Abstract

The methods for detecting and measuring autoantibodies have evolved markedly in recent years, encompassing three generations of analytical technologies. Many different immunoassay methods have been developed and used for research and laboratory practice purposes, from the early conventional (or monoplex) analytical methods able to detect single autoantibodies to the more recent multiplex platforms that can quantify tens of molecules. Although it has been in use for over 50 years, indirect immunofluorescence remains the standard method for research on many types of autoantibodies, due to its characteristics of diagnostic sensitivity and also to recent technological innovations which permit it a greater level of automation and standardization. The recent multiplex immunometric methods, with varying levels of automation, present characteristics of higher diagnostic accuracy, but are not yet widely diffused in autoimmunology laboratories due to the limited number of autoantibodies that are detectable, and due to the high cost of reagents and systems. Technological advancement in autoimmunology continues to evolve rapidly, and in the coming years new proteomic techniques will be able to radically change the approach to diagnostics and possibly also clinical treatment of autoimmune diseases. The scope of this review is to update the state of the art of technologies and methods for the measurement of autoantibodies, with special reference to innovations in indirect immunofluorescence and in multiple proteomic methods.

Keywords: autoimmune diseases; automation; indirect immunofluorescence; monoplex and multiplex immunoassay methods; standardization.

Introduction

Circulating autoantibodies against a wide number of structural and functional molecules present in ubiquitous or tissue-specific cells are considered useful markers of autoimmune diseases (AIDs). The identification of these antigen-antibody systems with the contemporary development of laboratory methods for the detection and measurement of autoantibodies is considered one of the milestones in the history of clinical immunology over the last 60 years.

In this field, two categories of methods, based on microscopy and biochemistry principles – and therefore defined as morphologic and immunologic methods – have had great importance in the progress of laboratory medicine. These technological advances were transferred to the field of autoimmunology since all the variants of the immunological methods have found applications in the diagnosis of autoimmune diseases. A synthetic classification of these technologies is based on the principle of multiplicity of detection and distinguishes among conventional monoplex methods and multiplex methods according to the capacity to measure one antibody or several simultaneously [1]. Table 1 shows a (probably incomplete) list of these methods.

The saga of immunodiagnostics originates in the late 1940s or early 1950s, with the discovery of demonstrable phenomena in vitro, made possible by the use of morphological methods. The first phenomenon connected with the presence of autoantibodies (LE phenomenon or

*Corresponding author: Nicola Bizzaro, Laboratorio di Patologia Clinica, Ospedale S. Antonio, Via Morgagni, 18, 33028 Tolmezzo (UD), Italy, Phone: +39 0433 488261, Fax: +39 0433 488697, E-mail: nicola.bizzaro@ass3.sanita.fvg.it
LE test – reaction of opsonization of anti-nucleosome antibodies) was discovered by means of the common techniques of light microscopy [2]. Next, following the first applications of the method for identifying bacteria using conjugated antibodies with fluorescent tracers, for the first time antinuclear antibodies (ANA) were detected in sera using the indirect immunofluorescence technique (IIF) on antigenic microspots coated to slides [3] and later on cellular substrates made from human tissues and from isolated cells [4].

Thus, IIF was the first monoplex technique applied to the detection of autoantibodies. In some applications however, (i.e., for ANA) it is effectively a multiplex method, given that it is able to detect more than 60 autoantibodies simultaneously, by means of the identification of at least 26 different cellular patterns [5].

Next to IIF, also belonging to the first generation, are other qualitative immunochromatographic methods (immuno-diffusion, complement fixation, passive agglutination, counterimmunoelectrophoresis, immunoprecipitation) developed during the year 1957 (the “golden year of immunodiagnostics”) [6–11]. During the 1970s and 1980s additional evolution took place in qualitative methods, with the introduction of immunoblot or dot-blot, and second-generation quantitative immunometric assays (IMA) were introduced [radioimmunoassay and its variants, immunoenzymatic assay and its variants, fluorimmunologic assay, and immunochemiluminescent assay, reviewed in [1]].

Since the turn of the millennium, research has produced an additional rapid advance in diagnostic technology for detection and quantification of autoantibodies. The reasons for this technological revolution have been given as:

(a) the increment of awareness of the physiopathogenetic and diagnostic role of autoantibodies in systemic and organ-specific autoimmune diseases (autoantibodies as diagnostic criteria);
(b) the refinement of procedures for identifying and purifying the target autoantigens of the autoimmune reaction, with special reference to recombinant DNA technologies;
(c) the application of quantitative determination of autoantibodies by automated analytic systems, initially introduced for determining hormones, tumor markers, metabolites, antibodies, etc.;
(d) the development of proteomic technologies, able to detect simultaneously a high number of autoantibodies in the same sample (multiplexing).

The occurrence of these processes in clinical laboratories has led to the advent of a new era in the measurement of autoantibodies, with exponential increase in analytic capacity and corresponding rise in the volume of test requests for the diagnosis of AIDs. This evolution has brought about the rapid disposal of 1st and 2nd generation immunoassay methods to the benefit of automated 3rd generation immunoassay methods [12] (Figure 1).

The aim of this review is to define the current state of technologies available in the autoimmunology laboratory (with special concern to the morphologic and proteomic technologies) with a view to updating our previous review [1].

**Multiplex immunoassay platforms: a real improvement of the diagnostic power of autoantibody testing?**

From the beginning of the third millennium, based on progress in the development of the genome, multiple measurement of genetic products has become a characteristic of laboratory methodology, defined with the suffix “omics”. Similar to other -omics, (e.g., ribonomics, metabolomics, etc.) proteomics – meaning the science which studies, on a large scale, the expression, the function and the interaction of protein – permits the parallel analysis of hundreds

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**Table 1** Classification of immunoassay methods for the detection and measurement of autoantibodies.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st generation monoplex</td>
<td>ID, CF, IIF, PHA, LPA, RIP, WB</td>
</tr>
<tr>
<td>2nd–3rd generation monoplex</td>
<td>RIA-IRMA, RRA, ELISA-IEMA, IB, DB, CLIA-ILMA, FIA-IFMA</td>
</tr>
<tr>
<td>Multiplex</td>
<td>MBA</td>
</tr>
</tbody>
</table>

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of different products, in this case antigens/antibodies, in minimal quantities of biological fluids. In this sense the discipline is held to represent the keystone for thoroughly investigating some acquired human diseases, in the fields of oncology, infectious diseases, immunology, and so on. Consequently, this has assisted the production of numerous analytic methods of both research and commercial application, for the simultaneous quantification of analytes of proteic nature (e.g., hormones, bio-humoral indicators, cytokines, antibodies, autoantibodies, etc.) by using new technologies, some of which can be considered as comprising a new category of immunometric methods [13].

Given the number and complexity of molecules involved in the activities of the immune system, the study of such molecules seemed an ideal field of application for proteomic techniques [14–16], the objective of which is to be able to study the entire autoimmune process rather than their single components. Among the many systems designed, some microarrays have found application for research on autoantibody profiling of AIDs and, in particular, planar and non-planar autoantigenic arrays (in suspension) [1, 17, 18].

Among the planar arrays we note systems made up of microspots on glass slides, on polystyrene microplates or nitrocellulose membranes and linear immunoblot systems on nitrocellulose membranes (Table 2). The first are based on techniques originally proposed by Ekins and Chu [19] and still hold a relevant importance for their simplicity and flexibility. There are not yet commercial applications available for these, but there are notable studies confirming their reliability [20–22].

The second are based on the linear Western blot technique (line immunoblot), with electrophoretic migration on nitrocellulose membranes. The method has found numer-

<table>
<thead>
<tr>
<th>Support</th>
<th>Format</th>
<th>Detection system</th>
<th>Autoantibodies tested (no. and type)</th>
<th>Estimated throughput (test/h)</th>
<th>Commercial product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>L</td>
<td>115</td>
<td>~10.000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>196</td>
<td>~10.000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>650</td>
<td>~10.000</td>
<td>–</td>
</tr>
<tr>
<td>Glass slide</td>
<td>Microspot</td>
<td>F</td>
<td>225</td>
<td>~10.000</td>
<td>–</td>
</tr>
<tr>
<td>Microplate</td>
<td>Microspot</td>
<td>C</td>
<td>15</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Microspot</td>
<td>C</td>
<td>30</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Line-blot</td>
<td>C</td>
<td>15 (ENA)</td>
<td>~50</td>
<td>Inno-LIA</td>
</tr>
<tr>
<td>Nitrocellulose membranes</td>
<td>Line-blot</td>
<td>C</td>
<td>13 (ENA)</td>
<td>~50</td>
<td>RecomLine</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Line-blot</td>
<td>C</td>
<td>11 (ENA)</td>
<td>~50</td>
<td>ANA-LIA</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Nanodot</td>
<td>L</td>
<td>10 (ENA)</td>
<td>ND</td>
<td>NALIA</td>
</tr>
</tbody>
</table>

Table 2: Planar autoantigen arrays for autoantibody detection.
C, colorimetry; F, fluorescence; L, chemiluminescence; ND, not declared.
uous commercial applications and there is clinical validation of the systems conducted on large sample numbers, including multicentric studies [23–27]. In general, line immunoblot offers diagnostic and clinical accuracy comparable to, if not superior to, conventional immunometric methods. Nevertheless it is our opinion that such technologies are not to be considered true microarrays, given the macroscopic dimensions of the transferred proteins and the limits to the number and variety of antigens applicable on the planar solid phase of these systems.

Among the non-planar arrays (Table 3) there have been developed systems in suspension that use micro-particles recognized by laser nephelometry [28] or laser fluorimetry in flow cytometry [29]. This last technology offers many commercial applications and has been validated widely by numerous clinical studies, conducted around the world. The principal commercial systems, based on fluorescent microbeads technology, are represented by the following: FIDIS (BioMedical Diagnostics, Marne la Vallee, France); AtheNA Multi-Lyte (Zeus Diagnostics, Raritan, NJ, USA); QuantaPlex (Instrumentation Laboratory, Barcelona, Spain); and BioPlex 2200 (Bio-Rad Laboratories, Hercules, CA, USA) (Table 3).

The FIDIS system, which has a reasonable level of automation and was the first to be introduced on the market, is able to determine diverse autoantibody profiles for connective tissue diseases, antiphospholipid syndrome, vasculitides, rheumatoid arthritis, celiac disease, autoimmune gastritis and autoimmune thyroidopathies. Many validation studies on its characteristics are published in the literature [30–37].

The AtheNA Multi-lyte system is able to do autoantibody profiling for rheumatic diseases, vasculitides, autoimmune thyroiditis and celiac disease. The automation level is similar to the FIDIS method. The many validation studies published attest to its good analytic reliability [36–48].

The QuantaPlex system is able to determine various autoantibody profiles for rheumatic diseases, celiac disease, vasculitides and autoimmune liver diseases and demonstrates, like the first two, high analytic reliability [35, 49–52].

The BioPlex 2200 system is of recent introduction, totally automated and with high analytic productivity, which is able to execute an autoantibody profile for rheumatic diseases, antiphospholipid syndrome, vasculitides and, more recently, celiac disease. Numerous clinical validation studies have been conducted on extensive case populations [53–65].

Enthusiastically welcomed at the time of its appearance in the clinical laboratory a few years ago, multiplexing should have allowed for overcoming several limitations of the conventional methods, including analytic problems (e.g., reduction of the volume of samples and reagents and containment of costs), logistical/managerial problems (e.g., simultaneous measuring in the same session and in the same analyte reaction environment, measured in real time with several methods), and physiopathological problems (e.g., association of markers in pathology-oriented or organ-oriented profiling) [16–18] and should have found extensive application in the routine activities of the autoimmunology laboratory. In any case, at this time the biomedical industry seems oriented to repropose in multiplex versions the autoantibody profiles already consolidated for the principal AIDs, with the only intention being to achieve diagnostic usage limited to the most common autoantibody specificities. This is the principal motive for the slow and extended penetration of these systems into routine diagnostics, where they are substituting for conventional methods. However, a truly flexible autoantibody profile is not yet available to cover the 30–50 most common and important autoantibodies. Such a profile would be used in the laboratory simply for evaluating the autoimmune status and autoantibody profile of the patient, for predictive, monitoring, and therapeutic purposes [66].

The renaissance of IIF

Thanks to the use of various tissue and cellular substrates (e.g., human laryngeal HEp-2 carcinoma cells, Crithidia luciliae hemoflagellates, human neutrophil granulocytes, triple-tissue substrate composed of liver, kidney,
and stomach of rat or mouse, monkey esophagus, human umbilical cord, human or animal pancreas, human or animal adrenal gland, pituitary, esophagus, bladder, monkey nerve tissue, etc.), IIF has permitted the detection over the years of an extensive series of specific autoantibodies directed against cellular autoantigens. This method, in time, became a consolidated and universally diffused procedure for detecting patients affected by AIDs, with differentiated use in the different AIDs, according to analytical sensitivity and specificity of the different types of substrates. Currently, the IIF method is considered the reference method for ANA and anti-neutrophil cytoplasmic antibodies (ANCA) screening and a confirmatory test for anti-DNA antibody detection [67–70]. However, the method is burdened by some unfavorable features: the need for expert morphologists, the subjectivity of interpretation, and the low degree of standardization and automation [71, 72]. Because of these limitations and the progressive increase of ANA test requests in autoimmunology laboratories, in the last 15 years technology innovation of analytical platforms has offered alternative solutions to the ANA-IIF test based on the manual or automated monoplex IMA, mainly of the ELISA type, with the use of solid phases made up by a mixture of nuclear-cytoplasmic antigens. Experience published in the literature has demonstrated that these manual [73–84] or automatic [85–87] systems do not provide the same analytic accuracy as IIF, in particular for the presence of false negative results (up to 35% of cases) in case of rare autoantibodies [79].

Therefore, it is maintained that the IMA monoplex methods do not represent a substitute for IIF, not even in unusual analytic conditions or when faced with a high volume of test requests [78].

The introduction of multiplex methods (multiplex immunoassays), automated in varying degrees, which are able to simultaneously measure several ANA-related antibodies, has produced – especially in the USA – the hypothesis that they can be used as a screening platform for ANA testing as an alternative to IIF. However, the sensitivity of the ANA screening test with multiplex immunoassay is not yet adequate and the presence of false negative results with respect to IIF is not dissimilar to the occurrence of the previously-discussed immunometric methods [35, 42, 56, 62], varying from 0.2% to 40.5% according to the population studied (Table 4). One of our recent studies conducted with a latest-generation multiplex system (BioPlex 2200) showed a higher sensitivity and a parallel specificity of the ANA-IIF (at 1:80 dilution) compared to the ANA screen on BioPlex 2200. In the study of 181 patients affected by autoimmune rheumatic diseases, the rate of false negatives has stabilized around 11.6% for the manual IIF method, vs. 14.9% for the BioPlex method (Table 5). In most cases, the patients had nucleolar or rare autoantibodies. In a group of 208 healthy and infectious disease patients, the specificity was similar between the two methods (Table 5).

Following the recent statement that the IIF technique should be considered as the standard screening method for the detection of ANA [69], the biomedical industry has proposed technological solutions which significantly improve the automation for the procedure, not only in the preparation of substrates and slides but also in reading under the microscope. This innovation is based on the principles of digitalization of fluoroscopic images and on the classification of patterns using standardized approaches (automatic positive-negative pattern interpretations) using expert

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Patients, n</th>
<th>IIF positive, %</th>
<th>MIA positive, %</th>
<th>Multiplex system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifli et al., 2006 [41]</td>
<td>910</td>
<td>17.3</td>
<td>17.1</td>
<td>AtheNA Multi-lyte</td>
</tr>
<tr>
<td>Bonilla et al., 2007 [42]</td>
<td>53</td>
<td>90.6</td>
<td>49.1</td>
<td>AtheNA Multi-lyte</td>
</tr>
<tr>
<td>Salamunic et al., 2008 [46]</td>
<td>897</td>
<td>27.9</td>
<td>23.6</td>
<td>AtheNA Multi-lyte</td>
</tr>
<tr>
<td>Hanly et al., 2010 [52]</td>
<td>192</td>
<td>81.3</td>
<td>75.5</td>
<td>BioPlex 2200</td>
</tr>
<tr>
<td>Op de Beeck et al., 2012 [65]</td>
<td>236</td>
<td>78.3</td>
<td>74.5</td>
<td>BioPlex 2200</td>
</tr>
</tbody>
</table>

Table 4 Diagnostic sensitivity of indirect immunofluorescence (IIF) and multiplex immunoassay (MIA) methods for ANA testing.

<table>
<thead>
<tr>
<th>Patients</th>
<th>n</th>
<th>IIF positive (1:80)</th>
<th>Bioplex positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>SLE</td>
<td>95</td>
<td>85</td>
<td>89.5</td>
</tr>
<tr>
<td>SSc</td>
<td>55</td>
<td>53</td>
<td>96.4</td>
</tr>
<tr>
<td>SS</td>
<td>18</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>DM/PM</td>
<td>8</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>160</td>
<td>88.4</td>
</tr>
<tr>
<td>Blood donors</td>
<td>120</td>
<td>7</td>
<td>5.8</td>
</tr>
<tr>
<td>Infectious disease</td>
<td>98</td>
<td>7</td>
<td>7.1</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>14</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table 5 Sensitivity of methods for ANA detection in AIDs: comparison between IIF and BioPlex 2200.

SLE, systemic lupus erythematosus; SSc, systemic sclerosis; SS, Sjögren’s syndrome; DM/PM, dermatomyositis/polymyositis.
Table 6 Patterns detected by the screening/recognition IIF automated systems.

<table>
<thead>
<tr>
<th>System</th>
<th>Screening −/+</th>
<th>Patterns, n</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aklides</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots</td>
</tr>
<tr>
<td>Europattern</td>
<td>Yes</td>
<td>6</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots, cytoplasmic</td>
</tr>
<tr>
<td>G-Sight</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots, mytochondrial</td>
</tr>
<tr>
<td>Nova-View</td>
<td>Yes</td>
<td>5</td>
<td>Homogeneous, speckled, nucleolar, centromeric, nuclear dots</td>
</tr>
<tr>
<td>Helios</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 7 Diagnostic sensitivity of manual and automated IIF methods for ANA testing.

<table>
<thead>
<tr>
<th>System</th>
<th>Patients Manual IIF no. positive (1:80)</th>
<th>Automated IIF no. positive</th>
<th>Agreement, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europattern</td>
<td>116</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Aklides</td>
<td>182</td>
<td>182</td>
<td>180</td>
</tr>
</tbody>
</table>

Conflicts of interest statement

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Renato Tozzoli was born in Venice, Italy, in 1951. He is Head of the Department of Laboratory Medicine and Medical Director at the Clinical Pathology Laboratory, St. Mary of Angels Hospital, Pordenone, Italy. He graduated in Medicine and Surgery at the University of Bologna, and specialized in Endocrinology in 1984 at the Modena University and in Clinical Pathology in 1988 at Padua University. He has published over 250 papers in national (Italian) and international journals, including several convention reports, books, and book chapters. He is General Chairman of the Italian Society of Laboratory Medicine Study Groups; Member of the Italian Association for Autoimmune Research; Founder and President of the Italian Interdisciplinary Forum for Research in Autoimmune Diseases (FIRMA) and Deputy Editor of Autoimmunity Highlights (Springer).

Chiara Bonaguri was born in Forlì, Italy in 1957. She obtained a PhD degree in Biology in 1981 at the University of Bologna; in 1991 she specialized in Biochemistry and Clinical Chemistry at the University of Parma and in Clinical Immunopathology (2008) at the University of Milan. Since 1992, she has worked at the Laboratory of Clinical Immunology of the University Hospital of Parma; and in 2009 headed the Autoimmune Serology Section. She has taught Allergology and Clinical Immunology since 1999 at the School of Medicine of the University of Parma. She is the author of several publications in peer-reviewed journals on autoimmune diseases and is a member of the Study Group on Autoimmune Diseases of the Italian Society of Laboratory Medicine.

Alessandra Melegari was born in Parma, Italy in 1961. She obtained her BSc in Biological Sciences at the Universita’ degli Studi di Parma in 1985 and her PhD in Food Chemistry and Technology in 1991. She was a biologist in various hospitals from 1992 to 1999 and a senior Diagnostic Biologist at the University Department of the Policlinico Hospital at Modena from 1999 to 2010. Since 2010 she has been a Senior Biologist in the Diagnostic Department of the NOCSAE at Modena Hospital and Head of Diagnostic Autoimmune Disease Activity. Alessandra is a teacher at the Post-graduate School of Rheumatology and Laboratory Medicine at the University of Modena where she instructs post-graduate students. She is active in many national and international congresses in laboratory medicine, and is the author and co-author of many articles on immunology, rheumatology and autoimmunity. Alessandra organized congresses on immunology in Modena in 2011 and 2012, and she has also organized courses for general physicians on autoimmune diseases.

Antonio Antico, was born in 1960. In 1987 he graduated in Medicine and later specialized in Clinical Pathology at the University of Padua. Currently he works as a medical doctor at the Laboratory of Clinical Pathology, General Hospital of Cittadella, Italy. Since 1993 he has mostly worked in the field of autoimmune/rheumatic diseases.

He is a member of the Study Group on Autoimmunology of the Italian Society of Laboratory Medicine. He has published many scientific papers in international journals on clinical-serological aspects of connective tissue diseases, autoimmune gastritis, celiac disease, autoimmune liver diseases, autoimmune blistering skin diseases and autoimmune neurological diseases.

Danila Bassetti graduated in Medicine and Surgery in 1978 at the Bologna University (Italy), where she specialized in Hygiene and Preventive Medicine (Public Health and Laboratory) and Health Law. Since 1981 she has directed the Serology section of the Public Health Laboratory in Trento (Italy) and since 1999 the Serology and Autoimmunity section at the Microbiology and Virology Unit of the S. Chiara Hospital in Trento. She is author of scientific articles on autoimmunity, infectiology and is Professor of Immunopathology at the Biomedical Laboratory Technician School of Verona University.

Nicola Bizzaro was born in Venice, Italy, in 1951. He is Head and Medical Director at the Laboratory of Clinical Pathology, San Antonio Hospital, Tolmezzo, Italy. He graduated in Medicine and Surgery at the University of Padua, and specialized in Clinical and Laboratory Hematology in 1987 and in Clinical Pathology in 1993 at Padua University. He has published over 440 papers in national (Italian) and international journals, including several convention reports, books, and book chapters. He is Chairman of the Italian Society of Laboratory Study Group on Autoimmune Diseases; Member of the Italian Association for Autoimmune Research; Founder of the Interdisciplinary Forum for Research in Autoimmune Diseases; Member of the Advisory Board of the International Congress on Autoimmunity; Chief Editor of the Italian Journal of Laboratory Medicine, the official Journal of the Italian Society of Laboratory Medicine; and Editor-in-Chief of Autoimmunity Highlights.