Kaleidoscope of autoimmunity – an update

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The spectrum of autoimmune diseases is constantly expanding, currently including more than 81 conditions [1]. Those clinical entities share a common background, including genetic predisposition, hormonal imbalance, environmental factors, underlying immune system defects and many others, together forming the "mosaic of autoimmunity" [2]. A change in one or more factors that constitute this unique mosaic may lead to a switch from one autoimmune disease to another, best described as the "kaleidoscope of autoimmunity" [3]. In the literature we encounter numerous reports of such phenomenon: development of sarcoidosis in patients with dermatomyositis [4], rheumatoid arthritis (RA) in systemic lupus erythematosus (SLE) -affected individual (Rhupus syndrome [5]), celiac disease and SLE in Hashimoto's thyroiditis patient [6] along with many others may serve as the flagship examples of this immune interplay.

Treatment and the kaleidoscope of autoimmunity: the double-edged sword

The shift of the kaleidoscope is mainly associated with a medical intervention, re-arranging the mosaic layout. Thus, treatment of one autoimmune disease may be associated with the emergence of another condition from this spectrum. This relationship may be illustrated by a recently published nationwide study conducted by Chang *et al.* in Taiwan [7], demonstrating that myasthenia gravis patients who underwent thymectomy had significantly increased risk of RA, progressive systemic sclerosis (pSS) and SLE in comparison to healthy individuals. Furthermore, in the medical literature we may encounter many case reports describing the kaleidoscope phenomenon, including myasthenia gravis and Hashimoto's thyroiditis induced in ITP patient following splenectomy [8] or antiphospholipid syndrome (APS) appearing two years after thymectomy performed in myasthenia gravis-affected woman [9].

Surgeries are not the only medical interventions associated with autoimmune kaleidoscope rotation. Occasionally drugs administered for one autoimmune disease may lead to the appearance of another condition. More than 30 years ago it was observed that D-penicillamine, previously widely prescribed as therapy for RA, has been associated with the induction of SLE, polymyositis and myasthenia gravis [10]. Other examples of this unique relationship include sulfasalazine treatment inducing SLE in ankylosing

spondylitis patient [11] or subacute cutaneous lupus erythematosus reported after leflunomide therapy [12]. The introduction of biologic treatment in the last 20 years has been a breakthrough in the treatment of many autoimmune diseases, although the phenomenon of de-novo treatment-induced autoimmunity emergence has not vanished. In the BIOGEAS registry, a multicenter project devoted to collecting data on the use of biological agents in patients with autoimmune diseases, nearly 13,000 cases of autoimmune disorders were reported, with psoriasis at the first place (6375 cases), followed by inflammatory bowel diseases (IBD, 845 cases) and central nervous system (CNS) demyelinating diseases (803 cases) [13]. The pharmacological scenario was highly heterogeneous with more than 30 different biological drugs involved pertaining to 8 different biologics groups. De-novo autoimmune diseases were most frequently reported after anti-TNF agents (9133 cases, from which 4154 after adalimumab therapy), B-cell targeted therapies (741 cases, 678 reported after rituximab) and anticytokine treatment (285 cases, 224 related to tocilizumab use). Based on their findings the authors calculated that an unexpected autoimmune disease may arise in around 8 out of 10,000 exposed patients. Psoriasis, IBDs and CNS demyelinating diseases are not the only presentations of biologic treatment-induced autoimmunity. In the review articles on this topic more than 50 different systemic and organ-specific autoimmune processes were encountered, including de-novo psoriasis, the paradoxical induction of uveitis and inflammatory ocular diseases, autoimmune hepatitis, interstitial lung disease, demyelinating CNS involvement as well as peripheral neuropathies, vasculitis, sarcoidosis, IBD, APS and other autoimmune diseases. [14]. Immune-related adverse events (irAEs) associated with cancer immunotherapy were recently added to the puzzle of kaleidoscopic autoimmunity [15]. The introduction of immune checkpoint inhibitors, blocking intrinsic down-regulators of immunity such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) or its ligand (PD-L1), is considered a breakthrough in the treatment of malignancies [16]. Nevertheless, the forthcoming era of this treatment is inevitably linked with a large spectrum of irAEs. [17]. This phenomenon is linked to PD-1 and its ligands impact on the inhibitory T cell signaling, mediating mechanisms of tolerance as well as providing immune homeostasis. Moreover, increasing evidence points to the fact that impaired PD-1: PD-L function plays an important role in many autoimmune diseases, including type 1 diabetes mellitus (T1DM), RA, SLE, systemic sclerosis, IBD, autoimmune hepatitis, Sjogren's syndrome and others. [18-20]. In the expanding spectrum of organ-specific immune-related adverse events we may encounter many rheumatic diseases (RA, psoriatic arthritis, Sjogren's syndrome, myositis and myalgia, lupus-like disease, scleroderma, giant cell arteritis), neurological disorders (myasthenia gravis-like syndrome, neuropathy, and transverse myelitis), dermatological diseases (rashes psoriasis, dermatitis with pruritus, vitiligo) along with many others (fatigue, autoimmune hepatitis, enterocolitis, endocrinopathies, T1DM, uveitis, sarcoidosis, immune cytopenias)[17-20].

The microbiome – the new puzzle of the mosaic

Over the past decade, our understanding of autoimmune diseases has been transformed by a growing appreciation of the pivotal role of the human microbiome. The microbiome composition has been reported to correlate with many autoimmune diseases, including IBD [21, 22], MS [23], and RA [24, 25], both in human studies and in animal models of these diseases [26]. The intestinal microbiota play a crucial role in the development of host immunity, and in turn the immune system also acts to regulate the microbiota through intestinal barrier maintenance and immune exclusion. Normally, these interactions are homeostatic, tightly controlled, and organized by both innate and adaptive immune responses. However, a combination of environmental exposures and genetic defects can result in a break in tolerance and intestinal homeostasis. The outcomes of these interactions at the mucosal interface have broad, systemic effects on host immunity and the development of chronic inflammatory or autoimmune disease [27].

Multiple microbes are able to alter the immune response; however, it is already known that changing the microbiome composition may on the one hand attenuate the original disorder, but on the other hand may induce another immunological disorder [28]. For example, lower abundance of *Prevotella* was observed in the fecal samples of MS patients. Enrichment of the *Prevotella* genera, corresponded with disease-modifying treatments of MS along with amelioration of the disease [29]. On the contrary, *Prevotella* has been found to be increased in feces samples of new-onset RA patients [30], suggesting that that different abundances of the same bacteria can yield opposite outcomes in different autoimmune diseases and thus an attempt to treat an autoimmune disease may result in the induction of another disorder from this spectrum.

The genetic kaleidoscope

Other features of the Kaleidoscope of Autoimmunity include the occurrence of multiple autoimmune diseases in one patient as well as the familial autoimmunity [31]. Those two phenomena indicate a substantial genetic component as well as a shared environmental component playing a crucial role in the pathogenesis of autoimmunity [32]. With the development of high-throughput sequencing technologies, genome-wide association studies (GWAS) have uncovered hundreds of risk loci for autoimmune diseases many of which overlap across different disorders [33]. The major histocompatibility complex (MHC) locus contributes to autoimmune disease risk more significantly than do any other known loci. In T1DM, 30% of disease liability is attributed to the MHC locus, compared with 9% for other loci discovered across the rest of the genome with GWAS [34]. Most associations are mediated by a handful of human leukocyte antigen (HLA) genes, which encode the receptors that are expressed by antigen-presenting cells to trigger the immune response.

It has been shown that specific polymorphisms in the HLA-DRB1 are shared between more than one autoimmune disease, namely the allele DRB1*04:01 is a strong risk factor for RA in multiple populations, as well as for T1DM whereas DRB1*03:01 has been shown to be associated with T1DM , SLE and Sjogren's syndrome, and DRB1*04:05 with T1DM, RA and autoimmune hepatitis [35]. Besides the MHC genes, many single nucleotide polymorphism (SNP) associated with autoimmune diseases were detected. For example, a non-synonymous variant in ***PTPN22*** was shown to be associated with many autoimmune diseases, including T1DM, RA, SLE and Graves disease [36]. ***CTLA4***, primarily linked to T1DM [37], was also found to be associated with RA [38] and alopecia areata [39]. Moreover, signal transducer and activator of transcription 4 (STAT4) has been recently identified as a susceptibility gene for multiple autoimmune diseases, including SLE, RA, systemic sclerosis and primary Sjogren's syndrome [40].

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Understanding and Addressing Gaps in Autoantibody Testing: Pragmatic Approaches to Precision Health

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There has been tremendous progress over the past half century since the introduction of antinuclear antibody (ANA) and other autoantibody (AA) testing to screen for and confirm the diagnosis of systemic autoimmune diseases (SAID). Despite this long history, there are several gaps in the understanding, utilization and interpretation of the ANA and AA test results (reviewed in (1)). These gaps include:

1) Widening Bandwidth: 'Clientele', Autoantibody: Discoveries and Technologies. Dating to the discovery of the LE cell and the development of the LE cell test (reviewed in (2)), followed by the ANA indirect immunofluorescence assay (IIFA) using cryopreserved rodent tissue sections (3), the initial autoantibody testing 'clientele' was largely restricted to rheumatologists who were most interested in a diagnostic test for the very complex clinical presentation of systemic lupus erythematous (SLE). However, since then the spectrum of clinicians utilizing ANA and AA testing has remarkably broadened (1). In some jurisdictions, primary care clinicians are responsible for ordering 75% of all ANA tests (4). Neurology and respirology are recent additions to the autoantibody "clientele" because of recognition that, for example, autoimmune encephalitis, neuropsychiatric conditions and pulmonary fibrosis have associations with specific AA (1).

2) Standardized Autoantibody Assay Parameters: A persistent limitation of ANA IIFA testing is standardization. Despite numerous studies and analyses, there is limited standardization of ANA test protocols, limited availability of appropriate controls, no universally accepted screening serum dilution (for adults or children) (5), different manufacturers use different secondary antibodies, the cell substrate (typically HEp-2 cells) are grown and fixed with differing protocols, the assay is semi-quantitative at best and the interpretation of various IIF patterns is highly observer dependent (6-8). Advances are being made in the performance of the IIF test through automated robotics and digital image analytics (9).

3) Nomenclature: It is well known that many of the autoantibody targets in SAID are not restricted to the nucleus (10). Hence, the term A**N**A is technically inaccurate and misleading because many of the SAID autoantibody targets are in the cytoplasm, directed to mitotic cells, and/or bind to extracellular targets (11;12). However, proposals to change the terminology from ANA to anti-cellular antibodies (ACA) has been met with resistance (10). By contrast, there is remarkable progress in standardization of the nomenclature of ANA IIF patterns and related technical issues (10;13). An example of nomenclature misuse is the perpetual lack of attention to well-defined autoantibody systems in SAID. For example,

current literature is replete with misnomers such as confusing the SSA/Ro60 antigen system with the Ro52/TRIM21 system. There is compelling evidence that we should bring to a close the notion that somehow SSA/Ro60 and Ro52/TRIM21 can be tested together or that they fit together into a clear-cut clinical paradigm.

4) Seronegative SAIDs: Despite the discovery and continuous description of numerous AA targets, a seronegative gap persists for many SAIDs. For example, the frequency of ANA-negative SLE ranges from 3-25% (7;12) despite more than 180 targets of AA being described in that disease alone (14). And the prevalence of ANA IIFA negative systemic sclerosis is 5-10% despite more than 60 autoantibody targets described (15;16). Evidently, only a handful (10-15) are used in diagnostic assays because most have perished in the innovation "valley of death" (17;18). The selection of certain AA as "clinically useful" and the rejection of others is dependent on their ability to meet SMAARTT criteria: **S**pecificity for disease balanced by acceptable **S**ensitivity, are they **M**easurable in conventional diagnostic platforms, are they **A**ctionable or associated with a clear clinical **A**dvantage or outcome (i.e. predictive, prognostic), are they **R**ealistic, **T**imely and **T**itratable (19).

5) Standardized Cohorts with Biobanks: One of the major challenges of AA assay development and validation is the lack of clinically-characterized SAID cohorts with accompanying serum/plasma and/or tissue biobanks. In addition to cohorts of established diseases, pre-clinical SAID cohorts are also important for studies designed to track the induction and evolution of SAIDs to definitive diagnosis. The American military serum and databank was used for the landmark study of Arbuckle et al who defined AA that appear years before the diagnosis of SLE (20;21). Other clinical databases with biobanks have historical roots, such as the Janus Serum Bank in Norway (22) and the National Serum Bank in Mexico (23). The Nurses' Health Studies in the USA has become a robust source of demographic, clinical and serological parameters that have been used to chart the course of SLE and identify risk factors for SLE development (24).

6) Value versus Cost: One of the prevailing concerns is the unabated increase in health care expenditures. In some jurisdictions health care expenditures approach 10% of the gross domestic product (GDP); in the Unites States of America it is approaching 20% of GDP. These economic concerns have prompted the Choosing Wisely movement, which reviewed conventional medical practice to determine areas in which savings could be achieved (reviewed in (25)). One target for savings in health care costs was a consensus by American and Canadian rheumatologists that excessive ANA tests were being ordered and various recommendations were offered to limit the use of ANA testing in patients with "aches and pains" or other non-specific signs or symptoms. While this is laudable, the recommendations are based on apparent misunderstanding of the widened bandwidth of ANA testing (i.e. it is not solely used to diagnose SLE) and in many jurisdictions rheumatologists are responsible for <30% of all ANA tests ordered (reviewed in (25;26)). In addition, most clinicians are unaware of the actual costs of ANA testing, or for the entire category of "*in vitro* diagnostics", which in most G8 nations accounts for <3% of all health care expenditures (27). The vagaries of HCE can be debated but one important perspective was championed by Denis Cortese the former CEO of Mayo Clinics, who suggested that HCE are not the most relevant consideration and that health care providers and managers would be better to focus on what he called the "value proposition" (28) where despite increased costs for some procedures and interventions, what is most important are the OUTCOMES of the patients where an evidence-based approach to health care might involve incremental spending (i.e. ANA testing to make an early diagnosis of SLE BEFORE they develop renal disease) while decreasing direct (emergency room visits, hospitalizations, expensive therapies) and indirect (lost wages due to poor health) costs. Perhaps a more meaningful approach to the Choosing Wisely diagnostic test hit list, is to focus on costs where a real difference can be made (25).

Closing the Gaps

1) Classification Criteria: Curiously, despite the limitations of the ANA IIFA test, it continues to be a key criterion in the classification of some of SAIDs, especially SLE. The most recently revised SLE classification criteria supported by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) uses the ANA IIF as a required entry criterion (29;30), apparently taking into consideration that the lack of specificity of the ANA test for SLE is counter-balanced by the other weighted clinical and serological findings. With respect to the new ACR/EULAR criteria, it should be noted that with respect to the ANA HEp-2 IIFA requirement, "an equivalent" assay is a permissible. This criterion may create misunderstanding because by not defining the characteristics of an "equivalent" test, it has no technical comparator definition except the assumption that this equivalence will relate to sensitivity and specificity of the ANA at a titer of ≥1/80. This may be a moot point because, as discussed below, there is a move to high-throughput solid-phase immunoassays (SPIA) testing that is not only equivalent to but exceeds the performance of the ANA IIFA test.

2) Technology: The broadening spectrum of SAID is intertwined with the appearance of newer SPIA such as addressable laser bead immunoassays (ALBIA), particle-based multi-analyte technology (PMAT) and line immunoassays (LIA) (31) and (reviewed in (32)). Despite the ANA IIFA being declared the "gold standard" (33;34), the lingering, and seemingly unresolvable limitations of this testing on HEp-2 cells portends a continuing replacement of this test with SPIA that are at least equivalent to or outperform it (8;35-39). In addition, automated ANA IIFA technologies have further closed technical and subjective interpretation gaps in ANA IIFA testing (reviewed in (9;40)). As has been proposed by inverting the 'pyramid' of reflex testing for ANCA testing (reviewed in (41)), there is a sense that ANA screening should follow suit and broad-spectrum screening tests be replaced by Multi-Analyte Arrays with Algorithmic Analyses (MAAAAs) (42). The MAA component of technology platforms are well developed and increasingly available but a gap to be closed is algorithmic analyses using artificial intelligence and deep neural networks to link big data to clinical care pathways (43-45). A word of caution: there should be no assumptions that a move to newer, high-throughput technologies such as MAAAA is nirvana (46). Indeed, many old challenges will persist, and new challenges will arise. Inter-manufacturer and inter-laboratory variability will continue to be a challenge, although standardization appears to be more easily attainable because purified components providing quantitative results are typically used in newer MAA platforms. Hence, a goal of standardized performance could be based on international reference standard sera and the assignment of results in 'international units" (47;48). This means that for every antigen in a MAA, an internal reference standard should be required, an important technical gap that needs to be and can be addressed.

3) Precision Health: Another factor widening the spectrum of ANA and increasing the gaps in AA testing is a concerted move to preventive medicine and precision health (PH) (49). Until recently, it has been assumed that the primary use of ANA and autoantibody testing is to support the diagnosis of a SAID with 'intent to treat' (25) and as criteria for entry into clinical trials (6;50). However, an emerging evidence-based approach to the identify very early SAID (pre-clinical SAID) and 'case finding' is focussing on 'intent to prevent' morbidity and mortality associated with SAIDs (25;51;52). In very early SAIDs, signs and symptoms do not always point to a single 'high pretest probability' disease, necessitating a paradigm shift in diagnostics where the focus is on testing individuals based on evidence-based risk factors and the earliest signs and symptoms (lower pre-test probability) of SAIDs. This will likely mean combining ANA and autoantibody testing with other 'omic biomarkers (i.e. cytokines) in MAAAA platforms. Real-time MAA data on patients starting at the earliest onset of disease has the potential to guide further investigations (biopsy, imaging, etc.), referrals to appropriate specialists, reclassification of patients based on molecular signatures and serve as a guide to treatment, predicting disease flares and confirming remissions (53-55).

4) Pragmatic Views on Precision Health and Artificial Intelligence: As noted above, there should be no assumptions that a move to newer, high-throughput technologies such as MAAAA is nirvana (46). Indeed, many old challenges will persist, and new challenges will arise. Inter-manufacturer and inter-laboratory variability will continue to be a challenge, although standardization appears to be more easily attainable because purified components providing quantitative results are typically used in newer MAA platforms. Hence, a goal of standardized performance could be based on international reference standard sera and the assignment of results in 'international units" (47;48). This means that for every antigen in a MAA, an internal reference standard should be required, an important technical gap that needs to be and can be addressed.

Currently, there is tremendous hype and hoopla about the power of 'artificial intelligence" (AI), "machine learning" and "deep neural networks" (DNN) to solve a vast array of problems in diagnostic and interventional medicine (45;53;56). Some of the utopian expectations of AI and DNN include elimination of drudgery, enablement humanism (more time with patients or doing medical tasks of importance), solve complex analytic problems, generate hypotheses and capitalize on widening availability and richness of medical research data (data clouds) and databases such as electronic medical records. This is counterbalanced by an appreciation that the limitations of AI/DNN includes loss of jobs, provision of 'robotic pseudocare', the GIGO problem (garbage-in-garbage-out) yielding black box answers to problems, commoditization of health information when there is growing perceptions that patients themselves should own their data (57;58). It is clear that for "image pattern" oriented medicine (radiology, pathology, genetics) that AI and DNN have already made significant advances (54;55;59;60). In AA laboratories we have already witnessed the power of AI with the advent of automated ANA IIFA pattern reading (9). The question is, what other tasks of 'drudgery' or those requiring analytic frameworks for huge information datasets (linking AA, cytokines, genomics into a precision diagnostic report) will AI and DNN assume in AA diagnostics, and when it does, what will be our "higher calling"?

Conclusions

Some of the gaps listed above persist despite knowledge available to fill them, while other gaps require additional effort and international collaboration. Arguably, the major change in autoantibody testing over the past half century has been the broadening spectrum of clinicians and health care providers that use and rely on autoantibody testing in their practices. In the future, gaps in autoantibody testing, especially the ANA IIFA and related AA tests should be driven by clinical approaches that focus on screening for SAIDs in patients with low pre-test probability and continuous evolution of diagnostic test platforms. SAIDs and other autoinflammatory syndromes and their widening spectrum of biomarkers have gained prominence in virtually all branches of medicine. In addition, with this widening spectrum of AA and clinician 'clientele', these tests will be increasingly used known as screening tests in low-pre-test probability clinical scenarios with intent to prevent disease sequalae and inroads to Precision Health.

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