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REVIEW

A scoping review of autoantibodies as biomarkers for canine autoimmune disease

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Abstract

Background: Autoantibody biomarkers are valuable tools used to diagnose and manage autoimmune diseases in dogs. However, prior publications have raised concerns over a lack of standardization and sufficient validation for the use of biomarkers in veterinary medicine.

Objectives: Systematically compile primary research on autoantibody biomarkers for autoimmune disease in dogs, summarize their methodological features, and evaluate their quality; synthesize data supporting their use into a resource for veterinarians and researchers.

Animals: Not used.

Methods: Five indices were searched to identify studies for evaluation: PubMed, CAB Abstracts, Web of Science, Agricola, and SCOPUS. Two independent reviewers (AET and ELC) screened titles and abstracts for exclusion criteria followed by full-text review of remaining articles. Relevant studies were classified based on study objectives (biomarker, epitope, technique). Data on study characteristics and outcomes were synthesized in independent data tables for each classification.

Results: Ninety-two studies qualified for final analysis (n = 49 biomarker, n = 9 epitope, and n = 34 technique studies). A high degree of heterogeneity in study characteristics and outcomes reporting was observed. Opportunities to strengthen future studies could include: (1) routine use of negative controls, (2) power analyses to inform sample sizes, (3) statistical analyses when appropriate, and (4) multiple detection techniques to confirm results.

Abbreviations: AChR, acetylcholine receptor; ADH, alcohol dehydrogenase; AHA, antihistone antibody; ANA, antinuclear antibody; ANCA, antineutrophil cytoplasmic antibodies; ARVC, arrhythmogenic right ventricular cardiomyopathy; cANCA, cytoplasmic antineutrophil cytoplasmic antibody; CCH, chromic chloride hemagglutination; CI, confidence interval; CNS, central nervous system; Dsg-1, desmoglein-1; EBA, epidermolysis bullosa acquisita; FFPE, formalin fixed paraffin embedded; GAD65, glutamic acid decarboxylase 65; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCH, glutaraldehyde chloride hemagglutination; GPT, gel precipitation test; HEp-2, human epithelial; hnRNPG, glycosylated heterogeneous nuclear ribonucleoprotein; IA-2, insulinoma antigen-2; IBD, inflammatory bowel disease; IFA, immunofluorescence assay; IHC, immunohistochemistry; IIF, indirect immunofluorescence; IMHA, immune-mediated hemolytic anemia; IMRD, immune-mediated rheumatic disease; ITP, immune-mediated thrombocytopenia; kDa, kilodaltons; LFT, latex fixation test; LISS, low ionic strength solution; MIR, main immunogenic region; MMM, masticatory muscle myositis; MUE, meningoencephalitis of unknown etiology; NME, necrotizing meningoencephalitis; NSDTRs, Nova Scotia Duck Tolling Retrievers; pANCA, perinuclear antineutrophil cytoplasmic antibodies; RA, rheumatoid arthritis; RF, rheumatoid factor; RIA, radioimmunoassay; RWT, Rose Waaler test; SARDS, sudden acquired retinal degeneration syndrome; SLE, systemic lupus erythematosus; T3, triiodothyronine; T4, thyroxine; TCH, tanned cell hemagglutination; Tg, thyroglobulin; TgAb, thyroglobulin antibodies; TPO, thyroid peroxidase.

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Conclusions: These findings provide a resource that will allow veterinary clinicians to efficiently evaluate the evidence supporting the use of autoantibody biomarkers, along with the varied methodological approaches used in their development.

KEYWORDS
autoantibody, autoimmunity, biomarker, dogs, immune-mediated diseases

1 | INTRODUCTION

Successful clinical management of autoimmune diseases relies on the ability to make timely and accurate diagnoses. Autoantibody biomarkers are an indispensable clinical tool for this purpose.[1-6] Use of autoantibody biomarkers in human medicine includes screening markers to predict disease onset,[7-14] diagnostic markers to confirm disease identity,[15-24] and prognostic markers to characterize disease progression,[25-28] severity,[29-35] or response to treatment.[36-39] Autoantibody biomarkers in veterinary medicine mainly are limited to diagnostic use, and less often are used in monitoring disease progression or response to treatment.[3-6] Despite the existence of many common autoimmune diseases in both dogs and humans with similar clinical features,[40-50] the smaller repertoire of autoantibody biomarkers for autoimmune disease in dogs suggests that biomarkers may be an underutilized or underdeveloped tool in veterinary medicine.

One obstacle to the widespread use of autoantibody biomarkers in veterinary medicine may stem from a lack of standardization with their development and use in veterinary settings. Concerns that biomarkers may be used in the clinic before robust validation, along with a lack of uniform standards for their use, have been raised in previously published review articles.[51,52] These authors urged veterinarians and researchers to scrutinize validation data before recommending specific biomarkers, underscoring the need for a resource to review the evidence supporting particular biomarkers used in veterinary medicine. However, these review articles addressed veterinary biomarkers across a range of diseases and lacked details specific to the use and performance of autoantibody biomarkers for autoimmune diseases.

To the best of our knowledge, no review articles are dedicated to evaluating the characteristics and performance of autoantibody biomarkers for all autoimmune diseases of dogs. We undertook this review to provide a resource for veterinary clinicians and researchers that synthesizes data from primary research on autoantibody biomarkers for autoimmune disorders in dogs and to provide insight into best practices in autoantibody biomarker discovery. Filling this gap is an important step in helping clinicians and researchers make judicious decisions on the use of autoantibody biomarkers in veterinary medicine and to understand the supportive data regarding their use. Our review also facilitates comparisons of autoantibody biomarkers between autoimmune diseases in dogs and humans, which is helpful for comparative animal model research. Such comparisons can lead to improved understanding and development of autoantibody biomarkers across species.

2 | METHODS

Our literature search followed practices consistent with the PRISMA statement.[53] This review was precluded from registration with PROSPERO because it does not directly impact human health.

2.1 | Literature search

Five reference databases (PubMed, SCOPUS, Web of Science, Agricola, and CAB Abstracts) were searched using the terms "autoantibodies" and "dogs" through April 28, 2020. Other search terms were tested, but abandoned after cross-checking manually curated papers showed no increase in relevant records. Results were imported into EndNoteX9,[54] and duplicates were removed. Remaining articles were exported to Microsoft Excel[55] for further filtering and data extraction.

2.2 | Study selection

Titles and abstracts were screened for the following exclusion criteria: (1) article not published in English; (2) nondog species focus of study; (3) case reports or <5 cases studied; (4) review articles and book chapters; (5) gray papers (eg, conference proceedings, abstracts); (6) nonautoimmune disease focus of the paper.

Two independent reviewers (AET and ELC) performed a full-text review of the remaining articles. Studies reporting only negative results were excluded from analysis, as our aim was to summarize data supportive of biomarkers.

Studies were first evaluated for relevance. Relevance criteria included: (1) evaluation of an autoantibody biomarker; (2) autoantibody evaluation with regard to a specific disease or organ system (vs studies of autoantibodies in autoimmune disease more broadly, such as cytotoxic T-lymphocyte-associated protein 4 [CTLA-4][56] or anticytokine antibodies)[57]; (3) autoantibody studied in the context of spontaneous autoimmune disease; (4) autoantibody used to discriminate between diseased and nondiseased state (eg, not studies measuring baseline concentrations of autoantibodies in certain populations or following autoantibody concentrations longitudinally in certain populations over time).

Relevant studies then were classified according to 1 of 3 study objectives: biomarker, epitope, or technique (Table S1). Biomarker studies included studies that aimed to discover or validate whether an

autoantibody biomarker or antibody-bound cells or tissues associated with a specific autoimmune disease. Epitope studies included those studies aimed at identifying the epitope targeted by autoantibody biomarkers for a specific autoimmune disease. Technique studies included studies aimed at developing novel detection techniques, comparing diagnostic accuracy between techniques, or optimizing existing detection methods for already described autoantibody biomarkers. Independent data extraction and synthesis were performed separately for each study objective classification group (biomarker, epitope, technique; Tables S2, S3, and S4, respectively).

Biomarker studies then were assessed against several quality metrics. Our quality criteria were defined as follows: (1) use of negative controls; (2) reporting specific numbers of cases and controls; (3) reporting raw data for autoantibody positive cases and controls as either numbers or percentages, or reporting significant differences between cases and controls; and (4) data not already reported in an existing publication. These quality metrics were informed by previously published veterinary meta-analyses[58-62] and published guidance for meta-analyses.[63,64] Biomarker studies that did not meet ≥1 of these criteria were excluded from downstream analysis.

Epitope and technique studies rarely provided adequate detail to evaluate these same quality inclusion criteria during full-text review. Therefore, epitope and technique studies were not required to meet the quality criteria for inclusion in the final analysis. We believed it was important to retain these studies to understand existing tests and to identify trends toward improvements in detection methods.

2.3 | Data extraction

Two independent authors (AET and ELC) collected data on study attributes including: study classification (biomarker, technique, epitope), first author, year of publication, organ system affected, autoimmune disease, antigenic target, breeds studied, detection technique, case and control numbers, percentage and number of antibody positive cases and controls, other relevant study groups, and statistical analyses performed. Data were tabulated in Microsoft Excel[55] by 2 authors (AET, ELC) independently and discrepancies were resolved after data extraction.

2.4 | Data reporting

In the biomarker analysis (Table S2), entries were created for every unique antigen or antibody-bound cell or tissue and detection technique pairing evaluated in a manuscript that had a positive result. Similar data were collected for epitope papers (Table S3) but also included a title column and a column describing the conclusions about the epitopes identified in the study. Technique papers (Table S4) were subcategorized as comparison, optimization, or new technique. Comparison papers focused on comparing different detection techniques for a particular antigen; optimization papers evaluated improvements to an existing technique; new technique papers included those attempting to detect a known autoantibody by a new method. Data on first author, year of publication, organ system affected, autoimmune disease, antigenic target, and technique were collected. A row was created for every unique comparison or optimization being tested. Additional data on the study objective (comparison, optimization, new), the comparisons or optimizations made, and the authors' conclusions also were reported in the analysis table.

3 | RESULTS

3.1 | Study selection

Our search returned 2286 records (616 from PubMed, 319 from CAB, 451 from Web of Science, 193 from Agricola, and 707 from SCOPUS). After removing 1167 duplicates, 1119 records remained. A review of abstracts and titles identified 760 additional articles for exclusion for the following reasons: non-English records (n = 134), nondog species (n = 163), case report or <5 cases studied (n = 99), review articles or book chapters (n = 170), gray papers (n = 42), and papers unrelated to autoimmune disease (n = 152). The remaining 359 records underwent a full-text review (Figure 1; created in Lucidchart).

During full-text review, 33 papers were found to report negative results and were excluded. Another 208 studies were removed for not meeting the relevance criteria defined in the methods section. Next, 26 biomarker studies were removed for not meeting the quality criteria defined in the methods section. Reasons for exclusion can be found in the "Gen Exclusion" column in Table S1. Specific reasons for exclusion based on quality are found in the "Specific Exclusion" column. A failure to use negative controls was the most common indication for exclusion based on quality criteria (19/26). Manuscripts qualifying for final analysis included 49 biomarker studies, 9 epitope studies, and 34 technique studies (Figure 1).

In the following sections, results are organized by study objectives: biomarker, epitope, or technique studies (Tables S2, S3, and S4, respectively). Within each objective classification, studies are further divided by organ system.

3.2 | Biomarker studies (n = 49)

3.2.1 | Study characteristics

Populations

Numbers of enrolled cases varied from 6[65] to 415.[66] Study size was classified by the size of the largest analysis cohort within a study. Small studies included <30 cases in any analysis cohort; medium, 30 to 99 cases in any analysis cohort; and large ≥100 cases in any analysis cohort. Most biomarker studies analyzed small case cohorts (24/49),[65,67-89] 18 studies were midsized,[90-107] and 7 were large.[66,108-113] We observed a variety of approaches for phenotyping and classification of cases. Some studies grouped cases based upon disease severity or other phenotypic characteristics,[80,85,93,94,112] such

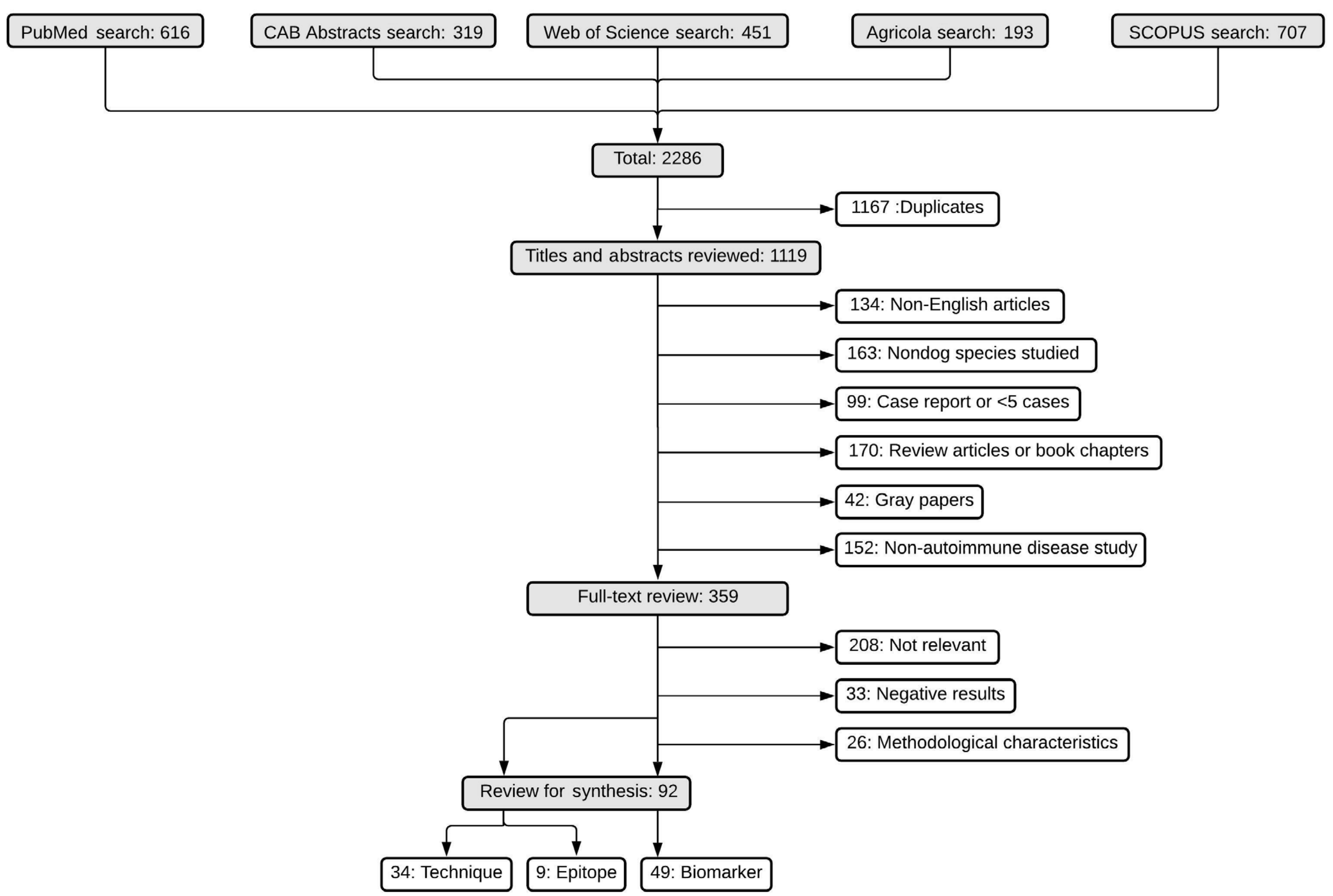

FIGURE 1 Flowchart of study selection process. Flow of papers from those that were originally identified from our electronic library search to those that were ultimately included in the manuscript. Created in Lucidchart, www.lucidchart.com

as subclinical or clinically confirmed disease. Other studies created cohorts based on the presence or absence of comorbidities.[79,86] A few studies cited data from smaller nested pilot studies in addition to reporting the findings of their larger study.[108,113]

Numbers of enrolled controls varied from 1[74,75] to 1057.[101] Most studies (31/49) used ≤30 controls in analysis cohorts[65-67,69-72,74-76,78-91,93,94,96,102,106,107,110]; including 12 studies with <5 controls but with sufficient numbers of cases to meet our inclusion criteria.[71,74,75,78,81,84,85,88,89,96,106,107] Another 12 studies used at least 1 midsized control cohort,[68,77,92,95,97-100,103,108,109,113] and only 6 studies included a large control cohort.[73,101,104,105,111,112]

The approach to phenotyping controls varied. The majority included dedicated healthy control populations. Some studies also included additional controls that were not necessarily healthy, such as hospitalized patients with different illnesses than that being studied.[73,95,101,104,113] These studies are denoted with an asterisk in the column "n (HC)" in Table S2. In many of these studies, patients with other related or unrelated diseases were analyzed as additional negative control groups independent of the healthy control group and are identified by a "Y" in the "Other controls used?" column in Table S2.

Studies diverged with regard to breed. A minority (n = 9), focused on a specific breed or breeds predisposed to the disease being studied.[67,74,79,80,89,93,94,103,108] Examples include Nova Scotia Duck Tolling Retrievers (NSDTRs) for immune-mediated rheumatic disease (IMRD),[108] Dobermans Pinschers for autoimmune hepatitis,[93,94] and Pugs for necrotizing meningoencephalitis (NME).[74] No single breed was overrepresented. Most studies did not report on breed or included a variety of breeds. A small number of studies used dogs from laboratory colonies,[80,82,88,100,110] generally Beagles[80,100] or Greyhounds,[88] as healthy controls in addition to client-owned pets. Nearly all studies sourced participants from client-owned pet populations.

Detection techniques

The techniques used were diverse, spanning at least 15 different techniques (Table S5). The Rose Waaler test, chromic chloride hemagglutination (CCH), and glutaraldehyde chloride hemagglutination (GCH) were counted as a single technique because they are all variations of hemagglutination. Most studies (n = 35) used at least 1 of the 4 most commonly used detection techniques: ELISA, immunofluorescence assay (IFA), Western blots, or radioimmunoassays (RIAs). Other techniques (eg, flow cytometry, immunoprecipitation) were used only once or twice across all of the biomarker studies evaluated. Thirty-three studies used only 1 detection technique (Table S5). An additional

13 studies used 2 detection techniques, and 3 studies used a combination of 3 detection techniques. In some of these studies, other techniques may have been used but produced negative results and were not included in our final analysis.

Statistics

The majority of studies did not use statistics to evaluate biomarker performance (eg, sensitivity and specificity, positive and negative percent agreement). When statistics were reported (18/49 studies[68,69,72,73,77,79,90-94,97-99,101,103,109,113]), the most common analysis was a test of significance comparing autoantibody positive cases to autoantibody positive controls with results reported as a *P*-value. Sensitivity and specificity were reported infrequently (5/49 papers),[91-94,109] but are not always appropriate, particularly when comparing the performance of tests for which a reference standard has been established. In these scenarios, using percentage positive and negative case agreement is preferred.[114,115] However, reports of percentage case agreement for biomarker studies were not observed, possibly because many of these studies identified nascent biomarkers for which there was no prior identification, few prior studies, or no established reference standard. Antibody performance generally was reported as numbers or percentages of cases that were autoantibody positive.

3.2.2 | Organ systems

Diseases affecting 9 organ systems were represented in our analysis of biomarker studies (Table S6).

Endocrine diseases (n = 11)

Studies on the endocrine system appeared most frequently (11/49) among the biomarker classification manuscripts. The specific endocrine diseases included hypothyroidism (n = 6),[77,99,101,102,107,110] diabetes mellitus (n = 4),[69,70,70,98] and hypoadrenocorticism (n = 1).[113]

Hypothyroidism studies typically were midsized or larger,[99,101,102,107,110] and most detected antithyroglobulin (Tg) autoantibodies. However, 1 study did not evaluate anti-Tg antibodies, but instead antibodies to triiodothyronine (T3) and thyroxine (T4) were used to define a population in which to test for the presence of a different antigen, thyroid peroxidase (TPO).[107] The most commonly used detection technique was ELISA, employed in (4/6) studies,[77,99,101,110] with anti-Tg positivity in cases ranging from 36% to 59%[99,101] and 0% to 6% for controls.[77,99,110] However, in the study with a large number of mixed controls (n = 1057), which included sick dogs with nonthyroidal illnesses, a 13% positivity rate was observed.[101] One of the ELISA studies reported antibody performance as the ability to detect combinations of 3 thyroid antigens: T3, T4, and Tg.[110] Statistical significance analyses were performed in (3/6) of these studies.[77,99,101] In 1 of the non-ELISA studies, tanned cell hemagglutination (TCH) and CCH were used to detect Tg autoantibody positivity. Autoantibody positivity in cases was 24% (6/25) when using TCH and 48% (12/25) when using CCH. Autoantibody positivity in healthy controls was 1/20 and 2/20 when tested by TCH and CCH, respectively. Statistical analysis was not performed. In the other non-ELISA study, Western blots were used to test for TPO antibodies in cases already determined to be autoantibody positive for Tg, T3, or T4. Seventeen percent of cases were anti-TPO positive. No autoantibodies were detected in controls. Statistical analysis was not performed.

Outcomes data and techniques used to detect autoantibodies in diabetes mellitus were heterogeneous. All 4 studies were small. Antigenic targets included insulin,[69,70] glutamic acid decarboxylase 65 (GAD65), and insulinoma antigen-2 (IA-2),[98] or islet cells.[86] No single autoantibody biomarker was pinpointed. Antibody positivity in cases ranged from 2/30 using RIA to simultaneously detect GAD65 and IA-2[98] to 18/25 using IFA on pancreatic islet cells.[86] Statistical significance tests were performed in 2/4 studies.[68,98]

Only 1 study focused on primary hypoadrenocorticism.[113] This study enrolled the largest number of cases among endocrine diseases for the biomarker category (n = 213) and used a single detection technique, RIA with recombinant antigenic proteins. Antibody positivity was congruent between the pilot study and the expanded cohort, 25% and 24%, respectively. Significant results were reported for autoantibodies against P450 side-chain cleavage autoantibodies when comparing cases to controls.

Rheumatic diseases (n = 8)

Studies on systemic autoimmune disease identified autoantibodies for systemic lupus erythematosus (SLE; n = 5),[83,90,104,105,111] rheumatoid arthritis (RA; n = 2),[100,112] and immune-mediated rheumatic disease (IMRD; n = 1).[108]

Multiple nuclear antigens were found in SLE, including histones,[90,104,111] DNA,[83,90,111] and other nuclear antigens.[83,105,111] Small,[83] midsized,[90,104,105] and large[111] studies were included in this group. Four studies used ELISAs either alone[83,90] or combined with other detection techniques.[104,111] Evaluation of antinuclear antibodies (ANAs) as a group, without specific antigens identified, was performed in 3 studies which all reported case positivity rates of 100%.[83,105,111] These studies used either ELISA[83] or IFA.[105,111] The IFA studies had 7.9% to 15.8% autoantibody positivity in healthy controls, whereas the ELISA had none. Statistical analyses were not performed.

Rheumatoid factor (RF) was the only autoantibody identified in 2 RA studies, including 1 midsized[100] and 1 large study.[112] Despite using different detection techniques, Rose-Waaler[112] and RIA,[100] both studies confirmed RF as an autoantigen in >70% of cases. Neither study reported statistics.

The single paper on IMRD identified multiple antigenic targets, including interleukin enhancer binding factors 2 and 3, RNA-binding motif protein X chromosome, and family with sequence similarity 134, member B protein (FAM134B).[108] Case cohorts ranged from 9 to 120 animals. Autoantibodies were not detected in healthy controls but were detected in 23% to 100% of cases. Statistics were not reported.

Dermatologic diseases (n = 7)

Most dermatologic studies were on pemphigus complex (n = 4).[81,95-97] One study also evaluated subepidermal bullous

dermatoses.[97] In these 4 studies, circulating serum autoantibodies against desmoglein-3,[97] desmocollin-1,[95,96] and keratinocytes were evaluated.[81,96] No common autoantibody was identified. All 4 studies were small to midsized. An IFA was most commonly used, either alone[95,96] or in combination with Western blotting.[81] Statistics were reported in 1 study.[97]

The remaining 3 dermatologic studies investigated subepidermal blistering dermatoses,[71] alopecia areata,[78] symmetrical lupoid onychodystrophy, and black hair follicular dysplasia.[79] Two studies initially identified autoantibodies bound to distinct skin cells or tissue from patient biopsy samples.[71,78] Follow-up experiments to detect circulating serum autoantibodies offered clues about antigen identity, such as molecular weight[78] or location of binding to morphological features (eg, basement membrane).[71] Both studies had small sample sizes, used multiple detection methods (IFA, Western Blots[71]; IFA, Western blotting, immunoprecipitation[78]), and did not perform statistical evaluations. The remaining study on black hair follicular dysplasia was midsized, used an ELISA, and reported a significant difference for autoantibody detection in cases vs controls.[97]

Gastrointestinal diseases (n = 6)

Gastrointestinal disease studies included those on hepatitis (n = 3),[82,93,94] inflammatory bowel disease (IBD; n = 2),[92,109] and pancreatitis (n = 1).[80] Many antigens were evaluated in the hepatitis studies, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and alcohol dehydrogenase (ADH)[94]; ANA, antihistone antibody (AHA),[93] liver membrane protein (LMP)[82]; and liver homogenate. No common autoantibody was detected. An ELISA was used in each of these papers, either alone[92] or in combination with other techniques.[93,94] All but 1 of these studies were small, and 1 was midsized.[93] Two of these studies reported statistical analyses. A sensitivity of 89.5% and a specificity of 100% was reported for detection of AHA in Doberman hepatitis.[93] In a separate study of Doberman hepatitis, anti-GAPDH and anti-ADH antibodies had sensitivities of 47.4% and 73.7% and specificities of 100% and 94.1%, respectively.[94]

The 2 IBD studies identified antineutrophil cytoplasmic antibodies (ANCA) or perinuclear antineutrophil cytoplasmic antibodies (pANCA). Each used a different detection method, either IFA[109] or ELISA.[92] In addition to detecting anti-ANCA antibodies, the ELISA study also detected a non-ANCA antibody, outer membrane protein porin C. Both studies reported significant differences in autoantibody concentrations between IBD and control groups. The midsized study using ELISA reported a sensitivity of 79% to 97% and specificity of 93% to 99% with a 95% confidence interval (CI) for the simultaneous detection of antipolymorphonuclear leukocyte antibody with antimembrane porin C antibody.[92] The large study using IFA reported a specificity of 88% to 94% (95% CI) at a 1:20 dilution in the assay.[109] Sensitivity was not reported.

In a small study of atrophic lymphocytic pancreatitis,[80] autoantibodies against an unspecified cytoplasmic protein in pancreatic acinar cells were detected in all cases, and none in controls. Statistical analyses were not reported in this manuscript.

Muscular and neuromuscular (n = 5)

Our analysis included 3 studies of muscular disease[75,85,89] and 2 studies of neuromuscular disease.[66,87] Two studies of masticatory muscle myositis (MMM) utilized Western blots and a secondary technique to detect autoantibodies to temporalis muscle fibers. One study reported 100% antibody positivity in cases,[75] and used liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify the antigen as a myosin-binding protein C family member protein. The other study used Western blotting[85] and had high positive staining for crude temporalis muscle protein fractions in both cases (15/16) and controls (2/3). When immunohistochemistry (IHC) was performed, presence of immune complexes varied from 25% to 100% of cases depending on temporalis muscle tissue features, such as neurogenic atrophy or presence of lesions. Circulating antitemporalis antibodies were found in 43% to 88% of cases, also varying with temporalis features. Statistical analyses were not reported in either study. In the third muscular disease study, a few polymyositis cases were evaluated for both immune complexes and circulating autoantibodies. Results from an IFA showed 5/15 cases had both immune complexes in muscle biopsy samples and circulating antibodies to the sarcolemma in muscle cells. Alternatively, ELISA detected that 8/17 cases had antisarcolemma antibodies. Only 1 healthy control was evaluated and was negative in all tests. Statistical analysis was not performed.

Two studies of myasthenia gravis evaluated various antigenic targets. One study found autoantibodies to ryanodine fusion protein and titin (specifically, epitope MGT-30). The titin cohort was the largest of any study evaluated in our entire analysis (n = 415) and used an ELISA. The ryanodine receptor cohort was midsized and used Western blotting.[66] Autoantibodies to titin epitope MGT-30 were found in 78/415 cases. In a separate analysis of 80 titin autoantibody positive cases, 13/80 also had autoantibodies for the ryanodine fusion protein. Autoantibodies were not identified in healthy controls.

The second study of myasthenia gravis tested a small number of animals for autoantibodies to the acetylcholine receptor (AChR) using a standard assay (Lindstrom 1977) and to skeletal muscle myofibrils using IFA.[87] The 13 cases included 3 dogs with a clinical presentation consistent with myasthenia gravis, another 8 dogs from a single litter, all of which exhibited transient signs of myasthenia gravis postvaccination and had spontaneously recovered, and another 2 dogs that did not exhibit signs of myasthenia gravis but were related to the litter of 8. Of these 13 cases, anti-AChR autoantibody was found only in the 3 dogs previously identified as having had myasthenia gravis. One of these 3 dogs also had antibodies bound to myofibrils in skeletal muscle. Healthy controls were not positive for either anti-AChR or antimyofibril autoantibody. Tests of significance were not performed.

Neurologic (n = 4)

Three studies on meningoencephalitis[74,76,91] and 1 on generalized autoimmune encephalitis were included in our analysis.[106] Two meningoencephalitis studies were small in size and reported 100% antiastrocyte autoantibody positivity in cases detected by IFA, with no autoantibody positive controls.[74,76] Neither study performed statistical analyses. The third meningoencephalitis study used ELISA and

found autoantibodies against glial fibrillary acidic protein. A statistically significant difference between cases and controls was reported and sensitivity and specificity were 91% and 73%, respectively.[91] The autoimmune encephalitis study used a commercially available IFA assay for humans to evaluate 6 neuronal cell surface antigens in cerebrospinal fluid (CSF) from dogs with various central nervous system (CNS) diseases. The number of inflammatory CNS disease cases was small (n = 19), and a response to only 1 antigenic target, anti-N-methyl-1-day-aspartate receptor 1, was found in 3 cases. Two of the autoantibody positive cases had a diagnosis of meningoencephalitis of unknown etiology (MUE). The third autoantibody positive case lacked a clear diagnosis but was suspected to have MUE. The 1 healthy control was tested and was not antibody positive. Statistical analysis was not performed.

Hematologic (n = 4)

We identified 4 studies related to hematologic disease, including immune-mediated hemolytic anemia (IMHA, n = 2),[73,88] immune-mediated thrombocytopenia (ITP, n = 1),[68] and immune-mediated neutropenia (n = 1).[65] Both IMHA studies identified antierythrocyte antibodies in at least 50% of cases and in <10% of controls. Detection techniques included ELISA, flow cytometry, and a direct enzyme-linked antiglobulin test. Only 1 IMHA study reported using statistical analysis.[73]

The ITP study found positive results for a panel of platelet-associated antigens detected by ELISA (phosphatidylserine, β-2 glycoprotein, phosphatidylcholine, cardiolipin, phosphatidylinositol, and noncardiolipin phospholipids). Antibody positivity varied from 40% to 100% of cases, varying by antigen, with 100% positivity found for noncardiolipin phospholipids. The number of cases was small (n = 20), and statistical analyses were reported for most of the antigenic targets.[68]

A small study on immune-mediated neutropenia using IFA found that 5/6 cases were positive for antineutrophil autoantibodies. However, a specific neutrophilic autoantigen was not identified. Statistics were reportedly used to compare cases and controls, but no specific calculation or *P*-value was reported.[65]

Other (n = 4)

Other organ systems studied included ocular (n = 2)[72,84] and cardiac (n = 2).[67,103] Both ocular studies were small and focused on sudden acquired retinal degeneration syndrome (SARDS). Different antigenic substrates, neuron enolase[72] and bovine eye fraction,[84] and different detection methods, Western blot[72] and ELISA,[84] were used. Autoantibody positivity was 25% when detected by Western blot[72] and 50% to 100% when detected by ELISA.[84] Only the Western blot study reported statistical significance.[72] The ELISA study also evaluated other ocular diseases suspected of having autoimmune etiology, including progressive retinal degeneration, Vogt-Koyanagi-Harada syndrome, and episcleritis. Antibodies in these cases also bound to soluble fractions of retina iris ciliary complex and lens, but with less frequency than in SARDS cases (2/6, 1/2, and 1/1, respectively).[84] Autoantibodies were not detected in healthy controls.

The 2 cardiac studies both investigated autoantibodies in patients with cardiomyopathy using Western blots. In the study on arrhythmogenic right ventricular cardiomyopathy (ARVC), autoantibodies to desmoglein-2 were reported in 100% of cases and in none of the healthy controls.[67] No statistical analyses were conducted, and case cohorts were small (n = 10). The other study was midsized and evaluated whether dilated cardiomyopathy patients had antibodies to 5 different antigens, including cardiac-specific (myosin heavy chain alpha isoform, alpha cardiac actin), housekeeping (GAPDH), mitochondrial (mitochondrial aconitate hydratase), and neuronal (brain glycogen phosphorylase) proteins.[103] Autoantibody positivity in cases ranged from 24.4% to 66.7%. Controls also had autoantibodies to all antigens, except for alpha cardiac actin. The difference in autoantibody positivity between cases and controls was not statistically significant for any of the 5 antigenic targets.

3.3 | Epitope studies (n = 9)

Nine epitope studies were included in our final analysis (Table S3). These included 4 studies for dermatologic disease,[116-119] 3 for endocrine disease,[120-122] and 1 each for neuromuscular[123] and rheumatic disease.[124]

3.3.1 | Dermatologic (n = 4)

The 4 dermatologic studies evaluated different diseases, including 1 each on pemphigus foliaceus,[119] pemphigus vulgaris,[116] bullous pemphigoid,[117] and epidermolysis bullosa acquisita (EBA).[118] The pemphigus foliaceus study was midsized[119] and the other 2 pemphigus studies were small-sized.[116,117] The study on EBA did not report the numbers of cases evaluated.[118] The study on pemphigus foliaceus used Western blotting and IFA to characterize the epitopes of desmoglein-1. Few cases were autoantibody positive (6%), and no controls were positive. The epitope was characterized as being conformation- and glycosylation-dependent. Statistical analyses were not performed.[119] In the pemphigus vulgaris study, IFA and Western blotting experiments showed that autoantibodies targeted desmoglein-3. No further characterization of the epitopes was reported. The percentage antibody positivity in cases varied from 45% to 91%, depending on antigenic substrate. Autoantibodies were not detected in healthy controls and statistical analysis was not performed.

In the bullous pemphigoid study, at least 4 techniques including cloning, IFA, Western blotting, and ELISA were used to define the NCA16A epitope for bullous pemphigoid antigen 2 in 4 cases.[117] These canine-derived autoantibodies bound the same epitope on human antigenic substrate, suggesting that the epitope is conserved across humans and dogs. No controls were used, and statistical analysis was not performed. A study on EBA used several techniques, including cloning, IFA, Western blotting, and electron microscopy to identify the epitope as the NC1 domain on type VII collagen. Numbers of cases and controls and the use of statistics were not reported.

3.3.2 | Endocrine (n = 3)

Endocrine studies included 2 on hypothyroidism[120,121] and 1 on lymphocytic thyroiditis, which studied dogs with increased antithyroglobulin antibodies but normal thyroid hormone concentrations.[122] The hypothyroidism studies were small[120,121] and included ≤10 cases and ≤5 controls. Both studies of hypothyroidism characterized the autoantibody epitopes for thyroglobulin using Western blots on tryptic peptides. One study found epitopes on 31, 32.5, and 43 kDa-sized fragments.[121] The other study concluded that epitopes were conformationally dependent and were not uniform across individuals.[120] Statistical analyses were not reported for either study. The lymphocytic thyroiditis study was midsized with 45 cases and 20 controls. A competition RIA was used to determine that a proportion of antithyroglobulin autoantibodies also recognized epitopes on T3. Statistical tests of significance were reported.

3.3.3 | Other (n = 2)

Of the remaining 2 epitope studies, 1 sought to characterize epitopes in 11 cases of SLE in German Shepherds. Autoantibodies to glycosylated heterogeneous nuclear ribonucleoprotein (hnRNPG) were found in all cases. Cloning and Western blotting identified an epitope characterized as a 33-amino acid sequence in the central region. Most cases (10/11) had autoantibodies to this epitope. A second epitope was discovered using immunoprecipitation of radiolabeled hnRNPG. Fewer cases were positive for this epitope, an RNA binding domain near the N-terminus. Statistical analyses were not reported in this study.[124] The other study was midsized, and used competitive RIA to evaluate 35 cases of myasthenia gravis.[123] Sixty-eight percent of these cases were anti-AChR autoantibody positive. The main epitope was identified on the alpha subunit distal from the acetylcholine binding site. This epitope also has been characterized for myasthenia gravis in humans and is known as the MIR (main immunogenic region). Other epitopes on the beta and gamma subunits of AChR were identified but were less prevalent than MIR. Statistical analyses were not reported.

3.4 | Technique studies (n = 34)

Thirty-four manuscripts were classified as technique papers, encompassing 5 organ systems: rheumatic (n = 10),[125-134] hematologic (n = 9),[135-143] dermatologic (n = 8),[144-151] endocrine (n = 5),[152-156] and gastrointestinal (n = 2; Tables S4 and S6).[157,158] Each comparison between techniques in a single study occupies a single row in Table S4. Several papers compared multiple techniques, and therefore these papers have multiple entries in Table S4.[126,127,131]

Statistics were not routinely used to evaluate the relationship between tests that were directly compared. Differences in detection of false positives and negatives, and sensitivity and specificity were sometimes discussed, and when reported, they are included in the conclusions column of Table S4. As previously discussed, reporting percentage positive and negative case agreement might have been appropriate, but such calculations were not observed. Kappa coefficients can be another appropriate statistic used to measure correlation, and sometimes were reported (Table S4).[159]

3.4.1 | Rheumatic (n = 10)

Ten studies on rheumatic disease investigated SLE (n = 6)[128,130-134] and RA (n = 4).[125-127,129] All SLE studies detected ANA. One study specifically detected antihistone antibodies,[134] and 2 also detected antiribonucleoprotein autoantibodies.[131,132] Objectives of rheumatic studies included optimization to the gold standard, IFA[128,130] (also referred to as indirect immunofluorescence [IIF-ANA]) or comparisons of IFA to alternate techniques.[131] Optimizations to IFA centered on the impact of the detection substrate. For example, 1 study using human HEp-2 cell substrate resulted in superior specificity and improved interpretability of the ANA staining pattern, in contrast to the historically used rodent organ substrate.[130] In a second study, simultaneous testing of multiple substrates followed by a Bonifacio-inspired multiparameter scoring system showed improved precision over standard IFA.[128] However, the authors cautioned that this approach was intended to supplement existing methods. Other SLE studies focused on different techniques such as ELISA,[131] line blots,[132,133] and flow cytometry[134] to identify specific nuclear antigens in cases already identified as IIF-ANA positive. However, the authors suggested that these techniques should not replace IFA, but rather supplement methods to classify SLE according to specific ANA target antigens, thereby allowing for increased diagnostic detail and more precise disease monitoring.

Studies on RA all detected rheumatoid factor (RF). Most papers employed a hemagglutination assay, such as the Rose Waaler test (RWT)[125-127] or latex fixation test (LFT).[126,127] Studies comparing performance among different types of hemagglutination tests showed poor correlation for sensitivity and specificity.[125,126] Alternate techniques such as the gel precipitation test (GPT)[126] and ELISA[127] also were compared to hemagglutination tests. Sometimes results were correlated,[127] other times the alternate tests resulted in false positives.[126,127] For example, 1 study compared GPT and LFT on serum vs synovial fluid. Gel precipitation was shown to be better at detecting RF in serum than LFT, but produced more false positive results.[126] Latex fixation was better at detecting RF in synovial fluid than both GPT and the Rose Waaler test.[126] Also, 2 studies used ELISA.[127,129] One compared ELISA to LFT and RWT.[127] Although RWT correlated highly with ELISA, LFT resulted in many false positives in control samples. However, heat inactivation of the serum before testing eliminated the false positives. The other ELISA study introduced human RF-positive serum as a novel reference standard.[129] The authors suggested doing so could allow for comparison of results across laboratories.

3.4.2 | Hematologic (n = 9)

Our synthesis included 9 hematologic studies. Seven investigated IMHA[135-137,140-143] and 2 investigated ITP.[138,139] All IMHA studies detected antierythrocyte autoantibodies. The Coomb's test, the historical standard for detection of antierythrocyte antibodies, was reported to be prone to false negatives.[137] In every comparison of the Coomb's test to alternate detection techniques, the alternate technique showed superior sensitivity.[135-137,140,143] These included 1 study that used papain low ionic strength solution (LISS),[136] 2 studies used ELISA,[135,143] and 1 used flow cytometry.[140] The ELISA was reported to have additional advantages over the Coomb's test, including improved ability to quantify results and less use of reagents. The advantages of flow cytometry included speed and objectivity in interpreting results. Both ITP studies evaluated antiplatelet autoantibodies. One compared flow cytometry to IFA.[138] The other compared direct ELISAs to indirect ELISAs.[139] No statistical difference was found for the sensitivity between flow cytometry and IFA. However, the direct ELISA was shown to have higher sensitivity (94%) and lower specificity (34%) than indirect ELISA (sensitivity 62%, specificity 80%).

3.4.3 | Dermatologic (n = 8)

Eight dermatologic studies were included in our analysis. Six related to pemphigus or pemphigus complex,[144-147,149,151] and 2 evaluated subepidermal blistering diseases including EBA.[148,150] Consistent with the biomarker studies, specific antigens were mostly unidentified.[146,147,149] Autoantibodies were defined by the morphological features of the skin that they targeted, such as epidermal stratum spinosum,[151] and intercellular or basement membrane.[144,145] However 1 study identified anticollagen IV autoantibodies.[148] All pemphigus complex studies either made comparisons between IHC and IFA,[144,145,151] or compared modifications to IFAs.[146,147,149] Most comparisons between IFA and IHC showed correlation between the test results.[144,151] However, 1 study showed that immunobridge peroxidase IHC was slightly more sensitive at detecting autoantibodies in pemphigus foliaceus but not in pemphigus vulgaris.[145] Other studies evaluated different substrates. For example, bovine esophagus yielded the best sensitivity and specificity to detect circulating autoantibodies using IFA out of 5 different substrates (bovine esophagus, bovine nose, bovine tongue, monkey esophagus, and canine nose).[146] Another study using IFA compared live MCA-B1 keratinocytes to canine lip and bovine esophagus. Live cells had the lowest sensitivity, and bovine esophagus had the highest sensitivity.[147] Lastly, the ability of direct vs indirect IFA to detect pemphigus foliaceus was compared. Indirect IFA had higher correlation with histopathology results than did direct IFA.[149]

The 2 studies on subepidermal blistering disease both studied EBA,[148,150] 1 of which also included cases of mucous membrane pemphigoid and bullous pemphigoid.[150] In the study on EBA only, immunoperoxidase IHC collagen IV staining on formalin-fixed, paraffin-embedded skin substrate was introduced to augment standard histopathologic analysis owing to its low cost and relatively high sensitivity.[148] The study that evaluated multiple subepidermal blistering diseases compared both intact and salt split preparations of 4 substrates for autoantibody detection: canine tongue, canine lip, canine dorsal haired skin, and ventral haired skin. Canine lip, either intact or salt split, had the lowest background staining, highest specificity, and was convenient to use because it was easy to section.

3.4.4 | Endocrine (n = 5)

All 5 endocrine studies sought to detect antithyroglobulin (TgAb) autoantibodies in hypothyroidism.[152-156] A study comparing the performance of chromic chloride hemagglutination (CCH), IFA, and ELISA determined that IFA had the highest sensitivity.[152] However the authors argued that the ELISA's convenience was well-suited for an early screening test or as a supplemental test. Nevertheless, they recommended against using any of these techniques for routine diagnosis because none exhibited an ideal combination of sensitivity, reliability, and simplicity. Two subsequent studies compared CCH and glutaraldehyde chloride hemagglutination (GCH).[153,154] Superior sensitivity was shown for CCH, but GCH had the practical advantage of being able to be used with sheep red blood cells that had undergone long-term freezing,[153] as well as increased cellular stability from glutaraldehyde fixation.[154] In the remaining 2 studies, an enzyme immunoassay (EIA, a technique similar to ELISA)[156] and an ELISA[155] were used. The ELISA study showed high reproducibility in detecting TgAb with high sensitivity and specificity. The EIA study showed high correlation with IFA. The EIA also had the advantage of detecting antibodies against T3 and T4, in addition to TgAb.

3.4.5 | Gastrointestinal (n = 2)

Two studies on gastrointestinal disease were included in our analysis. A study from 2002 sought to introduce IFA to detect pANCA in cases of IBD.[160] However, IFA was not compared to any other detection methods. Dogs with IBD were successfully differentiated from both dogs with diarrhea unrelated to IBD or healthy dogs. Specificity was reported as 0.82 to 0.95. In a later study, a commercially available human granulocyte mosaic biochip IIF assay was used to detect pANCA and cANCA in dogs with chronic enteropathies.[157] Although agreement of the results with samples previously tested using the canine granulocyte ANCA assay was strong (kappa = 0.77), the assay designed for humans was less sensitive (sensitivity, 0.61; 95% CI, 0.45-0.75). Although the sensitivity was not specifically reported for the canine granulocyte assay, it identified 3 additional positive samples that the assay designed for humans missed. Negative samples were fully concordant between the 2 assays. The authors emphasized the convenience of the assay designed for humans and noted several advantages: interpretation of results is less subjective, assay is faster, and assay has higher throughput.[157]

4 | DISCUSSION

Our aim was to provide a resource for the veterinary community to understand prior research supporting the use of autoantibody biomarkers for autoimmune diseases in dogs. We adopted methodology from systematic reviews for the following documented benefits: (1) a reduction in study selection bias; (2) the ability to aggregate and summarize data according to predefined criteria on study design and outcomes reporting; and (3) the ability to identify methodological challenges in the existing literature.[161,162] Prior reviews of autoantibody biomarkers in autoimmune diseases of dogs have focused on specific autoantibodies, such as ANA,[163,164] without regard to disease, or have discussed autoantibody biomarkers as part of a larger review of a specific disease.[51,52] In contrast, we conducted a comprehensive summary of the primary literature on autoantibody biomarker use in spontaneous cases of autoimmune disease in dogs.

We observed considerable heterogeneity among the studies included in our analysis. Autoimmune disorders include both organ-specific diseases such as hypothyroidism and pemphigus foliaceus, as well as systemic diseases affecting multiple organ systems such as SLE. Fundamental differences among these diseases translate into a range of study characteristics. For example, accessibility of substrate tissues and throughput of detection techniques influence study design, particularly in terms of sample size and the frequency of performing studies. Use of higher risk procedures such as a spinal tap to obtain autoantibodies from small volumes of CSF vs a venipuncture to collect circulating serum autoantibodies necessitates markedly different study designs. Accordingly, we observed differences in many study characteristics such as proportions and numbers of cases and controls, phenotyping standards, autoantibody detection techniques, outcomes reporting, and use of statistical tests.

We also observed different objectives for the studies evaluated, with 3 distinct categories identified: biomarker, epitope, and technique. The majority were conducted to discover or validate the association of autoantibodies or antibody-bound cells or tissues with a specific disease (biomarker studies). Fewer studies focused on characterizing the antigenic epitope (epitope studies). The third group had nearly the same number of studies as the biomarker group and sought to improve the technical aspects of detection for specific autoantibody biomarkers (technique studies). Data reported in studies from these different groups were not uniform. In order to organize and analyze this heterogeneous set of studies in a meaningful way, they were organized by shared objective and further analyzed by organ system and disease. Making direct comparisons among studies with similar objectives that reported similar types of data maximized our ability to draw useful conclusions. Further grouping by organ systems allowed us to extract meaningful conclusions for specific diseases.

Defining attributes of the biomarker studies included: most were small, meaning cohorts of ≤30 cases were analyzed, most did not use statistics to evaluate their findings, and most used a single detection technique. One of 4 detection techniques (ELISA, Western blot, IFA, and RIA; Table S5) were used in most studies. Twenty-six studies were excluded from the final analysis for quality concerns, with the most common reason being failure to include negative controls (19/26). Studies on endocrine disease were overrepresented compared to other organ systems. This outcome may be because endocrine diseases are common in dogs, sample tissue (blood) is easily obtained, and testing methods are scalable.

Findings from epitope studies were mostly similar to those for biomarker studies. Most epitope studies were small, statistical analysis was uncommon (only 1 study used statistics to analyze results),[122] and every study used at least 1 of the 4 most common detection methods mentioned in the discussion of biomarker studies. However, the use of cloning was a feature unique to epitope studies (4/9).[117-119,124] Cloning was used to express antigenic proteins or peptides for use in immunoassays[119,124] and various research tools.[117,118] Another difference is that studies of dermatologic disease were the most represented (n = 4) followed by endocrine studies (n = 3). An important finding for epitope papers was that epitopes showed variation in terms of the number found on a given antigen,[119-121,123,124] whether they were conformational[119,120,123] or linear,[121] and whether they cross-reacted with epitopes on a different antigen.[122]

Certain trends also can be observed for technique studies. Nearly all technique studies focused on 5 organ systems: rheumatic, hematological, dermatologic, endocrine, and gastrointestinal. This emphasis may reflect the organ systems most commonly affected by autoimmune diseases in dogs. Secondly, innovation resulting in old techniques being supplanted by new techniques has been limited. For example, endocrine studies showed a shift from hemagglutination assays to ELISAs.[152,155,156] However, technical improvements for the other organ systems (eg, rheumatic and dermatologic), have been less transformative. For example, dermatologic studies showed that IHC and IFA remain the enduring standards, with modifications to these techniques being the focus of these studies. Two techniques (ELISA[131] and line blot[132,133]) were explored as alternatives to IFA in SLE studies. However, the authors of these studies concluded that these newer techniques were best used to complement IFA. Lastly, studies on IMHA showed flow cytometry to be more sensitive, less expensive, and more objective than the gold standard Coomb's test,[140,141] but this method of identifying antierythrocyte antibodies is not widely used in clinical practice.

Our study had several limitations. Portions of the narrative around the initial discovery of certain autoantibody biomarkers may have been lost because studies of induced disease and case reports were excluded. Ideally, results from these types of studies would be followed up in larger cohorts in cases of spontaneous disease, which we would have captured in our literature review. We also acknowledge that the search terms we used may have limited the studies that were captured for inclusion. However, tests of other search term combinations did not yield additional relevant studies.

It may be argued that our quality inclusion criteria were too lenient. Had we adopted more rigorous criteria as found in formal meta-analysis quality assessment tools, very few studies would have

qualified.[53,63,64] Given that few veterinary studies include many of these characteristics, we opted for more inclusive variables. Nonetheless, the difficulty in applying many of these formal meta-analysis criteria to the studies of dogs we evaluated suggests there is room for increased methodological rigor in future work.

Barriers to adopting increased methodological rigor may arise from challenges unique to research in veterinary settings. For example, in comparison to human medicine, there is a lower availability of tissue biobanks[165] and large national or centralized study centers.[166] Additionally, less overall funding for studies in companion animals may pose a constraint to pursuing large studies that employ expensive or contemporary innovative technologies.

Our analysis provides a summary of research on the use of autoantibody biomarkers in autoimmune diseases of dogs, highlighting opportunities to improve future research. The number of studies omitting negative controls suggests that standardizing the incorporation of negative controls could be an important step toward increased rigor. Matching negative controls to cases by age, sex, and breed may further strengthen the methodological approach. Statistical comparisons of autoantibody positivity between cases and controls also should be considered when appropriate. Furthermore, using multiple detection techniques to discover autoantibodies can improve results.[167,168]

Our study serves as a reference to understand the contributions of primary research regarding the development of specific autoantibody biomarkers in autoimmune diseases of dogs. Future research that adopts additional methodological rigor has the potential to increase the repertoire of autoantibody biomarkers to detect autoimmune diseases in dogs and expand their utility for clinicians and patients alike.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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