterns, and titers. Of the total tests, 73.6% were negative, 21.6% positive, and 4.9% excluded due to complex mixed patterns. While annual HEp-2 IFA positivity rates showed statistically significant variation, the changes were small and did not follow a consistent trend linked to the pandemic.

The most frequent patterns were AC-4 (29.8%), AC-30 (26.5%), AC-2 (16.9%), AC-1 (5.7%), and the grouped nucleolar patterns AC-8/9/10 (4.6%). These patterns showed statistically significant but modest fluctuations over time. Notably, AC-4 peaked in 2021, AC-30 steadily increased, and AC-2

declined. The opposing trends of AC-2 and AC-30—despite their morphological similarities—may reflect improved recognition of AC-30 by analysts rather than a pandemic-related effect. Titer distributions also varied significantly over time. AC-4 was most often seen at 1:80, AC-30 at 1:160, and AC-2 and AC-1 at 1:640, with some shifts in frequency across the years.

A focused analysis was conducted on a subgroup of over 214,000 individuals who had both HEp-2 IFA and SARS-CoV-2 testing. Interestingly, HEp-2 IFA positivity was slightly lower in SARS-CoV-2-exposed individuals (22.2%) compared to non-exposed ones (22.9%), a statistically significant but minor difference. While the most common patterns (AC-4, AC-30, AC-2) were not significantly associated with SARS-CoV-2 exposure, the AC-6 was more frequent in exposed individuals, while the AC-19, AC-1, AC-31, and AC-25 patterns were less frequent. Pattern AC-6 in HEp-2 IFA testing is linked to autoantibodies targeting nuclear body proteins like Sp100 and PML, which are involved in antiviral defense (18,19). Viral infections, including SARS-CoV-2, can trigger immune responses that expose these nuclear antigens, potentially leading to autoantibody production. The observed increase in AC-6 pattern frequency among individuals exposed to SARS-CoV-2 may reflect viral disruption of nuclear body components, suggesting a possible connection between viral infection and autoimmunity.

In conclusion, the study suggests that while the overall HEp-2 IFA testing profile remained stable during the pandemic, subtle shifts in specific patterns—particularly those involving nuclear proteins linked to antiviral responses—may reflect a targeted immunological impact of SARS-CoV-2. However, limitations such as the retrospective design and lack of clinical data prevent definitive conclusions. Future research integrating clinical outcomes and functional assays is needed to clarify the clinical significance of these findings.

7. Competent- versus Expert-level patterns (presented by Luis Andrade)

ICAP classifies 32 HEp-2 IFA patterns into a structured tree with three main branches: nuclear, cytoplasmic, and mitotic apparatus. Each branch is further divided into two hierarchical levels: competent-level and expert-level patterns. Competent-level patterns are broader and expected to be recognized by all analysts, while expert-level patterns require more detailed interpretation and analyst experience. This two-tiered system acknowledges that HEp-2 IFA images exist on a continuous spectrum, influenced by the presence and concentration of multiple autoantibodies in a sample. As such, some patterns may fall between defined categories, making classification challenging—especially in low-titer samples, where fluorescence is dim and less distinct. In these cases, assigning a competent-level pattern may be more appropriate than forcing a specific expert-level classification.

Importantly, the terms “competent” and “expert” refer also to the complexity of the pattern, not entirely on the analyst’s skill. Even seasoned professionals may choose a competent-level classification when the image lacks clarity. To avoid confusion, the authors suggest reconsidering this terminology to better reflect the morphological complexity of patterns rather than implying a hierarchy of analyst expertise. Recognizing the continuous nature of immunofluorescence patterns and the limitations of discrete categories is essential for accurate and consistent reporting.

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Comparison of ICAP competence level AI-based ANA pattern interpretation with expert reading

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Objectives

Antinuclear antibody (ANA) assessment by indirect immunofluorescence (IIF) on HEp-2 cells is a screening test for the serological diagnosis of systemic autoimmune rheumatic diseases. Automated interpretation of ICAP competence level ANA patterns by novel artificial intelligence (AI)-aided pattern recognition was compared with expert reading under routine conditions.

Methods

Consecutive serum samples of 2671 individuals referred to a routine laboratory were analysed for ANA titers and patterns using the automated interpretation system akiron® NEO (Medipan, Germany). AI-based ANA detection was compared with independent classification by two experienced immunologists according to the international consensus on ANA (ICAP) competence level.

Results

Overall, a good agreement ($\kappa > 0.60$) between the different raters both for positive/negative classification of ANA fluorescence images as well as for the pattern classification of positive samples with a titer >= 1:320 was observed. Overall, positive/negative differentiation at different cut-offs revealed κ values from 0.584 to 0.760 whereas corresponding pattern recognition for interphase, metaphase and cytoplasmic patterns demonstrated κ values from 0.560 to 0.736.

Conclusions

AI-based ANA pattern interpretation showed a similar performance compared to human observers. AI-aided ANA image analysis can facilitate the diagnostic workflow of ANA IIF assays and reduce subjectivity during image classification.

What do physicians know about ICAP? Differences in ANA HEp-2 IFA pattern interpretation between rheumatologists and non-rheumatologists in Argentina

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Introduction

Autoantibodies determined by HEp-2 IFA are important biomarkers for the diagnosis of systemic autoimmune rheumatic diseases. The International Consensus on ANA Patterns (ICAP, www.anapatterns.org) provides standardized nomenclature and relevant clinical associations for more than 30 recognizable ANA patterns.

Objectives

We aimed to determine to which extent ICAP resources are known and utilized by physicians in their practice, influencing clinical decision making.

Materials and Methods

Cross-sectional online survey with 16 questions accessible through Google Forms, including ANA ordering, pattern interpretation, awareness of ICAP directives, and some basic clinical considerations. Chi-square tests applied to evaluate differences between 102 rheumatologists and 72 non-rheumatologists, all working in Argentina. The estimated response time was 5 to 6 minutes.

Results

Physicians reported 15 medical specialties, besides rheumatology, with an overrepresentation of specialties that normally ask for ANA tests: nephrology, internal medicine, immunology, dermatology. Most respondents were between 30 and 49 years old, although all ages between 30 and > 60 were represented. Anti-nuclear antibodies, Anti-nuclear factor, Anti-nucleocytoplasmic antibodies, and Anti-cell antibodies were distinct names commonly used for the HEp-2 IFA in Argentina. Rheumatologists were significantly more likely to know ICAP and use its website than non-rheumatologists (86.3% versus 37.5%, CI: [0.80 – 0.93] and CI: [0,26 – 0.49] respectively, $p<0.001$). Almost all respondents in both groups found the ANA titer clinically relevant (96.1% and 98.6% for rheumatologists and non-rheumatologists, respectively). Rheumatologists added some conditional statements, like clinical relevance depending on clinical findings, ANA final titer, or on the pattern. There was no significant difference between rheumatologists and non-rheumatologists regarding the clinical cutoff value used (1:80, 1:160 or ≥ 1:320), with 54% of all respondents considering 1:80 as acceptable. Nearly all respondents considered nuclear and mitotic patterns as clinically relevant (94.8% and 84% respectively), with no differences among medical specialties. Cytoplasmic patterns were considered ANA positive by 82.8% of all clinicians (144/174), with no difference observed between rheumatologists and other specialties.

Comments and conclusions

Most rheumatologists in Argentina are aware of and use ICAP resources. ANA titers and patterns are considered clinically relevant by all medical specialties. Only about a third of non-rheumatologists ordering ANA HEp-2 IFA are familiar with the ICAP initiative. This and many other shortcomings, like considering mitotic patterns as clinically relevant, or accepting 1:40 as a screening titer, must be the focus of educational interventions for rheumatologists and non-rheumatologists alike.

Less May Be More: When to Choose to Report at the Competent-Level HEp-2 IFA Patterns

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The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) is the most commonly used test for the screening of autoantibodies in patients suspect of systemic autoimmune diseases. The International Consensus on ANA Patterns (ICAP) is an initiative dedicated at promoting harmonization in reporting and standardization in the performance of the HEp-2 IFA test. ICAP consists of an international team of basic and clinical scientists with expertise and interest in autoantibody testing.

Over the last decade, ICAP has established 32 distinct immunofluorescence patterns observed in the HEp-2 IFA (1, 2). These patterns convey valuable clinical information, as they reflect the topographic distribution of the autoantigens targeted by autoantibodies in a given sample. Consequently, they provide important insights into the clinical relevance of a positive HEp-2 IFA result and guide the selection of follow-up antigen-specific immunoassays, taking into account the patient's clinical presentation.

The 32 ICAP patterns are organized into a classification tree (available at www.anapatterns.org) with three main branches that comprise the nuclear, cytoplasmic, and mitotic apparatus patterns, respectively (Figure 1). Within each of these branches, there are two hierarchically arranged tiers, comprising the competent-level and expert-level patterns, respectively. The nuclear branch comprises 23 categories (eight competent-level and 15 expert-level patterns). The cytoplasmic branch comprises 11 categories (five competent-level and six expert-level patterns). The mitotic apparatus comprises five expert-level patterns. Competent-level patterns are those that should be recognized by any analyst interpreting and reporting the HEp-2 IFA test. Expert-level patterns, on the other hand, are characterized by finer details that require greater expertise and/or well-defined image for accurate identification. In many cases, competent-level patterns serve as "umbrella classifications" for underlying expert-level patterns that closely resemble one another (Table 1).

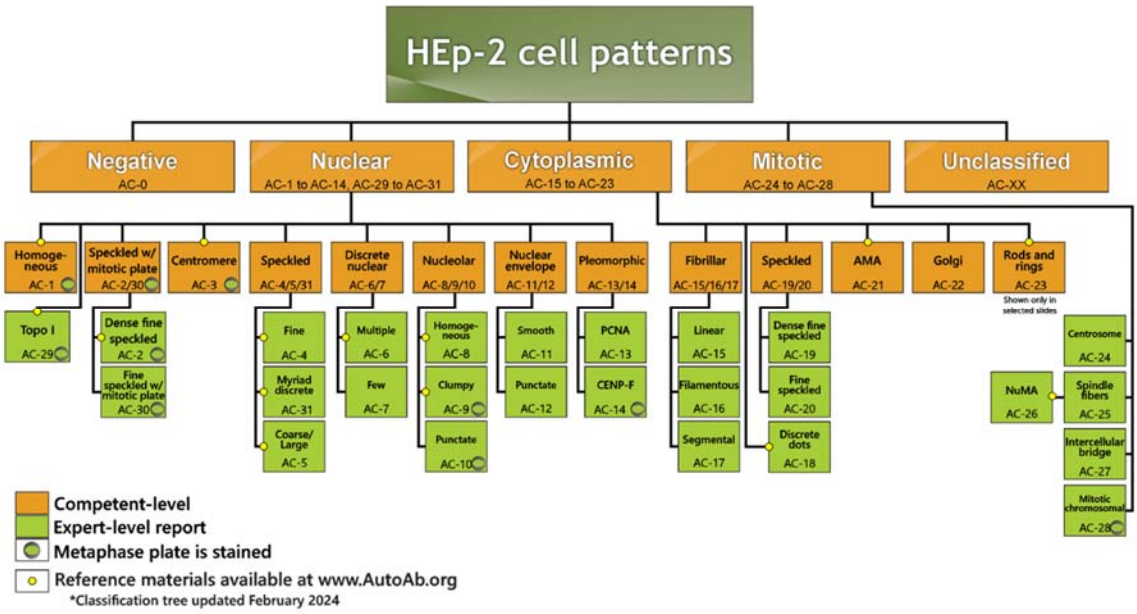

Figure 1. Current version of the ICAP classification tree (www.anapatterns.org)

Table 1. Alternative Designations for Competent and Expert-Level HEp-2 IFA Patterns

Overhanging Competent-Level Patterns	Underlying Expert-Level Patterns
Nuclear speckled with stained MP (AC2/AC30)	Nuclear dense fine speckled (AC-2) Nuclear fine speckled with MP (AC-30)
Nuclear speckled (AC-4/AC-5/AC-31)	Nuclear simple fine speckled (AC-4) Nuclear coarse speckled (AC-5) Nuclear myriad discrete fine speckled (AC-31)
Discrete nuclear speckles (AC-6/AC-7)	Multiple discrete nuclear speckles (AC-6) Few discrete nuclear speckles (AC-7)
Nucleolar (AC-8/AC-9/AC-10)	Nucleolar homogeneous (AC-8) Nucleolar clumpy (AC-9) Nucleolar punctate (AC-10)
Nuclear envelope (AC-11/AC-12)	Smooth nuclear envelope (AC-11) Punctate nuclear envelope (AC-12)
Nuclear pleomorphic (AC-13/AC-14)	PCNA-like (AC-13) CENP-F-like (AC-14)
Cytoplasmic fibrillar (AC-15/AC-16/AC-17)	Fibrillar linear (AC-15) Fibrillar filamentous (AC-16) Fibrillar segmental (AC-17)
Cytoplasmic speckled (AC-18/AC-19/AC-20)	Discrete cytoplasmic dots (AC-18) Cytoplasmic dense fine speckled (AC-19) Cytoplasmic fine speckled (AC-20)

The arrangement of ICAP classification tree into two hierarchical levels allows the analyst to choose whether to classify the image at the expert-level or at the broader, overhanging competent-level pattern. This approach acknowledges that the wide variety of images

encountered in the HEp-2 IFA routine testing follows a continuous gradient rather than a discrete format. This gradient arises from the fact that the images observed in any given HEp-2 IFA analysis reflect the combined effect of multiple autoantibodies present in the sample. Variations in the relative concentrations of these autoantibodies can produce a continuous array of closely resembling patterns. This contrasts with the discrete categorical system in the ICAP classification tree, which may occasionally make it difficult to classify images that fall between closely related categories. In this context, the competent-level patterns are particularly helpful, as they span broader segments of the continuous spectrum compared to the narrower, more specific expert-level patterns. A useful pictographic analogy to this concept is the contrast between discrete basic colors categories and the continuous spectrum of visible light, in which discrete color labels fail to capture many intermediate hues (Figure 2).

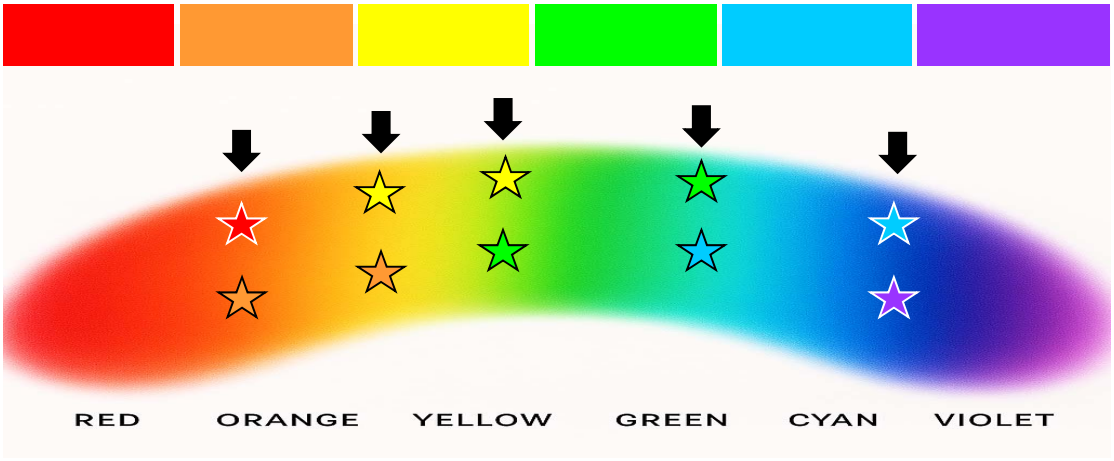

Figure 2. Discrete color categories versus the continuum spectrum of visible light. Arrows indicate intermediate areas within the continuous color spectrum between basic colors. Stars represent the color copy of the boxes (basic colors) at the top row.

The challenge in defining an image at the expert level is especially relevant in low titer samples, which produce dimmer and less distinct immunofluorescence images. In a pilot blinded controlled study, we evaluated the ability of experienced analysts to recognize HEp-2 IFA patterns in samples with high and low concentrations of autoantibodies. Eight highly experienced, blinded analysts examined HEp-2 slides probed with known samples diluted at 1/80 (producing a bright ++++/4 image) and at the second-to-last positive dilution (producing a dim +/4 image) of the same sample. The sample dilutions were randomized so that analysts were unaware of the distribution and nature of samples. A total of 12 samples were included, representing the following patterns: AC-1 (n=2), AC-2 (n=2), AC-3 (n=2), AC-5 (n=2), AC-21 (n=2), AC-26 (n=1), and AC-30 (n=1). Table 2 presents the pattern classifications assigned by the analysts for both the 1:80 dilution and the second-to-last positive dilution of each sample. Patterns AC-3, AC-21, and AC-26 demonstrated strong robustness, with all analysts correctly identifying them even at the lower dilution. In contrast, patterns AC-1, AC-2, and AC-5 were misclassified by some analysts at the second-to-last positive dilution. These results suggest that the recognition of some expert-level patterns can be challenging in low-titer samples. In such cases, it may be more appropriate to report the corresponding overarching competent-level pattern.

Table 2. Degree of confidence in assigning some HEp-2 IFA expert-level patterns is stronger in samples producing high as compared to those producing low immunofluorescence intensity

Sample	Dilution	Pattern assignment		Wrong pattern assignment
		Correct	Erroneous	
AC-1 #1	1/80	8	0	===///===
	Second to last	6	**2**	**AC-30**
AC-1 #2	1/80	8	0	===///===
	Second to last	4	**4**	**AC-30**
AC-2 #1	1/80	8	0	===///===
	Second to last	2	**6**	**AC-30**
AC-2 #2	1/80	8	0	===///===
	Second to last	3	**5**	**AC-30**
AC-3 #1	1/80	8	0	===///===
	Second to last	8	0	===///===
AC-3 #2	1/80	8	0	===///===
	Second to last	8	0	===///===
AC-5 #1	1/80	8	0	===///===
	Second to last	5	3	**AC4 + AC7**
AC-5 #2	1/80	8	0	===///===
	Second to last	2	6	**AC4 + AC7**
AC-21 #1	1/80	8	0	===///===
	Second to last	8	0	===///===
AC-21 #2	1/80	8	0	===///===
	Second to last	8	0	===///===
AC-26 #1	1/80	8	0	===///===
	Second to last	8	0	===///===
AC-30 #1	1/80	8	0	===///===
	Second to last	8	0	===///===

The classification of an image as representing a competent-level or expert-level pattern should not be viewed as inherently tied to the analyst's level of experience. Even seasoned analysts frequently encounter HEp-2 IFA images that are challenging to categorize within a specific expert-level pattern. In such cases, it may be more appropriate to assign the image to the corresponding overarching competent-level pattern. It is important to clarify that the terms "competent" and "expert" might unintentionally suggest that expert analysts must always use expert-level patterns, and vice versa. To avoid this misconception, an alternative nomenclature could be considered—one that reflects not the analyst's expertise, but rather the intrinsic complexity of the pattern's morphology and the difficulty of fitting a HEp-2 IFA image into the classification system. These alternative terms could be based in different criteria related to the immunofluorescence images, such as depicted in Table 3.

Table 3. Putative alternative designations to the so-called "Competent-level" and "Expert-level" patterns.

Attribute of reference	Possible alternative designations	
Structural complexity	Basic patterns	Refined patterns
Image resolution	Macro-level patterns	Micro-level patterns
	Broad-classification patterns	Fine-classification patterns
Recognition difficulty	Readily-recognizable patterns	Challenging-to-classify patterns
Resolution or detail	Coarse-level patterns	Fine-level patterns
Descriptive / intuitive	Fundamental patterns	Refined patterns
	Primary patterns	Advanced patterns
	Core patterns	Detailed patterns
Pattern clarity	Well-defined patterns	Subtle patterns
Visual complexity	Simple-morphology patterns	Complex-morphology patterns
	Straight-morphology patterns	Subtle patterns

Discrete classification systems are commonly used across many fields of human activity. ICAP has successfully developed a classification system for patterns observed in the HEp-2 IFA test, categorizing the most frequent images into 32 distinct patterns. Additionally, ICAP has introduced two hierarchical levels of patterns based on the difficulty of their recognition. This is a thoughtful approach, considering the continuous spectrum of possible HEp-2 IFA images, which are modulated by the multiple autoantibodies in the sample and their respective serum concentrations (titers). In practice, competent-level patterns offer a reliable alternative when analysts are uncertain between two closely resembling expert-level patterns. However, the terms "expert" and "competent" may unintentionally imply that the choice of pattern level is determined by the analyst's expertise, whereas it is often dictated by the inherent characteristics of the image itself. Therefore, it is proposed that alternative designations be considered for these two hierarchical categories—terminology that reflects the intrinsic features of HEp-2 IFA images rather than the analyst's level of experience.

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Anti-MDA5 antibodies produce a distinct cytoplasmic pattern on HEp-2 IFA A candidate AC 32?

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Melanoma Differentiation Associated gene-5 (MDA-5) is a cytosolic protein, essential for antiviral host immune responses. Physiologically, MDA-5 is an intracellular protein present in the cytoplasm of most cells with low tissue specificity. Initially identified in 2002, MDA-5 is considered as a key sensor of viral infection (Kang DC. et al. Proc Natl Acad Sci 2002). MDA-5 mediates the production of INF-I and the induction of other genes that collectively produce an antiviral host response.

The occurrence of anti-MDA-5 antibodies in dermatomyositis (DM) was first described by Sato S. et al. (Arthritis Rheum 2005) in Japanese cohort with Clinically Amyopathic DM (CADM) and rapidly progressive interstitial lung disease (RP-ILD). Currently, MDA-5 antibodies are included in the group of "myositis-specific autoantibodies" (MSA) that allow a better definition of patient subgroups in terms of clinical phenotypes, prognosis and response to treatment. The clinical presentation of DM with presence of anti-MDA-5 antibodies (Abs) is different from other DM with three distinct phenotypes according to the predominance of vascular, skin-articular or pulmonary symptoms. Anti-MDA-5 DM is a very rare disease (less than 2% of the idiopathic inflammatory myopathies [IIM] in Europe), and among the DM, the prevalence of anti-MDA-5 DM varies from 7% to 60% according to ethnicity.

From a biological point of view, the presence of anti-MDA-5 antibodies sera can be suspected by IIF on HEp-2 cells (HEp-2 IFA) with a particular cytoplasmic pattern defined as fine speckled cytoplasmic staining in rare clustered cells (Fig. 1). Specific tests for the identification of these anti-MDA5 antibodies are:

- Immunoprecipitation, but not used in current practice, because it is long, expensive and difficult to interpret due to comigration of the MDA-5 antigen with other antigens observed in DM (e.g., NXP2, TIF1 or OJ).
- Line blot assays (immunodot)
- Particle-based multi-analyte assays
- ELISA: a quantitative assay which allows a follow-up of the antibody levels during the course of the disease and therapy.

Actually, this pattern of fluorescence is classified as AC-XX and has to be differentiated from the AC19 pattern (See Educational Module 2 at ICAP website). As anti-MDA5 antibodies produce a distinctive cytoplasmic pattern, could it be a candidate for a new pattern in ICAP classification tree: the AC 32 pattern?

Several arguments allow this distinctive pattern to be a good candidate: 1) there are clear clinical associations/relevance, and usefulness for diagnosis or clinical subgroup classification. 2) this distinct pattern has a clear and novel morphological description and nice pictures are available. 3) we observed good reproducibility using different commercial slides and reproducibility in different laboratories. 4) MDA-5 is a known autoantigen. 6) we have data from solid phase assays supporting Ab specificity for this suggested pattern. 7) a survey among several ICAP members showed that the MDA5-like pattern was recognized by some of the participating laboratories. Of note, upon repeating the HEp-2 IFA test, the MDA5-like pattern was identified in most samples known to be anti-MDA5-positive.

But there are some limitations: 1) This distinct pattern was reported in only about 15% of patients with anti-MDA-5 DM. 2) Even when present, the MDA5-like pattern can be difficult to detect as only a few cells within a well are stained, and the staining intensity varies from + to +++. In some cases, the pattern was seen in just one or three positive cells, rather than in a cluster of cells (Fig.2). In fact, this pattern is difficult to detect in the traditional reading at the microscope and using Computer-Assisted Diagnosis (CAD) systems. 3) Sera from some patients with anti-MDA5 DM (Abs detected by specific assays), could also have additional HEp-2 IFA patterns like granular cytoplasmic pattern in all cells or nuclear speckled pattern, associated or not, with the typical MDA5-like cytoplasmic pattern. 4) Sometimes absence of fluorescence (AC-0) is reported in sera from patients with anti-MDA5 DM.

Because the MDA5-like pattern is observed in just a few scattered cell nests, it is very probable that this pattern is overlooked in the general HEp-2 IFA routine. Therefore, this pattern should be actively sought in samples from patients under suspicion of an IIM. In addition, the identification of this pattern can increase the assertiveness of a positive anti-MDA5 result in an antigen-specific immunoassay, especially when the positive reactivity is of low intensity, e.g., a faint band in the line blot assay.

In conclusion, despite these limitations, anti-MDA-5-like pattern is a good candidate to be integrated into the ICAP classification tree as a new cytoplasmic pattern, namely AC32. While ICAP is working in building a consensus on the matter, laboratories should be informed and analysts be aware of this distinct, rare and difficult to detect pattern, because its identification may be beneficial to the workout of autoantibody investigation in patients suspected of IIM. If such fluorescence is detected, by “chance” or following an active search, laboratories should provide a comment suggesting the search for anti-MDA5 antibodies using an antigenic-specific immunoassay due to the high predictive value of these autoantibodies.

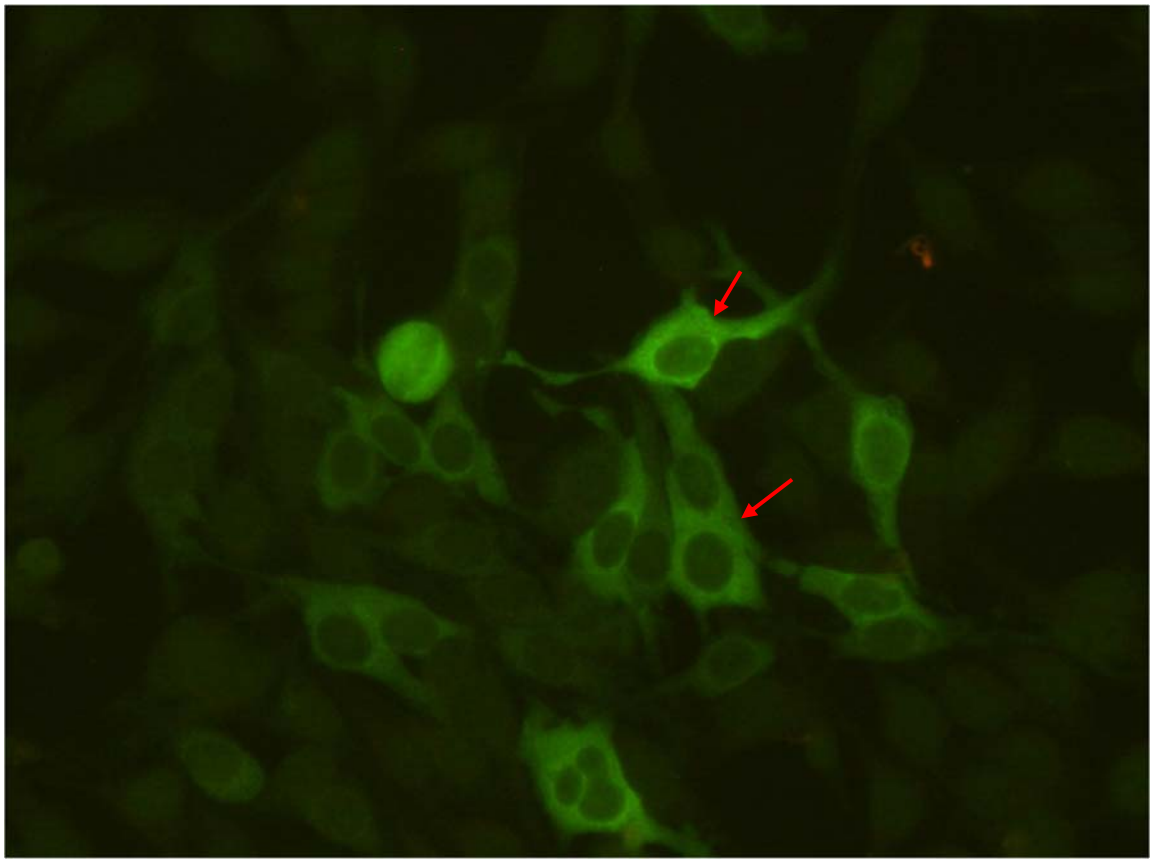

Figure 1. Indirect Immunofluorescence of a positive anti-MDA5 serum (diluted 1:80) on HEp-2 cells (ImmunoConcepts HEp-2000 ™)). Fine granular cytoplasmic staining in rare clustered cells.

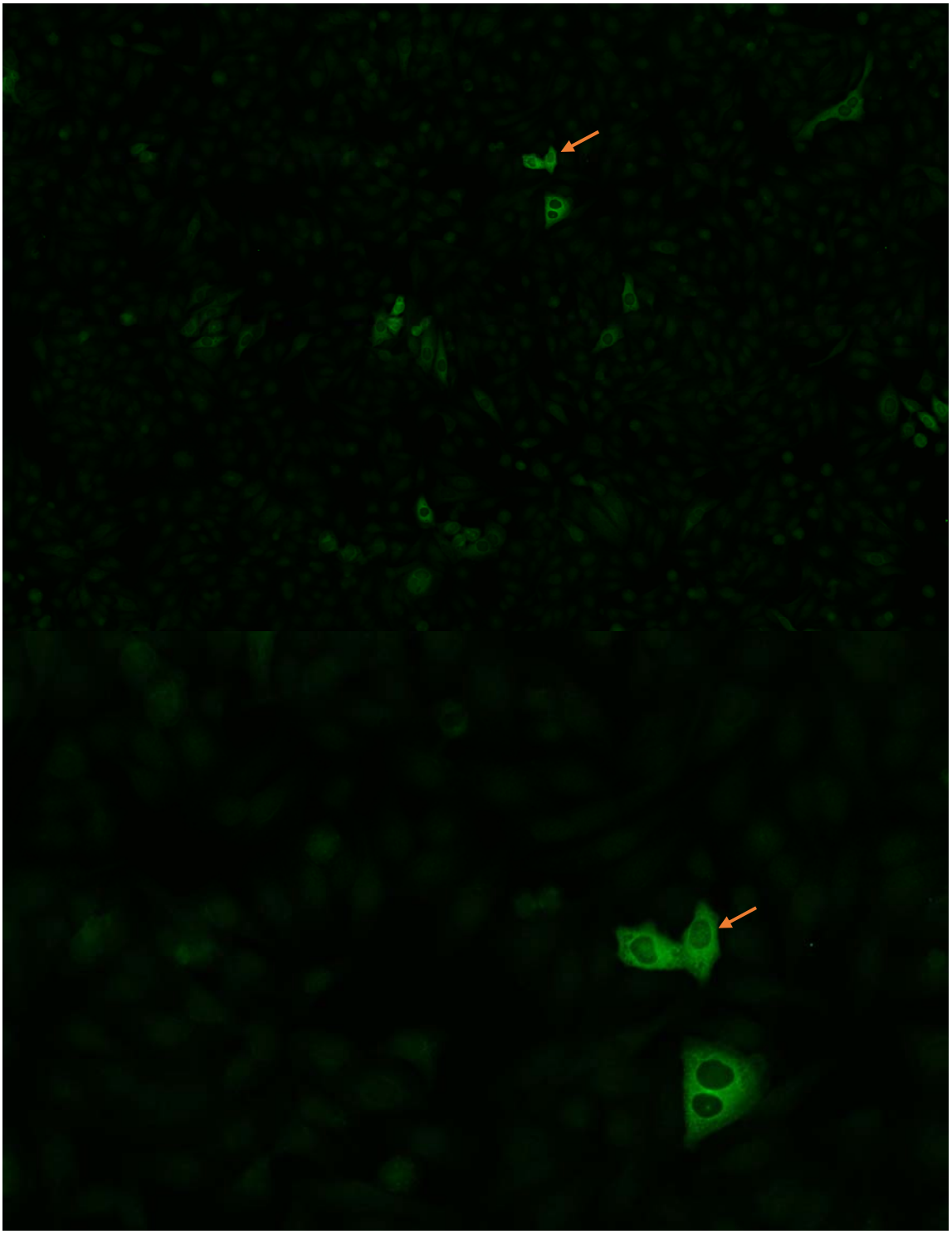

Figure 2. Indirect Immunofluorescence of a positive anti-MDA5 serum (diluted 1:80) on HEp-2 cells (AliveDx HEp-2 cells). A) Fine granular cytoplasmic staining in rare cells. B) Zoom on the positive cells observed in A.

(AliveDx ANA HEp-2 cells performed by the AliveDx LumiQ® system)

Correlation of anti-DFS70 autoantibody with HEp-20-10 cell immunofluorescence assay for nuclear dense fine speckled pattern AC-2

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Background

Over the past two decades, the identification and characterization of the dense fine speckled (DFS; ICAP AC-2) nuclear pattern in antinuclear antibody (ANA) testing-observed using HEp-2 or HEp-20-10 cells in indirect immunofluorescence assays (IFA)-has significantly influenced the landscape of ANA diagnostics. This specific AC-2 pattern is associated with antibodies targeting DFS70 (also known as lens epithelium-derived growth factor, LEDGF). Although the clinical significance of anti-DFS70 antibodies remains under investigation, emerging evidence suggests that high titers of these antibodies, particularly when present in isolation without other disease-specific autoantibodies, may be useful in excluding systemic autoimmune rheumatic diseases (SARDs) or ANA-associated rheumatic diseases (AARDs).(1,2) The correlation between the AC-2 pattern seen by IFA and the detection of anti-DFS70 antibodies through solid-phase assays continues to be an area of active research and clinical interest.

Methods

This study analyzed a total of 514 serum samples, including 464 samples previously characterized as ANA-positive and 50 as ANA-negative based on indirect immunofluorescence assay (IFA) using HEp-20-10 cells (Sprinter XL, EUROIMMUN AG). All samples were further evaluated using both the Anti-DFS70 ELISA (EUROIMMUN AG) and the EUROLINE ANA Profile line blot immunoassay, which includes targets such as DFS70, Mi-2, Ku, nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl100, Jo-1, CENP-B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-proteins, and AMA M2 (EUROBlotOne, EUROIMMUN AG). These assays were employed to detect anti-DFS70 antibodies as well as a broad panel of other disease-associated autoantibodies.

Results

This is the first known study to examine the correlation between anti-DFS70 antibodies, other SARD-associated autoantibodies, and ICAP-defined ANA patterns. Among samples exhibiting the AC-2 pattern, 99% were positive for anti-DFS70 antibodies as detected by either ELISA or EUROLINE. Anti-DFS70 antibodies were also identified in samples with other ANA patterns, including AC-1 (27.7%), AC-4/5/31 (15.9%), and AC-3 (18.2%) (Figure 1).

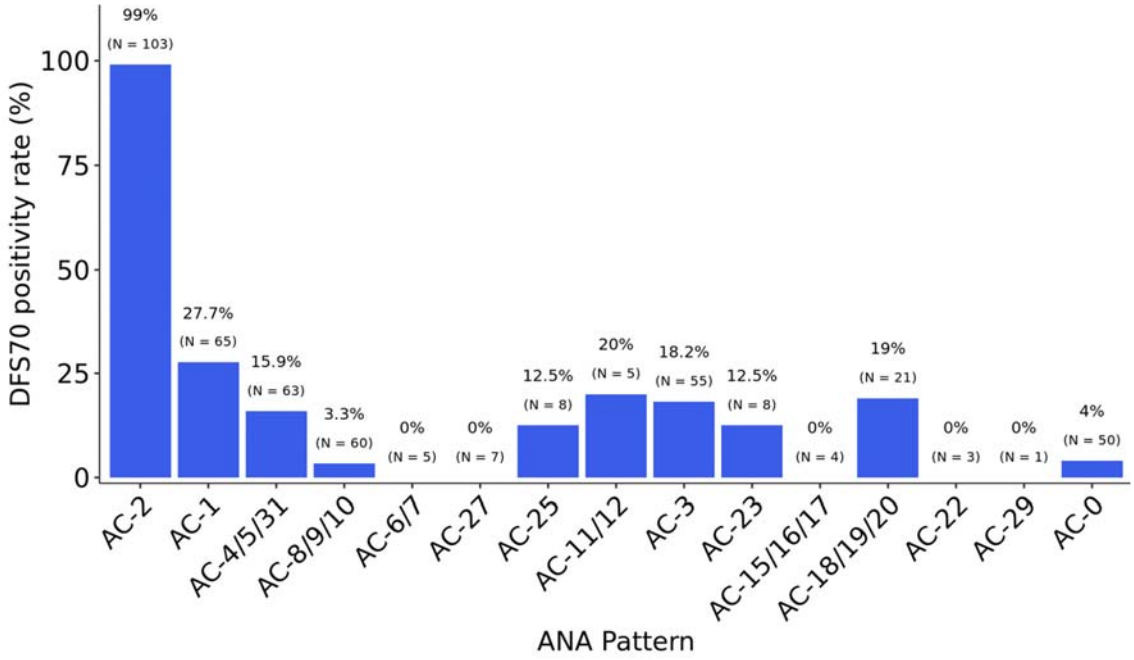

Figure 1. Anti-DFS70 positivity rate across all ICAP patterns

Notably, 4% of ANA-negative samples (AC-0) were positive for anti-DFS70 antibodies. Among all samples positive for anti-DFS70 by either assay, approximately 70% showed an exclusive AC-2 pattern on IFA. However, in samples where AC-2 was the sole ANA pattern observed, multiplex EUROLINE testing revealed that around 30% of patients also harbored additional SARD-associated autoantibodies-most commonly anti-dsDNA, anti-nucleosome, anti-Mi-2, anti-centromere B, anti-Scl-70, and anti-RNP-though these occurred at low frequency (Figure 2).

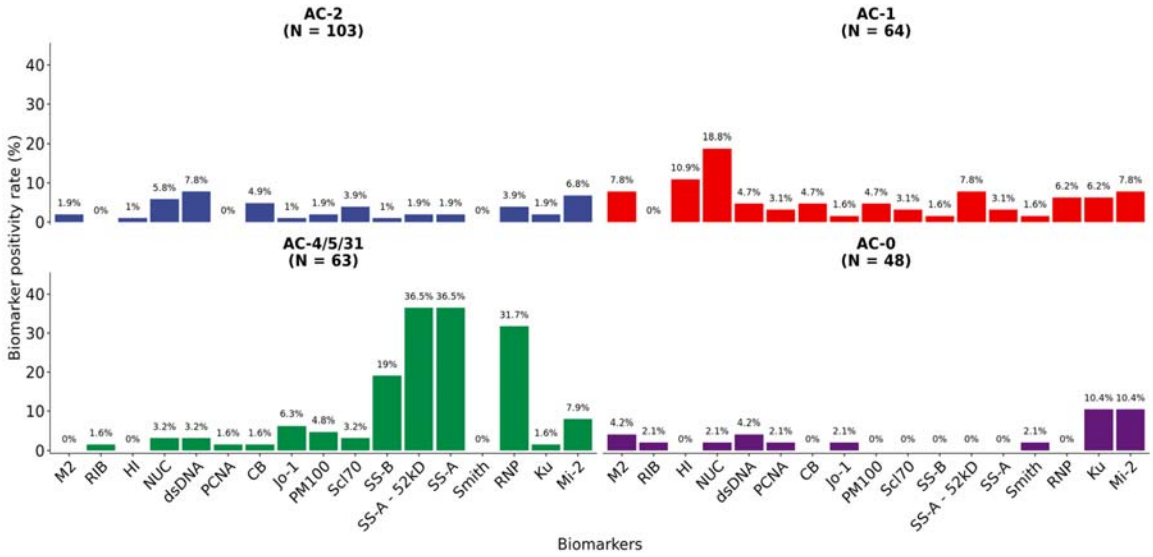

Figure 2. SARD biomarkers antibody positivity rates in AC-2, AC-1, AC-4/5/31, and AC-0

Conclusion

In this study, we demonstrated that nearly all samples exhibiting the AC-2 pattern on HEp-20-10 IFA were positive for anti-DFS70 antibodies, as detected by either ELISA or EUROLINE. While other autoantibodies were occasionally associated with the AC-2 pattern, their frequency was low. Notably, only 70% of AC-2 pattern samples showed anti-DFS70 reactivity in the absence of other SARD-associated antibodies.

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ICAP´s Educational Module on AC-XX Unclassified Patterns

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Background

The International Consensus on ANA Patterns (ICAP) was established in 2024 to harmonize ANA testing and reporting [1]. ICAP has since defined 32 fluorescence patterns, assigning codes AC-1 to AC-31, with AC-0 indicating the absence of specific staining on HEp-2 cells [2, 3, 4].

During routine ANA testing via indirect immunofluorescence assay (IFA), unusual ANA patterns occasionally arise that do not correspond to any known ICAP-defined pattern [3].

To address this, a new training module on the ICAP website has been developed to assist in distinguishing such undefined patterns from established single or composite patterns.

Objectives

This training module aims to clarify the rationale for coding atypical HEp-2 IFA staining patterns under the generic code AC-XX.

Results

AC-XX serves as a provisional code for reporting unclassified HEp-2 IFA patterns, facilitating their subsequent evaluation and classification by ICAP.

AC-XX serves as a temporary solution to report unclassified HEp-2 IFA patterns, allowing for further classification by ICAP. Although the clinical significance of these unclassified patterns remains to be fully elucidated, they may indicate autoimmune disorders and therefore should not be disregarded in diagnostic reports. The module presents eight examples of AC-XX patterns—four nuclear and four cytoplasmic—and provides guidance for differentiating them from ICAP-classified patterns. While providing detailed descriptions of AC-XX patterns is optional, it is recommended to accompany the AC-XX code with a brief pattern description and titer.

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Artificial Intelligence and HEp-2 IFA patterns

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Abstract

Artificial intelligence (AI) is rapidly transforming many aspects of medicine at an unprecedented pace, and rheumatology is no exception. Among its most promising applications is the use of deep learning for image analysis. The antinuclear antibody (ANA) test is traditionally performed using an indirect immunofluorescence assay (IFA) on HEp-2 cells. This method generates immunofluorescent patterns that must be manually interpreted under a microscope, which is a subjective process with considerable interrater variability.

The complexity of ANA pattern recognition has grown significantly over time. The International Consensus on ANA Patterns (ICAP) continues to expand its classification system, now encompassing a broad spectrum of patterns, many of which require substantial expertise and experience to interpret reliably. Laboratories are increasingly challenged by the need to recognize more patterns, which further increases the risk of diagnostic error and inefficiency.

This presentation will explore the emerging role of AI in improving the accuracy and efficiency of ANA pattern interpretation. We will introduce a novel machine learning model designed specifically to analyze ANA images and classify expert-level ANA patterns with a high degree of precision. The model is capable of distinguishing similar or mimicking ICAP patterns using significantly less time compared to manual reading, while maintaining expert-level accuracy. We will discuss the training and validation of this model, review its current performance metrics, and outline the potential implications for clinical practice.

Has there been a change in HEp-2 IFA results (positive tests, patterns, titers) since COVID-19?

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Introduction

Infectious diseases have long been recognized as potential triggers for autoimmune and autoinflammatory conditions, primarily through immunopathological mechanisms such as molecular mimicry, epitope spreading, and bystander activation of immune cells. Several viral agents—including Epstein–Barr virus (EBV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), human T-lymphotropic virus type 1 (HTLV-1), parvovirus B19, herpesviruses, and Zika virus—have well-established associations with the development of autoimmune diseases, acting as immunological triggers (Krishnan et al., 2025).

In this context, SARS-CoV-2 infection has garnered increasing scientific interest due to its potential association with the induction of autoimmune responses and the production of autoantibodies (Kocivnik & Velnar, 2022) (Darmarajan et al., 2022) (Damoiseaux et al., 2022)(Chang et al., 2021) (Sacchi et al., 2021). Studies have shown that individuals with COVID-19 exhibit significantly increased autoantibody reactivity compared to uninfected individuals, with a high prevalence of autoantibodies targeting immunomodulatory proteins, including cytokines, chemokines, complement components, and cell surface proteins. These findings suggest a potential pathological role for autoantibodies directed against the exoproteome in COVID-19, with important implications for immune system functionality and clinical outcomes (Mobasheri et al., 2022).

Motivated by reports indicating a possible association between SARS-CoV-2 infection and antinuclear antibody (ANA) positivity, several studies have sought to elucidate the clinical relevance of these findings (Bilgin et al., 2022) (Peker et al., 2021) (Galipeau et al., 2024). However, interpretation of these data requires caution due to methodological limitations, such as small sample sizes and heterogeneous inclusion criteria. Early studies conducted in 2020 reported ANA seroprevalence ranging from 25% to 57.5% in cohorts of infected individuals (Muratori et al., 2021) (Peker et al., 2021) (Peluso et al., 2022) (Dişli & Yıldız, 2025). Variability in positivity thresholds and the use of different detection methodologies across studies pose additional challenges to the comparison of results (Dişli & Yıldız, 2025) (Notarte et al., 2024) (Wang et al., 2020) (Antinuclear Antibodies Detected by Enzyme-Linked Immunosorbent Assay (ELISA) in Severe COVID-19: Clinical and Laboratory Associations, n.d.).

Conversely, investigations assessing clinically relevant autoantibodies associated with systemic rheumatic diseases—such as anti-dsDNA, anticentromere, antichromatin, anti-ribosomal P, anti-TROVE2/Ro60, anti-SS-B/La, anti-Sm, anti-RNP, anti-Scl-70, and anti-Jo-1—have indicated that acute SARS-CoV-2 infection is not associated with a high prevalence of these markers (Bossuyt et al., 2023). Longitudinal evidence suggests that the presence of these autoantibodies is comparable between patients with severe COVID-19 and uninfected individuals, supporting the hypothesis that autoantibody production may reflect nonspecific immune dysregulation associated with acute systemic inflammation, rather than a SARS-CoV-2-specific autoimmune response (Damoiseaux et al., 2022).

Additionally, population-based studies have reported an increased risk of newly diagnosed autoimmune diseases following the acute phase of SARS-CoV-2 infection. In a cohort of over 640,000 patients with COVID in Germany between 2020 and 2021, 1.1% of those without prior autoimmune diagnoses developed a first autoimmune disease within 3 to 15 months post-infection. Among individuals with pre-existing autoimmunity, 2.3% developed an additional autoimmune condition during the same period. This study employed two principal control cohorts: healthcare workers (HCWs) and pre-pandemic healthy individuals. The HCW group consisted of healthcare professionals who were longitudinally monitored for the development of autoantibodies after SARS-CoV-2 infection. Some participants were initially seronegative for anti-SARS-CoV-2 IgG and subsequently seroconverted prior to vaccination, while others were already seropositive at baseline and reported a range of post-COVID-19 symptoms. Therefore, this group included individuals with confirmed prior SARS-CoV-2 infection (Tesch et al., 2023). A study conducted in the Stockholm metropolitan area similarly identified the emergence of de novo autoantibodies with broad antigenic specificity following SARS-CoV-2 infection, which remained elevated for at least 12 months. These autoantibodies were more prevalent in severe COVID-19 cases and were associated with neuropsychiatric symptoms in the post-acute phase (Jernbom et al., 2024). Epitope mapping study revealed sequence similarities suggestive of molecular mimicry between the autoantibody-recognized epitopes and the conserved fusion peptide of the SARS-CoV-2 Spike glycoprotein (Nunez-Castilla et al., 2022).

Four years after the onset of the COVID-19 pandemic, considerable uncertainty remains regarding the impact of SARS-CoV-2 infection on the induction of autoantibodies. This study presents a retrospective analysis of morphological patterns observed in indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) results from 2019 to 2024, aiming to investigate potential shifts in overall positivity rates, fluorescence patterns—as classified according to the International Consensus on ANA Patterns (ICAP) (https://anapatterns.org/), and titers observed in HEp-2 IFA.

In addition, the HEp-2 IFA results were compared in a nested cohort of individuals with and without serologic and molecular evidence of SARS-CoV-2 infection.

Methodology and Dataset

All HEp-2 IFA results recorded in the database of a major clinical laboratory in Brazil, Grupo Fleury, from 2019 to 2024 were stratified into two cohorts: (1) HEp-2 IFA General, comprising all records with HEp-2 IFA results from 2020 to 2024, and (2) Records with HEp-2 IFA results associated with PCR and/or serological testing for SARS-CoV-2. This second group was further subdivided into Exposed (individuals with SARS-CoV-2 positive results by PCR and/or serological tests) and Non-Exposed (individuals with negative SARS-CoV-2 test results), referred to as SARS-CoV-2 Exp and SARS-CoV-2 Non-Exp, respectively. No data referring to identification of the individuals was made available to the researchers, thereby protecting their identity. Accordingly, informed consent was dismissed by the Ethics Committee.

For the purpose of temporal analysis, the study period was categorized into three distinct phases based on the global progression of the COVID-19 pandemic: (i) pre-pandemic (year 2019), representing the baseline period prior to the World Health Organization's declaration of the pandemic in March 2020; (ii) pandemic (years 2020–2022), encompassing the peak of viral transmission, implementation of public health measures, and mass vaccination campaigns; and (iii) post-pandemic (years 2023-2024), characterized by epidemiological stabilization at lower rates of SARS-CoV-2 infection. In both cohorts we analyzed the HEp-2 IFA results regarding frequency of positive results, pattern, and titer.

To evaluate differences in categorical variables—such as positivity rates and the distribution of fluorescence patterns over the assessed period—the chi-square test (χ^2 test) was applied. This statistical method is appropriate for investigating the presence of significant associations between two or more categorical variables, such as SARS-CoV-2 testing and HEp-2 IFA outcomes. The test compares the observed frequencies in each category with the expected frequencies under the assumption of independence, allowing for the determination of whether the observed differences are statistically significant. Conditional formatting was also employed to construct heatmaps, highlighting the gradient of HEp-2 IFA pattern frequencies over the evaluated time period.

The HEp-2 IFA was performed using slides from AESKU Diagnostics GmbH & Co. KG (Wendelsheim, Germany), strictly following the manufacturer's technical instructions. Whenever results confirmation was required, HEp-2 cell slides produced in-house by the laboratory staff were employed. As of 2024, AESKU slides were replaced by slides from EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany) into the routine, used in conjunction with the internally produced slides. The College of American Pathologists (CAP) proficiency testing program is regularly used as external quality control for HEp-2 IFA, with satisfactory performance throughout the evaluated period. Additionally, each testing session included a set of homemade internal controls, which were recorded daily using the SoftQC software. Readings were performed by a team of 12 independent analysts, all of whom underwent rigorous training and participate in a weekly harmonization meeting. Each sample was analyzed at the microscope by a minimum of two different analysts and conflicting cases were discussed with the senior analyst. Immunofluorescence analyses were conducted using Axioscope microscopes (Carl Zeiss

Microscopy GmbH, Jena, Germany), equipped with LED light sources and 40x objectives, ensuring high-resolution imaging and consistency in the interpretation of fluorescence patterns.

Results

A total of 1,262,824 HEp-2 IFA tests were processed between 2019 and 2024 (referred to as the HEp-2 IFA-General group), regardless of confirmatory testing for SARS-CoV-2 exposure. Among the results obtained, 929,075 (73.57%) were classified as negative, 272,310 (21.56%) as positive, and 61,439 (4.87%) were excluded from the analysis due to the presence of multiple fluorescence patterns, which pose greater classification complexity within the framework established by the ICAP system. The annual distribution of relative frequencies of positive and negative results revealed a small but statistically significant difference ($p < 0.0001$) along the years from 2019 to 2024 (frequency of positive results ranging from 22.01% to 23.26%), as illustrated in figure 1. However, this difference had an oscillating character across the years and did not show a consistent trend that could be related to the COVID-19 pandemic period.

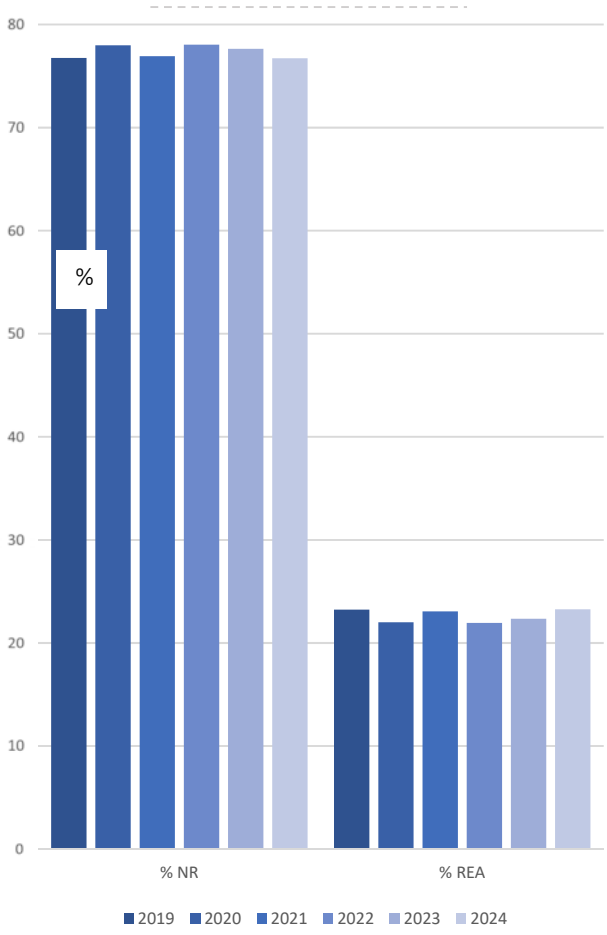

Figure 1. Annual distribution of the relative frequencies of positive and negative HEp-2 IFA results from 2019 to 2024. NR = non-reactive; REA = reactive. A statistically significant difference was observed across the years ($p < 0.0001$).

Based on the annual frequencies of results in the HEp-2 IFA-General group, the frequency of each ICAP pattern (AC-1 to AC-31) was calculated for the years 2019 through 2024. These results were compiled into a temporal table with conditional formatting using a heatmap with a color gradient—from dark blue (most prevalent) to light blue (least prevalent)—to visually highlight the distribution of pattern frequencies over time. Consequently, the patterns are not presented in ascending numerical order (AC-1 to AC-31), rather in a decreasing order of frequency of positive results. All data were subjected to statistical analysis to assess the significance of temporal variations in pattern frequencies (Table 1).

ICAP	2019	2020	2021	2022	2023	2024	AVERAGE
AC-4	30,03	29,12	32,46	28,48	27,25	31,16	29,77
AC-30	22,38	24,67	25,11	29,07	28,73	27,57	26,51
AC-2	19,97	19,81	16,81	16,08	16,83	13,56	16,85
AC-1	5,22	5,08	4,77	5,85	6,4	6,28	5,68
AC-8/9/10	5,08	5,93	6,04	3,47	3,37	4,3	4,59
AC-31	3,11	3,33	3,19	3,54	3,34	2,83	3,21
AC-3	2,12	2,15	1,98	2,14	2,12	1,98	2,08
AC-21	1,61	1,59	1,66	2,12	2,13	2,08	1,9
AC-5	1,44	1,42	1,32	1,42	1,47	1,27	1,39
AC-11/12	1,22	1,08	1,11	1,28	1,21	1,13	1,18
AC-7	1,06	0,81	0,68	1,06	0,98	1,1	0,96
AC-15/16/17	1,47	0,59	0,64	0,76	0,71	0,86	0,83
AC-18	0,87	0,65	0,64	0,78	1,01	0,78	0,8
AC-22	0,84	0,54	0,64	0,5	0,42	0,62	0,59
AC-19	0,63	0,62	0,56	0,53	0,52	0,62	0,58
AC-20	0,69	0,49	0,54	0,57	0,56	0,51	0,56
AC-26	0,55	0,53	0,45	0,49	0,56	0,44	0,5
AC-6	0,41	0,43	0,39	0,33	0,39	0,46	0,4
AC-27	0,11	0,17	0,12	0,4	0,79	0,57	0,39
AC-25	0,38	0,28	0,22	0,38	0,38	0,55	0,37
AC-29	0,31	0,29	0,29	0,31	0,33	0,25	0,29
AC-23	0,13	0,11	0,11	0,05	0,12	0,84	0,26
AC-24	0,29	0,2	0,15	0,22	0,23	0,14	0,2
AC-14	0,05	0,1	0,11	0,14	0,15	0,1	0,11
AC-13	0,02	0,01	0,02	0,02	0,01	0,01	0,01

Table 1. Annual frequency of HEp-2 IFA patterns in the HEp-2 IFA General group across the 2019-2024 interval. Conditional formatting using a color temperature scale, where darker shades of blue indicate higher frequency and lighter shades indicate lower frequency.

It was observed that all patterns exhibited statistically significant variations in their frequencies throughout the evaluated period ($p < 0.0001$), with the exception of pattern AC-13, whose frequency remained stable ($p = 0.721$), indicating no relevant fluctuation. Nevertheless, the magnitude of the differences in the frequency was small for each pattern along the years. The nuclear patterns AC-4 and AC-30 were the most prevalent, with average frequencies of 29.77% and 26.51%, respectively, followed by AC-2 (16.85%), AC-1 (5.68%), and the grouped nucleolar patterns AC-8/9/10 (4.59%).

Regarding the most prevalent HEp-2 IFA patterns (AC-4, AC-30, AC-2, and AC-8/9/10), we analyzed the dynamics of their frequencies across distinct phases of the evaluated timeline: Pre-pandemic (2019), Pandemic (2020 to 2022), and Post-pandemic (from 2023 onward). A statistically significant variation in these frequencies was observed across the pandemic phases ($p<0.0001$), indicating that the temporal distribution of frequency of these patterns was not homogeneous. Pattern AC-4 reached its highest frequency in 2021 (32.46%), followed by a decline in subsequent years and a new increase in 2024 (31.16%). Pattern AC-30 showed a continuous upward trend from 2019 to 2022, later stabilizing and exhibiting an accrual increase of 5.19 percentage points over the study period. In contrast, pattern AC-2 demonstrated a consistent downward trend, decreasing by 6.41 percentage points—from 19.97% in 2019 to 13.56% in 2024. Patterns AC-1 and AC-8/9/10 remained relatively stable throughout the analyzed years. These trends are illustrated in figure 2.

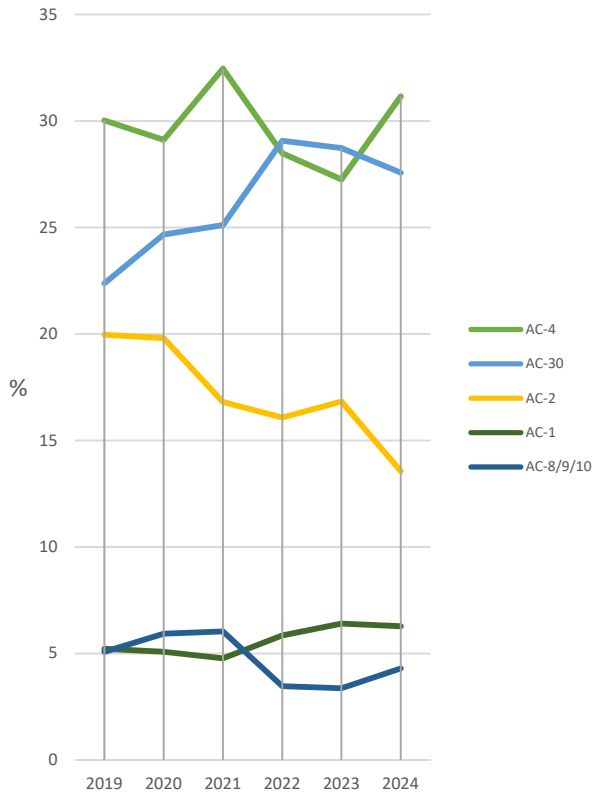

Figure 2. Temporal dynamics of the frequency of the most prevalent HEp-2 IFA patterns from 2019 to 2024 in the HEp-2 IFA General group: AC-4 $p<0.0001$; AC-30 $p<0.0001$; AC-2 $p<0.0001$; AC-1 $p<0.0001$; AC-8/9/10 $p<0.0001$.

Additionally, a ranking analysis was conducted to evaluate whether patterns AC-2, AC-4, and AC-30 exhibited similar temporal behaviors. It was observed that patterns AC-4 and AC-30 switched positions in the ranking during 2022 and 2023; however, in 2024, they returned to the same order observed between 2019 and 2021. In contrast, pattern AC-2 remained stable, consistently occupying the same position throughout the entire study period (Table 2).

Table 2. Relative position of AC-2, AC-4, and AC-30 patterns in the annual frequency ranking (2019–2024) in the HEp-2 IFA General group.

Year	1st place (%)	2nd place (%)	3rd place (%)
2019	AC-4 (30.02)	AC-30 (22.38)	AC-2 (19.65)
2020	AC-4 (29.19)	AC-30 (24.67)	AC-2 (19.80)
2021	AC-4 (32.46)	AC-30 (25.11)	AC-2 (16.80)
2022	AC-30 (29.07)	AC-4 (28.48)	AC-2 (16.07)
2023	AC-30 (28.73)	AC-4 (27.25)	AC-2 (16.83)
2024	AC-4 (31.15)	AC-30 (27,57)	AC-2 (13.56)

Considering the fact that the AC-2 and AC-30 patterns have close resemblance, and both stain the metaphase plate, it is interesting to observe that they showed an opposite trend along the period. As the acknowledgement of the AC-2 pattern antedates that of the AC-30 pattern, it is possible that the observed dynamics in AC-2/AC-30 patterns reflects the progressive acquaintance of the staff with AC-30 and, therefore, might not be related to the COVID-2 pandemics.

Among the most frequent HEp-2 IFA patterns, we also evaluated the distribution of titers across the different phases of the study period. A statistically significant variation was observed in the frequencies of all HEp-2 IFA pattern titers over the analyzed years ($p<0.0001$) (figure 3).

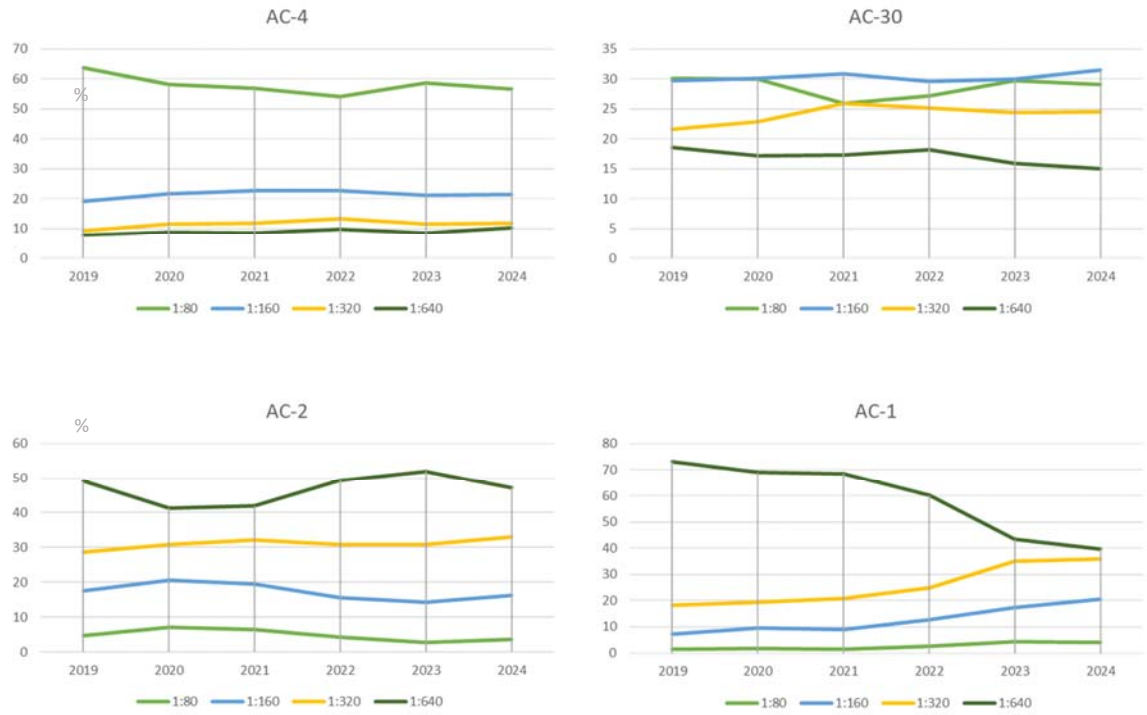

Figure 3. Relative frequency of titers associated with patterns AC-1, AC-2, AC-4, and AC-30 over the years. ($p<0.0001$).

For pattern AC-4, the 1:80 titer was the most frequent throughout the entire study period. In the case of pattern AC-30, the predominant titer was 1:160, consistently remaining the most prevalent across the years. Pattern AC-2 showed the highest frequency at the 1:640 titer, with a relatively stable distribution over time. Similarly, pattern AC-1 also exhibited 1:640 as the most frequent titer, although a reduction was observed in 2024.

The next stage of the analysis focused on the cohort with results from HEp-2 IFA and COVID-19 diagnostic tests (RT-PCR and/or serology), aiming to specifically investigate potential associations between SARS-CoV-2 exposure and HEp-2 IFA patterns. Among the 929,075 individuals with HEp-2 IFA tests performed between 2020 and 2024, 214,019 (23.04%) had requests for COVID-19 detection tests within six months before the HEp2-IFA test. The COVID-19 tests comprised RT-PCR and/or serology, which were used as criteria to confirm or exclude SARS-CoV-2 exposure. In this cohort, the frequency of positive HEp-2 IFA was 22.2% in the SARS-CoV-2-exposed individuals and 22.9% in the individuals with negative results for COVID-19 (SARS-CoV-2 non-exposed group), showing that individuals exposed to SARS-CoV-2 had a slight but statistically significant decrease in the rate of positive HEp-2 IFA results ($p<0.001$) (Table 3).

Table 3. Relative frequency of positive HEp-2 IFA among individuals exposed and unexposed to SARS-CoV-2, from 2020 to 2024.

SARS-CoV-2 Exposure	Positive HEp-2 IFA	Negative HEp-2 IFA
Yes	12,232 (22.2%)	42,923 (77.8%)
No	36,404 (22.9%)	122,460 (77.1%)

The annual relative distribution profile of negative and positive HEp-2 IFA results in SARS-CoV-2 exposed and non-exposed individuals remained stable throughout the 2020–2024 period (figure 4).

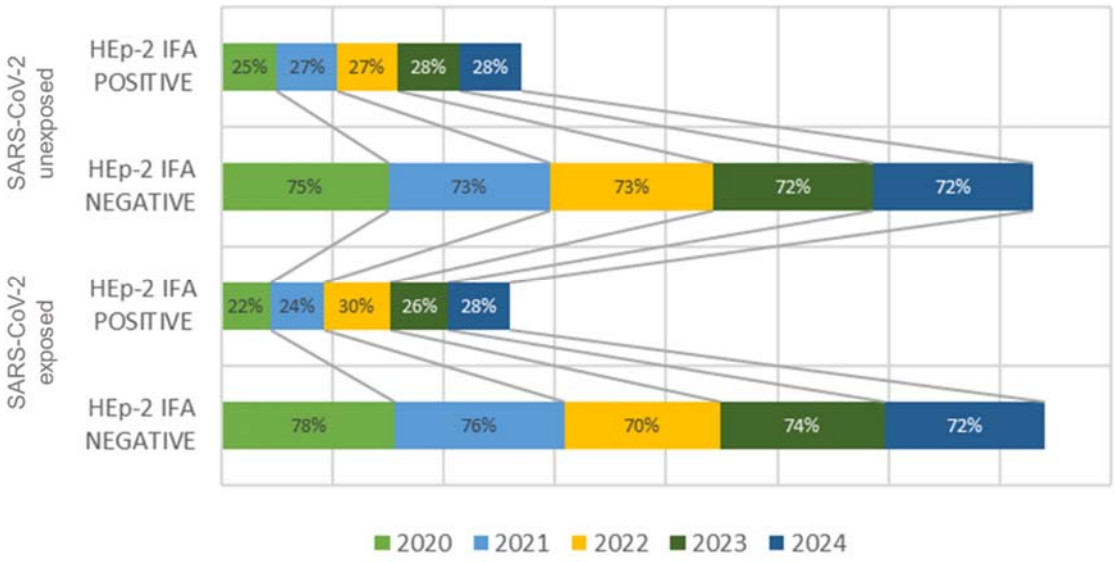

Figure 4. Annual relative distribution of the combinations between SARS-CoV-2 exposure status (based on positive or negative PCR and/or serology) and HEp-2 IFA results.

The analysis of the annual relative frequencies of HEp-2 IFA patterns in the SARS-CoV-2-Exp and SARS-CoV-2-Non-Exp groups revealed a general distribution similar to that observed in the overall HEp-2 IFA General group. Patterns AC-4 (28.94%), AC-30 (27.79%), AC-2 (18.95%), and AC-1 (5.09%) were the most prevalent, followed by the AC-8/9/10 combination (4.19%) (Table 4). In the SARS-CoV-2-Exp group, pattern AC-4 showed a declining trend over the period, decreasing from 33.2% in 2020 to 26.5% in 2023, with a slight recovery in 2024 (29.5%). Pattern AC-30 exhibited a marked increase, rising from 16.5% in 2020 to a peak of 30.1% in 2023, followed by a slight decrease in 2024 (27.7%). Pattern AC-2 displayed a continuous downward trajectory, dropping from 28.5% in 2020 to 17.2% in 2024, while pattern AC-1 showed a gradual and modest increase from 3.4% to 5.2% over the same period. The frequency of the AC-8/9/10 group remained relatively stable until 2023, with a more evident increase in 2024 (5.8%) (figure 5).

Table 4. Annual relative frequencies of HEp-2 IFA patterns in SARS-CoV-2 exposed and non-exposed individuals from 2020 to 2024 ($p<0.0001$).

	2020		2021		2022		2023		2024		AVERAGE
	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed	Exposed	Unexposed	
AC-4	33,25	31,55	33,68	32,27	27,5	27,88	26,47	25,9	29,48	29,55	28,94
AC-30	16,49	19,59	26,67	25,74	29,14	30,1	30,13	29,09	27,67	27,5	27,79
AC-2	28,53	26,06	20,08	19,82	20,54	17,51	18,88	19,77	17,18	16,4	18,95
AC-1	3,4	4,39	2,83	3,91	4,06	5,35	5,43	6,07	5,16	5,79	5,09
AC-8/9/10	3,66	3,84	3,66	3,91	3,9	3,66	3,57	4,22	5,82	4,84	4,19
AC-31	3,4	3,29	2,78	3,39	2,96	3,57	2,49	2,89	2,36	2,69	3,01
AC-21	1,57	1,48	0,93	1,46	2,16	1,97	1,8	1,92	1,5	2,06	1,8
AC-3	3,66	1,52	1,6	1,7	1,74	1,74	2,28	1,69	1,7	1,89	1,78
AC-11/12	1,05	1,22	1,13	1,02	1,45	1,12	1,29	1,05	1,15	1,01	1,11
AC-7	0,26	0,89	0,72	0,74	1,48	1,23	1,11	1,21	0,95	1,3	1,11
AC-5	0,79	1,1	1,18	1,14	1,13	0,96	1,2	0,82	1,07	0,9	1
AC-15/16/17	0,79	0,93	0,82	0,87	0,71	0,78	0,69	0,72	0,89	0,77	0,79
AC-18	0,52	0,68	0,72	0,59	0,64	0,67	1,05	0,91	0,81	0,63	0,73
AC-20	0,26	0,34	0,62	0,63	0,39	0,61	0,48	0,48	0,52	0,53	0,53
AC-27	0	0,17	0,26	0,15	0,32	0,37	0,81	0,83	0,78	0,58	0,5
AC-22	0,26	0,3	0,36	0,68	0,35	0,43	0,3	0,36	0,46	0,52	0,45
AC-19	0	0,89	0,46	0,56	0,26	0,45	0,21	0,41	0,23	0,44	0,43
AC-26	0,26	0,3	0,26	0,46	0,26	0,46	0,45	0,5	0,4	0,36	0,42
AC-25	0	0,59	0,05	0,24	0,32	0,36	0,27	0,29	0,2	0,47	0,32
AC-6	0,26	0,21	0,36	0,2	0,23	0,23	0,42	0,22	0,61	0,42	0,3
AC-23	1,05	0,04	0,36	0,05	0,06	0,05	0,03	0,11	0,66	0,75	0,25
AC-29	0,26	0,08	0,05	0,16	0,13	0,24	0,3	0,32	0,32	0,23	0,23
AC-24	0,26	0,21	0,15	0,21	0,26	0,19	0,24	0,18	0	0,24	0,2
AC-14	0	0,3	0,21	0,05	0,03	0,06	0,09	0,03	0,06	0,09	0,08
AC-13	0	0,04	0,05	0,04	0	0,02	0	0,01	0,03	0,03	0,02

Among individuals not exposed to SARS-CoV-2, similar trends were observed for patterns AC-4 and AC-2, both showing a downward trajectory until 2023, followed by a slight recovery in 2024. Pattern AC-30 increased until 2022 (30.1%), followed by a modest decline in the subsequent years. Pattern AC-1 exhibited a progressive increase, reaching 5.8% in 2024, while the AC-8/9/10 group remained stable throughout the period, with a slight and consistent year-over-year increase (figure 5).

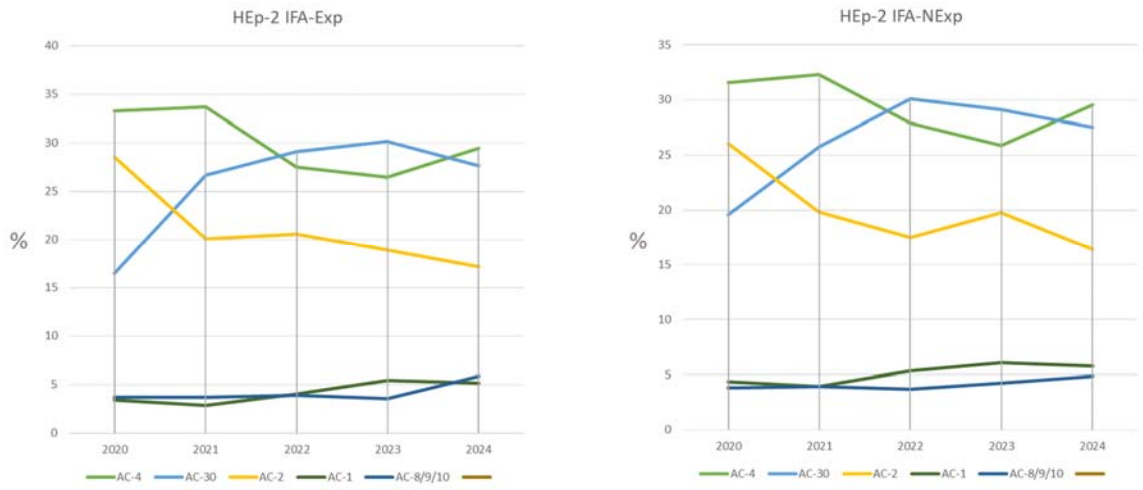

Figure 5. Annual relative frequencies of the most prevalent HEp-2 patterns in the SARS-CoV-2-Exp and SARS-CoV-2-Non-Exp groups.

Although HEp-2 IFA patterns AC-4, AC-30, and AC-2 were the most prevalent during the analyzed period—with relative frequencies of 28.94%, 27.79%, and 18.95%, respectively—statistical analysis indicated that none of these three prevalent patterns were significantly associated with SARS-CoV-2 exposure. In contrast, patterns AC-19, AC-1, AC-31, AC-6, and AC-25 presented p-values below 0.05, indicating a statistically significant association with exposure status (Table 5). Among these, the frequencies of AC-1, AC-19, AC-25, and AC-31 were negatively associated with exposure to SARS-CoV-2, whereas the frequency of AC-6 was positively associated with exposure to the virus (figure 6 & 7).

Table 5. Strength of a (Odds Ratio) between the frequency of HEp-2 IFA patterns and SARS-CoV-2 exposure.

ICAP	*p*-value	Odds Ratio	OR 95% CI Lower	OR 95% CI Upper
AC-19	<0,001	0,53	0,36	0,77
AC-1	<0,001	0,85	0,77	0,94
AC-31	0,01	0,84	0,74	0,95
AC-6	0,01	1,54	1,09	2,16
AC-25	0,02	0,61	0,40	0,93
AC-11/12	0,08	1,19	0,98	1,43
AC-5	0,10	1,18	0,97	1,44
AC-22	0,12	0,77	0,55	1,07
AC-21	0,19	0,90	0,77	1,05
AC-23	0,19	1,30	0,88	1,91
AC-26	0,20	0,80	0,57	1,13
AC-3	0,22	1,10	0,95	1,28
AC-18	0,22	1,16	0,92	1,46
AC-27	0,23	1,19	0,90	1,57
AC-30	0,23	1,03	0,98	1,08
AC-2	0,24	1,03	0,98	1,09
AC-24	0,36	0,79	0,48	1,30
AC-20	0,40	0,88	0,66	1,18
AC-8/9/10	0,44	1,04	0,94	1,15
AC-13	0,50	0,60	0,13	2,72
AC-7	0,67	0,96	0,79	1,17
AC-14	0,79	1,10	0,53	2,28
AC-29	0,84	0,96	0,62	1,48
AC-15/16/17	0,88	0,98	0,78	1,24
AC-4	0,99	1,00	0,96	1,05

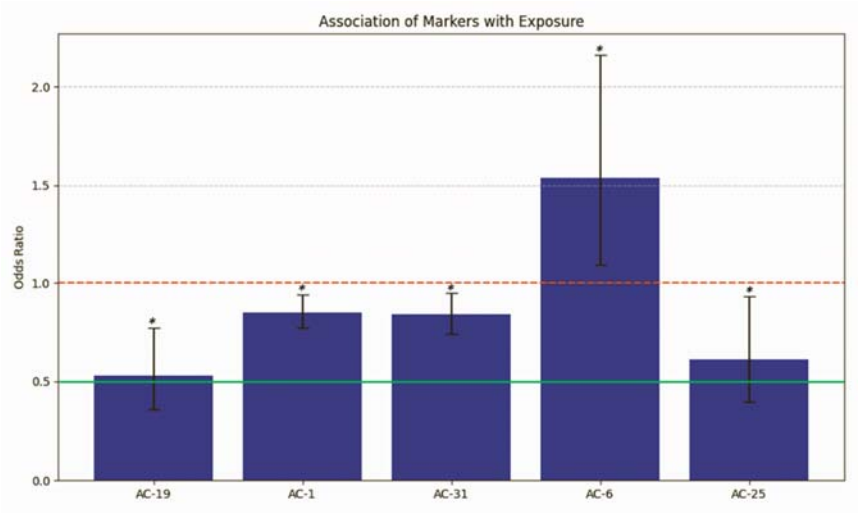

Figure 6. Strength of association (Odds Ratio) between the frequency of HEp-2 IFA patterns and previous exposure to SARS-CoV-2. Dark blue bars represent the odds ratio (OR) values for each HEp-2 IFA pattern. Vertical lines indicate the 95% confidence intervals (CI); The dashed red line at OR = 1 marks the point of neutrality (no association); * p<0.05.

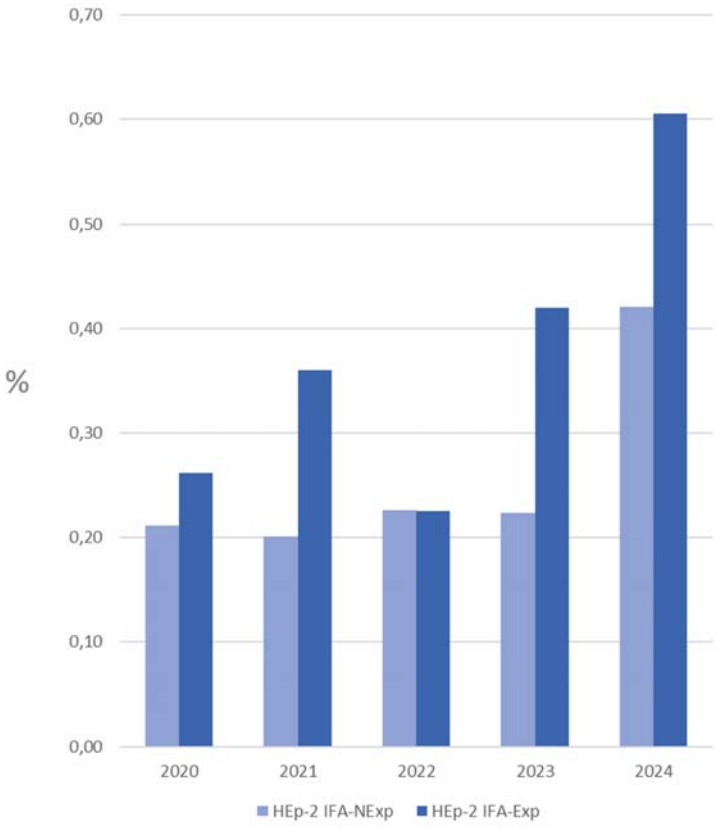

Figure 7. - Relative frequencies of the AC-6 pattern among the SARS-CoV-2-Exp, SARS-CoV-2-NON-Exp, and HEp-2 IFA General groups from 2022 to 2024.

Discussion

This large-scale retrospective analysis, based on over 1.2 million HEp-2 IFA results stratified into pre-pandemic (2019), pandemic (2020–2022), and post-pandemic (from 2023 onward) periods, provides unprecedented statistical power to explore subtle yet meaningful changes in positivity rates, fluorescence patterns, and autoantibody titers. However, it is essential that such findings be interpreted with caution, taking into account the distinction between causality and coincidence.

The temporal analysis revealed that the overall positivity rate of HEp-2 IFA-General group exhibited statistically significant fluctuations, though without a direct association with any specific year, suggesting relative stability in the global landscape and no evident association with the COVID-19 pandemics. In contrast, certain specific patterns—particularly AC-4, AC-30, and AC-2—demonstrated distinct dynamics across the pandemic phases. The AC-4 pattern peaked in 2021, followed by a decline and a slight rebound in 2024, potentially indicating a causal association with SARS-CoV-2 infection. The AC-30 pattern showed a continuous increase throughout the period; however, this trend may reflect a technical coincidence, possibly related to improved recognition of the fine speckled pattern with positive metaphase chromosomal plate (AC-30) by the reading team. The AC-2 pattern, in turn, exhibited a steady decline, reinforcing the possibility of improved accuracy in the identification and interpretation of AC-30 and AC-2 patterns. Meanwhile, the AC-1 and AC-8/9/10 patterns remained stable, with no evidence of causal or coincidental influence.

The comparison between individuals exposed and unexposed to SARS-CoV-2 (SARS-CoV-2-Exp and SARS-CoV-2-Non-Exp) revealed parallel trends in certain ICAP patterns, such as AC-4, AC-2, and AC-1, alongside subtle temporal differences. For instance, pattern AC-30 showed higher frequencies among exposed individuals starting in 2021, suggesting the influence of external or time-dependent factors. Pattern AC-1 exhibited a gradual increase in both groups, with a slight predominance among unexposed individuals in 2024. The AC-8/9/10 group remained stable across both contexts, with a modest increase more evident among exposed individuals in the final year analyzed. These findings suggest that most patterns followed similar trajectories regardless of viral exposure, although specific shifts may reflect immunological modulation associated with infection.

Statistical association analysis between SARS-CoV-2 exposure and specific ICAP patterns revealed a positive association for AC-6, while AC-19, AC-1, AC-31, and AC-25 showed inverse associations. Notably, pattern AC-6 may reflect the generation of autoantibodies targeting nuclear proteins such as Sp100, PML, and MJ/NXP-2. It is well established that viral infections can trigger systemic inflammatory responses characterized by excessive production of pro-inflammatory cytokines and polyclonal activation of B lymphocytes (Castleman et al., 2023). This dysregulated immunological environment favors the breakdown of central and peripheral immune tolerance, promoting the emergence of autoantibodies against nuclear antigens. The exposure of these autoantigens—often associated with cellular damage and nuclear body reorganization induced by viruses with nuclear tropism—enhances their immunogenicity and facilitates recognition by the adaptive immune system (Saheb Sharif-Askari et al., 2021).

Moreover, the molecular mechanisms linking these proteins to viral agents illustrate the complex interplay between host cellular defenses and viral evasion strategies. The nuclear bodies (NBs) formed by the PML protein serve as critical interaction hubs, influencing both viral replication and host immune responses. PML is essential for PML-NB formation and plays a pivotal role in antiviral

defense by interacting with viral components and participating in the organization of replication compartments, as observed in herpes simplex virus (HSV) infections (Xu & Roizman, 2017). Sp100 functions as a restriction factor against viruses such as HPV and is also targeted for degradation by viral proteins, including HSV's ICP0 (Guion et al., 2019). Several DNA viruses, including adenovirus and HPV, manipulate PML NBs to facilitate their replication, recruiting PML and Sp100 to promote viral transcription and neutralize their antiviral effects (Berscheminski et al., 2014). Both proteins are positively regulated by interferons, reinforcing their role in host immune responses, although many viruses induce their degradation as an immune escape strategy. This functional duality underscores the evolutionary dynamics between host defenses and viral countermeasures, constituting a molecular arms race. The documented interaction of viral proteins and PML-NB constituents may render sp100 and PML proteins autoimmunogenic, thereby justifying the observed association between SARS-CoV-2 exposure and increased frequency of the AC-6 pattern, which is known to represent the PML-NBs.

Finally, the annual stability in the distribution of HEp-2 IFA results relative to SARS-CoV-2 testing status suggests that, although the pandemic may have influenced specific autoantibody profiles, it did not fundamentally alter the overall landscape of results of the HEp-2 IFA testing. This finding supports the hypothesis that SARS-CoV-2 is not a primary driver of systemic autoimmunity, in line with longitudinal studies demonstrating transient elevations in autoantibodies without progression to overt autoimmune disease (Jernbom et al., 2024) (Saheb Sharif-Askari et al., 2021)

In conclusion, this study provides robust evidence that the COVID-19 pandemic may have influenced the dynamics of certain autoantibody patterns at the population level, particularly those associated with nuclear proteins involved in antiviral responses. However, the absence of structural changes in the global HEp-2 IFA positivity profile and the stability of several patterns suggest that these effects are specific rather than generalized. The retrospective design, lack of clinical data, and potential underreporting of SARS-CoV-2 exposure limit more definitive causal inferences. Future studies integrating clinical data, longitudinal follow-up, and functional assays will be essential to elucidate the underlying immunological mechanisms and determine the clinical relevance of these observations.

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Ten years of UK NEQAS external quality assessment on HEp-2 IFA: impact of ICAP and AI

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Abstract

External quality assurance (EQA) programs play a crucial role in monitoring laboratory practices, allowing each laboratory to evaluate the consistency of results across different methods as well the ability of individual laboratories to compare and improve over time their own performance. Autoimmune diagnostics has evolved considerably over the past decade, with advances in technology, improved standardisation, and enhanced harmonisation efforts.

The aim of our study was to analyze the UK NEQAS EQA reports for the “Antibodies to Nuclear and Related Antigens” program from 2013 to 2023, to assess the overall level of harmonization of the responses for anti-nuclear antibody (ANA) testing by indirect immunofluorescence (IIF), in terms of both pattern and titer consensus. As a second aim, we analyzed the impact of the introduction in UK NEQAS EQA reports of the International Consensus on ANA Patterns (ICAP) nomenclature and of the artificial intelligence (IA) by digital image recognition on the harmonization of the ANA HEp-2 IIF test.

The percentage of consensus for positive/negative results was significantly higher (90.9 ± 1.4) in 2023 than in 2013 (64.0 ± 7.8, $p < 0.001$). The consensus on pattern recognition was significantly lower for the homogenous pattern (70.5 ± 16.0) compared to the centromere (84.9 ± 14.9), the speckled (90.3 ± 12.3), and the negative (84.5 ± 18.6, $p < 0.001$) samples, while it was significantly higher for titers 1:80–1:320 than for titers > 1:320 ($p < 0.001$).

The difference between manual reading and digital reading was not significant (93.8 % vs. 92.4 %; $p = 0.078$), but it was significant between the pre- and post-use of the ICAP nomenclature (82.6 % vs. 93.8 %; $p < 0.001$).

Our study showed that the variability in ANA recognition and reporting is pattern (homogeneous > speckled > centromere) and titer (high titer > low titer) dependent. While we did not find any difference between the use of manual reading compared to digital reading, the adoption of the ICAP nomenclature greatly improved the harmonization of ANA reporting. Despite these results, challenges remain, particularly in achieving complete harmonisation across different laboratories and methodologies.

Harmonization of the Antinuclear Antibodies (ANA) HEp-2 test in Chile: workshops, courses, guidelines, working group and EQA for the national network of laboratories

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Abstract

Being ANA HEp-2 by indirect immunofluorescence (IFA) a gold standard method requested by the clinical laboratory for diagnostic support in various connective tissue diseases, the complexity of delivering a result based on observation with pattern recognition by the laboratory technician, involves multiple response possibilities if quality assurance requirements for the results are not implemented. This must be conducted hand in hand with the knowledge of advancements in antigen characterization, ANA patterns, clinical association, and IFA automation in order to complete a virtuous circle by specialized laboratory professionals together with the specialist physician so that it fulfills its clinical utility in supporting patient care.

Based on this background, the National and Reference Laboratory for Immunology (NRLI) of Public Health Institute of Chile (ISP), since the late 90s, has worked with the national network of immunology laboratories towards harmonization in methodological aspects, external quality assurance proficiency testing (EQA/PT), courses, and workshops in order to narrow the existing gap between the results for ANA and other autoantibody tests. Visualizing the harmonization initiatives in nomenclature and interpretation of patterns presented in the form of a didactic tree, the NLRI initially adopted the 2nd Brazilian Consensus on ANA HEp-2 and later the International Consensus on ANA Patterns (ICAP) in 2016 to disseminate it to the country's laboratories.

Thus, from 2016 to today, the work of promoting harmonization, carried out with the participation of approximately 60 laboratories that make up this national network, has consisted of conducting 6 courses that have trained 291 professionals, 2 symposia with 145 attendees, 3 participations in national congresses, 1 published guidelines "RECOMMENDATIONS FOR THE DETERMINATION OF ANTINUCLEAR ANTIBODIES" disseminated on the institutional website www.ispch.cl, incorporation of ICAP patterns into the "Autoimmunity" EQA/PT, and the formation

of an external working group non-profit (6 medical technologists, biochemists, and physicians with extensive experience) that actively collaborates in NLRI activities.

Regarding harmonization in Chile, the national laboratories practice aligned with the guidelines and recommendations, along with the participation of its professionals in courses and workshops (with an emphasis on quality assurance), the correlation between reported and assigned patterns in each round of the Autoimmune EQA/PT, and the collaboration of an expert working group, has fostered the adherence of laboratories to the ICAP consensus. In this same context, it is noteworthy to mention that Chile ranks 6th worldwide in the number of visits recorded on the website www.anapatterns.org so far in 2025, with 4,676 inquiries.

In light of the advancements in harmonization, it can be indicated that it is a process incorporated in laboratories at the national level, which requires continuous reinforcement and updating according to the state of the art.

Reproducibility of autoantibody titer assessment on HEp-2 cells in Brazilian Laboratories

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Background

The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) is the reference method for autoantibody screening, providing important information about the potential autoantibodies present in a sample. HEp-2 IFA patterns guide follow-up testing for antigen specificity, and some patterns have particular clinical significance. Another key aspect evaluated in this test is the autoantibody titer. Studies have shown that ANA titers ≥1:320, when requested by rheumatologists, are associated with systemic connective tissue diseases and have a higher positive predictive value (1,2). Experts have established initiatives for harmonizing and standardizing the HEp-2 IFA test, including the Brazilian Consensus on Antinuclear Antibodies (BCA HEp-2) in the year 2,000 (3) and the International Consensus on ANA Patterns (ICAP) in 2014 (4). Over the years, multiple consensus meetings have produced important recommendations for the execution and interpretation of the HEp-2 IFA. In 2009 (5), during the publication of the third BCA, Dellavance and colleagues recommended that laboratories calibrate the conjugate titer as a

key measure to adjust the amount of fluorochrome according to the microscope lamp intensity, in order to harmonize titer determination across different laboratories. They also suggested the use of reference sera with predefined concentrations in external quality assessment (EQA) protocols (5). Within this context, the present study aims to evaluate the reproducibility of autoantibody titer assessment on HEp-2 cells by analyzing the ability of 67 Brazilian clinical laboratories.

Methods

Fifteen samples were sent to 67 Brazilian clinical laboratories for titer characterization, and four reactivity ranges were defined: three negative samples, six low-reactivity samples (three at 1:160 and three at 1:320), three moderate-reactivity samples (1:640), and three high-reactivity samples (>1:640). A response was considered correct if the reported titer was one dilution below or one dilution above the nominal titer. All logistics related to the distribution of samples to the participating laboratories were managed by Controllab, a Brazilian enterprise dedicated to EQA programs for clinical laboratoris. Based on the titer results submitted by the laboratories for the f samples, the following evaluation indices were calculated: **Concordance Rate (CR):** number of laboratories with an acceptable result, calculated as the number of acceptable results divided by the total number of laboratories × 100. **Discordance Index (DI):** calculated by subtracting the CR from 100%. **Overestimation Index (OI):** proportion of laboratories that overestimated the titer above the acceptable range (OI = number of laboratories that classified above the acceptable range / total number of laboratories × 100). **Underestimation Index (UI):** proportion of laboratories that underestimated the titer below the acceptable range (UI = Number of laboratories that classified below the acceptable range / total number of laboratories × 100). The results for each index represent the average of the three evaluations performed for each reactivity titer range.

Results

The accuracy rate for identifying the titer of the three negative patterns had a mean Concordance Rate (CR) of 88.25%. On average, 11.8% of laboratories incorrectly categorized these negative samples by assigning a titer above equal to or greater than 1/80, as reflected by the Discordance Index (DI). Regarding the Concordance Rate across different reactivity ranges, the lowest accuracy was observed for titers 1/160 and 1/320, with CR values of 63.9% and 64.1%, respectively. In contrast, samples with titers of 1/640 and >1/640 showed higher concordance, with CR values of 72.4% and 93.2%, respectively. The Discordance Index indicated limited difficulty in identifying negative samples (11.8%) and samples with titers >1/640 (6.8%). However, samples with titers ranging from 1/160 to 1/640 exhibited higher discordance, with DI values ranging from 27.6% to 36.1%. Analysis of the Overestimation Index (OI), which reflects the proportion of laboratories reporting titers above the expected range, showed that this error was most prevalent for samples with a 1/160 titer. Conversely, the Underestimation Index (UI) indicated that the most affected samples were those with a 1/320 titer.

Table 1. Results of the average of three assessments of samples at different concentration ranges, expressed as low, intermediate, and high titers:

Sample	Concordance Rate (CR)	Discordance Index (DI)	Overestimation Index (OI)	Underestimation Index (UI)
Negative	88,25%	11,8%	-	-
1/160	63,90%	36,10%	27,6%	8,50%
1/320	64,1%	35,9%	7,4%	26%
1/640	72,40%	27,6%	11,0%	1,8%
>1/640	93,2%	6,8%	0,0	6,3%

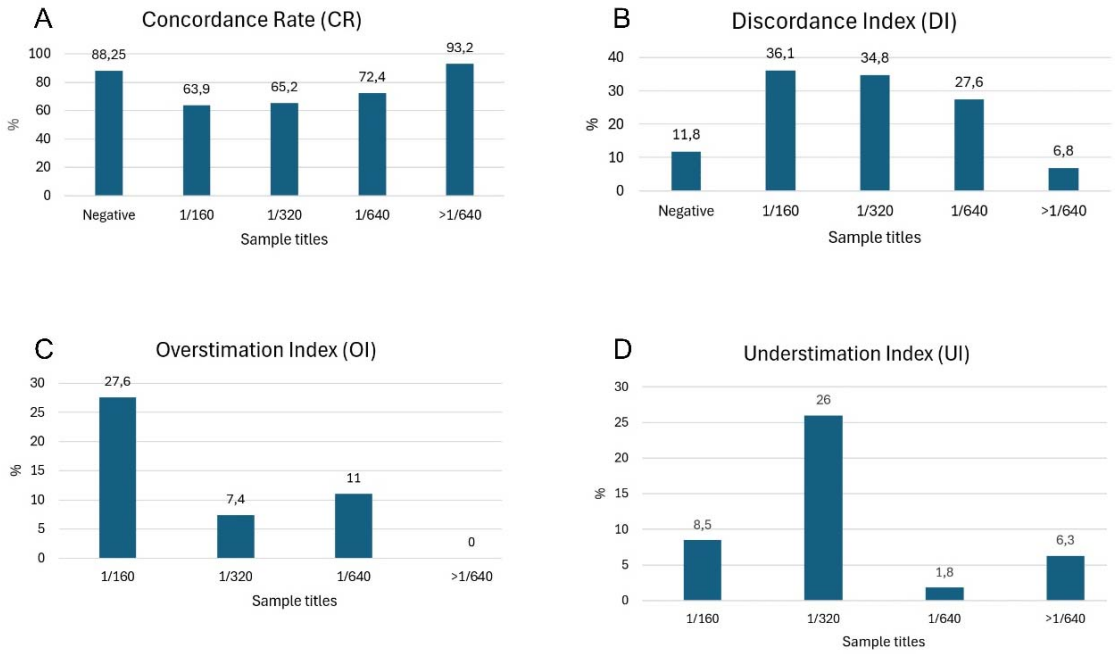

Figure 1. Average of three assessments of samples across different concentration ranges (negative, low, intermediate, and high titers), showing the concordance rate, discordance index, overestimation index, and underestimation index for titer classification among 67 Brazilian clinical laboratories.

Conclusion

The overall concordance in identifying negative samples was high (88.25%), indicating that most laboratories correctly classified non-reactive patterns. However, concordance varied according to titer levels. Lower titer (1/160 and 1/320) samples showed lower inter-laboratory agreement than higher titer (1/640 and >1/640) samples. Intermediate titer samples were more prone to misclassification, exhibiting higher discordance rates and suggesting greater challenges in

interpretation. These discrepancies, particularly in the intermediate ranges, highlight the need for improved standardization to enhance consistency across laboratories.

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Analytical performance of the HEp-2 substrate diagnostic kit for ANA as an initial step in the evaluation of a novel fully automated IFA analyzer in a laboratory in England

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Background

Indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2 IFA) remains a key tool in the diagnostic work-up of autoimmune connective tissue diseases (CTD). Traditionally performed manually, this method is labor-intensive, time-consuming, and subject to operator variability. While semi-automated and automated systems exist, there is still a need for fully automated platforms that integrate both slide processing and interpretation using computer-assisted image analysis. As a preliminary step in evaluating a novel fully automated IFA system (LumiQ®, AliveDx, Switzerland) in combination with the HEp-2 substrate Diagnostic Kit for ANA (investigational device), we assessed the analytical performance of the investigational device when used manually by a single operator, comparing results to historical IFA data from patients with suspected CTD.

Methods

Banked, de-identified human serum samples, previously manually characterized by the Mosaic Basic Profile (Euroimmun, Germany) (routine method) were included in the study. Samples were stored at -20°C for up to three months prior to being thawed for subsequent analysis with the investigational device. The study cohort consisted of 25 samples characterized as non-reactive and 60 samples characterized as reactive, with varying titers, covering a broad range of patterns, as per the International Consensus on ANA Patterns (ICAP). Samples were tested with the investigational device at a 1:80 screening dilution. The testing protocols for both investigational device and routine method were manually conducted following the manufacturer's instructions

for use (IFU), with all readings performed by a single operator using a manual fluorescent microscope to reduce inter-operator variability. Results were interpreted based on categorical classifications (reactive / non-reactive), pattern identification, and the grading of reactive samples from 1+ to 4+ at the screening dilution. Positive (PPA), negative (NPA) and overall percent agreement (OPA) were calculated for sample status (reactive or non-reactive). For reactive samples, percent agreement of each individual IFA pattern and overall pattern agreement were determined. From the 60 ANA reactive samples, 20 samples were selected to determine the end-point titer. Dilutions were prepared of these samples +/- one titer step around the pre-characterized end point titer (EPT).

Results

PPA was 100% (95% CI: 94.0, 100), with all 60 historically reactive samples yielding reactive results by the investigational device. NPA was 100% (86.3, 100), with all 25 historically non-reactive samples yielding non-reactive results by the investigational device. The overall agreement between methods was 100% (95% CI: 95.7, 100). The pattern agreement was 100% (95% CI: 86.3, 100). The end point titration of 20 positive samples showed EPT agreement of 100% (95% CI: 83.2, 100).

Conclusion

The manual preparation and readings using the investigational device showed high level of agreement with historical characterization. Future studies evaluating the fully automated capabilities of this solution will allow for an expanded assessment of the performance of the device and platform.

Frequency of ANA positivity and distribution of ANA patterns within the Croatian population referred for ANA testing

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Background

Antinuclear antibody (ANA) testing is a commonly performed diagnostic procedure in immunology laboratories, with indirect immunofluorescence (IIF) on HEp-2 cells being the gold standard method. The positive ANA result, along with its pattern type and titer, plays a crucial role in the diagnosis of autoimmune diseases.

Aim

To evaluate the frequency of ANA positivity and distribution of ANA patterns after applying the International Classification of ANA patterns (ICAP) and to compare these findings with the data obtained in 2012.

Materials and methods

A retrospective analysis was conducted on ANA test results between December 2022 and May 2025. ANA testing was performed using the IIF method on HEp-2 cells (Euroimmun, Lübeck, Germany), and patterns were visualized with an Olympus BX43 fluorescent microscope (Olympus, Tokyo, Japan). A cut-off titer 1:100 was used, with titration performed up to 1:640, and final results reported as >1:640 for higher ANA titers.

Results

Out of 16596 samples, 7242 (43.6%) were positive and 9354 (56.4%) were negative (AC-0). Among the positive samples, the frequencies of the ANA patterns were as follows: AC-1 (4.6%), AC-2 (19.6%), AC-3 (1.7%), AC-4/5 (38.9%), AC-6/7 (1.8%), AC-8/9/10 (14.8%), AC-11/12 (0.9%), AC-13/14 (0.2%), AC-15/16/17 (2.5%), AC-18/19/20 (1.5%), AC-21 (3.1%), AC-22 (1.2%), AC-23 (1.4%), AC-24/25/26/27/28 (2.6%), and AC-29 (0.3%). Additionally, 4.8% of samples exhibited an

undefined pattern, mostly with a borderline titer. Regarding titers, 29.6% of samples were borderline positive, 24.2% had a titer of 1:160, 16.6% 1:320, 8.0% 1:640, 12.9% >1:640, and 8.7% samples had patterns for which we do not report the titer. In comparison to the data obtained in 2012, the frequency of positive results increased significantly (43.6% vs 29%), with a notable rise in borderline results (29.6% vs 9%). The AC-4/5 pattern remained the most predominant, but there was a notable decrease in the frequency of the AC-1 pattern (4.6% vs 24%).

Conclusion

The study revealed a significant increase in the frequency of positive results, particularly in borderline cases. This change coincided with the introduction of a new microscope, but it may also be attributed to the early post-COVID period. These findings emphasize the importance of regularly monitoring the rates of negative versus positive results as part of ongoing quality control in ANA testing.

Evaluation of anti-nuclear antibody (ANA) patterns and their associations with autoimmune disease biomarkers

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Background

The presence of anti-nuclear antibodies (ANA) is a hallmark for many systemic autoimmune rheumatic diseases (SARD). In this study, we evaluated ANA testing performed at Labcorp from 2015 to 2023. We investigated the associations between ANA patterns obtained by indirect immunofluorescence assay (IFA) using HEp-2 cells with the commonly used autoimmune biomarkers in a large population to improve the understanding of ANA testing in SARD.

Methods

We conducted a retrospective review of de-identified patients results from 7,475,966 ANA tests performed at Labcorp from 2015 - 2023. We reviewed positivity rates for ANA by IFA at the following titers: 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 (EUROPattern, Euroimmun AG, Germany) and we compiled the frequency of the major patterns at these different titers. The patterns included speckled, homogeneous, centromere, nucleolar, nuclear dot, spindle apparatus, midbody, nuclear membrane, centriole, and proliferating cell nuclear antigen (PCNA). ANA-positive by IFA underwent reflex testing to various biomarkers indicative of various autoimmune diseases using a multiplex analyte testing platform (BioPlex 2200, Bio-Rad, USA). Available results of the reflex biomarkers testing for centromere, chromatin, dsDNA, Jo-1, ribosomal P, SS-A, SS-B, Scl70, Smith, Sm/RNP, and RNP were also included in the study.

Results

The positivity rates for ANA by IFA cut-offs were: 1:40; 85.6%, 1:80; 25.4%, 1:160; 16.5%, 1:320; 9.6%, 1:640; 5.5% and ≥1:1280; 2.9%. The most common patterns were speckled and homogeneous along with a few other patterns (centriole, spindle apparatus, and midbody) which

were identified at lower cutoffs under 1:320. The other patterns, specifically centromere patterns, were identified at higher titers at greater than or equal to 1:640. Almost all ANA-positive tests with centromere patterns had positive centromere antibody results. In speckled patterns, RNP, Smith/RNP, SS-A, and SS-B are expected to be present. We identified increased positivity rates of these biomarkers with increased titer sensitivity in speckled patterns particularly. In titers ≥1:1280, there were significant differences in positivity rates in centromere ($P < 0.01$), chromatin ($P < 0.001$), RNP ($P < 0.001$), Scl70 ($P < 0.001$), Smith ($P < 0.001$), Smith/RNP ($P < 0.001$), SS-A ($P < 0.001$), and SS-B ($P < 0.001$) biomarkers between homogenous and speckled patterns.

Conclusion

This is the first study we are aware of using such a large population. We investigated the distributions of ANA positivity rates using IFA by titer and associated patterns in used in routine diagnostic testing. Although recommendations for screening suggest using a titer of 1:40 as a cutoff, we suggest that using a cut-off of 1:80 or 1:160 may be more suitable for reporting positive results. Homogeneous and speckled are the most common patterns, especially at lower titers. Moreover, we identified the presence of both homogeneous and speckled patterns together in ANA-positive specimens. The less common patterns were identified at higher titers of 1:640 and above. Homogeneous and speckled patterns were associated with increased odds of having positive SARD-associated biomarkers except for centromere pattern which is mainly associated with a centromere biomarker.

Interference between nuclear homogeneous and centromere indirect immunofluorescence antinuclear antibody patterns

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Background

Antinuclear antibody (ANA) screening is recommended using the HEp-2 indirect immunofluorescence assay. Pattern recognition is particularly difficult if more than one ANA is present in the sample at a time.

Methods

Well-characterized samples having either a nuclear homogenous or centromere pattern were combined in various ratios to produce mixed patterns with known titers. The observed and expected pattern intensities were evaluated using three evaluation methods: conventional microscopy, on-screen reading, and EuroPattern (EPa) automated analysis.

Results

Homogeneous pattern was identified by conventional and on-screen reading in most samples even when titer of centromere pattern was high. Match% of homogeneous pattern by EPa declined significantly. The presence of homogeneous pattern markedly impaired detection of centromere pattern in all evaluation means. Furthermore, the correlation between expected and observed centromere intensities fell significantly as the homogeneous component rose.

Conclusion

Centromere pattern reduced accuracy of homogeneous pattern recognition, but the influence of nuclear homogeneous on centromere pattern was greater. Performance of EPa was inferior to

human reading, revealing limitations of current automated systems in recognizing mixed ANA patterns.

Comparative evaluation of IIF, ELISA, and Immunoblot in detecting autoantibodies in ANA-positive patients

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Objectives

We aimed to evaluate and compare the performance of indirect immunofluorescence (IIF) on HEp-2 cells, ELISA, and immunoblot (IB) in detecting specific autoantibodies among ANA-positive patients. By applying all three methods simultaneously, we sought to assess concordance and identify discrepancies in autoantibody profiles obtained through each technique, thereby informing their complementary or selective use in clinical immunodiagnostics.

Materials and methods

We examined 100 serum samples from Bulgarian patients referred for ANA screening (12 men, 88 women; mean age 47 years) using three methodologies: (1) HEp-2 cell IIF (BioSystems), with samples showing fluorescence >1:80 considered positive; (2) ELISA for detecting antibodies against SS-A, SS-B, Scl-70, and dsDNA-NcX using EUROIMMUN kits, and RNP/Sm, Jo-1, and AMA-M2 using Orgentec kits; and (3) IB for detecting IgG antibodies against 16 antigens (RNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl100, Centromere B, PCNA, dsDNA, nucleosomes, histones, ribosomal protein, AMA-M2, DFS70) using EUROLINE ANA Profile 3 plus DFS70 (EUROIMMUN).

Results

Forty-eight individuals demonstrated ANA titers >1:80. The most frequently detected antigens were SS-A (16/48), Ro-52 (15/48), Centromere B (11/48), and AMA-M2 (9/48). Anti-Scl-70 (ELISA) levels peaked at ANA titer 1:320 ($p = 0.012$), anti-centromere B (IB) levels increased sharply at

1:1280 and 1:2560 ($p = 0.014$), and anti-dsDNA (IB) levels were highest at 1:320 and declined at higher titers ($p = 0.020$), according to ANOVA analysis across ANA titers. In patents with higher ANA titers (≥1:1280) we detected an increased number of specific autoantibodies, particularly anti-dsDNA, anti-RNP/Sm, anti-SSA/Ro, and anti-Scl-70. A significant association was observed between anti-Centromere B antibodies by IB and ANA titer ($r = 0.480$, $p < 0.001$). Correlation analysis between methods revealed very strong correlations ($r > 0.9$) for RNP/Sm and Scl-70, strong correlations ($r = 0.7$-0.9) for SS-A and SS-B, and moderate correlations ($r = 0.5$-0.7) for nucleosomes, Jo-1, and AMA-M2.

Concordance analysis showed that 40 samples matched between IIF and IB, while 2 did not match. In 4 patients, positive staining was observed for antigens not represented in the IB panel, and 2 individuals showed no ICAP-defined IIF pattern for anti-Ro52. Concordance between ELISA and IB results ranged from 50-88% depending on antigen type, with the highest agreement observed for SS-A.

Conclusion

Our comparative study demonstrates that immunological methods for ANA determination provide complementary results with good correlation and agreement, despite differences in sensitivity and clinical relevance. The integration of multiple methodologies supplementary to each other enhances diagnostic accuracy in autoimmune disease evaluation.

Impact of preanalytical interference on detection of antinuclear antibodies (ANA) and antibodies to extractable nuclear antigens (ENA)

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Introduction

Detection of anti-nuclear antibodies (ANA) is an important diagnostic tool in systemic autoimmune disorders, and the correct identification of ANA patterns is critical for reflex testing and diagnostic evaluation. Extractable nuclear antigens (ENA) testing is used as a second-line test if ANA is positive and identifies specific autoantibodies (anti-RNP, anti-SS-A/Ro60, anti-Ro52, anti-SS-B, anti-Scl-70, anti-Jo-1, anti-Sm) associated with particular autoimmune diseases.

Aim

We aimed to assess whether hemolysis (H), lipemia (L), and hyperbilirubinemia (I) affect the results of ANA performance by indirect immunofluorescence (IIF) or ENA by chemiluminescent microparticle immunoassay (CMIA).

Materials and methods

ANA (IIF, Hep-2, Euroimmun), ENA7 (antibodies against RNP, SS-A/Ro60, Ro52, SS-B, Scl-70, Jo-1, and Sm antigens), and anti-SSA/Ro60 (all CMIA, Werfen) were performed in serum pools, positive and negative. The impact of each interfering substance (H, I, L) is investigated in positive and negative serum pools spiked with different amounts of hemoglobin, intralipid, and conjugated bilirubin. Three stock solutions were prepared, each containing a high concentration of the specific interfering substance. Measurements of HIL indices, hemoglobin, bilirubin, triglycerides,

and selected autoantibodies were performed in duplicate. Obtained results are compared with expected, and bias is calculated and evaluated according to predefined criteria (for ANA IIF, the same category of positivity or one titer difference; for ENA7 and anti-SSA/Ro60, the same category of positivity 30% and 40%, respectively, according to internal quality control criteria).

Results

The results show acceptable bias for ANA, ENA7, and anti-SSA/Ro60 antibodies in the presence of hemoglobin up to 11 g/L (H=11); bilirubin up to 1000 µmol/L (I=800), and triglycerides up to 364 mmol/L (L=60) in investigated positive and negative serum pools.

Conclusions

There is no influence of hemolysis, lipemia, and hyperbilirubinemia on the detection of ANA by indirect immunofluorescence, ENA7, and anti-SS-A/Ro60 by chemiluminescent microparticle immunoassay.

Antinuclear antibodies and anti-double stranded DNA autoantibodies testing in a clinical diagnostic laboratory in Singapore

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Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that disproportionately affects women. Asian populations report a prevalence rate of 3.7-127 per 100,000 persons as well as higher disease severity [1]. Current diagnostic criteria for SLE (European League Against Rheumatism/American College of Rheumatology 2019) require positive antinuclear antibodies (ANA) at ≥1:80 on HEp-2 cells as an entry criterion necessary to classify SLE, while positive anti-double stranded DNA (anti-dsDNA) antibodies using an assay with a specificity of ≥90% are an immunologic criterium carrying a high weightage of 6 out of 10 points required [2]. Current guidelines and publications suggest that testing may be carried out in a tiered approach using ANA IIF as the "gold standard" initial screening test, followed by specific autoantibody testing [3, 4], but actual practice on the ground may vary. In addition, there are many different modalities of measuring anti-dsDNA antibodies, including Farr Radioimmunoassay, ELISA, FEIA or Crithidia luciliae immunofluorescence test (CLIFT), all of which show high inter-method variability leading to discordant results [5, 6]. A specificity of ≥90% has been established in literature for modalities such as CLIFT and FEIA, but not for ELISA [7, 8].

Objective

To determine the prevalence of ANA and anti-dsDNA among patients tested in our laboratory and to explore how the correlation of the two tests and testing algorithms may be affected by the use of different ELISA anti-dsDNA assays.

Method

A retrospective data analysis of 6,241 records from patients who were tested for both ANA and dsDNA between December 2023 to November 2024 at the Immunology and Serology Laboratory, Singapore General Hospital was performed. Basic demographic data, ANA and dsDNA reported results were extracted from the Laboratory Information System (LIS). ANA were detected by indirect immunofluorescence on Hep-2 cells (ANA IFA HEp-20-10, EUROIMMUN Medizinische Labordiagnostika AG, Germany). A positive ANA has been defined in our laboratory as a titer ≥1:160. Assessment of autoantibody titers and patterns (Homogeneous, Speckled, Nucleolar, Centromere, Nuclear Membrane, Mitotic Spindle Apparatus, Nuclear dots, Centriole and PCNA) was visualized using a semi-automated system (EuroPattern, EUROIMMUN Medizinische Labordiagnostika AG, Germany) and determined by trained laboratory staff. For anti-dsDNA, serum samples received between December 2023 to June 2024 were tested using an ELISA method (Anti-dsDNA EIA, Bio-Rad Laboratories, USA), while samples received from July 2024 to November 2024 were detected by FEIA (EliA dsDNA, Phadia AB, Sweden). Statistical analysis of differences was determined by Fisher test while association between ANA and anti-dsDNA results for BioRad and Phadia EliA were determined via multivariable logistic regression. Analysis was performed using RStudio.

Results

98.0% of the study population had an ANA and anti-dsDNA test ordered concurrently in a single laboratory order, showing clinician preference for ordering ANA and anti-dsDNA in combination. The overall ANA positivity rate and anti-dsDNA positivity rate for our study population was 30.0% and 6.2% respectively. Similar to other studies, ANA positivity was more common among women (70%) than men (30%) (p-value = <0.01). On average, subjects positive for ANA were slightly older (mean age = 58.4 (±20.0)) compared to those negative for ANA (mean age = 55.2 (±20.1)) (p-value = <0.01). The two most common patterns of ANA were homogenous (43.7%) and speckled (33.3%). For anti-dsDNA, the positivity rate was higher by Biorad ELISA (7.7%) compared to Phadia EliA FEIA (4.2%).

In general, ANA positivity was associated with anti-dsDNA positivity (OR = 6.19, 95% CI: 4.96 – 7.74). Only 1.9% and 2.0% of subjects were positive for anti-dsDNA and negative for ANA, for BioRad ELISA and Phadia EliA respectively. When stratifying for a homogenous ANA pattern, the co-positivity rate for homogenous ANA and anti-dsDNA by Biorad ELISA (14.1%) was higher than the co-positivity rate for homogenous ANA and anti-dsDNA by Phadia EliA FEIA (4.3%) (p-value = <0.01). The lower co-positivity rate obtained for ANA and anti-dsDNA by Phadia EliA FEIA appears to be more closely aligned to the co-positivity rates described in literature [9, 10, 11 &12]. We suggest that this may be a function of the higher specificity inherent in the Phadia FEIA assay.

Conclusion

The prevalence of ANA and anti-dsDNA positivity in our study population was similar to study findings from other countries. Our clinicians show a strong preference for ordering ANA and anti-dsDNA together at the same time as an initial screening test. The known higher specificity of Phadia EliA FEIA compared to ELISA methods is reflected in a lower ANA and anti-dsDNA co-positivity rate which corroborates with rates described in literature. On one hand, this may reduce

non-specific or false-positive anti-dsDNA results resulting in better diagnostic accuracy. On the other hand, there may be a requirement for closer follow up and more comprehensive work-up for patients with a clinical suspicion of SLE but who are anti-dsDNA negative, to fulfil necessary criteria needed for diagnosis.

Hence, in accommodating latest diagnostic criteria, laboratories should be familiar and understand how different testing algorithms and assay methodologies may affect results in their patient population.

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9.4 Advances in the Differentiation of ANA-Specifities (Anti-dsDNA and A-ENA Antibodies)

Analytical performance of a fully automated multiplexed microarray immunoassay for the simultaneous detection of fifteen autoantibodies associated with connective tissue diseases in a reference laboratory in Southern France

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Background/Purpose

Autoantibodies are key diagnostic markers in autoimmune connective tissue diseases (CTD). Conventional testing often requires multiple single-measurand assays, which can be labor-intensive and time-consuming. We evaluated the analytical performance of a multiplex microarray immunoassay (MosaiQ AiPlex® CTDplus; AliveDx, Switzerland) for the simultaneous detection of 15 autoantibodies associated with CTD, used with its fully automated proprietary platform, compared to routine methods in a reference laboratory.

Methods

A cohort of analytically characterized sera (572 reactive and 2,686 non-reactive) were included in the study. Reference methods included a multiplex bead-based immunoassay for most measurands, indirect immunofluorescence assay (IFA) for DFS70, and a chemiluminescence assay for CCP. Samples were tested with the investigational microarray immunoassay that detects IgG autoantibodies to dsDNA, SS-A 60, TRIM21 (SS-A 52), SS-B, Sm, Sm/RNP, U1RNP, Jo-1, Scl-70, CENP B, Chromatin, Ribosomal P, DFS70, RNAP III, and CCP. Performance was assessed using positive (PPA), negative (NPA) and overall percent agreement (OPA). Additionally, Cohen's kappa coefficient was calculated.

Results

The microarray immunoassay demonstrated substantial agreement with the reference methods (overall Cohen's kappa: 0.72), with total PPA of 86.6% (95% CI: 83.4, 89.3), total NPA of 92.3% (95% CI: 91.2, 93.3) and total OPA of 91.3%. Individual measurands showed PPA ranging from 59.1% (Sm) to 100% (TRIM21, Jo-1), and NPA ranging from 72.6% (Chromatin) to 100% (Sm, Jo-1, CCP). Notably, high kappa values for almost perfect agreement were observed for SS-A 60 (0.92), TRIM21 (0.85) Sm/RNP (0.97), and CENP B (0.87). Cohen's kappa values for substantial agreement were observed for dsDNA (0.72), SS-B (0.75) and Sm (0.73); whereas U1RNP (0.49), Scl-70 (0.41) and Ribosomal P (0.43) showed moderated agreement. Finally, kappa values for fair agreement were found for Chromatin (0.33) and CCP (0.34). No samples were available for RNAP III and thus, no comparison was made. Due to limited sample availability, results for Jo-1 (reactive, overall) and CCP (non-reactive, overall) should be interpreted with caution. Detailed results are shown in Table 1.

Table 1. Agreement of the Microarray Immunoassay with Comparators

Measurand	Reactive results (n)	PPA (%)	95% CI	Non-reactive results (n)	NPA (%)	95% CI	OPA (%)	Cohen's kappa
dsDNA	49	87.2%	74.3, 95.2	186	90.7%	85.5, 94.2	90.0%	0.72
SS-A 60	88	98.9%	93.8, 100	179	95.0%	90.7, 97.4	96.3%	0.92
TRIM21	70	100%	94.9, 100	197	91.3%	86.4, 94.5	93.6%	0.85
SS-B	43	81.1%	64.8, 92	224	95.8%	92.1, 97.8	93.6%	0.75
Sm	26	59.1%	36.4, 79.3	241	100%	98.5, 100	96.6%	0.73
Sm/RNP	48	97.9%	88.9, 99.9	219	99.5%	97.5, 100	99.2%	0.97
U1RNP	78	82.2%	71.5, 90.2	189	73.0%	65.7, 79	75.7%	0.49
Jo1*	2*	100%	15.8, 100	265	100%	98.6, 100	100%	1
Scl-70	20	82.4%	56.6, 96.2	247	84.9%	78.8, 89.4	84.7%	0.41
CENP B	26	88.0%	68.8, 97.5	241	98.8%	96.4, 99.7	97.7%	0.87
Chromatin	73	63.9%	50.6, 75.8	194	72.6%	65.1, 78.7	70.2%	0.33
Ribosomal P	11	81.8%	48.2, 97.7	256	92.5%	88.5, 95.1	92.0%	0.43
DFS70	19	88.9%	65.3, 98.6	47	95.7%	85.2, 99.2	93.8%	0.85
RNAP III	-	-	-	-	-	-	-	-
CCP*	19	82.4%	56.6, 96.2	1*	100%	2.5, 99	83.3%	0.34
TOTAL	**572**	**86.6%**	**83.4, 89.3**	**2686**	**92.3%**	**91.2, 93.3**	**91.3%**	**0.72**

Equivocal not included. *Low number of samples. CI: confidence interval; NA: Not applicable; NPA: negative percentage agreement; OPA: overall percent agreement PPA: positive percentage agreement

Conclusions

This fully automated multiplex microarray immunoassay demonstrated overall substantial agreement with established methods for the detection of the autoantibodies featured in the assay. Its ability to simultaneously detect multiple autoantibodies in a single, automated run may streamline the serologic evaluation and support more efficient clinical workflows in autoimmune diagnostics.

A machine learning algorithm based on a 15-autoantibody profile by a novel fully automated multiplexed microarray immunoassay for the diagnosis of autoimmune connective tissue diseases

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Background

Detection of relevant autoantibodies is key in the identification of autoimmune connective tissue diseases (CTD). The evaluation of multiple autoantibodies for extended serological profiling may improve the diagnosis of these conditions. We evaluated the diagnostic utility, in patients with CTD and disease controls, of machine learning classifiers based on the 15-autoantibody profile performed by a novel, single-use, multiplexed microarray immunoassay, used with its fully automated high-throughput proprietary system for simultaneous the detection of IgG autoantibodies directed to dsDNA, SS-A 60, TRIM21 (SS-A 52), SS-B, Sm, Sm/RNP, U1RNP, Jo-1, Scl-70, Centromere B, Chromatin, Ribosomal P, DFS70, RNAP III and CCP2.

Methods

De-identified sera from 475 patients diagnosed with autoimmune CTD in accordance with current guidelines [127 patients with systemic lupus erythematosus (SLE), 74 with systemic sclerosis, 76 with Sjögren's syndrome (SjS), 71 with idiopathic inflammatory myopathies, 54 with mixed CTD, 73 with rheumatoid arthritis] and 652 patients with other disorders, who served as disease controls were analyzed using the investigational MosaiQ AiPlex® CTDplus (AliveDx, Switzerland) assay. Classification models were developed using all 15 autoantibodies or a selected subset, employing the RandomForest algorithm (XGBoost and Logistic L1 L2 were also used). Diagnostic performance was evaluated by receiver operating characteristic (ROC) curve analysis.

Results

A RandomForest classifier incorporating all 15 autoantibodies demonstrated robust performance in predicting SLE, achieving an area under the curve (AUC) of 0.92. In comparison, the individual SLE-specific markers dsDNA and Sm yielded lower AUCs of 0.68 and 0.60, respectively. For SjS, the 15-plex RandomForest classifier achieved an AUC of 0.83, outperforming a 3-plex RandomForest classifier based on SS-A 60, TRIM21, and SS-B autoantibodies, which had an AUC of 0.62. The individual AUCs for these markers were 0.63, 0.59, and 0.58, respectively. Similarly, for other CTDs, 15-plex classifiers consistently outperformed the individual disease-specific markers.

Conclusion

Multiplex autoantibody testing combined with machine learning algorithms has the potential to improve the diagnosis of autoimmune CTD.

Clinical utility of the detection anti-DFS70 autoantibodies by a novel fully automated multiplexed microarray immunoassay for the assessment of autoimmune connective tissue diseases

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Background

Detection of autoantibodies directed to Dense Fine Speckled 70 (DFS70) helps to confirm the DFS pattern observed in the indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA). While the clinical significance of anti-DFS70 remains a matter of debate, these autoantibodies have been reported to be negatively associated with autoimmune connective tissue diseases (CTD) when present in isolation [i.e., in the absence of autoantibodies to other common extractable nuclear antigens (ENA)]. We evaluated the clinical utility of this marker, with or without other autoantibodies associated with CTD, based on the 15-autoantibody profile performed by a novel, single-use, multiplexed microarray immunoassay, for simultaneous detection of IgG autoantibodies directed to dsDNA, SS-A 60, TRIM21 (SS-A 52), SS-B, Sm, Sm/RNP, U1RNP, Jo-1, Scl-70, Centromere B, Chromatin, Ribosomal P, DFS70, RNAP III and CCP2.

Methods

Banked, de-identified serum samples were analyzed from 667 patients diagnosed with ANA-associated CTD in accordance with current guidelines [171 patients with systemic lupus erythematosus (SLE), 135 with systemic sclerosis, 125 with Sjögren's syndrome (SjS), 152 with

mixed CTD (MCTD), and 84 with idiopathic inflammatory myopathies (IIM)]. Samples from 558 disease controls and 233 healthy blood donors were also included. All specimens were tested using the MosaiQ AiPlex® CTDplus microarray immunoassay (AliveDx, Switzerland). Prevalence of anti-DFS70 in the CTD, disease controls and blood donors' groups was calculated. The frequency of anti-DFS70 was assessed across all groups. Co-reactivity with other autoantibodies and its performance as a marker for discriminating CTD from non-CTD cases was evaluated.

Results

Anti-DFS70 antibodies were detected in 11/233 (4.72%) samples from blood donors, in 28/558 (5.01%) disease controls (including other autoimmune conditions such as autoimmune thyroiditis, anti-phospholipid syndrome and ulcerative colitis; infections: hepatitis B virus; and other conditions such as cancer, gout and osteoarthritis), and in 19/667 (2.84%) samples from patients diagnosed with CTD [SLE: 11/171 (6.43%), SSc: 4/135 (2.96%), SjS: 1/125 (0.8%), MCTD: 1/152 (0.65%), IIM: 2/84 (2.38%)]. Excluding equivocal and invalid results, 16 out 19 (84.2%) CTD samples reactive for anti-DFS70 also showed reactivity to ≥1 additional autoantibodies. Conversely, in the disease controls, all 22 anti-DFS70 reactive samples (100%) were non-reactive for the other 14 markers. Thus, isolated anti-DFS70 demonstrated a specificity of 100% (95% CI: 84.6%, 100%) and a sensitivity of 84.2% (95% CI: 60.4%, 96.6%) in discriminating CTD from non-CTD. When including blood donors in the non-CTD group, specificity was 94.1%; when considering blood donors alone, specificity was 80.0%

Conclusion

In the present retrospective cohort, isolated anti-DFS70 autoantibodies showed high specificity for the exclusion of autoimmune CTD. These findings support the clinical utility of anti-DFS70 as a negative marker for CTD when interpreted in the absence of other disease-associated autoantibodies.

Comparison of four different methods for determination of anti-dsDNA antibodies

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Background

Antibodies against double-stranded DNA (anti-dsDNA) are included in the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for systemic lupus erythematosus (SLE), and their levels correlate with disease activity. A positive anti-dsDNA result is typically associated with a homogeneous fluorescence pattern in the anti-nuclear antibody (ANA) indirect immunofluorescence (IIF) test. Therefore, a positive anti-dsDNA result with a negative ANA IIF test raises questions about its clinical significance. The inclusion criteria for this study were a positive anti-dsDNA result on the BioFlash analyzer and a negative ANA IIF result. BioFlash results were then compared with three other anti-dsDNA testing methods.

Methods

The study included 30 patients with negative ANA IIF results and positive anti-dsDNA results obtained using a chemiluminescent immunoassay (CLIA) on a BioFlash analyzer. The results for anti-dsDNA were compared with an enzyme-linked immunosorbent assay (ELISA) (Euroimmun anti-dsDNA-NcX ELISA), addressable laser bead immunoassays (ALBIA) (FidisTM Connective Profile) and IIF (NOVA Lite anti-dsDNA Crithidia Luciliae). Results were categorized as negative or positive and analyzed using kappa statistics and the intraclass correlation coefficient (MedCalc version 20.104)

Results

Of the 30 patients with negative ANA IIF and positive anti-dsDNA results on the BioFlash analyzer, 4 tested positive with the Euroimmun anti-dsDNA-NcX ELISA, 12 with FidisTM Connective Profile and 2 with NOVA Lite anti-dsDNA Crithidia Luciliae test.

Weighted kappa analysis showed poor to fair agreement among the methods. No agreement (kappa=0) was found between BioFlash and the other 3 tests. Fair agreement was observed between Euroimmun ELISA and FidisTM Connective Profile (kappa= 0.38 (95% CI: 0.09-0.66)) and between Euroimmun ELISA and NOVA Lite anti-dsDNA on Chritidia Luciliae (kappa=0.27 (95% CI: 0.24-0.77)). FidisTM Connective Profile showed fair agreement with NOVA Lite anti-dsDNA Chritidia Luciliae (kappa=0.19 (95% CI: -0,05-0.44)).

The intraclass correlation coefficient demonstrated moderate reliability of averages (0.55 (95% CI: 0.21-0.76)) and poor reliability of single ratings (0.23 (95% CI: 0.06-0.45)).

Conclusion

This study highlights significant variability among four different methods for detecting anti-dsDNA antibodies in patients with positive BioFlash results and negative ANA IIF tests. The lack of agreement, particularly between BioFlash and the other three methods, suggests potential differences in assay sensitivity, specificity, or antigenic targets. These findings underscore the importance of assay selection in clinical diagnostics and suggest that isolated positive results — especially in the context of negative ANA IIF — should be interpreted with caution. Further studies are needed to assess the clinical relevance of discordant anti-dsDNA results.

Evaluation of a new protein microarray for the multiplex detection of ANA in a retrospective study

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Introduction

Serology for detecting autoantibodies normally consists of a first test for screening and a second test - or multiple further tests - for the subsequent detailed determination of specific autoantibodies. Standard protocols recommend using an immunofluorescence assay (HEp-2 IFA) for screening followed by multiple tests (EIAs) for confirmation of borderline or positive results from the screening test. A new protein microarray for the multiplex serological detection of ANA, the ANA plus ViraChip®, presents a new multiplex microarray approach employing 17 autoantigens in one well of a microtiter plate. Thus, a broad spectrum of autoantibodies can be analyzed in one step to determine the specific autoantibodies, respectively.

Methods

In a retrospective evaluation study, a panel consisting of 205 selected serum specimens in a routine laboratory b was used. All specimens were characterized by HEp-2 IFA (184 reactives/positives; 21 negatives). Additionally, ELISA, Crithidia luciliae IFA and fluorescence based immunocup assays were used to detect specific antibodies. Furthermore, all specimens were analyzed with the new multiplex microarray method.

Results

The results show that the ANA plus ViraChip® displayed the following positive agreements:

88 % (Centromere), 91 % (dsDNA), 80 % (RNP/Sm), 55 % (Sm), 80 % (SSA52), 100 % (SSA), 56 % (SSB), 80 % (Jo-1), 75 % (Scl70). Negative agreement was determined as 100 %.

Conclusion

This study shows that the multiplex protein microarray analysis is a promising diagnostic tool for the determination of autoantibodies in a routine testing set up. Directly after a positive screening test just one well is needed to further find those autoantibodies specifically binding to the corresponding target antigens. Thus, laboratory routine processes can be further streamlined and standardized.

Anti-dsDNA autoantibodies - a 5 year review of first time positive results

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Background and Aims

Anti-dsDNA antibodies are important for the diagnosis and monitoring of Systemic Lupus Erythematosus (SLE) and are included among its classification criteria, since have been shown to associate with SLE disease activity and renal involvement. Due to their clinical relevance, these antibodies are routinely incorporated into screening algorithms for autoimmune diseases.

However, different methodologies for the detection of anti-dsDNA antibodies may give different results for the same samples, and this disparity is due to the diversity of autoantibodies, (that target different epitopes within the very complex structure of DNA and may have different clinical associations), and their different avidity.

In our laboratory, anti-dsDNA antibodies are first quantified by a chemiluminescent immunoassay (CLIA). Positive results are subsequently confirmed using Crithidia luciliae immunofluorescence and/or ELISA.

In this five-year retrospective analysis, we aim to evaluate the concordance between these three methodologies and its clinical association.

Methods

The authors present 5 years revised casuistic from 1st May 2020 to 30 April 2025. In 13224 samples tested by CLIA (Zenit Prime, Menarini), we reviewed the 110 first time positive samples. Confirmatory testing was performed using Crithidia luciliae immunofluorescence assay (Zenit IIF) and ELISA (DRG, Menarini). Clinical information was obtained from patient medical records to correlate with laboratory findings.

Results

The concordance between Critidia and ELISA results was 56%. Discordance occurred in 42 cases, and 90.5% of this discordance was due to Critidia negative or indeterminate results when ELISA was positive.

Among concordant results with both positive confirmatory tests, patients diagnosed with SLE were 61.7%, 8.8% diagnosed with other autoimmune diseases, and 29.5% with other diagnostics (drug-induced hepatitis, neoplasia, and others).

In patients with confirmatory concordant negative results, the percentages were 5.26% for SLE, 21.05% for other autoimmune diseases, and 68.42% for other diagnoses.

In discordant cases, the distribution of diagnoses was varied across categories without a clear predominance.

Conclusions

Anti-dsDNA antibodies are mostly present in SLE patients, as demonstrated by the high percentage of concordant positive results associated with this diagnosis. The moderate concordance between Crithidia luciliae immunofluorescence and ELISA assays indicates that using multiple confirmatory methods enhances diagnostic confidence.

Analytical comparison of different methods for the detection of autoantibodies to extractable nuclear antigens

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Objectives

The IIFA for the detection of ANA is still considered the reference method for ANA screening, and in practice laboratories perform additional, more specific anti-ENA tests together with the IIFA. There are many commercial tests (e.g., CLIA, Microblot Array – MBA, and dot/line immunoassays), but the only test that uses native antigens is the in-house counterimmunoelectrophoresis (CIE). Our aim was to determine the comparability between the assays routinely used in our laboratory (CIE and dot immunoassays - myositis (Myo) panel and scleroderma (SSc) panel) and other available methods for the detection of anti-ENA.

Methods

We analyzed sera samples of patients from the Department of Rheumatology, UMC Ljubljana, which were sent to our laboratory for routine testing (anti-ENA – CIE and/or Myo/Ssc panel (D-Tek, BlueDiver)). In addition, we analyzed samples using CLIA (BioFlash, Inova Diagnostics, Werfen) and MBA ANA plus (TestLine). Some autoantibodies from panels were not analyzed due to lack of positive samples or available comparable assays. The Kappa coefficient was calculated to determine the agreement between the methods.

Results

We tested a total of 272 samples from patients and compared the results between the methods. The first group includes autoantibodies associated with SLE and other CTDs. For Sm antibodies, moderate agreement was found between CIE and CLIA ($\kappa = 0.575$), while comparisons with MBA were limited due to antigen differences; however, for SmD (the more clinically relevant component), agreement between MBA and CIE/CLIA was similar ($\kappa \approx 0.55$). For RNP antibodies, there was substantial agreement between CIE and CLIA ($\kappa = 0.802$), with slightly lower values

when comparing CIE and CLIA with the SSc panel (κ ≈ 0.62). Among the RNP subtypes assessed with MBA, the best agreement with CIE/CLIA was found for RNP A (κ = 0.708–0.739), while RNP 68 and C showed weaker agreement. For Ku antibodies, the agreement ranged from substantial to almost perfect (κ = 0.665–0.866), depending on the method pair. Ro52 and Ro60 antibodies showed consistently high agreement (κ = 0.774–0.935), despite method differences in antigen composition. La antibodies also showed substantial and almost perfect agreement between methods (κ = 0.700–0.832).

In the myositis panel, Jo-1 showed almost perfect agreement across all methods (κ = 0.883–1.000). Other antibodies such as Mi-2, SRP54, MDA-5, SAE-2, and PL-12, showed moderate to substantial agreement (κ = 0.465–0.663) (comparing only MBA and Myo panel).

SSc-related antibodies showed variable agreement between methods. CENP B showed consistent substantial agreement throughout (κ = 0.737–0.747), while CENP A showed greater variability (κ = 0.375–0.943). Scl-70 showed substantial to almost perfect agreement between methods (κ = 0.714–1.000), with the best agreement between CLIA and the SSc panel. NOR-90, which was only determined using the MBA and the SSc panel, showed substantial agreement (κ = 0.721). PM/Scl-100 showed an overall lower agreement (κ = 0.244–0.663), especially for methods compared to CIE (antigen is whole PM/Scl), while PM/Scl-75 showed a slightly better agreement (κ = 0.487–0.567).

Conclusions

Our results show that diagnostic platforms have different levels of agreement. For some autoantibodies (e.g., Ro60, Ku, La, Jo-1, Scl-70) there is substantial or almost perfect agreement, while for others (e.g., Sm, RNP, Mi-2, PM/Scl) there are significant discrepancies. These results emphasize the importance of method selection in the clinical interpretation of autoantibody profiles.

Comparative evaluation of two commercially available anti-double-stranded DNA immunoassays for the diagnosis of systemic lupus erythematosus

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Background and aim

The determination of anti-dsDNA antibodies plays a significant role in diagnosis and management of systemic lupus erythematosus (SLE). According to the 2019 EULAR/ACR Classification Criteria for SLE, assay specificity ≥ 90% is required for use in the classification scoring system. In this study, we evaluate and compare the diagnostic performance of two available anti-dsDNA immunoassays.

Methods

Serum samples from 50 SLE patients and 100 disease controls were obtained from a sample bank. Within the SLE group, 11 patients had a diagnosis of lupus nephritis. Disease controls included cases of rheumatoid arthritis (RA), Sjögren's syndrome (SjS), systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIM), mixed connective tissue disease (MCTD), infections, and tumors. Samples were evaluated using the EliA™ dsDNA (Thermo Fisher Scientific, Germany) and Quanta Flash® dsDNA (Werfen, San Diego) assays. Pre-defined manufacturer cut-offs of 15 IU/mL for EliA™ and 35 IU/mL for the Quanta Flash® assays were used.

Results

The two assays demonstrated 91% sensitivity in the lupus nephritis sub-population, and the overall sensitivity in 50 SLE sera was 30% for EliA™ and 38% for Quanta Flash®. Specificities were 99% for EliA™ and 89% for Quanta Flash®. False positive results on the Quanta Flash® assay clustered to patients with RA, MCTD, or SjS, and the EliA™ assay reported a false positive result for a SjS case.

Conclusions

Analysis of predefined patient samples and disease controls indicates that the EliA™ dsDNA assay is more specific but less sensitive than the Quanta Flash® dsDNA. However, according to these results, the Quanta Flash® dsDNA assay does not meet the specificity threshold required by the ACR/EULAR for SLE classification purposes.

Evaluation of BioCLIA CTD Screen as a primary care screening test: Comparative analysis with immunofluorescence and EliA CTD Screen

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Background

Connective tissue diseases (CTDs) are systemic autoimmune conditions with increasing prevalence and a wide range of unspecific symptoms. General practitioners (GPs) are crucial for early identification or rule out and accurate referrals, essential for healthcare sustainability and improving patient quality of life. Therefore, GPs need access to reliable and accurate tests.

Objective

To evaluate the performance of the BioCLIA CTD Screen test (HOB Biotech Group Corp, China) in primary care settings.

Methods

This was a prospective study. A total of 845 consecutive samples received for ANA screening from primary care centers within our influence measured using the BioCLIA CTD Screen and showing positive results were included. Samples were subsequently evaluated with the EliA CTD Screen (Thermo Fisher Scientific, Sweden) and Hep-2 cells IIF (Menarini Diagnostics, Italy).

Results

When comparing BioCLIA positive samples, 72% (613) were negative with IIF and 77% (654) were negative with the EliA CTD Screen. Additionally, when EliA results were compared with IIF results, double negativity was confirmed in 68% (578) of the cases, indicating false most probably positive results from BioCLIA.

Conclusions

BioCLIA demonstrated an approximate 70% false positive rate at the manufacturer's cut-off value. This leads to a high number of positive results managed by primary care, potentially increasing the burden on specialized care and causing patient anxiety due to misdiagnosis. The usefulness of this test in primary care should be carefully evaluated in light of these results and manufacturers cut-off reconsidered.

A novel fiber optic SPR biosensor for the specific detection of high avidity anti-dsDNA antibodies to aid in the diagnosis of systemic lupus erythematosus

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Abstract

Systemic Lupus Erythematosus or SLE is a chronic autoimmune disease mainly detected in women during childbearing age. While early diagnosis is essential to prevent organ damage, it remains challenging due to the wide range of symptoms observed between and within these patients. Therefore, detection of SlE-specific biomarkers is needed for reliable diagnosis, among which high avidity anti-dsDNA antibodies are considered the most specific. Although a wide range of anti-dsDNA antibody tests are available, the easy, more automated assays preferred in clinical laboratories suffer from insufficient specificity, as low avidity anti-dsDNA antibodies are detected as well. There is thus a need for an easy, automated serological assay that can distinguish high from low avidity anti-dsDNA antibodies in a sensitive and specific manner, to aid in SLE diagnosis.

Therefore, we applied our in-house developed fiber optic surface plasmon resonance (FO-SPR) technology to establish a novel biosensor for specific high avidity anti-dsDNA antibody detection, as it offers real-time monitoring of binding interactions (in contrast to currently available tests), and thus is highly promising for distinguishing low from high avidity antibodies. We functionalized FO-SPR biosensors with dsDNA to capture anti-dsDNA antibodies, followed by introduction of gold nanoparticles (AuNPs), functionalized with anti-lgG antibodies that can bind to the pathogenic anti-dsDNA lgG antibodies from the sample, thereby achieving signal amplification.

With our FO-SPR test, we successfully obtained kinetic information about dsDNA-antibody binding in 10Jold diluted patient serum and could significantly differentiate serum pools made from

samples positive and negative for anti-dsDNA antibodies. Moreover, by introducing a labeling step with Au NPs, we obtained both an increased sensitivity (due to the added AuNPs mass) and specificity (by targeting the lgG isotype). Finally, we executed a preliminary cohort study (25 samples), and found that our FO-SPR biosensor could significantly distinguish serum samples from patients diagnosed with SLE (n=10) from disease (n=10) and healthy controls (n=5), both in the label-free association step as in the labeled amplification step with AuNPs. Furthermore, based on these preliminary results, we performed a Partial Least Squares Discriminant Analysis (PLS-DA), which indicated the potential of our assay for sensitive and specific detection of high avidity anti-dsDNA antibodies.

Overall, we clearly demonstrated the potential of the FO-SPR biosensor for sensitive and specific detection of high avidity anti-dsDNA antibodies in context of SLE diagnosis, which will be further validated using a larger clinical cohort screening with at least 100 patient samples.

Challenges of anti-dsDNA autoantibody measurement

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Abstract

Autoantibodies targeting dsDNA are essential biomarkers for diagnosis and disease monitoring of individuals with systemic lupus erythematosus (SLE). A wide range of anti-dsDNA autoantibody assays is available and used in clinical laboratories. However, result outcome is inconsistent between these assays, primarily due to differences in assay set-up (solid-phase versus fluidics, buffers,...) and differences in sequence, size and origin of the presented dsDNA molecule. This complicates assay standardization and harmonization as each assay favors detection of certain anti-dsDNA antibody subpopulations and missing others.

To study the influence of dsDNA sequence and size on the detected anti-dsDNA antibody levels in patients' serum, we developed a multiplexed anti-dsDNA antibody assay on a Luminex platform (Luminex200TM, Luminex Corporation) presenting 6 different synthetic dsDNA molecules with four different sizes (17, 42, 90 and 150 base pairs [bp]) and sequences (42(1) and 42(2) bp and 150(1) and 150(2) bp). Noteworthy, NaCl was added to the sample buffer to create stringent conditions favoring the binding of high avidity anti-dsDNA antibodies. Anti-dsDNA antibody levels were measured in two sera: (i) the WHO Reference Reagent Lupus anti-dsDNA antibodies (151174) and (ii) an in-house prepared Serum pool (n=5 samples). ln theory, a serum pool should represent an extended set of autoantibody specificities compared to the specificities present in a single sample.

We found that the detected mean fluorescence signals (MFl) were not only dependent on the size, but also on the sequence composition of the dsDNA molecule and the sample tested. ln addition, the relative MFI signals to the different dsDNA antigens varied between 151174 and the Serum pool, indicating that sample 151174 and the Serum pool show different anti-dsDNA antibody reactivities to the presented dsDNA.

Taken together, using a multiplexed assay approach, we showed that the binding of anti-dsDNA antibodies to dsDNA depends on the size and the sequence of the presented dsDNA molecule. This hampers assay standardization and could be related to the presence of (unique) antibody subpopulations favoring different dsDNA epitopes.

Evaluation of the analytical performance of ChLIA technology in the identification of antinuclear antibodies associated with SARD

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Abstract

Systemic autoimmune rheumatic diseases (SARDs) indicate an heterogenous group of disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome (SjS), and systemic sclerosis (SSc). The diagnosis and classification of these diseases is based on the detection of antinuclear antibodies (ANA). Indirect immunofluorescence (IIFT) on HEp-2 cells is still considered the gold standard for ANA screening, however other technologies such as chemiluminescence immunoassays (ChLIA) are gaining momentum. The aim of this study was to evaluate: a) the performance of ChLIA versus the currently employed methods indirect immunofluorescence (IIFT) and lineblot; b) the potential implementation of ChLIA technology into the routine laboratory diagnostic.

We considered a total of 114 serum samples (64 positives and 50 negatives). The samples were analyzed through a) IIFT (HEp-2010; Euroimmun AG, Luebeck); b) ChLIA ANA Screen (IDS) and ChLIA specific antigens (dsDNA, centromere B, Jo-1, Scl-70, SS-A/Ro, SS-B/La, Sm, U1-snRNP, IDS); c) lineblot (Ana profile+DFS0, Euroimmun AG, Luebeck).

IIFT and ChLIA ANA Screen have shown a general moderate agreement and as well lineblot and specific antigens ChLIA. The combination IIFT + lineblot gave a better analytical performance compared to ChLIA ANA screen + specific antigens. Overall, the combination of IIFT + ChLIA ANA Screen + lineblot, has shown a great performance (positive agreement:73%; negative agreement: 78%). Differently, IFA + ChLIA ANA Screen + ChLIA specific antigens highlighted a bare increase in sensitivity (80%), to specificity disadvantage (37%).

In our study, IIFT and lineblot have demonstrated the best analytical agreement. Furthermore, the addition of ANA Screen to those methods increases the sensitivity of about 6% and the specificity of 3%. Thus, we confirmed that ANA possible diagnostic algorithm could be divided in two phases: a) screening phase: IIF+ ChLIA Screen; b) confirmatory phase: lineblot.

Further studies will be necessary to confirm these results increasing the number of samples.

9.5 Advances of ANCA-Determination

Can immunoglobulin G (IgG) N-glycome changes in the anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis improve monitoring disease status?

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Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is an autoimmune disease in which immune dysregulation leads to necrotizing vasculitis. Monitoring disease status is crucial due to relapse possibilities as well as obtaining optimal remission. This pilot research investigates longitudinal IgG N-glycome changes in AAV patients under immunosuppressive treatment.

Materials and Methods

Serum samples from two female AAV patients (aged 63 and 67) were collected at multiple stages: at disease onset, after remission, and following maintenance therapy. One patient had myeloperoxidase (MPO)-AAV, the other proteinase-3 (PR3)-AAV, both with renal and pulmonary involvement. IgG was isolated, and N-glycans were enzymatically released, fluorophore-labeled, and analyzed using capillary gel electrophoresis. Glycan traits were calculated based on structural features.

Results

In both cases, a consistent increase in digalactosylated glycans and a decrease in agalactosylated structures were observed during remission, suggesting anti-inflammatory IgG glycosylation shifts. One patient also showed a significant decrease in afucosylated and sialylated glycans after Rituximab therapy. The other showed increased bisecting GlcNAc structure.

Conclusion

These case reports confirm that IgG N-glycome dynamically changes in response to immunosuppressive treatment in AAV. The consistent reduction in agalactosylation and elevation in galactosylation reflect anti-inflammatory remodeling of the IgG molecule. The increase of bisecting GlcNAc structure could point to anti-inflammatory status although literature data on its immunological role is still inconsistent. These preliminary findings suggest the potential of glycan profiling as a biomarker for disease monitoring and treatment response in AAV, meaning that the changes could be detected before anti-MPO and anti-Pr3 changes as well as the onset or the decrease of the symptoms. To discover the full potential of glycan status monitoring further investigation in larger patient cohorts are needed.

Analytical Performance of a Novel, Fully Automated Multiplexed Microarray Immunoassay Prototype for the Simultaneous Detection of Autoantibodies to GBM, PR3, and MPO: A Multicenter Evaluation

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Background/Purpose

Anti-neutrophil cytoplasmic antibodies (ANCA), including those directed to myeloperoxidase (MPO) and proteinase 3 (PR3) as well as antibodies against anti-glomerular basement membrane (anti-GBM) are critical markers in the diagnosis of small vessel vasculitides and anti-GBM disease, respectively. While some multiplex platforms are available, conventional testing often involves sequential single-analyte assays, which may delay diagnosis and increase workload. There remains a clinical need for fully automated multiplexed assays capable of simultaneously detecting these key markers. This multicenter study evaluated the analytical performance of a novel, fully automated multiplexed microarray immunoassay prototype (MosaiQ AiPlex® VAS microarray; AliveDx, Switzerland), designed for the simultaneous detection of IgG autoantibodies to PR3, MPO, and GBM, in comparison with routine singleplex and multiplex methods.

Methods

The study was conducted at four sites in the United Kingdom, including three public reference laboratories (two located in England and one in Wales) and a sponsor's site in Scotland. Comparator methods varied by site and included fluorescence enzyme immunoassay (FEIA), addressable laser bead immunoassay (ALBIA) and chemiluminescent immunoassay (CLIA). De-identified, banked serum samples previously characterized as reactive or non-reactive for PR3, MPO or GBM antibodies were tested using the investigational microarray prototype on its proprietary automated platform. Analytical performance was assessed by calculating positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) both in comparison to each site's routine method and in aggregate across all sites and platforms. One site using CLIA did not have anti-GBM reactive samples available for testing.

Results

The investigational microarray prototype consistently demonstrated high NPA across all measurands and comparator methods, ranging from 95.1% (anti-PR3 and anti-MPO, FEIA) to 100% (anti-PR3 at 1 ALBIA site and anti-GBM with FEIA, CLIA and at 1 ALBIA site). Overall NPA was 96.4% for anti-PR3 (95% CI: 94.5, 97.8), 96.1% for anti-MPO (95% CI: 94.0, 97.6) and 99.2% for anti-GBM (95% CI: 96.9, 99.9).

PPA ranged from 77.5% (anti-MPO, CLIA) to 100% (anti-GBM across methods). Overall PPA across sites and methods was 88.5% for anti-PR3 (95% CI: 81.6, 93.3), 89.1% for anti-MPO (95% CI: 82.4, 93.9) and 100% for anti-GBM (95% CI: 85.2, 100).

OPA across sites and methods ranged from 94.8% to 99.2%. Detailed results are summarized in Table 1.

Table 1. Agreement of the investigational microarray prototype with comparators

Site	Site 1 (Peterborough)			Site 2 (Newcastle)			Site 3 (Swansea)			Site 4 (Edinburgh)			All sites		
Method	FEIA			ALBIA			CLIA			ALBIA			All methods		
Measurand	anti-PR3	anti-MPO	anti-GBM	anti-PR3	anti-MPO	anti-GBM	anti-PR3	anti-MPO	anti-GBM	anti-PR3	anti-MPO	anti-GBM	anti-PR3	anti-MPO	anti-GBM
Reactive results (n)	11	15	2	32	37	5	52	40	NA	35	36	16	130	128	23
PPA (%)	**90.9**	**93.3**	**100**	**87.5**	**89.2**	**100**	**82.7**	**77.5**	**NA**	**97.1**	**100**	**100**	**88.5**	**89.1**	**100**
95% CI	62.3-99.5	70.2-99.7	17.8-100	71.9-95.0	75.3-95.7	56.6-100	70.3-90.6	62.5-87.7	NA	85.1-99.9	90.3-100	79.4-100	81.6-93.3	82.4-93.9	85.2-100
Non-reactive results (n)	143	143	43	92	87	57	178	190	41	107	121	96	520	541	237
NPA (%)	**95.1**	**95.1**	**100**	**95.7**	**95.4**	**96.5**	**95.5**	**95.3**	**100**	**100**	**99.2**	**100**	**96.4**	**96.1**	**99.2**
95% CI	90.2-97.6	90.2-97.6	91.8-100	89.4-98.3	88.7-98.2	88.1-99.4	91.4-97.7	91.2-97.5	91.4, 100	96.6-100	95.3-100	96.2-100	94.5-97.8	94.0-97.6	96.9-99.9
OPA (%)	**94.8**	**94.9**	**100**	**93.6**	**93.6**	**96.8**	**92.6**	**92.2**	**100***	**99.3**	**99.4**	**100**	**94.8**	**94.8**	**99.2**

ALBIA: addressable laser bead immunoassay, CI: confidence interval, CLIA: chemiluminescent immunoassay, FEIA: fluorescent enzyme immunoassay, NA: not applicable, NPA: negative percent agreement, OPA: overall percent agreement, PPA: positive percent agreement. *Limited to NPA (due to lack of anti-GBM reactive samples).

Conclusion

In this multicenter study, the investigational microarray prototype showed high concordance with routine singleplex and multiplex methods for the detection of anti-PR3, anti-MPO, and anti-GBM

autoantibodies. These findings support the potential utility of the assay as a single-platform solution for streamlined, simultaneous serologic evaluation of ANCA-associated vasculitis and anti-GBM disease. Future studies incorporating clinically characterized samples will enable expanded assessment of the assay's diagnostic performance.

Development of recombinant myeloperoxidase as an innovative approach for the detection of anti-MPO antibodies

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Background

Myeloperoxidase (MPO) is an enzyme that resides in the neutrophil primary granules and is one of the most important antigens targeted by anti-neutrophil cytoplasmic antibodies (ANCA). Anti-MPO antibodies are mostly found in a variety of vasculitides: idiopathic vasculitis, vasculitis-associated crescentic glomerulonephritis (CG), classic polyarteritis nodosa, Churg-Strauss syndrome and polyangiitis overlap syndrome without renal involvement. Precisely, anti-MPO antibodies were found in 77-100% of patients with idiopathic and vasculitis-associated CG. Moreover, levels of anti-MPO antibodies are significantly higher during active phases of disease compared to phases of remission, making them important markers for disease activity.

ANCA testing on Indirect Immunofluorescence Assay (IFA) is an important diagnostic tool for the screening of samples suspected of having various autoimmune mediated vasculitides. Several methods are available for the confirmation of ANCA, including but not limited to Enzyme-Linked Immunosorbent Assays (ELISA) and Chemiluminescence immunoassays (CLIA). For most autoantibody assays, native antigens have been replaced by recombinant proteins due to higher consistency and performance. However, due to the technical difficulty and/or low process yields, such a transition has not yet occurred for anti-MPO assays.

Objective

To generate and characterize, analytically and functionally, recombinant human MPO for the detection of anti-MPO antibodies that could be used as an alternative to the native antigen in some immunoassays, using QUANTA Flash® as example.

Methods

Several constructs comprising the full-length MPO sequence or a truncated version, with the addition of a terminal His-tag, were designed, cloned and transfected into mammalian cells. The cell culture supernatant was purified by a two-column process using an affinity and an ion exchange chromatography. The purified recombinant MPO antigen was then coupled to beads and assessed by QUANTA Flash®. A panel of 15 serum samples from patients with vasculitis and 15 serum samples from healthy donors was used to compare the functional performance of the recombinant MPO with the native antigen, as well as the lot-to-lot consistency. Moreover, up to 6 rounds of freeze/thaw (F/T) cycles were carried out to different antigen lots, which were then analyzed by SDS-PAGE and nano-Differential Scanning Fluorimetry (nanoDSF) to evaluate the antigen stability.

Results

The generated recombinant MPO showed a single band around 80kDa in an SDS-PAGE (in both reducing and non-reducing conditions), which corresponds to the monomeric form of the antigen. In contrast, native MPO shows two bands under reducing conditions, which correspond to the heavy chain (HC) and light chain (LC) of the antigen, and other several bands in non-reducing conditions that correspond to the intermediate subproducts of the protein and the dimeric form.

The purification process incorporated two chromatography columns and yielded >90% purity for all the generated antigen lots. Freeze/thaw studies showed that the protein was stable after 6 rounds of F/T cycles, maintaining the same electrophoretic profile and melting temperature (Tm).

Results showed that the process is highly reproducible as seen by a robust positive correlation between two antigen lots generated and coupled to beads (Pearson's correlation of r=0.998). Although the Pearson's correlation between the recombinant and the native MPO is lower (r=0.944), this is due to the fact that the recombinant antigen increases the reactivity for some positive samples.

Conclusions

We have been able to develop a robust process to generate recombinant MPO with high purity for the detection of anti-MPO antibodies. The new antigen showed similar clinical performance on QUANTA Flash® vs. the native counterpart currently being used in the assay. Although a bigger sample size should be tested for confirmation, recombinant MPO represents a promising antigen to improve the lot-to-lot consistency and avoid possible supply risks.

Changes in myeloperoxidase (MPO)-specific antineutrophil cytoplasmic antibody (ANCA) levels before and after treatment and their association with Birmingham Vasculitis Activity Score (BVAS)

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Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA), particularly myeloperoxidase autoantibodies (MPO-ANCA), are clinically relevant serological markers widely used in the diagnosis and management of microscopic polyangiitis (MPA)1. Despite limited evidence about the utility of MPO-ANCA levels in distinguishing active from inactive disease, as well as in predicting relapses, MPO-ANCA are frequently used in monitoring of patients. In this retrospective observational study, we evaluated a chemiluminescence immunoassay (CIA) for detecting MPO-ANCA IgG and its potential role in monitoring disease activity.

Methods

Remnant serum samples from Spanish patients diagnosed with MPA [n=57 unique patients with baseline (at time of diagnosis) and/or follow-up (post-treatment) samples], disease controls [systemic lupus erythematosus (SLE, n=20), mixed connective tissue diseases (MCTD, n=20), systemic sclerosis (SSc, n=20), infectious disease (ID, n=20), rheumatoid arthritis (RA, n=20)], and healthy individuals (HI, n=100) were tested on the QUANTA Flash® MPO (Inova Diagnostics, San Diego, USA; NOTE: intended use for diagnosis, not for monitoring) CIA. Among the MPA group, for only 41 patients both baseline and follow-up specimens were available. Information on the patients' Birmingham Vasculitis Activity Score (BVAS; >6 = active disease) and the time (in days) between baseline and follow up were available for all MPA patients at time point of blood

sampling. Spearman's rank correlation, paired t-test, and Wilcoxon-Mann-Whitney test were used in the data analysis.

Results

At diagnosis, MPO-ANCA were detected in 100.0% of MPA patients (41/41) and in none of the disease controls or healthy individuals. Among MPA patients with available paired results, notable differences were observed on MPO-ANCA levels between baseline and follow-up (mean: 831.5 vs 311.1 CU, p=0.0101). When comparing antibody levels between active vs. inactive disease, significant difference was also observed (mean: 764.0 vs. 521.1 CU, p=0.0479). A modest but significant correlation was found between MPO-ANCA levels and BVAS (Spearman's rho=0.275; p=0.0056). Moreover, when all available MPA datapoints were stratified by number of days after treatment (≤90 vs >90 days), MPO-ANCA levels were found to be significantly higher during the earlier period compared to the later period of treatment (median: 224.4 vs. 80.8 CU, p=0.0243).

Conclusion

In this Spanish cohort, MPO-ANCA IgG levels significantly decreased following treatment and were notably higher in patients with active disease compared to those with inactive disease. While these results show promise in MPA monitoring, cautious interpretation of MPO-ANCA titers in isolation is recommended. A prospective longitudinal study is warranted to further validate these findings and to expand the intended use of MPO-ANCA assays.

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Profiling of novel autoantibodies for prediction of disease activity in ANCA-associated vasculitis

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Introduction

ANCA-associated vasculitides (AAV) are a heterogenous group systemic autoimmune diseases characterized by necrotizing inflammation of small blood vessels requiring prompt initiation of immunosuppressive treatment to limit organ damage. Autoantibodies are the hallmark of AAV and is used in the clinic for diagnosis and classification. In addition, the pathogenic role of these autoantibodies has previously been suggested, and autoantibodies might therefore be used as biomarkers for disease activity.

As part of the PersonAlisation of RelApse risk in autoimmune DISEase (PARADISE) consortium, we aimed to investigate novel, and previously known autoantibodies in AAV to be used as biomarkers for disease activity.

Methods

Plasma samples (n=1221) from 535 individuals with AAV, and 66 healthy controls, as well as clinical information were collected from the RITA-Ireland Vasculitis Biobank, a national cohort across eight sites in Ireland. Samples were screened for IgG autoantibodies using our in-house multiplex antigen bead array including 369 antigens corresponding to 272 proteins.

Results

Novel autoantibodies with higher prevalence in the AAV group compared to healthy controls were identified. In addition, we could confirm autoantibody reactivities found in another independent cohort in previous studies by our group. Of the previously known anti-MPO positive individuals, we could confirm 98% of these, indicating that our assay is robust. Finally, the profiling of autoantibodies in samples collected from AAV patients at active disease versus remission also revealed a preliminary association of specific autoantibodies.

Conclusion

The autoantibody profiling in this study identified several novel autoantibodies, and the possible association with disease activity. Further analysis of the clinical impact of these findings still needs to be determined.

9.6 Biomarkers of intestinal mucosal inflammation

Autoantibodies and Biomarkers in the Follow-Up of Autoimmune Gastritis

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Abstract

Autoimmune chronic atrophic gastritis (A-CAG) is an immune-mediated inflammatory disorder that affects the corpus-fundus mucosa of the stomach [1, 2] leading to the loss of parietal cells, with these being replaced by atrophic and metaplastic tissue [3-5]. Hematological alterations represent the main clinical presentation, as a result of micronutrient deficiencies. Within the hematological findings, a common presentation is microcytic iron deficient anemia as a consequence of iron malabsorption for a reduced conversion of Fe^{3+} to Fe^{2+} due to decreased acid secretion into the stomach. Megaloblastic (also called pernicious) anemia generally represents a late-stage manifestation of A-CAG due to vitamin B12 malabsorption induced by intrinsic factor (IF) deficiency [6]. IF is responsible for binding vitamin B12 and facilitating uptake in the terminal ileum. When parietal cell loss occurs, the reduced bioavailability of this molecule is due to reduced production as well as autoantibodies targeting IF.

Increased interest in the pathogenesis and the evolution of A-CAG has led to the search for serological markers that can be used to detect changes in the gastric mucosa at an early stage and to monitor the course of the disease. Parietal cell autoantibodies (PCA) directed against the enzyme H^+/K^+-ATPase present on the surface of gastric parietal cells, have been proposed as suitable immunological markers of atrophic damage, as they can be detected in the serum when symptoms of gastritis are not yet present.

During the disease course, quantitative variations in PCAs titers can be observed, depending on the extent of gastric atrophy; they tend to gradually decline from early- to late-stage A-CAG and may eventually disappear in late-stage patients. This observation might be explained by considering that autoantibody levels increase progressively over time, reach a peak level, and then fall, as a result of the ongoing destruction of the gastric mucosa and the disappearance of the target autoantigen H+/K+-ATPase [1]. Hence, the absence of PCA does not exclude the possibility of late-stage atrophic damage.

Other autoantibodies that are detectable in the serum of patients affected by A-CAG are those directed against the IF. The IF is secreted by gastric parietal cells; under normal conditions, it binds through a high-affinity bond to vitamin B12, facilitating its transport to the terminal ileum, where it is absorbed. During A-CAG, the sequestration of the molecule by the related autoantibodies reduces intrinsic factor bioavailability, resulting in a lower or failed vitamin B12 absorption, with the consequent onset of pernicious anemia [7]. IFA are present in approximately 80% of patients with pernicious anemia; they are more specific than PCA (98.6% *vs*. 90%) for A-CAG, but the sensitivity is lower (37% vs. 81%), increasing during disease progression [8]. In the very advanced stages of A-CAG, IFA disappear, together with the disappearance of PCA due to the significant depletion of parietal cells.

However, the utility of measuring only the level of PCA and IFA in the follow-up of A-CAG does not appear to suffice. Recent evidence has suggested that, in monitoring A-CAG, PCA should be associated with an evaluation of gastric function through biochemical and hormonal tests, and the combined use of PCA with biomarkers of gastric function, such as pepsinogens I and II (PGI and PGII), and gastrin17 (G17), has been proposed [9]. The main reason is that under physiological conditions, PGI is secreted only by oxyntic glands of the corpus mucosa. Conversely, PGII is produced both in the corpus and in the gastric antrum and duodenum. In the presence of A-CAG, which is typically restricted to the corpus-fundus, serum levels of both PGI and PGII decrease even if, because of the antral sparing, PGII decline is less than that of PGI. As a result, there is a correlation between the decrease in the number of cells in the body-fundus of the stomach and the PGI/II ratio [6].

In turn, the destruction of parietal cells and the lowered PGI concentrations will reduce the secretion of hydrochloric acid and induce the compensatory increased production of G17 by the G-cells localized in the antrum [10]. Therefore, by associating pepsinogens with PCA for diagnosis and using G17 for follow-up, a much more complete picture of gastric function would be obtained, thus allowing for a more effective real-time monitoring of the state of the gastric mucosa and the progression of damage [11-14] (Table 1).

Finally, in the follow up it should be considered that A-CAG is a progressive disorder and since G17 has the potential to stimulate the proliferation of enterochromaffin-like cells [15, 16], patients with gastric atrophy should be monitored because they carry a high risk of this condition evolving into neuroendocrine (carcinoid) tumors.

Table 1. Clinical significance and utility of serological and immunological markers for autoimmune chronic atrophic gastritis (A-CAG) diagnosis and follow up (PGI: pepsinogen I; PGII: pepsinogen II; PGI/PGII: pepsinogen I/pepsinogen II ratio; G17: gastrin-17; PCAs: parietal cells antibodies; IFAs: anti-intrinsic factor antibodies).

Marker	Trend in A-CAG	Clinical significance	Diagnosis	Follow up
PGI	↓↓	A decrease is suggestive for atrophy of the stomach's body. Serum levels decrease in proportion to the severity of atrophy	+++	++
PGII	=	In A-CAG with progressive atrophy level declines less than of PGI due to its production mainly in the stomach's antrum and in duodenum	-	-
PGI/PGII	↓↓	The ratio decreases linearly with the reduction of PGI and with the severity of gastric atrophy	+++	++
G17	↑↑	Increases in response to a reduced acid production and stimulates the proliferation of enterochromaffin-like cells. It's a reliable marker of A-CAG evolution from the potential to an overt disease stage. Higher levels are associated with a more severe disease stage, possibly reflecting the extent of mucosal damage	++	+++
PCAs	↑↑	One of the first signal to suspect A-CAG. Present in 85-90% of A-CAG patients, but also in 2.5-9% of healthy adults, as well as in people with other autoimmune diseases. Detectable in the initial phases of A-CAG, tend to fluctuate over the course of the disease and progressively decrease until being undetectable in the later stages due to the depletion of parietal cells	+++	++
IFAs	↑	Present in approximately 80% of patients with pernicious anemia; more specific than PCAs (98.6% vs. 90%), but less sensitive (37% vs. 81%). Their detection becomes more pertinent during the advanced stages correlating with the atrophy stage. In the very advanced stages of A-CAG, IFAs disappear together with the disappearance of PCAs, due to the significant depletion of parietal cells	+	+

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Autoantibodies and biomarkers in follow-up of celiac disease

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Abstract

Celiac Disease (CD) is an autoimmune disease caused by an immune reaction to dietary gluten, a protein that is found in wheat, barley and rye. Gluten is widely used in the entire food chain because of its specific biochemical properties and not only found in obvious food such as bread and pasta. Eating food containing gluten triggers an HLA-DQ-2.5, -2.2 or -8 restricted CD4+ T-cell immune response in the small intestine of CD patients. This causes damage to the small intestine's epithelial lining, which will lead to a malabsorption syndrome. Most CD patients present at diagnosis with high titers of IgA auto-antibodies to the enzyme tissue transglutaminase (tTG-IgA). Together with changes in the mucosal architecture as observed in duodenal biopsies (villous atrophy), this establishes CD diagnosis. Current guidelines state that under certain conditions when the titer of tTG-IgA is high CD can be diagnosed without histological evaluation of duodenal biopsies. A strict gluten free diet (GFD) leads to healing of the intestinal mucosa and decline in tTG-IgA antibodies. Diagnostic criteria for CD are well defined and described in specific guidelines, but guidelines for monitoring of CD patients on a GFD are still lacking. Classical tools used to monitor the adherence and response to GFD, as well as novel non-invasive biomarkers that have been proposed for CD monitoring will be discussed.

The autoantigenic target glycoprotein 2 is a novel fecal marker for gut microbiota dysbiosis and systemic inflammation

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Objective

The antimicrobial autoantigenic target glycoprotein 2 (GP2) is an important component of the innate immune system which originates from the exocrine pancreas and intestinal as well as peribiliary glands. Mucosal loss of tolerance in the form of IgA to GP2 appears to be involved in the pathogenesis of autoimmune intestinal and liver disorders and related tumorigenesis. In this context, the interaction of GP2 with the intestinal microbiome and its implications are poorly understood.

Methods

Fecal samples from 2,812 individuals of the Study of Health in Pomerania (SHIP) were collected to determine GP2 levels by ELISA and gut microbiota profiles (16S rRNA gene sequencing). Data were correlated and associated with highly standardized and comprehensive phenotypic data of the study participants.

Results

Fecal GP2 levels were elevated in individuals with higher body mass index and smokers, whereas lower levels were found in case of preserved exocrine pancreatic function, female sex or a healthier diet. Moreover, higher GP2 levels were associated with increased serum levels of high-sensitivity C-reactive protein, loss of gut microbial diversity and an increase of potentially detrimental bacteria (Streptococcus, Haemophilus, Clostridium XIVa, or Collinsella). Additionally, predicted microbial pathways for the biosynthesis of beneficial short-chain fatty acids or lactic

acid were depleted in individuals with high fecal GP2. GP2 exhibited a stronger association to overall microbiome variation than the inflammation marker calprotectin.

Conclusion

Fecal GP2 is a biomarker of gut microbiota dysbiosis and associated with increased systemic inflammation. Pancreatic acinar cells do not appear to be solely responsible for the changes of fecal GP2 levels.

Newly developed ChLIA for detection of fluid calprotectin in serum

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Introduction

Calprotectin present in circulation serves as a biomarker indicative of the systemic activation of neutrophile granulocytes and monocytes. Measuring its level in serum is relevant for diagnosis and monitoring of systemic inflammations in autoimmune diseases and infections.

We present a newly developed chemiluminescence assay from Immunodiagnostic Systems Ltd (IDS), IDS Fluid Calprotectin, by reporting the results of calprotectin measurements in serum samples from healthy individuals and patients. Furthermore, the results of the IDS Fluid Calprotectin are compared with those of a newly developed Circulating Calprotectin ELISA from Euroimmun.

Methods

To establish the reference limit for the IDS Fluid Calprotectin, calprotectin levels were measured in 235 serum samples from healthy adults and 51 serum samples from healthy children (age range: 1-19).

To assess inflammatory status, calprotectin concentrations were quantified in a cohort comprising 378 serum samples from 50 healthy adults, 48 healthy children, 80 rheumatoid arthritis patients, 50 systemic lupus erythematodes patients, 50 Sjögren's syndrome patients, 50 systemic sclerosis patients, and 50 idiopathic inflammatory myopathy patients.

Quantitative results were compared with the Euroimmun Circulating Calprotectin ELISA, and agreement was assessed with Passing-Bablok regression.

Results

The reference limit for adults at 2.8 µg/mL was established as the 95% right side upper limit of normality following the guidance of CLSI C28-A3 robust method. Compared to healthy individuals, calprotectin concentrations were elevated in patient sera.

Passing-Bablok regression indicated constant and proportional differences between calprotectin concentrations: (EUROIMMUN = 0.75 (95% CI: [0.73, 0.78]) x IDS Fluid Calprotectin + 0.1 ([0.05, 0.014]); correlation coefficient r = 0.94; n=305.

Conclusion

The finding indicated high correlation with a quantitative bias between the results of the IDS Fluid Calprotectin and the Circulating Calprotectin ELISA (EUROIMMUN), which make it necessary to evaluate results with respect to the assay-specific reference limits. The IDS Fluid Calprotectin will be applied in studies aimed at exploring, for instance, the correlation with disease states associated with various systemic inflammations. Future research will determine whether fluid calprotectin is a more specific biomarker of inflammation than C-reactive protein (CRP).

Conflict of interest: MSS and HP are employees of IDS, a company that manufactures diagnostic tests and instruments. VBL, KH, and VH are employees of EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial gain as a result of this publication.

Newly developed ELISA for detection of circulating calprotectin in serum

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Introduction

Circulating calprotectin is a biomarker that reflects activation of the innate immune system. Measuring its concentration in serum or plasma is relevant for diagnosis and monitoring of systemic inflammations, autoimmune diseases, and infections.

Here we introduce a newly developed enzyme-linked immunosorbent assay (ELISA) from EUROIMMUN by presenting results of circulating calprotectin measurements in samples from healthy individuals and patients. Furthermore, the results of the ELISA are compared with those of a newly developed calprotectin chemiluminescence assay (ChLIA) from Immunodiagnostic Systems Ltd (IDS).

Methods

To determine the reference interval of the Circulating Calprotectin ELISA, calprotectin levels were measured in 136 paired serum and K_3 EDTA samples from healthy adults (74 male, 62 female, age range: [19-71] years).

For assessment of inflammatory status, calprotectin concentrations were measured in a panel comprising 441 serum samples from 136 healthy adults, 25 healthy children, 80 rheumatoid arthritis (RA) patients, 50 systemic lupus erythematodes (SLE) patients, 50 Sjögren's syndrome (SS) patients, 50 systemic sclerosis (SSc) patients, and 50 idiopathic inflammatory myopathy (IIM) patients with both the Circulating Calprotectin ELISA (EUROIMMUN) and the IDS Fluid Calprotectin ChLIA.

Quantitative results (n=305) of both immunoassays were compared, and possible bias was estimated with Passing-Bablok regression.

Results

The upper 95% reference limit of the Circulating Calprotectin ELISA was higher for serum (2.29 µg/mL) than for plasma samples (1.31 µg/mL).

Compared to healthy individuals, calprotectin concentrations were elevated in patient sera.

Passing-Bablok regression indicated small constant and proportional differences between calprotectin concentrations measured with ELISA versus ChLIA (n=307, regression: y=-0.13+1.33x, 95% confidence interval (CI) intercept: [-0.14, -0.10], CI slope: [1.32, 1.34]). In comparison, the calprotectin concentrations were lower when measured with ELISA.

Conclusion

Calprotectin concentrations measured with the Circulating Calprotectin ELISA (EUROIMMUN) aid in the serological assessment of inflammation status in adult, pediatric, and patient populations. Despite the high agreement between the Circulating Calprotectin ELISA (EUROIMMUN) and the IDS Fluid Calprotectin ChLIA, the results showed differences. It is therefore necessary to emphasize that the quantitative results must be evaluated with respect to the assay-specific reference intervals. Soon, the newly developed ELISA will be applied in clinical studies set out to determine reference values, likelihood ratios, and correlation with disease states of different systemic inflammations. Future research will determine whether circulating calprotectin is a more specific biomarker of inflammation than c-reactive protein (CRP).

Conflict of interest: MSS is an employee of IDS, a company that manufactures diagnostic tests and instruments. KH, VH, and VBL are employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

Role of assay-specific cut-offs for measurement of fecal calprotectin using chemiluminescence assays

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Introduction

Fecal calprotectin (FC) is a non-invasive biomarker specific for intestinal mucosal inflammation. Determination of FC concentration can support the serological diagnostics of inflammatory diseases of the gastrointestinal tract (inflammatory bowel disease (IBD), e.g., Crohn's disease or ulcerative colitis) and functional disease (irritable bowel syndrome (IBS)) in patients with chronic abdominal pain, as well as monitoring of IBD progression. Here we compared the agreement of FC concentrations measured with three commercially available chemiluminescence immunoassays (ChLIAs) processed automatically using random access technology.

Methods

After an extraction procedure, FC concentration was measured in remnant stool samples from 197 patients with suspected IBD who were examined at the university clinic of Liége following corresponding instructions for use of the different assays. Qualitative results of the Calprotectin ChLIA (Immunodiagnostic Systems Ltd, IDS) were compared with those of the fCAL turbo (Bühlmann) and the LIAISON Calprotectin (DiaSorin) using percent agreement and Cohen's kappa. Possible bias between quantitative results of the three methods was estimated with Passing-Bablok regression.

Results

The qualitative results of the IDS ChLIA were in almost perfect agreement with the other ChLIAs (κ>0.8, total percent agreement >93%). FC concentrations measured with the IDS and DiaSorin ChLIAs indicated a linear relationship, although results deviated at concentrations >200µg/g (n=127, regression: y=2.33+0.97x, confidence interval (CI) intercept: [-5.0, 10.5], CI slope: [0.8, 1.1]). FC concentrations measured with the IDS ChLIA showed a high proportional deviation compared to those obtained with the Bühlmann ChLIA which were significantly higher (n=115, regression: y=-7.9+1.6x, 95% CI intercept: [-21.3, 6.4], CI slope: [1.4, 1.9]).

Conclusion

The agreement between the qualitative ChLIA results and the clinical classification was high. However, the FC concentrations varied among the three ChLIAs. This finding underscores the necessity of interpreting quantitative results with respect to the assay-specific cut-offs. Measurement of FC concentration using ChLIAs presents a reliable and non-invasive tool to obtain high-quality results supporting serological diagnostics of IBD combined with the advantages of automated processing with random access technology.

Conflict of interest: MSS is an employee of IDS, a company that manufactures diagnostic tests and instruments. VBL is employed by EUROIMMUN, a company that manufactures diagnostic tests and instruments. None of the authors benefits from any potential or actual financial or non-financial gain as a result of this publication.

EDTA plasma for measuring circulating calprotectin: a matrix to avoid!

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Introduction

Circulating calprotectin (cCLP) gained interest as a biomarker of neutrophil-related inflammation. cCLP analysis, however, is hampered by pre-analytical and analytical confounders. Therefore, we aimed to define the best suitable matrix for cCLP measurements.

Materials and methods

Seven-point calibration curves were composed by spiking recombinant (rCLP) and native (nCLP) calprotectin in healthy control pools of serum, ethylenediaminetetraacetic acid (EDTA) plasma and citrate plasma. In parallel, eight patient samples in the respective matrices were analyzed. Measurements were performed using 4 different cCLP methods (EliA™ Calprotectin 2 (Thermo Fisher Scientific, serum/plasma protocol for research use only), BÜHLMANN sCAL® turbo (Bühlmann laboratories), QUANTA Flash® Circulating Calprotectin (Werfen), LIAISON® Calprotectin (DiaSorin S.P.A., serum/plasma protocol for research use only).

Result

For both serum and citrate plasma, rCLP and nCLP calibration curves were linear (R^2>0.99) with all methods. Serum and plasma cCLP concentrations were similar for both rCLP and nCLP curve samples with higher recoveries for nCLP compared to rCLP on all assays except Bühlmann (Table 1).

In EDTA plasma, the rCLP and nCLP recoveries were significantly lower than in serum and citrate plasma ($p<0.05$), which was most pronounced for the EliA™ and DiaSorin methods.

Patient samples showed cCLP concentrations in plasma that were half the corresponding serum cCLP concentrations. EDTA and citrate plasma samples yielded similar results for each individual method. In 3 out of 8 patient samples, cCLP levels in EDTA plasma were significantly higher than in the corresponding citrate plasma or serum samples.

Table 1. Median recovery of rCLP and nCLP

CLP formulation	Sample type	Median recovery of target concentrations (%)				
		Bühlmann	Werfen	Thermo Fisher	DiaSorin	Total median
rCLP	Serum	125,3	141,1	71,2	106,6	117,6
	Citrate plasma	123	129,9	74,1	110,9	115,8
	EDTA plasma	81,4	48,9	5,3	11,4	12,0
nCLP	Serum	118,5	156,9	121,8	139,3	133,8
	Citrate plasma	126,3	169,1	147,8	140,5	140,5
	EDTA plasma	67,2	52,6	4,7	7,2	7,5

Conclusion

Preliminary experiments revealed best recovery and reproducibility of cCLP results in serum and citrate plasma, excluding EDTA plasma as matrix of choice for measuring cCLP.

A new recombinant and native calprotectin formulation: a step forward towards standardization of circulating calprotectin assays

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Objective

Circulating calprotectin (cCLP) is an emerging biomarker for neutrophil-driven inflammation, especially in chronic inflammatory rheumatic diseases. However, significant inter-assay variability hampers its clinical utility. This study aimed to evaluate the standardization potential of recombinant calprotectin (rCLP) and native calprotectin (nCLP) calibration curves across four commercial cCLP assays.

Methods

Seven-point calibration curves were composed by spiking rCLP and nCLP stock solutions into healthy control pools of serum, EDTA plasma and citrate plasma. In parallel with the calibration curve samples, patient samples (serum: n=39; citrate plasma: n=37) were measured, spread over 3 days.

All cCLP tests were performed using four assays: EliA™ Calprotectin 2 (using a serum/plasma protocol for research use only, Thermo Fisher Scientific; FEIA), BÜHLMANN sCAL® turbo (Bühlmann laboratories; PETIA), QUANTA Flash® Circulating Calprotectin (Werfen; CLIA), and

LIAISON® Calprotectin assay (using a serum/plasma protocol for research use only, DiaSorin S.P. A.; CLIA).

cCLP results were recalculated using the respective rCLP and nCLP calibration curves, obtained in the same run and matrix.

Results

Very good rank correlations (r>0.9) were obtained across all assays and matrices for both original and recalibrated cCLP results. Passing-Bablok analysis however, indicated both systematic and proportional errors between methods. After rCLP recalibration, inter-assay agreement improved for most assays, except for EliATM and Bühlmann, which deviated further from consensus values. In contrast, nCLP recalibration consistently reduced inter-assay differences and harmonized results towards the trimmed mean (Table 1), all within maximum allowable uncertainty (MAU) thresholds (57,9% for serum and 43,5% for citrate plasma).

Table 1: Median relative difference towards trimmed mean for serum and citrate samples.

Serum samples	Median difference towards trimmed mean (%)		
	Original results	rCLP recalibrated results	nCLP recalibrated results
Bühlmann	-11,1	-19,5	-1,0
QUANTA Flash	13,4	-5,5	-2,0
EliA™	-2,9	35,9	2,4
Liaison	1,8	5,2	1,4
Citrate samples	Mean difference towards trimmed mean (%)		
	Original results	rCLP recalibrated results	nCLP recalibrated results
Bühlmann	-9,4	-21,9	1,2
QUANTA Flash	25,0	-0,4	-0,3
EliA™	-17,1	37,5	-0,9
Liaison	5,7	0,4	3,6

Conclusion

Both rCLP and nCLP calibrations improved alignment of cCLP assay results. However, nCLP demonstrated superior standardization potential across platforms. rCLP revealed potential as

cCLP calibrator, but assay-specific limitations, particularly with the EliA™ method, must be considered.

Anti-integrin αvβ6 IgG antibody as a diagnostic and prognostic marker in ulcerative colitis

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Introduction

Autoantibodies against integrin αvβ6 have recently been associated with ulcerative colitis (UC), its preclinical phase, and UC outcomes. However, data on the diagnostic utility of the autoantibody and its dynamics are scarce. Therefore, we aimed to examine the diagnostic

capacity of the anti-αvβ6 autoantibody. Moreover, we assessed its utility in defining a specific phenotype of UC and ascertained its temporal dynamics in relation to the disease course.

Aims & Methods

Using an in-house assay based on the EliA technology (Thermo Fisher Scientific, Uppsala, Sweden), anti-αvβ6 IgG autoantibodies were measured in serum from adult patients with suspected IBD in the Swedish inception cohort SIC IBD (discovery cohort, n=473) and the Norwegian population-based inception cohort IBSEN III (validation cohort, n=570). A cut-off of 400 µg/L was applied, corresponding to the lower calibrator for IgG (4 µg/L) and the 96th percentile of healthy individuals. The course of UC was categorized as indolent or aggressive at 12 months, based on a composite outcome of colectomy, hospital admission for active disease, treatment refractoriness towards ≥2 biological agents, or >2 courses of corticosteroids/cumulative dose of >2.5 g. Inflammatory proteins (n=92) were measured (Olink Proteomics, Uppsala). Welch's t-test was used with Benjamini-Hochberg correction, applying a 5% false discovery rate (FDR). Linear-mixed models were applied to investigate differences in autoantibody expression between diagnosis and three months follow-up.

Results

The discovery cohort comprised 327 IBD patients and 146 symptomatic controls without any discernible evidence of IBD, and the validation cohort included 366 IBD patients and 204 symptomatic controls. Patients with UC and those with colonic Crohn's disease in the discovery cohort had higher median anti-αvβ6 levels compared to symptomatic controls. The anti-αvβ6 autoantibody demonstrated a sensitivity of 73% and a specificity of 84% for distinguishing UC from CD and symptomatic controls in the discovery cohort and 79% and 91%, respectively, in the validation cohort.

Anti-αvβ6 positivity was associated with a severe UC phenotype, characterized by more extensive colitis (p=0.008), severe Mayo endoscopic activity (p=0.005), and pronounced systemic inflammation, defined as higher hsCRP (p=0.04) and lower albumin concentrations (p=0.01), in the discovery cohort. However, no association of f-Calprotectin was observed. Higher endoscopic activity in anti-αvβ6 positive UC patients was confirmed in the validation cohort. In both cohorts, an increasing extent of inflammation and endoscopic activity was observed with higher median levels of anti-integrin αvβ6.

Five proteins (SYND1, IL-17A, GZMB, MMP1, CXCL13) were significantly upregulated in anti-αvβ6 positive UC patients in the SIC-IBD, and two of them (IL-17A and GZMB) were validated in the IBSEN III cohort. Compared to patients with indolent UC, patients with an aggressive course presented with higher anti-αvβ6 levels at diagnosis in the discovery cohort (p=0.02) and potentially also in the validation cohort (p=0.13). A significant interaction of disease course was observed in autoantibody levels between diagnosis and 3 months in the SIC cohort (p<0.0001), as the levels decreased in patients with indolent UC but not in those with an aggressive course.

Conclusion

Anti-αvβ6 autoantibodies demonstrate a diagnostic capacity for UC and define a severe phenotype of the disease. Its temporal dynamics differ between patients with an aggressive and an indolent disease course.

Celiac Insider website: A diagnostic tool for improving celiac disease awareness and patient empowerment

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Background

Diagnosing celiac disease (CeD) poses significant challenges for healthcare professionals (HCPs) due to its diverse symptoms that often overlap with other gastrointestinal disorders. This leads to prolonged diagnosis times and impaired patient quality of life.

Objective

Our goal was to create an online platform to empower patients to get tested and raise awareness among HCPs to shorten the time to diagnosis of celiac disease.

Methods

Developed by Thermo Fisher Scientific (TFS), the website celiac insider was launched in English in November 2022. The site was promoted through SEM, social media and influencer campaigns. French and German versions were released in May 2024, with Italian and Spanish versions expected in July 2025.

Results

Celiac insider provides essential evidence-based resources to facilitate accurate diagnosis and support patients. It includes downloadable tools such as a food diary, symptom tracker, and brochures tailored for both audiences.

Since the introduction of the French and German website, the website has received more than 300,000 unique visits from more than 100 countries with an average visit time of 1 minute 38 seconds and 1:8 repeated visitors. As of May 2024, the French and German versions have received over 97,000 and 79,700 unique visits, respectively. With the upcoming launch of updated content

and additional language versions, we aim to double the total number of visitors and expand our reach to both patients and HCPs to the improve the diagnostic journey.

Conclusion

Celiac insider is contributing to improve to the diagnostic journey and quality of life for those living with CeD through education and empowerment. Its success is demonstrated by the strong engagement metrics.

Generation of monoclonal antibodies against tTG and mTG neo epitopes

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Introduction

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-neo and mTG-neo mAbs revealed novel patterns, previously not observed, and 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 serve as a tool to elucidate the pathogenic role of environmental triggers in the development of autoimmune diseases. Given their immunogenic potential, one must consider whether highly processed foods act as covert modulators of immune tolerance, thereby contributing to the rise in autoimmune disorders.

Novel epitopes and multiparametric diagnostics to eliminate unnecessary biopsies in pediatric celiac disease

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Background

The gold standard diagnosis of celiac disease relies on small bowel biopsies showing villous atrophy and lamina propria lymphocytic infiltration by 4-level Marsh score and the serological detection of anti tissue transglutaminase (tTG) and/or anti-endomysial IgA antibodies. To eliminate unnecessary and painful biopsies in children, and to reliably correlate autoantibody levels with clinical features and therapeutic outcomes a novel serological tool utilizing membrane-bound multiparametric enzyme immunoassay termed AESKUBLOTS® Gluten Related Disorders (GRD) has been developed for the overall quantitative determination of IgA antibodies in GRD.

Aims

To evaluate the clinical performance of AESKUBLOTS®GRD in Polish children presenting celiac disease

Methods

Antibody titers of AESKUBLOTS® GRD IgA; against the human tTG, neo epitopes of human tTG (tTG-neo), microbial transglutaminase (mTG), neo-mTg, deamidated gliadin specific peptides (DGP), gliadin, Frazer's fraction, human epidermal transglutaminase (TG3) and total IgA were quantitatively determined in a cohort of 71pediatric CD, and a control group of 29 cases of pediatric patients not related to GRD. The performance of the AESKUBLOTS was assessed by receiver operator characteristics (ROC)-curve analyses and the coefficients of correlation to

endomysium immune fluorescence endpoint titer (EPT), clinical history of the patients were calculated.

Results

The AESKUBLOTS® GRD IgA assay demonstrated excellent diagnostic performance. The tTG-neo IgA showed the highest sensitivity and specificity, reaching 91.38% (CI: 81.017–97.141%) and 100.00% (CI: 91.592–100.00%), respectively, with an AUC of 0.957 (CI: 0.896–0.987). Similarly, the mTG-neo IgA achieved a specificity of 100.00% (CI: 91.592–100.00%) and a sensitivity of 75.86% (CI: 62.831–86.130%), with an AUC of 0.879 (CI: 0.799–0.936). Both tTG-neo IgA and mTG-neo IgA strongly correlated with EMA EPT ($r^2 > 0.75$, $p < 0.001$). IgA deficiency (< 7 mg/dL) was reported in 7% of cases, confirmed by total IgA levels. Additionally, three CD patients showed elevated TG3 levels (> 20 U/ml to 300 U/ml).

Conclusion

The high specificity and diagnostic accuracy of tTG-neo IgA and mTG-neo IgA highlight their value in differentiating celiac disease in children from other autoimmune conditions, reinforcing their potential as reliable diagnostic biomarkers. Neo-epitopes are sensitive markers of GRD that can be integrated into routine laboratory serology for cost-effective and accurate diagnostics of pediatric celiac disease. Additionally, elevated TG3 levels (> 20 U/ml to 300 U/ml), found in some cases, may indicate Dermatitis Herpetiformis, a condition that should be considered in the clinical context. Future studies comparing neo-mTG and neo-tTG IgA in infants, toddlers, and older pediatric patients are necessary to address the reactivity of mTG-neo IgA, which may be influenced by exposure to mTG enzymes in processed foods, as well as dietary changes in children under supervised gluten avoidance.

Special case in celiac disease diagnostics: Detection of elevated endomysium or transglutaminase IgA-Ab despite selective IgA deficiency (sIgAD)

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Introduction

Celiac disease, a gluten-dependent autoimmune disease, primarily manifests itself in the perineal mucosa. Unlike most other autoimmune diseases, autoantibodies of the IgA type are therefore diagnostically relevant, in particular IgA antibodies against human tissue transglutaminase (TG-IgA), or endomysium IgA-Ab when tested in the IFT. If total IgA is detectable, IgG class antibodies are of little relevance, they are not sensitive enough and are often non-specific.

Selective IgA deficiency (sIgAD) is the most common congenital immunodeficiency, defined as serum IgA <0.06 g/l with inconspicuous IgG and IgM concentrations and no T-cell defect. However, sIgAD may only be diagnosed from the age of 4 years, as it is not uncommon for IgA maturation to be delayed. Secretory IgA (sIgA) is not taken into account in the diagnosis

Due to an HLA association, coeliac disease and sIgAD often occur together. If no IgA can be formed, the body switches to IgG-Ab during autoantibody formation, so that in the case of sIgAD, TG-IgG- or endomysium-IgG-Ab must also be examined.

Case description

In some cases, we were able to diagnose coeliac disease via elevated TG- or endomysium-IgA-Ab, although sIgAD was subsequently diagnosed - in some cases even before the diagnosis of coeliac disease. Without exception, these were children. In all cases, secretory IgA was detected in sputum or stool, but this is not included in the definition of sIgAD. In some cases, IgA could also be detected transiently in the serum during the florid phase of coeliac disease, but this fell back below the detection limit in parallel with the TG-Ig-Ab under a gluten-free diet. In at least one case, the spontaneous normalization of serum IgA in adulthood was demonstrated during long-term observation.

Discussion

The detection of sIgA in all our patients shows that they are in principle able to produce IgA-Ab. This explains why we were able to detect TG or endomysium IgA-Ab in these patients despite the serum IgA deficiency. Whether the detection of sIgA fundamentally contradicts the diagnosis of sIgAD or possibly represents a subgroup of sIgAD or is to be interpreted as an indication of above-average late maturation of IgA formation remains to be clarified in future studies. In at least one of our cases, however, the diagnosis of sIgAD was made too early, as the serum IgA was normal at the age of 18.

Conclusion

- The observed cases support the recommendation of the guideline to always start with IgA-Ab diagnostics in combination with total IgA and to only perform IgG diagnostics as a supplement if necessary.
- IgA maturation can also be very delayed, and the diagnosis of sIgAD should possibly not be made before adulthood.
- The inclusion of secretory IgA (sIgA) in the diagnosis of sIgAD should be considered.

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 bacterial survival factor and is widely used as a food additive in the processed food industry. As an enzyme, its activity is sensitive to temperature and pH.

Aim of the study was to investigate the temperature and pH ranges at which mTG operates effectively, specifically in its ability to cross-link gliadin peptides.

Methods

After optimizing conditions for mTG-mediated gliadin peptide cross-linking (Zedira, Germany), temperature and pH dose-response curves were evaluated. Gliadin peptides, mTG, and the cross-linked products were analyzed using SDS-PAGE.

Results

mTG exhibited optimal activity at 37–60°C, efficiently cross-linking gliadin peptides and producing high antibody titers in ELISA assays using CD patient sera. Beyond 60°C, mTG activity decreased sharply, resulting in a statistically significant reduction of about 65% in immunoreactive epitopes ($p<0.001$). Heating mTG to 80°C to denature it and subsequently cooling it to room temperature did not restore its activity. However, complexes formed at 37°C remained stable even when incubated at elevated temperatures, showing consistent immunoreactive ELISA values (1.19–1.17 OD). Regarding pH, mTG retained its activity at pH 4.0 and above.

Conclusions

During the preparation of processed foods, mTG cross-links gliadin peptides before heating or boiling. The resulting covalent isopeptide bonds are highly resistant to luminal proteases. Upon ingestion, gastric acidity is neutralized, with pH levels potentially rising to 4.5. Many children, adults on acid-suppressive medication, and infants or elderly individuals with higher gastric pH levels, as well as those with alkaline reflux, may be affected. Temperature and pH conditions during food preparation do not compromise mTG-induced cross-linking of gliadin peptides, allowing these cross-linked complexes to survive and reach the gut lumen. These stable cross-linked complexes exhibit heightened immunoreactivity in CD patients, potentially triggering or exacerbating immune responses associated with CD pathology.

9.7 Autoantibody analyses of autoimmune liver diseases

Made to aid in the diagnosis of autoimmune hepatitis: comparison of fluorescence enzyme immunoassay to immunoblot and immunofluorescence detecting anti-LKM-1 antibodies

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Background and aims

The presence of certain autoantibodies in blood samples of individuals suffering from autoimmune hepatitis (AIH) is an important criterion for the diagnosis of this chronic immune-mediated liver disease. Anti-liver kidney microsomal type 1 (LKM-1) antibodies are – aside from cross-reactivities observed for some hepatitis C (HCV) patients – a very specific biomarker for AIH type 2 (1). Besides indirect immunofluorescence testing (IIFT), solid phase assays are available to detect anti-LKM-1 antibodies. In this study, we compared an ELISA-based quantitative anti-LKM-1 test (the automated fluorescence enzyme immunoassay EliA™ LKM-1, Phadia AB, Thermo Fisher

Scientific, Uppsala, Sweden) with a qualitative immunoblot (EUROLINE Profile Autoimmune Liver Disease 8 Ag (IgG), EUROIMMUN, Lübeck, Germany) and IIFT (Mosaic Basic Profile 2, EUROIMMUN, Lübeck, Germany) in a large cohort of AIH patients and controls.

Methods

768 blood samples, comprising 189 from AIH patients and 63 from AIH/primary biliary cholangitis (PBC) or AIH/primary sclerosing cholangitis (PSC) overlap patients and 516 blood samples from disease and healthy controls (284 PBC, 63 PSC, 49 HCV, 20 non-alcoholic fatty liver disease (NAFLD), 30 alcoholic fatty liver disease/alcoholic liver disease (AFLD/ALD), 20 connective tissue diseases (CTD) patients and 50 healthy subjects) were measured on EliA LKM-1 (Thermo Fisher Scientific, Uppsala, Sweden), EUROLINE Profile Autoimmune Liver Disease 8 Ag (IgG) and IIFT Mosaic Basic Profile 2 (EUROIMMUN, Lübeck, Germany) according to manufacturers' instructions. Total, positive and negative agreements were calculated separately for the target disease group AIH (including AIH overlap patients) and for controls. Agreements were calculated with equivocal results judged as positive and with equivocal results judged as negative.

Results

EliA LKM-1 showed very good agreement with the EUROLINE LKM-1 immunoblot. In the target disease group, the total agreement was 100% with equivocal results interpreted as positive (99.6% with equivocal results interpreted as negative), positive agreement was 100% (85.7%) and negative agreement was 100% (100%). For the controls, we found 99.6% (99.4%) total, 33.3% (0.0%) positive and 100% (100%) negative agreement.

Comparison of EliA LKM-1 to LKM-1 IIFT in the target disease group resulted in a total agreement of 98.0% (98.4%), a positive agreement of 60.0% (60.0%) and a negative agreement of 99.6% (100%). In the control group, we found 99.8% (99.6%) total, 50.0% (0.0%) positive and 100% (100%) negative agreement.

Conclusions

EliA LKM-1 showed comparable performance to EUROLINE LKM-1 immunoblot as well as to IIFT, with a slightly higher specificity observed for EliA LKM-1.

Acknowledgements

The authors thank Thermo Fisher Scientific staff Alice Krayer, Yannik Finger and Katharina Weis for their professional support.

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Validation of F-actin on novel EUROLINE liver blot, a Dutch multicenter study

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Abstract

Autoimmune hepatitis (AIH) is a chronic liver disease of unknown etiology that is characterized by interface hepatitis on liver histology, hypergammaglobulinemia, circulating autoantibodies and a mostly favorable response to immune suppression. Detection of autoimmune liver disease-related antibodies is part of the diagnostic scoring system for AIH and, as such, is indicated for the diagnosis of AIH.

In 2023 EUROIMMUN (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) extended the antigens on their liver antibody line blot with F-actin. This initiated a Dutch multicenter validation study in order to compare the F-actin on the EUROLINE Autoimmune Liver Diseases blot with other solid phase (F-)actin-specific assays (Quanta lite® Actin IgG (Inova Diagnostics, San Diego, USA), BlueDiver Dot Liver10 IgG Immunodot (D-tek SA, Mons, Belgium)) and with indirect immunofluorescence tests (IFT) on rodent tissue (rat liver, kidney, stomach (LKS)), VSM47 cell line and HEp-2 cells (all EUROIMMUN). For the antigen-specific tests the

manufacturers cut-off was applied, borderline results were considered negative. For the IFT tests a titer equal or greater than 1:100 was considered positive.

Retrospectively, sera from 402 subjects (302 patients and 100 healthy controls) were analyzed. Selection criteria for patient samples included: diagnosis of AIH and/or diagnosis of PBC and/or request from gastroenterologist for autoimmune liver disease antibodies and hypergammaglobulinemia. The patient population consisted of 81 AIH patients of which 8 had AIH-PBC overlap, 59 PBC patients and 162 non-AIH non-PBC patients.

The three F-actin specific solid phase tests showed moderate/substantial agreement according to Cohen's kappa in both the AIH population and the overall study population.

F-actin assay	**Cohen's kappa (95% CI)**
EUROLINE Liver AND D-Dot Liver	67.5% (59.1-76.0)
EUROLINE Liver AND QuantaLite Actin	58.6% (49.4-67.8)
D-Dot Liver AND QuantaLite Actin	71.4 % (63.2-79.7)

The sensitivity to detect AIH patients in the total study population was between 0.58 – 0.73 for the antigen-specific tests versus 0.33 – 0.74 for IFT based tests. The specificity to detect AIH patients in the total study population was between 0.82 – 0.87 for the antigen-specific solid phase tests versus 0.82 – 0.93 for IFT based tests. Positive likelihood ratios varied between 4.10 – 5.34, indicating moderate contribution to a diagnosis AIH. Based on negative likelihood ratios the tests are not very useful to exclude AIH.

Method	**Sensitivity**	**Specificity**	**LR+**	**LR-**
VSM47 (IFT)	0.74	0.82	4.10	0.32
EUROLINE Liver (lineblot)	0.73	0.82	4.10	0.33
QuantaLite Actin (ELISA)	0.65	0.86	4.77	0.40
D-Dot Liver (Immuno dot)	0.58	0.87	4.33	0.48
LKS kidney GVT pattern (IFT)	0.38	0.93	5.34	0.66
ANA HEp-2 AC-15 pattern (IFT)	0.33	0.93	4.65	0.72

The specificity increased when an antigen specific solid phase test result was confirmed with a second test, irrespective of the nature of that second test, with a minimal to moderate increase

in the positive likelihood ratio. Combining test results to increase the specificity lowered the sensitivity, with a minimal increase in the negative likelihood ratio.

For this study population the test combination with the highest sensitivity was screening with the EUROIMMUN liver line blot and confirming positive F-actin test results using the VSM47 cell line. However, the positive likelihood ratio of this combination was only 6.41. The positive likelihood ratio seemed to increase most when solid phase testing was combined with the LKS tests included in this study.

In conclusion: the F-actin on EUROIMMUN liver lineblot detects anti-actin antibodies with comparable sensitivity and specificity as the IFT on VSM47. The advantage of the line blot is the simultaneous detection of multiple other autoimmune liver disease related autoantibodies.

Evaluation of a Microblot-Array for the detection of autoantibodies associated with autoimmune liver diseases

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Introduction

Autoimmune liver disease (ALD) is a chronic disease caused by an immune-mediated inflammatory response in genetically susceptible individuals. The different types of ALD are characterized by specific autoantibody profiles, which are crucial for diagnosis and classification. The aim of this study was to evaluate the performance of the Microblot-Array (MBA) for the detection of liver-related autoantibodies and to compare the results with those of other diagnostic methods (counterimmunoelectrophoresis (CIE) and line immunoassay (LIA)).

Methods

Samples routinely tested in our laboratory (Department of Rheumatology, UMC Ljubljana) for anti-PDH (in-house CIE) and autoantibodies in the liver LIA (LIA Liver, Human) were additionally analyzed with MBA Liver Profile (TestLine). The agreement between the methods was determined using the Kappa coefficient for antibodies against LKM, LC, SLA, gp210, Sp100, M2 and PDH (3E (BPO), OGDC-E2, PDC-E2).

Results

The agreement between the MBA and the CIE was assessed for anti-PDH, and for other autoantibodies the agreement between the LIA and the MBA was assessed based on the results of 85 samples tested.

The highest agreement was observed for anti-SLA ($\kappa = 0.935$), indicating almost perfect agreement. Anti-PDH ($\kappa = 0.808$), anti-gp210 ($\kappa = 0.804$), anti-M2 ($\kappa = 0.785$) and anti-Sp100 ($\kappa = 0.694$) showed substantial agreement, while anti-LKM antibodies showed moderate agreement

(κ = 0.557). The lowest agreement was found for anti-LC1 (κ = 0.234), indicating fair agreement between the methods.

Conclusion

The MBA showed good agreement with the CIE and LIA for most liver autoantibodies, especially for anti-SLA, anti-PDH, anti-gp210, anti-M2 and anti-Sp100. The lower agreement for anti-LC1 and anti-LKM shows the need for careful interpretation. Overall, the MBA appears to be a reliable tool for the detection of autoantibodies in autoimmune liver diseases.

Variability in the detection of autoantibodies in autoimmune hepatitis: A collaborative study by the Autoimmunity Group of the Catalan Society of Immunology

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Introduction

Autoimmune liver diseases (AILDs) are a group of three distinct conditions: autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). These conditions are characterised by chronic inflammation and progressive liver fibrosis. In this study, we focus on AIH, for which the detection of autoantibodies is fundamental to diagnosis, as with the other AILDs. Autoantibodies associated with AIH include anti-nuclear autoantibodies (ANA), anti-smooth muscle (SMA)/F-actin and anti-soluble liver antigen/liver-pancreas (SLA) for type 1 AIH and anti-liver-kidney microsomal type 1 (LKM-1) and anti-liver cytosol type 1 (LC-1) for type 2 AIH. Currently, a wide variety of techniques and commercial kits are available to identify the presence of AIH-related autoantibodies. Unfortunately, the sensitivity and specificity of these tests can vary, and in most cases, their performance depends on the brand of the commercial kit used.

The study aims to analyse the results of autoantibodies associated with AIH obtained in different hospitals belonging to the Autoimmunity Group of the Catalan Society of Immunology (CSI). In

addition, it also aims to provide physicians and laboratory specialists with useful information to improve the management of autoantibody detection in the diagnosis of AIH.

Patients & Methods

We have retrospectively analyzed the results obtained in the last 2 years in 6 hospitals belonging to the Autoimmunity Group of the CSI. The screening for AIH-associated autoantibodies was performed using indirect immunofluorescence assays on rat triple tissue (IFA-RTT) and antigen-specific techniques (AgST), such as immunoblotting (IB) or ELISA. Depending on the laboratory, different commercial kits were used for IFA-RTT: Werfen Inova (n=4), Euroimmun (n=1), and Aesku (n=1), and for AgST: for SMA/F-actin, ELISA (Werfen, n=2) or IB (Euroimmun, n=1, and D-Tek, n=1), and for LKM1 and LC1, IB (Euroimmun, n=4, and D-Tek, n=2). Anti-SLA autoantibodies were excluded because we only had results from one centre.

Results

A total of 568 results were found regarding SMA/F-actin. Most laboratories (5/6, 83.3%) performed IFA-RTT, followed by AgST, on samples with an anti-SMA pattern. An anti-SMA pattern was observed in 493 (86.8%) of the samples. F-actin positivity by AgST was confirmed in 121 out of 493 (24.5%) of these samples: 101/457 (22.1%) by ELISA (Werfen), 3/12 (25.0%) by IB (D-Tek), and 17/24 (70.8%) by IB (Euroimmun). In contrast, one laboratory performed AgST (IB, from Euroimmun) in parallel with IFA-RTT, obtaining 69/75 (92%) positive results for anti-F-actin by AgST in patients without an SMA-compatible pattern by IFA-RTT. Among them, 23/69 (33.3%) showed moderate/high intensity values (++/+++).

Concerning LKM1, 290 results were obtained. Among those samples with compatible IFA-RTT pattern, 28/35 (80%) were confirmed by IB. Regarding the manufacturer, 12/15 (80%) were detected by D-Tek, and 16/21 (76.2%) by Euroimmun. Furthermore, 34/255 (13.3%) of the samples were positive by IB (Euroimmun) despite not having a compatible IFA-RTT pattern. However, only five (14.7%) had moderate-to-high intensity values (++/+++).

Regarding LC1, 174 results were obtained, of which 171 (98.3%) were positive only by AgST with no compatible IFA-RTT pattern. Most of anti-LC1 were detected using the Euroimmun IB (168/171, 98.2%), of which 50/168 (29.8%) had moderate/high intensity values (++/+++).

Conclusion

Our data showed high variability in the detection of AIH-associated autoantibodies between techniques (IFA-RTT vs. AgST) and manufacturers. Regarding anti-LC1 antibodies, a large proportion of samples that tested positive by IB (using Euroimmun kit) were negative by IFA-RTT. These positive anti-LC-1 results should be confirmed when the clinical suspicion is high. To accurately assess any discrepancies found, it would be advisable to carefully review these results along with the patients' medical histories.

Italian guidelines for the immunological diagnosis of autoimmune liver diseases: a step forward from EASL guidelines

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Abstract

Autoimmune liver diseases, such as autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC), are characterized by chronic inflammation of the hepatocytes or bile ducts, respectively, not caused by infectious processes. If not identified and treated early, these diseases may progress to fibrosis and cirrhosis of the liver and lead to organ failure, requiring transplantation. In this context, laboratory contribution relies on the detection of disease-specific autoantibodies, playing a pivotal role in diagnosis and classification of these diseases. In fact, 95% of patients with AIH or PBC are positive for at least one of the autoantibodies typical of each disease. Among these, antinuclear antibodies (ANA), smooth muscle antibodies (SMA), liver-kidney microsomal antibodies (LKM), anti-liver citosol type 1 (LC1) antibodies, soluble liver antigens (SLA) antibodies and mitochondrial (AMA) antibodies, are considered key markers.

AIH is classically divided into two distinct subtypes: AIH type 1 and AIH type 2. Type 1 is the most common form and affects both children and adults, while type 2 AIH is a primarily pediatric disease. Type 1 AIH is characterized by the presence of ANA and/or SMA in the serum, while the presence of anti-LKM1 and/or anti-LC1 autoantibodies define type 2 AIH. The two forms are usually mutually exclusive.

Multiple international guidelines—including those from the European Association for the Study of the Liver (EASL), the American Association for the Study of Liver Diseases (AASLD), the International Autoimmune Hepatitis Group (IAHG), and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)—have consistently recommended the use of indirect immunofluorescence (IIF) on triple rodent tissue (liver, kidney, and stomach) sections for detecting ANA in suspected AIH patients, because this substrate may provide clues not only to ANA presence but also to overlapping antibodies, such as SMA and LKM, in a single assay run [1-5].

However, the global standard in most immunology laboratories has shifted toward the use of HEp-2 cells as the default substrate for ANA detection. This transition is largely due to logistical, economic, and technical reasons. HEp-2 cells provide a human-derived, standardized, high-throughput platform that is favored in commercial diagnostic kits, especially for systemic autoimmune rheumatic diseases. The cells' large nuclei and dense chromatin structures provide vivid and reproducible fluorescence patterns, facilitating pattern classification under the International Consensus on ANA Patterns (ICAP) guidelines [6]. In addition, laboratories favor automated platforms and standardized workflows, which often default to HEp-2 and higher dilutions to reduce background noise and improve throughput.

The continued reliance on triple tissue slides in lieu of HEp-2 cells according to recommendations represents a notable gap between international group guidance and real-world laboratory practice.

These discrepancies have prompted the Autoimmunology Study Group (GdS-AI) of the Italian Society of Clinical Pathology and Laboratory Medicine (SIPMeL) to update previous recommendations produced in 2009 [7] and to produce new guidelines for the laboratory diagnosis of autoimmune liver diseases [8]. The points of controversy with EASL and IAHG are described below.

Which method for ANA testing?

ANA are serological markers of autoimmune connective tissue diseases and, although low in specificity, represent one of the criteria for the diagnosis of type I AIH, being present in 60–80% of patients affected by the disease. IIF is the recommended analytical method for the detection of antinuclear autoantibodies [1]. However, the IAHG consensus statement [1] and the 2015 EASL guidelines [4] advise against the use of HEp-2 cells as a substrate for the screening test, reiterating that no fluorescence pattern and/or combination of patterns is characteristic of AIH and that, in any case, the definition of the pattern does not appear to have significant clinical implications. Instead, the detection of ANA, in combination with the detection of other associated autoantibodies, in rodent tissue sections (liver, kidney, and stomach) is recommended. This recommendation, already suggested in 2010 by the clinical practice guidelines proposed by the AASLD [3], has not been modified in the most recent revision of the same guidelines. [9, 10].

Conversely, a recent multicenter study demonstrated the validity of using both the IIF test on HEp-2 cells and the ELISA with HEp-2 nuclear extracts as a potentially reliable alternative to the recommended tests on triple murine tissue sections in the diagnostic workup of patients with suspected AIH [11]. The combinations of various methods and the cutoffs suggested by the results of this study, if confirmed by further research, will require a revision of the current diagnostic score.

The position of the GdS-AI SIPMeL confirms that, in the context of autoimmune liver diseases, the preferred substrate for ANA assessment by IIF is HEp-2 cells due to their greater diffusion and standardization and because, thanks to their greater sensitivity, they allow the evaluation of some autoantibody positivities that are otherwise not visible on triple rodent tissue [12], such as rim-like (AC-12) and multiple nuclear dots (AC-6).

Which screening dilution for ANA?

For the detection of ANA by IIF on triple rodent tissue, the starting serum dilution recommended by the IAHG is 1:40 in adults and 1:20 in children and adolescents [1]. However, we believe that on the HEp-2 cell line, these low dilutions cannot be applied in clinical laboratories as they would greatly increase false-positive results in healthy individuals and in patients affected by non-autoimmune diseases [13]. Furthermore, data available in the literature agree on the uselessness of using a screening dilution lower than that used for the adult population for children and adolescents [14, 15]. If HEp-2 cells are used as a substrate for ANA screening in AIH, recent studies suggest adopting a cut-off of ≥1:160, which guarantees sensitivity and specificity comparable to the 1:40 dilution on triple rat tissue [11]. This diagnostic approach comes close to real-life laboratory testing in which ANA screening for systemic autoimmune diseases is performed at a dilution of 1:80 or 1:160.

In suspected AIH, we suggest a starting serum dilution of 1:80 for both adults and children and adolescents. Furthermore, since the use of the HEp-2 substrate increases the analytical sensitivity of the test, we agree with the IAHG for the attribution of a halved score in the updated diagnostic criteria score for AIH: 1 point for ANA values of 1:80 and 2 points for values ≥1:160 [16] (Table 1), even if this possible suggestion of the correction factor has never been validated by comparative studies [17].

Table 1. Update of serological scores for the diagnosis of autoimmune hepatitis (modified from ref. 11).

Variable	Cutoff	Points
ANA or SMA/F-actin	1:40 on LKS	1
ANA	1:80 on HEp-2	1
ANA or SMA/F-actin	≥1:80 on LKS	2
ANA	≥1:160 on HEp-2	2
or LKM	≥1:40	2
or SLA	Positive	2
IgG	> UNL	1
	>1,1 times UNL	2
Evidence of hepatitis by liver histology	Compatible with AIH	1
	Typical AIH	2
Viral hepatitis (IgM anti-HAV, HBsAg, HBV DNA, HCV RNA)	Absent	2
	Probable AIH	≥6
	Definite AIH	≥7

The maximum score attributable to autoantibody positivity is 2. LKS, liver-kidney-stomach rodent tissue; UNL, upper normal limit

Which screening dilution for ASMA, AMA and anti-LKM on rodent triple tissue?

Another important area of discrepancy involves the initial dilution (titer) used in the detection of anti-smooth muscle antibodies and anti-liver-kidney microsomal antibodies. Both EASL and IAHG guidelines [1, 4] advocate the use of low starting dilutions, typically 1:20 (children) to 1:40 (adults), when screening for these autoantibodies using IIF on rodent tissue. This is due to the potential for clinically significant low-titer antibodies to be present in early or pediatric AIH [2, 4]. Diagnostic scoring systems like the simplified IAHG criteria reward points for presence, not titer strength, of ANA, SMA, and LKM antibodies [16]. Hence, the sensitivity of detection—especially at lower dilutions—is critical. However, this approach is debatable and carries several risks. A) Poor specificity: At such low dilutions, false positives are common, especially in elderly patients, healthy individuals, or during transient infections (e.g., EBV, HCV). B) Non-specific staining: At 1:20–1:40, background fluorescence and unspecific patterns increase, making interpretation difficult and subjective. C) Limited clinical relevance: Antibody titers below 1:80 are often clinically insignificant and not associated with established autoimmune diseases. D) Diagnostic confusion: Low titers may detect antibodies related to other conditions (e.g., connective tissue diseases), potentially misleading the diagnosis of AIH.

Galaski et al [11] recently reported that at a dilution of 1:40, the sensitivity is 86.9% and the specificity is 37.5%. This means that if the test is performed on a subject with a low pretest probability of AIH, as is often the case with requests to general laboratories, the positive predictive value (PPV) of the test is very low. At a titer of 1:80, however, the sensitivity drops slightly (80.3%), while the specificity increases to 69.4% and consequently also the PPV. Many clinical laboratories, therefore, use a higher threshold dilution (1:80) as a screening cutoff as higher dilutions reduce background reactivity and false positive rate.

The GdS-AI SIPMeL recommendations state that, due to the relative rarity of AIH and the resulting low pre-test probability of autoantibody requests to clinical laboratories, it is generally preferable to use 1:80 as a cutoff to ensure diagnostic specificity. Lower titers (e.g., 1:40) should be considered only in cases with strong clinical suspicion, especially in pediatrics. Routine use of low dilutions risks overdiagnosis and overtreatment.

For primary biliary cholangitis (PBC), anti-mitochondrial antibodies represent the most sensitive and specific marker for the disease, being detectable in approximately 90-95% of patients [18]. The IIF method on triple tissue sections is considered the gold standard for detecting AMA. Similar to what is recommended for other autoantibodies screened for on triple tissue, both EASL and IAHG recommend a starting dilution of 1:40 [19-21]. The GdS-AI SIPMeL instead proposes using a screening dilution of 1:40 only in specialized laboratories serving subjects with a high pre-test probability of PBC and 1:80 in general laboratories where the pre-test probability is much lower.

In conclusion, we believe that to be really effective, guidelines should be applicable to all clinical laboratories, not just the more experienced or reference ones, which probably constitute an insignificant portion of all clinical laboratories testing for antibodies in liver diseases.

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When being positive is not enough: The diagnostic dilemma of anti-Sp100 antibodies

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Background and Aims

Primary Biliary Cholangitis (PBC) is a chronic autoimmune liver disease characterized by progressive bile duct injury. While anti-mitochondrial antibodies (AMA) are the standard serological biomarker, anti-Sp100 antibodies have emerged as alternative PBC-specific biomarkers. However, their diagnostic relevance and clinical utility in everyday practice remain unclear. This study aimed to (1) evaluate the association between anti-Sp100 positivity and confirmed PBC diagnosis, (2) assess the added value of combining anti-Sp100 with other immunological markers, and (3) examine the prevalence of alternative autoimmune conditions among anti-Sp100-positive patients.

Methods

We retrospectively analyzed patients who tested positive for anti-Sp100 antibodies by immunoblot between 2019 and 2022. Antibody signal intensity was classified as weak, moderate, or strong. Clinical data included PBC diagnosis, classical symptoms, and presence of other autoimmune diseases. Biochemical and immunological markers included serum alkaline phosphatase (ALP), AMA, gp210 antibodies, and antinuclear antibody (ANA) patterns. Appropriate statistical tests (chi-square or Fisher’s exact test) were used to assess associations between variables.

Results

Of the 62 anti-Sp100-positive patients, 12 (19.4%) were diagnosed with PBC. Notably, all cases displayed the AC-6 multiple nuclear dots ANA pattern, consistent with Sp100 reactivity.

Strong anti-Sp100 positivity was found in 11 of the 12 PBC cases (91.7%) and in 11 of the 50 non-PBC cases (22.0%), showing a significant association with PBC (OR = 14.0 (IC 95% 1.68-116.86); p = 0.0103). However, despite this association, the diagnostic performance of strong anti-Sp100 positivity was limited, with a sensitivity of 91.7%, specificity of 56.0%, positive predictive value (PPV) of 33.3%, and negative predictive value (NPV) of 96.6%.

ALP was elevated in all PBC patients, compared to 24% of non-PBC cases (p<0.001). Combining anti-Sp100 positivity with elevated ALP improved diagnostic accuracy, achieving 100% sensitivity, 76.0% specificity, 50.0% PPV, and 100% NPV. The best performance was obtained when anti-Sp100, elevated ALP, and AMA or gp210 positivity were combined, yielding a sensitivity of 91.7%, specificity of 96.0%, PPV of 84.6%, and NPV of 98.0%.

Classical PBC manifestations were significantly more frequent among PBC patients: cholestasis (83.3% vs. 10.0%, OR=45 (IC 95% 7.61-266.11); p<0.001), pruritus (66.7% vs. 8.0%, OR=23.0 (IC 95%: 4.76-111.24); p<0.001), hepatomegaly (50.0% vs. 0%; p<0.001), and skin involvement (33.3% vs. 6.0%, OR=7.83 (IC 95%: 1.47-41.79); p=0.022).

Nearly half of anti-Sp100-positive patients without PBC (48%) had other autoimmune diseases.

Conclusions

Despite being considered PBC-specific markers, only one in five anti-sp100-positive patients had confirmed PBC diagnosis. However, strong anti-sp100 positivity combined with biochemical and serological biomarkers significantly improves diagnostic accuracy. The high prevalence of other autoimmune diseases in anti-sp100 positive patients suggests these antibodies may reflect broader autoimmune dysregulation rather than PBC-specific pathology. These findings emphasize the need for comprehensive clinical evaluation beyond isolated anti-sp100 positivity for PBC diagnosis.

10 Novel Therapies for Autoimmune Diseases and Biomarker for Therapy Response

AlloNK cell infusions in refractory SARDs

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Abstract

Natural Killer (NK) cells are lymphocytes belonging to the innate immune system with cytotoxic activity against virally infected and malignant cells. After activation, NK cells release cytotoxic perforin and granzymes, together with chemical modulators such as IFN-gamma and TNF-alpha. Humans characteristically carry CD-56+/CD3- NK cells, which target downregulated MHC class I cancer cells. This characteristic has clinical relevance for the immunotherapy of cancer and autoreactive B cells.[1]

Systemic autoimmune rheumatic diseases (SARDs) such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's Disease (SjD), systemic sclerosis (SSc) and Idiopathic Inflammatory Myopathies (IIMs) may show a protracted course, refractory to conventional therapies, with great morbidity and mortality. Also, the use of isolated anti-CD20 infusions (rituximab) may not lead to effective responses, due to suboptimal B cell depletion in tissues.[2]

Novel therapeutic resources, such as autologous chimeric antigen receptor (CAR) constructs, have shown deep depletion of B-cells and months of disease-free remission, without immunosuppressive drugs or glucocorticoids.[3-9] Impressive results were achieved in refractory SLE, RA, IIM, and SSc.[4, 10, 11] Nevertheless, the clinical use of CAR-T cells may be limited due to side-effects related to cytokine release syndrome and neurotoxicity, cost, quite complex logistics and manufacturing.[12]

The therapeutic potential of NK cells is expanding, as they are increasingly utilized as effector cells in combination with immune checkpoint inhibitors, NK cell engagers, CAR-NK constructs, and infusions of preactivated or genetically modified NK cell products (from autologous or allogeneic blood), utilizing diverse aspects of NK cell-mediated immunity.[13]

Therapy with allogeneic NK cells may lead to promising results. Combined with an anti-CD20 or -CD19 monoclonal antibodies, NK cells selected from donors with high-affinity CD16 (Fc receptor), then expanded in vitro and infused into patients, may enhance monoclonal antibodies lysis of pathogenic B cells.[14, 15] Previous evidence in patients with B-cell lymphoma suggested that the number of NK cells in circulation is important for antibody-based therapy with rituximab or obinutuzumab (anti-CD20 biologics).[15]

Potential advantages of by anti-B cell antibodies followed by allogeneic NK cell therapy are the outpatient administration, less patients experiencing light cytokine release syndrome (reclassified as infusion-related reactions in a recent B-cell-Non-Hodgkin Lymphoma trial)[16], no neurotoxicity, no graft-versus-host disease, no apheresis needed, no genetic engineering required if used with monoclonal antibodies, use of cryopreserved vials and off-the-shelf availability.

Table 1 shows ongoing clinical trials in phases 1 and 2, registered on clinicaltrials.gov.

Therapy	**Indications Covered**	**ClinicalTrials.gov Identifier**
Allogeneic NK cells	Systemic lupus erythematosus / Lupus nephritis	NCT06265220 *(Phase 1/1b, allo NK + rituximab or obinutuzumab)*
Allogeneic NK cells	Rheumatoid arthritis, Sjögren's disease, idiopathic inflammatory myopathies, systemic sclerosis	NCT06991114 *(Phase 2a, allo NK + rituximab)*
Allogeneic NK cells	Rheumatoid arthritis, systemic lupus erythematosus, granulomatosis with polyangiitis, pemphigus vulgaris	NCT06581562 *(Phase 1 investigator-initiated trial)*
CAR-NK	Lupus nephritis, primary membranous nephropathy	NCT06557265 *(Phase 1: allogeneic CAR-NK targeting CD19)*
CAR-NK	Systemic lupus erythematosus	NCT06518668 *(Phase 1: investigator-sponsored study)*
CAR-NK	ANCA-associated vasculitis, systemic sclerosis, idiopathic inflammatory myopathies	NCT06733935 (*Phase 1: allogeneic CAR-NK targeting CD19)*

Table 1. Clinical trials using NK cells in refractory systemic autoimmune rheumatic diseases and registered on clinicaltrials.gov (as of August 2025).

Key questions are open, until current trials using allogeneic NK cells in SARDs deliver results:

- Is there a reset of the immune system after B-cell depletion using the sequence of conditioning >> anti-CD20 infusion >> allogeneic NK cells, or using CAR-NK targeting CD-19?
- What is the positive therapeutic durability, including remission?
- How do side-effects and efficacy compare to CAR constructs-based therapies?
- Specifically, how frequent and severe are cytokine release syndromes and immune effector cell-associated neurotoxic syndromes (ICANS)?
- Which is the response on long-lived CD19-negative B cells, refractory to biologic treatment? Will CD20-negative B cells (plasmablasts and short-lived plasma cells) respond to allogeneic NK cell therapies?

- Which is the best possible conditioning treatment? Do we need lymphodepletion chemotherapy at all?
- How will distinct SARDs respond in relation to clinical and immunology improvement?

NK cells-based immunotherapies are promising avenues for exploration in refractory SARDs, with great expectations for safety and efficacy from ongoing trials.

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CAR T-cell therapy in autoimmune diseases: current stage and perspectives

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy, initially designed for hematological malignancies, is rapidly evolving as an innovative therapeutic option for autoimmune diseases (AIDs). Pathogenic autoantibodies (Pat-AAbs) are hallmarks of many AIDs, including the systemic AIDs (for example systemic lupus erythematosus, systemic sclerosis, Sjögren's, Dermato/polymyositis, among others), and the organ-specific AIDs, such as Myasthenia Gravis, Pemphigus Vulgaris, Hashimoto's, Graves', Goodpasture, Celiac Disease, among various others. Conventional treatments often fail to achieve sustained remission, prompting exploration of novel, targeted strategies such as CAR T-cell therapies. Although we will use "CAR T-cell therapy" for simplicity, a more accurate term would be "Autologous Engineered Cytolytic-cell Immunotherapy" (AECI), as the cytolytic cells ($CD8^+$ T or NK cells) are of the patient's own origin and genetically engineered to express the CAR, that targets specific antigens.

Here we briefly discuss the current status, clinical outcomes, unique toxicities, and emerging approaches within the CAR T-cell therapeutic landscape in autoimmune diseases.

Currently, CD19-directed CAR T-cell therapy (CAR-CD19) is the most widely studied approach for various AIDs, depleting B lymphocytes. CAR-CD19 broadly deplete B cells, including pathogenic autoreactive clones. In clinical trials involving refractory SLE patients, CAR-CD19 has shown efficacy, characterized by sustained, drug-free remission, complete B-cell depletion, significant autoantibody reductions, and long-term immune repertoire resetting. Conversely, in diseases like SSc, where fibrotic mechanisms predominate over humoral immune abnormalities, therapeutic benefits have been more moderate, primarily halting progression or partially reversing early fibrosis rather than achieving complete disease remission.

Safety profiles in autoimmune settings are distinct from oncology. Common CAR T-associated toxicities, including cytokine release syndrome (CRS) and immune effector cell-associated

neurotoxicity syndrome (ICANS), occur less frequently and generally manifest in milder forms. However, unique adverse events have been identified, notably what is call “local immune effector cell-associated toxicity syndrome” (LICATS). LICATS presents as transient inflammatory exacerbations in previously affected organs shortly after CAR T-cell infusion, typically resolving spontaneously or after short-term corticosteroid treatment. Clinical recognition of LICATS is crucial for preventing unnecessary escalation of immunosuppressive therapies.

Despite promising results, multiple barriers impede the broader adoption of CAR T-cell therapies in AIDs. These include high production costs, complex logistics, infection risks associated with prolonged B-cell depletion and hypogammaglobulinemia, and limitations inherent in current clinical evidence derived primarily from small, uncontrolled trials. Therefore, randomized large-scale controlled trials are essential to further validate therapeutic effectiveness and long-term safety profiles. A comprehensive review of the ongoing trials for CAR T applications in AIDs can be found elsewhere (1).

Future perspectives emphasize precision and specificity. Chimeric autoantibody receptor (CAAR)-T cells represent a significant advancement toward precise targeting and elimination of autoreactive B cells without broader immunosuppression. Initial results with CAAR-T therapy, such as targeting desmoglein-3 in pemphigus vulgaris, highlight potential advantages of this targeted approach (2). Further promising avenues include combining CAR T-cell therapies targeting different antigens and bispecific T-cell engagers (BiTEs), or employing CAAR-T and advanced gene-editing technologies to disrupt specific autoreactive clones selectively. For example, a dual-targeting approach presented success in a recently published case report, where in addition to the CAR-CD19, CAR T-cell targeting BCMA was infused, depleting not just the B lymphocytes but also de plasma cells (3).

Overall, while Autologous Engineered Cytolytic-cell Immunotherapy offer promising therapeutic potential where conventional and/or immunobiological-based therapies have failed, further research is essential—particularly to advance precision medicine approaches for eligible selection of patients, as well as refine protocols to enhance safety and efficacy, and reduce costs to broaden accessibility.

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Below CAR T cells – news on therapies targeting B and plasma cells

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Abstract

Chimeric antigen receptor (CAR) T cells directed against CD19 have led to apparent, at least partly spectacular, success in systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), autoimmune myositides, and systemic sclerosis (SSc). Bispecific antibodies to B cell antigens and CD3, such as teclistamab, which binds both CD3 and BCMA, have started to likewise show efficacy in individual cases. Demonstrating an essential role of B cells and plasmablasts carrying CD19 or BCMA, these results have renewed interest in other therapeutic approaches targeting B cells and plasma cells. B cell depletion by rituximab and other antibodies against CD20, found on a narrower population of B cells than CD19, is standard in ANCA associated vasculitis, rheumatoid arthritis, but also multiple sclerosis, and included in current recommendations for SLE, SSc, and myositides. Obinutuzumab, a novel anti-CD20 antibody with optimized depletory function, has successfully finished phase 2 and phase 3 randomized controlled trials (RCTs) in lupus nephritis, and will presumably soon become available for this indication. Inebilizumab, an antibody targeting CD19, like the CAR T cells, was successful in a phase 3 RCT in IgG4-related disease, which is also regarded as an autoimmune disease today. More indirectly, by stopping a feedback loop promoting B cell survival, the anti-BlyS/BAFF antibody belimumab now comes with more than a decade of routine experience in non-renal SLE, and more recently also demonstrated additional benefit in lupus nephritis. For Sjögren's disease, the anti-BAFF receptor (BAFFR) antibody Ianalumab, which combines BAFFR blockade with the depletion of B cells carrying this receptor, is soon expected after the first ever successful phase 3 RCTs in this condition. And despite setbacks based on safety concerns, several approaches targeting the CD40-CD40 ligand co-stimulation of B cells appear promising for SLE, but also other diseases. Among the targeted synthetic DMARDs, Bruton's tyrosine kinase (BTK) inhibitors missed success in SLE and rheumatoid arthritis, but the BTK inhibitor remibrutinib was successful in a phase 2 trial in Sjögren's disease. Together with the plasma-cell-targeting anti-CD38 antibody daratumumab, which improved severe SLE and dermatomyositis at least in case studies, a large spectrum of B cell-targeting and plasma-cell-targeting therapies are in clinical trial programs, and many of them are likely to improve the management of patients with systemic autoimmune diseases in the near future. The differential

effects of these medications will also improve our understanding of the differential pathophysiology of the diseases.

Biomarkers for Treatment Response in Rheumatoid Arthritis: Insights from EU-Funded Projects

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Abstract

Rheumatoid arthritis (RA) remains a highly heterogeneous autoimmune disease with substantial unmet needs, including the absence of robust, clinically applicable biomarkers for predicting treatment response and managing difficult-to-treat RA (D2T-RA). Current therapeutic selection often relies on trial-and-error, neglecting patient preferences and contributing to poor adherence. To address these challenges, the European Commission has funded four Horizon Europe initiatives aiming to advance precision medicine in RA. **SQUEEZE** focuses on identifying, validating, and integrating clinical, laboratory, molecular, digital, and behavioural biomarkers to optimize disease-modifying antirheumatic drug (DMARD) selection, dosing, and delivery, supported by a digital-enabled care model to enhance shared decision-making and adherence. **STRATA-FIT** develops stratification tools for D2T-RA, including decision aids, mechanistic insights, and a federated Learning Healthcare System for secure data sharing. **MDR-RA** aims to define clinical and molecular phenotypes of multi-DMARD-resistant RA, integrate multidimensional patient data into the iCare-RA predictive algorithm, and validate its clinical utility in a randomized trial, alongside early cost-effectiveness modelling and stakeholder engagement. **ID-DARK-MATTER-NCD** investigates the hidden infectious and molecular triggers of immune-related noncommunicable diseases, including RA, through large-scale antigen screening (>600,000 antigens), deep multi-omics profiling of 6,000 patients, causal testing in gnotobiotic mouse models, and AI-driven disease prediction. Collectively, these projects seek to transform RA management through biomarker-driven, patient-centered, and data-integrated strategies, bridging the gap between mechanistic discovery and clinical application.

The pathological role of a novel fibroblast-like synoviocyte targeting autoantibody in therapy response in a mouse model of arthritis

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Background/Purpose

One third of rheumatoid arthritis (RA) patients do not respond to first-line treatment with classical synthetic disease-modifying anti-rheumatic drugs (csDMARD), such as methotrexate (MTX), and short-term glucocorticoids. We previously identified a panel of 3 antibody biomarkers against University Hasselt (UH)-RA peptides, that were associated with the lack of first-line therapy response. One of these antibodies, anti-UH-RA.329 antibody, showed binding to fibroblast-like synoviocytes (FLS) in RA synovial tissue. This study aimed to biologically characterize this antibody by determining its pathological effects on therapy response in a mouse model of arthritis.

Methods

To establish an MTX-induced remission model, collagen-induced arthritis (CIA) was induced in 8-week-old DBA/1 mice. Three MTX dosing regimens were tested (n=5/group): 2.5 mg/kg subcutaneously (SC) twice/week, 20 mg/kg SC once/week, and 35 mg/kg intraperitoneally (IP) twice/week, alongside untreated arthritic and healthy control groups. Visual arthritis scores (VAS), ankle, and paw thickness were monitored over 70 days. The optimal MTX regimen (35 mg/kg IP) was used in a subsequent experiment evaluating the pathological effects of anti-UH-RA.329 antibody. Thus, DBA/1 mice were divided into four groups (n=7/group): arthritic mice, arthritic mice treated with MTX, arthritic mice treated with MTX and polyclonal rabbit anti-UH-RA.329 antibody, and arthritic mice treated with MTX and rabbit IgG control antibody. These mice were monitored for 70 days and the VAS, ankle and paw thickness were determined throughout the study. At day 70, mice were sacrificed and ankles were harvested for histological scoring.

Results

The MTX-induced remission CIA model was optimized, with a dose of 35 mg/kg MTX IP twice/week showing the lowest arthritis incidence (80%) and clinical severity. This model was used to test the pathological effect of the anti-UH-RA.329 antibody on therapy response. In mice receiving anti-UH-RA.329 antibody alongside MTX, arthritis severity was high with an average VAS of 3.8 out of 4, and average ankle thickness of 3.4 mm. Histological analysis confirmed severe joint pathology, with a mean score of 11.6 out of 12, characterized by pronounced synovial hyperplasia, inflammatory infiltrates and cartilage destruction. In contrast, MTX-only treated mice displayed significantly milder disease when compared to the anti-UH-RA.329 antibody group (VAS: 1.8/4, $p < 0.0001$; ankle thickness: 2.7 mm, $p = 0.005$; histology score: 4.1/12, $p < 0.0001$). Similarly, mice treated with MTX and control IgG antibody also demonstrated reduced disease severity compared to the anti-UH-RA.329 antibody group (VAS: 2.0/4, p: <0.0001; ankle thickness: 2.8 mm, $p = 0.002$; histology score: 4.4/12, $p = 0.001$).

Conclusion

Passive transfer of the anti-UH-RA.329 antibody abolished the effect of first-line MTX treatment in the CIA model, both in comparison to animals treated with MTX only and those treated with MTX and IgG control antibodies. These results highlight the functional relevance of the anti-UH-RA.329 antibody in treatment response and/or RA pathology in vivo.

Autoantibodies and biomarkers for treatment response in systemic lupus erythematosus and sub-analysis in lupus nephritis

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Objectives

This study aims to evaluate serological, urinary, and cellular parameters as biomarkers for treatment response in systemic lupus erythematosus (SLE) and lupus nephritis (LN).

Methods

Conducted retrospectively at a single tertiary care center, the study encompassed SLE patients monitored according to routine clinical protocols, with follow-up intervals of 1, 3, 6, or 12 months contingent upon disease activity. Monitoring continued until either the last recorded visit or achievement of remission as defined by the revised DORIS criteria. Biomarker data were obtained at baseline—corresponding to therapy initiation or intensification—and during subsequent follow-ups when available. These data included autoantibodies (e.g., anti-dsDNA, anti-chromatin; Bioplex 2200TM), serum proteins (e.g., C3, C4, CH50, albumin), urinary biomarkers (e.g., protein-to-creatinine ratio [PCR], urinary soluble CD163 normalized to creatinine [usCD163/Cre], estimated glomerular filtration rate [eGFR]), and interferon-gamma release following 24-hour phytohemagglutinin stimulation (IGRA-PHA).

Results

Nine biomarkers demonstrating efficacy in distinguishing active SLE and/or LN from inactive disease states were identified; these comprised anti-dsDNA and anti-chromatin antibodies, C3C4/CH50 complement activation markers, serum albumin levels, reduced PHA-induced IFN-γ release (<8 IU/ml), and for active LN specifically, urinary PCR and usCD163/Cre ratios. Subsequent analyses at therapy initiation or intensification revealed via log-rank testing that prolonged time to remission was significantly associated with a high-risk serological profile characterized by double positivity for anti-dsDNA/anti-chromatin antibodies combined with complement activation (DP-C+ group; median time to response: five years) and low IGRA-PHA responses (≤1.6 IU/ml; median time to response: 3.5 years). Notably, these parameters exhibited independence in multivariate regression models. Following the initiation of therapy in the DP-C+ subgroup, reductions in anti-dsDNA and chromatin antibody levels below 40 IU/mL and 3.5 AU/mL, respectively, were correlated with a complete therapeutic response. Furthermore, categorization of usCD163/Cre into low, medium, and high levels at three- and six-months post-therapy initiation demonstrated superior predictive capacity compared to protein-to-creatinine ratio (PCR) for distinguishing complete, partial, and non-responders.

In **conclusion**, these results substantiate the role of the dsDNA/chromatin/complement complex within immune complexes, and exhausted CD8+ T cells (indicated by markedly reduced IGRA-PHA levels) in the pathophysiology of SLE and for LN CD163-positive macrophages (M2c phenotype). These findings advocate for the integration of these biomarkers into standard SLE management protocols to enhance prediction of therapeutic outcomes.

11 Miscellaneous

Reviving Autoimmune diseases and Allergy

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Introduction & Objective

Increased IgE levels in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases have been described for the first time almost 40 years ago but are largely understudied. Recent studies have shown that elevated Ig E titrations are not associated with higher prevalence for atopy and allergy in autoimmune diseases patients. Anti-IgE isotype antibodies referred as self-damaging autoantibodies with important contribution to disease pathogenesis and progression may be de cause of increased IgE levels in some autoimmune diseases like SLE.

In a previous study we analysed the casuistry of our clinical laboratory over the past five years, regarding Ig E elevations, correlating with increasing severity of specific type antibodies in SLE, Systemic sclerosis, Myositis, Autoimmune Liver Disease, Inflammatory Bowel Disease and in Rheumatoid Arthritis (RA),but we haven't found in our positive population any association between IgE titration and IIF results, so we decided to separate elevations in IgE titrations and look for correlations in different Systemic Autoimmune Diseases, mainly SLE and RA.

Methods

Total IgE serum measurement by ImmunoCAP™ Total IgE.

ANA screening with IIF in Hep-2 cells (Euroimmun™); ds-DNA by FEIA (Thermofisher ™) with confirmatory IIF with Crithidia luciliae (Euroimmun™). Immunoblotting for SARDS (Euroimmun™). Liver autoimmune diseases study by IIF in Liver mosaic 9 (Euroimmun™) and liver immunoblotting profile by Euroimmun™. Systemic Sclerosis and Myositis autoantibody profile by immunoblotting (Euroimmun™). Rheumatoid Arthritis anti-CCP2 (2nd generation anti-cyclic citrullinated peptide) (EliA™ Thermofisher ™).

Results

From January 2020 to December 2024 (5 years) were performed 42492 Total IgE with autoimmunity requests [ANA screening (44,52%); ENA profile (15,24%), ds-DNA antibodies

(20,06%); Autoimmune Liver Diseases (3,59%), ANCA antibodies (2,12%) and Rheumatoid Arthritis (14,47%)].

In the positive population we found a higher Total IgE median in ds-DNA, FEIA (71,90 UI/mL) and in the Rheumatoid Arthritis group (63,95 UI/mL). In RA, Total IgE median in the positive group is higher than negative group [Mood's Median test: P-Value < 0,05].

Conclusions

Although we did not find any association between IgE titration and autoimmune disease severity, future areas of research and anti-IgE therapies might bring additional data on IgE autoantibodies pathogenic activity in autoimmunity.

Relapsing polychondritis in Brazil: epidemiology, diagnosis, treatment, and patient perspectives on symptoms and complications

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Introduction

Relapsing polychondritis (RP) is a rare, chronic, immune-mediated disease characterized by recurrent inflammation of cartilaginous tissues, primarily affecting the ears, nose, joints, and respiratory tract. While existing data from North America and Europe have advanced understanding of RP, epidemiological and patient-centered data in Brazil remain limited.

Objective

This study aimed to assess the clinical characteristics, diagnostic pathways, treatment patterns, and patient perspectives of RP across Brazil.

Methods

A total of 111 patients from all five Brazilian regions participated in an online survey designed to gather both medical and personal insights into RP.

Results

The cohort was predominantly female (76.6%) with a mean age of 47.9 years. Most respondents were from the Southeast (52.3%) and South (18%) regions, with a more frequent racial

distribution of 64.9% white and 28.8% mixed-race (pardo). Diagnosis was primarily made by rheumatologists (75.7%), although many patients experienced delayed recognition, with nearly half (49.5%) consulting more than three physicians and 66.7% requiring emergency care before diagnosis. While 44.1% received a diagnosis within one year of symptom onset, 9% waited over a decade. The most common pre-diagnosis symptoms included ear inflammation/pain (90.1%), voice changes (41.4%), eye inflammation (40.5%), and joint pain (35.1%). After treatment initiation, some symptoms such as ear inflammation and eye issues improved, but others—including hand/wrist pain, shortness of breath, and nose inflammation—became more prevalent. Notably, 21% reported worsening hearing loss. Major complications included disability (32.4%), pneumonia (20.7%), and airway complications, including tracheomalacia (5.4%) and intubation (8.1%). Patients identified several factors associated with symptom flares: dietary triggers (gluten, sugar, alcohol), environmental factors (climate changes, sleep deprivation), emotional stress, and major life events such as financial difficulties (57.6%) and bereavement (47%). Infections, particularly of the upper respiratory and gastrointestinal tracts, were linked to flares in over half the participants. Hormonal influences also played a role, with 91.2% of women reporting symptom worsening during menstruation and 33.3% of postpartum women experiencing flares. Despite the disease's disabling impact, only 22.5% of patients applied for social security benefits, and one-third of those applications were denied.

Conclusions

This is the most comprehensive study to date on RP in Brazil, highlighting diagnostic delays, complex symptom patterns, treatment challenges, and significant unmet patient needs. The findings emphasize the need for heightened physician awareness, targeted research, and improved healthcare and social support strategies.

ENO1 Surface Localization and Autoantibody Response in Prostate Cancer

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Abstract

Prostate cancer (PCa) is a leading cause of cancer death among men worldwide, with men of African ancestry (AA) exhibiting higher mortality rates compared to men of European ancestry (EA) men. Treatment for metastatic prostate cancer includes taxane-based chemotherapy and prostate specific membrane antigen (PSMA)-targeted therapies. However, resistance and limited PSMA expression in a subset of patients, particularly those with neuroendocrine-like (NEPC-L) features, highlight the need for alternative therapeutic targets. Enolase-1 (ENO1), a glycolytic enzyme with emerging non-glycolytic roles, has been detected on the surface of various cancer types and is the target of autoantibody responses in cancer patients, highlighting its potential as both tumor-associated antigen (TAA) biomarker and therapeutic target. Its roles in PCa progression and immunobiology have not been extensively investigated. Western blotting analysis of subcellular fractions, flow cytometric analysis, and confocal microscopy confirmed the presence of ENO1, but not ENO2, on the surface of docetaxel-resistant NEPC-L cell lines, with differential surface abundance patterns likely dependent on glucose regulatory mechanisms. For instance, under high-glucose conditions—mimicking the tumor microenvironment—surface ENO1 levels were increased, likely through c-MYC-mediated transcription and suppression of MBP1, an ENO1 splice variant that negatively regulates c-MYC. Additionally, we evaluated the prevalence of autoantibodies specifically targeting ENO1 or ENO2 in 307 PCa patients and 200 controls using ELISA and multiplex Western blotting. Results showed significantly higher frequency and levels of ENO1 autoantibodies in PCa patients ($p < 0.05$), with even greater levels observed in AA patients compared to European American (EA) patients ($p < 0.001$). In contrast, ENO2 autoantibody levels showed no significant differences between patients and controls. Survival analyses demonstrated that elevated ENO1, but not ENO2, autoantibody levels were associated with poorer overall and cancer-specific survival, and advanced tumor stage, in AA patients. Together, these findings highlight ENO1 as a surface TAA in aggressive, therapy-resistant

PCa subtypes, and a target of autoantibodies that correlates with poor clinical outcomes, especially among AA patients. The dual role of ENO1—as a metabolic regulator and immunogenic surface antigen—warrants further investigation into the utility of this surface TAA and its associated autoantibody response in PCa prognosis and targeted therapies.

Immunomonitoring in severe COVID-19 patients with anti-IFN-I auto-antibodies

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Abstract

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) infection manifests with different degrees of severity, from asymptomatic to severe pneumonia with fatal outcomes. Different studies reported high prevalence of auto-antibodies neutralizing type I interferons (IFN) in patients suffering from life-threatening coronavirus disease 2019 (COVID-19). Some studies suggested that these auto-antibodies may worsen the early stages of COVID-19, leading to hospitalization in the intensive care unit (ICU). However, to date, immunological alterations in patients with auto-antibodies remain poorly described.

To assess these alterations, we performed cellular immunophenotyping (absolute count of T, B, NK cells and monocyte HLA-DR expression) and cytokine measurements (IL-6, IL-10, TNF-α, IFN-γ) at 3 time-points in a cohort of 64 patients suffering from life-threatening COVID-19 hospitalized in ICU in Lyon (France).

Among the 64 patients, 4 (6.25%) were positive for auto-antibodies neutralizing type I IFN. Lymphopenia was frequent and affected all lymphocyte subsets, with a median of 0.9 Giga/L in

patients with or without auto-antibodies. CD4+ and CD8+ T lymphocytes, B cells and NK cells counts did not show any difference between the 2 groups at baseline. Expression of HLA-DR on monocytes was moderately decreased in both groups. IL-6 tended to be higher in patients with auto-antibodies (211.6 versus 107 pg/mL) while IL-10, TNF-α and IFN-γ did not differ between groups.

Overall, while auto-antibodies appear to influence the severity of the early stages of the disease, the present findings suggest that, at an immunological level, once severe acute respiratory distress syndrome has developed, these auto-antibodies likely do not further impair the immune system or drive progression to a more severe form of the disease. Thus, the identification of auto-antibodies neutralizing type I IFN may be a good prognostic biomarker, when tested in the very early stages of the disease, to identify patients that may require intensive care and that may benefit from early targeted anti-viral treatment.

Assessment of the impact of PM 2.5 and NO2 exposure in a European air pollution hotspot for air pollution on autoantibody status in rheumatoid arthritis

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Background

Despite extensive research, the exact etiology of rheumatoid arthritis (RA) remains unknown but is postulated to be multifactorial, involving both genetic and environmental factors. Tobacco smoking is the best characterized environmental risk factors, but recently different work in literature have evaluate the relationship between air pollutant (AP) exposure and rheumatoid arthritis (RA) autoantibody status (1, 2) but the heterogeneity of the published studies hampers clear conclusions. Results are actually discordant with different limitations such as type of air pollutant analyzed, number of persons involved, time of exposure and method of analysis used (1,3,4).

Aim of our work is to evaluate the relationship between PM2.5 and NO2 exposure and RA autoantibody status of a population resident in an area recognized as a air pollution hot spot in Europe. Despite air quality in Europe has improved over the last 20 years, the European Environment Agency calculated that in 2023 there were more than 253000 premature deaths caused by fine particulate matter (PM2.5) and 52000 deaths caused by Nitrogen Dioxide (NO2), beyond the guideline's values suggested by the World Health Organization (5). Among European regions, the Po Valley is known for its challenges in maintaining air quality standards despite emission reduction efforts (5). The area is recognized as a significant air pollution hotspot in Europe, characterized by high levels of pollutants such as NO2 and PM, mainly from traffic, industrial activities, and agricultural practices. The basin-like topography bounded by the Alps and the Apennines, the stagnant air masses, and thermal inversions trap pollutants in the lower atmosphere, leading to persistent poor air quality identified cities in the Po Valley as having the highest mortality burden from PM2.5 exposure in Europe (5).

Methods

We performed a cross-sectional study utilizing enrollment data from a group of 2153 person that have performed - anti-cyclic citrullinated peptide antibody (ACPA) in the last 10 years. Mean exposure levels were obtained for NO2 and PM2.5 from air quality monitoring stations at patients' residential zip codes at the moment of analysis.

Statistical analyses were performed with RStudio software (version 4.2.1). All tests were two sided. We used log transformation to calculate geometric means for each age group. Plasma levels of ACPA were illustrated using box plots with 1%, 25%, 50%, 75%, and 99% on the log scale. Cumulative incidence curves were estimated by the method of Kaplan-Meier and Fine-Gray. Time-varying Cox proportional hazards models were used to assess the relationships of ACPA (2012–2021) with each of the air pollutants PM2.5 and NO2, adjusted known risk factors, accounting for censoring and individual differences. Logistic regression models were used to assess the relationship between ACPA and PM2.5 and NO2 covariates, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Results and Conclusion

The cohort included 2153 samples (543 male and 1610 female), with mean age of 60 years, 501 ACPA positive and 1652 negative respectively (23%, and 77%). The results shown evidence of association for ACPA exposures to $PM_{2.5}$ [21.7,27.5] in any period analyzed [OR:1.08(1.00−1.16, p=0.043)], with a modest increased risk of ACPA, for the period analyzed. No significant association for ACPA exposures to NO_2[25.2,31.8] was observed [OR:1.02(0.96−1.08, p=0.548)].

In conclusion, exposure to PM2.5 particulate concentration increase the risk of developing ACPA positive biomarker and RA. Further study of fine particulate matter in the pathogenesis of RA is warranted.

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Molecular Mimicry Study of the L1 Protein of Human Papilloma Virus

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Abstract

Vaccine-related adverse events that trigger autoimmune diseases can be associated with molecular mimicry between the host and the organism, as a mechanism. Accordingly, we inspected autoimmune disease associated human proteins' shared pentapeptide sequences within the L1 protein sequence of the human papilloma virus (HPV) types 6, 11, 16, 18, 31, 33, 45, 52, and 58. The disorder of interest was post-orthostatic tachycardia syndrome (POTS), as the Vaccine Adverse Event Reporting System (VAERS) Request tool of the Center for Disease Control and Prevention (https://wonder.cdc.gov/vaers.html) reveals that around 1/3 (0.34) of the VAERS with post-orthostatic tachycardia syndrome (symptom code 10063080) is involving HPV as the vaccine type. We selected the human proteins to inspect for shared sequences, through the literature. G-protein coupled receptors with autoantibodies associated with POTS were among the inspected protein sequences. We also looked into immune epitope database (IEDB at iedb.org) for the experimentally-validated epitopes containing the shared peptide sequences, and autoimmune disease associated ones among those. In support, other conditions like alopecia areata, celiac disease, Crohn's Disease, fibromyalgia, Graves' disease, Hashimoto's thyroiditis, Guillain–Barré syndrome, and narcolepsy also involve associated autoantigens, sequences of which share pentapeptides with the L1 protein of the HPV types of interest.

As a result, the muscarinic acetylcholine receptor (ACM)3 protein shared-sequence AGLQA was present as a part of autoimmune disease associated epitope (IEDB: 527351), sourced from the Spectrin alpha chain, non-erythrocytic 1 (UniProt: Q13813). The other peptide sequences (RAGVM and STATL) shared by the same protein were serving as such epitopes in the citrullinated form (IEDB: 959810 and 981767). There was a similar situation for the shared-sequences KKKKV of ACM5 (IEDB: 1393456), while its STSTT sequence had multiple such epitopes (IEDB: 656869) with overlapping sequences, sourced by the protein Hom s 5 (UniProt: P02538).

The autoimmune disease related epitope (IEDB: 972215) containing the shared-sequence VFKVV of the alpha-1B adrenergic receptor, was belonging to acidic fibroblast growth factor intracellular-binding protein (UniProt: O43427). Finally, the pentameric SPSPS sequence shared with the beta-1 adrenergic receptor was an autoimmune disease-related epitope (505465), sourced by the SH2 domain-containing protein 3C (UniProt: Q8N5H7). Here, one exemplary epitope was only mentioned, for brevity.

The remaining part of the work led to similar findings as outlined above, for the other diseases under study, and their associated autoantigen protein sequences with shared pentapeptides. In sum, the results supported the possible role of molecular mimicry in the induction of POTS as an autoimmune response, through molecular mimicry.

Note: The IEDB IDs given in the abstract are selected manually from multiple IDs as representative.

Positive Measles-Rubella-Zoster Reaction (MRZR) prevalence in Croatian patients with Multiple Sclerosis and positive oligoclonal bands (OCBs)

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Background

Positive Measles-Rubella-Zoster Reaction (MRZR) indicates intrathecal specific antibody synthesis. A polyspecific antiviral response within the CNS is considered present when at least two antibody specificity indices (ASI) are ≥1.5 and is highly specific for multiple sclerosis (MS), with reported positivity rates ranging from 70% to 89%.

Aim

We aimed to assess MRZR positivity in Croatian patients treated at University Hospital Center Sestre milosrdnice, Zagreb, from December 2023 to February 2025.

Matherial and methods

We retrospectively analyzed data from 293 patients (180 females, 113 males) tested for MRZR with Herpes Simplex Viruses 1/2 (HSV) extension (MRZHR) and ASI for Borrelia burgdorferi (BB) using ELISA (Euroimmun, Germany). Testing included adults with positive oligoclonal bands (OCBs) and all pediatric patients regardless of OCBs positivity. OCBs were considered positive when ≥2 bands were detected in the CSF compared to the paired serum sample, corresponding to Type 2 and Type 3 patterns. BB ASI was performed to exclude neuroborreliosis as a cause of positive OCBs.

Results

Positive OCBs were found in 83/293 patients, and MS was confirmed in 51/293 patients. For 4/51 MS patients who were OCBs negative MRZHR or BB was not performed. MRZHR was positive only in 9/47 (19%) MS patients with positive OCBs. Rubella and Varicella were positive in 6/9, Morbilli and Rubella in 2/9, Morbilli and Varicella in 1/9 patients. HSV was not positive in any case with more than 2 positive ASI. MRZ reaction (MRZHR) and BB tests were performed in 12 pediatric patients, including 8 with OCBs Type 1, 2 with OCBs Type 2, and 3 with OCBs Type 4. None of these patients had a positive MRZHR or a confirmed diagnosis of MS. Six of 83 OCB-positive patients showed BB intrathecal synthesis, but none had MS.

Conclusions

Although MRZHR is considered useful and highly specific for MS, in our cohort, it showed significantly lower positivity (19%) than reported. Extending MRZR with HSV appears unnecessary for our population. Not a single child tested MRZHR positive. According to our results, MRZHR has limited added value if OCBs and MRI strongly support the MS diagnosis.

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