Editorial introductions

Current Opinion in Rheumatology was launched in 1989. It is one of a successful series of review journals whose unique format is designed to provide a systematic and critical assessment of the literature as presented in the many primary journals. The field of Rheumatology is divided into 15 sections that are reviewed once a year. Each section is assigned a Section Editor, a leading authority in the area, who identifies the most important topics at that time. Here we are pleased to introduce the Journal's Section Editors for this issue.

SECTION EDITORS

John Varga

John Varga MD is the John and Nancy Hughes Distinguished Professor at Northwestern University's Feinberg School of Medicine in Chicago, Illinois, USA.

Coming to the United States as a refugee, Dr Varga attended Columbia University, and obtained his medical degree from New York University. Following Rheumatology Fellowship in Boston, USA, he pursued post-doctoral research training with Professor Sergio Jimenez at the University of Pennsylvania, USA. In 2004, he joined the faculty at Feinberg School of Medicine, USA, where he founded and directs the integrated Scleroderma Program. His research focuses on the pathogenesis and treatment of scleroderma and fibrosis, and bridges clinical and laboratory-based investigation.

Dr Varga has mentored over 20 trainees, several of whom are now independent academic investigators. He is the author of more than 350 original articles, 40 book chapters and four books. His research has been continuously funded by the National Institutes of Health. He is an elected member of the Association of American Physicians, and is Master of the American College of Rheumatology.

Anna Ghirardello

Anna Ghirardello, DSc, PhD, graduated in Biological Sciences from the University of Padova, Italy where she also qualified in Clinical Pathology. She received her PhD degree and post-doctoral fellowship at the University of Padova. She is Biologist at the University Hospital of Padova. Her main fields of interest include the pathogenesis and laboratory diagnostics of connective tissue diseases, focused primarily on systemic lupus erythematosus and idiopathic inflammatory myopathies. Dr Ghirardello is a member of the Italian Society of Rheumatology (SIR), Council member of the Italian Interdisciplinary Forum for the Research in Autoimmune Diseases (FIRMA), and member of the EuroMyositis Registry. She has authored over 120 ISI publications.

Andrea Doria

Andrea Doria is Professor of Rheumatology and Head of the Unit of Connective Tissue Disease and Rare Rheumatic Diseases, Division of Rheumatology, Department of Medicine, University of Padua, Italy. He is Head of the Rheumatology Unit, University of Padua and Director of Rheumatology postgraduate medical school.

Professor Doria received his medical degree and qualification in Rheumatology from the University of Padua. He was Council member of the Italian College of Rheumatology (CRO) between 1999 and 2005 and a Council member of the Italian Society of Rheumatology (SIR) from 2007 to 2010 and from 2013 until now. He is also a member of American College of Rheumatology (ACR).

Professor Doria has organised over ten international conferences on autoimmunity and was involved as ''expert'' in the EUropean League Against Rheumatism (EULAR) Standing Committee for the development of clinical and therapeutic recommendations: (1) EULAR recommendations for the management of systemic lupus erythematosus (SLE)—Assessment of the SLE patient (2008–2009); (2) EULAR recommendations for the management of SLE Part II—Neuropsychiatric disease (2008–2009); (3) Joint EULAR and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis (2012). Professor Doria is a member of the Lupus Academy Steering Committee and co-Chaired the 4th Annual Meeting held in Rome 27th February to 1st March 2015. He was the chair of the 10th European Lupus Meeting, held in Venice (Italy) 5–8th October 2016.

Professor Doria is on the Editorial Boards of several rheumatology and immunology journals, including *Lupus, Autoimmunity, Clinical and Experimental Rheumatology, Autoimmunity Reviews, Journal of Autoimmunity, Experimental Biology and Medicine, Rheumatology Reports, Journal Autoimmunity Highlights and Reumatismo* (the official journal of Italian Society of Rheumatology).

He has authored over 250 ISI publications on SLE and other connective tissue diseases. These include clinical studies describing new manifestations or subgroups of autoimmune disorders, prognostic risk factors, diagnostic tests and therapeutic interventions, as well as immunochemical studies that evaluate autoantibodies, epitopes and complementary epitopes of autoantigens. In addition, he has authored and co-authored three books, over 90 book chapters and conference proceedings, and over 500 abstracts for national and international conferences.

Professor Doria has long-standing experience of the clinical management of patients with connective tissue diseases. The Unit in which he works is a tertiary referral rheumatology centre, within Italy, for the diagnosis and management of patients affected with systemic connective diseases. In addition, he has expertise in the management and follow-up of pregnant patients with systemic rheumatic diseases. Professor Doria has also trained over 30 students in Rheumatology.

Mariele Gatto

Dr Mariele Gatto, MD, is Rheumatologist and currently attending her last year of PhD course in Clinical and Experimental Sciences at Padova University, Italy. Dr Gatto performs both clinical activity and laboratory research at Padova University, with a major focus on development and treatment of systemic lupus erythematosus (SLE) and other connective tissue diseases. So far, her *Cursus Studiorum* was carried out between Padova University and other foreign institutions where Dr Gatto could acquire and improve research skills, particularly at Zabludowicz Center for Autoimmune Diseases in Tel Aviv, Israel and at Charité Hospital in Berlin, Germany, with a major focus on B cells in lupus.

Dr Gatto is actively involved in patient recruitment and follow-up within randomized controlled trials, investigating novel therapeutics in SLE, inflammatory myositis and Sjogren syndrome, as well as in training of younger fellows and students at Padova Medical School.

Dr Gatto has attended several national and international meetings and symposia as speaker and was awarded so far with four prizes (CORA young researcher award 2015; prize of the Italian Society of Rheumatology 2016; CORA award 2019; DIMAR 2019 award at Medicine Department of Padova university) for best abstract presentation.

She is author or co-author of 58 publications available in PubMed.

Amr H. Sawalha

Dr Amr H. Sawalha, MD, is Professor of Pediatrics, Medicine, and Immunology at the University of Pittsburgh School of Medicine. He holds the Vincent Londino Endowed Chair and is Director of the Division of Rheumatology at Children's Hospital of Pittsburgh. Dr Sawalha is Director of the Lupus Center of Excellence that spans the clinical and research enterprises of the University of Pittsburgh Medical Center and the University of Pittsburgh. He is a graduate of Jordan University of Science and Technology. He completed his residency training at the University of Oklahoma Health Sciences Center and his

fellowship in Rheumatology at the University of Michigan. Dr Sawalha was on faculty at the University of Oklahoma Health Sciences Center and the Oklahoma Medical Research Foundation before he returned to Michigan in 2012, where he was Professor of Internal Medicine and Marvin and Betty Danto Research Professor of Connective Tissue Research, Director of the NIH-funded rheumatology training grant, and Associate Director of the NIH-funded University of Michigan Basic Autoimmunity Center of Excellence. He moved to the University of Pittsburgh in the Spring of 2019.

Dr Sawalha's research program focuses on elucidating genetic and epigenetic contribution to the pathogenesis of systemic autoimmune and inflammatory diseases. His team applies state-of-the-art genomic, epigenomic, and bioinformatics methodologies, and subsequent functional studies using both in vitro and in vivo systems to identify and characterize genetic loci and pathways involved in the pathogenesis of immune-mediated diseases.

Dr Sawalha has authored over 170 manuscripts, book chapters, and review articles. He is on the editorial board of several journals in his field and serves as a reviewer for numerous scientific journals in rheumatology, genetics, and immunology. He is a member of the Medical Scientific Advisory Council of the Lupus Foundation of America and member of the Vasculitis Foundation Medical and Scientific Advisory Board. He currently serves as Chair of the Abstract Oversight Committee of the American college of Rheumatology. Dr. Sawalha has received numerous awards including the Edmund L Dubois, MD, Memorial Lectureship Award from the American College of Rheumatology in recognition for his work in lupus, the Henry Kunkel Young Award from the American College of Rheumatology, and has been elected as member of the American Society for Clinical Investigation.

Raynaud's phenomenon and digital ulcers: advances in evaluation and management

Ariane L. Herrick

Purpose of review
The aim of this review is to give an update on advances in evaluation and management of systemic sclerosis (SSc)-related Raynaud's phenomenon and digital ulceration, focusing on reports from the last 18 months. The increasing recognition of the huge impact of Raynaud's phenomenon and of digital ulceration on the everyday lives of patients with SSc has sparked enthusiasm internationally to develop better outcome measures and better treatments, and so a review is timely.

Recent findings
There have been recent advances in the development of patient reported outcome instruments [e.g. the Hand Disability in Systemic Sclerosis-Digital Ulcers (HDISS-DU) instrument] and also in noninvasive imaging techniques, including thermography and laser Doppler methods. Improved outcome measures will facilitate future clinical trials, both early phase proof-of-concept and later phase trials. New insights have been gained into mechanisms of action and methods of administration of 'conventional' therapies, for example phosphodiesterase inhibitors and intravenous prostanoids. New treatment approaches are being investigated, including topical and procedural therapies.

Summary
Clinicians can look forward to seeing these advances translating into clinical benefit over the next 5 years. To help ensure this, they should strive whenever possible to recruit patients with SSc-related digital vasculopathy into observational studies and clinical trials.

Keywords
digital ulcers, outcome measures, Raynaud's phenomenon, systemic sclerosis, treatment

INTRODUCTION

Almost all patients with systemic sclerosis (SSc) experience Raynaud's phenomenon and in the order of 50% will develop at least one digital ulcer (Fig. 1) during the course of their disease [1▪]. Recent studies have confirmed that in patients with SSc, both Raynaud's phenomenon and digital ulcers can have a very major impact on everyday activities or quality of life [2,3].

Since this topic was last reviewed in 2016 [4], progress has been made in the quest for improved outcome measures and better, safer treatments. This review outlines recent advances in the evaluation and treatment of Raynaud's phenomenon and digital ulcers, mainly in the context of the patient with SSc and focusing on reports in the last 12–18 months (since October 2019). In each subsection, Raynaud's phenomenon and digital ulcers will be considered separately, always remembering that both are part of the spectrum of SSc-related digital vasculopathy and therefore certain common principles apply.

EVALUATION

Expert consensus is that annual assessment of patients with SSc should include Raynaud's phenomenon and digital ulceration [5]. In the clinic, the severity of digital vasculopathy is judged by the frequency, severity and duration of Raynaud's attacks, and by whether digital ulcers (or critical ischaemia) have developed. In research studies, precise measures are required, in order to assess disease progression and/or treatment response.

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KEY POINTS

- The treatment of SSc-related Raynaud's phenomenon and of digital ulceration remains unsatisfactory for many patients: therefore, new approaches to therapy are required.
- Developments in new patient-reported outcome measures for SSc-related Raynaud's phenomenon and for digital ulceration are likely to facilitate future Phase II and Phase III clinical trials.
- Advances in noninvasive imaging will facilitate early phase, proof-of-concept studies.
- New treatment approaches currently under investigation include topical therapies and procedural treatments including botulinum toxin injections.
- Long-term studies are needed of combination therapies and of potential vascular remodelling therapies.

Raynaud's phenomenon

The Raynaud's Condition Score (RCS) [6] measured on a scale of 0–10, Raynaud's attack frequency and attack duration tend to be the outcome measures currently most used in multicentre later phase clinical trials. A concern about patient-reported outcome (PRO) measures is that although highly feasible, by their nature they are subjective, and clinicians recognize the limitations of the RCS [7]. Studies of Raynaud's phenomenon have been plagued by a marked placebo response [8,9], which may at least in part be attributable to their subjective end-points. Challenges include different patient-related factors: RCS is influenced, for example, by coping strategies and catastrophizing [10▪]. In recent years, Pauling *et al.* [11] have highlighted the multifaceted patient experience of Raynaud's phenomenon and have undertaken several studies 'setting the scene' for the development of a PRO instrument, which will capture this 'broad' patient experience of Raynaud's phenomenon [12,13]. A key factor in developing this new instrument has been the high level of patient involvement throughout [12].

For early phase, proof-of-concept studies, noninvasive imaging techniques for measuring finger blood flow [14▪] are attracting increasing interest and may be the way forward. Recent advances have been made with both infra-red thermography (which measures surface temperature and which therefore gives an indirect measure of blood flow) and laser Doppler techniques [single point laser Doppler flowmetry, laser Doppler imaging and laser speckle contrast imaging (LSCI)]. Pauling *et al.* [15▪▪] recently conducted a systematic review of studies incorporating laser Doppler methods, and Melsen *et al.* [16▪] reviewed laser Doppler flowmetry. As with thermography [17,18], there has been a lack of standardization in imaging protocols and methodologies across laser Doppler studies. It is worth highlighting that of the 29 studies reviewed by Pauling *et al.* [15▪▪], all but one were single-centre, perhaps underscoring the current 'individuality' of protocols and studies. Therefore, an important step forward has been the validation, in a multicentre study of 159 patients from six UK centres [19], of a standard cold challenge test, measuring blood flow response by both thermography and LSCI. Thermography has the advantage of being easier to use and less expensive than LSCI, especially if mobile phone thermography can become established. Finger temperature measurements using mobile phone

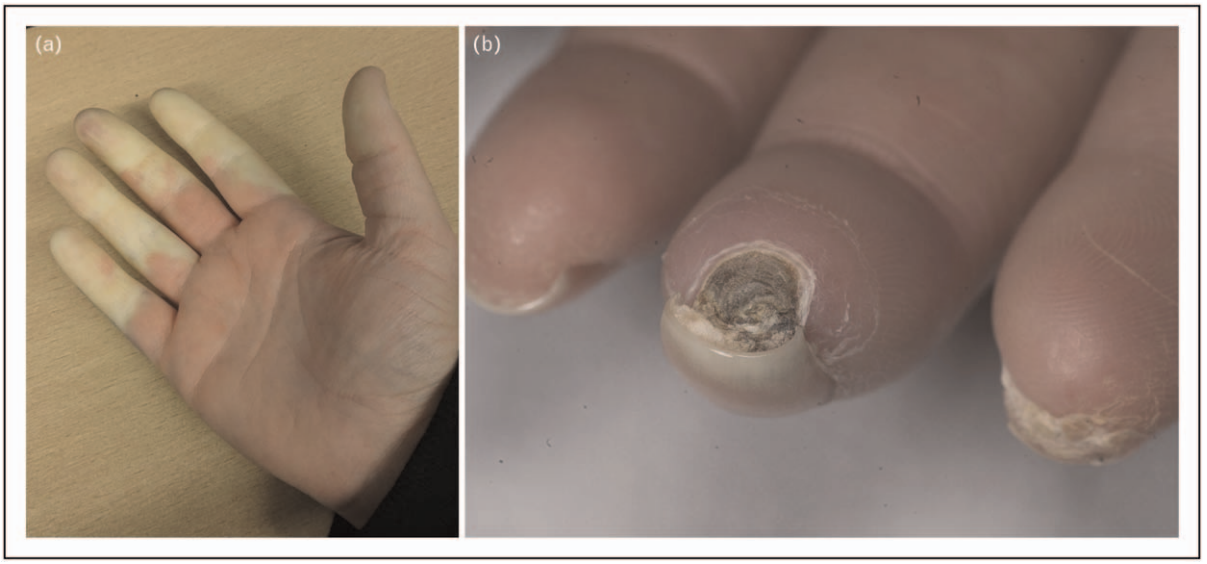

FIGURE 1. Raynaud's phenomenon, showing pallor phase (a) and an example of digital ulceration (b).

thermography have recently been shown to correlate with those obtained using the gold standard (but more cumbersome) thermocouple technique [20▪]. However, mobile phone thermography is not yet validated for use in an ambulatory setting.

More sophisticated methods of studying the finger vasculature are being developed, including optical coherence tomography, multispectral imaging and photoacoustic imaging [14▪]. Photoacoustic (optoacoustic) imaging is particularly exciting because in addition to measuring oxygenation [21,22▪], it provides three-dimensional visualization of nailfold capillaries and a means to measure capillary volume [23▪].

Digital ulcers

Although clinicians recognise the need to evaluate digital ulcers [24], defining and measuring digital ulcers and ulcer healing is challenging [25]. In 2017, a World Scleroderma Foundation definition for SSc-related skin ulcers was proposed [26]. As with Raynaud's phenomenon, there is recognition that the impact on the patient is multifaceted [27▪,28▪] with increasing interest in PRO instruments. The Hand Disability in Systemic Sclerosis-Digital Ulcers (HDISS-DU) PRO instrument [29▪▪], developed in accordance with US Food and Drug Administration Guidance, has been shown to have excellent internal consistency reliability (Cronbach's alpha 0.97–0.98) and test-retest reliability (intraclass correlation coefficients > 0.80) and is sensitive to change: it therefore holds promise as a new outcome measure. Other outcome measures are also being developed/researched including a composite score (the Digital Ulcer Clinical Assessment Score) [30].

Clinical photography allows measurement of ulcer size [31] and if photographs are taken by patients themselves using mobile phones [32] then this allows very frequent assessment in an ambulatory setting and, by extrapolation, potentially a more accurate timing of 'ulcer healing' than has hitherto been possible. Different noninvasive imaging modalities have been proposed to assess digital ulcers, including ultrasound [33,34], LSCI and laser Doppler imaging [35▪,36]. Blood flow is usually reduced at the site of ulceration but can be increased if the ulcer is infected [35▪]. As with Raynaud's phenomenon, noninvasive imaging could be a way forward in early phase studies.

TREATMENT

Although a recent study suggested that the majority of patients prescribed a calcium channel blocker, an endothelin receptor antagonist or sildenafil continue on these drugs after 4 years [37▪], many patients with SSc experience only minimal (if any) benefit from current treatments for their digital vasculopathy or discontinue these due to inefficacy. A recent systematic review and network meta-analysis [38▪▪] concluded that the only drugs for which there was some evidence of efficacy in secondary Raynaud's phenomenon were calcium channel blockers and phosphodiesterase type 5 (PDE5) inhibitors, and even for these the evidence level was low. New approaches to treatment for both Raynaud's phenomenon and digital ulceration are required.

Key points underpinning treatment (shown diagrammatically in Fig. 2) are that:

(1) In Raynaud's phenomenon, the normal balance between vasoconstriction and vasodilation is altered in favour of vasoconstriction, therefore effective therapies should either reduce vasoconstriction or increase vasodilation.
(2) In SSc-related Raynaud's phenomenon, vasospasm occurs 'on top of' structural vascular abnormalities (not present in primary Raynaud's phenomenon) at the level of both the digital artery and the microcirculation, leading to severe vascular compromise and (often) irreversible tissue damage. Therefore, it is in patients with SSc that the need for effective treatments is greatest.
(3) The 'golden' question 'Can we prevent and/or reverse structural digital vascular disease?' is yet to be satisfactorily answered, but we may be edging a little nearer.

The focus of this review is recent advances: current treatment of Raynaud's phenomenon and digital ulceration are described fully elsewhere [39–43]. However, to orientate the reader, a stepwise approach to management is summarized in Fig. 3. Although there is some variation in practice in the use of PDE5 inhibitors, of bosentan and of intravenous (i.v.) iloprost, depending on centre/country/drug availability [44,45▪], all are now widely used by clinicians with an interest in SSc [46]. It should be noted that few of the treatments advocated in guidelines for the treatment of Raynaud's phenomenon or of SSc-related digital ulceration are licenced for these indications.

Raynaud's phenomenon – new insights

Different 'categories' of treatment are considered in turn.

Oral vasodilator therapies

One of the main advances reported in 2016 [4] was the increasing trend to prescribe a PDE5 inhibitor as

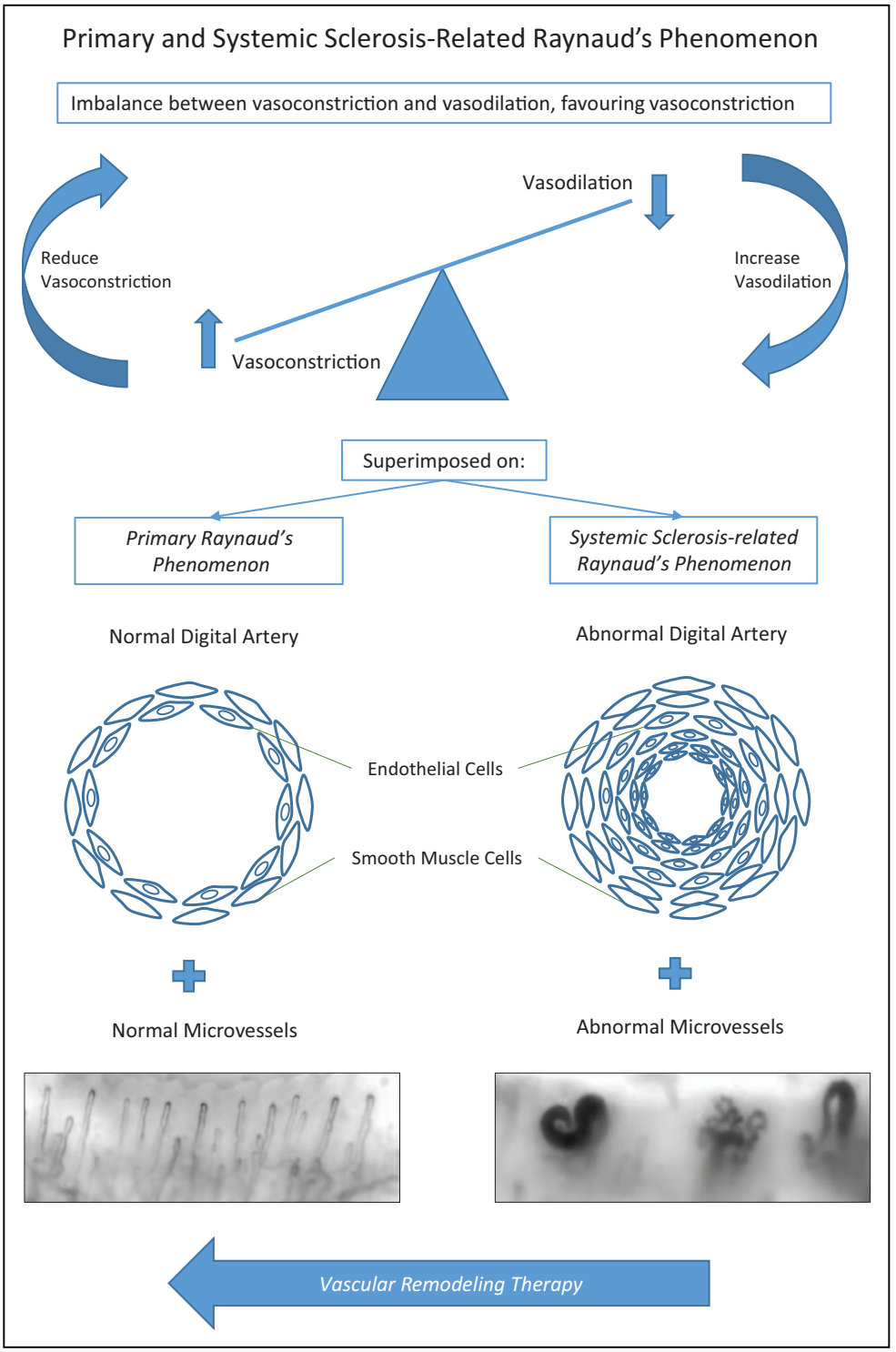

FIGURE 2. The pathophysiology of Raynaud's phenomenon and the broad principles of drug treatment. Drug treatment should be aimed at either reducing vasoconstriction or increasing vasodilation. In primary Raynaud's phenomenon, the vasculature is structurally normal, with normal digital arteries and normal nailfold capillaries (as visualized noninvasively by nailfold capillaroscopy). However, in SSc-related Raynaud's phenomenon, the digital arteries are abnormal, resulting in a narrow lumen, and the nailfold capillaries are abnormal: in the example shown, there is a reduced capillary density (compared to in primary Raynaud's phenomenon), and those capillaries which are present are enlarged. Treatments are needed that will remodel the abnormal digital arteries and abnormal microvessels.

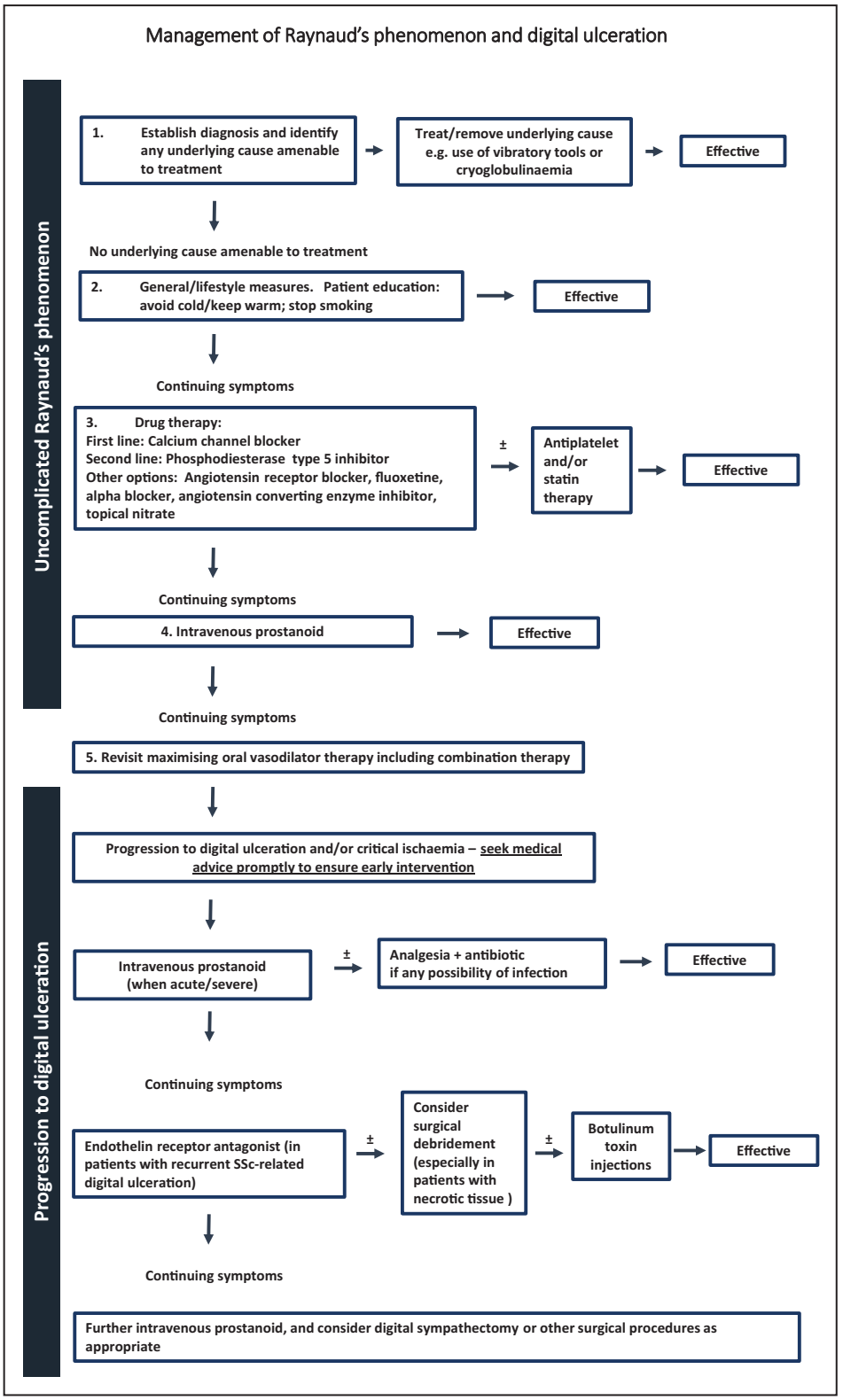

FIGURE 3. The principles of management of 'uncomplicated' Raynaud's phenomenon, and of systemic sclerosis related digital vasculopathy, which has progressed to digital ulceration.

second-line treatment following on from (and possibly combined with) a calcium channel blocker. Five years later, this trend continues. Roustit *et al.* [47] conducted a series of n-of-1 trials of on-demand sildenafil versus placebo, and although no definite benefit from active treatment was shown (probably

because of the heterogeneous response between patients), there was a 'high probability' that sildenafil administered in this way conferred benefit. This study was important not only because on demand sildenafil 'makes sense' and deserves further research, but because of the n-of-1 study design, which could be very applicable in future treatment studies of Raynaud's phenomenon. Sildenafil may have beneficial properties in addition to vasodilation: it may reduce oxidative stress [48▪], and could therefore have some disease-modifying effect. PDE5 inhibitors increase the effect of nitric oxide. The nitric oxide pathway can also be supplemented via L-arginine, as reviewed by Curtiss *et al.* [49], an approach warranting further research.

Prostaglandin analogues

Intravenous prostanoid therapy is well established for the treatment of severe Raynaud's phenomenon and digital ulceration. Recent reports have described different protocols [44,50,51▪], and a systematic review of i.v. iloprost highlighted the lack of consensus between experts regarding the optimal regime [52]. Barsotti *et al.* [51▪] in a retrospective single-centre study of 47 patients compared a continuous 48-h infusion with 3–5 h/day outpatient infusions (0.5–2.0 ng/kg/min) for 2 days: costs were comparable, but the outpatient infusions were less well tolerated. An interesting recent development is the suggestion that i.v. therapy can be administered outside the hospital setting [53,54]. A new suggested mechanism of action of iloprost [55▪▪], namely stabilization of adherens junctions, may explain the prolonged clinical benefit experienced by many patients.

Topical therapies

Raynaud's phenomenon is a local phenomenon of the extremities. Therefore, topical therapies, applied locally to the fingers (and possibly the toes) seem a logical way forward, minimizing the risks of systemic vasodilatory adverse effects. Although the use of topical glyceryl trinitrate for Raynaud's phenomenon was suggested as far back as 1951 [56], progress with topical therapies has been very slow. It is hoped that recent systematic reviews on topical nitrates [57,58] herald a wave of new interest, with several publications on topical therapies over the last 3 years [59–61,62▪,63–65,66▪]. Wasan *et al.* [62▪] reported a preclinical study investigating the formulation of a novel nifedipine cream stable to ultraviolet light. Topical PDE5 inhibitors are also being researched [63,64] and topical econazole nitrate, an antagonist of Transient Receptor Potential Melastatin 8 has been studied *in vitro* [61,66▪]. These are all important steps forward towards long-awaited clinical trials of topical treatments.

Botulinum toxin injections

Botulinum toxin injections are used by many clinicians in the treatment of refractory Raynaud's phenomenon and digital ulceration, and there have been several recent reviews [67,68▪,69▪,70]. A placebo-controlled randomized controlled trial (RCT) [71] in 40 patients with SSc failed to detect benefit as assessed by laser Doppler imaging, although there was some improvement in secondary endpoints, and it was suggested that the long disease duration in some patients contributed to the overall negative results. In the last 2 years, several small series including patients with SSc have reported beneficial effects, including in terms of pain and functional improvement [72▪,73,74,75▪]. These reports demonstrate how different investigators use different injection protocols and different outcome measures in different patient populations, making it difficult to make comparisons between studies. It has recently been suggested that a dorsal approach may be preferable to the more conventional palmar route [72▪], and that ultrasound-guided injections may improve accuracy of administration, reducing the risk of muscle weakness and potentially allowing smaller doses to be used [76]. Larger, randomized RCTs are required to establish the role of botulinum toxin injections.

Other potential therapies which have been investigated recently

Beetroot juice [77▪] was shown to have beneficial effects on the peripheral vasculature in patients with Raynaud's phenomenon. An exercise programme was associated with some reduction in Raynaud's-related pain in patients with SSc [78]. Procedural therapies continue to attract interest, with a recent randomized study suggesting benefit from galvanic current electrical stimulation [79▪]. Minimally invasive, single-port thoracoscopic sympathicotomy improved hand perfusion in a study of eight patients, one of whom had SSc [80▪]. These treatments all deserve to be further researched.

If will be interesting to see whether new therapies are identified via drug repurposing, as discussed by Putkaradze *et al.* [81▪].

Digital ulcers – new insights

There have been no large-scale treatment studies of digital ulcers reported in the last 18 months.

Oral vasodilator therapies

An analysis of baseline data (collected between March 2015 and November 2016) from the European multicentre DeSScipher study [82▪] reported

that calcium channel blockers were by far the most commonly prescribed drugs for digital ulcers and that very few patients were on combination therapy: among the 905 patients with current or previous digital ulcers, only 399 (44.1%) were on two or more vasoactive agents. Chang *et al.* [83▪] in a 24-week observational study (nonrandomized, open-label) reported that the number of new digital ulcers was reduced in the 49 patients treated with bosentan compared with in the 11 patients treated with a PDE5 inhibitor, but time to ulcer healing was similar between groups. These two studies [82▪,83▪] serve as a reminder that to inform optimal management of SSc-related digital ulceration we require studies comparing endothelin receptor antagonists and PDE5 inhibitors, and examining combination therapies.

Topical treatments

As for Raynaud's phenomenon, topical treatments make sense. Why give a vasoactive therapy systemically when the lesion is at the tip of a finger? Glyceryl trinitrate, applied directly on to SSc-related digital ulcers, increases blood flow and is well tolerated [84]. A recent retrospective study of 4 weeks' of tadalafil cream applied topically to the web spaces of fingers with new digital ulcers (the cream was not applied directly to the ulcers) [63] concluded that the treatment could promote ulcer healing and that randomized trials were required. Guigui *et al.* [85▪] reported a safety study of iontophoresis of treprostinil hydrogel, including five patients with SSc-related digital ulceration. The hydrogel was applied directly over the ulcer: efficacy studies are eagerly awaited.

Procedural including surgical treatments

Botulinum toxin injections have already been discussed under Raynaud's phenomenon, and have been advocated specifically for digital ulceration [68▪]. The main surgical treatments attracting interest in the last few years are digital sympathectomy and autologous fat grafting. Recently, Pignatti *et al.* [86] reported that digital ulcers refractory to other treatments healed in eight out of nine patients following autologous fat grafting: results of RCTs are required to establish the role of this approach to therapy.

New procedural approaches continue to be explored. Low-level light therapy [87], which has the advantage of being a local treatment, was feasible and well tolerated in a study of 14 digital ulcers in eight patients. Korsten *et al.* [88] reported a case of extensor digital ulcer healing with rheopheresis (a double-filtration plasmapheresis), the rationale being that blood viscosity may be increased in patients with SSc and that reducing viscosity may improve finger blood flow.

Remodelling the vasculature

As demonstrated in Fig. 2, our aspiration is to prevent progression of structural vascular disease and ideally reverse this. Bruni *et al.* [89▪] concluded from a retrospective study of 134 patients with SSc that prostanoids, sildenafil and angiotensin receptor blockers might confer some vascular protective effect, indicating that the time is right to embark on large-scale prospective studies of vascular protective/remodelling therapies.

CONCLUSION

Progress is being made in the quest for new treatments. Advances in noninvasive imaging should allow rapid throughput of early phase, small scale laboratory-based clinical trials, allowing prioritization of the most promising candidate drugs to be taken forward to expensive multicentre studies, thus allowing efficient use of resources. Advances in PRO measures, and in mobile phone technologies should facilitate these later phase trials. We can look forward to trials of new approaches to treatment (including topical therapies) over the next 5 years, and also to studies guiding us in optimizing our use of existing drugs, including trials of combination therapies.

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Conflicts of interest

A. Herrick has received consultancy fees from Boehringer-Ingelheim, Camurus, CSL Behring and Gesynta Pharma, speaker fees from Actelion and Janssen, and research funding from Actelion and Gesynta Pharma.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

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14. ■ Herrick AL, Dinsdale G, Murray A. New perspectives in the imaging of Raynaud's phenomenon. Eur J Rheumatol 2020; 7(Suppl 3):S212–S221.

This review gives an update on the different imaging methods, which can be used to assess Raynaud's phenomenon, with a focus on not only nailfold capillaroscopy, thermography and laser Doppler methods, but also covering emerging technologies including photoacoustic imaging.

15. ■■ Pauling JD, Hackett N, Guida A, Merkel PA. Performance of laser-derived imaging for assessing digital perfusion in clinical trials of systemic sclerosis-related digital vasculopathy: a systematic literature review. Sem Arthritis Rheum 2020; 50:1114–1130.

This systematic review includes detailed tables summarising 29 studies (26 of Raynaud's/finger blood flow, three of digital ulcers), all of which included a laser Doppler-based outcome: laser Doppler flowmetry (especially in earlier studies), laser Doppler imaging and laser speckle contrast imaging. It provides the reader with a comprehensive update of application of these techniques to the assessment of SSc-related digital vasculopathy.

16. ■ Melsens K, Van Impe S, Paolino S, *et al.* The preliminary validation of laser Doppler flowmetry in systemic sclerosis in accordance with the OMERACT filter. Sem Arthritis Rheum 2020; 50:321–328.

This review focused on 18 studies, which assessed fingertip blood flow in patients with SSc, and concluded that laser Doppler flowmetry is not yet fully validated as a tool to assess finger blood flow in this population.

17. Pauling JD, Shipley JA, Harris ND, McHugh NJ. Use of infrared thermography as an endpoint in therapeutic trials of Raynaud's phenomenon and systemic sclerosis (Review). Clin Exp Rheumatol 2012; 30(Suppl 71):S103–S115.
18. Murray A, Pauling JD. Noninvasive methods of assessing Raynaud's phenomenon. In: Wigley FM, Herrick AL, Flavahan NA, editors. Raynaud's phenomenon. A guide to pathogenesis and treatment. New York: Springer Science+Business Media; 2015. pp. 199–242.
19. Wilkinson JD, Leggett SA, Marjanovic EJ, *et al.* A multicentre study of the validity and reliability of responses to hand cold challenge as measured by laser speckle contrast imaging and thermography: outcome measures for systemic sclerosis-related Raynaud's phenomenon. Arthritis Rheumatol 2018; 70:903–911.
20. ■ Herrick AL, Heal C, Wilkinson J, *et al.* Temperature response to cold challenge and mobile phone thermography as outcome measures for systemic sclerosis-related Raynaud's phenomenon. Scand J Rheumatol 2021; doi: 10.1080/03009742.2021.1907926 [Epub ahead of print]

Although this was a small pilot study, the findings are of interest because measurements of finger skin temperature obtained in two different ways before and during i.v. iloprost (by continuous thermocouple recording and by mobile phone thermography) showed a strong estimated latent correlation, suggesting that mobile phone thermography shows promise as an outcome measure.

21. Eisenbrey JR, Stanczak M, Forsberg F, *et al.* Photoacoustic oxygenation quantification in patients with Raynaud's: first-in-human results. Ultrasound Med Biol 2018; 44:2081–2088.
22. ■ Daoudi K, Kersten BE, van den Ende CHM, *et al.* Photoacoustic and high-frequency ultrasound imaging of systemic sclerosis patients. Arthritis Res Ther 2021; 23:22.

This study demonstrates the applicability of photoacoustic imaging to the study of the digital vasculature in patients with SSc: oxygen saturation was reduced in patients with SSc.

23. ■ Nitkunanantharajah S, Haedicke K, Moore TB, *et al.* Three-dimensional optoacoustic imaging of nailfold capillaries in systemic sclerosis and its potential for disease differentiation using deep learning. Sci Rep 2020; 10:16444.

This study demonstrated the ability to visualize nailfold capillaries in patients with SSc in three dimensions using raster-scanning optoacoustic mesoscopy, which could therefore provide new insights into pathogenesis and measurement of SSc-related microvascular disease.

24. Blagojevic J, Bellando-Randone S, Abignano G, *et al.* Classification, categorization and essential items for digital ulcer evaluation in systemic sclerosis: a DeSScipher/European Scleroderma Trials and Research group (EUSTAR) survey. Arthritis Res Ther 2019; 21:35.
25. Hughes M, Tracey A, Bhushan M, *et al.* Reliability of digital ulcer definitions as proposed by the UK Scleroderma Study Group: a challenge for clinical trial design. J Scleroderma Relat Disord 2018; 3:170–174.
26. Suliman AS, Bruni C, Johnson SR, *et al.* Defining skin ulcers in systemic sclerosis: systematic literature review and proposed World Scleroderma Foundation (WSF) Definition. J Scleroderma Relat Disord 2017; 2:115–120.
27. ■ Hughes M, Pauling JD, Jones J, *et al.* Multicenter qualitative study exploring the patient experience of digital ulcers in systemic sclerosis. Arthritis Care Res 2020; 72:723–733.

This study includes a 'conceptual map' of five inter-related themes, which contribute to the patient experience of digital ulcers, and will inform the development of a new PRO instrument.

28. ■ Jones J, Hughes M, Pauling J, *et al.* What narrative devices do people with systemic sclerosis use to describe the experience of pain from digital ulcers: a multicentre focus group study at UK scleroderma centres. BMJ Open 2020; 10:e037568.

This study expanded on the work of ref. [27■], exploring the theme of pain and includes several direct quotes from patients, exemplifying the burden of digital ulcers.

29. ■■ Mouthon L, Poiraudeau S, Vernon M, *et al.* Psychometric validation of the Hand Disability in Systemic Sclerosis-Digital Ulcers (HDISS-DU) patient-reported outcome instrument. Arthritis Res Ther 2020; 22:3.

The development of the HDISS-DU included a strong emphasis on patient input. It is likely that the HDISS-DU will become adopted as an outcome measure in future clinical studies (including RCTs) of SSc-related digital ulceration.

30. Bruni C, Tanaka Ngcozana T, Francesca Braschi F, *et al.* Preliminary validation of the Digital Ulcer Clinical Assessment Score in systemic sclerosis. J Rheumatol 2019; 46:603–608.
31. Simpson V, Hughes M, Wilkinson J, *et al.* Quantifying digital ulcers in systemic sclerosis: reliability of computer-assisted planimetry in measuring lesion size. Arthritis Care Res 2018; 70:486–490.
32. Dinsdale G, Moore TL, Manning JB, *et al.* Tracking digital ulcers in systemic sclerosis: a feasibility study assessing lesion area in patient-recorded smartphone photographs. Ann Rheum Dis 2018; 77:1382–1384.
33. Hughes M, Moore T, Manning J, *et al.* A pilot study using high-frequency ultrasound to measure digital ulcers: a possible outcome measure in systemic sclerosis clinical trials? Clin Exp Rheumatol 2017; 35(Suppl 106):S218–S219.
34. Suliman YA, Kafaja S, Fitzgerald J, *et al.* Ultrasound characterization of cutaneous ulcers in systemic sclerosis. Clin Rheumatol 2018; 37:1555–1561.
35. ■ Barsotti S, d'Ascanio A, Valentina V, *et al.* Is there a role for laser speckle contrast analysis (LASCA) in predicting the outcome of digital ulcers in patients with systemic sclerosis? Clinical Rheumatol 2020; 39:69–75.

This study is of interest because it presents further data to suggest that laser Doppler methods may provide objective measures by which to assess digital ulcers, and highlights that blood flow measurement will be influenced by presence of infection.

36. Marjanovic E, Moore TL, Manning JB, *et al.* Systemic sclerosis-related digital ulcers: a pilot study of cutaneous oxygenation and perfusion. Rheumatology 2020; 59:3573–3575.
37. ■ Panopoulos S, Chatzidionysiou K, Tektonidou MG, *et al.* Treatment modalities and drug survival in a systemic sclerosis real-life patient cohort. Arthritis Res Ther 2020; 22:56.

The interest of this study from the Raynaud's phenomenon/digital ulceration perspective lies in the high proportion of patients continuing on vasoactive therapy after 2 years. The authors acknowledge the limitations of the retrospective design, and that the drug survival analysis related to a single centre.

38. ■■ Khouri C, Lepelley M, Bailly S, *et al.* Comparative efficacy and safety of treatments for secondary Raynaud's phenomenon: a systematic review and network meta-analysis of randomised trials. Lancet Rheumatol 2019; 1:e237–246.

This network meta-analysis examined efficacy and tolerability of treatments for secondary Raynaud's phenomenon: 58 RCTs (3867 patients) and 15 classes of drugs were included. There was low-level evidence of moderate efficacy for calcium channel blockers and PDE5 inhibitors, but findings did not support the use of any other drugs, underscoring the need for new treatments.

39. Hughes M, Ong VH, Anderson ME, *et al.* Consensus best practice pathway of the UK Scleroderma Study Group: digital vasculopathy in systemic sclerosis. Rheumatology 2015; 54:2015–2024.

40. Kowal-Bielecka O, Fransen J, Avouac J, *et al.* Update of EULAR recommendations for the treatment of systemic sclerosis. Ann Rheum Dis 2017; 76:1327–1339.

41. Herrick AL. Evidence based management of Raynaud's phenomenon. Ther Adv Musculoskel Dis 2017; 9:317–329.

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43. Fernandez-Codina A, Canas-Ruano E, Pope JE. Management of Raynaud's phenomenon in systemic sclerosis: a practical approach. J Scleroderma Relat Disord 2019; 4:102–110.

44. Negrini S, Magnani O, Matucci-Cerinic M, *et al.* Iloprost use and medical management of systemic sclerosis-related vasculopathy in Italian tertiary referral centers: results from the PROSIT study. Clin Exp Med 2019; 19:357–366.

45. de Vries-Bouwstra JK, Allanore Y, Matucci-Cerinic M, Balbir-Gurman A.
■ Worldwide expert agreement on updated recommendations for the treatment of systemic sclerosis. J Rheumatol 2020; 47:249–254.

Results of a survey circulated to systemic sclerosis experts suggested that although most agreed with the European League Against Rheumatism recommendations for the treatment of systemic sclerosis, there is some variation in practice regarding the use of PDE5 inhibitors, bosentan and i.v. iloprost for digital vasculopathy.

46. Fernandez-Codina A, Walker KM, Pope JE. Treatment algorithms for systemic sclerosis according to experts. Arthritis Rheumatol 2018; 70:1820–1828.

47. Roustit M, Giai J, Gaget O, *et al.* On-demand sildenafil as a treatment for Raynaud phenomenon: a series of n-of-1 trials. Ann Int Med 2018; 169:694–703.

48. Di Luigi L, Sgro P, Duranti G, *et al.* Sildenafil reduces expression and release
■ of IL-6 and IL-8 induced by reactive oxygen species in systemic sclerosis fibroblasts. Int J Mol Sci 2020; 21:3161.

The interest of this study lies in the finding that sildenafil reduces oxidative stress. Therefore, sildenafil could reduce vascular injury and thereby have a disease-modifying (as well as a vasodilatory) effect in patients with SSc-related digital vasculopathy.

49. Curtiss P, Schwager Z, Lo Sicco K, Franks AG Jr. The clinical effects of l-arginine and asymmetric dimethylarginine: implications for treatment in secondary Raynaud's phenomenon. J Eur Acad Dermatol Venereol 2019; 33:497–503.

50. Law ST, Farber HW, Simms RW. Use of intravenous epoprostenol as a treatment for the digital vasculopathy associated with the scleroderma spectrum of diseases. J Scleroderma Relat Disord 2017; 2:208–212.

51. Barsotti S, Lorenzoni V, Di Battista M, *et al.* Prostanoids in scleroderma
■ microangiopathy: clinical and pharmacoeconomic comparison between two intravenous regimens. Scand J Rheumatol 2021; 50:307–313.

This retrospective single-centre study describes experience in switching from in-patient to out-patient administration of iloprost and so may be relevant in the Covid-19 era when access to hospital beds can be difficult. The authors correctly emphasize the limitations of the study design.

52. Ingegnoli F, Schioppo T, Allanore Y, *et al.* Practical suggestions on intravenous iloprost in Raynaud's phenomenon and digital ulcer secondary to systemic sclerosis: systematic literature review and expert consensus. Semin Arthritis Rheum 2019; 48:686–693.

53. Fraticelli P, Martino GP, Murri M, *et al.* A novel iloprost administration method with portable syringe pump for the treatment of acral ulcers and Raynaud's phenomenon in systemic sclerosis patients. A pilot study (ILOPORTA). Clin Exp Rheumatol 2017; 35(Suppl 106):173–178.

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■■ ism of prostacyclin analog iloprost in reversing vascular dysfunction in scleroderma. Arthritis Rheumatol 2021; 73:520–529.

Mechanisms of action of iloprost were explored in studies of dermal endothelial cells from 10 patients with diffuse cutaneous SSc and 13 healthy controls. The authors provide a rationale for a prolonged vascular protective effect of iloprost involving stabilization of endothelial cell adherens junctions, normalization of angiogenesis and inhibition of endothelial-to-mesenchymal transition.

56. Kleckner MS, Allen EV, Wakim KG. The effect of local application of glyceryl trinitrate (nitroglycerine) on Raynaud's disease and Raynaud's phenomenon: studies on blood flow and clinical manifestations. Circulation 1951; 3:681–689.

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58. Qui Q, Chan T, Luen M, *et al.* Use of nitroglycerin ointment to treat primary and secondary Raynaud's phenomenon: a systematic literature review. Rheum Int 2018; 38:2209–2216.

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60. von Schoen-Angerer T, Deckers B, Henes J, *et al.* Effect of topical rosemary essential oil on Raynaud phenomenon in systemic sclerosis. Complement Ther Med 2018; 40:191–194.

61. Bahl D, Daftardar S, Devi Bachu R, *et al.* Evaluation of topical econazole nitrate formulations with potential for treating Raynaud's phenomenon. Pharmaceut Dev Technol 2019; 24:689–699.

62. Wasan EK, Zhao J, Poteet J, *et al.* Development of a UV-stabilized topical
■ formulation of nifedipine for the treatment of Raynaud phenomenon and chilblains. Pharmaceutics 2019; 11:594.

The authors point out that for nifedipine to be delivered topically, a photoprotectant is required to prevent photodegradation. Their experiments suggested that a combination of quercetin and avobenzone was able to protect nifedipine in vitro from UVA radiation-induced decomposition.

63. Fernandez-Codina A, Kazem M, Pope JE. Possible benefit of tadalafil cream for the treatment of Raynaud's phenomenon and digital ulcers in systemic sclerosis. Clin Rheumatol 2020; 39:963–965.

64. Naef R, Tenor H, Koch G. TOP-N53: a clinical drug candidate for the treatment of nonhealing wounds. Chimia 2020; 74:814–817.

65. Pintea Bentea G, Wauters A, Wautrecht J-C, Cogan E. Laser Doppler imaging evaluation of nitroglycerin patch application in systemic sclerosis patients. Vasc Med 2020; 25:559–568.

66. Daftardar S, Bahl D, Boddu SHS, *et al.* Ultrasound-mediated topical delivery
■ of econazole nitrate with potential for treating Raynaud's phenomenon. Int J Pharm 2020; 580:119229.

This in-vitro study evaluated the effect of low-frequency ultrasound on the permeability (and toxicity) of formulations of econazole nitrate, in studies of porcine skin. Drug permeation was significantly increased with ultrasound treatment, suggesting that ultrasound-assisted transdermal delivery may be a way forward for the treatment of Raynaud's phenomenon.

67. Freeman MD, Margulies IG, Sanati-Mehrizy P, *et al.* Nonaesthetic applications for botulinum toxin in plastic surgery. Plast Reconstr Surg 2020; 146:157–170.

68. Lautenbach G, Dobrota R, Mihai C, *et al.* Evaluation of botulinum toxin A
■ injections for the treatment of refractory chronic digital ulcers in patients with systemic sclerosis. Clin Exp Rheumatol 2020; 38(Suppl 125):S154–S160.

This study, which includes a systematic review of the literature prior to August 2017, gives a detailed discussion of different approaches to botulinum toxin injections, and highlights the challenges in assessing digital ulcer response to treatment.

69. Martina E, Diotallevi F, Radi G, *et al.* Therapeutic use of botulinum neurotoxins
■ in dermatology: systematic review. Toxins 2021; 13:120.

A comprehensive review of the use of botulinum for different indications including Raynaud's phenomenon.

70. Gallegos JE, Inglesby DC, Young ZT, Herrera FA. Botulinum toxin for the treatment of intractable Raynaud phenomenon. J Hand Surg Am 2021; 46:54–58.

71. Bello RJ, Cooney CM, Melamed E, *et al.* The therapeutic efficacy of botulinum toxin in treating scleroderma-associated Raynaud's phenomenon: a randomized, double-blind, placebo-controlled clinical trial. Arthritis Rheumatol 2017; 69:1661–1669.

72. Dhaliwal K, Griffin MF, Salinas S, *et al.* Optimisation of botulinum toxin type a
■ treatment for the management of Raynaud's phenomenon using a dorsal approach: a prospective case series. Clin Rheumatol 2019; 38:3669–3676.

Forty patients with SSc were studied prospectively. Points of most interest were that using a dorsal approach for the injections, no patients experienced muscle weakness and improvement was demonstrated in several outcome measures, including pain, Raynaud's symptoms, hand function and finger temperature.

73. Habib SM, Brenninkmeijer EE, Vermeer MH, *et al.* Botulinum toxin type A in the treatment of Raynaud's phenomenon. Dermatol Therapy 2020; 33:e14182.

74. Nagarajan M, McArthur P. Targeted high concentration botulinum toxin A injections in patients with Raynaud's phenomenon: a retrospective single-centre experience. Rheumatol Int 2021; 41:943–949.

75. Goldberg SH, Akoon A, Kirchner HL, Deegan J. The effects of botulinum
■ toxin A on pain in ischemic vasospasm. J Hand Surg Am 2021; 46:513.e1–513.e12.

Ten of the 20 patients reported in this retrospective study had SSc. Of interest was that only patients with abnormal photoplethymographic testing (which was performed before and after cold water immersion) were included, and the authors suggested that those patients with the greatest warming response (indicating reversibility in their perfusion deficit) were those most likely to experience a good clinical response to botulinum toxin.

76. Lobb DC, Pierce J, Perry M, DeGeorge B. The use of ultrasound guidance for the treatment of Raynaud disease of the hand with botulinum toxin. Ann Plastic Surg 2020; 84(Suppl 5):S386–S388.

77. Shepherd AI, Costello JT, Bailey SJ, *et al.* 'Beet' the cold: beetroot juice
■ supplementation improves peripheral blood flow, endothelial function, and anti-inflammatory status in individuals with Raynaud's phenomenon. J Applied Physiol 2019; 127:1478–1490.

This study examined a new approach to supplementing the nitric oxide pathway, via dietary inorganic nitrate supplementation. Four out of the 23 patients studied had SSc.

78. Mitropoulos A, Gumber A, Crank H, *et al.* Exploring the feasibility of an exercise programme including aerobic and resistance training in people with limited cutaneous systemic sclerosis. Clin Rheumatol 2020; 39:1889–1898.

79. Tapia-Haro RM, Garcia-Rios MC, Toledano-Moreno S, *et al.* The complementary effects of galvanic current electrical stimulation associated with conservative treatment to increase vasodilation in patients with Raynaud's phenomenon: a randomized trial. Clin Rehab 2020; 34:595–606.

This RCT showed benefits in the experimental group (including in number of Raynaud's attacks), of whom three of 17 had SSc. The authors acknowledged the limitations of the lack of blinding.

80. Van Room AM, Kuijpers M, van de Zande SC, *et al.* Treatment of resistant Raynaud's phenomenon with single-port thoracoscopic sympathicotomy: a minimally invasive endoscopic technique. Rheumatology 2020; 59:1021–1025.

Although describing a small study of only eight patients and with only a short follow-up, this study is of interest because of the definite improvement in perfusion of the hand of the treated 'side', and the potential advantage of a procedural approach with a low risk of adverse effects.

81. Putkaradze Z, Roustit M, Cracowski J-L, Khouri C. Drug repurposing in Raynaud's phenomenon through adverse event signature matching in the World Health Organization pharmacovigilance database. Br J Clin Pharmacol 2020; 86:2217–2222.

This study highlights the potential usefulness of a drug repurposing approach to identify potential new treatments for Raynaud's phenomenon. Results of a cluster analysis suggested that fumaric acid could be effective in secondary Raynaud's phenomenon.

82. Blagojevic J, Abignano G, Avouac J, *et al.* Use of vasoactive/vasodilating drugs for systemic sclerosis (SSc)-related digital ulcers (DUs) in expert tertiary centres: results from the analysis of the observational real-life DeSScipher study. Clin Rheumatol 2020; 39:27–36.

This study is of interest because it bench-marks clinical practice vis-à-vis treatment of SSc-related digital ulceration between 2013 and 2016. A key question is whether the use of bosentan and PDE5 inhibitors has increased as per current guidelines/recommendations.

83. Chang SH, Jun JB, Lee YJ, *et al.* A clinical comparison of an endothelin receptor antagonist and phosphodiesterase type 5 inhibitors for treating digital ulcers of systemic sclerosis. Rheumatology 2021. doi: 10.1093/rheumatology/keab147. [Epub ahead of print]

This study's conclusions have to be interpreted with caution, as patients were not randomized and treatment was open label. However, it highlights the need for a head-to-head randomized comparison of bosentan versus PDE5 inhibition.

84. Hughes M, Moore T, Manning J, *et al.* Reduced perfusion in systemic sclerosis digital ulcers (both fingertip and extensor) can be increased by topical application of glyceryl trinitrate. Microvasc Res 2017; 111:32–36.

85. Guigui A, Mazet R, Blaise S, *et al.* Treprotinil hydrogel iontophoresis in systemic sclerosis-related digital skin ulcers: a safety study. J Clin Pharmacol 2020; 60:758–767.

This safety study of treprostinil hydrogel iontophorseis had two parts: the first in 12 healthy controls (three skin locations) and the second in five patients with SSc-related digital ulcers. The interest in the study lies in its detailed tackling of the problem as to how to deliver vasoactive therapy locally to the ulcer site.

86. Pignatti M, Spinella A, Cocchiara E, *et al.* Autologous fat grafting for the oral and digital complications of systemic sclerosis: results of a prospective study. Aesth Plast Surg 2020; 44:1820–1832.

87. Hughes M, Moore T, Manning J, *et al.* A feasibility study of a novel low-level light therapy for digital ulcers in systemic sclerosis. J Dermatol Treat 2019; 30:251–257.

88. Korsten P, Muller GA, Rademacher J-G, *et al.* Rheopheresis for digital ulcers and Raynaud's phenomenon in systemic sclerosis refractory to conventional treatments. Front Med (Lausanne) 2019; 6:208.

89. Bruni C, Cometi L, Gigante A, *et al.* Prediction and primary prevention of major vascular complications in systemic sclerosis. Eur J Int Med 2021; 87:51–58.

This study of 134 patients with SSc but without major vascular complications at study 'entry' had the limitations inherent of a retrospective series, and numbers of patients treated with individual drugs/classes of drugs were small. Nonetheless, the study is of interest because it highlights the need for prospective studies examining vascular protection.

Contribution of monocytes and macrophages to the pathogenesis of systemic sclerosis: recent insights and therapeutic implications

Alain Lescoat[a,b,c,d], *Valérie Lecureur*[b], *and John Varga*[c,d]

Purpose of review
To discuss recent studies addressing the role of monocytes and macrophages in the pathogenesis of systemic sclerosis (SSc) based on human and mouse models.

Recent findings
Studies indicate that monocyte adhesion could be increased in SSc secondary to an interferon-dependent loss of CD52, and chemotaxis up-regulated through the CCR3/CCL24 pathway. Beyond the conventional M1/M2 paradigm of macrophage subpopulations, new subpopulations of macrophages have been recently described in skin and lung biopsies from SSc patients. Notably, single-cell ribonucleic acid sequencing has provided evidence for SPP1+ lung macrophages or FCGR3A+ skin macrophages in SSc. Impaired pro-resolving capacities of macrophages such as efferocytosis, i.e. the ability to phagocyte apoptotic cells, could also participate in the inflammatory and autoimmune features in SSc.

Summary
Through their potential pro-fibrotic and pro-inflammatory properties, macrophages are at the cross-road of key SSc pathogenic processes and associated manifestations. Investigative drugs targeting macrophage polarization, such as pan-janus kinase inhibitors (tofacitinib or ruxolitinib) impacting both M1 and M2 activations, or Romilkimab inhibiting IL-4 and IL-13, have shown promising results in preclinical models or phase I/II clinical trials in SSc and other fibro-inflammatory disorders. Macrophage-based cellular therapy may also represent an innovative approach for the treatment of SSc, as initial training of macrophages may modulate the severity of fibrotic and autoimmune manifestations of the disease.

Keywords
chemotaxis, JAK inhibitor, macrophages, monocytes, scleroderma, systemic sclerosis

INTRODUCTION

Systemic sclerosis (SSc or scleroderma) is a chronic autoimmune disease characterized by a triad of pathogenic processes including widespread vasculopathy, immune dysregulation and fibrosis of the skin and internal organs [1,2]. The fibrotic features of the disease include life-threatening manifestations such as interstitial lung disease (SSc-ILD) [3]. To date, there is no disease-modifying drug impacting the entire SSc disease process and equally benefiting all SSc patients. A better understanding of SSc pathogenesis and identification of new therapeutic pathways are thus needed [3,4]. Serum autoantibodies targeting nuclear antigens (antinuclear antibodies; ANA) are virtually always detected in SSc patients, implicating adaptive immunity in pathogenesis, notably represented by B-cells and the process of co-stimulation [5–7]. Innate immunity also plays an important role in SSc-related tissue damages. Recent studies have identified monocytes and macrophages as key drivers of the inflammatory and fibrotic manifestations of SSc [8].

Macrophages are a heterogeneous population of mononuclear cells comprising tissue-specific

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KEY POINTS

- Gene expressions associated to M1 pro-inflammatory macrophages and to M2 pro-fibrotic macrophages are upregulated in the tissues from patients with systemic sclerosis.
- Pro-resolving properties of macrophages, such as efferocytosis - i.e. their capacity to phagocyte cellular debris and apoptotic cells - could be impaired in systemic sclerosis.
- Monocytes adhesion could be increased due to an IFN dependent loss of CD52 expression and chemotaxis could also be increased notably through the CCR3/CCL24 pathway.
- Beyond the M1/M2 paradigm, new sub-populations of SSc macrophages have been recently described based on single-cell ribonucleic acid sequencing, notably including SPP1+ macrophages in the lung that may show specific proliferating capacities or FCGR3A+ macrophages in the skin expressing pro-fibrotic markers such as CCL18 or IL-6.
- Therapies targeting M2 polarization such as Romilkimab or simultaneously impacting M1 and M2 polarizations such as pan-janus kinase inhibitors (tofacitinib, ruxolitinib) may represent promising treatments for the fibrotic and inflammatory manifestations of systemic sclerosis.

resident macrophages and blood monocytes-derived macrophages that are recruited in inflammation or fibrosis [9]. Macrophages can adopt various activation states, also called polarization profiles, depending on their cytokine microenvironment [10]. High levels of Th1derived mediators such as type II interferon (IFN) or toll-like receptors (TLR)4 agonists (such as LPS or tenascin-C) induce a classical pro-inflammatory polarization of macrophages, also called M1 or M(IFNγ); M(LPS) macrophages [11–14]. On the other extreme of this macrophage activation spectrum, the Th2-derived interleukins (IL)-4 and IL-13 can induce an alternative M(IL-4 and/or IL-13) or M2a polarization of macrophages. These M2a macrophages can participate in fibrosis through the secretion of profibrotic mediators transforming growth factor-β (TGFβ), CCL18, Platelet-derived growth factor (PDGF), and mediators from the fibroblast growth factor family, that induce fibroblast proliferation or collagen production [13,15]. A remarkable recent study documented that close functional interaction of macrophages with myofibroblasts is also fostered by a long-range macrophage/myofibroblast communication system based on the transmission of myofibroblast-generated mechanical forces through fibrillar extracellular matrix (ECM). Macrophages can thus perceive and respond to dynamic changes of the fibrillar collagen matrix induced by myofibroblasts [16].

By sitting at the crossroads of inflammation and fibrosis, macrophages are essential, but hitherto underappreciated and poorly understood, players in SSc (Fig. 1). It is now increasingly recognized that macrophages represent potential targets for disease-modifying therapies in SSc. Recent results from single-cell ribonucleic acid (RNA) sequencing of fibrotic tissues in idiopathic pulmonary fibrosis (IPF) and SSc also identify new macrophage subpopulations demonstrating increasing complexity and heterogeneity within the M1 and M2 populations [17▪▪]. This update of the fast-evolving field of research will discuss emerging insights on the origins, identity, functional heterogeneity, mechanisms and pathogenic roles of monocytes and macrophages in SSc. We will highlight the potential therapeutic pathways and investigative drugs that could impact macrophages in SSc based on recent results from studies with humans and murine models of scleroderma.

MONOCYTES SUBPOPULATIONS AND CHEMOTAXIS IN THE PATHOGENESIS OF SYSTEMIC SCLEROSIS

Human blood monocytes are classified into three subsets based on their membrane expression of CD16 and CD14: classical monocytes (CD14++, CD16-), intermediary monocytes (CD14+, CD16+) and nonclassical monocytes (CD14+, CD16++) [9]. Their precise properties may vary depending on tissue and clinical contexts. Nonclassical monocytes constitute less than 15% of human blood monocytes in normal conditions and may represent a subset of pro-fibrotic monocytes, although some authors also highlight their pro-inflammatory properties [18]. Nonclassical monocytes patrol blood vessels and participate in endothelial monitoring, and would only be secondarily recruited into tissues where they could differentiate into profibrotic macrophages, whereas classical monocytes would be recruited earlier [19]. In SSc, CD16-positive (nonclassical) monocyte count was higher than in controls in several studies and was associated in some of them with more severe fibrotic disease manifestations, including ILD and a higher modified Rodnan Skin Score (mRSS) [20,21]. A higher proportion of CD16-positive (CD16+) monocytes expressing CXCL10, an IFN-inducible marker, has also been demonstrated in SSc suggesting that IFN signature may contribute to monocytes activation in early SSc [22,23].

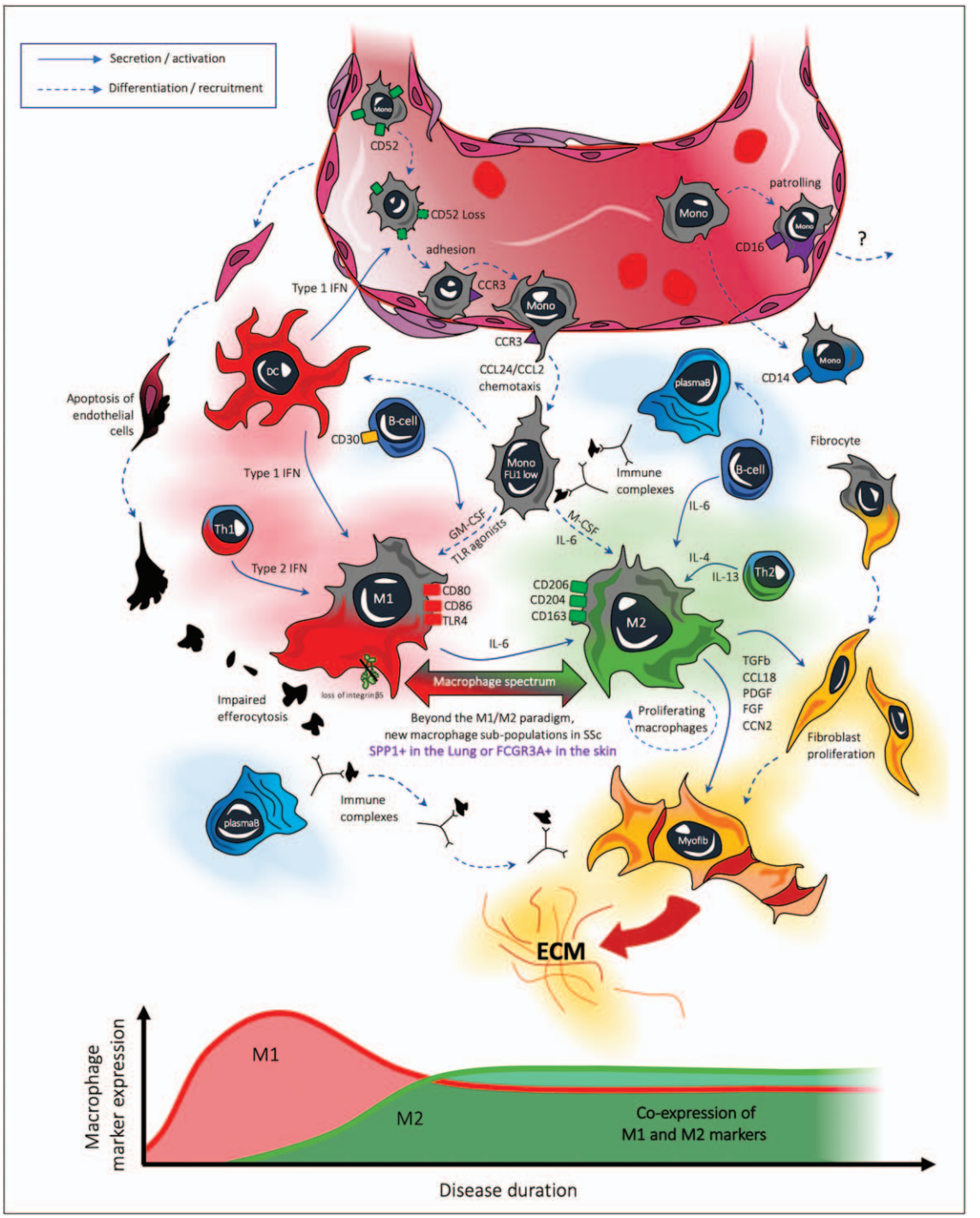

FIGURE 1. Contribution of monocytes and macrophages to the pathogenesis of systemic sclerosis: The decreased expression of CD52 may participate in monocyte adhesion. Increased expression of CCR3 and other chemotaxis receptors induces monocyte recruitment. Under the influence of colony-stimulating factors (GM-/M-CSF) and cytokine environment (Th1 and Th2), macrophages in SSc adopt a mixed polarization profile including M1 and M2 markers. M1 polarization may be especially increased at the very early stage of the disease. Pro-resolving properties of macrophages such as efferocytosis could be impaired in SSc due to M1 polarization. M2 polarization will participate in the fibrotic manifestations of the disease through the stimulation of fibroblasts proliferation and myofibroblast activation. Beyond the M1/M2 paradigm, new sub-populations of SSc macrophages have been recently described based on single cell RNA sequencing, notably including SPP1+ lung macrophages that may show specific proliferating capacities or FCGR3A+ skin macrophages expressing pro-fibrotic markers such as CCL18 or IL-6. DC, dendritic cells; ECM, extracellular matrix; Mono, monocytes; Myofib, myofibroblasts; PlasmaB, plasma cells; RNA, ribonucleic acid.

CD52 is a glycosylphosphatidylinositol (GPI) anchored protein notably expressed on monocytes that might participate in regulating monocyte adhesion and inflammatory responses. The role of CD52 in SSc has been recently explored in an *in vitro* study showing down-regulated CD52 expression in SSc monocytes [24]. Decreased CD52 expression in SSc was mediated by type I IFN signaling and may promote monocyte adhesion to endothelial cells with potential increased recruitment into tissues. Notably, specific janus kinase (JAK)-1 inhibition by itacitinib resulted in a partial restoration of CD52 expression, strengthening the role of type I IFN in the regulation of CD52 since JAK-1 participates in IFNα signaling. Interestingly, SSc patients treated with immuno-modulatory drugs in routine care in this study show restored expression of CD52 [24].

Following monocyte adhesion to endothelial cells, chemokine signaling contributes to monocyte recruitment into tissue. The chemokine receptor CCR3 is up-regulated in SSc monocytes [25]. This receptor can bind CCL24 (also called eotaxin) a chemokine already implicated in monocyte recruitment into inflammatory and/or fibrotic tissues. In the bleomycin model, CCL24 knockout mice had reduced inflammatory cell numbers in bronchoalveolar lavage (BAL) fluid [26]. Blocking CCL24 by CM-101, a therapeutic monoclonal antibody targeting this chemokine, similarly reduced skin and lung fibrosis, as well as decreased pulmonary infiltrate and white blood cell count in BAL fluid. Blockade of monocyte recruitment may be an appealing novel therapeutic approach to limit fibrotic manifestations of SSc. This hypothesis is also supported by the effects of mycophenolate mofetil (MMF) in patients with SSc, as the down-regulation of the chemokine CCL2 by MMF was associated to a lower count of skin macrophages with improved or stable mRSS [27]. Interestingly, lower levels of serum CCL2 after treatment with MMF were also associated with lower monocyte migration assessed with a fluorescence-based Chemotaxis Cell Migration Assay. Blocking monocyte chemotaxis may not only limit the number of monocyte-derived-macrophages (MDM) in damaged tissues but could also participate in reducing the number of monocyte-derived dendritic cells (DC), notably plasmacytoid DC which are potential producers of type 1 IFN. Their precise ontogeny in humans is nonetheless still debated [19]. Monocytes might also be precursors of fibroblast-like cells that directly contribute to the pool of myofibroblasts responsible for ECM production, although these cells may derive from bone marrow myeloid cells and not directly from blood monocytes [28].

MONOCYTES-DERIVED MACROPHAGES AND MACROPHAGE DIFFERENTIATION IN SYSTEMIC SCLEROSIS

The heterologous differentiation of blood monocytes from healthy donors treated with SSc patient plasma *in vitro* induced MDM mimicking the phenotype and profibrotic properties of SSc MDM cultured under autologous conditions [29▪]. These results suggest that plasma factors are key determinants of monocyte differentiation and the future fate of MDM in SSc. Two growth factors play essential roles in regulating blood monocyte differentiation into MDM *in vitro* : Macrophage colony-stimulating factor (M-CSF) and Granulocyte-macrophage CSF (GM-CSF) [30,31]. These growth factors also influence monocyte differentiation *in vivo* in tissues, and may partially determine their activation state, since GM-CSF primes MDM for classical polarization and M-CSF for alternative polarization. Despite this potential initial M-/GM-CSF-dependent priming, MDM differentiation and polarization can be influenced by the local cytokine environment including Th1 or Th2 mediators [11]. Th2 cytokines such as IL-4 could induce CD30+ effector B-cells characterized by the secretion of GM-CSF in SSc, suggesting that despite a pro-fibrotic Th2 environment a GM-CSF depend pro-inflammatory priming of macrophages may occur [32]. B-cells could also directly participate in the differentiation and polarization of M2 macrophages as in the bleomycin mouse models, B-cell depletion decreased the expression of the M2 marker CD206, in the lung and skin. *In vitro*, co-culture of B-cells from bleomycin mice with macrophages from untreated mice induced M2 polarization in an IL-6 dependent manner [33▪▪]. Immune complexes from SSc patients can induce the secretion of osteopontin (SPP1) by monocytes [34▪]. Osteopontin can then promote lung fibroblast migration and activation with subsequent pro-fibrotic effects. Interestingly, immune complexes also favored an autocrine production of M-CSF and IL-6 by monocytes. Moreover, in lung samples from SSc patients, the production of osteopontin overlapped with the production of CCL18, a key M2-associated marker [34▪]. Based on these results, we hypothesize that immune complexes participate in the differentiation of monocytes into profibrotic macrophages in an M-CSF-dependent manner, strengthening the relevance of exploring the impact of M- and GM-CSF on monocytes *in vitro* and *in vivo* [31]. The promising results of tocilizumab (therapeutic monoclonal antibody targeting IL-6 receptor) in SSc-ILD may partly be explained by its impact on this IL-6-dependent M2 polarization [35]. A population of SPP1+ macrophages has been recently identified using single cell-RNA sequencing

approaches in lung tissue from patients with IPF, demonstrating that these SPP1+ macrophages constitute a subpopulation of CD163+ M2 macrophages which was notably over-represented in the lower lobes where the fibrotic damages were more prominent [17▪▪]. These SPP1+ macrophages have also been identified in SSc, and may have specific proliferating properties [17▪▪,36▪▪]. Single cell transcriptome analysis from dcSSc skin biopsies also demonstrates the expansion of newly identified myeloid subpopulations, including FCGR3A+ macrophages, FCN1+ monocyte-derived DCs and a subset of proliferating macrophages [37▪▪]. FCGR3A+ macrophages identified in the skin of dcSSc patients expressed profibrotic cytokines and chemokines such as IL-6 or CCL18. These FCGR3A+ macrophages (presumably derived from MARCO+ skin resident macrophages) were characterized by the expression of M2 polarization markers such as CD204 (MSR1), CD163 or MS4A4A and the upregulation of signaling pathways such as 'response to lipopolysaccharide' which are classically associated with M1 polarization [37▪▪,38].

DUALITY OF MACROPHAGES POLARIZATION IN SYSTEMIC SCLEROSIS: A MIXED M1-M2 SIGNATURE

Although initial analyses from the lung, skin and blood from SSc patients suggested a prominent M2 profibrotic signature of monocyte/macrophages, recent studies highlight the existence of a mixed phenotype sharing M1 and M2 properties in SSc [39,40]. The duality of M-/GM-CSF-dependent priming on the one hand and cytokine/chemokine-dependent activation on the other hand, may participate in blurring the clear distinction between M1 and M2 macrophages in SSc [30,31]. An increased population of circulating myeloid cells co-expressing the M2 markers CD204, CD163, CD206 and the M1 markers CD80, CD86, TLR4 was thus identified by flow cytometry in blood from SSc patients [41]. Similar conclusions were driven from the analysis of the phenotype of M-CSF-derived MDM from SSc patients [42]. Interestingly this subpopulation of circulating myeloid cells from the monocyte/macrophage lineage co-expressing M1 and M2 markers was especially associated with SSc-ILD, suggesting that this myeloid population may characterize patients with a more severe phenotype [43].

Gene expression analysis of skin biopsies from patients with early diffuse cutaneous SSc (dcSSc), a disease subset characterized by widespread skin fibrosis, demonstrated that the 3 major cell subtypes over-represented in these patients in comparison with healthy donors were M1-, M2-macrophages and fibroblasts [44▪▪]. M1-related gene expression was also significantly up-regulated in patients with very early dcSSc as compared to patients with longer disease duration, suggesting that pro-inflammatory signature is prominent during the early stages of the disease [44▪▪]. This increased M1 signature in early skin disease with secondary more balanced M1/M2 gene expression has also been demonstrated in the hypochlorous acid (HOCl)-induced mouse model of SSc [45▪]. In this model, lung macrophages also showed higher expression of both M1 (NOS2, IFi44, CXCL10) and M2 (Arg1, Flt1, Chi3L3) makers even in late-stage disease, a feature that was also described in SSc-ILD in humans [40]. Recent data have also suggested that blood monocytes from SSc patients show decreased expression of Fli1, a transcription factor from the Ets family known to protect mice from SSc-associated vasculopathy, as demonstrated by conditional knockout of Fli1 in endothelial cells. The down-regulation of Fli1 in human myeloid cells and in mouse peritoneal macrophages led to a mixed polarization profile characterized by the expression of M2 markers (CD163) with concomitant up-regulation of type I and type II IFN pathway-related genes (CXCL10), which are classically associated with M1 polarization [46].

Beyond these M1 and M2 profiles, recent data suggest that CXCL4 could trigger the secretion of profibrotic PDGF-bb by monocytes and macrophages [47]. CXCL4 is upregulated in the serum of patients with SSc and can induce M(CXCL4) polarization of macrophages (M4), although the role of this M4 macrophage sub-population in SSc is still to be determined [48,49].

TARGETING M1 AND/OR M2 POLARIZATION AS A THERAPEUTIC APPROACH IN SYSTEMIC SCLEROSIS

M1 signature is driven by a JAK/STAT-dependent type II INF signaling, whereas M2 signature is associated with JAK/STAT-dependent IL-4/IL-13 signaling. Broad JAK/STAT inhibition with pharmacological pan-JAK inhibitors may therefore represent a relevant therapeutic approach to simultaneously target both M1 and M2 macrophages in SSc. In the HOCl-induced mouse model of SSc, concomitant blockade of IFN and IL-4/IL-13 pathways with the pan-JAK inhibitor ruxolitinib decreased both M1 and M2 markers and successfully prevented skin and lung fibrosis [45▪]. Comparable results were obtained in a preventive therapeutic protocol with tofacitinib, another pan-JAK inhibitor, in the bleomycin-induced disease model [50▪]. Despite these promising preclinical findings, tofacitinib failed to decrease lung fibrosis in a therapeutic approach,

suggesting that such treatment should be considered in patients with early disease, before the onset of fibrotic manifestations, or only to mitigate their progression [50▪]. When considering M2 polarization only, the concomitant inhibition of IL-4 and IL-13 with Romilkimab, a therapeutic monoclonal antibody, has shown promising results in a phase II trial with a significant difference on mRSS between active therapy and placebo in favor of Romilkimab [51▪]. However, the direct impact of Romilkimab on the polarization profile of skin macrophages was not evaluated in this study. Moreover, JAK inhibitors and Romilkimab do not solely target macrophages and their impact on other cell subtypes such as myofibroblasts may participate in the observed effects.

MACROPHAGES AND IMPAIRED RESOLUTION OF INFLAMMATION IN SYSTEMIC SCLEROSIS

Beyond their role in tissue damage, macrophages also show pro-resolving properties that might play a role in the pathogenesis of SSc. Resolution of inflammation involves phagocytosis of cellular debris and apoptotic bodies through a process called efferocytosis, which is performed by professional phagocytes such as macrophages [52]. Efferocytosis involves the specific recognition and binding of phosphatidylserine from apoptotic cells by macrophage receptors (such as integrin, BAI, TIM, CD300 or TAM tyrosine kinases receptors) directly or indirectly via an interaction of phosphatidylserine with bridging molecules such as MFG-E8 and GAS6 [53]. Mice with a genetic defect in MFG-E8 show autoimmune features and decreased efferocytosis [54]. Interestingly, MDM from SSc patients also show decreased efferocytosis capacity that could be linked to their mixed M1/M2 polarization profile [55▪]. M1 macrophages have impaired efferocytosis capacities due to a decreased expression of integrins such as ITGβ5 which is also down-regulated in SSc MDM in comparison with healthy donors [55▪]. The impaired efferocytosis capacities of SSc MDM could also result from cytoskeleton remodeling driven by overactivity of the intracellular RhoA/ROCK pathway [56]. In this regard, ROCK inhibitors enhance efferocytosis capacities of SSc MDM and prevent fibrotic and autoimmune processes in a mouse model induced by HOCl [57], highlighting that targeting macrophages through this pathway may be promising in SSc [58].

Apoptotic bodies express nuclear antigens at their surface, and their persistence due to impaired efferocytosis may promote circulation of autoantigens. Although this hypothesis has been well demonstrated in systemic lupus erythematosus, it still remains to be confirmed in SSc [59,60]. Impaired efferocytosis could also lead to secondary necrosis and release of intranuclear proteins. This circulation of nuclear proteins could participate in the formation of immune complexes composed of ANA and nuclear autoantigens [34▪,59,61]. Immune complexes could in return induce SPP1 secretion by monocytes and exacerbate profibrotic pathways, as previously discussed. This impaired efferocytosis could also explain the accumulation of apoptotic cells in the skin of SSc patients and mice [62,63].

CONCLUSION AND PERSPECTIVES: MONOCYTE/MACROPHAGE-BASED CELLULAR THERAPY IN SYSTEMIC SCLEROSIS

Beyond their pathogenic role in tissue damage, macrophages could also have beneficial effects. Their therapeutic potential is suggested in a recent study exploring trained immunity in a preclinical model of SSc [64▪▪]. Trained immunity is an emerging concept suggesting that macrophages and other cells comprising the innate immune system, such as natural killer (NK) cells, can acquire immune memory, showing a different quality and intensity of response after the second exposure to a microorganism in comparison with the response after a first exposure to the same microorganism [65]. Metabolic and epigenetic cellular reprogramming induced by the initial exposure are thought to play a key role in trained immunity [66]. Applying this concept to SSc, this study based on the SSc-like fibrosis induced by HOCl subcutaneous injections, demonstrated that immune training of mice with injection of LPS or BCG (Bacillus Calmette–Guérin vaccine) prior to the induction of scleroderma with HOCl injections could modulate disease severity [64▪▪]. Specifically, mice in the BCG-treated group showed more severe lung and skin fibrosis than control (i.e. nonpretreated) mice. On the contrary, mice that had an LPS challenge as immune training prior HOCl injections showed a less severe phenotype than control mice without prior immune training. The authors hypothesized that these effects could be mediated by modulating the activation state of macrophages. They conducted a cellular therapy based on weekly intra-peritoneal injections of *in vitro* trained bone marrow-derived macrophages (BMDM) in HOCl-mice. Interestingly, HOCl mice that received LPS-*in vitro*-trained BMDM showed decreased fibrotic and autoimmune features in comparison with control HOCl-mice whereas mice receiving BCG-*in vitro*-trained BMDM showed more severe manifestations. Although the precise

mechanism underlying these striking effects remain unclear at this time, and the applicability of these phenomena to patients with SSc is unknown, these results suggest that *in vitro* trained M(LPS) macrophages are antifibrotic, and this approach could constitute a novel promising cellular therapy in SSc.

Through their potential profibrotic and proinflammatory properties, macrophages are at the crossroads of the canonical SSc pathogenic processes. Defective pro-resolving activities of SSc macrophages could also participate in the inflammatory and autoimmune features of the disease. Investigative drugs targeting macrophage polarization, such as JAK inhibitors or Romilkimab, have shown promising results in preclinical models or phase I/II trials [51▪,67]. The evaluation of these effects in larger clinical trials is awaited. Macrophage-based cellular therapy may represent an exciting future approach for the treatment of SSc.

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V.L. and J.V.: none.

Conflicts of interest

A.L. and V.L.: Nothing to disclose

J.V.: J.V. reports consulting agreements/advisory for Boehringer Ingelheim, TeneoBio, Mitobridge, Emerald Pharma, Horizon Therapeutics, Formation Biologics, Astellas, Forbius, Abingworth and research funding from Pfizer, Bristol-Myers-Squibb, Teneo-Bio, Takeda, Sun Bio.

Off-Label Use/Unapproved Drugs or Products:
The following drugs are still investigational in SSc:
-JAK inhibitors (Ruxolitinib, tofacitinib)
-CM-101
-Romilkimab
-ROCK inhibitors

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New mechanism-based approaches to treating and evaluating the vasculopathy of scleroderma

Nicholas A. Flavahan

Purpose of review
Utilizing recent insight into the vasculopathy of scleroderma (SSc), the review will highlight new opportunities for evaluating and treating the disease by promoting stabilization and protection of the microvasculature.

Recent findings
Endothelial junctional signaling initiated by vascular endothelial-cadherin (VE-cadherin) and Tie2 receptors, which are fundamental to promoting vascular health and stability, are disrupted in SSc. This would be expected to not only diminish their protective activity, but also increase pathological processes that are normally restrained by these signaling mediators, resulting in pathological changes in vascular function and structure. Indeed, key features of SSc vasculopathy, from the earliest signs of edema and puffy fingers to pathological disruption of hemodynamics, nutritional blood flow, capillary structure and angiogenesis are all consistent with this altered endothelial signaling. It also likely contributes to further progression of the disease including tissue fibrosis, and organ and tissue injury.

Summary
Restoring protective endothelial junctional signaling should combat the vasculopathy of SSc and prevent further deterioration in vascular and organ function. Indeed, this type of targeted approach has achieved remarkable results in preclinical models for other diseases. Furthermore, tracking this endothelial junctional signaling, for example by assessing vascular permeability, should facilitate insight into disease progression and its response to therapy.

Keywords
adherens junctions, angiopoietins, Tie2, vascular endothelial-cadherin, vascular endothelial protein tyrosine phosphatase

INTRODUCTION

Scleroderma (SSc) is associated with functional and structural deterioration of the microcirculation that precipitates clinically significant events including Raynaud's phenomenon (RPh), ischemic injury and digital ulcers, pulmonary hypertension, and renal crisis [1▪]. Microvascular injury and endothelial modulation are primary events in the initiation and propagation of the disease process [2,3]. Recent excellent reviews discuss SSc pathogenesis including current and emerging treatments [4▪▪,5▪▪,6,7▪▪]. For SSc vasculopathy and RPh, therapeutic options remain centered on vasodilatation [5▪▪,6]. Improving nutritional blood flow must remain a primary goal in SSc. However, rather than focusing on vasodilatation, this review will utilize recent insight into SSc vasculopathy to highlight new opportunities for evaluating and treating the disease by promoting stabilization and protection of the microvasculature. Vascular quiescence and stability are active processes, reflecting integrated signaling within and between vascular cells [8]. Although transient destabilization is necessary, for example for acute inflammatory responses or angiogenesis, chronic destabilization threatens vascular integrity with loss of microvasculature (rarefaction) and development of arterial lesions [8,9,10▪]. There is compelling evidence that mechanisms underlying vascular stability and quiescence are disrupted in SSc, resulting in chronic destabilization that drives progression of the disease.

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KEY POINTS

- The protective activity evoked by endothelial junctional signaling of vascular endothelial-cadherin (VE-cadherin) and Tie2 is reduced in SSc microvascular endothelial cells, which will not only diminish vascular protection, but also trigger pathological signaling that is normally held in check by these mediators.
- Vasculopathy in SSc likely reflects a chronic state of destabilization resulting from this change in signaling, which precipitates the characteristic changes in vascular function and structure and contributes to further progression of the disease including fibrosis and organ injury.
- Targeted therapy to restore protective endothelial junctional signaling and stabilize the vascular system should be a powerful approach to combat the vasculopathy and prevent further progression of the disease process.

AN INTRIGUING CONNECTION: VE-CADHERIN, ANGIOPOIETINS, AND CAPILLARY DERANGEMENT IN SCLERODERMA

Increased vascular permeability leading to tissue edema is an early feature of SSc vasculopathy that precedes fibrosis [11–14]. Edematous puffy fingers along with RPh and antinuclear antibody positivity are the 'red flags' raising suspicion of very early SSc, and puffy fingers/hands are independent predictors of RPh evolving into SSc [15]. Increased permeability in SSc can occur in capillaries with normal morphology, and in normal and fibrotic tissue [14,16,17]. This hyperpermeability reflects gap formation between endothelial cells [16,17]. There is also reduced pericyte coverage and glycocalyx in SSc capillaries [18,19], which normally protect and stabilize these essential structures [20,21]. Indeed, there is a characteristic progressive change in SSc capillaries with dilatation as the earliest event followed by rarefaction [22].

The major determinant of systemic vascular permeability is adherens junctions, which are formed by engagement and clustering of VE-cadherin molecules between adjacent endothelial cells [8,23–25]. VE-cadherin clustering establishes signaling complexes that enhance endothelial cellular adhesion and stabilize vascular structures [8]. Transient junctional disruption and gap formation is necessary for increased permeability and inflammatory cell extravasation during acute inflammatory responses. Indeed, inflammatory cells and mediators stimulate VE-cadherin degradation and junctional disruption [8,24,26], whereas protection of VE-cadherin junctional clustering blocks inflammation-induced increases in permeability and inflammatory cell extravasation [8,27,28]. VE-cadherin acts in an integrated manner with the angiopoietin (Angpt)–Tie2 system, which comprises competitive ligands (Angpt1, Angpt2) and endothelial Tie2 receptors. Angpt1 is released by pericytes and activates Tie2, whereas Angpt2 is produced by endothelial cells and can block Tie2 [10▪]. Angpt1 protects endothelial adherens junctions by reducing VE-cadherin internalization and by promoting cortical actin formation [29–33]. In turn, VE-cadherin junctional signaling inhibits Angpt2 expression [10▪]. However, inflammatory stimuli increase the expression and Tie2 antagonistic activity of Angpt2, which contributes to VE-cadherin degradation, junction disruption, and increased permeability [10▪,32,34–36]. Angpt1 also increases vascular stability by promoting pericyte recruitment and retention and by protecting the capillary glycocalyx, whereas Angpt2 promotes pericyte loss and glycocalyx degradation [35,37]. Indeed, during chronic inflammation, the reduced signaling by VE-cadherin and Tie2 causes pericyte loss and precipitates capillary dilatation and rarefaction, as well as increased microvascular permeability and edema [35,38,39].

Expression of VE-cadherin is selectively decreased in SSc microvascular endothelium [19,40,41▪▪]. Moreover, circulating and microvascular levels of Angpt2 are increased and evident at an early stage of the disease [42,43▪]. Circulating levels of Angpt1 and the microvascular expression of Tie2 are also reduced in SSc patients [42]. Indeed, the pathological changes in structure and function of SSc capillaries are consistent with diminished VE-cadherin and Tie2 junctional signaling. The former would destabilize adherens junctions resulting in increased permeability and edema, whereas Angpt2 and reduced Tie2 activation would amplify this junctional dysfunction and precipitate degradation of the glycocalyx and loss of pericytes, resulting in capillary dilatation and rarefaction. Indeed, chronic disruption of endothelial junctions threatens the stability and integrity of the microvasculature [8,9,44].

THE PROTECTIVE IMPACT OF ENDOTHELIAL JUNCTIONAL SIGNALING

The junctional activity of VE-cadherin and Angpt1:-Tie2 promote vascular stability by regulating numerous signaling pathways (Fig. 1).

An important primary pathway stimulated by junctional clustering of VE-cadherin or junctional localization of Angpt1:Tie2 is phosphoinositide-3-kinase (PI3K)/Akt signaling [8,45–47]. Junction-dependent Akt activity supports vascular homeostasis in part by increasing expression of Kruppel-like factors 2 and 4 (KLF2, KLF4) [47]. KLF2/4 are powerful transcriptional promoters of endothelial and vascular

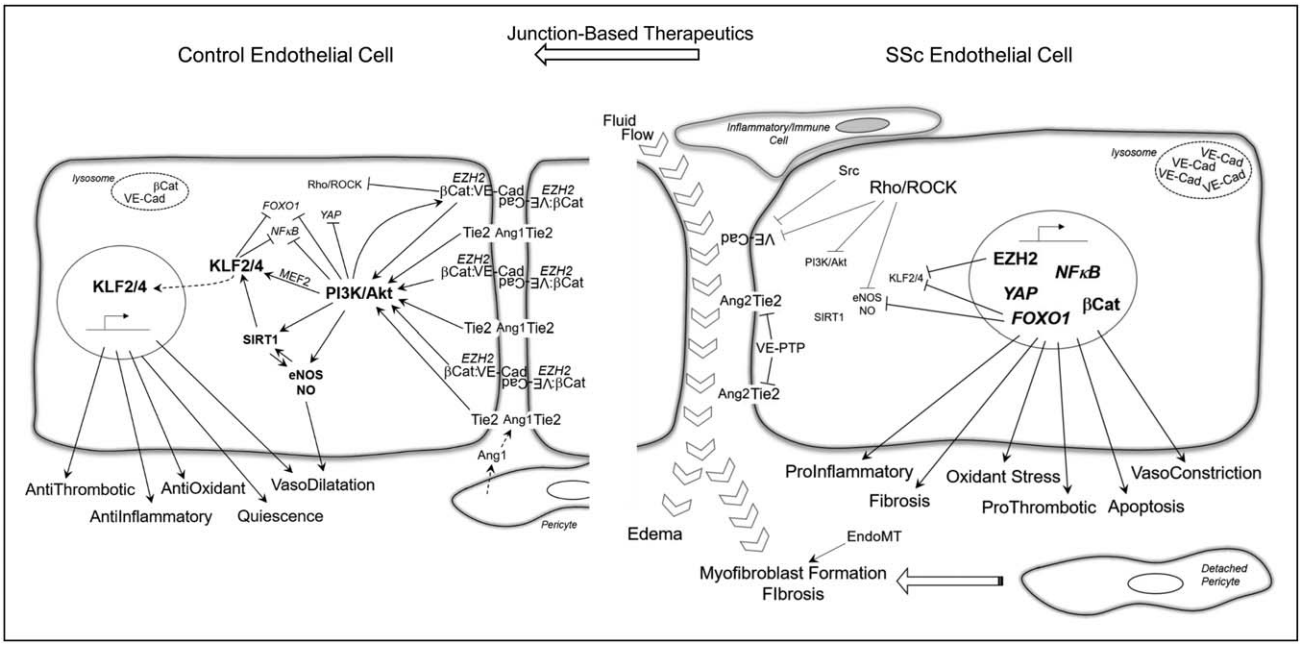

FIGURE 1. Schematic representation highlighting the potential importance of endothelial junctional disruption to the scleroderma disease process. The left panel, representing normal endothelial cells, presents some of the protective pathways initiated by junctional signaling of VE-cadherin clustering and Tie2 activation by Angpt1, whereas the right panel, representing scleroderma cells, presents some of the signaling processes accompanying chronic disruption of endothelial junctions. Restoration of protective junctional signaling (junction-based therapeutics), for example VE-PTP inhibition or amplification of junctional VE-cadherin or Tie2 activity, should stabilize the scleroderma vasculature and combat progression of the disease process. Ang1, angiopoietin-1; Ang2, angiopoietin-2; KLF2/4, Krüppel-like factor 2 and 4; MEF2, myocyte enhancer factor 2; PI3K, phosphoinositide-3-kinase; SIRT1, sirtuin 1; VE-Cad, VE-cadherin; VE-PTP, vascular endothelial protein tyrosine phosphatase; YAP, yes-associated protein; βCat, βcatenin.

stability, ensuring physiological regulation of vascular tone [e.g., increasing endothelial NO synthase (eNOS) expression, decreasing endothelin-1 (ET1) expression], inhibiting inflammation [e.g., decreasing vascular cell adhesion molecule-1 (VCAM-1), IL-6, IL-8 expression, and nuclear factor kappa-B (NFκB) activity], inhibiting thrombosis (e.g., increasing thrombomodulin, decreasing PAI-1 and tissue factor expression), and reducing oxidant stress (e.g., increasing catalase expression) [48,49]. KLF2/KF4 also increases VE-cadherin expression and suppresses Angpt2 expression [50]. Junction-dependent Akt activity also exerts protective activity by phosphorylation of FOXO1 and yes-associated protein (YAP), inhibiting their nuclear translocation and transcriptional activity [21,45,51–53]. When active, FOXO1 decreases eNOS expression and increases endothelial inducible NOS expression, which will impair endothelium-dependent dilatation and precipitate oxidant stress [54,55]. Indeed, FOXO1 activity may be responsible for this switch in NOS expression in SSc endothelium and contribute to endothelial dilator dysfunction in SSc [56]. In dysfunctional endothelial cells from severely obese individuals, FOXO1 inhibition restored eNOS expression and activity, and NO production [57]. Endothelial FOXO1 can also disrupt vascular stability and homeostasis by activating target genes, including ET1, intercellular adhesion molecule 1 (ICAM-1), VCAM-1, and by repression of KLF2/4 [58–60]. Likewise, transcriptionally active YAP increases endothelial inflammatory and fibrotic responses by increasing expression of Angpt2, connective tissue growth factor (CTGF), VCAM-1, and ICAM-1 [51,61]. YAP activity is increased in SSc fibroblasts where it may contribute to SSc fibrotic responses in part by increasing expression of CTGF [6,7▪▪].

PI3K/Akt signaling is an important pathway for activating eNOS, and junctional signaling by VE-cadherin or Angpt1:Tie2 increases eNOS activity and endothelium-dependent dilatation [24,46,62,63]. VE-cadherin is also a crucial component of the mechanotransduction pathway that enables shear stress to modulate endothelial function, including flow-mediated dilation [8]. In addition to the anti-inflammatory effects of increasing KLF2/4 expression and inhibiting FOXO1/YAP activity, PI3K/Akt activation by Angpt1:Tie2 directly inhibits NFκB activity [64,65]. Likewise, PI3K/Akt signaling by VE-cadherin or Angpt1:Tie2 directly inhibits endothelial apoptosis

and blocks the proapoptotic activity of FOXO1 [24,66,67].

Clustering of VE-cadherin at adherens junctions sequesters the transcription regulators, EZH2 and β-catenin, preventing their nuclear translocation and activity [8,53]. EZH2, the catalytic subunit of the polycomb repressor complex 2, mediates trimethylation of lysine 27 in histone 3 (H3K27me3), a repressive epigenetic mark [68]. EZH2 activity and H3K27me3 levels are increased in SSc endothelial cells [41▪▪,68], consistent with reduced VE-cadherin activity. Endothelial EZH2 activity has been implicated in promoting multiple aspects of endothelial cell dysfunction [69▪]. Importantly, EZH2 binds to and silences the *KLF2* gene resulting in marked reduction in KLF2 levels and suppression of its protective activity [70]. Moreover, EZH2 inhibition significantly increased angiogenic activity in normal microvascular endothelial cells and the impaired activity in SSc cells [68]. β-Catenin is the binding partner of VE-cadherin and is instrumental in junctional signaling of VE-cadherin. Following disruption of adherens junctions, β-catenin can translocate to the nucleus and regulate transcription, including increased expression of ET1, the renin–angiotensin system, IL-6, TNFα, and iNOS [8]. Moreover, junctional disruption and β-catenin nuclear activity are key steps in EndoMT, the transdifferentiation of endothelial cells to a mesenchymal cell phenotype [8]. EndoMT contributes to generation of myofibroblasts and fibrosis in SSc [71–73]. Elevated nuclear levels of β-catenin are observed in multiple cell types in SSc skin and lung tissue [6,74–77]. However, because of β-catenin's pro-fibrotic activity in (myo)fibroblasts, analysis has so far been concentrated on these cells [6,74–77].

Therefore, junctional signaling by VE-cadherin clustering or Angpt1:Tie2 activity maintains vascular health by inhibiting inflammatory activity, thrombosis, oxidant stress and apoptosis, and by preserving the physiological regulation of nutritional blood flow. In contrast, disruption of junctional signaling will not only diminish this protective activity, but will increase the destabilizing activity of FOXO1, YAP, EZH2, and β-catenin to promote a proinflammatory, prothrombotic, and fragile vasculature that is unable to adequately regulate blood flow [8,9] (Fig. 1). Importantly, SSc endothelial cells have reduced Akt activity [78], consistent with decreased junction-based signaling from VE-cadherin and Angpt1:Tie2, and Angpt2 antagonism of Tie2. Diminution in junctional signaling would be expected to precipitate many of the key pathological mechanisms and clinical features of SSc and contribute significantly to pathological progression of the disease process.

MECHANISMS UNDERLYING DISRUPTION IN VE-CADHERIN JUNCTIONAL SIGNALING IN SCLERODERMA

Mechanism(s) responsible for decreasing VE-cadherin and disrupting endothelial junctions in SSc have not been defined. Inflammatory mediators target VE-cadherin predominantly by increasing its phosphorylation, internalization, and degradation, with key roles for Src and RhoA/ROCK signaling [8]. The relative balance between RhoA/ROCK-mediated inhibition and PI3KAkt-mediated augmentation of adherens junctions determines VE-cadherin activity and junctional status during early development [8]. This balance appears to reset toward junctional disruption in SSc endothelium with increased RhoA/ROCK and decreased Akt signaling [78,79]. Inflammatory and fibrogenic mediators known to have increased activity in SSc could contribute to VE-cadherin degradation and junctional disruption, including Angpt2, oxidant stress, thrombin, IL-4, and transforming growth factor beta (TGFβ) [6,8,80–83]. Canonical Wnt signaling, which is hyperactivated in SSc [75–77], also causes endothelial dysfunction, oxidant stress, and junction destabilization [84–86]. Reduced VE-cadherin levels may also reflect reduced expression, for example by decreased activity of KLF2/4. Also, expression of Fli1, which promotes VE-cadherin expression, is reduced in SSc endothelium [19,71,87]. Endothelial Fli1 deficiency causes diminished expression of VE-cadherin, Tie2, PDGFB, and the sphingosine 1-phosphate type 1 receptor, S1P1 [19,87]. These latter mediators are important in promoting endothelial-pericyte interactions, and S1P1 and Tie2 signaling are also important in promoting VE-cadherin clustering at endothelial junctions [8,19,87]. Not surprisingly, endothelial Fli1 deficiency is associated with increased vascular permeability, pericyte loss, and capillary dilatation [19].

SCLERODERMA VASCULOPATHY AND ACCELERATED AGING

Fibroblast senescence and accelerated aging may contribute to SSc fibrosis [6,7▪▪,88]. There are also remarkable parallels between the vasculopathy of SSc and vascular aging. Aging causes widespread destabilization of the vasculature, ranging from microvascular rarefaction and impaired angiogenesis to intimal lesions in proximal arteries [8,89]. VE-cadherin levels are decreased in aging arteries and microcirculation, which is associated with junctional disruption and increased permeability [24,35]. This reflects oxidant-induced, Src-dependent degradation of VE-cadherin [24,80]. Angpt2 expression is also increased in aging microvascular endothelium, which is associated with pericyte loss, dilated and abnormally shaped

capillaries, and capillary rarefaction [35,90]. Dissociation of capillary pericytes is accompanied by their differentiation to myofibroblasts and tissue fibrosis [90]. Aging endothelial cells are chronically stressed, producing and responding to pathological mediators, resulting in endothelial frailty and senescence that are important instigators of vascular aging [8]. Endothelial levels of protective antiaging mediators including histone deacetylase sirtuin 1 (SIRT1) are decreased [91]. SIRT1 expression and activity is also reduced in SSc, although because of its antifibrotic activity, analyses have focused on fibroblasts [6,92]. In endothelial cells, SIRT1 exerts important protective activity including inhibiting FOXO1, increasing KLF2/4 expression, inhibiting NFκB, and activating eNOS [91,93]. Therefore, decreased endothelial SIRT1 activity could amplify vascular dysfunction in SSc. Gastrointestinal microbial dysbiosis may contribute to aging-induced vascular deterioration, through increased intestinal permeability and entry of microbial products [94]. Although gastrointestinal involvement is an early feature of SSc [3,4▪▪], the role of microbial dysbiosis has not been defined [95▪▪].

MICROVASCULAR PERMEABILITY AND INTERSTITIAL FIBROSIS

Increased vascular permeability and edema are very early features of SSc that precede tissue fibrosis [11–15]. Increased microvascular permeability is considered an important component of fibrogenic responses [14], and the onset of interstitial fibrosis is often accompanied by microvascular disease [96]. This could reflect the generation of fibrogenic mediators following decreased VE-cadherin and Angpt1:-Tie2 junctional signaling [8]. In addition, reduced Angpt1:Tie2 signaling causes pericyte loss from capillaries, which can contribute to fibrosis by differentiating to myofibroblasts [39,90,96], while at the same time leaving vulnerable capillaries prone to instability [96]. Indeed, loss of capillaries occurs in association with progression of fibrosis [39]. Moreover, junctional disruption can promote EndoMT and further generation of myofibroblasts. The link between vascular permeability and interstitial fibrosis may also be more direct. For example, increased interstitial flow created by increased permeability and swelling can stimulate fibroblast-to-myofibroblast differentiation [97]. Interstitial flow is increased in SSc [16,17].

Regardless of the mechanistic linkage between junctional disruption/vascular permeability and interstitial fibrosis, interventions aimed at restoring normal junctional signaling would be expected to decrease permeability and reduce fibrosis. Indeed, in preclinical models, increasing endothelial Tie2 activity or VE-cadherin signaling reduced tissue fibrosis, which was associated with decreases in fibrogenic mediators and myofibroblast content [37,98–102]. In contrast, enforced reductions in endothelial Tie2 activity or VE-cadherin increased fibrosis [38,39,101].

VASCULAR STABILIZATION AS VASCULAR THERAPY

Therapeutic amplification of endothelial junctional signaling by VE-cadherin and Tie2 should be a powerful mechanism to restore protective signaling and vascular stability, and prevent initiation and progression of vascular diseases, including SSc [8,103]. Indeed, molecular and pharmacological strategies to increase endothelial junctional signaling have shown promise in numerous preclinical disease models, preventing pathological changes in vascular dynamics, vascular and tissue remodeling, edema, inflammation, and organ function, and reducing mortality [8,10▪,27,28,34,104–109].

The approach could be targeted to individual pathological mechanisms (Fig. 1) following confirmation of their involvement in SSc. Indeed, some of these potential mechanisms are being considered as antifibrotic targets in SSc, including Rho/ROCK, YAP, oncostatin M, thrombin, Wnt, β-catenin, and Toll-like receptor 4 (TLR4) [6,7▪▪]. However, the most effective approach will likely be direct amplification of junctional VE-cadherin and/or Tie2 signaling. This was highlighted by the action of iloprost to restore junctional clustering of VE-cadherin in SSc endothelial cells, which was accompanied by improved barrier function and angiogenic activity, and a reduction in EndoMT [41▪▪]. However, it remains unclear if this protective activity of iloprost can be achieved during clinical therapy [41▪▪]. Therefore, direct modulation of these processes, for example blocking Angpt2 or activating Tie2 (Angpt1 and mimics) should be more successful [10▪,27,28,34,37,38,63,98–102,104–106]. A highly promising target is vascular endothelial protein tyrosine phosphatase (VE-PTP).

VE-PTP is selectively expressed in endothelium and reduces tyrosine phosphorylation of Tie2, which inhibits its activity, and VE-cadherin, which prevents its internalization [110▪▪,111]. VE-PTP inhibition stabilized endothelial junctions and blocked vascular leak caused by Angpt2 or inflammatory mediators [110▪▪]. However, after Tie2 gene deletion, the effect was reversed to junction destabilization [110▪▪]. Therefore, beneficial effects of VE-PTP inhibition on Tie2 override its potential direct negative impact on VE-cadherin. Importantly, VE-PTP inhibition, by decreasing the threshold for Tie2 activation, converts Angpt2 from a Tie2 antagonist (and poor partial

agonist [103]) to an effective agonist [112]. Therefore, by increasing basal and Angpt1-dependent Tie2 activity and by converting Angpt2 from antagonist to agonist, VE-PTP inhibition is a highly appealing therapeutic strategy to improve endothelial junctional signaling. Indeed, in preclinical studies of renal diabetic injury, VE-PTP inhibition increased Tie2 and Akt activity, caused nuclear exclusion of FOXO1 and decreased expression of FOXO1 target genes, and reduced renal injury and fibrosis [111]. In addition, VE-PTP inhibition increased blood flow, in association with eNOS phosphorylation [111]. VE-PTP inhibition also stabilized the ocular vasculature in preclinical studies and is being assessed in clinical trials for treatment of diabetic macular edema [110▪▪].

Endothelial junctional signaling is an amplification nexus for both protective and pathological signaling [8]. For example, junctional signaling by VE-cadherin and Tie2 activates PI3K/Akt to promote junctional stability and further protective signaling [8,83]. In contrast, pathological mediators disrupt junctional signaling resulting in destabilizing activity (e.g., FOXO1, YAP) and expression of mediators (e.g., Angpt2) that sustain junctional and vascular disruption. By interrupting such pathological amplification, protection of endothelial junctions may reset endothelial and vascular homeostasis toward protective signaling and vascular stability. Indeed, short-term or pulsed treatment may provide long-lasting benefit, as has been suggested for the therapeutic effects of iloprost and prostanoids [41▪▪,113].

Confirmation of specific mechanisms underlying vascular destabilization in SSc (Fig. 1) may identify important biomarkers for use in clinical analyses. Moreover, because endothelial barrier protection represents a fundamental role of junctional signaling, clinical analyses would benefit from directly assessing vascular permeability, for example using albumin-binding contrast agents [14] or fluorescent dyes [16,17]. Although nailfold videocapillaroscopy is a key component of SSc diagnostics, it provides only limited structural information [1▪,114▪▪]. Because changes in vascular function generally precede structural alterations, increased functional analysis, in particular endothelial junctional signaling, should provide much earlier insight into disease progression or diagnosis, and the response to therapeutic interventions.

Stabilizing junctional signaling will also protect nutritional blood flow in SSc. Analyzing nutritional blood flow in hands or digits is challenging because of a high density of arteriovenous anastomoses (AVAs), which dominate cutaneous blood flow without contributing to nutritional blood flow [115]. Although there are similarities in regulatory mechanisms of AVAs and nutritional arterioles, there are also remarkable differences [115]. For example, isoproterenol increases finger blood flow solely by dilating AVAs without affecting nutritional blood flow [116]. To be clinically relevant, assessment of cutaneous blood flow must be able to distinguish nutritional blood flow from AVA-dependent flow. Approaches such as thermography, laser Doppler imaging lack that resolution, and laser speckle contrast imaging can also be impacted by AVA flow [117–119]. Restricting analysis to dorsal surfaces, with their reduced AVA density, is not devoid of AVA interference, which may reflect extensive connections between palmer and dorsal circulations. The introduction of an automated quantitative approach to quantify changes in structure and perfusion of SSc nailfold capillaries could represent a highly significant advance to assess the nutritional vasculature [114▪▪,120]. Additional approaches include optical coherence tomography-based techniques, which have been used to provide remarkable high-resolution images of capillary morphology and quantitation of their perfusion [121,122▪▪,123,124].

CONCLUSION

SSc is associated with reduced activity of VE-cadherin and Tie2 in microvascular endothelial cells. These endothelial junctional mediators have key roles in maintaining vascular stability and normal physiological function, whereas chronic disruption in their activity can promote pathological changes in vascular function and structure, and threaten the integrity of the vasculature. Indeed, the vasculopathy of SSc likely reflects a chronic state of endothelial junctional destabilization that contributes to disruption of normal function and structure including altered hemodynamics and impaired nutritional blood flow, microvascular rarefaction and impaired angiogenesis, vascular lesion development, and intravascular thrombosis. This vascular destabilization contributes to some of the earliest features of the disease and is likely a key stimulus for progression of the disease including organ injury and interstitial fibrosis. Targeted therapy to restore this protective endothelial junctional signaling should be a highly effective approach to combating progression of the disease.

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Conflicts of interest

There are no conflicts of interest.

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Biomarkers in systemic sclerosis: mechanistic insights into pathogenesis and treatment

Joseph R. Arron

Purpose of review
Systemic sclerosis (SSc) is heterogenous on molecular, cellular, tissue, and clinical levels. Although many biomarkers have been described in clinical studies, few have been rigorously mapped to specific molecular pathways, tissue pathologies, and clinical manifestations. A focused assessment of peripheral blood levels of C–C Motif Chemokine Ligand-18 (CCL18) and periostin illustrates how biomarkers can link molecular mediators to clinical outcomes.

Recent findings
CCL18 is produced by pulmonary macrophages in response to type 2 cytokines and IL6. Elevated serum CCL18 is associated with interstitial lung disease (ILD) in SSc patients and is prognostic for ILD progression. It is pharmacologically modulated by IL6 inhibition, and associated with stabilization of lung function decline but not with improvements in skin fibrosis. Periostin is produced by dermal fibroblasts in SSc in response to type 2 cytokines and transforming growth factor-beta. Elevated serum periostin is associated with cutaneous disease in SSc patients but not ILD. Other cell- and tissue-specific biomarkers detectable in peripheral blood and informative with respect to SSc pathogenesis include KL-6 and SP-D in lung epithelium, osteopontin in lung macrophages, and cartilage oligomeric matrix protein in dermal fibroblasts.

Summary
Blood biomarkers related to specific molecular mediators, cell types, and tissues of origin can help to link therapeutic targets to treatable traits in SSc.

Keywords
biomarker, C–C Motif Chemokine Ligand-18, periostin, systemic sclerosis

INTRODUCTION

A biomarker can be defined as any objectively measurable attribute that provides information about a physiological state. In systemic sclerosis (SSc) patients, commonly used types of biomarkers may include: clinical symptom and/or functional metrics such as the Modified Rodnan Skin Score (MRSS), Combined Response Index in SSc, St. George's Respiratory Questionnaire, spirometry, or diffusing capacity; clinical imaging modalities such as high-resolution chest computed tomography (CT) or skin ultrasonography; cellular indicators such as the composition of peripheral blood or tissue biopsies; or molecular markers such as germline genetics, gene expression levels, or proteins measured in tissue by histology or secreted into biological fluids [1]. Although several of these attributes will be considered, the main focus of this review will be on recent insights derived from selected molecular biomarkers, how they relate mechanisms of disease pathogenesis to clinical attributes of SSc in observational and interventional clinical studies, and projections of how these insights can be extrapolated more broadly.

TYPES AND USES OF BIOMARKERS

Biomarkers can be used in five main ways: diagnostic, prognostic, predictive, pharmacodynamic, and surrogate [2]. Diagnostic biomarkers are assessed at a given point in time to establish a diagnosis of a disease; particular autoantibody patterns (e.g., antinuclear, antitopoisomerase, anticentromere, etc.) have long been associated with subtypes of SSc and are part of its diagnostic workup [3]. Prognostic

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KEY POINTS

- Systemic sclerosis (SSc) is heterogeneous; biomarkers can provide insight into molecular, cellular, and tissue-specific manifestations of the disease.
- In SSc, blood levels of CCL18 correspond to IL6-driven macrophage activity and interstitial lung disease, whereas blood levels of periostin correspond to TGFβ-driven fibroblast activity and skin fibrosis.
- Prognostic biomarkers are useful for stratifying patients by treatable traits in clinical studies.
- Pharmacodynamic effects of different interventions provide insights into mechanisms of action.
- A pharmacodynamic effect on a prognostic biomarker has the potential to be developed into a surrogate biomarker in future studies.

biomarkers are assessed at a given point in time and are used to forecast future changes in clinical manifestations of disease, e.g., elevated levels of a biomarker associated with lung epithelial damage may be associated with an increased likelihood of decline in lung function over the subsequent 12 months. Predictive biomarkers are assessed at a given point in time and can inform on the likelihood of a clinical response to a particular intervention, e.g., a gene signature associated with activity of the profibrotic cytokine in skin biopsies may identify patients with a greater likelihood of improved MRSS upon treatment with a pharmacologic inhibitor of that cytokine. Pharmacodynamic biomarkers are assessed at multiple points in time and change with treatment, indicating that a particular intervention has engaged its target and is exerting biological effects; a pharmacodynamic biomarker may simply indicate that a molecular pathway has been engaged or it may also read out downstream physiological effects of engaging that pathway [4]. Surrogate biomarkers can be thought of as a combination of prognostic and pharmacodynamic biomarkers, in that a short-term treatment-induced change in a prognostic biomarker may be associated with a long-term improvement in how a patient feels, functions, or survives [2], e.g., decreased hemoglobin A1C levels with glycemic control agents are associated with reduction in microvascular complications in patients with diabetes [5]. A given biomarker may have utility for one or more of these five attributes. Although autoantibody patterns are a useful part of the diagnostic criteria in SSc [3], the remainder of this review will focus on how selected biomarkers *within* the population of patients carrying a clinical SSc diagnosis can be used to understand the relationships between molecular mediators, cellular and tissue pathology, and clinical manifestations.

RECENT BIOMARKER INSIGHTS IN SYSTEMIC SCLEROSIS: C–C MOTIF CHEMOKINE LIGAND-18 AND PERIOSTIN

SSc is a complex and heterogeneous disorder, presenting with a pathologic triad of autoimmunity, vasculitis, and tissue fibrosis manifesting clinically in the skin in the vast majority of patients and variably progressing to other tissues such as lung and kidney in subsets of patients [6]. Recent clinical studies have identified several biomarkers that have promise for understanding disease pathogenesis, prognosis, and treatment for SSc patients [7]. Because SSc is a multiorgan disease involving the interplay between numerous cell types, insights into the tissues and cells from which soluble systemic biomarkers are produced and the stimuli that produce them have shed light on disease mechanisms. Importantly, while many therapeutic targets are measurable, biomarkers that reflect the activity of those therapeutic targets on specific cells in specific tissues may ultimately be more informative than the targets themselves. For example, whereas transforming growth factor-beta (TGFβ) is widely implicated as a pro-fibrotic cytokine involved in SSc [8], it has multiple other functions and is produced in an inactive 'latent' form, often attached to cell surface proteins and/or extracellular matrix components, which can in turn be activated via various context-dependent methods that differ across TGFβ isoforms, cell types, and tissues [9]. Simply measuring TGFβ protein or gene expression levels in a biological matrix may therefore be substantially less informative as to its functional relevance than a biomarker that is induced as a direct consequence of TGFβ receptor signaling in a specific cell type relevant to disease biology. Rather than provide a compendium of reported biomarkers in SSc patients (recently reviewed in [1]), we will focus primarily on two selected biomarkers, C–C Motif Chemokine Ligand-18 (CCL18) and periostin, which have been informative into SSc pathogenesis and treatment in their own right, but which also serve as examples of a more comprehensive approach to identifying and interpreting multiple nuanced facets of biomarkers in SSc that can be applied more broadly.

CCL18 and periostin provide clear illustrations of how biomarkers reflecting the activity of candidate therapeutic targets on specific cells in specific tissues can be useful and informative. CCL18 is an orphan chemokine with no murine ortholog, thus understanding its function has been elusive [10]. Serum or plasma CCL18 levels are elevated in SSc

patients with ILD, and in multiple studies, higher levels of CCL18 at a given point in time have been associated with subsequent declines in lung function and ILD-related mortality [11,12▪▪,13–18]. This prognostic property for CCL18 as a biomarker of progressive ILD is not confined to SSc; similar effects are seen in patients with idiopathic pulmonary fibrosis (IPF) [19]. Periostin is a matricellular protein found in connective tissues [20]. Peripheral blood periostin is elevated in patients with both diffuse and limited cutaneous SSc, independent of evidence of ILD. Moreover, serum periostin levels are strongly and continuously correlated with MRSS, thus they may serve as a proxy for the extent of active cutaneous disease [21]. Interestingly, while certain lung conditions such as asthma and IPF are associated with elevated serum periostin, they present with mild elevations, typically up to less than 20% above levels seen in healthy controls [22]. However, in SSc patients, periostin levels may be up to 3-fold higher than those seen in healthy controls [21]. Thus, while both CCL18 and periostin are often found at increased levels in peripheral blood of SSc patients, they are related to different tissues of origin and different clinical manifestations.

Probing deeper into CCL18 and periostin as SSc biomarkers, there is clear evidence that they are produced by different cell types. CCL18 expression is largely restricted to hematopoietically derived cells of the myeloid lineage, and is particularly highly expressed in monocyte-derived macrophages in the lung in chronic respiratory diseases [10]. It has been implicated as a marker of 'alternatively activated' or 'M2' macrophages that can be induced by exposure to type 2 cytokines such as interleukin-4 (IL4) and IL13 [23]. However, recent single-cell transcriptomic studies of primary myeloid cells in diseased tissue have suggested that the 'M1/M2' paradigm for macrophages is overly simplistic and largely based on in vitro studies; populations of CCL18+ macrophages have been identified in multiple pulmonary disorders [24▪,25▪,26▪]. Intriguingly, while IL6 itself does not induce substantial CCL18 production from undifferentiated macrophages, it 'hyperpolarizes' macrophages that have been treated with type 2 cytokines [27▪]. Periostin is not expressed in hematopoietically derived cells; rather its expression is highest in mesenchymal cells such as fibroblasts and osteoblasts, with lower expression in some epithelial cells [20]. Like CCL18, periostin expression can be regulated by type 2 cytokines, but it is also induced by TGFβ in mesenchymal cells and may synergize with TGFβ to induce extracellular matrix production [28,29]. Therefore, elevated CCL18 levels may indicate activity or numbers of a particular subset of macrophages, whereas elevated periostin levels may indicate activity or numbers of fibroblasts in tissues of SSc patients. Furthermore, as detailed above, in SSc patients, CCL18 may inform on macrophage biology in the lung whereas periostin may inform on fibroblast activity in the skin.

CCL18 and periostin have been evaluated as pharmacodynamic biomarkers in recent studies of tocilizumab, a monoclonal antibody against IL6R that inhibits IL6 signaling, in SSc patients [30,31▪]. Unlike prior SSc therapies such as cyclophosphamide, mycophenolate, methotrexate, and nintedanib that have broad effects on multiple pathways, tocilizumab selectively targets IL6 and therefore offers an opportunity to more precisely dissect the effects of a single molecular target in a complex disease. In phase 2 and phase 3 studies, tocilizumab treatment failed to meet its primary outcome measure of significantly reducing MRSS in SSc patients, but it had a highly significant and clinically meaningful impact on lung function as measured by forced vital capacity (FVC) as compared to placebo-treated patients, wherein placebo-treated patients lost an average of 4.6% of FVC over the 48-week treatment period while tocilizumab-treated patients only lost 0.4% of FVC on average in the phase 3 study [31▪]. These results led to tocilizumab's approval for the treatment of SSc-associated ILD. Patients in the phase 2 study had active disease, with a mean baseline MRSS of 26, and had substantially elevated baseline levels of serum CCL18 and periostin, approximately 2–3-fold above the average levels observed in healthy controls. Tocilizumab treatment was associated with profound pharmacodynamic effects on serum CCL18, with levels reduced by more than 1/3 within 3 weeks of treatment [30]. However, despite substantially elevated baseline serum periostin, there was no pharmacodynamic effect of tocilizumab on periostin levels [30]. As CCL18 in SSc patients is associated with ILD and produced by lung macrophages, whereas periostin in SSc is associated with skin fibrosis and produced by dermal fibroblasts, these pharmacodynamic effects are consistent with the inference that IL6 contributes to the phenotype of 'fibrosis-associated' macrophages but less so to dermal fibroblasts, and may provide at least a partial mechanistic explanation of the discordant outcomes in lung and skin in these studies. On the other hand, romilkimab, a bispecific antibody against IL4 and IL13, resulted in a statistically significant reduction in MRSS compared to placebo and a reduction, albeit not statistically significant, in serum periostin levels [32▪]. There was a modest but not significant benefit on lung function; romilkimab previously failed to demonstrate benefit on lung function and had no

pharmacodynamic effect on serum periostin in an IPF trial [33]. Whereas tocilizumab treatment quickly and substantially reduced CCL18 levels in SSc patients, periostin levels in romilkimab-treated SSc patients remained elevated despite the modest pharmacodynamic effect, whereas effects on CCL18 were not reported. Taken together, these findings suggest that different molecular interventions may have different effects across clinical manifestations of SSc.

Although IL6 is a pleiotropic cytokine that has been implicated in multiple processes that could contribute to SSc pathophysiology [34], the pharmacodynamic and clinical effects of tocilizumab treatment in SSc patients have clarified the key mechanisms whereby IL6 drives SSc, and, just as importantly, mechanisms where it may not directly contribute to disease pathogenesis. These observations can help to identify additional molecular pathways that may contribute to the residual burden of disease in the context of IL6 inhibition. One pathway that appears to be a key contributor to fibrogenesis in the skin and other tissues is TGFβ [8]. TGFβ levels and activity are substantially upregulated in skin and lung of SSc patients with active disease, and TGFβ inhibitors have been evaluated in SSc. TGFβ activity on dermal fibroblasts leads to increased expression of periostin and cartilage oligomeric matrix protein (COMP) [28,35], among other markers of fibroblast activation and myofibroblast differentiation. In a small mechanistic study, patients with active cutaneous SSc were assessed via serial skin biopsies before and 3, 7, and 24 weeks after a single dose of 5 mg/kg or two doses of 1 mg/kg fresolimumab, a mAb that inhibits TGFβ [36]. Although it was not a placebo-controlled study, reductions in MRSS were observed, along with reductions in the expression of TGFβ-inducible genes in dermal fibroblasts such as COMP and SerpinE1 in skin biopsies. However, levels of CD163, corresponding to macrophages, were not significantly reduced in fresolimumab-treated patients, suggesting that TGFβ inhibition may have more of an effect on fibroblast biology than on myeloid cell biology in the specific context of SSc skin. Although not directly comparable, skin biopsies from tocilizumab-treated patients suggested a greater effect of IL6 inhibition on macrophage biology than on fibroblast biology in that tissue [30]. Unfortunately, as TGFβ exerts pleiotropic homeostatic functions, fresolimumab studies were not extended further, possibly due to on-target toxicity concerns of bleeding, anemia, and keratoacanthomas [36,37]. Emerging approaches to target TGFβ signaling more selectively to maximize therapeutic index are currently under investigation [38].

INSIGHTS FROM OTHER BLOOD BIOMARKERS IN SYSTEMIC SCLEROSIS

Among additional biomarkers related to distinct cell and tissue biology in SSc, Krebs von der Lungen-6 (KL-6) and surfactant protein D (SP-D) are expressed by alveolar epithelial cells and may be released into systemic circulation upon cellular injury or death. Elevated levels of both KL-6 and SP-D in peripheral blood have been associated with the presence and extent of ILD in SSc patients as well as in other interstitial lung diseases (ILD) [11]. Unlike CCL18 and periostin which are primarily produced by macrophages and fibroblasts in SSc, these biomarkers may inform on alveolar epithelium and thus capture a related but distinct aspect of SSc-ILD pathogenesis. Osteopontin (OPN/SPP1) is expressed in a number of cell types associated with fibrotic diseases but is particularly elevated in fibrosis-associated macrophages and can be induced by the presence of immune complexes, M-CSF, and IL6. Elevated OPN levels are associated with the presence of ILD in SSc patients and prognostic for lung function decline. OPN's expression patterns on a single cell level are overlapping, but not identical, with those of CCL18, and tocilizumab treatment is associated with pharmacodynamic effects on serum OPN levels [39]. Endothelial cell-derived biomarkers such as endostatin, endothelin, E-selectin, and vascular cell adhesion molecule have been associated with vascular manifestations of SSc such as pulmonary artery hypertension and renal crisis [1]. Taken together, these and other biomarkers may provide an integrated picture of the extent and type of tissue and organ involvement in SSc patients and careful assessment of the pharmacodynamic effects of various interventions on these biomarkers may help to further elucidate mechanisms of disease.

BENEFITS AND LIMITATIONS OF BLOOD BIOMARKERS

It is important to note several nuances that underlie the benefits and limitations of peripheral blood protein biomarker levels in SSc. First, as is it not always feasible to directly sample diseased tissue, a blood biomarker may integrate the total burden of disease across that tissue; however, few biomarkers are perfectly specific for a given stimulus, cell type, or tissue. Second, while strong pharmacodynamic effects of a specific molecular intervention on a given biomarker may confirm the relevance of that biomarker to the targeted pathway, many stimuli that could be active in disease may regulate its expression and an observed pharmacodynamic effect on a biomarker could be an indirect consequence of pathways downstream of the molecular

target. Third, most peripheral blood protein biomarkers observe a continuous normal or skewed-normal distribution in patients that often overlaps at least partially with the distribution observed in non-diseased control subjects, thus it may be counterproductive to apply an arbitrary cutoff value to declare a given patient as 'high' or 'low' for a biomarker, despite regulatory pressure to do so [40]. Finally, sample type, collection, and processing and assay detection reagents, standards, and instrumentation for most biomarkers discussed here have not been rigorously standardized, thus while comparing biomarker levels within a given study can be informative, it is dangerous to interpret 'absolute' biomarker values provided across studies where measurement techniques may not be identical. Overall, these concerns are a significant reason why few biomarkers have been validated for the diagnosis and treatment of SSc and incorporated into treatment guidelines [3]. However, these limitations should not be prohibitive to using biomarkers to elucidate mechanisms of disease pathogenesis, prognosis, and treatment response to improve the treatment and management of SSc patients.

CONCLUSION/FUTURE PERSPECTIVES

Biomarkers can yield insights linking molecular mediators, cellular targets, tissue pathology, and clinical manifestations in SSc. CCL18 is produced by fibrosis-associated macrophages in the lung in response to type 2 cytokines and IL6, and its systemic levels correspond to the extent and prognosis of ILD. Pharmacologic inhibition of IL6 exerts pharmacodynamic effects on CCL18 and clinical effects on lung function but not skin fibrosis. Periostin is produced by fibroblasts in response to type 2 cytokines and TGFβ and its systemic levels correspond to the extent of skin fibrosis in SSc (Fig. 1). Although prospective validation will be needed to substantiate the following proposition, it is possible to imagine a scenario wherein a biomarker like CCL18 could be used in future SSc patient management in multiple ways, e.g., prognostic for progressive ILD, predictive for benefit from IL6 inhibition, pharmacodynamically modulated in response to IL6 inhibition, and as a surrogate for lung function stabilization. In the near future, emerging technologies such as single-cell transcriptomics, proteomic methods, and quantitative clinical outcome measures along with

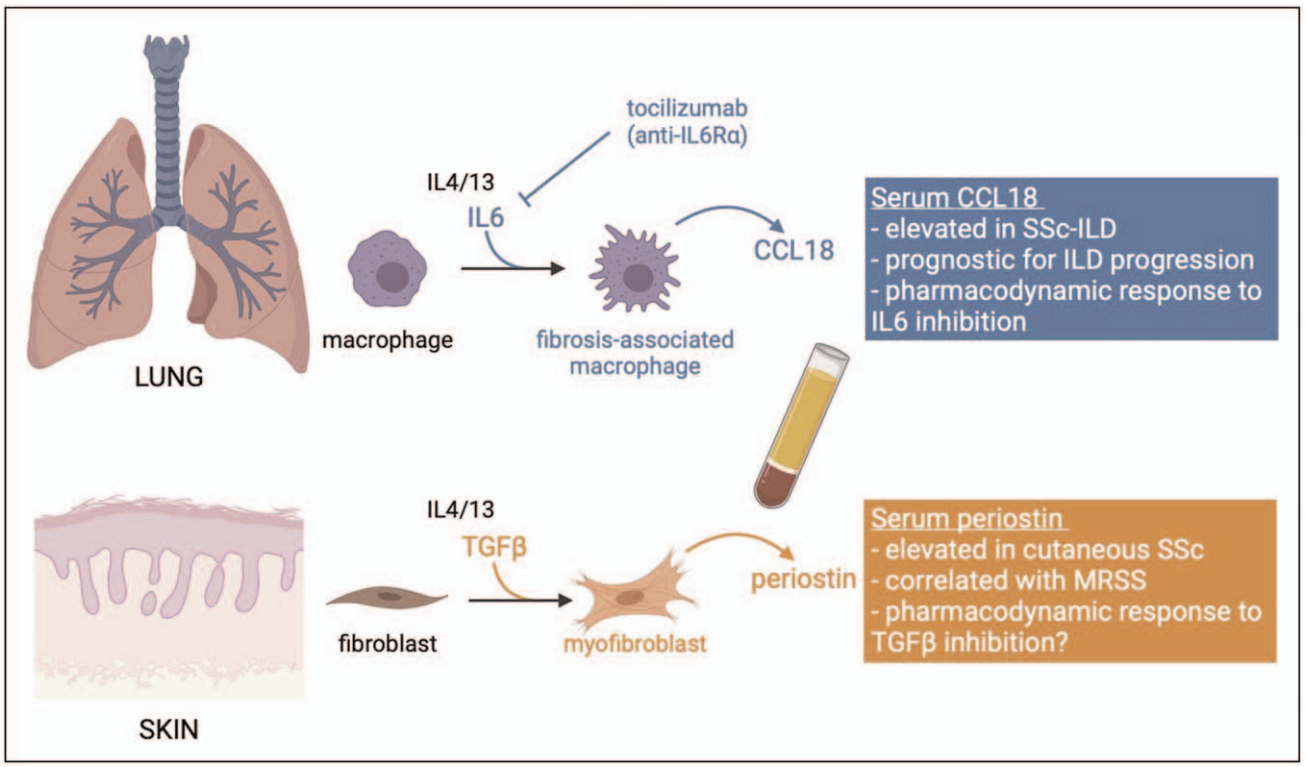

FIGURE 1. Examples of peripheral blood protein biomarkers in systemic sclerosis. CCL18 (top) is induced by type 2 cytokines and IL6 in fibrosis-associated macrophages in lung tissue. Periostin (bottom) is induced by type 2 cytokines and TGFβ in myofibroblasts in skin. Both biomarkers are soluble secreted proteins that can be readily detected in peripheral blood. Elevated levels of each biomarker relate to distinct molecular pathways, cellular sources, and tissue pathologies in SSc patients. Figure created using BioRender. C–C Motif Chemokine Ligand-18 (CCL18); SSc, systemic sclerosis; TGFβ, transforming growth factor-beta.

multiple new candidate therapeutics in clinical trials will present opportunities to use biomarkers to systematically dissect key pathogenic mechanisms and address evolving unmet needs in SSc.

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Conflicts of interest

J.R.A. is an employee of Genentech, Inc., holds stock/options in the Roche Group, and is a named inventor on patents pending related to biomarkers in fibrotic disorders.

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Insights into origins and specificities of autoantibodies in systemic sclerosis

Eleni Tiniakou, Jonathan Crawford, and Erika Darrah

Purpose of review
Autoantibodies are hallmark findings in systemic sclerosis (SSc), often present prior to disease onset. Clinical diagnosis and prognosis of SSc have long relied on the antitopoisomerase – anticentromere – anti-RNA polymerase antibody trichotomy. However, many more autoantibodies found in SSc are being actively investigated for insights into triggering events, mechanisms of tolerance break, and connections to tissue damage. This review examines recent studies on SSc autoantibodies and the early events that lead to their development.

Recent findings
Recent work has elucidated potential connections between human cytomegalovirus infection, silicone breast implants, and malignancy to SSc autoantibody development. At the level of the dendritic cell:T cell interaction, where tolerance is broken, new studies identified shared motifs in the peptide-binding domains of SSc-associated human leukocyte antigen alleles. Immunological analysis of SSc patient B cells has uncovered several anomalies in the regulatory capacities of SSc naïve and memory B cell populations. Expanding efforts to uncover new SSc autoantibodies revealed anti-CXCL4, anticollagen V, and other autoantibodies as potential players in disease pathogenesis.

Summary
Further research into the role of autoantibodies in SSc development may uncover new mechanism-guided therapeutic targets. In addition, a better understanding of autoantibody associations with SSc disease outcomes will improve clinical care.

Keywords
antigen processing, autoantibodies, B cells, human leukocyte antigen, systemic sclerosis/scleroderma

INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disease characterized by vasculopathy, fibrosis and immunologic derangements [1]. Autoantibodies have become a cornerstone for SSc diagnosis, prognosis and classification into clinically informative subsets. However, little is known about their origin and role in SSc pathogenesis. It was recently shown that autoantibodies are present in ~52% of SSc patients prior to the onset of symptoms [2], signifying the presence of unknown initiating events leading to the breach of immune tolerance to a specific set of autoantigens. In combination with the discovery of new autoantibody specificities, recent studies aimed at understanding the molecular basis of the immunogenetic risk in SSc could reveal important insights into the evolution of the immune response, starting from the processing of autoantigens by dendritic cells (DCs), to the role of B cells, and culminating with the development of autoantibodies and clinical presentation of SSc. The role of this review is to examine recent studies that focus on SSc antibodies with an emphasis on the early events and upstream factors that lead to their development (Fig. 1).

TRIGGERS/INITIATING EVENTS

Infectious agents/pathogens

Most SSc autoantigens are ubiquitous proteins with homologs in diverse microbial commensals and infectious agents. The molecular mimicry hypothesis suggests that T or B cells specific for pathogen-

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KEY POINTS

- Recent work has elucidated potential relationships between triggering events and SSc autoantibody development: HCMV infection with ARA and ACA autoantibodies; silicone exposure with ATA autoantibodies; and malignancy with ARA and anti-Th/To antibodies.
- A shared motif in the peptide-binding groove of diverse SSc-associated HLA-DR variants may drive the presentation of common topoisomerase epitopes in ATA-positive SSc.
- Multiple B cells populations have been found to be overexpressed in SSc, which have altered immunologic and signaling profiles consistent with a less regulatory phenotype
- New autoantibody specificities, including Th/To, vinculin, gAchR, CXCL4, CXCR3, CXCR4, and collagen V have been found in SSc patients, each potentially offering novel clinical and mechanistic insights.

derived antigens could potentially cross-react with self-antigens, leading to the development of autoimmunity [3]. In SSc, the discovery of higher levels of antibodies against human cytomegalovirus (HCMV) proteins in SSc compared to healthy controls has led to further investigation of a possible association between anti-HCMV immune responses and SSc [4,5]. When comparing the repertoire and magnitude of antibodies against different CMV proteins between patients with SSc and control groups (healthy controls and patients with multiple sclerosis), a potential association of anti-UL83 and UL44 antibodies with SSc autoantibodies (anti-RNA polymerase III(ARA) and anticentromere (ACA) frequency, and ACA levels) was unveiled [6], as well as an association of anti-UL83, UL-44 and p38 with anti-Ro52 positive SSc [7]. Previous epidemiologic data had also demonstrated a connection to *Helicobacter pylori*, but this was not verified in a recent study, possibly due to the fact that the comparator controls had a much higher than expected prevalence of *H. pylori* infection [8].

Recently, the largest ever cohort of African American patients with SSc demonstrated that specific *HLA* alleles are strongly associated with distinct SSc autoantibodies [9▪▪]. The authors went on to identify potential epitopes from known SSc autoantigens (i.e. topoisomerase I, fibrillarin, and centromere) with high predicted binding affinity for these SSc-associated human leukocyte antigen (HLA) class II variants and bioinformatically investigated which proteins from microorganisms had homologous sequences. They observed significant homology with viruses of the *Mimiviridae* and *Phycodnaviridae* families, which are known to infect amoeba and algae, respectively, but not humans [9▪▪]. Although these associations and the molecular mimicry hypothesis are intriguing, it remains to be demonstrated how these infectious agents could initiate immune responses in humans leading to the breach of tolerance to SSc autoantigens.

Environmental factors (silica, silicone breast implants, smoking)

Several epidemiologic studies have identified environmental factors linked to the development of SSc. Silica exposure has been identified as a potential risk factor for SSc since the beginning of the twentieth century, identified in multiple cohorts across the world [10,11]. A recent study from Australia reiterated this association, especially in SSc patients with antitopoisomerase-1 (ATA) autoantibodies [12]. With the increasing use of silicone breast implants, it has been postulated that silicone could also lead to an increase in autoimmunity by creating a chronic inflammatory state and/or functioning as an adjuvant, thus lowering the threshold for the loss of self-tolerance. Separate case studies from Italy confirmed a higher frequency of silicone breast implants [13] and a higher risk for rupture [14] specifically in patients with ARA-positive SSc. Although smoking has been identified as a potential trigger for autoimmunity in general [15], and a risk factor for worse prognosis in patients with SSc [16], ATA were found to be negatively associated with smoking (OR 1.77, 95% CI 1.04–2.99, $P = 0.034$) [17]. This is an unexpected finding of a detrimental environmental factor acting as a protective influence against developing specific autoantibodies in SSc and merits further evaluation.

Cancer

Multiple studies have confirmed a temporal association between cancer and scleroderma emergence, especially in patients with ARA [18]. The hypothesis that malignancy can be a trigger for SSc has been further solidified by the demonstration that antigen-specific CD4+ T cells against mutated RNA polymerase III (POLR3) found in the tumor can cross-react to the wild type POLR3 autoantigen in patients with ARA-positive SSc [19]. Interestingly, it was recently found that amongst SSc patients with ARA, those without cancer were more likely to have additional autoantibodies against another member of the RNA polymerase complex (POLR1) [20]. This study suggests that a broad autoimmune response

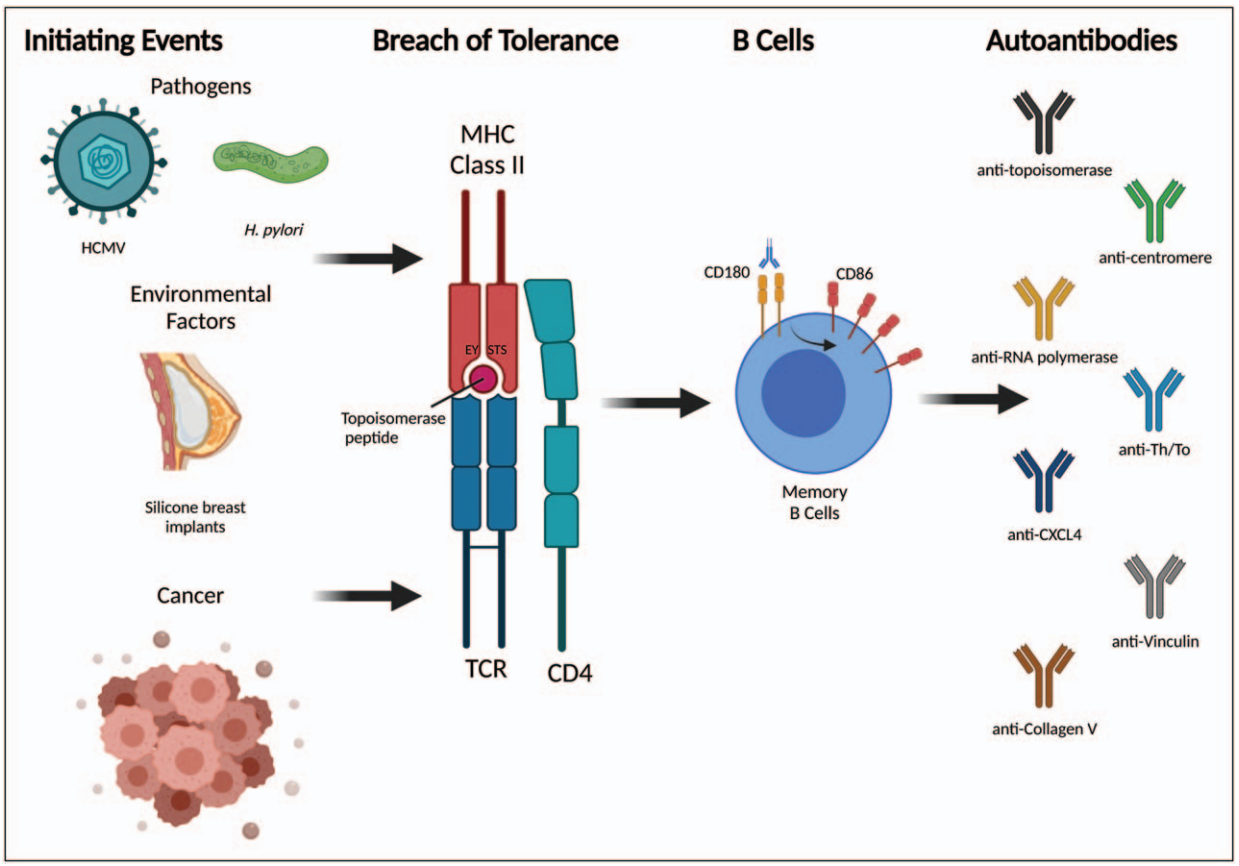

FIGURE 1. Overview of autoantibody development in SSc. Viral infections, silica, and malignancy have recently been studied as potential triggers for autoantibody development [4–8,10–14,18–20,21▪]. These initiating events lead to a breach of tolerance at the level of DC:T cell interactions, which may be influenced by SSc-associated motifs in the DRB1 peptide-binding groove [26▪▪,27]. Downstream of the initial breach of tolerance, B cells coordinate the autoimmune response as antigen-presenting cells and as producers of autoantibodies, and recent work explores alterations in the signaling profiles and regulatory capacity of multiple B cell subsets [26▪▪,30,31▪]. Finally, newly-discovered autoantibodies may have predictive value or be involved in disease pathogenesis [35,36,43–53]. SSc, systemic sclerosis.

against RNA polymerase subunits could represent a successful antitumor immune response. Similarly, anti-Th/To antibodies seemed to exert a protective role against the emergence of malignancy [21▪], implicating tumorigenesis as a trigger for multiple SSc autoantibodies.

The strong correlation of autoantibody specificity in SSc with cancer raises the question of whether these autoimmune responses represent a universal reaction to malignancy regardless of the clinical manifestations of an autoimmune disease. Interestingly, in a study of people without SSc, antinuclear antibodies (ANA) with a nucleolar pattern exhibited an increased relative risk (RR 1.5, 95% CI 1.03–2.3) for cancer, especially in the presence of ATA and ARA [22▪]. Although a more targeted study of patients without SSc who had breast cancer failed to detect the presence of ARA [23], this could be limited by the small size of the cohort and further studies are warranted.

EARLY EVENTS AND BREACH OF TOLERANCE

Given the findings that immune tolerance is broken to autoantigens years to decades before the onset of clinical symptoms [2], defining early events leading to the initiation of autoimmunity has been challenging. Recent attempts to define the earliest steps in the development of immune responses in SSc have focused largely on the role of DCs, their interactions with T cells, and potential roles of these T cells in disease pathogenesis [24]. Ultimately, autoantibody development can be traced back to the DC:T cell interaction that gives rise to autoreactive CD4+ T helper responses that promote autoantibody

secretion by autoreactive B cells. This is exemplified in a study in which repeated administration of DCs pulsed with topoisomerase peptides in mice, induced the production of ATA, as well as inflammation and fibrosis in the skin and lungs [25].

Despite the central role of DCs in sampling antigens from the extracellular environment and presenting selected peptides in the grove of HLA class II molecules to CD4+ T cells to initiate immune responses, little is known about the processing and presentation of SSc autoantigens. A recent study examined the processing and presentation of topoisomerase by monocyte-derived DCs using a novel natural antigen processing assay [26▪▪]. This study revealed that despite having a diverse array of *HLA-DRB1* alleles, patients with ATA-positive SSc, presented the same core set of immunogenic topoisomerase peptides. These peptides stimulated CD4+ T cells, and the breadth of the T cell response to multiple epitopes was associated with the severity of interstitial lung disease. Further analysis revealed that this phenomenon was driven by unique features present on both sides of the peptide:HLA-DR interaction. Common motifs were found to be present within the peptide binding groove in the HLA-DRβ chain (i.e. EYSTS/GE and FLEDRRAA/L), which presumably allowed binding of the common set of peptides (Fig. 2). In addition, while some topoisomerase peptides were predicted to bind in the same register to all HLA variants, another set was predicted to possess the ability to bind in different registers to different HLA-DRs. In a different study, *in silico* molecular modeling was utilized to examine the molecular dynamics of the topoisomerase peptide:HLA-DR interaction [27]. Here, it was observed that HLA-DR variants linked to SSc were predicted to bind a topoisomerase peptide shown to contain an ATA epitope with high affinity that resulted in an energetically stable peptide:HLA complex. In contrast, in a variant not associated with SSc, the topoisomerase peptide was predicted to be more flexible and mobile.

Interestingly, both studies suggested novel HLA-DR variants that have not previously been associated with SSc as having novel features in their peptide binding grooves that may promote the presentation of topoisomerase peptides [26▪▪,27]. In line with this, recent evidence suggests that similar principles may also be operative for additional HLA class II molecules, including specific HLA-DP variants [28]. A study evaluating the amino acid sequences in the peptide binding groove of HLA-DP molecules in patients with ATA-positive SSc found that variants that harbored more negatively-charged amino acid triplets were more strongly associated with disease and were predicted to bind more stably to a positively-charged topoisomerase peptide. The phenomenon of distinct HLA variants with common sequences in their peptide binding grooves associating with the development of an autoimmune response has been noted in rheumatoid arthritis since 1987 when it was dubbed the 'shared epitope'

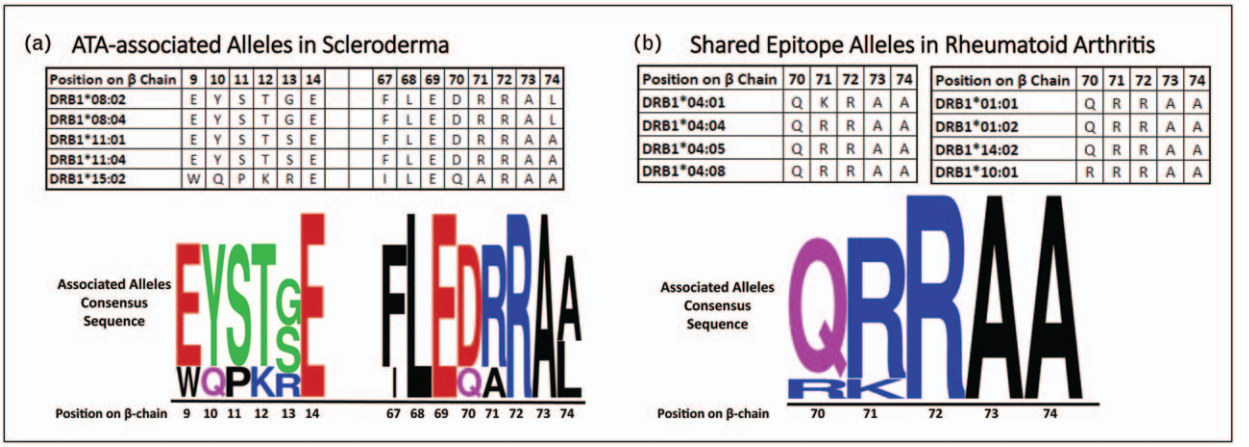

FIGURE 2. Common motifs in SSc-associated HLA-DRB1 alleles are reminiscent of the RA-associated shared epitope. (a) Two recent studies have suggested that common motifs in the β-chain of the peptide-binding groove encoded for by HLA-DRB1 alleles associated with SSc may play an important role in autoantibody development. (b) In rheumatoid arthritis, a group of distinct HLA-DRB1 alleles have been identified that contain a shared amino acid sequence in the β-chain of the peptide binding groove, termed the 'shared epitope' (SE). SE alleles are implicated in RA pathogenesis, particularly in individuals that make autoantibodies to citrullinated proteins. The parallels between antibody-associated HLA-DRB1 variants in RA and SSc suggest the existence of an ATA-associated SE in SSc and perhaps a similar paradigm in other autoantibody-subsets in SSc. ATA, antitopoisomerase; SSc, systemic sclerosis.

(SE) hypothesis (Fig. 2). Together, these studies suggest that a deeper analysis of HLA variants, characterizing common motifs in their peptide binding grooves and their presentation of autoantigenic peptides in SSc, may identify SSc-associated SE variants, and offer unique insights into disease initiation.

B CELLS

Since the two main immunologic roles of B cells are to make antibodies and act as antigen presenting cells (APCs), new insights into B cells subsets and effector functions in SSc may shed light into disease pathogenesis. This is particularly important since, unlike other APCs, B cells primarily and efficiently present specific antigens taken up via binding to their antigen-specific B cell receptor (BCR) [29]. Therefore, B cell populations found differentially expressed in SSc may have direct roles in orchestrating the antigen-specificity of the immune response in SSc.

B cells from SSc patients with diffuse skin disease have recently been shown to express lower levels of the toll-like receptor (TLR) homologue CD180 than healthy donor B cells at both the protein and transcriptional levels [30]. This was most pronounced in the CD27−IgD− and CD27−IgD+ naive B cell populations [31▪]. Although CD180 is a negative regulator of TLR4 and lacks an intracellular signaling domain, antibody cross-linking of CD180 was shown to induce activation, IL-6 production, and secretion of ATA IgM by non-SSc tonsillar B cells [30]. In a follow-up study, cross-linking of CD180 on class-switched and nonswitched memory B cells resulted in a robust increase in the expression of the costimulatory molecule CD86 by SSc B cells compared to those from healthy donors [31▪]. The same study also identified additional anomalies in signaling downstream of CD180 ligation in B cells from SSc patients compared to healthy controls including reduced basal and inducible expression of the anti-inflammatory cytokine IL-10 and decreased inducible phosphorylation of NF-κB. Another study found that SSc B cells had lower expression of P-selectin glycoprotein ligand-1 (PSGL-1), and that SSc B cells expressing PSGL-1 expressed lower levels of IL-10 [26▪▪]. Together, these findings suggest that dysregulated populations of B cells with decreased levels of immunomodulatory receptors and altered signaling profiles lead to a less regulatory phenotype, which may contribute to the development of autoreactivity in SSc. Although the antigen specificity of these cells in SSc is currently unknown, further analysis of these subsets may uncover additional roles for autoantigen-specific B cells in SSc pathogenesis, outside of their role as antibody-producing cells.

AUTOANTIBODY SPECIFICITY

Autoantibodies are detected in over 90% of SSc cases, and are useful biomarkers for diagnosis, definition of phenotypic subgroups and risk stratification [32]. The classical autoantibodies associated with SSc – ATA, ACA, ARA – have been well characterized and studied for associations with disease manifestations [33]. However, 10% of patients are negative for these antibodies, and more recently described antibodies are emerging in clinical practice to facilitate subcategorization of patients with SSc. Once example are antibodies to Th/To, a nuclear macromolecular RNAase complex. Anti-Th/To antibodies are present in 3–5% of SSc patients in association with limited cutaneous disease and pulmonary involvement [34,35].

Not all disease nuances can be predicted by the classic SSc antibodies, and that has led to a reverse approach to antibody discovery, i.e. examining organ-specific targets. For example, antibodies against vinculin, a cytoskeletal protein expressed at high levels in endothelial cells and the gastrointestinal (GI) tract [36], and antibodies against the nicotinic acetylcholine receptor in autonomic ganglia (gAchR) [37], a protein important for GI motility, have been found to correlate with GI manifestations in SSc. These antibodies were discovered in small cross-sectional studies, lacking the time component that would reveal whether these antibodies are byproducts of the ongoing SSc immune response or precede the genesis of GI symptoms. Further investigations of longitudinal cohorts could provide valuable information and potentially identify immunotherapy targets before the progression to the end stage of fibrosis. Additional recently discovered antibody specificities and associations are included in Table 1.

Although most known SSc autoantigens are nuclear proteins, in recent years, several groups have discovered new SSc-associated antibodies targeting nonnuclear antigens. One example is CXCL4, which has been identified as biomarker of severe SSc [38]. CXCL4 was recently shown to possess the ability to organize DNA into immune complexes that can activate plasmacytoid DCs (pDCs) via TLR9 and lead to type I interferon (IFN-I) secretion, which is associated with disease severity in SSc [39]. This observation led to the hypothesis that CXCL4 itself may be an autoantigen and that the associated autoantibodies may be actively involved in the SSc pathogenesis through upregulation of this pathway. Two separate studies from Italy verified the existence of

Table 1. Recently discovered antibody specificities in SSc

Autoantigen	Reference	Patient population	Prevalence	Clinical associations
CXCR3	[42]	327 SSc, 234 HC	12.0%	ILD
CXCR4	[42]	327 SSc, 234 HC	17.0%	ILD
CXCL4	[40]	Discovery cohort: 34 SSc, 35 UC, 29 HC, 14 SLE	53% SSc in discovery cohort	Disease duration, serum/plasma IFNα
		Replication cohort: 32 SSc, 20 HC, 12 SLE	31% SSc in replication cohort	ILD, disease score and mRSS in patients with active disease
	[41]	42 SSc, 25 HC, 79 VEDOSS (Discovery cohort: 31, Replication cohort: 48)	31% early SSc, 32% long-standing SSc, 21% VEDOSS	Long-standing SSc: ILD, skin manifestations, IFNα
CXCL4-L1	[41]	42 SSc, 25 HC, 79 VEDOSS (Discovery cohort: 31, Replication cohort: 48)	12% VEDOSS (not reported for other groups)	Long-standing SSc: skin manifestations
RNPC3	[47]	Discovery cohort: 75 SSc	14% in severe GI dysfunction vs. 3% in patients without GI dysfunction	Male, black, moderate-severe GI disease, ILD
		Confirmatory cohort: 117 SSc	18% in severe GI dysfunction vs. 6% in patients without GI dysfunction	
	[48]	447 SSc	4%	ILD, end stage lung disease,
				Higher use of nonglucorticoid immunosuppressive medications, lower event-free survival
Th/To	[34]	202 SSc, 159 disease controls	5% limited cutaneous SSc, 0% controls anti-Rpp38 (subunit of Th/To)	
		(15 SLE, 5 SjS, 27 DM/PM, 20 HBV, 21 HCV, 18 HIV, 20 syphilis, 33 other)		
	[35]	6 SSc		Limited skin disease, pulmonary involvement
		402 SSc (literature review)	3%	
	[21▪]	804 SSc (401 SSc without cancer, 403 SSc with cancer)	8%	Limited skin disease, pulmonary involvement, less likely to develop cancer within 2 years of SSc onset
Vinculin	[36]	83 SSc (GI enriched group), 72 SSc (vascular enriched group), 88 HC	37% GI enriched group, 32% vascular enriched group, 13.64% HC	GI-VAS score
gAChR	[37]	50 SSc: 19 with GI manifestations, 31 without GI manifestations	14%	SSc with GI manifestations: digital ulcers, VEGF expression, higher levels of hAChR higher endostatin levels
Annexin V	[49]	70 SSc	16% IgG, 14% IgM	ILD, PAH, digital microangiopathy
C1q		124 SSc, 25 SjS, 29 RA, 38 SLE, 53 HC	16% SSc, 4% SjS, 3% RA, 6% HD, 34% SLE	ILD, PAH, male sex
Saccharomyces cerevisiae	[51]	74 SSc, 57 HC	IgG: 43% of SSc, 2% HC; IgA: 16% SSc, 5% HC	IgG: African background, IgA: negatively associated with Medsger score
eIF2B	[52]	118 SSc, 8 myositis, 2 overlap SSc/myositis, 4 UCTD, 3 HC	2% SSc	ILD
Centriole	[53]	2 SSc		PAH
Collagen V a1 chain	[45]	17 early SSc	41%	

Table 1 (*Continued*)

Autoantigen	Reference	Patient population	Prevalence	Clinical associations
Immunoglobulin G galactosylation	[54]	93 LSc, 298 SSc, and 436 HC		dcSSc, modified Rodnan skin score, ESR
Progranulin	[55]	8 lsSSc, 31 dcSSc, 22 SjS, 3 MCTD, 33 DM/PM, 15 APLS, 11 UCTD	25% lsSSc, 32% dcSSc, 41% SjS, 33% MCTD, 12% DM/PM, 40% APLS, 18% UCTD	
ANCA	[56]	1303 SSc	9% ANCA positive, 14% PR3, 11% MPO	ILD in ANCA and PR3, PE in ANCA, increased mortality
ACE2	[57]	27 SSc, 23 HC	18% SSc, 9% HC	Decreased levels of plasma angiotensin-(1–7)
Alpha-enolase	[58]	38 SSc	47%	No clinical associations
Collagen type V	[46]	81 female SSc, 19 HC female	33% early SSc, 17% defined SSc, 5% HC	Shorter disease duration, younger age

ACE2, angiotensin-converting-enzyme-2; ANCA, antineutrophil cytoplasmic antibodies; C1q, complement component 1q; CXCL, C-X-C motif ligand; CXCL4-L1, differs from CXCL4 by three C-terminal amino acid substitutions; CXCR, C-X-C motif receptor; DM, dermatomyositis; eIF2B, Eukaryotic Initiation Factor 2B; ESR, erythrocyte sedimentation rate; gAchR, ganglionic Acetylcholine receptor; GI, gastrointestinal; HBV, hepatitis B virus; HC, healthy control; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFNα, interferon α; ILD, intestitial lung disease; MCTD, mixed connective tissue disease; MPO, myeloperoxidase; PAH, pulmonary arterial hypertension; PM, polymyositis; PR3, proteinase 3; RA, rheumatoid arthritis; RNPC3, RNA-binding region-containing protein 3; SjS, Sjogren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UC, ulcerative colitis; UCTD, undifferenciated connective tissue disease; VAS, visual analog scale; VEDF, vascular endothelial growth factor; VEDOSS, very early diagnosis of systemic sclerosis.

such autoantibodies in ~30% of SSc patients, and their association with IFN-I serum levels, lung fibrosis and skin inflammation [40,41]. The clinical associations of these autoantibodies suggest that they could be involved in disease pathogenesis, and their correlation with disease duration implies that the elevated CXCL4-DNA/RNA complexes most likely promote this novel humoral autoimmune response during SSc progression [40,41]. Similarly, antibodies against CXCR3 (the receptor for CXCL4) and CXCR4 have been found and are associated with progressive lung disease [42]. Moreover, anti-CXCR3 antibodies in SSc recognize intracellular epitopes more than similar antibodies found in healthy controls [43], suggesting altered antigen processing of CXCR3 in SSc.

Given that fibrosis is the major clinical manifestation of SSc, different fibrotic models have been investigated as a surrogate to understand disease mechanism. Collagen V, a significant component of the extracellular matrix, has been specifically interrogated, and mice immunized with collagen V recapitulated the cutaneous, vascular and pulmonary manifestations of SSc [44]. Interestingly, anticollagen V antibodies were found in SSc patients, with a prevalence of 17–41% depending on disease stage, and were more prevalent early in disease and in younger patients [45,46]. Moreover, lung biopsies from SSc patients were found to have increased reactivity when stained with anticollagen V antibodies isolated from early SSc patients, compared to control tissue [45]. Although this does not establish a causative relationship, as this could also be an epiphenomenon triggered by fibrosis itself, these studies highlight how the search for novel antigen specificities in SSc may reveal new insights in into disease mechanism.

CONCLUSION

Numerous novel SSc-specific autoantibodies have been described and investigated for their associations with different clinical manifestations of the disease. Although there is no strong direct evidence of their pathogenicity as of yet, studying the origin and specificity of autoantibodies in SSc can shed light on the earliest events in the genesis of autoimmune responses in SSc and provide important missing pieces of the pathogenic puzzle. Future research examining the effect of antibodies on target tissues and their development at different stages of the disease will further advance our understanding of this complex disease and reveal potential targets for personalized antigen-specific immunotherapy.

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Conflicts of interest

There are no conflicts of interest.

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The dynamic organelle primary cilia: emerging roles in organ fibrosis

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Purpose of review
Primary cilia, the antenna-like organelles on most mammalian cells, host key components of multiple morphogen signal transduction pathways. Mutations in genes responsible for primary cilia assembly and function generally result in pathological conditions known as ciliopathies, which underlie several diseases, including various forms of fibrosis. Primary cilia modulate cellular responses to extracellular cues, including TGF-β and morphogens, such as Hedgehog. Aberrant morphogen signaling is recognized as essential for the transition of mesenchymal progenitor cells to myofibroblasts, the key step in fibrosis. This article aims to provide a critical overview of recent developments and insights in primary cilia biology relevant to fibrosis.

Recent findings
Several studies have highlighted the association of altered primary cilia with various forms of fibrosis. In a rather complex manner, the presence of primary cilia seems to be required for initiation of myofibroblast transition, whereas its loss promotes myofibroblast transition at a later stage. Recent evidence also suggested that noncanonical functions of ciliary transport proteins may influence, such cellular transitions independently of primary cilia. The possibility of opposing signaling regulations being topologically separated between primary cilia and plasma membrane could also be critical for fibrosis.

Summary
Recent progress in the field suggests that primary cilia are critical mediators of the pathogenesis of fibrosis. Understanding the potential role of primary cilia in fibrosis and the underlying mechanisms may pave the way for entirely new approaches for fibrosis prevention and treatment of SSc.

Keywords
ciliopathies, fibrosis, myofibroblasts, primary cilia, systemic sclerosis

INTRODUCTION

Fibrosis is an outcome of a dysregulated tissue repair in response to chronic inflammatory injury. Deposition of extracellular matrix (ECM) components is normal during wound healing of all organs. However, if the injury is repetitive or severe, ECM components accumulate, causing organ fibrosis, which in turn leads to disruption of tissue architecture, organ dysfunction, and ultimately organ failure [1]. Systemic sclerosis (SSc) is associated with fibrosis occurring synchronously in multiple organs [2].

Transition or reprogramming of quiescent mesenchymal cells to biosynthetically and mechanically activated myofibroblast is accepted as key to the pathogenesis of fibrosis in SSc and other fibrotic diseases [3]. However, the origin of myofibroblasts has been a subject of debate, and the list of myofibroblast progenitor cells includes epithelial cells, endothelial cells, pericytes, bone marrow-derived monocytes as well as adipocytes. Irrespective of the origin, growth factor-mediated signaling pathways are known to serve as pivotal triggers for conversion to myofibroblasts. Most prominently, Hedgehog, Wnt, TGF-β, PDGF, and other soluble extracellular cues and mechanical signals have been shown to be important in triggering fibrosis and SSc. It is noteworthy that in addition to the default localization of cell plasma membrane and immediate cytoplasmic vicinity, components of almost all of the intracellular signaling pathways are also found to be concentrated in primary cilia.

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KEY POINTS

- Primary cilia function as an extracellular sensory organelle, a cellular antenna hosting components of various signaling pathways important for multiple physiological processes.
- Aberration in primary cilia biogenesis and functions, broadly known as ciliopathy, has been reported to be associated with many diseases, including fibrosis.
- Primary cilia seem to be required to initiate myofibroblast transition, a critical step for the pathogenesis of fibrosis, whereas its loss promotes myofibroblast transition at a later stage.
- The counterbalance of opposing signaling regulation mediated by plasma membrane-based and primary cilia-based components of the same signaling pathway could be crucial to disease pathogenesis.

Cilia are the oldest known organelles [4], primarily known for their motility function. A distinct sensory role of the organelle was attributed [5] in the late 19th century to its solitary variant [6], and named primary cilia [7]. Primary cilia are organelles present in almost all vertebrate cells. Ciliopathies are a heterogeneous group of disorders characterized by abnormal formation and function of cilia [8,9]. Several ciliopathies have been reported to be associated with fibrotic outcomes, raising the question of how ciliopathy, commonly a genetic phenomenon, is associated with acquired pathogenic fibrotic diseases [10].

Surprisingly, to date, there are only a few studies available that mechanistically evaluate the association between alterations in primary cilia and fibrosis. Here, we summarize recent progress in identifying and characterizing such association, and evaluate literature relating to the emerging role of primary cilia as a potential regulator of fibrosis.

PRIMARY CILIA STRUCTURE AND CILIARY TRANSPORT

The primary cilia is a specialized organelle with a microtubule-based inner core known as axoneme that develops from the mother centriole, and extends out from the cell surface in a nondividing cell. In addition to axoneme, primary cilia contain a transition zone that serves as a diffusion barrier and the basal body, an overlapping region with the mother centriole. The axoneme contains nine semi-doublet microtubules situated along the circular periphery. In contrast to motile cilia, the central pair complex is absent. Cells repurpose their microtubule generating machinery, the centriole, to produce primary cilia when they are not engaged in the cell cycle. As a result, primary cilia remain as a deterrent to the cell cycle. More precisely, primary cilia only assemble during the G1–G0 phase of the cell cycle, reaching maximum length followed by a disassembly phase through the transition from the G phase into the S phase. Primary cilia disappear entirely before cell entry into mitosis [11,12].

Primary cilia form a dynamic molecular device along with centriole and Golgi apparatus. Although the centriole provides the structural support by dynamically generating the microtubule tracks, Golgi supplies the train of cargo vesicles through a complex secretory pathway involving BBsomes (Fig. 1). These cargo vesicles enter primary cilia through the diffusion barrier, travel along the microtubule track of cilia to the tip, and return to the ciliary base. While at the tip, the cargos are released, and returning cargos are loaded on returning vesicles, which hop back onto the reverse train of motors traveling towards the ciliary base on the reverse journey [13]. The intraflagellar transport (IFT) system is a remarkably complex cellular machinery that mediates bi-directional intraciliary transport with the help of numerous IFT proteins and motors (Table 1). The IFTB protein complex mediates the upward (centripetal) journey, whereas the IFT-A complex mediates the reverse journey from the ciliary tip to the base [14]. Once at the ciliary base, the cargo vesicles exit the primary cilia to continue their cellular journey to various destinations via the secretory pathway [15].

Apart from the ciliary structural components, a number of cargo proteins are components of signal transduction pathways. Primary cilia have been termed cellular antennae because of their elongated shape and their ability to sense extracellular chemical, environmental and physical cues. Their unique geometric structure makes them suitable and enriched in receptors and signal transducers that coordinate the output of various cellular signaling pathways[16▪▪]. It is, therefore, not unexpected that mutations that lead to dysfunctional primary cilia will affect the genesis or function of these signaling pathways, ultimately causing disease.

PRIMARY CILIA AS A HUB FOR SIGNAL TRANSDUCTION PATHWAYS

Primary cilia hosts components of several key signal transduction pathways, including those triggered by TGF-β, Hedgehog, and Wnt (Table 2). Details of these pathways have been documented previously [4,10,17].

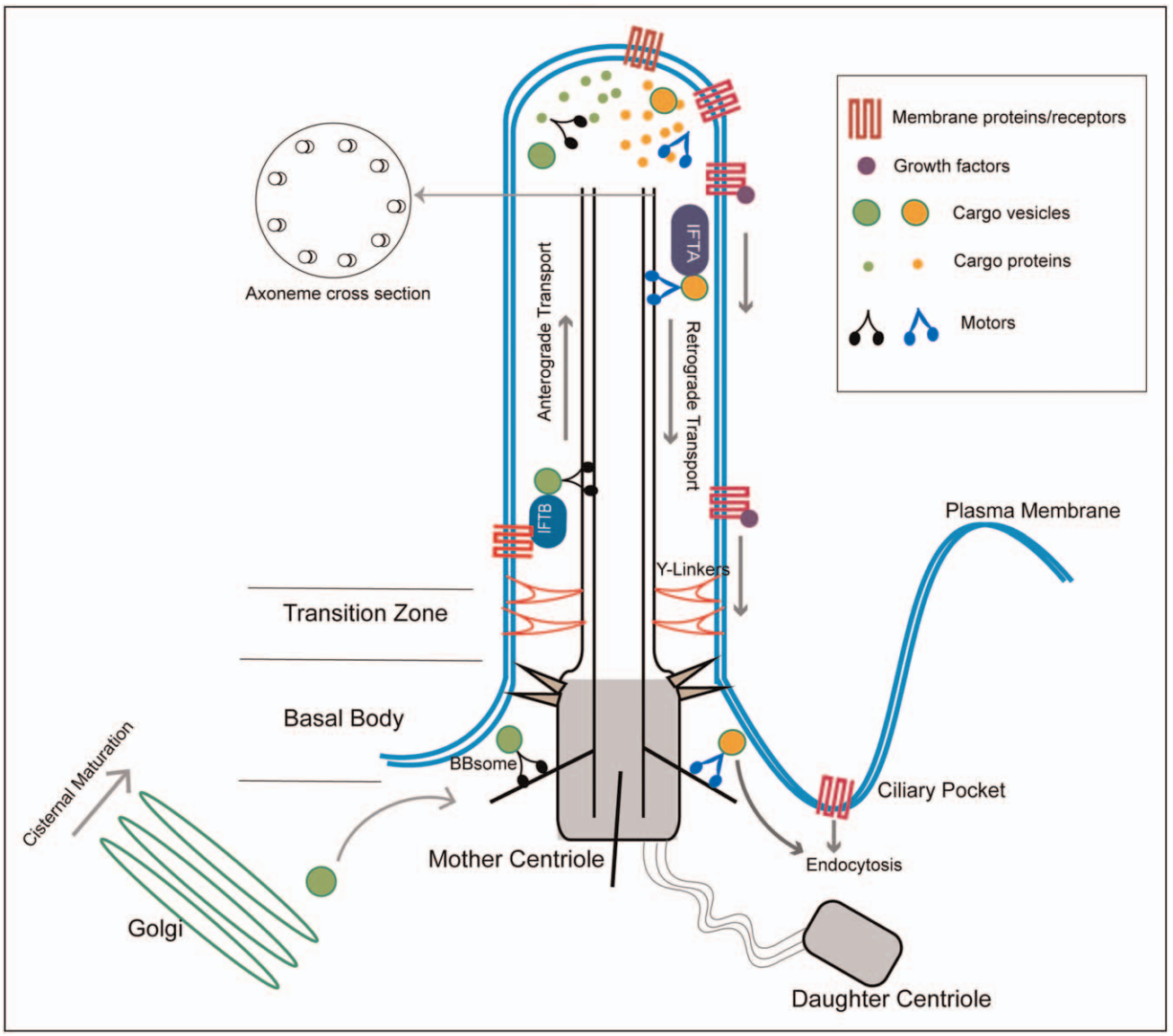

FIGURE 1. The primary cilium and ciliary transport. A schematic representation of primary cilia structure along with ciliary transport. Golgi, centriole, and primary cilia together form a dynamic intracellular device. Nine doublets of microtubules represent the axoneme of primary cilia. The ciliary membrane covers the axoneme. The transition zone is characterized by Y-shaped linkers that mediate interaction with the ciliary membrane. The basal body is composed of mother and daughter centrioles. Cisternal maturation of Golgi yields cargo vesicles in the form of BBsome, which enters through the transition zone. These cargo vesicles enter primary cilia through the diffusion barrier, travel along the microtubule track of cilia to the tip, and return to the ciliary base. Although at the tip, the cargos are released, and returning cargos are loaded on returning vesicles, which hop back onto the reverse train of motors traveling towards the ciliary base on the reverse journey. Various morphogen-signaling receptors also travel through the IFT train while being embedded in the membrane. The receptors usually are internalized through endocytosis at ciliary pockets.

Canonical TGF-β signaling involves heterotetrameric RI and RII serine/threonine kinase TGF-β receptors. These TGF-β receptors arrive and are inserted in the primary cilia membrane by the IFT transport pathway just like other primary cilia receptors. Upon binding to ligands, the receptors travel down via the ciliary surface near the ciliary pocket and are internalized through clathrin-mediated endocytosis, inducing downstream pathways of phosphorylation and activation of transcription factors SMAD2/3 [18]. This signaling pathway is modulated by the inhibitory effect of SMAD7/iSMAD, which has been reported to be selectively localized to the primary cilia [19]. Notably, primary cilia-associated TGF-β receptors can activate Hedgehog signaling via crosstalk with smoothened (SMO) [20▪].

Table 1. Trafficking pathways relevant to primary cilia

Pathway	Human genes	Function/site of action
Intraflagellar transport IFTA-complex network	*IFT43, IFT121, IFT122, IFT139, IFT140,* and *IFT144*	Retrograde ciliary transport
Intraflagellar transport IFTB-complex network	*IFT20, IFT22, IFT25, IFT27, IFT38, IFT46, IFT52, IFT54, IFT56, IFT57, IFT70, IFT74, IFT80, IFT81, IFT88,* and *IFT172*	Anterograde ciliary transport
IFT complex A accessory	*TULP3*	Retrograde ciliary transport
IFT complex B accessory	*CLUAP1, TTC26*	Anterograde ciliary transport
BBSome	*BBS1* *BBS2* *BBS3 BBS4BBS5 BBS6 BBS7 BBS8 BBS9 BBIP10*	Golgi to cilia
Exocyst/Par complex	*Sec10, cdc42*	Golgi to cilia
Kinesin-2	*KIF3A* *KIF3B* *KAP3, KIFAP3, KIF17*	Anterograde ciliary transport
Cytoplasmic dynein 2	*DYNC2H1* *WDR34, DYNC2LI1* *DYNLL1*	Retrograde ciliary transport

Wnt ligands bind to frizzled receptors in the canonical pathway that are located in the cell membrane covering the primary cilia [21]. Activation of Wnt signaling is associated with the expression of genes critical for cell proliferation, differentiation, and survival [22]. Importantly, the Wnt signaling pathway has been implicated in SSc [23,24].

Hedgehog signaling pathways regulate several cellular processes during development and tissue homeostasis and have been implicated in fibrotic myofibroblast transition in the skin [25,26]. Transduction of the Hedgehog signal is mediated through the transmembrane proteins Patched1 (Ptch1) and SMO. Both Ptch1 and SMO are present along the primary cilia membrane [27]. In the absence of sonic hedgehog ligand (SHH), Ptch1 inhibits the pathway by inhibiting SMO activity. Whenever SMO is inactive, the GLI transcription factors are proteolytically processed to a repressor that binds to Hedgehog target genes and blocks their transcription. In the presence of ligands, Ptch1 is inhibited, resulting in activation and enrichment of SMO, which then promotes the conversion of full-length GLI into a transcriptional activator (GLIA), followed by induction of targeted gene expression [28].

A recent report documented that primary cilia-based G-protein coupled receptor (GPCR) systems interpret incoming cues differently than their plasma membrane-based counterpart [16▪▪]. The authors argued that cylindrical geometric shape with a high membrane to lumen ratio would differentiate relative availability of the ciliary signaling components with each other as compared with their plasma membrane counterpart. On the basis of this theory, the authors demonstrated that only ciliary Hedgehog signaling components repress the pathway while the plasma membrane components activate it. It appears that the cell prefers to keep counterbalancing regulatory forces that are topologically located in two different cellular locations, plasma membrane and primary cilia (Fig. 2). It would be interesting to investigate whether

Table 2. Major signaling pathways in primary cilia

Signaling pathway	Function
TGF-β	Cell proliferation, migration, differentiation, apoptosis, ECM remodeling, immune functions, and tumor metastasis and is one of the major signaling pathways associated with myofibroblast transition and fibrosis
Hedgehog (Hh)	Tissue development, homeostasis, and repair as well as in regulating morphogenesis of the skin during embryogenesis
Wnt	Cell morphology, migration, and oriented cell division, skin development and maintenance, tissue regeneration and repair
Notch	Development and homeostasis of many types of tissues, such as the nervous system, the vascular system, the hematopoietic system, somites, the muscle, the skin, and the pancreas
Other G-protein-coupled receptors (GPCRs)	GPCRs, the largest class of proteins, regulate numerous functions in the cells

ECM, extracellular matrix.

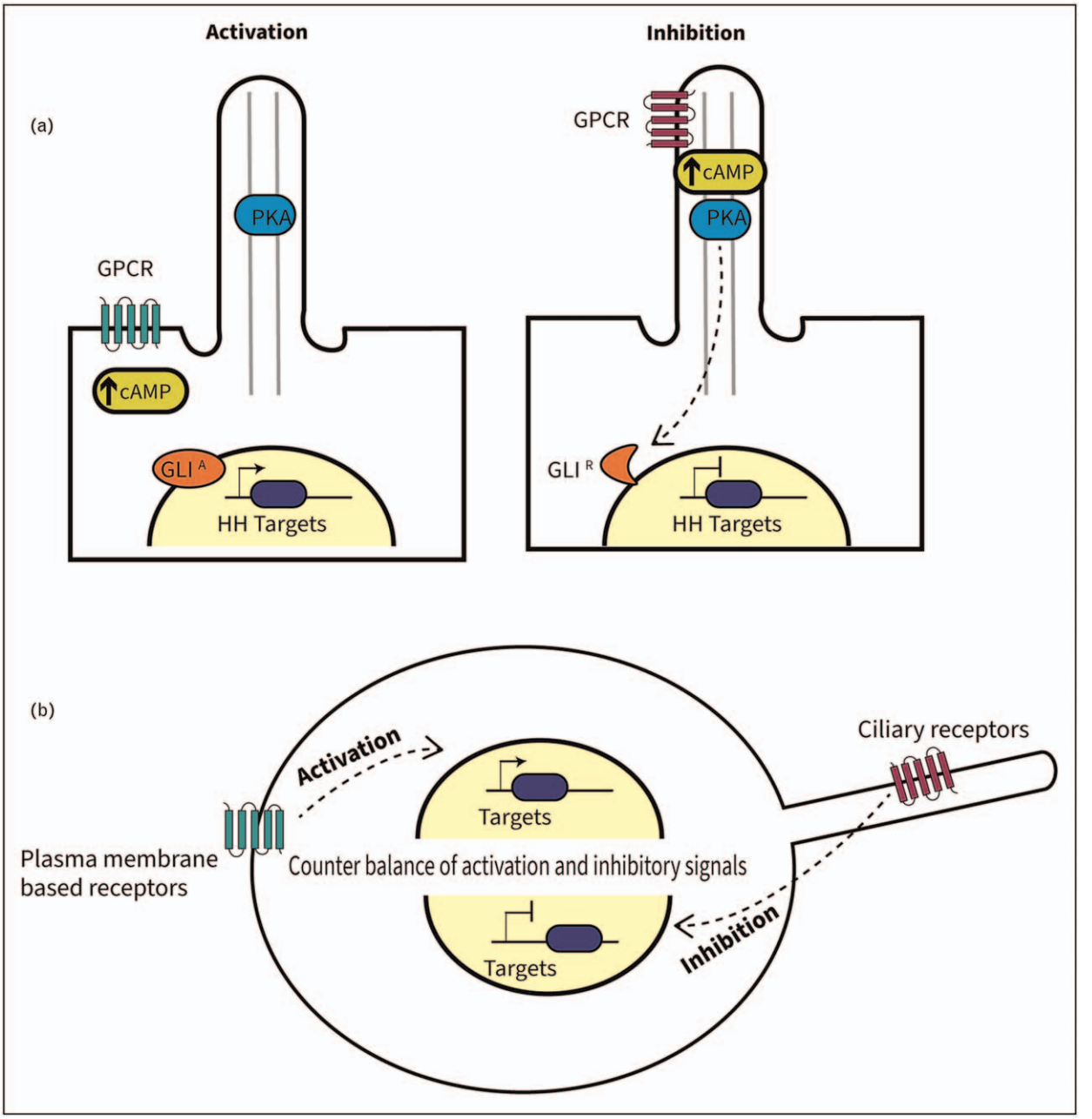

FIGURE 2. Geometric shape of primary cilia enables cell topologically separate activation and inhibitory components of signal transduction pathways between primary cilia and plasma membrane: (a) Hedgehog signal transduction interpretation in primary cilia and plasma membrane. Ciliary cAMP regulated by ciliary GPCRs locally activates ciliary PKA, phosphorylating GLI to generate its transcriptional repressor form (GLIR). As a result, the ciliary hh signal always inhibits the hh pathway. Equivalent amounts of cAMP produced by GPCRs in the plasma membrane do not activate ciliary PKA. Thus, upon Hedgehog stimulation and in the absence of ciliary PKA activity, GLI assumes its transcriptional activator form (GLIA) and induces Hedgehog target gene expression. (b) Schematic representation for a hypothetical model of morphogen signaling transduction regulated by the primary cilium. Data from Truong *et al.* (2021) [16▪▪] indicated the potential counterbalance of activation and inhibitory signal originating from two different cellular locations. The geometric shape of primary cilia, more specifically the high membrane to lumen ratio, potentially enables it to interpret various other morphogenic signals differently. As a result, a cell can generate both activation and inhibitory signal at the same time from two topologically distant intracellular locations. The net effect of the counterbalance of these two opposing signals will determine the fate of resultant and effective signaling. As the cilia length changes dynamically, its contribution to the counterbalance also changes.

components from the TGF-βpathway, the key signaling mechanism responsible for fibrosis, behave the same way or not.

CILIOPATHIES AND FIBROSIS

More than 950 cilia-related genes and their respective ciliary proteins have been reported to date (van Dam *et al.* [29]). Dysfunctional primary cilia result in pleiotropic manifestations reflected by abnormal distribution and varied functionality. Broadly speaking, ciliopathy defines morphological/and or functional alteration of cilia. A broad spectrum of human genetic diseases categorized under 'ciliopathies' have been linked to hundreds of established ciliopathy-associated proteins [30]. Several forms of cancer have been reported to be associated with ciliary decapitation [31]. The phenotypes of complete loss or dysfunction of primary cilia are remarkably heterogeneous and complex. Total or partial loss of mature primary cilia might lead to multiorgan disorders, while defects of morphologically normal primary cilia often associate with late-onset polycystic kidney disease (PKD) [20▪]. The most common inherited cause of kidney failure, autosomal dominant PKD (ADPKD), is a systemic ciliopathy that presents with progressive renal cyst expansion and extrarenal manifestations that result from loss-of-function mutations in the genes *PKD1* and *PKD2* [32].

Remarkably, ciliopathies also manifest fibrosis in various organs. For example, PKD, shows several features, including tubulointerstitial fibrosis in the kidneys as well as liver fibrosis [33]. Primary cilia has been reported to regulate ECM composition [34].

It was recently reported that primary cilia in fibroblasts are required for cardiac fibrosis [35]. The authors reported that fibroblasts harboring primary cilium exist in the developing and adult mouse heart and adult human myocardium. These ciliated fibroblasts were shown to accumulate at the site of myocardial injury. They further documented that primary cilia and polycystin-1 (PC1) participate in critical signaling functions, including TGF-β1 responsiveness, SMAD activation, ECM biogenesis, and fibrogenesis.

Knockout of genes encoding proteins required for transport of ciliary proteins to the primary cilia has been associated with fibrosis. Knockout of Cdc42, an exocyst-associated GTPase, resulted in fibrosis in the kidney in mice [36]. Exocysts are required to build primary cilia by targeting and docking vesicles carrying ciliary proteins. In addition to being found near the tight junction, exocyst proteins were also localized to the primary cilia in kidney cells. Exocyst cannot target/dock transport vesicles without Sec10, a key component, and would disintegrate and be degraded instead. Knockdown of Sec10 in MDCK cells abrogated ciliogenesis, whereas Sec10 overexpression enhanced ciliogenesis. A possible mechanism to target the exocyst to nascent primary cilia to participate in ciliogenesis is through the Par complex. Cdc42, a 'Par' complex associated GTPase, regulates exocyst formation by regulating polarized exocytosis. Synergistic genetic interaction between zebrafish cdc42 and sec10 indicated that cdc42 and sec10 function in the same pathway. Cdc42 conditional knockout kidneys showed increased interstitial fibrosis, along with all features of the nephronophthisis form of PKD. These data support a model in which Cdc42 localizes the exocyst to the primary cilium. The exocyst then targets and docks vesicles carrying proteins necessary for ciliogenesis; if this does not occur, abnormal ciliogenesis and PKD result.

IFT88 is a critical component of the IFT-mediated ciliary protein transport system. IFT88 was recently implicated in fibrosis. Knockout of IFT88 in the mouse has been shown to lead to periportal fibrosis in the liver [37]. Using a congenital mouse model of cilia dysfunction (IFT88Orpk) to study the importance of macrophages in hepatorenal fibrocystic disease (HRFCD), expansion of the bile duct region, and development of fibrosis in the liver was documented. Cilia dysfunction in these mice was accompanied by the accumulation of infiltrating macrophages. The presence of dysfunctional primary cilia on cholangiocytes was associated with persistently low levels of injury, increased production of macrophage chemoattractant cytokines along with increased numbers of infiltrating macrophages. The authors proposed that infiltrating macrophages promoted the expansion of biliary fibrosis in IFT88Orpk mice.

SPAG17 is a poorly characterized microtubule-associated protein that is involved in primary cilia formation and function. Downregulation of SPG17 in multiple cell types was implicated in systemic sclerosis [10]. The suppression of SPAG17, potentially because of epigenetic mechanisms, is associated with stunted cilia formation and enhanced myofibroblast transformation and reprogramming of various mesenchymal precursor cells. SPAG17 thus might be an additional primary cilia-associated protein whose dysfunction is associated with fibrosis and seems to represent a distinct acquired ciliopathic condition.

It is evident from these studies that the alteration of primary cilia structure and function are associated with various forms of fibrosis. This form of ciliopathy that is associated with fibrosis is rarely

genetic, rather acquired, suggesting epigenetic regulations. Ciliary proteins (or variants) that play noncanonical roles could be critical in such epigenetic regulation.

Multiple ciliary proteins, including IFT88, KIF3A, TTBK2, and NPHP4, have been reported to function outside their native site of actions in a cilia-independent noncanonical capacity (Mc Fie *et al.* [38▪]). A recent study demonstrated that disruption of ciliary intraflagellar transport (IFT) altered the cellular response to IL-1β, supporting a putative link emerging between cilia and inflammation. IFT88 depletion in cultured cells affected cytosolic NFκB translocation dynamics. RNA-seq analysis indicated that IFT88 regulated one-third of the genome wide targets, including the pro-inflammatory genes *Nos2*, *Il6*, and *Tnf*. Importantly, these altered NFκB dynamics were independent of the assembly of a ciliary axoneme; moreover, depletion of IFT88 was found to inhibit inflammatory responses even in nonciliated macrophages. These results indicate that ciliary proteins, including IFT88, KIF3A, TTBK2, and NPHP4, act outside the ciliary axoneme to tune cytoplasmic NFκB signaling specifies the downstream cell response. This is, thus, a noncanonical function for ciliary proteins in shaping cellular inflammation.

The nature of morphological alteration or dysfunctional cilia assembly is not the same in all forms of fibrosis. In fibrotic skin cells from patients with systemic sclerosis, primary cilia length appears to be decreased (unpublished data). In contrast, in the lungs in idiopathic pulmonary fibrosis (IPF) and fibrotic hearts, primary cilia frequency either increases (Lee *et al.* [39]) or primary cilia are required in cardiac fibrosis, as elaborated earlier (Villalobos *et al.* [35]). The lungs from patients with IPF showed an increase in the number of primary cilia in alveolar cells and fibroblasts (Lee *et al.* [39]). In addition, an increase in the expression of ciliogenesis-related genes, such as IFT20 and IFT88 was observed in IPF and was associated with the upregulation of SHH signaling.

Such phenotypic differences among various fibrotic diseases suggest the fibrotic processes are complex and may differ from organ to organ. In the light of Truong *et al.*'s study [16▪▪] in 2021, counterbalancing of opposing signaling regulation may explain such an apparent anomaly. If activation and inhibition of TGF-βpathway are being manifested in two different cellular locations, plasma membrane and primary cilia (or vice versa), counterbalance of such opposing regulation will tip-off with any type of morphological alteration of primary cilia potentially resulting in fibrosis.

MYOFIBROBLAST TRANSITION AND PRIMARY CILIA

Quiescent tissue-resident cells, including fibroblasts, preadipocytes, monocytes, pericytes, endothelial cells, and epithelial cells, transform into activated myofibroblasts responsible for ECM accumulation and fibrosis, but the underlying mechanism are still enigmatic (Bhattacharyya *et al.* [40]; van Caam *et al.* [41]). Signaling pathways, including TGF-β, Hedgehog, and Wnt, have been implicated in driving or sustaining pathological myofibroblast transition [23,42]. As primary cilia harbor a significant proportion of these signaling components, it is expected that primary cilia play an active role in modulating myofibroblast transition (Teves *et al.* [10]).

Morphological alteration of primary cilia has been demonstrated to accompany myofibroblast transition but the extent differs depending on the cellular progenitor source. Absence of primary cilia have been documented to play a critical role in the key fibrotic process of endothelial to mesenchymal transition or EndoMT (Egorova *et al.* [43]). In endothelial cells, primary cilia function by transducing local blood flow information into functional responses, such as nitric oxide production and initiation of gene expression. Cilia are present on endothelial cells in areas of low or disturbed flow and absent in areas of high flow. In the embryonic heart, a high-flow regime applies to the endocardial cushion area, and the absence of primary cell here coincides with EndoMT. Mutation in IFT88, an IFT protein homolog known to be critical for ciliogenesis, renders endothelial cells prone to shear stress-mediated mesenchymal transition and activated TGF-βsignaling [43].

Moreover, EndoMT attributed to loss of IFT88 have also been shown to exacerbate bleomycin-induced pulmonary fibrosis (Singh *et al.* [44▪▪]). In a recent study, the authors silenced *Ift88* in endothelial cells *in vitro* and generated endothelial cell-specific Ift88-knockout mice (Ift88endo). In cultured *Ift88*- silenced ECs, endothelial markers were significantly down-regulated with a concomitant increase in expression of mesenchymal markers, including type I collagen, and increased proliferation. Cardiac and pulmonary vascular endothelial cells isolated from the Ift88endo mice lacking IFT88 demonstrated spontaneous EndoMT. Importantly, bleomycin treatment caused exacerbated pulmonary fibrosis in Ift88endo mice. The results indicate that primary cilia loss in ECs was associated with EndoMT, which then contributed to the development of pulmonary fibrosis. IFT88 knockout mice similarly displayed exacerbated doxorubicin-induced cardiotoxicity and fibrosis [45].

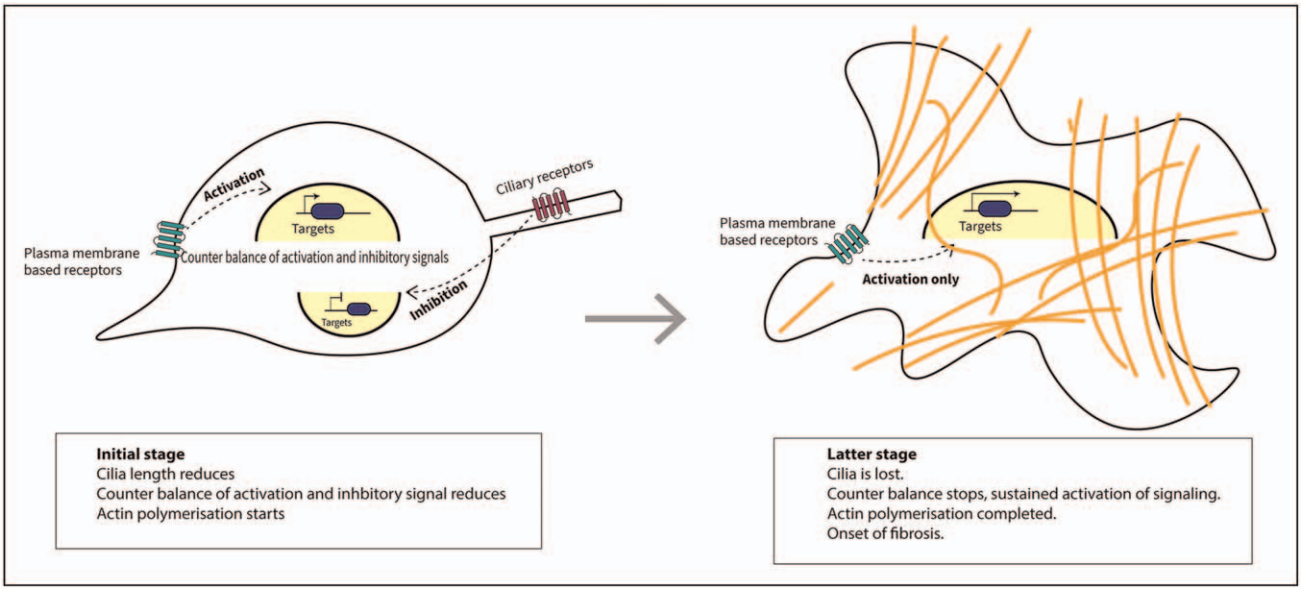

FIGURE 3. The primary cilium modulates progenitor–myofibroblast transition by being present at the initial stage and absent later. The progenitor to myofibroblast transition possibly happens in two phases. In the initial stage, presence of primary cilia facilitates the initial change causing primary cilia length to reduce. This would accompany actin polymerization. The hypothetical counterbalance of activation and inhibitory signals of morphogen signaling pathways will shift towards more activation. In the latter stage, complete loss of cilia will initiate sustained activation of signaling and a high degree of actin polymerization. This would make an irreversible myofibroblast transition resulting in fibrosis.

Divergence and complexity in myofibroblast transition is progenitor cell type-dependent. Irrespective of the progenitor type, whether fibroblast or epithelial cells, transition to myofibroblast results in loss of primary cilia but its initial presence may be essential for the transition (Rozycki *et al.* [46]). Human adipose progenitors, on the other hand, retain a shortened primary cilium after TGF-β-mediated myofibroblast transition [47]. A similar phenotype has been displayed by lung fibroblasts as well [48]. It would be interesting to see the morphological alteration of cilia in live conditions inside a living animal during fibrosis. Visualization of dynamics of primary cilia in mouse internal organ tissue is challenging. However, window chamber-based intravital imaging has been successful in circumventing this issue [49].

Actin filaments are organized into bundles and networks attached to the cell membrane by diverse cross-linking proteins. During cell migration, actin filament bundles form either radially at the leading edge or as axial stress fibers. Prior studies demonstrated that loss-of-function mutations in ciliopathy genes increased stress fiber formation and impaired ciliogenesis [50,51], whereas pharmacological inhibition of actin polymerization promoted ciliogenesis [52–54]. These studies suggested that polymerization of the actin cytoskeleton, F-actin branching, and the formation of stress fibers, all hallmark of myofibroblast transition, negatively influence primary cilium formation, whereas depolymerization or depletion of actin enhances ciliogenesis [55,56]. In short, myofibroblast harboring all its agonist features for an active, robust form displays a progressively downgraded form of primary cilia.

CONCLUSION

The precise mechanisms underlying progenitor cell-to-myofibroblast transition and sustained activation underlying pathological fibrosis remain poorly understood. We summarize recent progress suggesting that primary cilia is a critical factor of fibrosis pathogenesis by regulating progenitor cell transformation, including EndoMT and similar fibrotic reprogramming events. Understanding the potential role of primary cilia and their mechanism of action in fibrosis may pave the way for developing entirely new approaches for fibrosis prevention and treatment. Over the past decade, several groups discovered crucial cellular signaling pathways that function via primary cilia. Recent discoveries suggest that the unique geometric shape with a high membrane to lumen ratio enables the cilia to be in an advantageous position to regulate many cellular processes uniquely.

As organ fibrosis is a mostly acquired phenomenon while ciliopathy is mostly genetic, the question

arises whether epigenetic regulations are involved in fibrosis-related ciliopathy. Ciliary proteins may have both canonical and noncanonical roles, and the latter being involved in regulating morphogen signaling [38], potentially leading to fibrosis. It would be interesting to investigate the cellular benefit of using noncanonical variants of cilia-associated proteins (e.g. IFT88 and SPAG17) to regulate disease-causing transcription regulatory signaling pathways.

The cellular ingenuity of repurposing MTOC (microtubule organizing center) function of centriole in nonmitotic conditions by attaching it to the membrane and producing axoneme structure for cilia display energy conservation and optimization. The intimate relationship between cilia and the cell cycle is unsurprising as cilia, and mitotic spindles are microtubule-based, and both use components of the same molecular machinery. Whereas the mitotic spindle is essential for cell cycle progression and cytokinesis, cilia supposedly prevent cells from entering the cell cycle. What happened to this device in a differentiated cell? Primary cilia in nondividing cells are well positioned to form a signaling hub outside of the nucleus. Such a center could integrate information to initiate responses and to maintain cellular homeostasis if cell survival is jeopardized. These more discrete functions may remain undetected until differentiated cells are confronted with emergencies [57], such as fibrosis.

Recently, as it has been shown that the ciliary Hedgehog signaling component only represses the Hedgehog pathway whereas its plasma membrane counterpart activates it. The report hypothesized and proved that cells interpret the same signaling pathway differently by sequestering them in primary cilia or cell membranes [16]. This raises the possibility that various signaling pathway components that reside in the primary cilia might be acting differently than so far perceived. For example, it may be possible that ciliary TGF-β components may only repress the TGF-β pathway and act to counterbalance the activation-only mode of plasma membrane resident TGF-β components. The diminishing length of primary cilia depletes the cell of such counterbalance resulting in constant activation of the TGF-β pathway, which in turn causes fibrosis (Fig. 3). A previous report of the association of SMAD7 with TGBRI in primary cilia in selective conditions supports such possibility [19].

In the light of this theory of opposing signaling regulations being topologically separated between cilia and plasma membrane, EndoMT also needs to be re-analyzed (Fig. 3). As discovered by Rozycki *et al.* [46], the transition is biphasic – an initial phase when primary cilia presence is necessary and a latter phase when its absence promotes the transition. This result suggests that the regulatory counterbalance is needed for the initial phase of the transition but not for the later stage.

The role of ciliogenesis and altered ciliary function in SSc and other fibrotic diseases has not been established. Further investigations in the light of new evidence will shine the light on these unexplored issues.

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Conflicts of interest

There are no conflicts of interest.

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Adipose tissue and adipose secretome in systemic sclerosis

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Purpose of review
Adipose tissue is closely associated with systemic sclerosis (SSc)-pathology, both anatomically and functionally. This review focuses on local effects of adipocytes in the context of adipose to mesenchymal transdifferentiation (AMT), effects of the adipose stromal vascular fraction on SSc pathogenesis and systemic effects of adipose tissue secretome.

Recent findings
Novel populations of fibroblasts evolving from adipose tissue were identified– for example COL11+ cancer-associated fibroblasts differentiated from adipose-derived stromal cells. Lipofibroblasts in human lungs were described using nonconventional markers that allow more effective population identification. These findings could make an important contribution to further clarification of adipocyte involvement in SSc.
Recent studies confirmed that lipolysis contributes to fibrogenesis through AMT differentiation and release of fatty acids (FA). Unbalanced metabolism of FA has been reported in several studies in SSc. Other adipose tissue secretome molecules (e.g. lysophosphatidic acid), novel adipokines and extracellular vesicles from adipose mesenchymal stem cells make important contributions to the pro-/antifibrotic balance.

Summary
There is a growing evidence of important contribution of adipose tissue and its secretome to SSc pathogenesis. Novel techniques such as single-cell RNA sequencing (scRNAseq) and metabolomics, albeit challenging to use in adipose tissue, will provide further evidence.

Keywords
adipocyte, adipokines, adipose tissue, lipokines, metabolism, systemic sclerosis

INTRODUCTION

Adipose tissue is a critical contributor to the pathogenesis of systemic sclerosis (SSc), even though it is not the primary site of disease manifestation. Adipocytes have been shown to contribute directly to SSc pathogenesis through the process of adipocyte-myofibroblast transition (AMT) and are sources of myofibroblast precursors at various tissue sites (e.g. dermal white adipose tissue (dWAT) in skin, lipofibroblasts in lung) (Fig. 1). Other cells in adipose tissue, called stromal vascular fraction (SVF), contain adipose-derived mesenchymal stem cells which may also play an important role in regulating vasculopathy, fibrogenesis and immune responses in SSc, as shown by the beneficial effects of their substitution in therapies.

Beyond the direct effects of adipocytes and cells of the SVF, adipose tissue is an important regulator of whole-body metabolism. Alterations in metabolism are characteristic disease components of SSc. In addition, adipose tissue secretes adipokines, lipokines and extracellular vesicles with profound paracrine and endocrine effects.

DIRECT CONTRIBUTION OF ADIPOSE TISSUE TO SYSTEMIC SCLEROSIS PATHOLOGY – ADIPOCYTES

Increasing evidence suggests that WAT is directly involved in the development of fibrotic lesions in SSc. It was shown that degradation of intradermal

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KEY POINTS

- Adipocyte to mesenchymal cell transdifferentiation represents an important source of myofibroblasts in fibrosis, however further lineage-tracing on a single-cell level is needed to dwell into the detailed mechanisms and subpopulations.
- Promising effects of local fat grafting and SVF-based therapies in SSc.
- Dysregulation in fatty acids metabolism and lipokines represent the characteristic metabolic alterations in SSc, suggesting a central role of the adipocyte secretome.
- There are a number of adipokines and their cleavage products whose role in SSc has not yet been elucidated.
- Adipose tissue-derived extracellular vesicles and their cargo plays a significant role in the pathogenesis of SSc.

adipose tissue precedes the onset of dermal fibrosis, leading to the hypothesis that myofibroblasts originate from adipocytes undergoing AMT (Fig. 1). The authors confirmed that myofibroblasts in the bleomycin-treated model originate from adiponectin positive intradermal progenitors as observed by the transition of adipose mesenchymal stem cells (ADSC) to myofibroblast *ex vivo* [1]. In mouse skin wound healing studies, two distinct origins of SMA^{+} and Col I^{+} myofibroblasts were described, one of them originating from adipocyte progenitors (expressing $CD26^{High}$ and CD9 protein) and is evenly distributed in the wound [2]. Recently, it was reported that mature adipocytes adjacent to the wound can dedifferentiate into myofibroblasts through the process of lipolysis, enabling the release of fatty acids (FA) that attract macrophages and influence the timeline of revascularization [3▪▪]. Interestingly, inhibition of lipolysis (genetically or with DPP4/Wnt inhibition) in mature adipocytes enabled exacerbation of bleomycin-induced skin fibrosis. This reveals additional important role of adipocytes (besides their role as myofibroblast precursors) in regulating extracellular matrix production through the release of various FA [4].

It is speculated that immature and mature adipocytes in skin fibrosis produce distinct fibroblast-like cells that differ in their synthetic activity. This suggests that the ratio between immature and mature adipocytes may be important in SSc pathogenesis [5,6]. In a recent single-cell study comparing skin tissues from SSc and healthy controls, Tabib *et al.* identified preadipocyte cluster that was similarly distributed in tissue from healthy and SSc patients. However, no transcriptional overlap was observed between preadipocytes and myofibroblasts, which would suggest a transition of these cell types [7].

In many cancers, activated cancer-associated fibroblasts at the tumor margin develop from adipose-derived stromal cells co-expressing both fibroblastic genes such as lumican and decorin, as well as adipose-related genes such as apolipoprotein D, protaglandin D2 synthase and complement factor D (CFD). These cancer-associated fibroblasts express COL11A1 as a cell marker. This population may not consistently express ASMA, pointing to importance of a different, non typical myofibroblast population in cancer [8▪▪]. The COL11+ fibroblast population, recently also described in skin tissue [9], was reported to correspond to differentiated myofibroblasts, expressing fibrocartilage and myofibroblast gene signature. Interestingly, in healthy skin tissue, Tabib *et al.* described the COL11+ DPEP1+ RBP4+ fibroblast population [10], with enriched GO processes associated with tendon, muscle and extracellular matrix development, suggesting its pluripotent potential. Additionally, it expressed retinol binding protein 4 (RBP4), an adipokine that regulates adipocyte progenitor differentiation. In a scRNAseq study of SSc skin, COL11/ACTA2 expressing fibroblasts were described in healthy and SSc skin, representing a minor fibroblast population, though to represent dermal sheath cells (lining of epithelium of hair follicle from bulge to dermal papilla) [7], the dermal structure strongly interacting with dWAT.

Lung lipofibroblasts, local equivalents of lung adipocytes, express common adipocyte genes, including Pparg, Plin2, Fabp1, Fabp4, Fabp5, Lpl, and Lipa and also demonstrate the ability to dedifferentiate into myofibroblasts and contribute to lung fibrosis [11▪▪,12▪▪]. They are differentiated from lung fibroblast progenitors, expressing platelet derived growth factor receptor and are able to dynamically transdifferentiate between myofibroblast and lipofibroblast upon stimuli [13]. The lipofibroblast population was consistently described in rodent lung but rarely in human lung, leading to controversy in the literature regarding its existence, identification, and relevance to human disease. Recently, Schipke *et al.* confirmed the presence of lipofibroblasts in structurally normal, fibrotic, and emphysematous human lungs [14] using specific electron microscopy approach. Using scRNAseq Habermann *et al.* [15▪] described upregulation of PLIN2+ lipofibroblast-like group in patients with idiopathic pulmonary fibrosis, which expressed lower levels of COL1A1 compared to adjacent fibroblasts, confirming their distinct gene signatures compared to myofibroblasts, whereas Valenzi *et al.* [16] did not

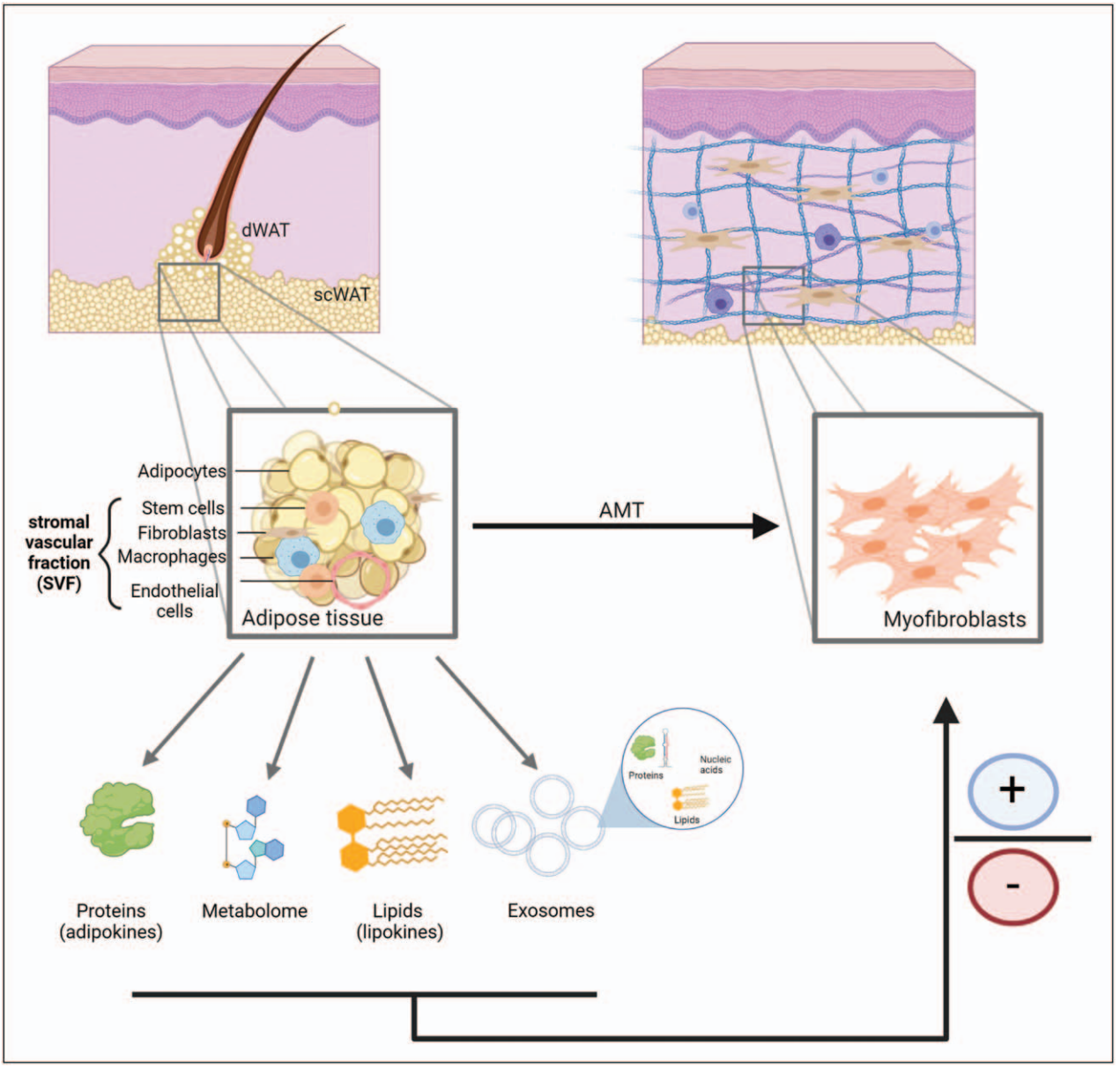

FIGURE 1. Adipose tissue composition and secretome. Adipose tissue is consisting of adipocytes and stromal vascular fraction (including preadipocytes, immune cells, hematopoetic and adipose stem cells, fibroblasts, pericytes, endothelial cells, smooth muscle cells) and excreting secretome with endo- and paracrine effects, consisting of adipokines, lipokines and extracellular vesicles. Adipose tissue secretome can inhibit or accelerate fibrogenesis, depending on 'fitness' of adipocytes. *Created with BioRender.com*.

detect lipofibroblast population in SSc-associated interstitial lung disease. In healthy and idiopathic pulmonary fibrosis tissue, Liu *et al*. were able to describe the lipofibroblast population, however observed that canonical lipofibroblast markers were ineffective in identifying lipofibroblast clusters in human lung and proposed TCF21 as a new lineage marker for lipofibroblasts [12▪▪]. This finding may explain the difficulty in identifying human lung fibroblasts in vivo using conventional marker genes.

Detailed tissue preparation and protocol procedures should be considered when analyzing and interpreting the presence of fat cells in the context of scRNAseq. First, when digesting for example skin tissue, adipose tissue is usually removed from rest of the tissue as adipose tissue could interfere with enzymatic digestion. Second, adipocytes cannot be easily detected in scRNAseq assays due to their large size [17]. This could explain the difficulties in identifying adipose tissue-related cells using conventional tissue preparation protocols.

REGENERATIVE AND REPARATIVE CAPABILITIES OF STROMAL VASCULAR FRACTION

The beneficial effects of adipose tissue supplementation have been investigated using fat grafting and SVF/ADSCs injections in various preclinical and clinical models of SSc as recently reviewed by Rosa *et al.* [6].

Administration of human ADSC in bleomycin-induced pulmonary fibrosis improved alveolar cuboidal epithelial cell hyperplasia, infiltration of immune cells and tissue fibrosis. Furthermore, treatment with ADSCs resulted in suppression of epithelial cell apoptosis and reduced expression of transforming growth factor beta in fibrotic lung tissue. Similarly, in models of skin fibrosis, it was observed that locally injected ADSCs or aspirated fat enriched with SVF significantly reduced the established skin fibrosis. Systemic application of allogeneic ADSC attenuated skin and lung fibrosis in graft versus host disease. Fat grafting and SVF/ADSC-based treatments were also tested in several clinical trials in SSc. For treatment of SSc-related facial impairment, autologous fat grafting was shown to improve functional (perioral fullness, facial expression, mastication, oral hygiene, mouth opening), and esthetic outcomes in SSc patients. Similarly, few reports suggest the beneficial effect of ADSC-infiltration on facial skin fibrosis improving manifestation of skin disease (e.g. dyschromia, sensitivity, mouth opening). For hand-related SSc complications, autologous fat grafting or local injections of ADSC-containing SVF were shown to be beneficial and safe for treatment of Raynaud's phenomenon and improved healing of digital ulcers with improvement in neo-vascularization, wound healing and hand functionality [6].

This points toward a safe and efficient profile of local fat grafting and ADSC/SVF-based treatment in SSc. It is yet to be determined whether allogenic source of fat tissue might be more efficient compared to autologous, due to described changes in composition/function of ADSC in SSc patients.

SYSTEMIC EFFECTS OF ADIPOSE TISSUE - METABOLIC CHANGES

Metabolic alterations are increasingly recognized as an important process underlying tissue fibrosis in multiple organs, including skin [18], lung [19], heart [20], liver [21] and kidney [22]. Patients with systemic sclerosis exhibit metabolic alterations at both systemic and tissue level. Patients with SSc have an impaired lipid profile, as shown in many studies. The most recent study, which included 73 SSc patients, reported low levels of total cholesterol, high and low density cholesterol, apolipoprotein A1 and high triglycerides in serum [23].

Adipocytes regulate the storage and release of lipids in response to systemic metabolic demands. Insulin signaling causes glucose uptake followed by *de novo* lipogenesis, and influences the hydrolysis of lipoproteins and entry of released free FA into the adipocyte where they are stored in the form of triacylglycerol in lipid droplets. During fasting, fat mobilization occurs, glucagon and cateholamines/norepinephrine cause lipolysis and release of free FA from adipocytes [24]. It was reported that white unstimulated adipocytes predominantly secrete proteins that regulate carbohydrate metabolism, whereas stimulation with norepinephrine associated with SSc pathophysiology [25] induces proteins that regulate lipid metabolism [26]. Metabolic changes toward an imbalanced lipid metabolism have been reported in few metabolomic studies of SSc. Specifically, in sera of SSc patients, Otria *et al* identified dysregulated metabolomic signature [27▪▪]. They observed an altered FA and carnitines profile (the molecule that enables FA transport into mitochondria), suggesting impaired mitochondrial beta-oxidation of FA and excessive amino acid consumption for ATP generation. Moreover, perturbations in beta-oxidation of FA and amino acid pathways were found also in urine of SSc patients [28]. Transcriptomic analysis of adipose tissue from mice fed with a high-fat diet revealed no significant changes in SVF transcriptome. However, they observed that adipocytes exihibited more fibroblast-like phenotype along with suppression of adipocyte programs [29▪].

ADIPOKINES

Adipokines are bioactive polypeptides secreted by adipose tissue. They are very heterogeneous in origin (Fig. 2a shows phylogenetic trees), structure and function. They are involved in the response to infection, immunity, inflammation and metabolic regulation and act as vasoactive substances (Fig. 2c). Adipokines can be tissue specific such as adiponectin or secreted by various tissues such as adipsin (complement factor D) (Fig. 3), in the latter case other tissues may also contribute to their elevated serum levels in SSc (Fig. 2d). The same is true after inflammatory stimulations, where adipocytes can be one of the sources of cytokines, such as IL6, TNFα or the acute phase protein orosomucoid (alpha-1 acid glycoprotein), an abundant serum protein, that was shown to be upregulated in adipose tissue [30] and was able to limit adipose tissue fibrosis by activating AMP-activated protein kinase (AMPK) [31▪▪].

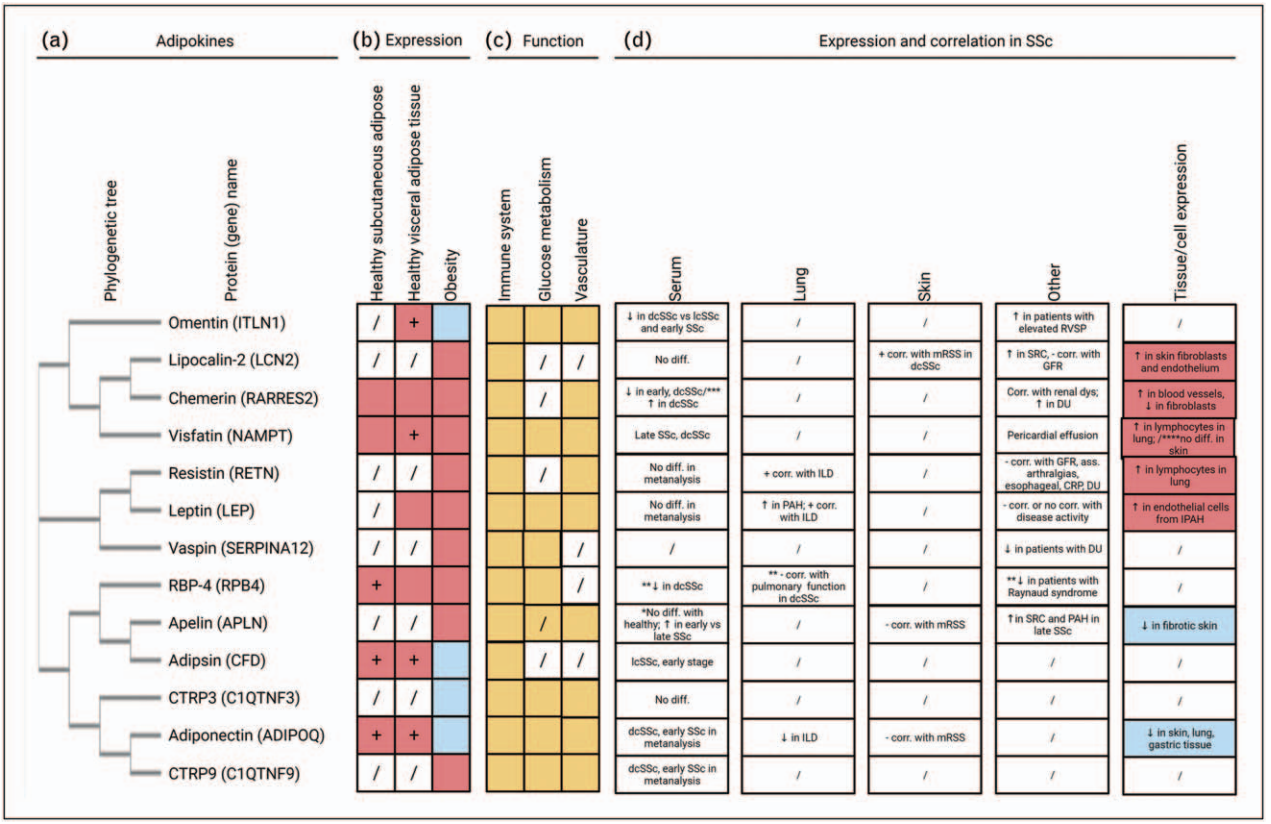

FIGURE 2. Adipokines and their association with SSc. Adipokines studied in SSc (a) Phylogenetic tree created by ClustalOmega; (b) Expression in healthy adipocytes as reported in GTEx database (white no expression; red upregulated expression up to 1000 TPM; red+ upregulated more than 1000 TPM) and expression in obesity/insulin resistance (blue – upregulated; red – downregulated) (from [32]); (c) Involvement in biological processes (Gene ontology): Immune function, glucose regulation and vascular processes; created by STRING Analysis of a network of adipokines related to SSc; (d) Correlation with SSc-pathology serum, in skin, lung or other tissues or cells [35] except from: * [59]; ** [60]; *** [61]; **** [37▪▪]. Created with BioRender.com. SSc, systemic sclerosis.

Moreover, adipokines are predominantly synthesized in adipocytes (such as adiponectin) or other cells, eg. omentin secreted by SVF [32] which may explain the difference in their value as biomarkers for adipocyte loss in SSc. Similarly, CTRP9, the closest paralog of adiponectin was shown to have elevated serum levels in SSc in contrast to decreased adiponectin (Fig. 2) [33]. The observed difference may be due to the low expression of CTRP9 in unstimulated adipose tissue in contrast to adiponectin (Fig. 3), such that the disappearance of dWAT in SSc attenuates adiponectin, but not CTRP9.

Serum levels and possible functions of three adipokines – adiponectin, leptin and resistin were well studied in association with SSc (Fig. 2d). An antifibrotic role of adiponectin with selective dWAT expansion and protection from skin and peritoneal fibrosis was observed in adiponectin-transgenic mice, whereas adiponectin-knockout mice developed exaggerated dermal fibrosis upon bleomycin treatment [34]. Leptin and resistin showed opposite effects than adiponectin in cell culture and animal models [35].

Visfatin (nicotinamide phosphoribosyltransferase, NAMPT) is another adipokine, with low tissue specificity (Fig. 3). Extracellular NAMPT increases the proinflammatory effects of macrophages, neutrophils and lymphocyte proliferation, possibly by regulating adenine signaling [36]. Intracellularly, NAMPT functions as rate-limiting enzyme in the conversion of nicotinamide mononucleotide, that is further converted to NAD+, which is excessively catabolized in SSc (shown by upregulation of CD38 and NNMT enzymes) leading to multiorgan fibrosis. Although NAD+ salvage pathway and NAMPT expression were not shown to be changed in SSc skin [37▪▪], serum levels in SSc were found to be elevated (Fig. 2d) and *in vitro* experiment demonstrated the ability of visfatin to inhibit collagen expression in dermal fibroblasts [38].

In both, mice [26] and humans [39], the secretome of WAT also consists of extracellular matrix

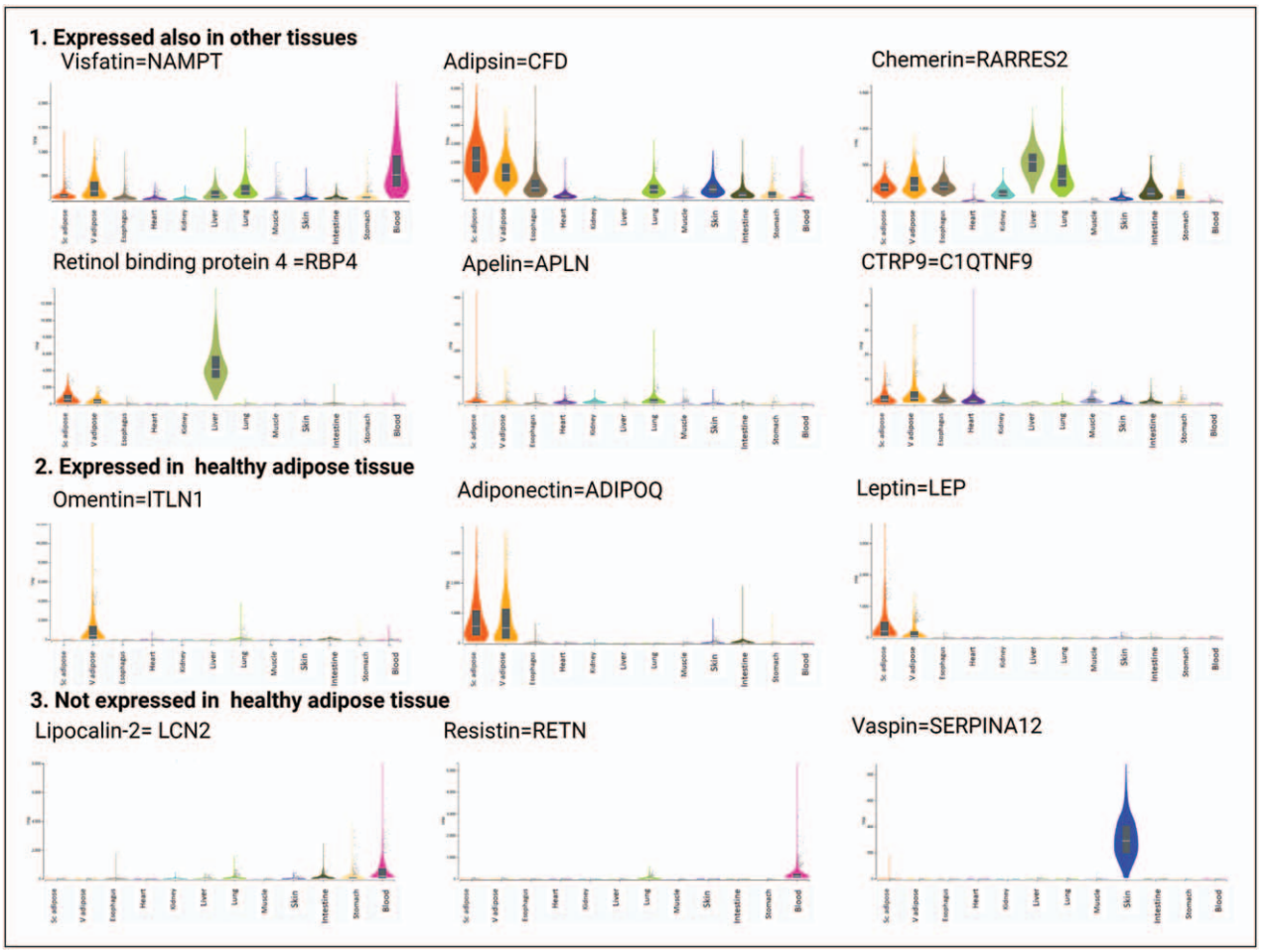

FIGURE 3. Expression of adipokines in human tissues. Adipokines can be divided into three subgroups: those with low tissue specific expression, those with major expression in healthy adipose tissue (some of them with decreased expression after stimulation such as adiponectin and other increase such as leptin) and those that are not expressed in healthy adipose tissue, but do highly express after stimuli. Sc adipose – subcutaneous adipose tissue; V adipose-visceral adipose tissue (omentum); Esophagus-Gastroesopaheal junction; Heart-left ventricle, Kidney-cortex; Skin-suprapubic; Small intestine-terminal ileum; (data are from GTEx portal https://www.gtexportal.org/home/). Created by BioRender.com.

proteins, such as COL12A1, COL18 and fibrilin-1, which are important for adipocyte differentiation and remodeling [40]. Parts of these secreted extracellular matrix molecules can also have their own biological functions. The cleaved C-terminal part of COL18 is circulating antiangiogenic peptide endostatin. Cleavage of the C-terminal profibrilin-1 results in asprosin, produced predominantly by WAT [40]. Asprosin has been studied in ischemic heart disease, where injection of asprosin-pretreated mesenchymal stromal cells into infarcted hearts inhibited myocardial fibrosis [41]. Progranulin [32] is another adipokine with variety of cleavage products, capable of promoting wound healing, dermal fibroblast division, migration and endothelial tube formation [42]. It is thought to have anti-inflammatory effects as it binds competitively to TNF receptors. Autoantibodies against progranulin were found in 25% lcSSc and 32% dcSSc patients [43].

In addition, other matricellular proteins associated with SSc pathology were also reported to be expressed by adipocytes, such as tenascin C [39] and the secreted protein acidic and rich in cysteine (SPARC) family proteins SPARC and FSTL1 [32]. All were found elevated in the SSc circulation and exert profibrotic effects on dermal fibroblasts and endothelial cells [44], but it is currently unclear what proportion of their elevated levels in SSc are contributing adipocytes.

LIPOKINES

Lipokines are lipid molecules from adipose tissue that are actively secreted and act as metabolic regulators in various distant tissues. One of the first

lipokines identified was lysophosphatidic acid (LPA), followed by palmitoleate, FA esters of hydroxyl FA (FAHFA) [45].

LPA was originally identified as factor secreted by differentiated adipocytes that could promote preadipocyte proliferation. Later, plasma lysophospholipase D (autotaxin (ATX)/ENPP2 ectonucleotide pyrophosphatase/phosphodiesterase 2) was identified as adipocyte secreted enzyme able of LPA synthesis on the extracellular side of adipocytes. Adipose-specific ATX knockout mice exhibited a ~40% reduction in circulating LPA levels, demonstrating that adipose tissue contributes significantly to total extracellular LPA [45], whereas norephineprine potently induces autotaxin/ENPP2 in adipocytes [26]. LPA actions were shown to be involved in the development of dermal and pulmonary fibrosis via the LPA1 receptor [46]. Moreover, increased arachidonoyl (20:4)-LPA levels in skin and serum and increased LPA1 receptor expression in dermal fibroblasts and skin were found in patients with SSc [47,48]. LPA receptor antagonists (SAR100842) and ATX inhibitors (BBT 877) are under intense investigation in SSc. Recently, the phase 3 clinical trial of the LPA receptor antagonist GLPG1690 has been terminated due to poor benefit-risk profile [49].

A metabolomic study of idiopathic pulmonary fibrosis tissues revealed increased levels of long and medium chain FA, including palmitoleate [19]. Palmitoleate is one of the abundant FA in serum, which is mainly synthesized in the cis isoform by *de novo* lipogenesis from acetyl-CoA followed by desaturation by stearoyl-CoA desaturase 1 (SCD1) in adipose tissue (and liver) [24]. It has been shown to protect endothelial cells [50] and inhibit NFkB activation and M1 polarization via AMPK activation [51]. Long-term feeding of mice with two FAHFA induced liver fibrosis in some mice, which was possibly developed due to the induction of *de novo* lipogenesis [52].

Since the lipidomic studies are only in its advent, more is to be discovered in the coming years. It is clear that extensive perturbation in FA profile is observed in SSc patients and their regulation might represent future therapeutic targets.

EXTRACELLULAR VESICLES

Adipose tissue is an important source of extracellular vesicles: exosomes, which are nano-sized vesicles that form intarluminaly in multivesicular bodies, and larger microvesicles, formed by blebbing of the plasma membrane. In mice, WAT releases 1–2% of its lipid content daily via exosomes [24]. These vesicles serve as cargo for various components such as proteins, nucleic acids and lipids, but may also serve as a cellular waste disposal [53]. In the human secretome of brown and white adipocytes almost 30% peptides had no signaling peptide, which means that they are nonclassically secreted from adipocytes through exosomes or microvesicles [39]. Analysis of serum from ADicer KO mice exhibiting defect in miRNA processing in adipose tissue and serum from patients with lipodystrophy showed that adipose tissue is a major source of circulating exosomal miRNAs in both mice and humans [54].

Exosomes derived from human ADSCs injected into the vein of mice were reported to be recruited to the wound area and stimulate early stage wound healing processes. The study showed that exosomes from human ADSC enter dermal fibroblasts and promote their migration, proliferation and collagen synthesis. [55]. Recent study confirmed improved wound healing and reduced collagen deposition using ADSC exosomes in BALB/c excision wound model. ADSC exosomes attenuated proliferation, collagen deposition and transdifferentiation of fibroblasts-to-myofibroblasts in fibroblasts isolated from hypertrophic scar via the miR-192-5p/IL-17RA/Smad axis [56▪]. They report higher miRNA-192-5p presence in exosomes from ADSC than in cell culture. This is consistent with Rozier *et al.* who recently reported that extracellular vesicles from ADSC exerted a stronger antifibrotic effect in TGFβ1 stimulated fibroblasts and SSc fibroblasts than ADSC in coculture [57].

In the last two years, there have been a few reports that exosomes from ADSC successfully ameliorate cardiac, liver and lung fibrosis in animal models [56▪]. However, exosomes from ADSCs have been shown to carry active STAT3 to induce M2 macrophage polarization [58]. This highlights that careful studies are required before this can be translated to therapeutic use.

CONCLUSION

Different adipose tissue depots play important roles in regulating fibrogenesis at systemic and tissue site level. Recent scRNAseq data suggests that adipocyte-derived myofibroblasts may represent a unique transcriptomic population and further studies are needed to elucidate their detailed role in fibrogenesis. Despite the crucial role of adipose tissue in SSc pathogenesis, specific studies focusing on adipose tissue and its secretome in SSc are still lacking.

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Conflicts of interest

There are no conflicts of interest.

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Environmental triggers for connective tissue disease: the case of COVID-19 associated with dermatomyositis-specific autoantibodies

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Purpose of review
The aim of the present review is to analyze the link between autoimmune diseases and environmental factors, in particular severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (COVID-19) as it shares numerous features with the interstitial lung disease associated with connective tissue diseases positive for rare autoantibodies directed at highly specific autoantigens (i.e., MDA5 and RIG1) among the intracellular sensors of SARS-CoV-2 in the innate response against viruses.

Recent findings
As shown in recent publications and in our original data, specific autoantibodies may be functionally relevant to COVID-19 infection. We evaluated sera from 35 hospitalized patients with COVID-19 to identify antinuclear antibodies and autoantibodies directed against specific antigenic targets, and we identified antinuclear antibodies (ANA) in 20/35 of patients with COVID-19 (57%), in patients with need for supplemental oxygen (90% vs. 20% in ANA-negative cases; $P < 0.0001$). In 7/35 COVID-19 sera, we detected anti-MJ/NXP2 ($n = 3$), anti-RIG1 ($n = 2$), anti-Scl-70/TOPO1 ($n = 1$), and anti-MDA5 ($n = 1$), overall associated with a significantly worse pulmonary involvement at lung computerized tomography scans. Eleven (31%) patients were positive for antibodies against the E2/E3 subunits of mitochondrial pyruvate dehydrogenase complex.

Summary
Viral infections such as COVID-19 are associated with ANA and autoantibodies directed toward antiviral signaling antigens in particular in patients with worse pulmonary involvement.

Keywords
antinuclear antibody, connective tissue disease, mitochondria, SARS-CoV-2

INFECTIONS AND AUTOIMMUNITY

Environmental factors are involved in the onset of systemic autoimmune rheumatic diseases in genetically predisposed individuals, as observed for numerous conditions [1]. In particular in the case of idiopathic inflammatory myositis (IIMs), such as poly- and dermatomyositis (PM and DM) environmental factors such as smoking, sun exposure, infections, medications, vaccines, stressful life events and physical activity seem to be responsible for disease onset or flare [2]. During the recent COVID-19 pandemic, the role of infections has become even more clearly associated with the onset of auto-inflammatory and autoimmune manifestations including myalgia and myositis [3▪▪,4]. Since the earliest reports, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been associated with autoimmunity also from the serological point of view [4,5▪▪], in some cases associated with chronic conditions [6] and features typical of connective tissue diseases (CTD), DM in particular [7]. First, numerous cytokines are shared by the macrophage activation syndrome observed in CTD and the cytokine release syndrome characterizing severe COVID-

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KEY POINTS

- Antinuclear antibodies are detected in 57% of patients with COVID-19.
- AntiMJ/NXP2, antiScl-70/TOPO1, antiMDA5, antiRIG1 are cumulatively found in 20% of cases.
- Anti-E2/E3 pyruvate dehydrogenase antibodies are positive in 31% of patients.
- Autoantibody-positive patients have worse COVID-19 pulmonary involvement.

19 [8,9], in both cases culminating in endothelial dysfunction, vasculopathy, and thrombotic manifestations. Second, both sets of disease benefit from anti-inflammatory treatments, consisting of glucocorticoids and anticytokine agents [10]. Third, the COVID-19 interstitial lung disease resembles what was observed in CTD [11,12]. Fourth and most relevant to our study, some CTD-associated autoantibodies are directed toward molecules involved in the innate immune response against viruses. In fact, antimelanoma differentiation-associated gene 5 (anti-MDA5) antibodies are linked to a specific form of DM, generally presenting as a clinically amyopathic cutaneous vasculopathy with a predominant and rapidly progressive interstitial lung disease. Fifth, MDA5 is a pattern recognition receptor of the RIG1-like receptor (RLR) family that recognizes intracellular viral RNA and triggers type I interferons, together with additional viral RNA sensors such as RIG1 or the DHX58 component in the DHX58-TBK1 pathway that may be induced by a viral trigger [13]. When complexed with viral RNA and overexpressed during infections, MDA5 may be recognized by antigen-presenting cells and ultimately lead to autoantibody production, similarly to the production of anti-RIG1 or anti-DHX58 autoantibodies [14▪▪]. Furthermore, three immunogenic linear epitopes with high sequence identity to SARS-CoV-2 proteins have been identified in patients with DM [15] thus pointing at molecular mimicry to link COVID-19 and autoimmune manifestations. Similar mechanisms could be hypothesized also for other autoantibodies directed against intracellular proteins hyper-expressed or massively released in the extra-cellular space due to tissue damage. We hypothesized that COVID-19 may be associated with novel and established tissue disease-associated autoantibodies, particularly those directed against antigens involved in the antiviral immune response, and that these may be associated with a more severe form of infection.

The scope of the present review is to describe COVID-19 infection as the prototype of the environmental factor associated with the production of autoantibodies so far identified in DM patients affected by rapidly progressive interstitial lung disease.

AUTOANTIBODIES IN COVID-19 VIRAL INFECTION: OUR EXPERIENCE

Viral agents may be involved in the onset of IIMs through several possible mechanisms, from changes in the host cellular proteins, no longer recognized by the host immune system, to stimulation of autoantibody production, carrying pathogenic idiotypes [16]. The infection and also its timing are relevant related to PM/DM disease onset, as most infections develop 3 months before the clinical onset of myositis [17,18] in particular in juvenile forms. Based on these elements, we investigated sera from 35 patients with a proven SARS-CoV-2 infection, i.e., a positive real time-polymerase chain reaction (RT-PCR) on a nasal swab or bronchoalveolar lavage, that were consecutively hospitalized in the Rheumatology Department (dedicated to low-intensity care of COVID-19 cases) at Humanitas Research Hospital between October 1st and November 30th, 2020. The work has been conducted in accordance of the Helsinki Declaration. Informed consent was obtained from all the patients. We collected data about demographic, clinical, and serologic characteristics, COVID-19 duration (i.e., date since the onset of symptoms), pharmacological treatments received during hospitalization, blood oxygenation at admission (i.e., the ratio between the partial pressure of oxygen and the fraction of inspired oxygen) and the highest level of oxygen support required, the need for intensive care unit admission, and mortality. Comorbidities and thromboembolic events (i.e., pulmonary embolism, deep and superficial vein thrombosis) were also recorded for all patients. In the clinical setting, local protocols allocated patients to receive dexamethasone if oxygen support was required (one patient received methylprednisolone, 40 mg) or to receive remdesivir if symptoms occurred less than 10 days before admission in the absence of renal or liver failure. No patient in this cohort was treated with other immune-modulators, including monoclonal antibodies.

Anti-nuclear antibodies (ANA) and cytoplasmic antibodies were tested by indirect immunofluorescence (IIF) on HEp-2 slides (INOVA Diagnostics, San Diego, CA, USA) as described [19], and defined according to official standards (ICAP; https://www.anapatterns.org). Immunoprecipitation-Western Blot (IP-WB) was used to test for anti-MDA5, anti-MJ/NXP-2, antieIF2B, and anti-E2/E3 antibodies as previously described [20,21]. ELISA was used to test for

anti-Scl70/TOPO1, anti-RNA Pol III, and anti-Jo1 (QUANTA Lite Jo-1, INOVA Diagnostics, San Diego, CA, USA) according to the manufacturer protocols [22]. Anti-RIG1 and anti-DHX58 were tested by ELISA using recombinant proteins *(unpublished data)*, to evaluate the presence of these two different autoantibodies induced by the SARS-CoV-2 viral infection. RNA-IP was used to detect anti-Sjogren's syndrome-related antigen A (SSA)/Ro, anti-SSB/La, anti-RNPs (U2, U3, U1), anti-SRP, anti-Th/To, anti-Sm, anti-PL7, anti-PL12, and other antiaminoacyl tRNA synthetases as previously described [23]. Data were analyzed using the GraphPad Prism version 7.01 for Windows (GraphPad, San Diego, CA), and P values < 0.05 were considered statistically significant.

In our cohort of COVID-19 patients, 63% were males with a median age of 74, one patient had rheumatoid arthritis and one skin psoriasis. Patients came to the Emergency Department referring a median duration of COVID-19 symptoms of 4 days and the following hospital stay had a median duration of 15 days, with 28/35 patients (80%) requiring oxygen supplementation. Dexamethasone 6 mg intravenously for 10 days was administered to 27/28 patients who required oxygen support. Eleven patients also received remdesivir 200 mg IV for 5 days.

Results of ANA and cytoplasmic antibodies and specific autoantibodies are shown in Tables 1–3. We classified ANA and cytoplasmic positivity based on the predominant immunofluorescence staining, even though the coexistence of both patterns is possible. In fact, in 7/15 cytoplasmic positive patients (46.6%) we also observed a concomitant weaker nuclear fluorescence. Twenty/35 COVID-19 sera (57%) were ANA positive, in 95% at titers $\geq$1:160 most frequently with speckled (45%), nucleolar (30%) and homogeneous (15%) patterns. In 7/20 of ANA-positive patients (35%), specific autoantibodies were detected (Fig. 1 panels a–g), in particular IP-WB confirmed 3/35 anti-MJ/NXP2 (Fig. 1 panel h), 2/35 anti-RIG1, 1/35 anti-MDA5 (Fig. 1 panel h), and one anti-Scl70/TOPO1 (Fig. 1 panel g).

Table 1. ANA titers and patterns in hospitalized patients with COVID-19

	***n*:20**		***n*: 15**
ANA pattern		Cytoplasmatic pattern	
Speckled	9 (45%)	Speckled	5 (33.3%)
Nucleolar	6 (30%)	Reticular	9 (60%)
Homogeneous	3 (15%)	Polar/Golgi-like	1 (6.6%)
Mitotic spindle	1 (5%)		
Nuclear dots	1 (5%)		
Titer		**Titer**	
1:80	1 (5%)	1:80	1 (6.6%)
1:160	6 (30%)	1:160	2 (13.3%)
1:320	3 (15%)	1:320	4 (26.6%)
1:640	6 (30%)	1:640	2 (13.3%)
1:1280	4 (20%)	1:1280	6 (40%)

ANA, anti-nuclear antibodies.

Eleven out of 15 COVID-19 sera (73%) with cytoplasmic pattern were also positive for antibodies against the E2/E3 subunit of the pyruvate dehydrogenase complex, known as part of the antimitochondrial antigenic complex. No reactivity for additional myositis-specific antibodies belonging to the antiaminoacyl tRNA synthetases family, myositis-associated antibodies, and antibodies linked to other rheumatological diseases was observed. The proportion of patients requiring oxygen support was significantly higher among ANA-positive patients (90% vs. 20% ANA-negative; $P < 0.0001$) and, as expected, remdesivir was also more frequently used in ANA-positive patients (35% vs. 26.6%; $P = 0.025$). ANA-positive patients had longer hospitalization and higher extent of lung involvement including both poorly and nonaerated patterns. Anti-E2/E3-positive patients were significantly older than negative patients, and despite the association of this autoantibody with primary biliary cholangitis, only 3/11 (27%) anti-E2/E3-positive patients had elevated transaminases. The anti-E2/E3 status did not influence lung scores at imaging, blood oxygenation, and oxygen requirement for SARS-CoV-2.

THE EVOLVING SCENARIO OF COVID-19 AND AUTOIMMUNE DISEASES

Although there is growing evidence of the role of autoimmunity in the severity of COVID-19, the prevalence of serum autoantibodies, their influence on clinical outcomes, and the implications on patient management remain elusive. We report herein that over 50% of hospitalized patients with COVID-19 are positive for ANA with 20% of these manifesting additional autoantibodies commonly identified in CTD and other autoimmune diseases, in both cases associated with lung involvement and with a potential mechanistic link based on the target antiviral signaling antigens, namely anti-Scl-70/TOPO1, RIG1, MDA5, and E2/E3.

Variable prevalence rates have been described for autoantibodies in COVID-19, as represented by retrospective studies in Chinese patients with severe COVID-19 having positive ANA (50%), anti–52 kDa SSA/Ro (20%), and anti–60 kDa SSA/Ro (25%) [24] or in Italian patients with 45% being positive for at least one autoantibody associated with poorer prognosis [5▪▪]. Furthermore, among 29 critically ill COVID-19 patients from Greece almost 70% of

Table 2. Comparison of ANA (+) and (−) hospitalized patients with COVID-19

	20 ANA (+)	15 ANA (−)	*P* value
Female	12 (60%)	5 (33.3%)	0.22
Age	74 (58–61)	76 (73–80)	0.44
Comorbidities			
Hypertension	8 (40%)	11 (73.3%)	0.11
Coronary heart disease	3 (15%)	5 (33.3%)	0.98
Diabetes mellitus	1 (5%)	3 (20%)	0.72
Cancer	3 (15%)	5 (33.3%)	0.76
Symptom duration days	5 (2–7)	3 (2–7)	0.7
Days of hospital stay	17.5 (9–24)	11 (8–18)	0.4
Medical therapy			
Glucocorticoids	17 (85%)	12 (80%)	0.15
Remdesivir	7 (35%)	4 (26.6%)	0.025
O2 requirement	18 (90%)	3 (20%)	<0.0001
PaO2/FiO2 at admission	299 (242–325)	309 (276–380)	0.2
Pulmonary thromboembolism	1 (5%)	4 (6.6%)	0.18
NIPPV	2	0	0.59
Lung segmentation			
Abnormal	14% (9–18)	9% (8–19)	0.4
Hyperinflated	2.5% (1–13)	7% (2–12)	0.3
Poorly aerated	11% (6.5–13)	7% (6–15)	0.6
Nonaerated	3% (1–5)	2% (2–3)	0.8
Compromised ≥ 20%	4 (20%)	2 (13.3%)	0.94
Blood tests			
Hemoglobin (g/dL)	14.2 (12.5–15.6)	13.3 (11.4–14)	0.2
Platelet count (10^3/mm^3)	173.5 (148.25–214.25)	200 (160–277)	0.2
Neutrophil count (10^3/mm^3)	4.05 (3.175–6.425)	4.5 (3.3–6.6)	0.6
Lymphocyte count (10^3/mm^3)	0.9 (0.725–1.4)	0.75 (0.375–0.925)	0.03
Eosinophil count (10^3/mm^3)	0 (0–0)	0 (0–0)	0.4
Creatinine (mg/dL)	0.9 (0.8–1.1)	0.9 (0.6–1.3)	0.9
GPT (IU/L)	26 (15–60)	20 (16–42)	0.4
ALP (U/L)	82 (67.75–106.25)	82 (68.25–105.25)	0.45
Gamma-GT (UI/L)	40 (24.75–59.25)	39 (23.75–56.75)	0.08
Troponin (ng/L)	8.25 (4.8–10.8)	12.1 (6.3–34.3)	0.03
CK (U/L)	81 (38–170)	134 (92–203)	0.07
BNP (pg/mL)	41 (22–59)	148 (77–456)	< 0.0001
Inflammatory markers			
C-reactive protein (mg/dL)	4.9 (1.9–8.1)	9.1 (4.8–12.5)	0.09
D-dimer (ng/dL)	292 (209–699)	312 (282–1330)	0.3
Ferritin (ng/mL)	314 (214–640)	294 (162–642)	0.6
LDH (IU/L)	299 (249–385)	263 (243–380)	0.6
IL-6 (pg/mL)	37 (24–68)	51 (23–63)	0.9
Procalcitonin (mg/dL)	0.1 (0.06–0.35)	0.09 (0.05–0.16)	0.3
C3 (mg/dL)	140 (138–149)	136 (129–147)	0.3
C4 (mg/dL)	51 (40–63)	39 (30–54)	0.2
COVID-19 serology			
Anti-SARS-CoV-2 (S1/S2) IgG	6 (30%)	5 (33.3%)	0.98
Anti-SARS-CoV-2 (S1/S2) IgG titer	5.6 (3.8–18.7)	6.2 (3.8–27.1)	0.9
Anti-SARS-CoV-2 (RBD) IgM	4 (20%)	5 (33.3%)	0.51
Anti-SARS-CoV-2 (S1- RBD) IgM titer	1.37 (0.5–12.2)	0.4 (0.1–10.5)	0.2

ANA, anti-nuclear antibodies; BNP, brain natriuretic peptide; CK, creatine kinase; IQR, interquartile range; LDH, lactate dehydrogenase; NIPPV, noninvasive positive pressure ventilation; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Table 3. Comparison of SAA (specific autoantibodies) (+) and (−) groups among hospitalized patients with COVID-19

	7 SAA (+)	8 SAA (−)	*P* value
Female	5 (71.4%)	8 (28.57%)	0.025
Age	80 (72.5–81.5)	74 (65–77.25)	0.18
Comorbidities			
Hypertension	2 (28.57%)	17 (60.7%)	0.12
Coronary heart disease	2 (28.57%)	6 (21.4%)	0.68
Diabetes mellitus	1 (14.2%)	3 (10.7%)	0.79
Cancer	2 (28.57%)	6 (21.4%)	0.68
Days of symptom onset before admission	5 (3–7)	3.5 (2–7)	0.7
Days of hospital stay	19 (9–24)	15 (8.75–18.75)	0.42
Medical therapy			
Glucocorticoids	6 (85.7%)	23 (82.14%)	0.82
Remdesivir	1 (14.2%)	10 (35.7%)	0.27
O2 requirement	6 (85.7%)	22 (78.57%)	0.49
PaO2/FiO2 at admission	276 (233–324)	311.5 (273.5–364.75)	0.23
Pulmonary thromboembolism	1 (14.2%)	4 (14.2%)	0.99
NIPPV	1 (14.2%)	1 (3.57%)	0.27
Lung segmentation			
Abnormal	19% (14–23.5)	12% (8–16)	0.04
Hyperinflated	1% (1–2)	6% (2–14)	0.01
Poorly aerated	13% (11–16.5)	8% (6–13)	0.02
Nonaerated	3% (3–6)	2% (2–3)	0.02
Compromised $\geq$ 20%	3 (42.85%)	3 (10.7%)	0.043
Blood tests			
Hemoglobin (g/dL)	14 (12.9–14.35)	13.8 (12.4–14.85)	0.68
Platelet count (10^3/mm^3)	163 (152.5–253.5)	185 (159–226.75)	0.59
Neutrophil count (10^3/mm^3)	3.5 (3–3.9)	4.2 (3.7–6.75)	0.13
Lymphocyte count (10^3/mm^3)	0.8 (0.75–1.25)	0.9 (0.7–1.1)	0.66
Eosinophil count (10^3/mm^3)	0 (0–0)	0 (0–0)	0.82
Creatinine (mg/dL)	0.94 (0.89–0.99)	0.9 (0.74–1.18)	0.59
GPT (IU/L)	15 (14.5–43–5)	26 (18.5–47)	0.47
ALP (U/L)	72 (59–88.5)	85 (70.5–108)	0.37
Gamma-GT (UI/L)	32.5 (21–58.25)	40.5 (25.2–52.2)	0.79
Troponin (ng/L)	8.7 (5.45–12.02)	9.3 (5.6–17.17)	0.76
CK (U/L)	81 (57.5–152)	113 (60–188)	0.99
BNP (pg/mL)	51 (18.5–67.5)	77 (37–189.55)	0.39
Inflammatory markers (median – IQR)			
C-reactive protein (mg/dL)	4.92 (3.72–6.73)	7.69 (2.27–12.45)	0.79
D-dimer (ng/dL)	634 (303.5–1447)	302 (226.5–508.5)	0.52
Ferritin (ng/mL)	256.2 (211.3–288.35)	332.1 (206.25–641.2)	0.47
LDH (IU/L)	334 (260.5–388.5)	279 (243.24–324.25)	0.14
IL-6 (pg/mL)	47 (39.5–65)	36 (23–63)	0.22
Procalcitonin (mg/dL)	0.1 (0.05–0.11)	0.14 (0.05–0.2)	0.42
C3 (mg/dL)	139 (138–148)	139.5 (131.5–147)	0.48
C4 (mg/dL)	47 (35–48)	46 (35.75–57.75)	0.68
COVID-19 serology			
Anti-SARS-CoV-2 (anti-S1, S2) IgG positivity	3 (42.85%)	8 (28.57%)	0.33
Anti-SARS-CoV-2 (anti-S1, S2) IgG [AU/mL]	9.15 (4.25–16.2)	5.44 (3.8–20)	0.66
Anti-SARS-CoV-2 (S1- RBD) IgM positivity	3 (42.85%)	6 (21.4%)	0.21
Anti-SARS-CoV-2 (S1- RBD) IgM [Index]	8.35 (3.55–16.35)	0.49 (0.17–3.95)	0.99

BNP, brain natriuretic peptide; CK, creatine kinase; IQR, interquartile range; LDH, lactate dehydrogenase; NIPPV, noninvasive positive pressure ventilation; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

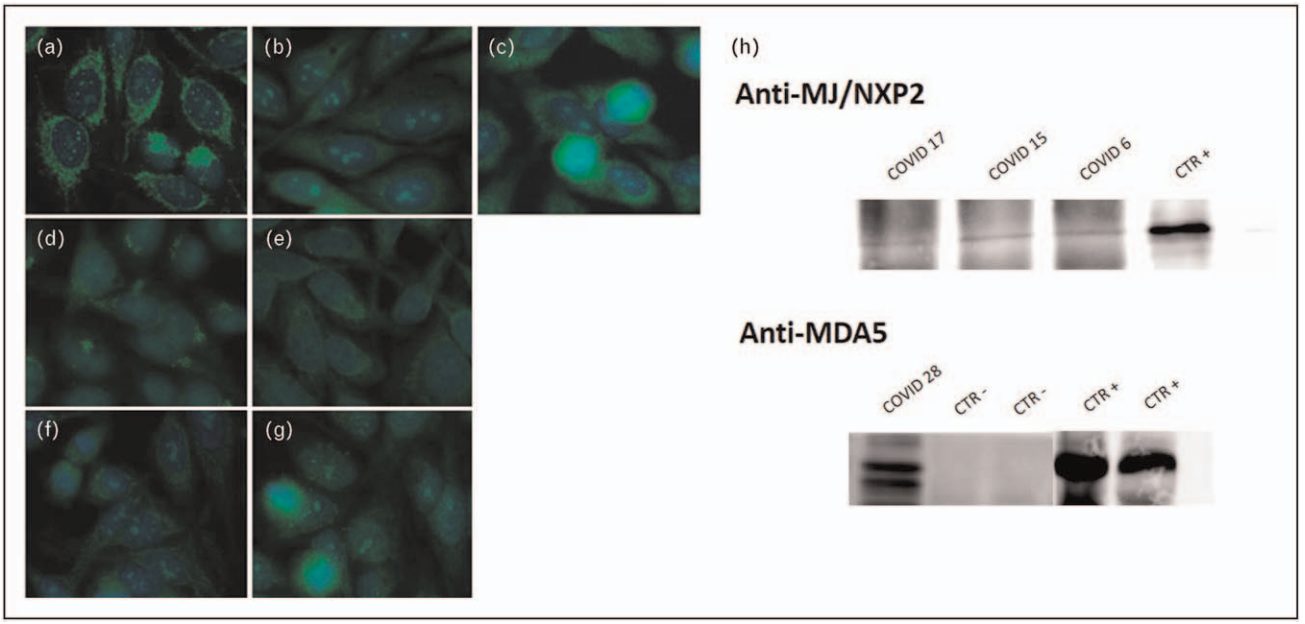

FIGURE 1. Immunofluorescence staining pattern of specific autoantibodies from patients with COVID-19. Serum dilution, 1:160. (a) Anti-MJ/NXP2: few nuclear dots and cytoplasmatic reticular AMA-like. (b) Anti-MJ/NXP2: nucleolar clumpy and cytoplasmatic speckled. (c) Anti-MJ/NXP2: nucleolar punctate and cytoplasmatic speckled. (d) Anti-RIG1: weak nuclear homogenous and cytoplasmatic polar/Golgi-like patterns. (e) Anti-RIG1: weak nuclear speckled. (f) Anti-MDA5: nucleolar clumpy and cytoplasmatic reticular AMA-like. (g) Anti-Scl70/TOPO1: weak nuclear homogeneous. (h) IP-Western blot analysis of COVID-19 patients with autoreactivity for anti MJ/NXP2 ($n = 3$) and -MDA5 ($n = 1$) antibodies (*CTR+: positive control, CTR−: negative control*).

patients had ANA (34.5%), antiphospholipid antibodies (antiβ2 glycoprotein I 34.5% and anticardiolipin 24.1%), p-antineutrophil cytoplasm antibodies (ANCA) (6.9%), and c-ANCA (6.9%) [25]. A recent meta-analysis confirmed that the presence of 'latent autoimmunity', defined as the isolated presence of autoimmune antibodies without sign or symptoms fulfilling classification criteria for autoimmune disease, correlates with a more critical disease and longer hospital stay, and the most represented antibodies in this condition include ANA (43%), rheumatoid factor (57%), and antiphospholipid antibodies (57%). This is in agreement with our data, as patients with COVID-19 and ANA are more frequently females and have a more severe lung impairment, requiring more frequently supplemental oxygen and antiviral therapy, which partially accounts for the longer hospital stay also associated. We hypothesize that in the context of an acute viral infection as the one induced by COVID-19, autoantigens can be exposed leading to the loss of tolerance and to the development of less-specific autoantibodies, such as ANA, which may worsen the tissue damage created by the inflammatory response to the virus.

Regarding more specific autoantibodies, Chang *et al.* [26] reported that 50% of patients had autoantibodies recognizing autoantigens associated with CTD, namely transient autoantibodies against MDA5 and Jo1, and permanent autoantibodies against Scl70/TOPO1. Our data confirm the significant correlation between positive anti-MJ/NXP2, anti-RIG1, anti-MDA5, and anti-Scl70/TOPO1 and the severity of COVID-19, mainly in terms of pulmonary disease at lung imaging without any sign of a *de novo* or preexisting rheumatic disease.

Additional rare specificities have been recently reported in a review by Liu *et al.* [7], for which autoantibodies against contactin-associated protein 2 (anti-Caspr2), ganglioside GD1b (anti-GD1b) and myelin oligodendrocyte glycoprotein (anti-MOG) have been shown in case reports or retrospective studies with unclear meaning in the COVID-19 disease onset and manifestations such as neurological impairment [27,28].

In our cohort of COVID-19 patients, all the antigens recognized by patients' autoantibodies are relevant to the pathogenesis of SARS-CoV-2 viral infection. First, MDA5 is the main sensor of SARS-CoV-2 infection in lung cells [29▪], in which interferon-induced genes, such as the one encoding for the different components MDA5 and RIG1, are upregulated during COVID-19 [30,31]. Second, double-stranded RNA (dsRNA) molecules are produced during coronavirus infections and are recognized by pattern recognition receptors, such as the RLR, which in turn undergo a conformational change

that allows their interaction with mitochondrial antiviral signaling (MAVS) protein located in the outer mitochondrial membrane. MAVS is a fundamental signaling molecule to link the upstream viral RNA recognition by RLR to downstream activation of the NF-kB transcription pathway. In MDA5- or MAVS-knockout cells, the number of cells infected by the Coronavirus was higher due to a significant decrease in interferon production [32]. Third, both Scl-70/TOPO1 and MJ/NXP2 are important players in host responses to viruses, being involved in activating inflammatory response against infectious agents and in RNA metabolism [33,34], respectively. Fourth, the mitochondrial dysfunction observed during COVID-19 and its possible role in host defense escape mechanisms fit with the observed prevalence of antibodies directly against mitochondrial antigens (E2/E3) without an association with pulmonary and liver disease severity.

We speculate that the SARS-CoV-2 infection may lead to the hyper-expression of proteins involved in the response against the virus, with more severe and longer infection further contributing to this process. This may on the one hand indicate a stronger antiviral response against the more severe infection and on the other hand that the underlying virus-induced immune modification, leading to the development of autoantibodies to specific proteins involved in the antiviral response in a subset of patients, may worsen the lung damage or even induce long-term complications. Moreover, such a complexity in the immune landscape of COVID-19 can possibly account both for the inter-individual variability of the disease manifestation and for the persistence of symptoms time after healing (known as 'long COVID').

We currently live a moment in which vaccination campaigns against COVID-19 are playing a fundamental role in the prevention of this disease. A recent report on a case of myositis related to COVID19 vaccination has been published [35], with muscle inflammation related to the site of vaccine injection and not with systemic involvement, and also this aspect plays a key role to avoid COVID-19 inflammatory and autoimmune disease manifestations.

CONCLUSION

One of the hallmarks of COVID-19 infection is the complex host-pathogen interaction that governs the innate immune signaling circuits. In this scenario, viral RNA sensors such as MDA5 and RIG1 seem to be crucial players in setting a type I interferon response. Nonetheless, the dyskinetic interferon response which occurs late with respect to viral replication, does not allow to control over the infectious process but rather it triggers the expression of numerous genes which likely contribute to COVID-19 immunopathogenesis. Further investigation on the innate immune system will likely provide additional insights regarding the molecular basis of severe disease outcomes and enable new therapeutic strategies for the treatment of patients with COVID-19.

On the other hand, the dysregulation of the immune response is the inflammatory milieu from which autoantibodies can emerge. The immune-mediated tissue damage that results from the production of self-reactive autoantibodies can further contribute to the unfavorable outcomes in COVID-19, as we detected in our study population. This may even explain the wide range of disease manifestation severity, as well as some of the signs and symptoms observed in the so-called '*long COVID*'. In that case, autoantibodies could even be used as predictive markers of a more critical disease with additional sequelae.

It is still unclear whether the positivity to autoantibodies is a transient epiphenomenon in the acute infection, or whether SARS-CoV-2 itself is a trigger of autoimmunity. Further studies will help understanding if this auto reactive state persists in time, thus converting into a self-sustaining autoimmunity that precedes the onset of connective tissue diseases. If this is the case, a rheumatologic follow up would be indicated in COVID-19 patients who developed autoantibodies, in order to detect the first manifestations of rheumatic diseases and propose early intervention strategies.

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Conflicts of interest

There are no conflicts of interest.

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Lymphocyte immunophenotyping in inflammatory myositis: a review

Chiara Franco, Mariele Gatto, Luca Iaccarino, Anna Ghirardello, and Andrea Doria

Purpose of review
This is a comprehensive review of the current knowledge on predominant immune cell phenotypes involved in idiopathic inflammatory myopathies (IIM).

Recent findings
Major circulating immune cell subpopulations described in IIM encompass the lymphocyte compartment. An unbalance in T cell subsets seems to consistently affect the peripheral and muscle compartment, with a predominance of CD4+ T and B cells in dermatomyositis, CD8+ T cells in polymyositis/inclusion body myositis (IBM) and novel findings highlighting novel proinflammatory T subsets, that is, CD8+Tbet+ and CD28− T cells across different IIM subsets. On the other hand, an impairment in Treg cells number and function has been described especially across polymyositis/dermatomyositis and IBM. Total T follicular helper (Tfh) cells, increased in immune-mediated necrotizing myopathy, skewed toward Tfh2 and Tfh17 in dermatomyositis, polymyositis, and juvenile dermatomyositis. B cell compartment is more rarely described in IIM, yet an unbalance in this pool is as well likely. Evidence of plasma cells increased in polymyositis, dermatomyositis, IBM, and Bregs decreased in dermatomyositis have been reported. Perturbations in the memory and naïve subsets are common in dermatomyositis/polymyositis and antisynthetase syndrome.

Summary
Protean immune cell abnormalities characterize different IIM subsets, reflecting the complexity of these autoimmune conditions. A deeper understanding of B-cell and T-cell immunophenotyping may promote early diagnosis and identification of new potential therapeutic targets.

Keywords
idiopathic inflammatory myopathies, immune system, peripheral and tissue lymphocytes

INTRODUCTION

Idiopathic inflammatory myopathies (IIM) are a group of rare and heterogeneous muscle inflammatory diseases, which differ from each other in muscle involvement and extra-muscular manifestations [1]. Myositis are autoimmune diseases characterized by both inflammatory and non-inflammatory mechanisms, immune abnormalities [2] and nonimmune mechanisms [3], sustained by genetic and environmental predisposing factors [2]. Disease subsets encompass dermatomyositis [2,4], polymyositis, immune-mediated necrotizing myopathy (IMNM), antisynthetase syndrome, with a distinctive feature of lung involvement [5], overlap syndrome with myositis, inclusion body myositis (IBM), and cancer-associated myositis (CAM) [2]. Growing evidence demonstrates an interaction between the immune system and skeletal muscle injury in IIM, following different pathogenetic mechanisms [6▪]. Indeed, the histopathological features of IIM share the presence of mononuclear cell infiltrates and muscle fiber necrosis [7]. Although both the innate and adaptive immune systems appear to be involved in the pathogenesis of IIM [8], lymphocytes seem to play a central role [9], where adaptive immunity to self-antigens is induced [10,11]. T and B cells are major components, recognizing specific antigens and generating specific cell-mediated or antibody-mediated responses [12▪▪]. T cells are predominant

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KEY POINTS

- Lymphocytes play a central role in myositis pathogenesis, where adaptive immunity to self-antigens is induced.
- An unbalance in T-cell and B-cell subsets seems to affect the peripheral and muscle compartment of myositis patients.
- The understanding of immune cells abnormalities in the different subsets of IIM may promote early diagnosis and identification of new potential therapeutic targets.

in muscle inflammatory infiltrates, with differences in T cell subpopulations according to myositis subset, while B cells are rare [10]. T-cell subsets include CD4+ T helper (Th) cells, which recognize major histocompatibility complex (MHC) class II-presenting peptides, and CD8+ cytotoxic T-cells, recognizing MHC class I-restricted peptides [10]. Consequently, CD4+ T cells generate a repertoire of effector T cells, including Th and T follicular helper (Tfh) cells, that activate target cells, besides regulatory T (Tregs) cells with immunosuppressive function [12▪▪]. Significantly, it has been reported an association between HLA alleles and autoantibody specificities in IIM patients [13]. Despite their predominance in biopsy [8], the precise role of T cells in the pathogenesis of IIM has not been clarified; nevertheless, the presence of T lymphocytes expressing restricted T cell receptor (TCR) families suggests that clones capable of recognizing autoantigens effectively participate in the pathophysiology of the disease [6▪]. Consistently, myositis-specific (MSAs) and myositis-associated autoantibodies profile represents a diagnostic tool for adult and juvenile polymyositis/dermatomyositis [14–17]. MSAs are associated with CAM, peculiar skin lesions or pulmonary involvement [14] in IIM, and they are useful in the definition of disease subsets [16]. This review aims to overview the major lymphocyte subsets in IIM.

T LYMPHOCYTES

Tissue T lymphocytes

The classical infiltrating lymphocyte pattern highlights CD4+ T cells and B cells to be prevalent in the perimysium in dermatomyositis [8], with CD4+ Th cells and B cells infiltrating endomysial capillaries [9] too, while CD8+ T cells predominantly populate the endomysium in polymyositis [8]. CD8+ T cells in polymyositis directly attack muscle fibers expressing MHC class I-presenting antigens [9,6▪], with consequent release of cytotoxic molecules, tissue destruction, and release of autoantigens [6▪]. It has been observed that CD8+ T cells infiltrating IIM muscle express perforin-1, granzyme B [10], and IFN-γ [18], indicating their muscle cytotoxicity [10]. The subset CD8+CD57+ T cells exhibit enhanced cytotoxic potency and impaired proliferative capability [18]. Significantly, it has been observed an expansion of these cells in IIM patients compared with healthy controls, particularly in IBM. On the contrary, this population is not increased in antisynthetase syndrome anti-Jo-1+ patients [19▪]. Specifically, it has been observed an increased frequency of CD8+ T cells expressing high levels of T-bet (T-box expressed in T cells) and senescent marker CD57 (CD8+T-bet+CD57+ $CD28^{null}CD27^{null}CD127^{null}$) in both the muscle and blood of sIBM. The presence of also nonsenescent cells (CD8+T-bet+ CD57− $CD28^{low}CD27^{low}CD127^{low}$CD38+HLA-DR+) in the patients suggests continuous proliferative capacity and effector functions of these cells, explaining the progressive and destructive nature of IBM [20▪]. Finally, CD8+T-bet+ cells have been suggested as IBM biomarker [20▪], thus partially justifying the resistance of IBM to glucocorticoid treatment, since terminally differentiated effector T cells seem to be resistant to glucocorticoids [6▪]. Another peculiar infiltrating pattern has been identified in the IBM muscle, that encompasses a signature of highly differentiated cytotoxic CD8+ T cells (effector memory cells, TEMs, and terminally differentiated effector memory cells, TEMRAs) [12▪▪], responsible for myofibers destruction [20▪,21]. The interaction of various inflammatory cytokines and chemokines can lead to an imbalance between regulatory [e.g., regulatory T (Tregs)] and inflammatory (e.g., Th17) cells [11,22], as well as abnormal autoantigen clearance mechanisms and antigen presentation [11]. Tregs reduction is observed within the muscle of IBM patients [23], while in juvenile dermatomyositis (JDM) patients muscle Tregs seem to be increased, despite their loss of function in regulation of the immune system [23,24]. In accordance, many studies report functional deficiency of Tregs in all IIM clinical subsets [25].

A novel highly differentiated CD28− T-cell subset has been found in peripheral blood and inflammatory infiltrates of IIM patients [8], as predominant muscle-infiltrating T-cell phenotype [8,12▪▪]. This long-lived pro-inflammatory T cell [10] is proposed to arise from prolonged T cells stimulation [8], as a result of a chronic inflammatory stimulus [10]. CD28− T cells might display strong myotoxicity [8] because of their high-IFN-γ secretion and degranulation potential [12▪▪]. In comparison with CD28+ T cells, CD28− T cells are

hypersensitive and able to release large amounts of cytokines as well as cytolytic granules [12▪▪]. Specifically, CD28− T cells are increased in the muscle of dermatomyositis, polymyositis, and IBM patients [12▪▪]. In polymyositis, CD28−, CD4+, CD8+ T cells can induce a greater degree of muscle cell death. Furthermore, myotubes are more sensitive to CD28− T cell lethality than myoblasts, possibly owing to muscle-specific antigens during differentiation [12▪▪]. In addition, these cells are less responsive and resistant to apoptosis and to immunosuppressive therapies [8]. Indeed, CD28− T cells proliferation and function are only partly suppressed by glucocorticoids and Tregs in dermatomyositis/polymyositis patients [12▪▪].

Circulating T lymphocytes

Increased CD4+ T cells are also represented in peripheral blood of dermatomyositis patients, while both dermatomyositis and polymyositis patients exhibit decreased circulating CD8+ central memory T-cells [12▪▪]. On the other hand, Shimojima *et al.* [9] found decreased CD4+, CD8+, and CD3+ T cells within the peripheral blood lymphocytes of polymyositis/dermatomyositis patients with active disease. Among peripheral blood lymphocytes, the proportion of activated Th cells was significantly increased in both polymyositis and dermatomyositis, when compared with controls, while natural killer and activated B cells were significantly decreased [9]. Immediately after muscle injury, the immune response is dominated by the arrival of Th1 lymphocytes and the stimulation of proinflammatory cells [6▪]. The increase in the percentages of activated Th1 and Th2 cells, with a decrease of Th17 cells, leads to increased Th2/Th1, Th2/Th17, and Th1/Th17 ratios in patients compared with controls, and reflect disease activity but not severity in polymyositis/dermatomyositis [9]. By contrast, a recent review reported increased Th17 cell subset in the peripheral blood of both adult and JDM [9,12▪▪]. Th2/Th17 in both polymyositis and dermatomyositis, and Th2/Th1 in dermatomyositis, significantly decreased after clinical remission compared with those observed before treatment in patients who received prednisolone with or without immunosuppressive agents [9].

The increase in CD8+T-bet+ observed in the muscle is confirmed in the blood of sIBM. Moreover, consistently with the decrease in Treg cells in inflamed muscle [6▪], the decrease in peripheral Tregs [8] in polymyositis/dermatomyositis patients would fail to prevent autoimmunity and control inflammation, contributing to the pathogenesis of these diseases. Tregs reduction could be aggravated by conventional therapies, especially immunosuppressive agents, consequently increasing patients' risk of malignancies and infections [26]. The lack of significant differences among peripheral blood lymphocytes subsets in JDM make them poor predictors of disease activity. However, it has been reported a higher Th/T suppressor cell ratio in JDM patients with respect to healthy controls [21], and activated CD3+CD69+ T cells decrease in association with a decreased disease activity [21].

The levels of circulating Tfh cells precursors are found to increase in IIM patients compared with controls, and the dysregulation of Tfh cells and its associated cytokine (IL-21), may cause loss of immune tolerance of B-cells [22]. The circulating Tfh CXCR5+CD4+ T cells are specialized to support B cell maturation in germinal centers. Tfh cells are skewed toward Tfh2 and Tfh17, as opposed to Tfh1 cells, in dermatomyositis, polymyositis, and JDM patients, and this cell differentiation has been linked to disease activity and the number of blood plasmablasts. In addition, Tfh cells are increased in IMNM patient with positive HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase) target [12▪▪,27] autoantibody and subsequently declined after immunosuppressive therapy, with an improved clinical outcome [12▪▪].

The increase of CD28− T cells in the peripheral blood of dermatomyositis, polymyositis, and IBM patients is lower compared with the frequencies detected in inflamed muscle, indicating active recruitment, local proliferation or preferential retention of CD28− T cells in the tissue [8,12▪▪].

B LYMPHOCYTES

Tissue B lymphocytes

B-cell activating factor (BAFF) up-regulation in muscle biopsy, especially in patients with anti-Jo-1 antibodies and dermatomyositis, suggests that a local maturation of B cells to antibody-producing plasma cells may occur in myositis, where B cells may act as antigen-presenting cells [10]. Moreover, BAFF Receptor is expressed in skeletal muscle inflammatory cells, and BAFF expression may be associated with an increased number of CD4+ T cells and CD19+ B cells in dermatomyositis, suggesting that BAFF/BAFF-R pathway contributes to both T and B cell responses [28]. Consistently, both B cells [29] and terminally differentiated plasma cells [21], have been reported in muscle tissue of polymyositis [8,10], dermatomyositis, sIBM [10], and IBM [8] indicating their likely role in muscle inflammation [10]. More recently, it has been shown that plasma cells can be identified in all subtypes of IIMs and may undergo modifications (clonal expansion, class

switch recombination, and somatic hyper mutation) that support an antigen-driven response, while B cells are found in the perivascular infiltrate of dermatomyositis patients [24].

Within the muscle biopsies of anti-Jo1 antisynthetase syndrome patients, it has been found perifascicular infiltrations of memory B cells. The dysregulated homeostasis of memory B cells between tissue and blood compartments suggests that they target the muscle, where they carry out effector functions [19▪]. Consistently to peripheral blood, IgM+ Jo-1-binding B cells are detected in the muscle biopsy of antisynthetase syndrome [30▪].

Circulating B lymphocytes

It is well known that autoantigens, such as Jo-1 and Mi-2, drive a B-cell antigen-specific immune response in muscles [10], which reflects the presence of MSA as circulating markers of disease entities within the spectrum of myositis [15], including polymyositis/dermatomyositis patients [14,24]. In the peripheral blood of JDM patients with active disease, immature transitional B cells, presenting an inflammatory phenotype, are expanded [30▪]. Conversely, circulating transitional B cells did not differ in dermatomyositis compared with controls, yet their level decreased after treatment [12▪▪]. Circulating naïve and memory B cells are abundant in dermatomyositis/polymyositis patients [12▪▪], while a decrease in memory B cells together with an increase in naïve B cells have been reported in anti-Jo1 syndrome [12▪▪,19▪].

A recent research highlighted that in antisynthetase syndrome patients, the majority of Jo-1-binding B cells were IgM+ (not class-switched) with a higher percentage of autoimmune-prone $CD21^{lo}$ cells related to disease severity, compared with non-Jo-1-binding B cells. Moreover, $CD21^{lo}$IgM+IgD-CD27+ memory B cells were increased, showing a reduced capacity to differentiate into antibody-secreting cells. Specifically, the detection of IgM+ Jo-1-binding B cells in peripheral blood, consistent with antisynthetase syndrome/IIM patient muscle biopsy findings, suggests that IgG class switch and terminal differentiation of Jo-1-binding B cells occurs at the site of attack. Authors reported that IgG class-switching of Jo-1-binding B cells is not restricted to tissue and that they exit tissues to recirculate after undergoing IgG class switch [30▪]. Consistently, in vitro data showed a reduced frequency of Jo-1-binding B cells differentiated into CD38hiCD24− plasmablasts compared with non-Jo-1-binding B cells [30▪].

To date, the evidence about the role of $CD19+CD24^{high}CD38^{high}$ regulatory B cells (Bregs) with immunosuppressive properties is not fully elucidated and limited to dermatomyositis [31,12▪▪]. It has been observed a significant decrease of Bregs in blood samples of dermatomyositis patients in comparison with healthy controls and patients affected by other autoimmune diseases [31,32], showing a relationship with MSA, pulmonary interstitial fibrosis and global disease scores [12▪▪]. In fact, dermatomyositis patients with positive MSA had lower Bregs levels than negative patients, and lower level of Bregs was also found in dermatomyositis patients with than in those without interstitial lung disease. Indeed, dermatomyositis patients in remission, had Breg levels significantly increased after treatment [31].

NOVEL POTENTIAL CIRCULATING MARKERS IN IMMUNE-MEDIATED INFLAMMATORY MYOSITIS

Recently, it has been compared the number of lymphocytes with that of neutrophils, players of adaptative and innate immunity, respectively. The value of neutrophil-to-lymphocyte ratio (NLR) in adult patients with polymyositis/dermatomyositis was investigated in survived and nonsurvived patients. Polymyositis/dermatomyositis patients in nonsurvivor group exhibited a significantly higher baseline NLR value compared with that in the survivor group. The research revealed that high-NLR value is an independent risk factor for survival in patients with polymyositis/dermatomyositis, especially in association with lung involvement. Furthermore, it has been suggested to be associated with disease activity in many malignant and nonmalignant diseases, including systemic autoimmune diseases [33].

Significantly, extracellular vesicles has been reported as participating in abnormal activation of the autoimmune system [11]. These heterogeneous lipid bilayer nanoparticles, naturally released from cells [34,35], mediate cell-cell communication. Extracellular vesicles play a role in different immune-related processes: antigen presentation, T-cell stimulation, cell killing, cytokine transport, and Treg cells differentiation (Fig. 1). Moreover, they induce antigen-specific tolerance and establish allograft tolerance [36]. Extracellular vesicles express peptide-MHC [36], thus they can present intracellular self-antigens to activate autoreactive T cells [11], including both CD8+ and CD4+ T lymphocytes [36], which in turn mediate the development of the disease [11]. Furthermore, TCR-enriched extracellular vesicles released by T cells are activation-competent, highlighting a novel form of contact-independent APC-T cell crosstalk [36]. It is noteworthy that extracellular vesicles derived from Treg cells

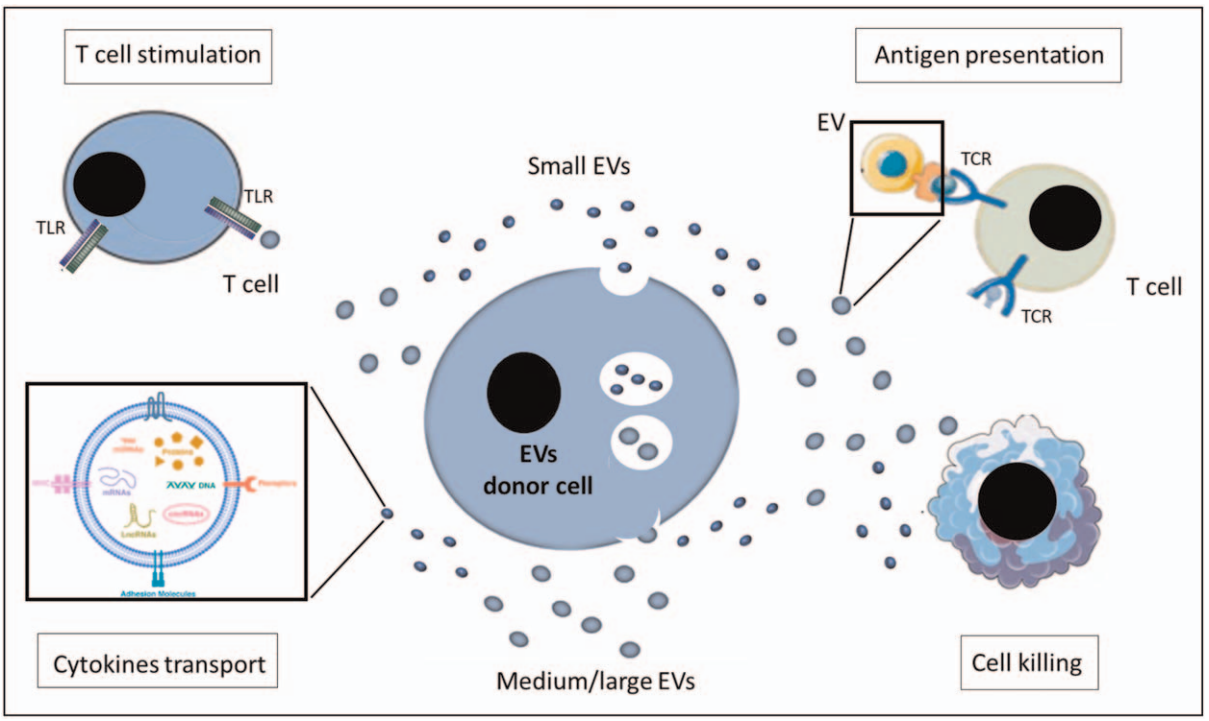

FIGURE 1. Role of extracellular vesicles in immune-related processes. The extracellular vesicles released from cells participate in abnormal activation of the autoimmune system acting at level of T-cell stimulation, antigen presentation, cytokine transport and cell killing. EVs, extracellular vesicles; TCR, T-cell receptor; TLR, Toll-like receptor.

could promote other T cells polarized to the Treg phenotype [37]. Indeed, Treg cells inhibit the proliferation and function of Th1/Th17 cells through direct cell–cell contact, producing anti-inflammatory cytokines, and releasing extracellular vesicles with regulatory activity [36]. On the other hand, extracellular vesicles from endothelial cells possess the modulation ability which blocks T cells activation and dampens tissue chronic inflammation [37]. In addition, immune cell-derived extracellular vesicles not only promote immunity but can also reduce immune activity [11], for example modulating the suppressive function of Treg cells [36]. Finally, mesenchymal stem cells release immunosuppressive extracellular vesicles, which are actually used to treat autoimmune diseases [37].

THERAPEUTIC OPTIONS

Most of therapeutic strategies for IIM are directed to suppressing or modifying immune cells activity [10] as immunotherapy treatment [38]. Current treatment in polymyositis/dermatomyositis is focused on the efficacy of glucocorticoid-sparing immunosuppressive agents. Glucocorticoids are used empirically as first-line treatment despite their various adverse effects. However, the concomitant treatment with glucocorticoid-sparing immunosuppressive agents, even as combined multitarget treatment, successfully reduces the initial glucocorticoid dose for remission induction, the relapse risk during glucocorticoid tapering, and adverse effects of glucocorticoids [39]. Finally, biologics drugs seem promising in some IIM patients [39]. Indeed, a recent study conducted in polymyositis and dermatomyositis patients highlighted that absolute numbers of Tregs is restored by low-dose IL-2, which also allows a modest increase of other circulating lymphocytes populations. This treatment, coupled with conventional therapy, leads to clinical remission reducing muscle tissue inflammation and chemokines secretion by fibroblasts, thus decreasing peripheral lymphocytes infiltration [26]. Finally, a suggested therapy against antisynthetase syndrome (Jo-1+) should target both nonclass-switched Jo-1-binding B cells and IgG class-switching to more effectively block cross-talk with autoreactive T cells [30▪].

CONCLUSION

The growing interest in the characterization of lymphocyte populations involved in the pathogenesis of myositis aims to use them for differential

Table 1. Abnormalities in lymphocyte subsets in idiopathic inflammatory myopathies (IIM)

Tissue T lymphocytes	
DM	↑ CD4+ T cells; CD28− T cells
PM	↑ CD8+ T cells; CD28− T cells
IBM	↑ TEMs and TEMRAs cells; CD28− T cells; CD8+T-bet+ cells
	↓ Treg cells
JDM	↑ Treg cells
Tissue B lymphocytes	
DM	↑ B cells; plasma cells
PM	↑ B cells; plasma cells
IBM	↑ B cells; plasma cells
ASS	↑ IgM+ Jo-1 binding B cells; memory B cells
Circulating T lymphocytes	
DM	↑ CD4+ T cells; Th1, Th2 cells; Tfh2, Tfh17 cells; CD28− T cells
	↓ CD8+ T cells; CD8+ memory T cells; CD3+ T cells
PM	↑ Th1, Th2 cells; Tfh2, Tfh17 cells; CD28− T cells
	↓ CD8+ T cells; CD8+ memory T cells; CD3+ T cells; Th17 cells; Treg cells
IBM	↑ CD28− T cells; CD8+T-bet+ cells
JDM	↑ Th17 cells; Tfh2, Tfh17 cells
Circulating B lymphocytes	
DM	↑ Naïve B cells; memory B cells
	↓ Breg cells
PM	↑ Naïve B cells; memory B cells
JDM	↑ Immature transitional B cells
ASS	↑ Naïve B cells; CD21lowIgM+IgD-CD27+ memory B cells
	↓ Memory B cells

Characterization of T and B lymphocytes in tissue and circulating compartments in myositis subsets. Up and down arrows indicate expanded and reduced population, respectively. ASS, antisynthetase syndrome; Breg cells, regulatory B cells; DM, dermatomyositis; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathies; JDM, juvenile dermatomyositis; PM, polymyositis; TEMRAs, terminally differentiated effector memory cells; TEMs, effector memory cells; Tfh cells, T follicular helper cells; Th cells, T helper cells; Treg cells, regulatory T cells.

diagnosis of disease subsets (Table 1). Moreover, the recent correlations between lymphocytes and neutrophils (NLR), as well as the study of extracellular vesicles role in autoimmune diseases indicate the continuous research for new disease-specific biomarkers. Myositis therapeutic strategies are constantly evolving with the aim of affecting the immune response. Further understanding of the lymphocyte populations that dominate myositis will help to manage the differential diagnosis and appropriate therapy for these diseases.

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Conflicts of interest

There are no conflicts of interest.

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Skeletal muscle immunohistochemistry of acquired and hereditary myopathies

Olof Danielsson and Bo Häggqvist

Purpose of review
The continued development in the field of immunohistochemistry (IHC) has improved the ability to diagnose muscle diseases. Many hereditary diseases are diagnosed by the absence or abnormal localization of proteins. Detection of secondary pathological protein expression is also used in diagnostics, and to study disease processes. We relate and discuss recent reports, where IHC has been an important tool in the investigation of muscle diseases.

Recent findings
In idiopathic inflammatory myopathies, IHC has extended its role to diagnose subgroups. This is most evident concerning immune-mediated necrotizing myopathy and antisynthetase syndrome. The availability of new antibodies has increased the sensitivity of a muscle biopsy to diagnose several hereditary myopathies. The introduction of protein restoration therapies in muscular dystrophies also comes with the need to detect and measure protein levels. For the study of disease processes at the protein level, in both acquired and hereditary myopathies IHC, often combined with gene studies, PCR-based methods, western blotting and electron microscopy, continues to bring forth interesting results.

Summary
IHC is an integrated tool in muscle pathology, where recent studies contribute to improved diagnostic skills and increased insights into disease processes.

Keywords
acquired myopathies, hereditary myopathies, immunohistochemistry, inflammatory myopathies

INTRODUCTION

Immunohistochemistry (IHC) has an essential role in diagnosing and studying diseases in muscle. The availability of antibodies, directed against epitopes of proteins involved in muscle disease, allows diagnosis at the protein level for a growing number of diseases [1]. Many recessive diseases are characterized by a weakened expression or the absence of proteins, whereas other proteins may be upregulated. In several, often dominant, diseases, there are an abnormal localization or aggregation of proteins [2▪▪].

The major reasons for performing a muscle biopsy are to diagnose diseases, evaluate treatment or to study disease processes. It is worth considering, that some diseases manifest in muscle tissue as a whole, whereas others show multifocal or, in time and space, varying degrees of involvement. IHC is commonly used together with stains for morphology and histochemistry, and sometimes immunoblotting or electron microscopy (EM) are added. In this review, we discuss recent publications, where IHC was an important tool in the investigation of human muscle biopsies.

Due to the length of muscle fibres, serial transverse sectioning of fibres is a convenient method to study the expression of multiple proteins in single fibres. Many primary antibodies are integrated in the diagnostic routine, and many more are applied in targeted approaches, for diagnosis or research purposes. In an effort to address the varying results and secure the quality of different laboratories, the *EURO-NMD pathology working group* recently presented *Standards for Muscle Pathology* [1]. The report contains valuable recommendations for the most commonly used antibodies. For a more general discussion of IHC in muscle pathology, the reader is

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KEY POINTS

- Immunohistochemistry is an essential tool in muscle pathology.
- Immunohistochemistry facilitates differential diagnosis of inflammatory myopathies and enables diagnosis at the protein level for many hereditary diseases.
- Immunohistochemistry is increasingly applied to evaluate genetic restoration therapy and to study disease processes at the protein level.

well advised to turn to *Muscle Biopsy – A Practical Approach* [2▪▪].

INFLAMMATORY MYOPATHIES

Xu *et al.* investigated muscle pathology and muscle specific autoantibodies (MSA) in blood in 111 patients with idiopathic inflammatory myopathy (IIM) [3]. There were 34 patients with an immune-mediated necrotizing myopathy (IMNM), 6 with polymyositis (PM) and 16 with nonspecific myositis (NSM). Patients with antisynthetase antibodies (ASA) were classified as dermatomyositis (DM), which constituted the largest group (56 patients). The authors noted that a perifascicular necrosis was particularly found in patients with Jo-1 antibodies in blood, and punched out fibres, only in non-ASA DM. IHC included typing of inflammatory cells and MHC I. MHC II and anti-C5b-9 (membrane attack complex; MAC) were not used. The results of this study support that IIM patients with Jo-1 antibodies have a distinctive pathology.

Day *et al.* compared the distinguishing features of IMNM patients (n = 62) with other IIM and normal controls (n = 17) [4▪]. They highlighted a clinical and histological heterogeneity within the IMNM group, and that patients with SRP antibodies presented with a more severe phenotype. They found that MAC not just stained the sarcoplasm in necrotic fibres, but also exhibited a granular sarcolemmal stain of nonnecrotic and regenerating fibres. The authors point out that the MAC staining characteristics lend support to a complement-associated cytolysis as the major cause of myotoxicity. However, not all degenerating fibres were stained with MAC, indicating the presence of other modes of cytotoxicity. The group used a graded scale for the expression of MHC I, MAC, LC3 (marker of autophagy), neonatal myosin heavy chain (MHCn) and perifascicular MHC I, similar to one used in our lab [5]. The same group investigated the High Mobility Group Box Protein 1 (HMGB1) in muscle tissue and blood, in patients with IIM [6]. HMGB1 is a ubiquitous nonhistone nuclear DNA-binding protein that under different physiological and pathological conditions undergoes extra-nuclear translocation, where it may act as a signal of tissue damage and a pro-inflammatory mediator. They used serial sections, stained with routine stains and IHC, including anti-HMGB1. They further measured HMGB1 in patient serum, using an ELISA kit. The authors concluded that HMGB1 shows increased expression in the sarcoplasm, associated with diverse pathological processes. The serum levels of HMGB1 were elevated in patients with IIM, with the exception of inclusion body myositis (IBM), compared to controls. The authors conclude that the release of HMGB1 from necrotic fibres could trigger further local muscle cytolysis, and thus have a deleterious role in IIM, and that measurement of HMGB1 in serum has a potential for evaluation of disease activity.

Ayaki *et al.* described the muscle pathology of three patients with Nakajo-Nishimura syndrome (NNS) [7▪], which is a rare genetic disease, caused by a recessive mutation of the PSMB8 gene that encodes the immunoproteasome subunit β5i. Immunoproteasome subunit β1 and β5 have been reported to regulate the expression of MHC I in IIM. The patients develop episodes of periodic fever, skin rash and progressive myopathy. This syndrome and two others are now referred to as proteasome-associated autoinflammatory syndrome. They found a mononuclear cell infiltration in the endomysium and in the perivascular area, consisting of $CD4^+$- and $CD8^+$- T cells, and an overexpression of MHC I in myofibres, confirming that NNS causes an inflammatory myopathy. As this genetic inflammatory myopathy has pathological resemblances with IBM and a poor response to corticosteroids, further study of this disease may gain insights to the more common IBM. This and the preceding study present support for an alternative chain of events, leading to IIM. In contrast to a starting point, with the production of antibodies to a yet unknown antigen, aberrations in processes, more often referred to as the innate immune response, may secondarily trigger adaptive immune responses, as further discussed in a review by Day [8].

We investigated apoptosis in IIM with partial invasion [9▪]. The infiltrates of these diseases are dominated by $CD8^+$ (cytotoxic) T-cells, also staining for granzyme B and FAS-ligand, known to induce apoptosis of target cells. We used serial sectioning of muscle, a panel of antibodies and the TUNEL assay to detect apoptosis. We found $TUNEL^+$ myonuclei almost exclusively in IIM with partial invasion, not in DM or healthy controls. Similar to earlier studies, we did not find signs of apoptosis in partially

Table 1. Typical pathology in IIM subgroups

IIM	Infiltrate	Blood vessels	Muscle fibres[a]	Perifascicular region	Inflammatory cells	MHC I[b]	MHC II[b]	MAC[b]
IBM	Endomysial	–	Partial invasion, vacuoles, mitochondrial changes	–	Cytotoxic T-cells, macrophages	+++	0	0
PM	Endomysial	–	Partial invasion	–	Cytotoxic T-cells, macrophages	++	0	0
DM	Perivascular	Capillar swelling, vasculitis[c]	Punched out fibres, ghost fibres	Atrophy, connective tissue fragmentation	Helper T-cells, B-cells	++	+	Capillaries:++, sarcolemma: +
IMNM	Endomysial	–	Widespread necrosis	-	Macrophages, T-cells sparse	+	0	Capillaries: +, sarcolemma: ++
AS	Perimysial	–	–	Necrosis, connective tissue fragmentation, atrophy	Macrophages, helper T-cells	++	++	0
NSM	Perivascular	–	–	–	T-cells, macrophages	++	+	0

AS, antisynthestase syndrome; DM, dermatomyositis; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathy; IMNM, immune-mediated necrotizing myopathy; NSM, nonspecific myositis; PM, polymyositis.
[a]Necrotic fibres are present in all types of IIM, and are not considered here.
[b]Grading (0 - +++) signifies the 'common' grades of protein expression in the group.
[c]Vasculitis is mainly found in juvenile DM.

invaded fibres, but in other fibres, also surrounded by $CD8^+$ and granzyme B^+ cells. The importance of apoptosis in IIM is uncertain, but its presence highlights that many pathogenic processes are ongoing, and need to be considered, when studying these diseases.

Xiaoyu Hou *et al.* 2021 investigated 24 patients with juvenile DM (JDM) and 12 with overlap myositis (OM) with IHC, Western blot and RT-PCR [10]. They found that the negative regulator of type I interferon ISG15, was more strongly expressed in muscle of JDM patients, compared to OM and controls, and that MHC II expression was upregulated in perifascicular muscle fibres of OM patients. The type 1 interferon signature in DM and the perifascicular MHC II upregulation in OM, similar to adults with antisynthetase syndrome, are distinguishing pathological features of these diseases. The presence of peripheral T helper cells (Tph) in 26 adult DM patients was studied by Cyrille Hou *et al.* [11]. They used IHC for typing of inflammatory cells and flow cytometry for cell typing in blood. They found that Tph and B cells were expanded and co-aggregated in muscle, whereas there was a reverse relation of Tph cells to disease activity in blood. The authors mention Tph cells as potential markers of disease activity, and that these cells could have a key role of forming ectopic lymphoid structures in muscle.

The classification of IIM has been an evolving discussion, based on clinical features, pathology and, increasingly on MSA detection in blood. The localization of the inflammatory infiltrates, the type of affection of muscle fibres, connective tissue and blood vessels, help to diagnose different IIM subgroups, as do different IHC stains [12]. Five main pathological entities of IIM have been recognized: DM, PM, necrotizing autoimmune myopathy (NAM, the alternate designation of IMNM), IBM and NSM [12]. Lately, a strong case has been made to also add antisynthetase syndrome to that list [13–15], consistent with the perimysial pathology, earlier described in this journal by Pestronk, and further investigated in a more recent study [16,17]. In contrast, the PM-entity has been vigorously debated [18–20] (see separate contribution in this issue). With a reminder of the not uncommon overlap between and variations within groups [19], the typical pathological features, differentiating IIM, are summarized in Table 1, and illustrated in Fig. 1.

Immune checkpoint inhibitors have significantly improved the treatment of a variety of cancers, but immune related side effects are not uncommon. Matas-Garcia *et al.* reported a distinct myopathology in nine thus treated patients, who had developed an inflammatory myopathy [21].

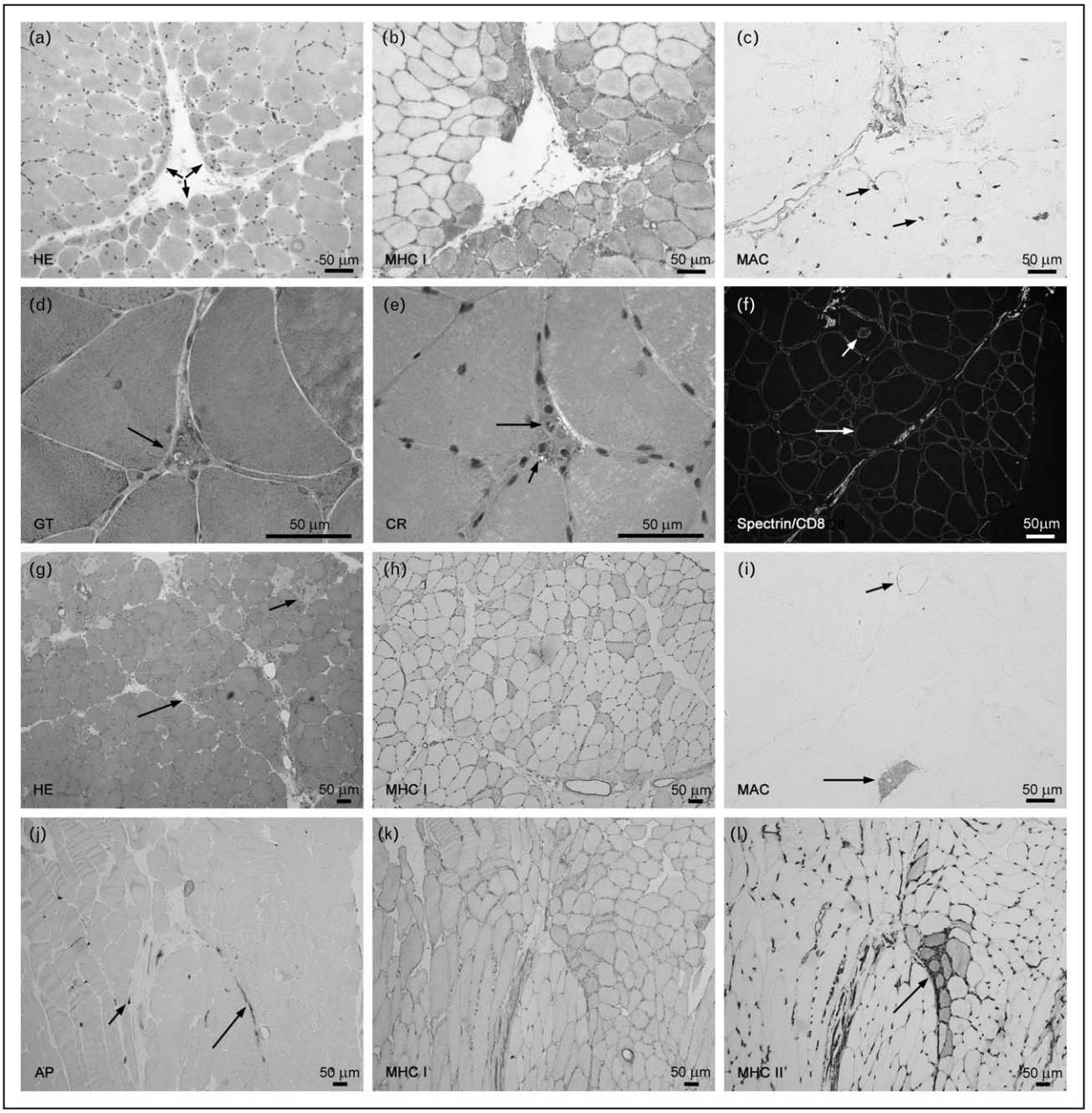

FIGURE 1. Distinguishing pathology in IIM. Dermatomyositis: In the haematoxylin-eosin (HE) stain, a perifascicular atrophy is evident (arrows, a), and the area shows a strong upregulation of MHC I (b), and intensively C5b-9 (MAC)-stained capillaries (arrows, c). Inclusion body myositis (IBM): A fibre with rimmed vacuoles (arrow) is shown in modified Gömöri trichrome stain (GT, d). The same fibre in Congo red stain (CR) shows amyloid substance (long arrow), which shows birefringence in polarized light (short arrow, e). Partial invasion of a fibre (demarcated by spectrin) with $CD8^+$ T cells (short arrow) is mandatory for the diagnosis IBM, but it is also seen in polymyositis (immunofluorescence, f). Immune-mediated necrotizing myopathy: A biopsy from a patient with HMGCR-antibodies in blood, shows several fibres in different stages of necrosis (paling, small arrow; macrophage invasion, large arrow; g). The MHC I stained section shows a non-uniform pattern of upregulation in fibres (h). MAC not only stains a necrotic fibre (large arrow), but also sarcolemma of a non-necrotic fibre (small arrow, i). Antisynthetase syndrome: In a patient with Jo-1 antibodies in blood, the stain for alkaline phosphatase (AP) shows intense stain in the perimysium (large arrow) and of interstitial cells (small arrow, j). In the perifascicular area, where an inflammatory infiltrate is seen, the fibres strongly upregulate both MHC I (k) and MHC II (arrow, l).

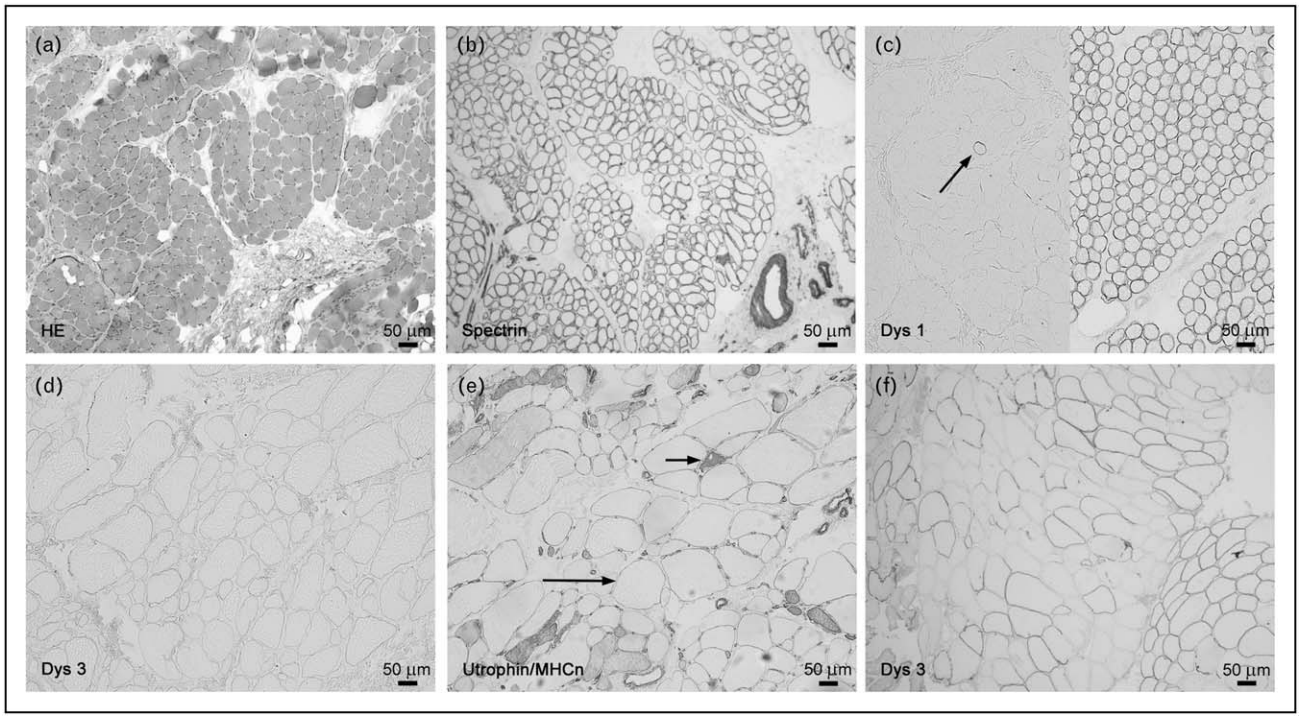

FIGURE 2. The pathology of dystrophinopathies. The haematoxylin-eosin (HE) stained section from a patient with Duchenne myscular dystrophy is shown (DMD, a). There are round fibres, increased endomysial tissue, and some necrotic fibres. A spectrin stain shows normal expression except for some necrotic fibres (b). A stain with antibody against the rod domain of dystrophin (Dys 1) shows no expression (c, left), except in one fibre, which has spontaneously remutated (''revertant fibre'', arrow). A normal control is also shown (c, right). The dystrophin expression in a biopsy from a Becker patient is reduced (d). Here, an antibody against the N-terminus is used (Dys 3). Double stain against utrophin and neonatal myosin heavy chain (MHCn), shows upregulation of utrophin in regenerating (MHCn-positive, small arrow) as well as in non-regenerating fibres (MHCn-negative, large arrow) (e). Due to aberrant allele inactivation in a symptomatic carrier (mother of a child with DMD), the expression of dystrophin is disturbed (f).

The inflammatory infiltrates were in all cases focally clustered, and dominated by $CD68^+$ macrophages in a pseudo granulomatous pattern. MHC I expression predominated in the perifascicular zone, and MHC II fibre expression was found in three cases.

DYSTROPHIC MYOPATHIES

Frank *et al.* reported a phase 1, dose escalation study in patients with Duchenne muscular dystrophy (DMD), using an antisense oligonucleotide designed to induce exon 53 skipping [22]. They compared the expression of dystrophin in muscle prior to treatment and after 48 weeks, using Western blot, RT-PCR and immunofluorescence. Successful exon skipping was detected in all patients. The median restored dystrophin expression was 1% of the normal amount. To translate to a meaningful clinical gain for patients, levels in the range of 15–40% should probably be aimed for [23]. Mendell *et al.* conducted a phase 1/2a, nonrandomized controlled trial, with a single systemic infusion of rAAVrh74.MHCK6.micro-dystrophin in patients with DMD [24▪]. Minimal side effects were observed and promising short time functional results. Post treatment expression, using immunofluorescence and western blotting of dystrophin, showed near normal expression. These results are certainly encouraging. In contrast to treatments using exon skipping, gene transfer therapy is not restricted to patients carrying specific mutations. On the other hand, the successful transfection of micro-dystrophin will at best lead to a disease, similar to a mild Becker muscular dystrophy. In addition to diagnose and differentiate diseases, caused by disturbances of dystrophin expression (dystrophinopathies), IHC offers an essential tool to evaluate treatments, aiming for dystrophin restoration. Figure 2 illustrates the pathology of these diseases.

VACUOLAR AND TRIPLET REPEAT MYOPATHIES

Two groups investigated patient biopsies with hereditary hypokalemic periodic paralysis (hypo-

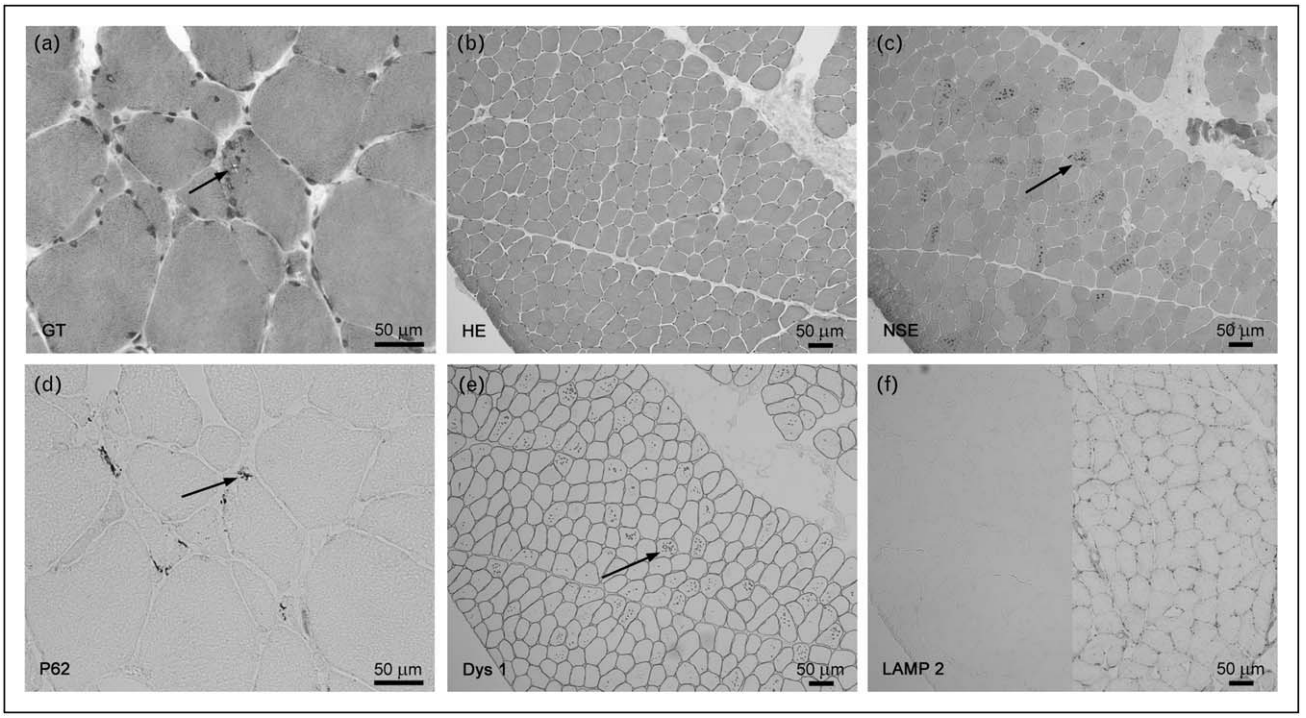

FIGURE 3. Two vacuolar myopathies. In section from a patient with Welander muscular dystrophy (a), there are fibres with rimmed vacuoles (arrow, modified Gömöri stain (GT)). The detection of the P62 protein identifies disturbance of the ubiquitin-proteasome pathway (d). Sections b, c, e, f show a biopsy from a boy with Danon's disease (i. e. LAMP-2 deficiency). The HE stain shows only slight pathological changes (b). The stain for non-specific esterase (NSE) shows esterase positive vacuoles (c), which are enveloped by dystrophin (Dys 1, e). No LAMP-2 expression is found, which confirms Danon's disease (f, left). Normal expression is seen in a control (f, right).

PP) due to CACNA1S mutations. With IHC, Nagasaka *et al.* investigated the localization of several proteins related to excitation-contraction coupling, in biopsies from two patients [25]. Using immunofluorescence, they found co-localization of L-type calcium channels and the ryanodine receptor at the margins of vacuoles, which together with ultrastructural findings indicate that the observed vacuoles originate from the sarcoplastic reticulum. Holm-Yildis *et al.*, using histochemistry, IHC and EM, detected vacuoles containing glycogen in both fibre types in biopsies, also from patients not yet experiencing paralytic attacks, highlighting a possibility of a hypo-PP when these type of vacuoles are detected [26].

Palmio *et al.* described eight patients with a dominant mutation in the J domain of DNAJB6, presenting with a distal myopathy [27]. Similar to patients with G/F domain DNAJB6 mutations with a limb-girdle phenotype (LGMD D1 DNAJB6-related), these biopsies, investigated with immunofluorescence, showed aggregation of DNAJB6, and markers of autophagy in the rimmed vacuolar fibres.

Oculopharyngeal muscular dystrophy (OPMD) is a late onset disease, characterized by ptosis, dysphagia and proximal weakness caused by a trinucleotide repeat expansion in the poly-A binding protein nuclear 1 gene (PABPN1). Using immunofluorescence, Galimberti *et al.* reported high sensitivity and specificity of detecting nuclear PABPN1, using a recombinant monoclonal anti-PABPN1 antibody for diagnosing OPMD, also in biopsies without vacuoles, and from patients without classical symptoms [28▪]. The approach described by the authors holds promise to become an additional tool, diagnosing OPMD. Xi *et al.* investigated a group of patients with Oculopharyngodistal MD (OPDM), not carrying the earlier known LRP12 gene expansion [29]. They found a 5'UTR CGG repeat expansion in the GIPC1 gene in 30, mostly unrelated, patients. Using routine stains and IHC, they investigated muscle biopsies in 17 cases. All biopsies contained dystrophic changes, and 14 had p62 (marker of autophagy) positive rimmed vacuoles, in the sarcoplasm and in intranuclear inclusions. The detection of vacuoles in muscle facilitates diagnosis of acquired and hereditary myopathies. IHC may help to demonstrate the content and probable origin of the vacuoles. Figure 3 shows pathological findings in two vacuolar myopathies: Welander muscular dystrophy and Danon's disease.

CONGENITAL MYOPATHIES

Ogazawa *et al.* used immunofluorescence antibodies against the ryanodine receptor 1 (RYR 1) in congenital neuromuscular disease with uniform type 1 fibre (CNMDU1) and central core disease [30]. They further found evidence of an evolution of core development with disease progression, and the results suggested that CNMDU1 due to RYR1 mutation, is a *de facto* core myopathy. Perrin *et al.* reported two siblings with a congenital myopathy with minicores, carrying three not earlier described titin mutations, causing a secondary loss of fast myosin heavy chain [31]. Two missense mutations were located in the I and A band of titin, domains critical for protein stability and interaction with myosin, respectively. No truncated protein was detected, indicating that the degeneration of the frame shift allele product was due to the third frame shift mutation.

Bouman *et al.* investigated 18 Dutch patients with the rare nemaline myopathy type 6 (NEM 6), caused by a mutation in KBTBD13 gene [32]. The disease is characterized by a peculiar slowness in movement and progressive proximal muscle and, particularly, neck flexor weakness, starting in childhood. NEM6 myopathology hallmarks are, in addition to rods, including *ring-rods fibres*, prominent cores and nuclear clumps. KBTBD13 protein binds to actin, and the mutated protein is suggested to *stiffen* the thin filament and thereby impair muscle-relaxation kinetics. Rods were immunoreactive for α-actinin and myotilin. The authors suggest that KBTBD13- related congenital myopathy ought to be classified as a rod-core myopathy. Evangelista *et al.* reported a patient with a heterozygous mutation in the Filamin C gene (FLNC), predicted to affect the actin-binding domain of the protein [33]. The clinical phenotype and MRI findings were strongly suggestive of a myofibrillar myopathy, related to a FLNC mutation. However, standard stains and antibodies against desmin, myotilin, αB-crystallin and ZASP did not show protein aggregation, but instead rods, ring fibres and type 1 predominance. These reports illustrate that pathological, clinical and genetic overlap is not uncommon in muscle diseases, and that some types of pathology evolve with time. The absence of fibre types is important to note in congenital diseases. Different protein domains affected by mutations in the same gene, may have different effects on pathology, as well as on clinical symptoms.

METABOLIC MYOPATHIES

The classic methods to diagnose mitochondrial disease in muscle have been modified Gömöri stain for detecting ragged-red fibres, and cytochrome oxidase (COX)/succinate dehydrogenase double stain for detecting COX-negative fibres. Recently, antibodies against the specific protein complexes of the respiratory chain have been applied to add diagnostic precision and sensitivity. This is shown in the study by Visuttjai *et al.*, describing two new mutations in the MT-TN gene [34], and Lu *et al.* detecting a weaker stain of antibodies against complex I and IV in three MELAS patients with normal histochemistry [35].

CONCLUSION

In idiopathic inflammatory myopathies, IHC has expanded its role to diagnose subgroups, most evident for immune-mediated necrotizing myopathy and antisynthetase syndrome. IHC is valuable for the evaluation of gene mutations with uncertain pathogenicity or deviating phenotypes, and of gene restoration therapy. Further, IHC, together with western blotting, ultrastructural investigations and PCR-based methods, continues to bring interesting insights into the disease processes at the molecular level.

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Conflicts of interest

There are no conflicts of interest.

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Polymyositis: does it really exist as a distinct clinical subset?

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Purpose of review
To summarize information on polymyositis; diagnosis, definitions, published data and opinions.

Recent findings
Polymyositis originally referred to inflammatory muscle diseases presenting with muscle weakness and inflammatory cell infiltrates on muscle tissue visible by microscopy. Over time and with improved technology to immunophenotype infiltrating inflammatory cells and characterize muscle fibres, the meaning of polymyositis changed and became more specific. There is ongoing controversy over the term polymyositis, with proponents for a strict definition based on histopathological and immunohistochemical features on muscle biopsies whereas others advocate for a broader clinical and histopathological phenotype. Over the past decades, the discovery of several myositis-specific autoantibodies together with distinct histopathological features have enabled the identification of new subsets previously labelled as polymyositis notably the antisynthetase syndrome and the immune-mediated necrotizing myopathies thus reducing the number of patients classified as polymyositis.

Summary
There are still a small number of patients among the idiopathic inflammatory myopathies that can be classified as polymyositis as discussed in this review but the entity is now considered relatively rare.

Keywords
antisynthetase syndrome, idiopathic inflammatory myopathies, immune-mediated necrotizing myopathy, polymyositis

The Master said, . . . If names are not correct, language is not in accordance with the truth of things. If language is not in accordance with the truth of things, affairs cannot be carried out to success. . .. Therefore, a superior man considers it necessary that the names he uses be spoken appropriately. . .. What the superior man requires, is just that in his words there may be nothing incorrect.

Confucius, Chinese sage and philosopher, 551–479 BC, from Book XIII

With courtesy of Dr FW Miller

INTRODUCTION

Classification of diseases is essential to discuss treatment and prognosis with patients, to understand the pathophysiology of a condition and to perform clinical trials. This might be straightforward in conditions where the underlying cause is known, such as infections, for example, coronavirus [coronavirus disease 2019 (COVID-19)] infection. For diseases with undefined or multifactorial causes, such as idiopathic inflammatory myopathies (IIM), definitions need to be agreed upon. This is where classification criteria can help clinicians and researchers define groups of patients that share clinical and laboratory characteristics with high sensitivity and

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KEY POINTS

- Polymyositis is histopathologically characterized by an endomysial inflammatory T cell infiltrate surrounding and/or invading nonnecrotic muscle fibres.
- This entity is rare and should be considered after careful assessment for alternate diagnoses including inclusion body myositis, antisynthetase syndrome, immune-mediated necrotizing myopathies, overlap myositis and non-inflammatory myopathies.
- Meticulous classification of IIM based on serology, histopathology and perhaps novel biomarkers, such as gene expression will hopefully facilitate research and improve disease outcomes.

specificity. Disease classification should help healthcare providers, patients, patient-support groups, funding and regulatory agencies, classification coding systems, insurers, and health delivery systems communicate and understand each other. It should define consistent groups for comparisons of clinical cases, research studies, meta-analyses, assessment of trends and future healthcare needs. But most importantly, classification criteria should reflect subsets that share similar clinical phenotypes, prognosis, and therapeutic response. The controversy surrounding IIM classification and especially whether polymyositis represents a distinct entity remains and will be discussed in this article where we will summarize current opinions [1,2].

A BRIEF HISTORICAL NOTE ON POLYMYOSITIS

The name polymyositis made its appearance in the scientific literature around 1860 to label a disorder characterized by skeletal muscle weakness and abnormalities in muscle fibres visible with early microscopes [3]. In 1863, patients with a similar presentation but a striking skin rash were reported, and their condition named dermatomyositis [4]. For many years thereafter polymyositis referred to a wide range of disorders often interchangeably with dermatomyositis, regardless of existing skin rash. Other names were used to designate this unspecific condition, such as menopausal muscular dystrophy or late-onset progressive muscular dystrophy. In 1958, Walton and Adams published an attempt at a more systematic polymyositis definition (Table 1) [5]. They described characteristic clinical features of IIM, including limb girdle muscular weakness, pain, arthralgias, fevers, and possible overlap with connective tissue diseases, such as systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis. They also emphasized the importance of muscle biopsies in the clinical evaluation of patients with IIM although mentioning that histopathological changes in polymyositis could be discrete and unspecific [6]. At this time, the difference between polymyositis and dermatomyositis lied in the presence of absence of skin rashes, which was also the case for the diagnostic and classification criteria proposed by Bohan and Peter [7,8] in 1975. Since then, several IIM classification criteria have been proposed including polymyositis as a subset with varying definitions (Table 2) [1,7–15].

SUBSETS PREVIOUSLY CLASSIFIED UNDER POLYMYOSITIS

Autoantibody discovery and histopathology advances have completely overturned this classic dichotomous approach to IIM classification. Myositis-specific autoantibodies (MSA) are intimately associated with the clinical course and phenotypes of IIM. On the basis of clinicoserological approaches, subsets, such as antisynthetase syndrome, immune-mediated necrotizing myopathy and overlap myositis emerged.

In addition to myositis and antibodies to aminoacyl-tRNA synthetases, patients with antisynthetase syndrome have overlapping features, such as interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, mechanic's hands, and systemic symptoms in the form of fever, especially early in the disease. Sometimes, ILD or arthritis may even dominate the clinical picture. The disease is so characteristic clinically and laboratory-wise that it

Table 1. Walton and Adams definition

Group I: polymyositis acute (with myoglobinuria) or subacute/chronic (in childhood, early, middle or late adult life)
Group II: polymyositis with dominant muscular weakness and evidence of an associated collagen disease or dermatomyositis with severe muscular disability and minimal or transient skin changes
Group III: polymyositis complicating severe collagen disease [e.g. rheumatoid arthritis (RA)], or dermatomyositis with florid skin changes and minor muscle weakness
Group IV: polymyositis complicating malignant disease (including 'carcinomatous myopathy' and dermatomyositis occurring in patients with malignant disease)

Table 2. Comparison of different polymyositis definitions

Authors	Myopathic muscle weakness	Elevated muscle enzymes	Myopathic EMG	Other features	Histopathology features
Bohan and Peter [7,8]	X	X	X	–	Necrosis, phagocytosis, regeneration with basophilia, large vesicular sarcolemmal nuclei and prominent nucleoli, perifascicular atrophy, variation in fiber size, and inflammatory exudate often perivascular
Dalakas [11,12]	X	X	X	–	Primary inflammation with CD8/MHC1 complex and no vacuoles OR ubiquitous MHC-1 expression but no CD8-positive infiltrates or vacuoles
van der Meulen [1]	X	X	–	–	Mononuclear cells surrounding and preferably invading individual nonnecrotic muscle fibers in the endomysium
Tanimoto [13]	X	X	X	Anti-Jo1 Overlap features[a]	Inflammatory infiltration with degeneration or necrosis of muscle fibers, active phagocytosis, central nuclei, or evidence of active regeneration
ENMC criteria [14]	X	X	X	–	Endomysial inflammatory T cell infiltrate surrounding and invading nonnecrotic muscle fibres OR endomysial CD8 T cells surrounding, but not definitely invading nonnecrotic muscle fibres, or ubiquitous MHC-1 expression
EULAR/ACR [15]	X	X	X	Anti-Jo1 Overlap features[a]	Endomysial infiltration of mononuclear cells surrounding, but not invading, myofibers

[a]Fever, arthralgias/arthritis.

is now considered a distinct syndrome. Patients with antisynthetase syndrome have fairly typical muscle biopsy findings that include perifascicular necrosis and perimysial fragmentation as the most characteristic features thus distinguishing them from the classical description of polymyositis with lymphocytes invading or surrounding nonnecrotic endomysial muscle fibres [14,16,17]. Moreover, unique gene expression profiles in muscle tissue were found in antisynthetase syndrome when compared with inclusion body myositis, MSA-defined dermatomyositis, and immune-mediated necrotizing myopathy [18▪▪]. Similarly, a small study on gene expression in whole blood showed that activation of interferon signalling, and T-helper cell pathways were significantly more upregulated in a subset of anti-Jo1 patients ($n = 5$) when compared with controls than polymyositis and dermatomyositis [19▪▪]. Prognosis of patients with aminoacyl-tRNA synthetases autoantibodies is closely related to ILD severity, appearing to be worse in Non-anti-Jo1 patients than those with anti-Jo1 [20].

Patients with immune-mediated necrotizing myopathy have severe and often refractory muscle disease, which dominates the clinical picture, although some may have other clinical manifestations, such as dysphagia, cardiac, or pulmonary involvement. The predominant biopsy finding is necrosis of muscle fibres, often without any inflammatory infiltration. However, some patients demonstrate variable lymphocytic infiltrates in addition to abundant necrotic fibres, making the differentiation with polymyositis based on muscle biopsy features difficult if currently available criteria are used [7,8,14,15]. In these cases, it is the presence of IMNM-associated autoantibodies to signal recognition particle (SRP) or 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) that is exclusionary for the diagnosis of polymyositis. Eng *et al.* [21▪] used a machine-learning algorithm called similarity

network fusion on clinical and biological data from 168 patients originally included in the Rituximab in Myositis trial to predict disease outcomes in refractory IIM. Cytokine and chemokine profiles, MSA and disease activity measures were integrated in the analysis and five subgroups emerged. Interestingly, when these new data-driven subgroups were compared with traditional classification, polymyositis patients were found mostly in the low IgM and anti-SRP subgroups. This suggests again that several patients previously classified as polymyositis can now be classified as having immune-mediated necrotizing myopathy.

Patients who have autoantibodies associated with overlap myositis, such as anti-RNP, anti-Ku or anti-PM/Scl are often diagnosed as such but in some cases as polymyositis with another connective tissue disease depending on the predominant clinical organ involvement. Very heterogeneous histopathologic manifestations have been described in overlap syndromes, including sometimes those described as diagnostic for polymyositis [22]. However, recent studies on myositis-associated with systemic sclerosis, or scleromyositis have revealed distinguishing histopathological features in this subset called minimal myositis with capillary pathology [23▪,24]. This suggests that within the overlap myositis subset there are entities that share specific histopathological features, which are distinct from polymyositis.

INFLAMMATORY MYOPATHY AND IMMUNE CHECKPOINT INHIBITORS

Recently, several cases of inflammatory myopathies have been described in patients treated with immune checkpoint inhibitors. In addition to classical symptoms of myositis, atypical features, such as oculobulbar involvement, myocarditis, and/or myasthenia gravis can co-occur in these individuals and lead to fatal outcomes [25,26,27▪]. The diagnosis of inflammatory myopathy is usually made based on muscle weakness and high creatine kinase levels. Only a minority of these patients have myositis-specific or myositis-associated autoantibodies [28]. Given the precarity of their condition, muscle biopsies are not always performed in these patients, and therefore, such data is only available for a small number of cases. The findings are variable, showing necrotizing myopathic changes with either lymphocytic infiltrates of mainly CD4 T cells and CD20 cells, lymphocytic infiltration with both CD8 and CD4 T cells or greater infiltration of CD8-dominant T cells compared with CD4 T cells in the muscle fibres [28,29▪,30,31]. The muscle histopathology of immune-checkpoint inhibitor-associated myopathy can also show minimal inflammatory infiltrates and closely resemble immune-mediated necrotizing myopathies, yet recent studies suggest that multifocal clusters of necrotic fibres are unique to the entity [27▪]. Altogether, although there are similarities between immune-checkpoint inhibitor-associated myopathy and polymyositis, the atypical clinical manifestations, and characteristic histopathological findings similar to immune-mediated necrotizing myopathies do not support the entity as a 'model' of polymyositis, but rather forms a separate entity.

A CONTEMPORARY POLYMYOSITIS DEFINITION

So, are there still any patients who can be classified as polymyositis? Following the publication of the 2017 EULAR/ACR classification criteria, several cohorts tested their performance confirming their high sensitivity for IIM identification [15,32,33▪]. However, the IIM subsets assigned by the new criteria differed significantly from expert opinions and clinicoserological approaches. Loarce-Martos *et al.* when carefully reviewing patients classified as polymyositis with the 2017 EULAR/ACR criteria in their IIM cohort (37/255) found that only nine remained classified as polymyositis whereas the others were re-classified as immune-mediated necrotizing myopathy, connective tissue disease-associated myopathy, unspecific myopathy, dermatomyositis, cancer-associated myopathy, and noninflammatory myopathy [34▪▪]. All the remaining polymyositis patients in this study were seronegative.

Indeed, in practice, many patients without myositis-specific or myositis-associated autoantibodies are categorized as polymyositis if they do not have dermatomyositis rashes. This is also the case for patients positive for anti-Ro52, a nonspecific autoantibody that can be found in several connective tissue diseases. Anti-Ro52 without anti-Ro60 is up to 10 times more common in inflammatory myopathies than in other connective tissue diseases and its presence is a sign of autoimmunity [35]. In IIM, anti-Ro52 is often found in combination with other myositis-specific or myositis-associated antibodies. Their presence is associated with higher prevalence of ILD and carries a poorer prognosis [36▪,37–39]. Still, in some IIM cases, anti-Ro52 is the only autoantibody found. Single anti-Ro52 positivity combined with muscle weakness, biopsy pathology, electromyogram (EMG) findings, elevation of muscle enzymes, and absence of any laboratory or clinical signs suggestive of other entity from IIM may be assigned the diagnosis of polymyositis. Our own experience suggests that although not very frequent, such patients exist. Of note, some

clinicoserological approaches consider anti-Ro52 positivity as an overlap feature that would imply an overlap myositis [40,41]. In the cases where patients have no other convincing overlap features, this remains controversial.

Novel autoantibodies, such as those against the survival of motor neuron (SMN) complex were initially described in polymyositis cases but now suggest an overlap myositis diagnosis with their presence being associated with an overlap with systemic sclerosis [42▪,43]. Antibodies against four-and-a-half-LIM-domain 1 (FHL1) were described in patients with severe muscle involvement with atrophy, frequent dysphagia, and vasculitis [44]. The incidence of anti-FHL1 in a cohort of 141 IIM patients was 25% with 58% of positive patients classified as polymyositis using the Bohan and Peter classification. This autoantibody is not, however, specific for polymyositis, as some of the patients with anti-FHL1 are diagnosed as dermatomyositis, juvenile dermatomyositis, or inclusion body myositis. Routine detection of this autoantibody is not yet available, and more studies are needed to understand if it plays a role in identifying polymyositis patients, which does not seem to be the case at this moment. Antieukaryotic initiation factor 3 has been described in three seronegative polymyositis patients [45▪▪]. Still, the histopathological findings of the three cases were partial (no inflammatory cell immunophenotypes in 2/3) and one case displayed overlap features. Validation in larger studies will be required to confirm that this new autoantibody is specific to polymyositis.

The highest risk of a polymyositis misdiagnosis remains in patients who have no known autoantibodies. Polymyositis patients without autoantibodies exist but careful exclusion of mimics is essential. First, inclusion body myositis needs to be excluded, which can occasionally be difficult and require careful examination and repeated muscle biopsies. Imaging modalities, such as quantitative computed tomography, magnetic resonance imaging, positron emission tomography, and ultrasound may in certain cases help to differentiate IBM from polymyositis or other neuromuscular disorders [46,47,48▪,49]. However, this might only be true for established disease and may be difficult to implement outside specialized centers. In patients with suspected polymyositis, the lack of response to immunosuppressive therapy should raise the suspicion for another muscle disease, such as muscle dystrophy, metabolic myopathies, endocrinopathies, channelopathies, toxic myopathies and a number of other rare conditions. Similarly, the absence of biopsy features often associated with polymyositis diagnosis, such as endomysial T cell infiltration and MHC-1 expression on muscle fibers should suggest nonimmune origin [50]. Therefore, looking for family history of muscle weakness, asymmetric, and significant distal weakness, muscle pain as a main symptom, sudden onset of muscle weakness, episodic muscle weakness after exercise, fasting, or illness, ocular and facial muscle involvement, early muscle atrophy or hypertrophy, presence of myotonic discharges on EMG or clinical myotonia, neuropathy, fasciculations, or cramping and very high or normal muscle enzymes is recommended to exclude noninflammatory conditions that may clinically present as polymyositis.

CONCLUSION

Polymyositis does exist as a distinct entity but scientific development including discovery of several new MSAs and the identification of distinct gene expression patterns in muscle biopsies have made possible the description of new and more homogenous phenotypes previously labelled as polymyositis, thus narrowing the spectrum of this once very heterogenous and large IIM subset. Careful physical examination, extended autoantibody testing and systematic histopathology assessments are necessary to support a polymyositis diagnosis. The 2017 EULAR/ACR classification criteria are sensitive and specific for IIM identification but not its subsets. The challenge for future criteria will be to integrate the antisynthetase syndrome, immune-mediated necrotizing myopathies and overlap myositis subsets that have distinct autoantibody profiles, histopathology and clinical course.

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Conflicts of interest

I.E.L. has received research grants from Astra-Zeneca and has served as consultant for Argenx, Corbus Pharmaceuticals, Inc., Janssen, Kezaar, Octapharma,

Orphazyme, EMD Serono Research & Development Institute, Sobi and has stock shares in Roche and Novartis. J.V. has been on Speakers Bureau of Abbvie, Biogen, MSD, Pfizer, Roche, Sanofi, UCB, Werfen and has consulted for Abbvie, Argenx, Boehringer, Eli Lilly and Octapharma. V.L. and A.N. declare no conflicts of interest.

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Differential diagnosis of necrotizing myopathy

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Purpose of review
Necrotizing myopathy is a broad term. It includes patients with the recently described immune-mediated necrotizing myopathies (IMNM) who have specific antibodies, such as anti-hydroxy-3-methylglutaryl-CoA reductase or anti-signal recognition particle, seronegative phenotypes that can be associated with cancer, and other types of myositis and connective tissue diseases involving necrotic muscle fibers as a characteristic pathologic feature. Necrotizing myopathies that are not immune-mediated, such as those caused by drugs, dystrophies, infections, or even hypothyroidism are also included. The purpose of this review is to address the differential diagnosis of these disorders.

Recent findings
New IMNM have been described over the last few years, some of them related with checkpoint inhibitors, drugs that are being increasingly used in cancer treatment. Necrotizing myopathy has also been reported in association with specific phenotypes and autoantibodies (e.g. anti-Mi2 dermatomyositis, antisynthetase syndrome, and myositis associated with antimitochondrial antibodies). Rarer cases associated with graft-versus-host disease and severe acute respiratory syndrome coronavirus 2 infection are also emerging.

Summary
Differentiation between patients with IMNM and those without the superimposed autoimmune phenomena helps clinicians determine the best individualized approach to use and the appropriate immunosuppressive therapy, whenever needed.

Keywords
checkpoint inhibitors, differential diagnosis, immune-mediated necrotizing myopathy, necrotizing myositis, severe acute respiratory syndrome coronavirus 2

INTRODUCTION

The first definition of necrotizing myopathy, established in 2003, included a key pathological criterion: the presence of numerous necrotic muscle fibers as the main pathological feature [1]. Long before that time, Bohan and Peter [2] had noted a predominance of necrotic muscle fibers in some patients diagnosed with idiopathic inflammatory myopathy, and alerted clinicians to a possible association with malignant disease. This observation likely referred to what is now known as seronegative immune-mediated necrotizing myopathy (IMNM).

IMNM is a heterogeneous condition, only recently recognized as an individual entity. It is the most common myositis subtype and can be divided into three main groups. The first two include myositis patients who test positive to one of two specific autoantibodies, anti-signal recognition particle (SRP) or anti-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and the last includes those with no known autoantibodies, categorized as seronegative. This classification, which was set up in 2013, has the advantage that muscle biopsy can be avoided in patients with typical clinical features (high creatine kinase levels, proximal muscle weakness, absence of systemic disease) and positive testing for these antibodies [3].

In addition to IMNM, in which muscle is the main target and patients have specific autoantibodies or are seronegative, certain inflammatory myopathies can also show signs of widespread necrosis, and these findings can lead to misdiagnoses if other systemic manifestations are not considered [4]. This

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KEY POINTS

- Necrotizing myopathy is a broad term that includes immune-mediated and nonimmune-mediated myopathies.
- Dystrophies, drug toxicity, and infections must be considered in the differential diagnosis of necrotizing myopathy.
- In addition to the immune-mediated necrotizing myopathy (IMNM) group focused on skeletal muscle (seronegative or positive to anti-signal recognition particle or anti-hydroxy-3-methylglutaryl-CoA reductase antibodies), other autoimmune disorders related with specific autoantibodies, such as antisynthetase, anti-Mi2, anti-Ku, or antimitochondrial antibodies can also develop necrotizing myopathy features.
- Severe acute respiratory syndrome coronavirus 2 infection (COVID-19) should be included as a condition associated with IMNM.

can occur with anti-Mi2-associated dermatomyositis [5▪▪], anti-Ku-associated myositis [6], and antisynthetase syndrome [7]. Moreover, although infrequent, certain connective tissue diseases, especially systemic sclerosis [8▪], may develop pathological features indistinguishable from IMNM.

Recently, other forms of IMNM have been reported, occurring in patients receiving checkpoint inhibitors [9▪,10,11▪], in patients who develop graft-versus-host disease (GVHD), and in association with certain infections, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [(coronavirus disease 2019 (COVID-19 disease)] [12–14]. Differentiation between the immune-mediated disorders, which usually require treatment with immunosuppressive agents, and nonimmune-mediated necrotizing myopathies resulting from drug toxicity [15,16], genetic disorders (e.g. dystrophies) [17–19], and even common disorders, such as hypothyroidism [20] is essential to avoid toxicity and effectively treat these diseases (Table 1).

The aim of this review is to address the differential diagnosis of patients with necrotizing myopathy.

IMMUNE-MEDIATED OR NONIMMUNE-MEDIATED, THAT IS THE QUESTION

Several features of muscle biopsy specimens, such as evidence of MHC class I upregulation on sarcolemma of nonnecrotic muscle fibers and membrane attack complex (MAC) deposition on sarcolemma, can help identify immune-mediated forms of necrotizing myopathy (Table 2 and Figs. 1 and 2) but these indicators are not always found and clinicians have to rely on other approaches, such as clinical manifestations,

Table 1. Conditions associated with necrotizing myopathy

Immune-mediated necrotizing myopathy
Preferential muscle involvement (nonsystemic)
Antibody-positive (anti-SRP or anti-HMGCR)
Seronegative (no specific myositis autoantibodies)
Malignancy
Connective tissue diseases (or specific autoantibodies)
Myositis (antisynthetase antibodies, anti-Mi2 antibodies, and antimitochondrial antibodies)
Systemic sclerosis, Sjögren syndrome, systemic lupus erythematosus
Checkpoint inhibitor-associated myopathy
Infections
Graft-versus-host disease
Other types of necrotizing myopathy (nonimmune)
Dystrophies (e.g. dysferlinopathy[a], facioscapulohumeral muscular dystrophy)
Drug toxicity (e.g. statins, bevacizumab…)
Infections (e.g. influenza, SARS-Cov2…)
Hypothyroidism (severe)

[a]An inflammatory infiltrate of the polymyositis type (CD8+ lymphocytes) together with upregulated MHC-I are occasionally seen.
HMGCR, hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle.

autoantibody testing, and MRI features. Necrosis can be a nonspecific finding, and the diagnosis should not be made on histology evidence alone.

It should also be noted that some situations (e.g. statin use and some viral infections) can lead to both types of phenotypes, nonimmune or immune-mediated. The reason for this different disease expression is unknown, but from the clinical viewpoint it is important to know that either presentation is possible. In the case of statins, the presence of anti-HMGCR antibodies may be of help to determine,

Table 2. Myopathological features for the differential diagnosis of immune-mediated necrotizing myopathy vs. nonimmune necrotizing myopathy

IMNM	Nonimmune IMNM
Necrotic myofibers scattered through the muscle biopsy	Idem
Myofiber regeneration	Idem
Macrophage infiltrates	Absent
MHC class I on sarcolemma of nonnecrotic muscle fibers	Absent (mostly)
MAC (C5b-9) deposition on sarcolemma	Absent (mostly)
MHC class II on sarcolemma[a] (overlap myositis syndrome, connective tissue diseases…)	Absent (mostly)

IMNM, immune-mediated necrotizing myopathy; MAC, membrane attack complex; MHC, major histocompatibility complex.
[a]Unusual feature.

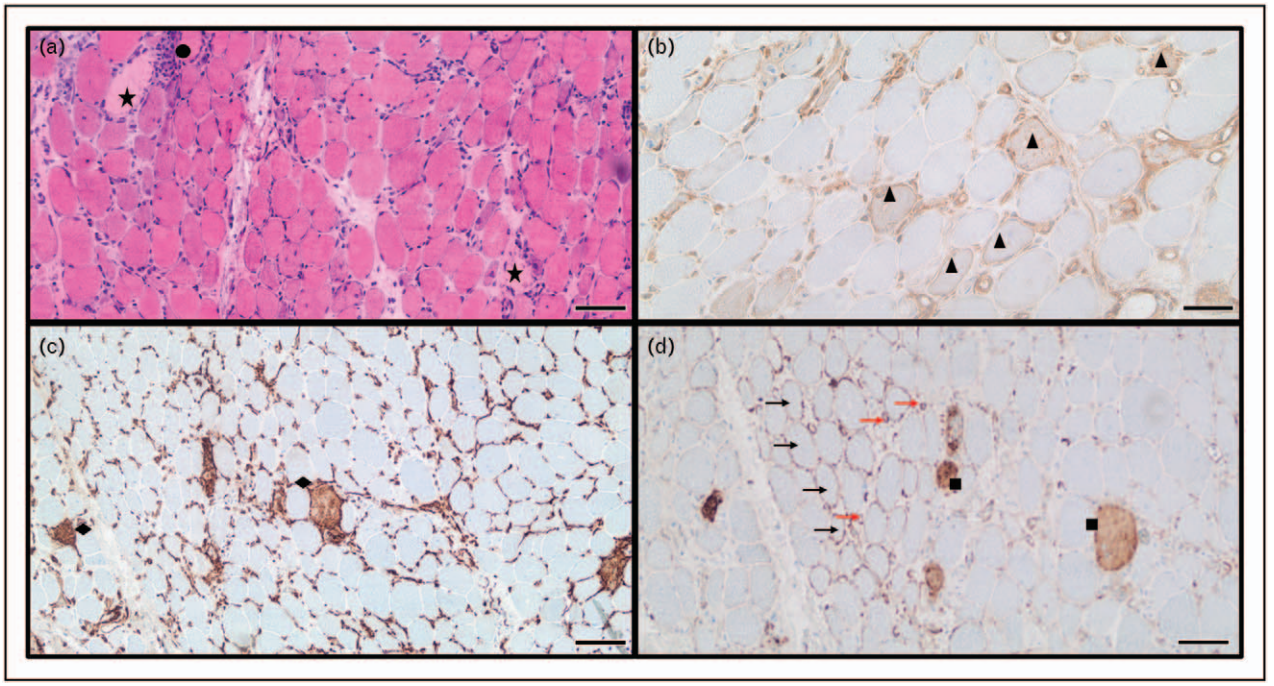

FIGURE 1. Necrotizing immune-mediated myopathy, HMGCR related. (a) Necrotic fibers (stars) with minimal inflammatory infiltrate (circle). Hematoxilin-eosin (H&E) in frozen tissue. (b) Random positivity of MHC-I (triangles). Class I of the major histocompatibility complex (MHC) antigens immunochemistry (IHQ) in frozen tissue. (c) Random positivity of MHC-II. Class II of the MHC antigens IHQ in frozen tissue. Clear positivity in some muscle cell (diamonds), together with endothelial cells (normal reaction). (d) Clear positivity of some muscle cells either in sarcolemma (black arrow) or sarcoplasmic (squares), as well as positivity of some capillaries (red arrows). Membrane attack complex (C5b-9) IHQ staining in frozen tissue. 100 μm ▬▬. HMGCR, hydroxy-3-methylglutaryl-CoA reductase.

which phenotype is expressed, but unfortunately, in other scenarios these helpful data are not available.

IMMUNE-MEDIATED NECROTIZING MYOPATHY

IMNM usually affects the skeletal muscle and spares other organs and systems, which contrasts with other myositis phenotypes, such as dermatomyositis and antisynthetase syndrome. Three main subtypes of this muscle-targeted disease have been identified: two IMNM forms associated with specific autoantibodies, either anti-SRP or anti-HMGCR, and one in which no known autoantibodies are detected as markers of the disease, referred to as *seronegative*. It is well recognized that this last group can be associated with malignant disease, and a recent study has suggested that cardiac and respiratory complications are not uncommon in these patients [21▪]. In a retrospective analysis, researchers identified 109 patients with IMNM belonging to one of the three groups (anti-SRP, anti-HMGCR, or *seronegative*) and found that cardiorespiratory abnormalities were not infrequent, although most cases were related to muscle effects (e.g. respiratory compromise mainly because of restrictive lung disease associated with muscle impairment). Only 6% of the patients developed some type of interstitial lung disease. Left ventricular diastolic dysfunction was the most common echocardiographic finding, although it was impossible to know whether myocarditis had contributed to the condition.

In a study performed in a cohort of South Australian patients with IMNM, again including the three main subtypes, the heterogeneity of the diagnosis was confirmed and the disease features described: most patients (67%) were severely weak at presentation and complement deposition on muscle capillaries was associated with the severity of the condition. High-creatine kinase values, usually greater than 5000 IU/l were the rule in these patients [22▪].

OTHER MYOSITIS PHENOTYPES AND CONNECTIVE TISSUE DISORDERS

Muscle fiber necrosis is an important feature in several myositis phenotypes and it can appear in some connective tissue disorders. In these cases, other clinical manifestations and pathological features, as well a specific autoantibody pattern can be useful to reach the final diagnosis. For example, in antisynthetase syndrome, patients may show a

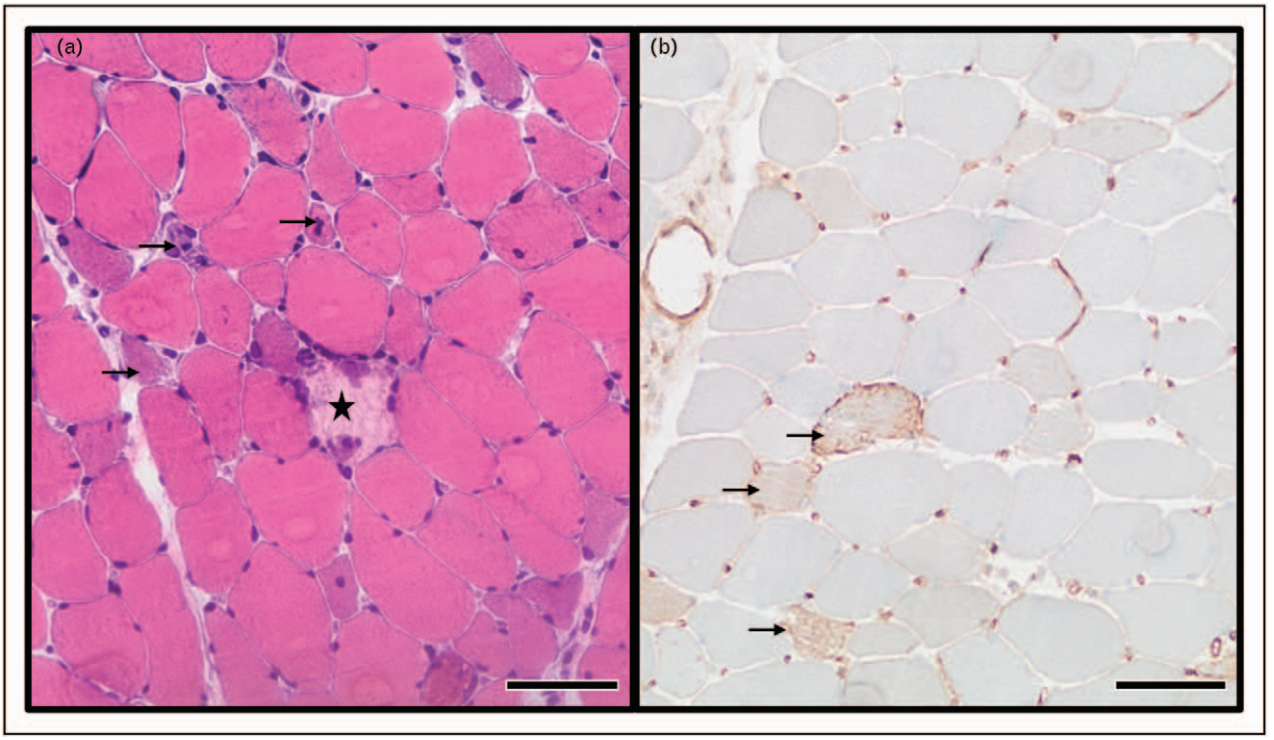

FIGURE 2. Toxic necrotizing nonimmune-mediated myopathy. (a) Necrotic cell (star) and some basophilic cells in appearance (regenerating cells) (arrows). H&E in frozen tissue. (b) Positivity of MHC-I only in necrotic cells (arrows). Class I of the major histocompatibility complex (MHC) antigens IHQ in frozen tissue. 100 μm ▬▬.

more systemic disease with interstitial lung involvement, capillaroscopic changes, fever, or the typical 'mechanic's hands'. Muscle biopsy shows perimysial inflammatory infiltrates with perifascicular atrophy, but perifascicular myofiber necrosis may also be present [7,23].

Other myositis phenotypes, particularly those related to specific autoantibodies, are also associated with myofiber necrosis as a prominent muscle biopsy finding. Tanboon *et al.* [5▪▪] reviewed 188 muscle biopsies of proven dermatomyositis patients with sarcoplasmic expression of myxovirus-resistance protein A, considered to be the hallmark of the pathological diagnosis of dermatomyositis. The authors found that perifascicular necrosis was a major finding in patients testing positive to anti-Mi2 antibodies. An etiopathogenic mechanism similar to that of antisynthetase syndrome was suggested by the authors of this research.

Several studies published in the last few months have focused on the role of antimitochondrial antibodies as a marker of a specific myositis phenotype with myocardial involvement, and all of them report muscle fiber necrosis as the main pathology finding [24–26,27▪]. In a study from Johns Hopkins University in Baltimore [26], four of seven patients with inflammatory myopathy and antimitochondrial antibodies were diagnosed with IMNM. In a large cohort of 1167 patients with idiopathic inflammatory myopathy, more than half of the 23 patients with isolated antimitochondrial antibodies had a histopathological diagnosis of IMNM [27▪]. Thus, this phenotype should be considered when IMNM is the pathological diagnosis.

Myofiber necrosis has also been reported in connective tissue disorders where myositis is rare, such as Sjögren syndrome [28] and lupus [29]. Nonetheless, systemic sclerosis patients are those who mainly show IMNM as the pathological signature of the disease. In a recent study [8▪], researchers from Canada analyzed close to 600 muscle biopsies in systemic sclerosis patients, and necrosis was reported in more than half the specimens, mainly from patients positive to anti-Ku antibodies, although only 16% met the ENMC (European Neuromuscular Center) criteria for IMNM.

CHECKPOINT BLOCKADE-ASSOCIATED MYOPATHY

Immune checkpoint blockade is a smart strategy to eliminate some cancers, particularly melanomas and nonsmall cell lung cancer. Unfortunately, activation of the immune system, which is good for the treatment of cancer, can produce adverse immunological events. One of these is checkpoint inhibitor-

associated myopathy. A study from the Mayo Clinic compared the characteristics of 24 patients with this condition to those of 38 IMNM patients who had not been exposed to checkpoint inhibitors [9▪]. The muscle pathology findings in the two groups were similar and could be categorized as IMNM but there was a higher rate of mitochondrial anomalies in the group receiving checkpoint inhibitors. Patients exposed to these agents showed some striking clinical differences: ocular muscle involvement (unrelated to the neuromuscular junction) and myocarditis (occasionally fatal) were more common. Well known IMNM-related autoantibodies (anti-HMGCR and anti-SRP) tested positive in two-thirds of patients with IMNM but in none of those exposed to checkpoint blockade. Lymphopenia and low serum levels of creatin kinase were also more frequent in patients who received checkpoint inhibitors.

Researchers from Barcelona have described a characteristic clinical and pathologic phenotype in patients treated with these agents. Matas-Garcia *et al.* [11▪] studied nine patients treated with anti-PD1 or anti-PD1L inhibitors who had developed muscle weakness. The authors concluded that an axial pattern of muscle disease (*dropped head* syndrome) together with extraocular muscle involvement and proximal weakness are hallmarks of a distinct and characteristic muscle phenotype. Muscle biopsy disclosed features of IMNM (MHC-I sarcolemma upregulation and MAC capillary deposition) combined with a predominant macrophage infiltrate of a pseudogranulomatous type (Fig. 3), which were

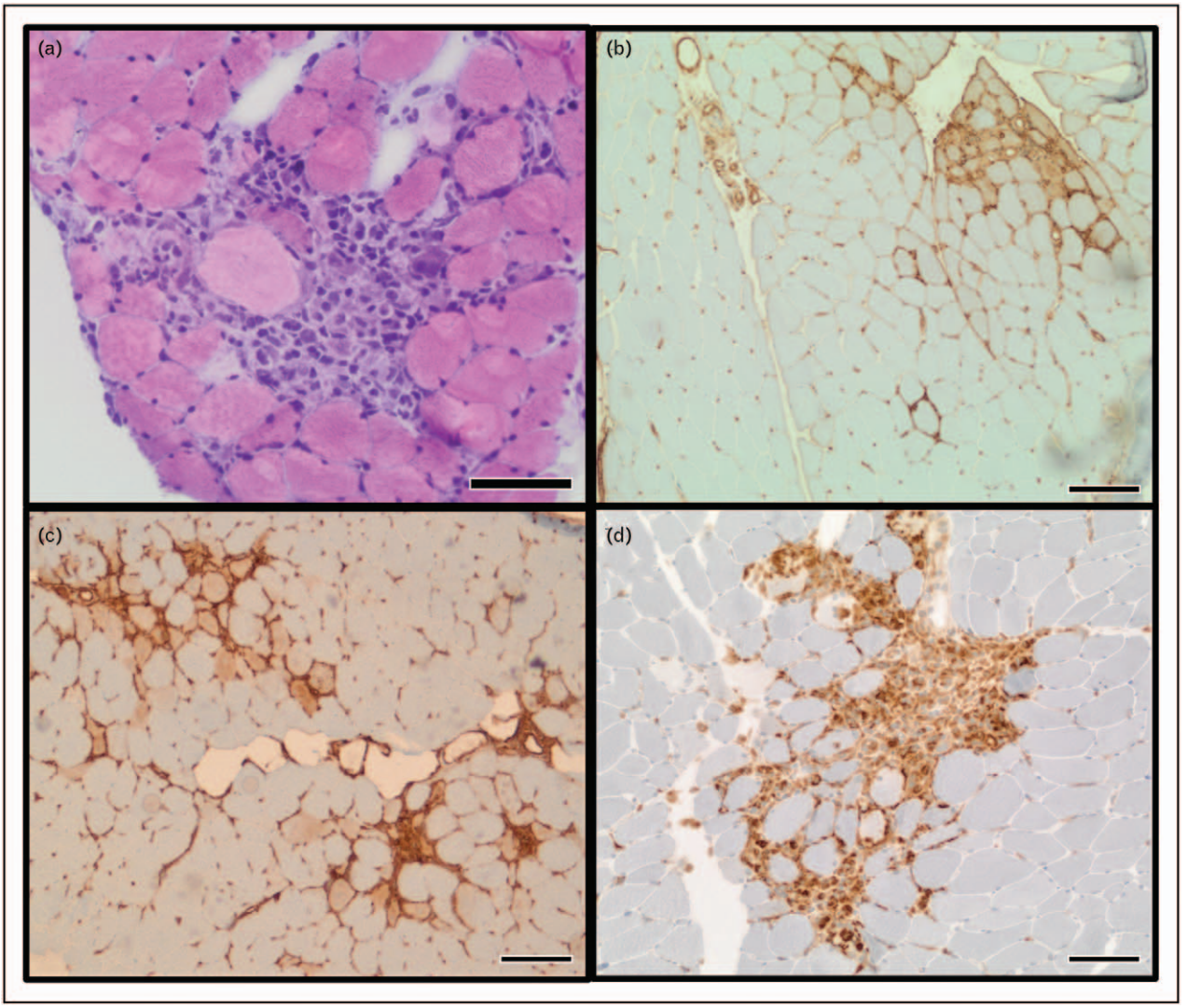

FIGURE 3. Immuno-mediated necrotizing myopathy PD1/PD1L related. (a) Pseudogranulomatous reaction in muscle biopsy. H&E in frozen tissue. (b) Random positivity of MHC-I. Class I of the MHC antigens IHQ in frozen tissue. (c) Random positivity of HCM-II. Class II of the MHC antigens. IHQ in frozen tissue. (d) Positivity of macrophagic cells with CD68 immunostaining. CD68 positivity. IHQ in frozen tissue. 100 μm ▬▬.

characteristic and found in other affected patients. PD1 staining in muscle biopsy material was detected in one of three patients reported by Vermeulen *et al.* [10], but the authors acknowledged that the technical sensitivity to detect PD1/PD1L staining is controversial.

One of the most difficult scenarios for the clinician is when a patient with malignant disease treated with checkpoint inhibitors develops muscle weakness and IMNM. Is it a seronegative form of IMNM, in which an association with malignancy is well recognized, or is an adverse event related to the checkpoint blockade? Although therapy includes immunosuppressive agents in both cases, the approach may be different. If the IMNM is considered a paraneoplastic feature, the cancer should be treated intensively but if it is because of the immunotherapy, the risk/benefit of administering a new shot of immunotherapy should be carefully considered. Certain data, such as the clinical phenotype and muscle biopsy findings of a pseudogranulomatous macrophage infiltrate may help in the decision.

CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GVHD occurring in allogeneic hemopoietic stem cell transplantation is a multisystem disorder of immune dysregulation. The muscle may become a target of the immune response, and myositis is a well known manifestation of the disorder [30]. IMNM may occasionally be the pathological substrate in these patients, although is difficult to distinguish between GVHD-associated myositis, which usually appears several months after transplantation and is accompanied by involvement of other organs (e.g. liver and skin), and de novo myositis, which seems to be more frequent in these patients than in the general population [31]. In a recent study reported by Saw *et al.* [32], 8 of 17 patients diagnosed with GVHD and immune-mediated myopathy had some degree of muscle fiber necrosis, which ranged from mild to severe. Axial involvement with dropped head was present in nearly half the patients but proximal muscle weakness was the main feature.

INFECTIONS

A recent report described IMNM with anti-SRP antibodies, apparently triggered by influenza virus infection [33]. In addition, several case studies have reported SARS-Cov-2 infection as a cause of IMNM [12–14]. In an analysis of 35 patients who died from SARS-Cov-2 infection, histopathological findings at autopsy showed IMNM in 9 patients, with MHC-1 expression observed in all of them [34▪▪]. Nevertheless, in other such cases with an immune-mediated mechanism, necrosis was not a predominant feature on muscle biopsy. One possibility to consider is whether or not virus-induced type I interferonopathy might be implicated in these cases [35].

NECROTIZING MYOPATHY

Dystrophies

Several features of muscular dystrophies can be similar to those of IMNM; hence, differentiation between the two conditions may be challenging. Myofiber necrosis and regeneration are typical pathological findings in both dystrophies and IMNM [17]. Scapular winging is suggested to be a characteristic feature in patients with various types of dystrophies, in particular those with facioscapulohumeral dystrophy [18]. However, it is not exclusive to these conditions, as patients positive for anti-SRP antibodies can have scapular winging because of a tendency to considerable muscle atrophy, mainly in the shoulder girdle [19]. This could also be the case of some patients with IMNM and anti-HMGCR antibodies. Moreover, some forms of nongenetically classified pediatric dystrophies ultimately have been identified as juvenile forms of anti-HMGCR-related IMNM, which responds to immunosuppressive therapy. A similar scenario has been reported in adult patients [36]. Tests to detect this antibody are warranted because of the possibility to treat these patients with immunosuppressive agents.

Other limb girdle muscular dystrophies, such as type IIb, also known as dysferlinopathy, can also masquerade as IMNM because of the presence of myofiber necrosis. The absence of sarcolemma MHC-I upregulation and MAC deposit on muscle capillaries in immunohistochemistry studies favors the diagnosis of dystrophy. Facial and extraocular muscle involvement, an asymmetrical pattern of weakness, and familial disease reinforce the suspicion of genetic or heritable myopathies. Genetic testing is the cornerstone for the diagnosis.

Whole-body MRI can be of help in the differential diagnosis of IMNM. A recently published study analyzed the pattern of muscle involvement in 42 patients diagnosed with IMNM [37]. Muscles from the axial, lumbar, and pelvifemoral regions were the most highly affected, specifically in patients positive for anti-HMGCR or anti-SRP antibodies. This pattern of muscle damage differed from that observed in a control group of 60 patients diagnosed with sporadic inclusion body myositis.

Electrophysiologic features can also be useful in the differential diagnosis of necrotizing myopathy. Triplett *et al.* [38] analyzed the electrophysiological assessment of 119 patients diagnosed with IMNM

(17 with anti-SRP, 49 seronegative, and 53 with anti-HMGCR) and compared the data with those obtained in a cohort of 938 patients with other types of myopathy. They found that electrical myotonia (i.e. myotonic discharges, defined as repetitive 20–80 Hz discharges, waxing and/or waning in amplitude and frequency) without clinical myotonia was significantly more common in the IMNM group than in the remaining myopathies, and was five-fold more likely than in patients with limb girdle muscular dystrophies. The presence of myotonia seems to predict a good response to immunotherapy in IMNM patients.

DRUGS AND TOXICITY

Drug-induced myopathy has a wide range of clinical presentations. Myalgia, cramps, or weakness because of true muscle involvement in the form of necrotizing myopathy are some of the possible presentations. Rhabdomyolysis is the most severe form of drug-induced toxicity. Statins, mentioned above as a cause of IMNM, can also provoke pure toxic myopathy with myofiber necrosis and muscle cell regeneration, and without MHC-I expression in normal cells or MAC deposit on capillaries. Thus, this drug may be implicated in two different expressions of muscle disease, and detection of anti-HMGCR antibodies is essential to differentiate between them. Other drugs classically involved in muscle toxicity include fibrates, alcohol, and heroin. Drug-induced toxicity and necrotizing myositis have also been reported after pregabalin therapy [15,16]. In all these cases, discontinuation of the drug treatment is mandatory while waiting for muscle regeneration, which usually requires several weeks.

CRITICAL CARE MYOPATHY

In addition to muscle atrophy, development of acute necrotizing myopathy is well recognized in ICU patients. Wash and Carvalho [39] recently reviewed this topic. Selective loss of thick myofilaments in muscle fibers detected by electron microscopy seems to contribute to the pathogenesis of this condition. High-dose steroid administration, use of neuromuscular junction-blocking agents, and systemic infection are associated with critical care myopathy.

CLUES FOR AN ACCURATE DIAGNOSIS IN PATIENTS WITH NECROTIZING MYOPATHY

Necrosis is a nonspecific finding. Even though a predominance of this feature on muscle biopsy and an absence of inflammatory infiltrate suggest a diagnosis of necrotizing myopathy, these criteria

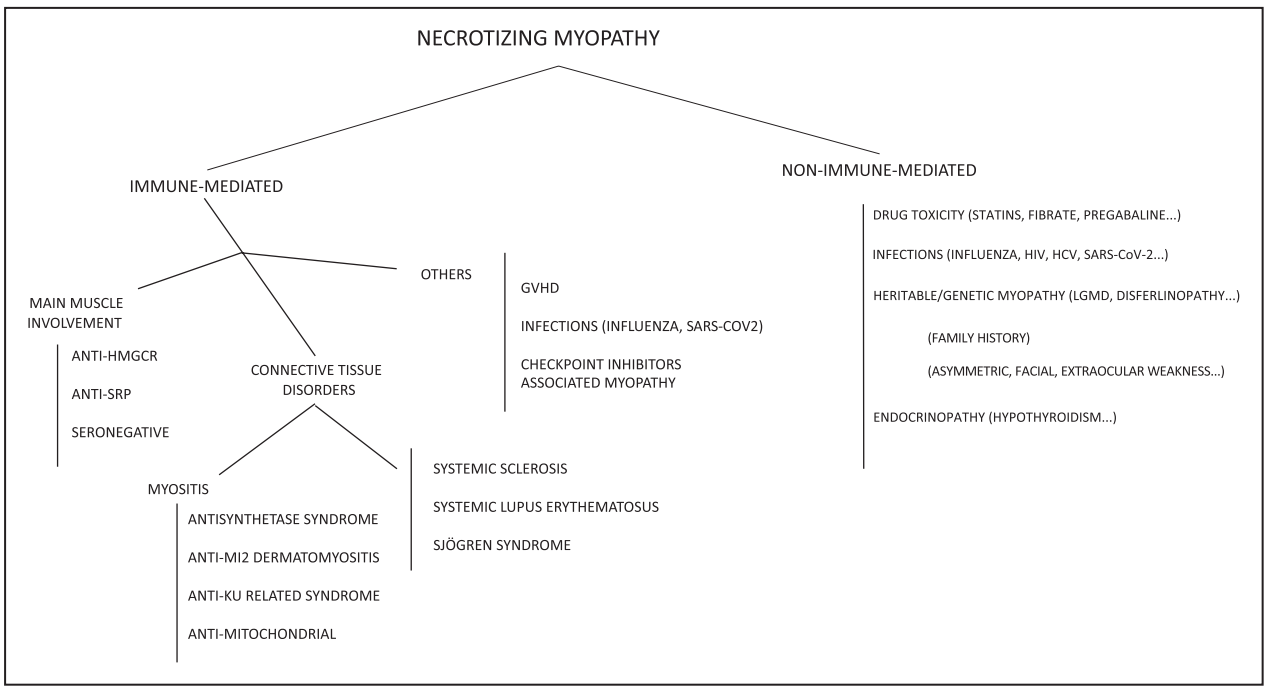

FIGURE 4. Differential diagnosis of necrotizing myopathy. GVHD, graft-versus-host disease; HMGCR, hydroxy-3-methylglutaryl-CoA reductase; LGMD, limb girdle muscular dystrophy; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SRP, signal recognition particle.

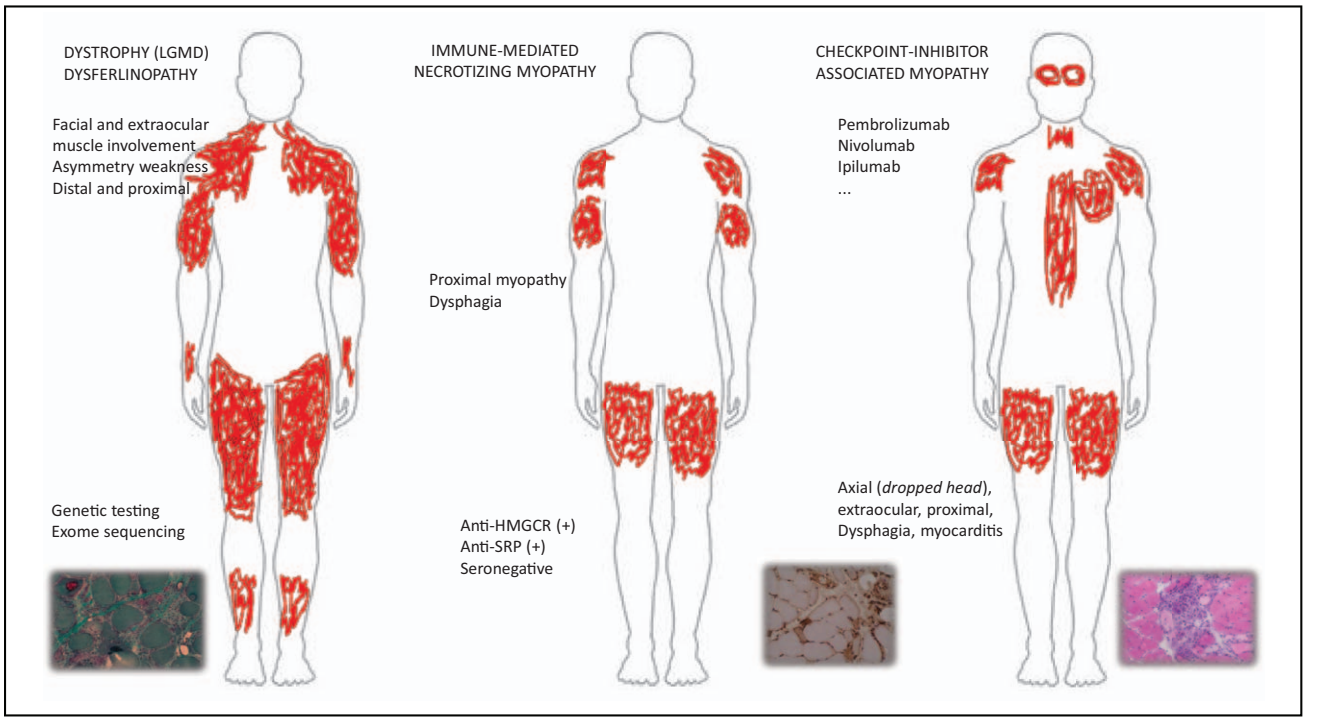

FIGURE 5. Muscle involvement patterns in three types of necrotizing myopathy: checkpoint inhibitor-associated myopathy, dystrophies, and immune-mediated necrotizing myopathy. Dystrophy: marked variability in fiber size. Gomori's trichrome in frozen tissue. IMNM: sarcolemmal and cytoplasmatic positivity of the Class I MHC antigens in frozen tissue. Checkpoint-inhibitors: severe perivascular pseudogranulomatous reaction (macrophages). H&E in frozen tissue.

are not infallible. To a certain extent, upregulation of MHC-I antigen and MAC deposit on capillaries and sarcolemma of nonnecrotic muscle fibers are hallmarks of immune-mediated myopathy but there are also exceptions. Thus, clinicians attending patients with suspected myositis and abundant myofiber necrosis, degeneration, regenerations, and minimal (if any) inflammatory infiltrate on muscle biopsy have to rely on other features to establish the correct diagnosis. Differential diagnosis is summarized in Fig. 4. Fig. 5 shows different muscle patterns in IMNM.

CLINICAL GROUNDS

The first approach will include an anamnesis and clinical examination. The history of drug exposure (statins and others) should also be included. Development of subacute, progressive, proximal-dominant muscle weakness or an acute presentation and high serum creatine kinase levels after treatment with a given drug may help in the diagnosis. Exclusive muscle involvement and the presence of other systemic features, such as characteristic skin lesions (heliotrope rash and Gottron sign in dermatomyositis, and sclerodactyly, telangiectasia, and skin thickening in systemic sclerosis), interstitial lung disease, and arthritis (in antisynthetase syndrome) may help to identify the disease.

In contrast, when the clinical examination reveals extraocular muscle involvement, facial weakness, or distal, asymmetrical weakness in a patient with other affected family members, the possibility of a heritable myopathy, such as dystrophies, disferlinopathies, and any type of limb girdle muscle dystrophy should be considered. Among the endocrinopathies, severe hypothyroidism should be borne in mind as a cause of necrotizing myopathy, and in this case, Woltman's sign (delayed relaxation of the ankle jerk reflex) may be of great help [40].

IMMUNOLOGIC PROFILE

The autoantibody profile can be of value to properly categorize the disease but it is usually the combination of all available data that enables the astute clinician to achieve the correct diagnosis. Whenever autoantibodies are present, other features can be sought and correctly identified. Patients with anti-Mi2 antibodies and necrotizing myopathy may be easy to diagnose if heliotrope rash or Gottron papules are found, as occurs in a patient with anti-Ku or antisynthetase antibodies.

CONCLUSION

Necrotizing myopathy is associated with numerous disorders and is a frequent finding in patients with muscle disease. It can occur in conditions with immune-mediated phenomena, such as primary muscle autoimmune diseases with or without autoantibodies, in other myositis types, or in connective tissue disorders. Cases related to immunotherapy, GVHD, and some viral infections, such as SARS-CoV-2 can also be included in the group of IMNM. It is important to keep in mind that not all necrotizing myopathies are of the immune-mediated type, as other diseases, drug toxicity, and infections are sometimes the cause, leading to misdiagnoses. When the condition is not immune-related, it makes no sense to treat it with immunosuppressive agents, as there is a risk of causing more harm than benefit.

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Conflicts of interest

There are no conflicts of interest.

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CURRENT OPINION

Anti-HMGCR myopathy: clinical and histopathological features, and prognosis

Takashi Kurashige

Purpose of review
This review aims to describe clinical and pathological features, prognosis and treatment in patients with anti-HMGCR antibody positive immune-mediated necrotizing myopathy (HMGCR-IMNM) based on recent findings.

Recent findings
Using advances in diagnostic modalities that can confirm the presence of anti-HMGCR antibody, the clinical and pathological manifestations of HMGCR-IMNM were found to be broader than previously reported. Although only a small percentage of HMGCR-IMNM patients present with atypical manifestations, some of these patients show slow disease progression and clinical symptoms, which are similar to those of limb-girdle muscular dystrophies. Other atypical HMGCR-IMNM patients have skin conditions similar to dermatomyositis-like skin rush or dermatological presentations of Jessner-Kanoff disease or cutaneous lymphoma, whose pathological changes including CD8-positive and bcl-2-positive lymphocytic accumulations, similar to Jessner-Kanoff lymphocytic infiltration of skin or low-grade cutaneous lymphoma, which are observed in muscle and skin.

Summary
Anti-HMGCR autoantibodies define unique populations of IMNM patients. Recent studies have revealed that clinicopathological manifestations of HMGCR-IMNM, especially extramuscular symptoms and pathological manifestations, are more common than previously recognized.

Keywords
anti3-hydroxy-3-methylglutaryl-CoA reductase, Jessner lymphocytic infiltration, myopathy, skin condition

INTRODUCTION

Idiopathic inflammatory myopathies (IIMs) are a rare group of autoimmune diseases that can cause chronic inflammation of skeletal muscle and/or organs, including the skin, joints, lungs, gastrointestinal tract and heart. The muscle involvement of IIMs may cause muscle weakness, and extramuscular manifestations may lead to life-threatening complications [1]. IIMs were initially classified as dermatomyositis or polymyositis by the presence of a characteristic skin rash [2]. Today, IIMs are classified into five categories: polymyositis, dermatomyositis, immune-mediated necrotizing myopathy (IMNM), sporadic inclusion body myositis (sIBM) and nonspecific myositis by clinicopathological manifestations [3–5]. In addition to histological patterns, there are more than 15 myositis-specific antibodies (MSA) against factors considered to be important in the mechanism underlying IIMs [6,7]. IMNM is also frequently associated with antisignal recognition particle (anti-SRP) and anti3-hydroxy-3-methylglutaryl-coA reductase (anti-HMGCR) antibodies], which were found to be characteristic of IMNM [4,5,8,9].

IMNM induced by statin was initially described in patients on statin therapy, who developed a persistent myopathy in spite of statin discontinuation, and were responsive only to immunosuppression [10].

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KEY POINTS

- A small percentage of HMGCR-IMNM patients show atypical clinicopathological symptoms and slow disease progression.
- HMGCR-IMNM patients with skin conditions show dermatomyositis-like skin rush, pruritic erythematous papules with photosensitivity and/or dermatological presentations of Jessner-Kanoff disease or cutaneous lymphoma.
- Pathological findings of HMGCR-IMNM patients present bcl-2- and CD8-positive lymphocyte infiltration to muscle (including endomysium) and perivascular areas of skin. Lymphocytic infiltrations sometimes form accumulations similar to Jessner-Kanoff lymphocytic infiltration of skin or low-grade cutaneous lymphoma with bcl-2- and CD8-positive lymphocytes.

The anti-HMGCR antibody revealed the characteristics of myopathy including very high serum levels of creatine kinase, widespread damage visible through MRI [8,11–13], the presence of the sarcolemmal and capillary membrane attack complex (MAC) deposition on muscle biopsy [11,14–16], and the necessity of intense immunosuppressive treatment [11,15–20]. The clinical manifestations of anti-HMGCR antibody-positive IMNM (HMGCR-IMNM) have recently been defined as the presence of proximal weakness and elevated creatine kinase levels by published reports [21–23] and the 224th European Neuromuscular Centre (ENMC) International Workshop [24]. Interestingly, limb-girdle muscular dystrophy (LGMD) presentation [25] and isolated hyperCKemia [16] were also included in the spectrum of HMGCR-IMNM. In addition, recent studies revealed the small percentage of HMGCR-IMNM patients have dermatological manifestations [26▪–29▪]. Here, we review manifestations of HMGCR-IMNM, and highlight recent clinical advances in HMGCR-IMNM.

ANTI-HMGCR ANTIBODY AND EPIDEMIOLOGY

HMGCR is a glycoprotein catalyzing the conversion of HMG-CoA to mevalonic acid, an essential step in cholesterol biosynthesis [30,31]. HMGCR is inhibited by statins (HMGCR inhibitors), which suppress serum cholesterol levels and markedly reduce overall cardiovascular events [32]. The cytoplasmic catalytic domain of HMGCR links to an endoplasmic reticulum membrane-embedded domain [31]. The intracellular C-terminal part of the enzyme is recognized by anti-HMGCR antibody [8]. Although genetic variants of HMGCR are associated with different levels of low-density lipoproteins and cardiovascular risks [33], the coding region for the C-terminal part of HMGCR has no known genetic variants. This suggests that variants in the C-terminal part of HMGCR may be lethal. Indeed, liver-specific HMGCR knockout mice die before 6 weeks of age [34] and skeletal muscle-specific HMGCR knockout mice exhibit rhabdomyolysis [35]. However, although up to 20% of patients exposed to statins experience muscle symptoms [36], the large majority of these symptoms are due to statin-induced direct toxicity [37–41] and do not develop anti-HMGCR autoantibodies [38].

The prevalence of HMGCR-IMNM ranges from 6 to 20% of IIMs [8,15,26▪,43–45]. HMGCR-IMNM occurs more frequently in women after 40 years of age [8,43,46,47]. In juvenile IIMs patients, the prevalence of HMGCR-IMNM is 1% [48–50]. The target of anti-HMGCR antibody is the same as that of statins, which leads to the hypothesis that statins may be a factor in HMGCR-IMNM pathogenesis. The percentage of statin exposure in different patient groups ranges from 15 to 65% [8,51], depending on geographic origin, ethnicity and age. The percentage of statin exposure is low in Asia [51], moderate in Europe [15] and high in the USA [8]. In addition, 90% of HMGCR-IMNM patients who are over 50 years of age have been exposed to statins [11].

Genetic background may also play a role in HMGCR-IMNM pathogenesis. The presence of the MHC class II allele DRB1∗11 : 01 confers a strong immunogenetic predisposition to HMGCR-IMNM in adults [42,52,53], whereas this risk is associated with DRB1∗07 : 01 in infants [48].

Malignancy is the most frequent comorbidity of IIMs. The risk of malignancy was found to be slightly increased in HMGCR-IMNM patients in two different studies [54,55] but was not increased in other studies [19,26▪]. There is no increased risk of malignancy in patients with anti-SRP-positive IMNM (SRP-IMNM) [54,55]. The greatest increase in cancer risk was found for seronegative IMNM [51,56,57]. Thus, patients with seronegative IMNM are at an increased risk of malignancy, and HMGCR-IMNMs may be considered relatively muscle-predominant diseases.

CLINICAL SYMPTOMS: FOCUS ON THE DIFFERENCE BETWEEN HMGCR-IMNM AND SRP-IMNM

Muscular phenotype

IMNM patients usually have a proximal bilateral and symmetrical weakness of the upper and lower limbs, which is usually a severe muscle deficit. Muscle weakness appears more predominantly in lower limbs than upper limbs [44,47,58]. However, muscle weakness of HMGCR-IMNM patients is usually milder than that of

Table 1. Clinical history and symptoms of HMGCR-IMNM compared with those of SRP-IMNM

	HMGCR-IMNM	SRP-IMNM
Statin exposure	+∼++	±
Childhood onset	+	+
Severe muscle weakness	+	+++
Cervical muscle weakness	++	+++
Shoulder-girdle weakness	+	+
Myalgia	+	++
Dysphagia	++	+++
Cardiac involvement	-	±∼++
Respiratory failure	-	+
Interstitial pneumonia	±	±
Raynaud phenomenon	-	±
Skin condition	±	±

+++ - +: more common ∼ less common, ±: rare, -: none.
HMGCR, anti3-hydroxy-3-methylglutaryl-coA reductase; IMNM, immune-mediated necrotizing myopathy; SRP, signal recognition particle. Adapted from [15, 17, 26, 44, 47, 58].

SRP-IMNM patients (Table 1) [43,55]. In addition, the proximal pattern of muscle weakness is notable in HMGCR-IMNM patients due to the slow disease onset, in which lower limb weakness precedes upper limb weakness [43,49,58]. Myalgia is also observed in IMNM patients. The incidence of myalgia in HMGCR-IMNM patients is less than that in SRP-INMN patients [15,20,43,44,47,58]. Dysphagia was observed in up to a quarter of patients with anti-HMGCR-positive IMNM [15,19], which was less than the incidence of dysphagia in SRP-IMNM patients [43].

Patients with HMGCR-IMNM show high serum creatine kinase levels, which are sometimes 30 times higher than the upper limit of normal [43,55]. Their serum creatine kinase level correlates with the percentage of necrotic muscle fibres [22]. Because the serum creatine kinase level correlates with muscle mass, a high serum creatine kinase level may become lower over time, notably in patients with a long disease duration and severe muscle atrophy that is verified by a low creatinine level.

HMGCR-IMNM was considered to be a condition with an acute–subacute onset similar to other subtypes of IIMs. More than two-thirds of patients with seropositive IMNM have an acute (within a few weeks) or subacute (in <6 months) onset [54]. Meanwhile, the remaining patients positive for anti-HMGCR antibodies have a slowly progressive onset. They have a long disease duration (over years) and are diagnosed with LGMD [8,25,59]. Interestingly, HMGCR-IMNM patients with slow disease progression, especially young patient, sometimes show scapular winging though muscle atrophy as observed frequently in SRP-IMNM [25,43]. These findings suggest that it may be difficult to distinguish between HMGCR-IMNM and LGMD, especially if the disease course is slowly progressive [25]. In young patients suspected to have LGMD who show high levels of serum creatine kinase and no extramuscular manifestations, testing for anti-HMGCR autoantibody should be as valuable as genetic testing to ensure that efficacious treatments are not delayed.

In typical HMGCR-IMNM cases, MRI showed focal or diffuse abnormal signals in the trunk and limb muscles on T2-weighted images (T2WI) or short tau inversion recovery (STIR) images, which were more pronounced on gadolinium-enhanced T1 weighted images (Gd-T1) [11,60] (Fig. 1a, b). The most affected muscle groups in the proximal lower extremities are the posterior thigh, gluteal, and medial thigh compartments [19,50,61]. Distal muscles in the lower leg compartment have been less studied. In contrast, abnormal T2WI/STIR signals and muscle atrophies were rarely observed in muscles of HMGCR-IMNM patients, similar to LGMD in the early phase [25,62▪▪] (Fig. 1c). After disease progression in HMGCR-IMNM patients, MRI showed muscle atrophy and diffuse abnormal T2WI/STIR signals, which were not enhanced by Gd-T1 [25] (Fig. 1d).

Extramuscular phenotype

For HMGCR-IMNM patients, the frequency of extramuscular manifestations was low. However, several studies have reported skin conditions in these patients, including Raynaud phenomenon, a nonspecific skin rash and dermatomyositis-like skin eruptions [11,15,26▪–29▪,50]. HMGCR-IMNM patients with skin conditions usually develop pruritic erythematous papules with photosensitivity (Fig. 1E, F) [28▪,29▪,50] and sometimes show the dermatological presentations of Jessner-Kanoff disease or cutaneous lymphoma [26▪,27▪]. However, the incidence of anti-HMGCR antibody in Jessner-Kanoff disease or cutaneous lymphoma is still unknown. Other extramuscular manifestations including cardiac and pulmonary involvement have been considered to be part of HMGCR-IMNM on rare occasions [15,26▪,27▪,43,45,60].

HISTOPATHOLOGICAL MANIFESTATIONS

Typical myopathological findings

Haematoxylin and eosin staining and modified Gomori trichrome staining revealed muscle fibres in different stages of necrosis and regeneration, which are characteristic of IMNM (Fig. 2a, b) [24]. Alkaline phosphatase staining also showed

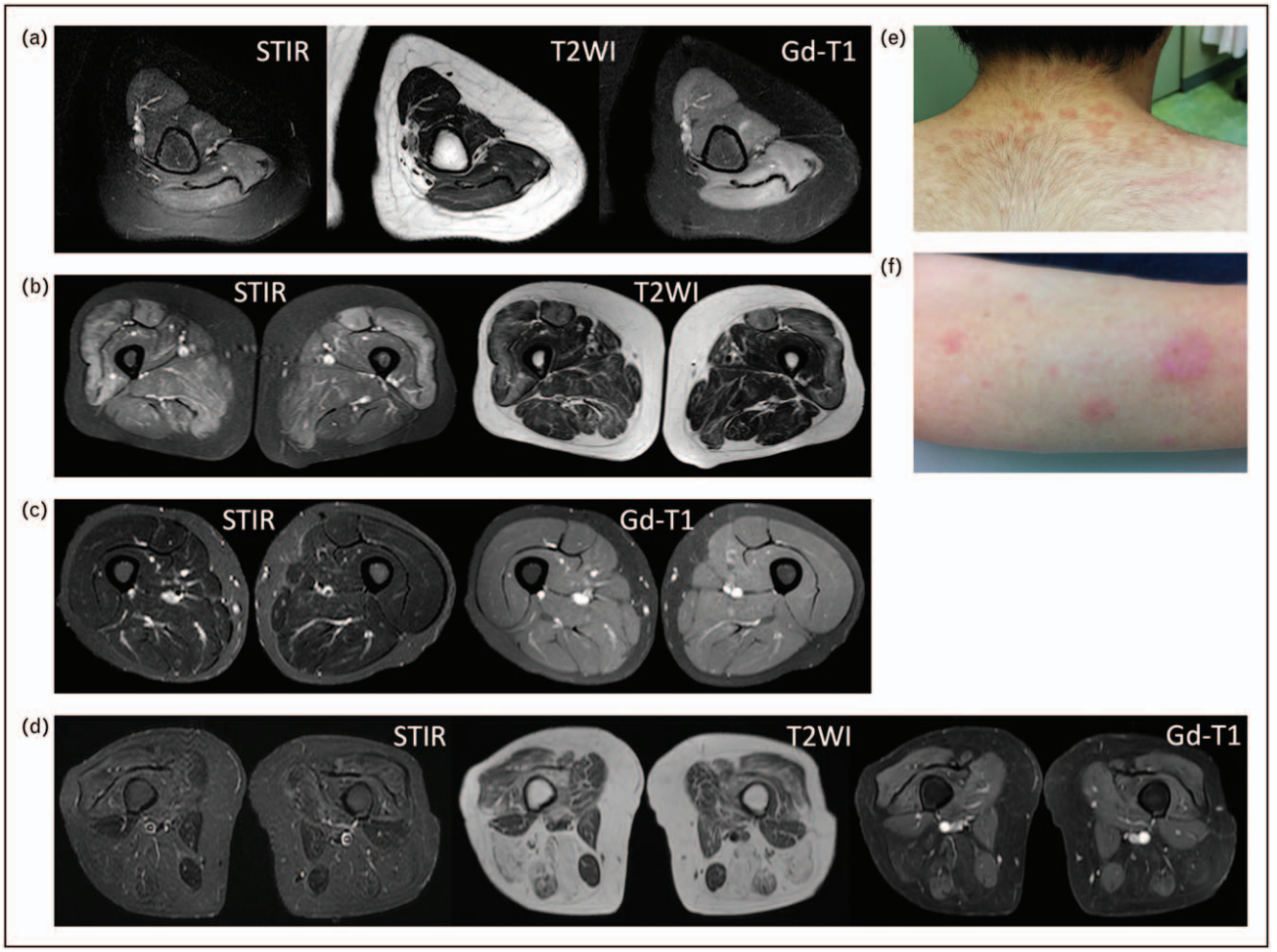

FIGURE 1. Magnetic resonance imaging and skin manifestation of HMGCR-IMNM. (a, b) The trunk and limb muscles on short tau inversion recovery (STIR), T2 weighted images (T2WI), and gadolinium-enhanced T1 weighted (Gd-T1) images of typical HMGCR-IMNM patients. (c) T2WI and STIR images of muscles of HMGCR-IMNM patients mimicking early phase LGMD. (d) T2WI, STIR, and Gd-T1 images in patients with HMGCR-IMNM mimicking late phase LGMD. (e, f) Pruritic erythematous papules with photosensitivity in HMGCR-IMNM patients with skin conditions.

regenerating muscle fibres (Fig. 2c). Quantitatively, necrotic muscle fibres represent between 1 and 20% of all fibres [22] and are randomly distributed throughout the muscle fascicles [15,22]. Endomysial lymphocytic infiltration in muscle biopsy specimens from IMNM patients is usually milder than in specimens from patients with other IIMs [22].

MHC class I was usually observed on the sarcolemma, while MHC class I expression in the cytoplasm was rare except for necrotic fibres (Fig. 2d) [24]. Lymphocytes infiltrating to the endomysium were usually positive for CD3 and CD8 (Fig. 2e), with B cells and plasma cells being the exception [63]. Notably, in about 20–30% of biopsies from patients with IMNM and a high proportion of necrotic myofibers, the signs of T-lymphocytic infiltration are similar to those in other IIMs [22,24]. A recent study showed an increased presence of apoptosis marker bcl-2-positive T-lymphocytes in muscle specimens from HMGCR-IMNM patients (Fig. 2f) [26▪], which might be a unique feature of HMGCR-IMNM patients.

Sarcolemmal C5b–9 (MAC) deposits are observed on a variable number of muscle fibres [22,24]. IMNM muscle fibres also present with fine granular and homogeneous staining of the autophagy marker SQSTM1/p62 in the sarcoplasm, but no coarse, squiggly or 'plaque-like' p62 positivity [64,65]. These features are not associated with the SQSTM1/p62 immunopositivity with large, rimmed vacuoles, but are instead characteristic of sIBM [64].

Atypical pathological findings in muscle and dermis

In HMGCR-IMNM patients with slow progression and clinical features similar to LGMD, it was difficult to observe necrotic and regenerating muscle fibres

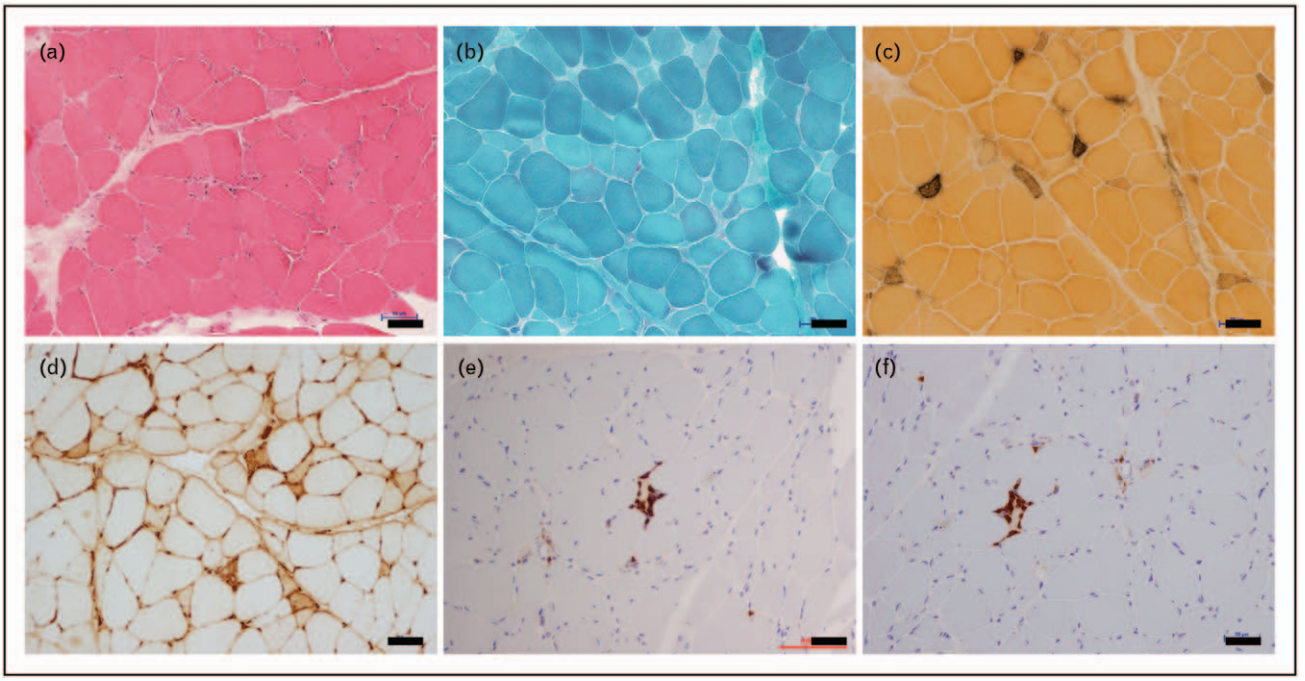

FIGURE 2. Myopathological findings of HMGCR-IMNM patients with typical clinical and pathological presentations. (a, b) Hematoxylin and eosin (HE) staining and modified Gomori trichrome staining. (c) Alkaline phosphatase staining. (d) MHC class I immunostaining. (e) CD8-positive lymphocytes infiltrating into the endomysium. (f) The high frequency of the apoptosis marker bcl-2-positive T-lymphocytes in muscle specimens from HMGCR-IMNM patients. Scale bars: 50 μm.

(Fig. 3a, b). There were several HMGCR-IMNM patients whose muscle specimens had muscle fibers with centrally placed nuclei (Fig. 3c) and regenerating fibres identified as type 2C fibres based on ATPase staining (Fig. 3d) instead of necrotic muscle fibres [24]. In addition, in patients with severe inflammation, inflammatory cells formed accumulations similar to lymphocytic accumulations (Fig. 3e), which were composed of lymphocytes positive for CD8 (Fig. 3g), CD20 (Fig. 3h) and bcl-2 (Fig. 3i) [26▪]. Although these lymphocytic accumulations sometimes showed similarity to lymphoma, lymphocytes that stained positively for the proliferation marker ki-67 were rare.

In HMGCR-IMNM patients with skin conditions, skin biopsy specimens usually showed nonspecific superficial perivascular dermatitis (Fig. 4a) with CD8-positive T-lymphocytes (Fig. 4b). About a half of T-lymphocytes were positive for bcl-2 (Fig. 4c) [26▪]. Interestingly, some patients with skin conditions histologically showed a dermal lymphocytic inflammatory infiltrate with perivascular arrangement and accumulation, which were mainly composed of small lymphocytes with histiocytes (Fig. 4d) [26▪–28▪]. Infiltration consisted of CD8- and bcl-2-positive T-lymphocytes without plasmacytes (Fig. 4e, f). These lymphocytic infiltrations are similar to Jessner-Kanoff lymphocytic infiltration or low-grade cutaneous lymphoma [26▪,27▪].

PROGNOSIS AND TREATMENT

Among IIMs, except for IBM, patients with seropositive IMNM including HMGCR-IMNM have the most severe disease in terms of muscle-related morbidities [56]. In IMNM patients, muscle atrophy and consecutive weakness frequently occur early in the disease course for untreated patients, or at long-term follow-up when treatment has been insufficient. In addition, HMGCR-IMNM patients show poor recovery of muscle strength after immunotherapy. Less than half of patients recover normal muscle strength within 2 years of disease onset, and only two-thirds reach that level of improvement within 4 years [19]. However, the outcome partially depends on the age of the patient at disease onset, with only half of the younger patients (<50 years) reaching normal muscle strength 4 years after disease onset, compared with the majority of older patients (>60 years) [19].

The disease duration for HMGCR-IMNM is long and a large majority of patients require immunosuppressants or immunomodulatory drugs for many years after diagnosis [15], which causes the side effects and comorbidities of these treatments to accumulate. The severity of muscle damage is dependent on the time from symptom onset to treatment initiation, and on disease duration [66▪▪]. IMNM results in a higher proportion of atrophied thigh muscles with fatty replacement, and this muscle damage begins early in the course of the disease (Fig. 1c, d). Muscle

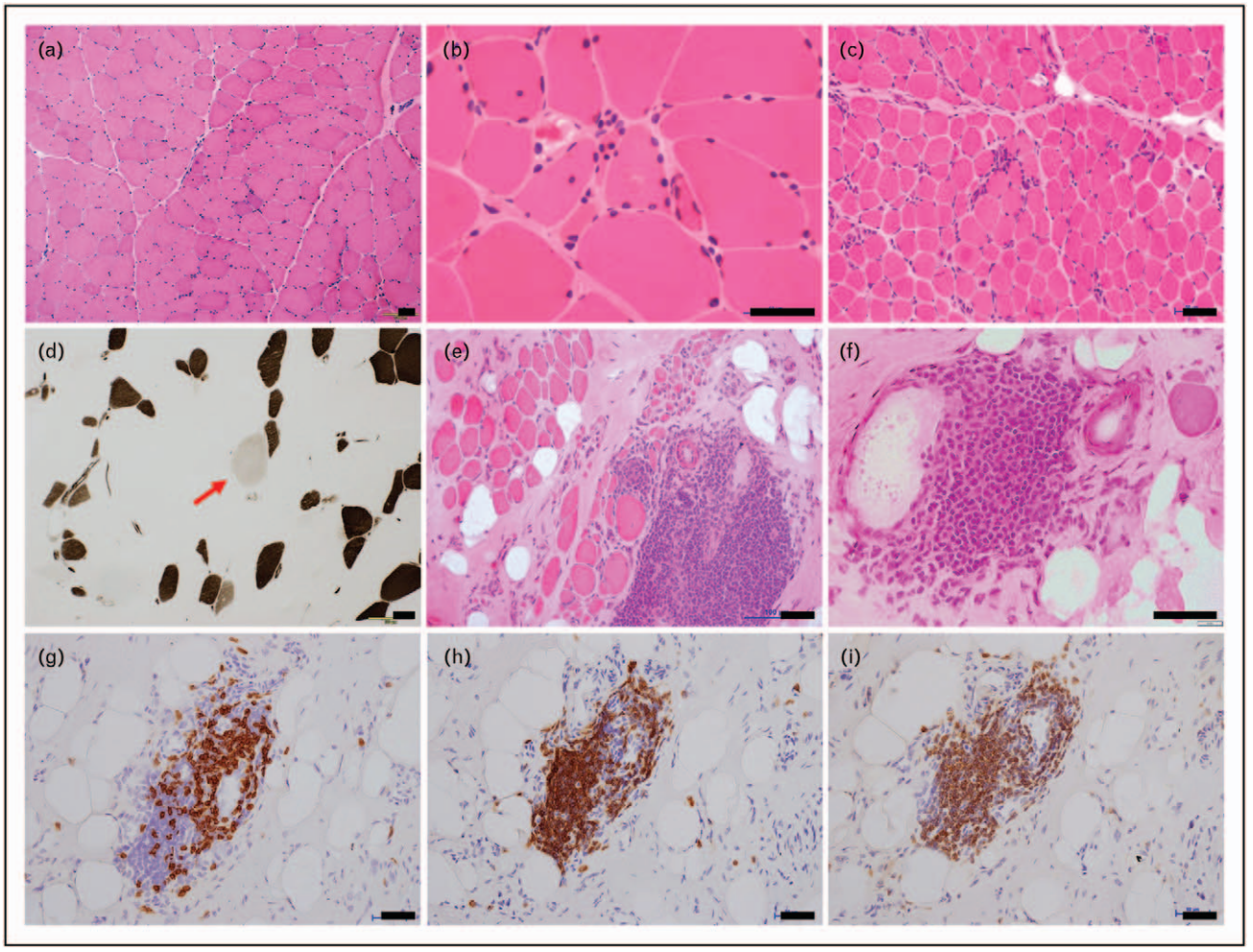

FIGURE 3. Myopathological findings of HMGCR-IMNM patients with atypical presentations. (a–c) Hematoxylin and eosin (HE) staining images of HMGCR-IMNM patients with slow progression and clinical features similar to LGMD. (d) Regenerating fibers identified as type 2C fibers based on ATPase staining. (e, f) HE staining images of patients with severe inflammation forming accumulations similar to lymphocytic follicles without any abnormal lymphocytes. (g–i) Lymphocytes were positive for CD8 (g), CD20 (h), and bcl-2 (i) Scale bars: 50 μm.

atrophy and fatty replacement in HMGCR-IMNM patients are milder than in SRP-IMNM patients [47]. The muscle damage can be predicted by disease duration, which also affects muscle damage burden [66▪▪]. Importantly, muscle damage does not involve axial muscle [66▪▪].

Unfortunately, no randomized, blinded, controlled trials have been published for patients with IMNM, but there is an ongoing trial for IMNM referenced on ClinicalTrials.gov (A Phase 2, Multicenter, Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Safety, Tolerability, and Efficacy of Zilucoplan in Subjects With Immune-Mediated Necrotizing Myopathy) [67]. Thus, current treatments for HMGCR-IMNM are mostly empirical, based on previous experience with treating IIMs, the results of retrospective case series and expert consensus [24]. For polymyositis and dermatomyositis, therapeutic approaches start with corticosteroids (given intravenously or orally) [24]. Corticosteroids are recommended in combination with a corticosteroid-sparing agent such as methotrexate [24]. Prednisone monotherapy is insufficient to control disease activity in most patients [68]. The large majority of patients require a second-line agent in addition to corticosteroids within 6 months of starting treatment [18]. Interestingly, steroid-free induction strategies are thought to be efficacious for IMNM cases in which anti-HMGCR-associated myopathy with elevated levels of serum creatine kinase is diagnosed early, before muscle weakness has occurred [69▪▪]. Other observational studies report that steroids failed to control disease in the large majority (92–100%) of patients with anti-

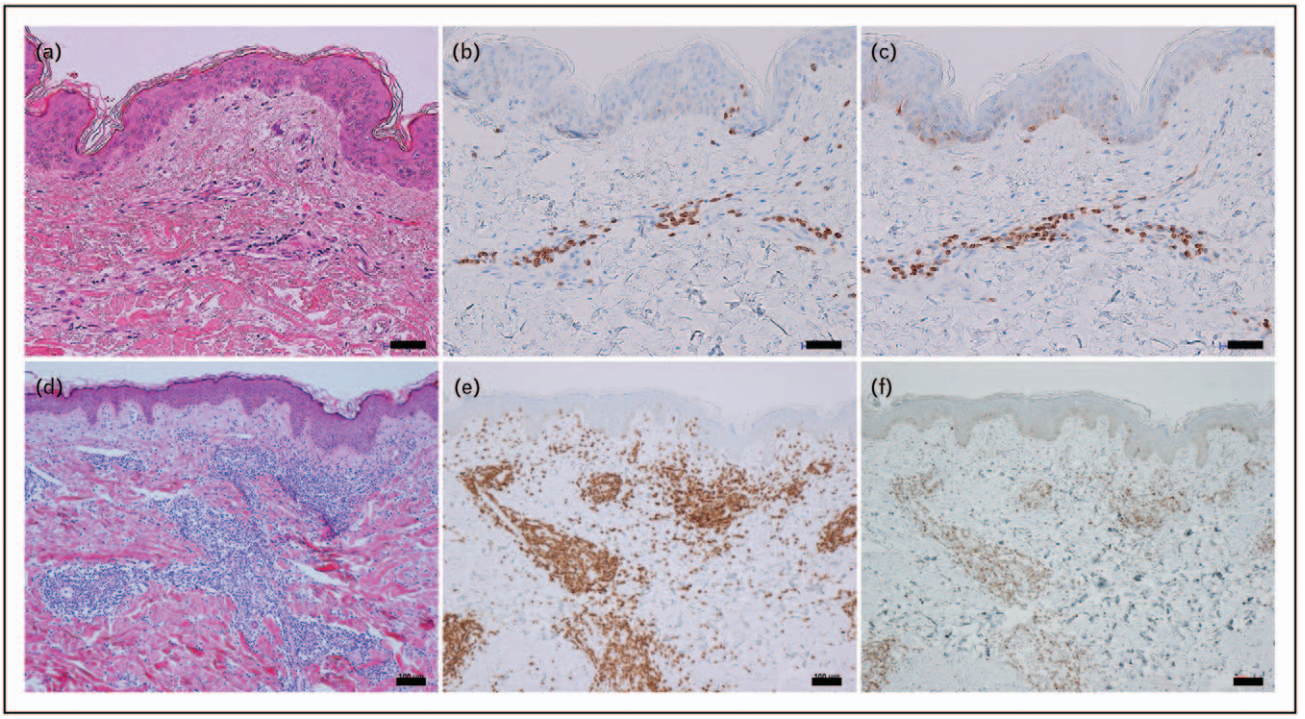

FIGURE 4. Dermatopathological findings of HMGCR-IMNM patients with atypical presentations. (a) Non-specific superficial perivascular dermatitis was usually observed. (b, c) Lymphocytes were positive for CD8 (b) and bcl-2 (c). (d) A dermal perivascular arrangement and accumulation of lymphocytes were sometimes observed. (e, f) Infiltration consisted of lymphocytes positive for CD8 (e) and bcl-2 (f). Scale bars: 50 μm (a–c), 100 μm (d–f).

HMGCR-positive IMNM [17,70,71]. The consensus statement produced by the ENMC workshop states that IMNM should be treated with both corticosteroids and an immunosuppressant within 1 month of initial presentation, and proposed methotrexate as an initial immunosuppressant for IMNM [24]. If no adequate response is observed within 6 months of treatment, intravenous immunoglobulins (IVIGs) should be added to the above-mentioned treatments for HMGCR-IMNM patients [24]. Clinicians should be aware that rituximab does not have a notable effect on HMGCR-IMNM [20,71,72].

In HMGCR-IMNM patients previously prescribed statins, continuation of statin therapy would be a major problem. If necessary, proprotein convertase subtilisin kexin type 9 inhibitors may be a well tolerated alternative to statins for lowering cholesterol levels [73].

CONCLUSION

HMGCR-IMNM is usually characterized by acute-progressive proximal muscle weakness that mainly affects the lower extremities. Recently, the clinical and pathological manifestations of HMGCR-IMNM were found to be broader than previously reported. Atypical HMGCR-IMNM patients show slow disease progression and clinical symptoms which are similar to those of LGMD. Other atypical HMGCR-IMNM patients have skin conditions similar to dermatomyositis-like skin rush or dermatological presentations of Jessner-Kanoff disease or cutaneous lymphoma. bcl-2-T-lymphocytic infiltration and accumulation are observed in both muscle and skin. Unfortunately, treatment for HMGCR-IMNM has made almost no progress in recent years. Additional research is needed to clarify the immunopathologic mechanisms involved in disease pathogenesis, and to test additional therapeutic choices.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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Physical exercise for the management of systemic autoimmune myopathies: recent findings, and future perspectives

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Purpose of review
The aim of this review is to present the main pieces of evidence, recent literature and to present future perspectives on the use of exercise/physical training in the treatment and improvement of the quality of life of patients with systemic autoimmune myopathies.

Recent findings
In the last decades, knowledge about the relevance of physical exercise training in preventing and treating chronic diseases and improving quality of life has grown. Following the global trend exemplified by the expression 'exercise is medicine', the importance of exercise/physical training has also grown in myopathies. However, the science of exercise has a lot to collaborate on and improve patients' quality of life with myopathies by appropriating new technological tools, including accessible and low-cost devices and smartphone apps.

Summary
Physical exercise, as already consolidated in the literature, is an effective, well tolerated, and low-cost strategy for patients with myopathies. The use of wearable devices, smartphone apps, and online training prescriptions must accompany the global scenario, bringing new research fields and expanding the options for access to training for the individualized basis, and prescribed by qualified professionals.

Keywords
inflammatory myopathies, myositis, physical exercise, physical training

INTRODUCTION

In the last century, the importance of physical exercise and its effects on health have been established, such as in the treatment of blood pressure [1,2], diabetes mellitus [3,4], dyslipidemia [5], metabolic syndrome [6], and cardiovascular diseases. More recently, in the last few decades, rheumatic diseases and specifically systemic autoimmune myopathies (or idiopathic inflammatory myopathies) have seen exponential growth in the evidence related to the benefits of physical exercise [7–9]. Systemic autoimmune myopathies are a heterogeneous group of diseases, including dermatomyositis, polymyositis, immune-mediated necrotizing myopathy (IMNM), inclusion body myopathy (IBM), and antisynthetase syndrome (ASSD). Their main similar characteristics are the muscular inflammatory process, functional deficit, reduced aerobic capacity, presence of fatigue, and weakness, leading to the emergence of other chronic diseases, reduced quality of life, increased physical inactivity, and morbidity/mortality [10–13]. Therefore, this review proposes examining the recent findings on the role of physical exercise in systemic autoimmune myopathies' treatment, with regard to inflammatory responses, disease activity, and functional capacity, as well as present perspectives on the use of technology to promote physical exercise, leading to improved quality of life.

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KEY POINTS

- Physical training protocols seem to be well tolerated and effective in the treatment of systemic autoimmune myopathies.
- Physical training does not alter inflammatory markers and does not reactivate the disease.
- We must encourage: training protocols based on PROs; develop and validate wearable devices and smartphone apps with a focus on exercise and PROs; the home-based training.

METHODS

Selection criteria

For the topic 'current perspectives', we selected articles published in the last 18 months, through a bibliographic search in the electronic database PubMed, Google Scholar, ScienceDirect.

Were included articles in English, published between 6 January 2019 and 28 March 2021, using the following keywords and Boolean operators: *exercise* OR *'physical exercise'* OR *'resistance training'* AND *myositis** AND *myopathy* NOT (*'mitochondrial myopathy'* AND *'metabolic myopathy'*).

We consider uncontrolled randomized and non-randomized clinical trials and case studies. In addition, we included studies that presented a detailed description of the physical exercise and the patients included, which met the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR 2017) classification criteria [14] or criteria of Connors *et al.* [15] for ASSD.

We excluded articles published outside the period described above, conference papers, dissertations, theses, literature reviews, and studies not published in English. In addition, we excluded studies carried out with patients with other myopathies (mitochondrial myopathy and metabolic myopathy), as shown in Fig. 1.

Historical perspective

From the initial studies by Thomas Delorme [16,17] regarding the use of strength training for musculoskeletal rehabilitation of ex-combatants of the second world war, a new field of application and a great interest in the therapeutic effects of physical exercise in rehabilitation and mainly its use in the treatment/rehabilitation of several comorbidities has emerged [16].

However, nearly five decades later, the pioneering Hicks *et al.* [18] and Escalante *et al.* [19] began to study the effects of physical exercise on myopathies, since then, interest has grown and physical exercise training has been included in the treatment of classic symptoms, such as muscle weakness, muscle atrophy, and weight loss, arousing interest in its use to maintain autonomy and mainly to improve the quality of life of patient with systemic autoimmune myopathies.

In this context, strength training, which is characterized by voluntary muscle contraction aiming to produce strength against external resistance, is well known to provide neurological and morphological adaptations, such as increase in muscle strength, muscle hypertrophy, and, consequently, improving body composition [20,21]. Therefore, based on these

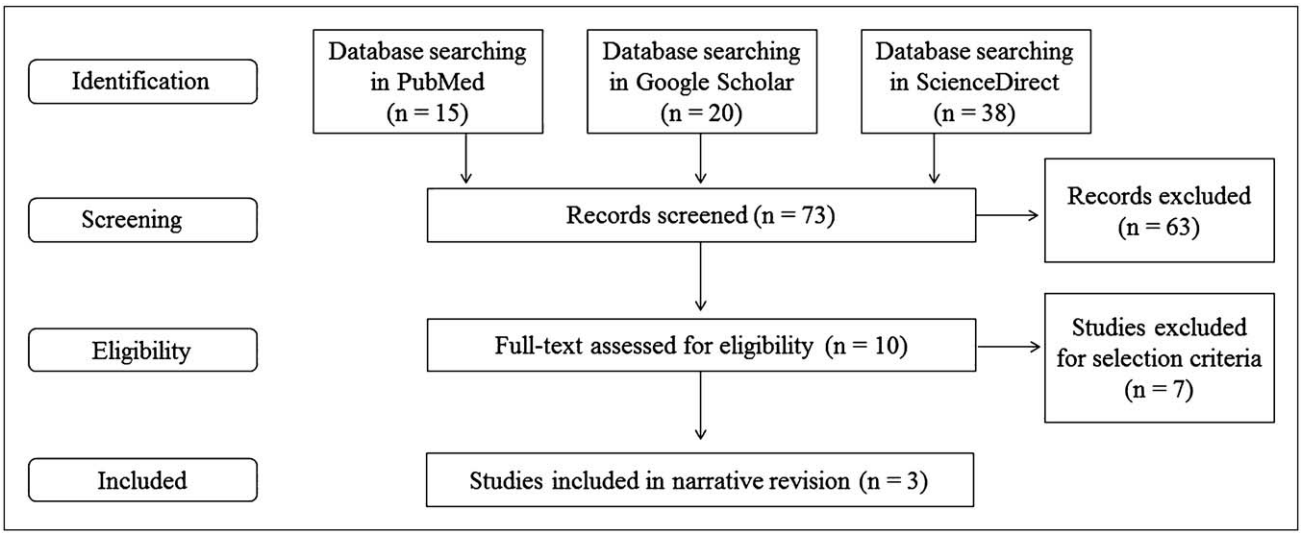

FIGURE 1. Representation of the study selection process by the flowchart.

adaptations to strength training, it has emerged as an obvious choice to combat the main symptoms of systemic autoimmune myopathies.

Hicks *et al.* [18], in their innovative study with isometric strength training carried out on a patient with polymyositis, observed changes in inflammatory markers and showed increased muscle strength after 4 weeks of training. In the same year, Escalante *et al.* [19] corroborated these findings by observing increased muscle strength without altering creatine phosphokinase (CPK) in active disease patients after 2 weeks of strength training.

Aerobic exercise is characterized by exercises that use larger muscle groups and is performed in a cyclic manner, which can be continuous or intermittent, leading to physiological adaptations, such as increased mitochondrial biogenesis, higher capillary density, and increased ability to capture, transport, and use oxygen, among other cardiovascular benefits, reducing the risk of cardiovascular diseases, metabolic syndrome, and blood pressure [22,23].

Following the recognized benefits of aerobic exercise in healthy individuals, Wiesinger *et al.* [24,25], in their two initial studies applying aerobic exercise in patients with polymyositis and dermatomyositis, observed the efficiency of this type of intervention by the improvement in aerobic capacity, functional capacity, and muscle strength. In addition, the patients did not present CPK changes, enabling the inclusion of this type of exercise as one of the tools used in the rehabilitation of patients with polymyositis and dermatomyositis.

When aerobic exercise and strength training were combined, as designed, as Alexanderson *et al.* [26], the authors found that this type of exercise training was well tolerated and improved the functional capacity, walking distance, and quality of life of patients with dermatomyositis and polymyositis. Additionally, a highlight of this study was that patients performed a home-based protocol.

Several studies have demonstrated the best-known effects of creatine supplementation, which are increased strength, muscle power, and consequently increased muscle mass [27]. In addition, some clinical conditions may benefit from its use, such as hereditary defects of creatine synthesis, muscular dystrophies, muscular atrophy, depressed mood disorder, and sleep deprivation [27,28]. On the basis of this assumption, the concomitant use of creatine and performance of strength training seems to be a coherent strategy in view of the described effects of creatine supplementation and the symptoms of in myopathies.

In this regard, Chung *et al.* [29] evaluated 37 patients (22 with polymyositis and 15 with dermatomyositis) randomized into two groups (placebo and physical exercise, and creatine and physical exercise) throughout a 6-month protocol. The protocol was performed according to a previous study [26], including strength training of lower and upper limbs, 15 min of aerobic training, and stretching (i.e. combined training). Interestingly, the group that received creatine supplementation associated with physical exercise showed greater benefits, with emphasis on the improvement of functional capacity, and muscle strength, without altering quality of life, pain, depression, or CPK.

Another type of protocol in which interest increased over the past few decades is the strength training associated with vascular occlusion as it was shown to improve the strength and cross-section area of the occluded muscle, even using a reduced overload [30,31].

From this conceptual point of view, Gualano *et al.* [32] used strength training associated with vascular occlusion in myopathies, and specifically in IBM patients, in a case report study. The authors obtained positive responses regarding the quality of life, muscle strength, muscle cross-sectional area, and time in the Up-And-Go Test, confirming the improvement of IBM patient' muscular function. This was an outstanding finding as positive effects were not previously reported in this population, and therefore, a possible therapy was provided to attenuate the traditional atrophy of the disease without producing a disease flare [32].

Subsequently, and trying to trace the process of this area of intersection, another important randomized study (e.g. exercise group or nonexercise group) was conducted by Jorgensen *et al.* [33] with 22 IBM patients, using a 12-week protocol of strength training associated with vascular occlusion.

The results did not show changes in the primary outcome of the study, namely, physical function assessed through the 36-item Short-Form Health Survey [34]. However, the nonexercise group showed an approximately 9% decline in knee extension strength, differently from what was found in the exercise group, demonstrating a possible protective effect of this type of intervention on muscle strength decline observed in IBM patients [33].

Our group [35] was the pioneer in showing the safety of a combined training protocol in eight patients diagnosed with IMNM. In this study, we found increased aerobic capacity, strength, and muscle function after combined exercise training. Regarding safety, the patients did not present changes in inflammatory markers or worsening of clinical features based on the International Myositis Assessment and Clinical Studies Group [36].

Despite these promising results regarding the role of physical exercise in patients with systemic

autoimmune myopathies, this is a heterogeneous group of diseases with a complex diagnosis process, which requires invasive techniques (e.g. muscle biopsy) and specific autoantibodies identification. Moreover, each systemic autoimmune myopathy presents different comorbidities requiring specific treatment, and, consequently, specific exercise training protocols [37,38].

A clear example can be observed for patients with ASSD, a disease that is still underdiagnosed in clinical practice and presents significant cardiopulmonary impairment, leading to a reduction in aerobic power [13,15].

However, it is important to highlight the effects of exercise that are little discussed presently when it comes to patients with myopathies, such as improving the quality of life and improving the ability to perform activities of daily living [24,25,39–43]. In addition, studies suggest effects of exercise training in reducing pain, fatigue, depression, and anxiety [44–47], the prevalence of which increased because of the coronavirus disease 2019 (COVID-19) pandemic, even more so in infected patients, who might possibly have even greater reflections on health-related quality of life in the coming years [48].

Collectively, studies assessing physical exercise in patients with systemic autoimmune myopathies observed that the clinical picture does not worsen, or that CPK does not change with the practice of training or physical exercise [11,26,43,49]. Additionally, physical exercise is already well studied, mainly in relation to its anti-inflammatory effect and in modulation of cytokines, such as interleukin-6 [50,51], justifying the importance of such practice for patients with systemic autoimmune myopathies.

In summary, we highlight Infographic 1, http://links.lww.com/COR/A51, presenting the historical perspective and the main advances regarding physical training in systemic autoimmune myopathies.

Moreover, we highlight the importance of multiprofessional intervention (e.g. integration between a rheumatologist and clinical exercise physiologist), which can increase patient engagement in the practice of physical exercise, thus improving the quality of life and physical functioning of this population, as well as reducing physical inactivity and sedentary behavior, which are frequent in rheumatologic patients [52,53]. Therefore, a wide area of knowledge has been built by diverse explorers, although there are still many pathways to reveal now and in the future.

Current perspectives

In the last 2 years, mostly because of the COVID-19 pandemic, there have been few advances in understanding the effects of physical training on autoimmune myopathies.

Among the studies previously selected according to the eligibility criteria, after the review performed (A.M.S. and R.G.M.), only three articles were selected and reviewed in detail.

Most studies were related to other neuromuscular diseases or had an unclear diagnosis. However, we highlight the low scientific production in this period.

In 2019, Jensen *et al.* [54▪], in a randomized study, using a low load strength training protocol associated with vascular occlusion in patients with IBM, evaluated the inflammatory responses at the muscle level from the biopsy. This protocol has been shown not to exacerbate the chronic inflammatory process presented in this disease and may be beneficial in combating muscle weakness and muscle atrophy, which traditionally lead to reduced ability to perform activities of daily living [54▪].

In that same year, Oliveira *et al.* [55] evaluated the effect of combined training in nine patients with systemic autoimmune myopathies, with emphasis on the inclusion of two patients with ASSD. The study demonstrated reduced insulin resistance and improved β-cell function, indicating that 12 weeks of physical training can improve metabolic dysfunction, providing a possible reduction in cardiovascular risk.

Additionally, this same study demonstrated that patients with systemic autoimmune myopathies stable according to IMACS scores can benefit from physical training in improved muscle strength, functional capacity, and increased time to exhaustion, and improvement in time to achieve that respiratory compensation point on the cardiopulmonary treadmill test, with no change in acute phase reagents or clinical worsening [55].

Akulwar [56] conducted a case study with a dermatomyositis patient. In a 12-week combined training protocol, the patient showed improvement in all scores evaluated in the IMACS, with an average of approximately a 23.3% increase in the domains of this score, in addition to improved quality of life based on the SF-36 questionnaire, functional capacity, strength, aerobic capacity (increased cycling and treadmill total time), reduced muscle pain, and fatigue. Finally, no change in acute phase reactants or disease reactivation was found in this dermatomyositis patient.

The current studies corroborate the findings of classic studies that evaluated the effects of physical exercise on systemic autoimmune myopathies, further strengthening the use of this tool.

Summary of these findings are presented in Table 1.

Table 1. Current studies in physical exercise for the management of myopathies

Authors	Year	Disease	Number of patients	Randomization (yes or no)	Disease status	Characteristics of the protocol and training			Inflammatory marker/ response	Summary of results
						Type of exercises	Time (weeks)	Variables evaluated		
Jensen *et al.* [54▪]	2019	IBM	21	Yes	No activity	Strength training with vascular occlusion	12	Immune response (T cells, macrophages, and dendritic cells)	↔	No change in inflammatory response
De Oliveira *et al.* [55]	2019	DM, PM, ASSD	9 (6 DM; 2 ASSD, 1 PM)	No	No activity	Combined training	12	Strength; aerobic cap.; functional cap.; quality of life; insulin resistance; body composition	CPK ↔	Strength ↑; aerobic cap. ↑; functional cap. ↑; quality of life ↑; insulin resistance ↓; body composition ↑
Akulwar [56]	2021	DM	1	No	No activity	Combined training	12	Strength; aerobic cap.; functional cap.; quality of life; muscle pain; fatigue; balance; IMACS Score; HAQ	CPK, the transaminases; lactate dehydrogenase; and aldolase ↔	Strength ↑; aerobic cap. ↑; functional cap. ↑; quality of life ↑; muscle pain ↓; fatigue ↓; balance ↑; IMACS Score ↓; HAQ ↓

ASSD, antisynthetase syndrome; combined training: strength and aerobic training; Cap., capacity; CPK, creatine phosphokinase; DM, dermatomyositis; IBM, inclusion body myositis; MMT, Manual Muscle Testing; PM, polymyositis; ↔: no change; ↑: increase; ↓: reduction.

Future perspectives

Despite the numerous and essential advances concerning the safety and effectiveness of exercise training for different systemic autoimmune myopathies, several gaps remain regarding the efficacy of exercise training on the patient-reported outcomes (PROs), mainly in the pain and fatigue perception in these diseases [57▪,58–60], leading us to highlight the lack of a validated tool available to measure exercise effectiveness in PROS producing a significant challenge in clinical research settings [57▪].

Currently, the COVID-19 pandemic leads to an urgent call for actions to mitigate the negative effects observed in several studies on psychological health, pain, and quality of life, as seen similarly in systemic autoimmune myopathies [57▪,61,62▪▪].

Another challenge that remains is approaches to change negative behavior, such as physical inactivity and sedentary behavior. Recently, low-cost wearable devices gained attention as a promising strategy to counteract and monitor the negative effects related to the COVID-19 pandemic [63–65,66▪▪,67], with emphasis on the Franssen *et al.*'s study [66▪▪] that demonstrated that wearable devices can be an important tool in the treatment of chronic diseases, helping to reduce important markers in these comorbidities.

In this context, the available data on physical activity in systemic autoimmune myopathies measured using an accelerometer showed several limitations because of sample size and poor association with disease parameters [68]. Furthermore, to our knowledge, no studies have validated the use of low-cost wearable devices (e.g. by assessing reliability and reproducibility of the data) to measure physical activity and sedentary behavior in patients with systemic autoimmune myopathies.

In addition, as a global trend in this scenario of coping with the pandemic COVID-19, strategies and research aiming to develop programs, smartphone apps, and online platforms, specific to patients with systemic autoimmune myopathies, should be promoted, which will support the treatment and quality of life of this population [69].

CONCLUSION

Physical exercise is a well tolerated and low-cost strategy for the management of patients with myopathies, producing mainly improved quality of life. Additionally, the creation of protocols based on patient-reported outcomes, and home-based are extremely necessary during the current COVID-19 pandemic. Furthermore, wearable devices and smartphone apps should be developed and encouraged in prescribing physical training to these populations.

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Conflicts of interest

There are no conflicts of interest.

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Optimizing reproductive health management in lupus and Sjogren's syndrome

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Purpose of review
People with childbearing capacity who are diagnosed with systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS) have specific and important reproductive health considerations.

Recent findings
Recommendations from the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) provide rheumatologists and other clinicians with guidance for reproductive health management of patients with rheumatic diseases. Patient-centered reproductive health counseling can help clinicians to operationalize the EULAR and ACR guidelines and enhance patient care.

Summary
Disease activity monitoring, risk factor stratification, and prescription of pregnancy-compatible medications during pregnancy help to anticipate complications and enhance pregnancy outcomes in SLE and SS. Assisted reproductive technologies are also safe among people with well-controlled disease. Safe and effective contraceptive methods are available for patients with SLE and SS, and pregnancy termination appears to be safe among these patients.

Keywords
family planning, pregnancy, reproductive health, Sjogren's syndrome, systemic lupus erythematosus

INTRODUCTION

This review describes the optimal reproductive healthcare and management of patients with childbearing capacity who are diagnosed with systemic lupus erythematosus (SLE) and Sjogren's Syndrome (SS). The framework of the review is provided by the European League Against Rheumatism (EULAR) and the American College of Rheumatology Reproductive Health Guideline (ACR RHG), which have provided evidence-based guidance for the care of patients across their reproductive lives [1,2▪▪]. This review also presents patient-centered approaches that the rheumatologist may use to advance the family planning care of patients with childbearing capacity.

PREPREGNANCY RISK ASSESSMENT AND PREGNANCY MANAGEMENT

Systemic lupus erythematosus

Pregnancy outcomes in systemic lupus erythematosus

People with SLE account for approximately 4500 pregnancies in the United States [3]. Although many of these pregnancies are viable and healthy [4], individuals with SLE have at least twice the rate of miscarriage, a five-fold greater risk of preeclampsia and eclampsia, and higher rates of maternal thrombosis and unplanned cesarean sections as compared to people without SLE [5]. Fetal complications are also more common, including preterm birth, intrauterine fetal growth restriction and stillbirth [6▪▪,7,8,9▪,10]. Pulmonary hypertension and history of or active lupus nephritis at the time of conception are particularly strong risk factors for adverse maternal and fetal outcomes [11,12▪▪]. The presence of antiphospholipid antibodies (aPL) or syndrome (APS) also increase the risk of miscarriage and

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KEY POINTS

- Pregnancy outcomes can be enhanced with laboratory monitoring and clinical assessment during SLE and SS pregnancy.
- Prepregnancy counseling is important to facilitate risk factor stratification and early referral to maternal-fetal medicine.
- Assisted reproductive technologies are safe for people with SLE and SS, assuming their diseases are stable; anticoagulation should be considered if the patient has antiphospholipid antibodies or antiphospholipid antibody syndrome.
- Safe and effective contraceptive methods are available for patients with SLE and SS, and estrogen-containing contraception can be considered among people with well-controlled SLE and no antiphospholipid antibodies.
- Pregnancy termination is likely safe for patients with SLE and SS based on limited data.

preeclampsia [4], and the presence of anti-Ro/SSA and/or anti-La/SSB antibodies increases the risk for neonatal lupus [1].

Although these statistics are troubling, an analysis of the National Inpatient Sample found that SLE pregnancy-associated outcomes have improved over a 17 years period [13▪], including a decline in maternal mortality. Better outcomes may result from clinical advances, including increasing prescription of hydroxychloroquine (HCQ) during pregnancy and better anticipatory care and pregnancy management [14]. However, improvements in pregnancy outcomes are not yet equitable in SLE [15▪▪]. In the PROMISSE study, a prospective observational cohort of pregnant patients with SLE and/or APS, Black and Hispanic women were twice as likely to experience adverse pregnancy outcomes as white women, an effect that was attenuated with adjustment for socioeconomic status (SES) [16]. Irrespective of SES, some Black and Hispanic people who are pregnant may experience structural racism and discrimination in their healthcare and unequal access to treatment [15▪▪,17]– social determinants of health that may contribute to the persistent disparities in pregnancy outcomes observed in SLE [18].

Clinical and laboratory monitoring in systemic lupus erythematosus pregnancy

Clinical and laboratory assessment of the pregnant SLE patient can be challenging for the rheumatologist, as the physiologic changes of pregnancy may be difficult to differentiate from active SLE [19]. Active SLE may present with inflammatory arthritis, rash, mucosal ulcers, alopecia, and serositis, anemia, thrombocytopenia, proteinuria, hypocomplementemia, and elevated inflammatory markers [20]. However, up to 50% of pregnancies among people in the general population are complicated by anemia due to hemodilution related to increased blood volumes. Thrombocytopenia occurs in approximately 10% of healthy pregnancies. Increased blood flow through the kidneys may lead to proteinuria that is not related to active lupus nephritis. Laboratory evidence of SLE activity may be masked by complement levels, which may increase by 10–50% due to increased hepatic protein synthesis during pregnancy. Conversely, erythrocyte stimulation factor increases during a normal pregnancy due to increased fibrinogen levels and therefore may not be a reliable marker of disease activity [21].

To help to better anticipate and manage SLE disease activity, the ACR RHG strongly recommends that pregnant patients are monitored at least once per trimester with clinical history and examination and laboratory testing [22]. Single-time laboratory testing should include anti-Ro/SSA, anti-La/SSB, and aPL antibodies (i.e., lupus anticoagulant, and anticardiolipin and antibeta-2 glycoprotein antibodies) checked prior to pregnancy or in early stages of pregnancy [1,2▪▪] (Table 1). Other labs should be checked at least once a trimester: complete blood cell count with cell differential, urinalysis, urinary protein and creatinine ratio, antidouble-stranded DNA, complement 3 (C3) and 4 (C4) levels. Some centers also recommend testing for liver function tests and uric acid levels at least at baseline, as uptrending levels, as well as rising blood pressures, may be early indicators of preeclampsia [23,24] (Table 1).

Sjogren's syndrome

Pregnancy outcomes

Maternal outcomes in SS have been rarely studied, although they generally seem favorable [25]. A minority of SS patients have interstitial disease and/or pulmonary hypertension [26], which are independently associated with high rates of maternal and fetal mortality [27,28]. Neonatal lupus is probably the most concerning complication associated with SS pregnancy, and is discussed separately in Section Neonatal lupus detection and management.

Clinical and laboratory monitoring in pregnancy

Similar to SLE, the ACR RHG recommends testing for anti-Ro/SSA and anti-La/SSB antibodies and aPL

Table 1. Laboratory and procedural testing for SLE and Sjogren's Syndrome

One-Time Testing SLE and SS prior to or early in pregnancy		
Neonatal Lupus Risk	Anti-Ro/SS-A	If anti-Ro/SS-A+: Serial echocardiography between weeks 16/18 through week 26 (screening intervals not defined)
	Anti-La/SS-B	
	RNP	
Thrombotic Risk	Lupus anticoagulant	
	Anticardiolipin antibody IgG, IgM	
	Beta-2 glycoprotein antibody IgG, IgM	
Testing at Least Once a Trimester		
SLE Disease Activity and Preeclampsia risk		Double-stranded DNA
		Complement 3
		Complement 4
		Urinalysis
		Urine protein/creatinine ratio
		Complete blood count
		Complete metabolic panel
		Uric acid level

RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS, Sjogren's syndrome.

antibodies once before or early in SS pregnancy (Table 1) [2▪▪].

Neonatal lupus detection and management

Neonatal lupus is a syndrome that may occur in fetuses exposed to maternal Ro/SSA antibodies, which are commonly found in patients with SLE and SS [29]; it is more rarely associated with maternal La/SSB or ribonucleoprotein antibodies [30]. Approximately 10% of infants with neonatal lupus develop a nonscarring rash that may be observed at birth or after sun exposure, and typically resolves within four months after birth as maternal antibody levels decrease. Cytopenias and transaminitis may affect approximately 20–30% of neonates, but usually improve within four months. The most serious manifestation of neonatal lupus is cardiac conduction abnormalities, which occur in 2% of pregnancies of primigravid people with Ro/SSA+ and up to 20% of pregnancies among people with a prior pregnancy complicated by neonatal lupus [31]. Congenital heart block (CHB) is caused by transplacental passage of Ro/SSA+ between weeks 12 and 24 of gestation, which can induce fetal myocarditis and disrupt the nascent cardiac conduction system. In 17.5% of cases, CHB causes death of the fetus or neonate [32], and up to 70% of surviving children will require a pacemaker [32].

Among pregnant people with Ro/SSA or La/SSB antibodies who are primigravid or have not had a prior pregnancy complicated by CHB, the ACR RHG conditionally recommends serial fetal echocardiography among patients with starting between 16 and 18 weeks through week 26; specific screening intervals are not specified [2▪▪]. The RHG also conditionally recommends *weekly* fetal echocardiography between weeks 16 and 18 and continuing through week 26 among people who have had a prior pregnancy complicated by CHB. If first- or second-degree heart block is detected, the ACR RHG conditionally recommends treatment with oral dexamethasone 4 mg daily, which can cross the placenta and theoretically reduce the inflammation of the myocardium [2▪▪]. This is somewhat controversial: First- and second-degree block may revert to normal sinus rhythm after treatment with dexamethasone but can also revert spontaneously without treatment [33]. Furthermore, dexamethasone treatment may cause spontaneous abortion, endocrinopathies, hypertension among pregnant people, and oligohydramnios, intrauterine growth restriction, adrenal insufficiency, and delayed neuromotor development in fetuses/neonates [34]. Because of these considerations, some clinicians and centers choose not to treat first- or second-degree block [35]. There is greater consensus about holding dexamethasone in cases of complete heart block, for which effective medical treatment has not been determined [31]. Other therapies, such as intravenous immunoglobulin, are being studied but have not been consistently found to prevent or reverse cardiac conduction abnormalities [36].

Table 2. Medication safety during pregnancy

	Experts' perceptions of safety
Prednisone (10 mg or less)	Safe
Hydroxychloroquine	Safe
NSAIDs (nonselective)	Safe
Tacrolimus	Safe
Azathioprine	Safe
Methotrexate	Not safe
Leflunomide	Not safe
Cyclophosphamide	Not safe, particularly in first trimester, but may be considered in second or third trimesters if life-threatening disease
Mycophenolate mofetil	Not safe
Rituximab, belimumab	Unclear safety profile; rituximab may be considered if life-threatening disease

Expert opinion was abstracted from the ACR Reproductive Guideline.

Emerging data suggest that HCQ is effective at reducing the risk of CHB among people who have had a prior pregnancy complicated by CHB. HCQ reduced the risk of CHB by 60% among pregnant patients with Ro/SSA+ antibody [37]. A recent clinical trial by Izmirly and colleagues found that HCQ prevented recurrent CHB by over 50% among mothers who had a prior pregnancy complicated by neonatal lupus [38▪▪].

Medication management during pregnancy and breastfeeding for systemic lupus erythematosus and Sjogren's syndrome

Pregnant people are often excluded from randomized controlled or other high-quality trials that test the treatment efficacy and safety of medications. Although meant to protect the patient and fetus, this approach may have undermined the identification of safe and effective treatment options for pregnant patients. However, safe and effective medications do exist, and patients should continue to use these during pregnancy and lactation to optimize disease control and facilitate healthier outcomes (Table 2). In contrast, medications with teratogenic potential must be discontinued prior to pregnancy (Table 2). The medication safety of several therapies commonly used in SLE and SS are discussed in greater detail below:

Hydroxychloroquine

HCQ improves pregnancy outcomes among patients with SLE and prevents the development of neonatal lupus. A meta-analysis including nine retrospective studies and 1132 pregnancies reported that preeclampsia, gestational hypertension, and preterm birth were significantly lower among pregnant patients with SLE who used HCQ [39]. Given its favorable risk profile, the ACR RHG suggests that people with SLE and/or with Ro/SSA+ antibody should be prescribed HCQ during pregnancy [2▪▪]. A recent administrative study assessed outcomes of 2045 neonates exposed to HCQ *in utero* versus nearly 3.2 million unexposed neonates. Considering patient-level risk factors, the adjusted relative risk for malformations among HCQ-exposed neonates was 1.26 (95% CI 1.04–1.33) and seemed to be greater among patients whose HCQ dose was 400 mg or greater. No specific pattern of malformations was observed, although oral clefts, respiratory issues, and urinary anomalies were noted [40]. However, another meta-analysis found that HCQ had no deleterious effect on fetal outcomes, congenital malformations, or risk of stillbirth, low birth weight, or premature birth [41]. At this point, unless there is a contraindication such as allergy, we recommend that HCQ is initiated even if the patient with SLE or SS is asymptomatic from their disease; however, possible risks of HCQ therapy must be explored in future work.

Azathioprine and tacrolimus

Azathioprine is often used for a variety of clinical manifestations of SLE and SS, and tacrolimus is commonly used in the context of lupus nephritis. Neither medication is first-line treatment for lupus nephritis, but are safer to use in this context than cyclophosphamide or mycophenolate [2▪▪,42].

Low-dose aspirin

The American College of Obstetricians and Gynecologists (ACOG) recommends that all pregnant people with 'autoimmune diseases' should use low-dose aspirin at a dosage of 81 daily during pregnancy as prophylaxis against preeclampsia starting at the end of the first trimester (approximately 12 weeks of gestation) [43]. Pregnant

patients with APS can safely use baby aspirin and anticoagulation to prevent thrombosis and preeclampsia, and combination therapy does not seem to increase the risk of peripartum hemorrhage [44]. There is less guidance about when to discontinue aspirin in the context of delivery; whereas some trials have discontinued aspirin at 36 weeks of gestation, the ACOG recommends that aspirin is continued through delivery as data do not indicate an increased risk of hemorrhagic complications.

Rituximab and belimumab

The efficacy of rituximab in SLE or SS is unclear, although many clinicians use it for refractory thrombocytopenia, lupus nephritis, or interstitial lung disease. The manufacturer's recommendation that it should be discontinued one year before conception is probably conservative. The ACR RHG recommends that rituximab is discontinued at the point of conception but can be continued in cases of life- or organ-threatening disease. A study from the MotherToBaby Pregnancy registry reported that among 19 pregnant patients who received rituximab within a year of conception or after they became pregnant, one infant had multiple hemangiomas, but no other offspring had major congenital abnormalities or defects. No pregnancies ended in miscarriage or stillbirth [45▪▪]. One of the main concerns about rituximab use during pregnancy is B cell depletion of the neonate and infection risk. B cell depletion was observed in 9 of 23 neonates exposed to rituximab in utero in one review; by six months, however, all nine neonates had normal cell counts [46].

Belimumab, which is Food and Drug Administration-approved for SLE, is also not recommended in pregnancy at this time due to insufficient safety data. Belimumab was transferred cross-placentally in primate studies [47], although like rituximab, its large molecular size may limit much cross-placental transfer during the early stages of pregnancy.

FERTILITY AND ASSISTED REPRODUCTION

Although women with rheumatic diseases often believe they are subfertile as a result of their diseases [48], data suggest that their fertility potential is comparable to healthy people [49]. An exception is that subfertility is more common among patients who have been treated with cyclophosphamide [50,51]. Assisted reproductive technologies (ART) generally appear to be safe among people with well-controlled rheumatic diseases [52▪▪]. ART procedures include ovarian stimulation, which requires estrogens and other exogenous hormones; intrauterine insemination; in vitro fertilization; and embryo transfer [52▪▪]. As pregnancy is the intended outcome of ART, many of the factors that predict healthy pregnancies in SLE and SS—including quiescent disease and use of pregnancy-compatible medications—determine the optimal timing for ART. Laboratory testing for anti-Ro/SSA, anti-La/SSB, and aPL antibodies should be conducted before ART. Prophylactic anticoagulation with heparin or low molecular weight heparin is strongly recommended by the ACR RHG for people with APS and history of miscarriages, and conditionally recommended for pregnant people with aPL but no history of thrombosis. The ACR RHG also recommends that medical treatment is continued among people who undergo ovarian stimulation, even if they use medications that are typically discontinued in pregnancy (i.e., methotrexate or mycophenolate) [2▪▪].

CONTRACEPTION

Contemporary contraceptives appear safe for people with SS and SLE (Table 3). Two key randomized controlled trials assessing the safety of combined estrogen-progestin contraception (COCs) among women with SLE found that COCs did not exacerbate disease flares among patients with well-controlled SLE [53,54]. The Centers for Disease Control Medical Eligibility Criteria consider COCs to be reasonably safe alternatives for women with SLE, including those who use immunosuppressive medications and/or have thrombocytopenia [55]. However, due to the potential thrombotic risks associated with estrogen, estrogen-containing contraception should be avoided among people with SLE with apL antibodies or with unknown aPL antibody status [55]. COCs are also not preferred for women with highly active SLE or lupus nephritis due to insufficient safety data.

Progestin-only contraception is considered less thrombogenic than estrogen-containing contraception, particularly the highly effective progestin-containing intrauterine device and subdermal implant [2▪▪]. Emergency contraception, which contains progestin only, is also safe and effective for use among people with SLE. The progestin-containing contraceptive injection (i.e., depot medroxyprogesterone, or the 'Depo Shot') has been found to increase the risk of thrombosis by three-fold in the general population [56]; therefore, ACR does not recommend this injection for use among people with positive aPL given their elevated baseline risk for thrombosis.

Although safety and efficacy are often prioritized by clinicians [57], we note that people may also preference contraceptive methods for other

Table 3. Selected contraceptive methods[g]

	Highly effective methods			Moderately effective methods				Least effective methods	
					Estrogen-containing				
	Female/male sterilization	IUD[a,b]	Implant[b] (Arm)	Injection ('Depo')	Pill	Patch	Ring	Female/male condom	Diaphragm
Efficacy (*Pregnancies per 100 women in first year of use*) [55,65]	Less than 1 out of 100			4 out of 100	7 out of 100			Female: 21 out of 100 Male: 18 out of 100	12 out of 100
Schedule and use	Permanent	Copper: Lasts ≥12 years [66] LNG[c]: Lasts up to 7 years [26]	Lasts up to 5 years [24]	Shot every 3 months[e]	Daily	Weekly	Monthly	Use with sex	
Return to fertility	Rare	Rapid[d]	Rapid[d]	10 months (median) [38▪▪]	Rapid			N/A	N/A
Benefits	Permanent	Convenient LNG: Lightens periods Copper IUD: No hormones	Convenient Lightens periods	Maximally discrete	Lightens periods	Lightens periods	Lightens periods	No hormones Reduces transmission of STIs[f] No prescription required	No hormones
Rheumatic disease considerations		Progestin-based treatments generally safe with active SLE, APS, thrombosis [20,21,23]			Contraindicated if active SLE, history of APS, or thrombosis [20,21,23]				
Possible side effects	Pain, bleeding, infection, surgical complications	Some pain with placement		Weight gain	Nausea, breast tenderness, spotting for first few months			Allergic reaction, irritation	
Other considerations	Permanent	Safe for immuno-suppressed and nulliparous women [23] Irregular periods possible Copper IUD: Heavier periods possible Requires placement by trained provider	Irregular periods Injection site reactions are rare Single rod etonogestrel implants require placement by trained provider	Bone mineral density transiently decreases [30]	Avoid if age≥35 years and cigarette smoking, severe hypertension, migraine with aura Avoid if history of breast or endometrial cancers, stroke or cardiovascular disease				
				May reduce risk of ovarian and uterine cancers May improve acne					

Reproduced with permission [59].
[a]IUD, intrauterine device.
[b]May be removed for any reason whenever desired.
[c]LNG, levonorgestrel (a progestin)
[d]Rapid return to fertility: Most women are able to become pregnant within 1–3 menstrual cycles after cessation of method [39].
[e]Subcutaneous injection can be self-injected, subcutaneous or intramuscular forms can be administered by provider.
[f]STI, sexually transmitted infection.
[g]Other methods include emergency contraception, contraceptive sponge, natural family planning, and withdrawal.

reasons, including convenience, side effects, discretion, and noncontraceptive benefits.

PREGNANCY TERMINATION

Although understudied, pregnancy termination appears to be safe for people with SLE and SS. A recent study found that among 284 people who underwent pregnancy termination, none experienced disease flares or complications requiring hospitalization [58].

OPTIMIZING FAMILY PLANNING COUNSELING

We recommend that clinicians should initiate family planning counseling with every reproductive-age patient with childbearing capacity regardless of gender, sexual orientation, race, and ethnicity. Rheumatologists should ascertain an individual's childbearing capacity (e.g., history of sterilization), pregnancy history, interest in and preferred timeline for future pregnancy, desire to avoid pregnancy, risks for unintended pregnancy, exposure to potentially teratogenic medications, contraception needs, and access to reproductive health providers.

We find that open-ended phrasing is helpful when initiating these conversations: 'Tell me a bit about your life and where pregnancy fits in your plans. I ask because I'd like to work with you to try to make sure things turn out the way you'd like them to' [59]. The Healthy Outcomes in Pregnancy with SLE Through Education of Providers website (HOPSTEP; www.lupuspregnancy.org) also presents language that rheumatologists may use to engage patients in shared decision-making around reproductive health. Patients appreciate when these conversations are initiated by the rheumatologists early and often during their clinical care [60].

Patients with SLE or SS who wish for pregnancy soon should be assessed by the rheumatologist for evidence of disease activity (Table 1). The rheumatologist should prescribe a prenatal vitamin, switch from fetotoxic to pregnancy-compatible medications, and initiate a discussion about the optimal timing of pregnancy. Pregnancy is rarely only a clinical decision for patients, and some patients will choose to pursue pregnancy even in cases of severe disease burden or active disease. Rheumatologists should refer patients to maternal-fetal medicine specialists for additional clarification of patients' pregnancy-associated risks and to facilitate individualized health screening and care (e.g., vaccination) prior to pregnancy.

Rheumatologists should also screen patients for risk factors for unplanned pregnancy. In one survey of reproductive-age women with SLE, over half of the respondents engaged in unprotected heterosexual sex [61]. In another study of pregnant people with SLE, 40% of the pregnancies were unplanned and were more likely to have been conceived during a period of active disease and culminate in adverse outcomes than planned SLE pregnancies [62▪▪]. Unplanned pregnancy is also an independent predictor of fetal loss among women with SLE [63]. Rheumatologists may wish to become cognizant of factors that contribute to unplanned pregnancies among people with SLE and SS, including patient and provider misperceptions about the safety of hormonal contraception; patients' underestimation of their own fertility potential; patients' preferences to avoid contraception (e.g., due to polypharmacy, unacceptable side effect profiles, or personal preference); conflicted thoughts and feelings about pregnancy; and lack of access to reproductive health providers [48,57,64]. Rheumatologists should also avoid prescribing medications with teratogenic potential to patients who are at risk for unintended pregnancy whenever possible. Rheumatologists may refer patients who do not desire pregnancy but are at risk for unintended pregnancy to primary care providers or gynecologists for family planning care. In the meantime, rheumatologists may advise patients to use emergency contraception if needed, which is safe for all patients with SLE and SS and is available over the counter or in online pharmacies.

CONCLUSION

Many people with SLE and SS may be reassured that they can experience healthy pregnancies. ART seem to be safe for people with well-controlled disease. Safe and effective contraceptive methods are available for patients who wish to avoid pregnancy. Finally, emerging data suggest that pregnancy termination is safe for people with rheumatic diseases. With the development of evidence-based resources by EULAR and the ACR, rheumatologists are well poised to help people with SLE and SS realize their reproductive goals and safely transition through their reproductive lives.

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Conflicts of interest

M.E.B.C. has received funding from Pfizer, Janssen, UCB Pharma for work unrelated to the current project; she is a

consultant for UCB and GSK; Duke University has reviewed independent medical education funding from GSK for lupuspregnancy.org. M.B.T. and K.P.H. do not have any other financial interests or conflicts of interest to disclose.

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Transcriptomics data: pointing the way to subclassification and personalized medicine in systemic lupus erythematosus

Erika L. Hubbard, Amrie C. Grammer, and Peter E. Lipsky

Purpose of review
To summarize recent studies stratifying SLE patients into subgroups based on gene expression profiling and suggest future improvements for employing transcriptomic data to foster precision medicine.

Recent findings
Bioinformatic & machine learning pipelines have been employed to dissect the transcriptomic heterogeneity of lupus patients and identify more homogenous subgroups. Some examples include the use of unsupervised random forest and k-means clustering to separate adult SLE patients into seven clusters and hierarchical clustering of single-cell RNA-sequencing (scRNA-seq) of immune cells yielding four clusters in a cohort of adult SLE and pediatric SLE participants. Random forest classification of bulk RNA-seq data from sorted blood cells enabled prediction of high or low disease activity in European and Asian SLE patients. Inferred transcription factor activity stratified adult and pediatric SLE into two subgroups.

Summary
Several different endotypes of SLE patients with differing molecular profiles have been reported but a global consensus of clinically actionable groups has not been reached. Moreover, heterogeneity between datasets, reproducibility of predictions as well as the most effective classification approach have not been resolved. Nevertheless, gene expression-based precision medicine remains an attractive option to subset lupus patients.

Keywords
bioinformatics, machine learning, precision medicine, systemic lupus erythematosus, transcriptomics

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease affecting multiple organ systems with significant morbidity and mortality primarily affecting women of childbearing age. The development of well tolerated and effective therapies has been challenged by heterogeneous disease presentation, resulting in numerous unsuccessful clinical trials. Over the past decade, there has been a considerable focus on gene expression profiling as a potential means of addressing clinical heterogeneity. The goal is to identify relevant disease endotypes that could be employed as the basis of individualized precision medicine.

On the basis of earlier suggestive results, more recent work has employed more sophisticated bioinformatics, machine learning, and artificial intelligence methodologies in an attempt to uncover hidden patterns in lupus transcriptomics and dissect patient clinical heterogeneity into subgroups with similar molecular pathogenesis. It is generally assumed that many genetic and genomic abnormalities can underlie the clinical lupus phenotype and that understanding the precise molecular pathways engaged in an individual patient is essential to foster precision and personalized medicine. Moreover, new and improved patient stratification is believed to be essential for successful clinical trial testing of potential new therapies, and transcriptomic-based patient sub-setting could be the basis of identifying patients for a clinical trial, and, thereby improve the

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KEY POINTS

- Transcriptomics has enabled increased insight into the pathogenesis of SLE but translation of these findings into clinical tools has been slow.
- Bioinformatics and machine learning approaches are useful in SLE patient stratification but proper deconvolution of transcriptomic effects from therapies and ancestry is needed.
- A global consensus of SLE endotypes has not been reached and is challenged by study design, analytic approach, and data availability.

likelihood of success. This review will highlight recent studies that use transcriptomic signatures as the basis for SLE patient subclassification and studies that otherwise attempt to further precision medicine in lupus.

ADDRESSING SYSTEMIC LUPUS ERYTHEMATOSUS HETEROGENEITY

The first real clue that lupus patients could be subsetted based on gene expression profiling emerged from analysis of the interferon (*IFN*) gene signature (IGS), a group of genes known to be induced commonly by type I IFN. Approximately 50–75% of SLE patients express the IGS [1–5]. IGS+ lupus patients initially were thought to have greater disease activity or severity but more recent studies have indicated that the IGS is persistent in individual patients despite immunosuppressive therapy, does not correlate well with disease activity as measured by the SLE Disease Activity Index (SLEDAI), is correlated with the presence of activated myeloid cells, and appears to be associated with the presence of anti-RNP and/or anti-DNA autoantibodies [1,6▪]. One group recently reported the stratification of lupus patients into IGS hi and lo in an Indian SLE cohort exhibiting renal and neuropsychiatric disease manifestations, although type I *IFN* gene expression persisted despite changes in disease activity [7▪]. Another group additionally carried out stratification of a diverse, multiethnic SLE patient cohort and noted that gene expression and co-expression profiles of *IL1RN* and *TNFSF13B* changed in coordination with the IGS longitudinally [8▪], suggesting IGS status might be informative for selection of therapy.

The IGS has also been used to distinguish SLE patients from healthy participants and from patients with other autoimmune, inflammatory, and infectious diseases. A 93-gene MetaSignature from blood transcriptomic data was developed and reported to persist across numerous tissues and cell types [9▪]. Of the 93-gene signature, most were members of the IGS, although 14 were characterized as 'noninterferon, nonneutrophil' and suggested to be previously underappreciated genes of therapeutic and diagnostic interest.

Indeed, other biomarkers in addition to the IGS have been identified, such as CD38, which demonstrated heterogeneous expression by peripheral blood immune cell subsets in a lupus cohort [10]. This supports the contention that gene signatures other than the IGS could be assessed for their capacity to sub-set lupus patients into meaningful endotypes.

EFFORTS TOWARDS SYSTEMIC LUPUS ERYTHEMATOSUS PATIENT STRATIFICATION

Machine learning and artificial intelligence are commonly used methodologies to improve precision medicine outcomes in lupus and other autoimmune diseases, especially in studies involving high-throughput sequencing data [6▪,11–13]. Xing *et al.* in 2021 [14▪] employed a comprehensive approach combining transcriptomic data with clinical risk factors of atherosclerosis to build a predictive risk model in Chinese patients with SLE. Using differential gene expression analysis of RNA-seq data from blood, LASSO and multivariate logistic regression were employed to build a probabilistic model of the risk of atherosclerosis in SLE patients based on the expression level of the *KRT10* gene, age, and the presence of hyperlipidemia. This model exhibited reasonably good performance with an Area Under the Curve (AUC) in ROC analysis of 0.922 and sensitivity and specificity of 85 and 87.2%, respectively. The model could provide benefit to predicting atherosclerosis in Chinese SLE, particularly in asymptomatic patients. However, limitations of this study include the dependence on variance-stabilized, transformed raw count data for the single gene, *KRT10*, identified by RNA-seq and included in the model. Whether inclusion of a single gene will be sufficient to permit this model to perform in other clinical settings remains to be determined.

Several recent studies have turned to cell type-specific gene expression profiles to glean useful information that can be applied to SLE patient stratification. Employing a classic bioinformatic approach, Andreoletti *et al.* (2021) [15▪▪] utilized unsupervised k-means clustering, differential expression analysis, gene network co-expression analysis, and random forest classifiers to reveal immune cell-specific transcriptomic signatures and to improve SLE patient stratification into more clinically actionable groups based on the inclusion of multiple ethnicities. Using

bulk RNA-seq data derived from sorted immune cells from the blood (CD14+ monocytes, CD19+ B cells, CD4+ T cells, and NK cells) in SLE patients of Asian Ancestry (AsA) and European Ancestry, the analysis yielded three distinct groups of patients based on CD4+ T cells and CD14+ monocytes and two groups each of B cells and NK cells, with individual clusters correlating differently and significantly with ACR classification criteria for SLE. Although the authors hoped to improve upon previous subclassification studies by accounting for ethnic diversity among lupus patients, they largely focused on two ancestral groups (European Ancestry and AsA), and much of their results from k-means clustering, differential expression, and co-expression analyses highlighted potential differences in SLE pathogenesis between AsA and European Ancestry patients. Ultimately, they demonstrated a machine learning classifier of reasonable predictive value (AUC = 0.8) using CD4+ T-cell or CD19+ B-cell transcriptomic data that could categorize SLE patients as having high or low disease activity, if of European or Asian ethnicity. Whereas the authors accounted for possible transcriptomic changes contributed by patient medications and sex, most of the differences between the groups were modest. One of the CD4+ T-cell clusters positively correlated with SLEDAI score, whereas another CD4+ T-cell cluster and one CD19+ B-cell cluster positively correlated with Systemic Lupus International Collaborating Clinics (SLICC) classification score. Like other studies of this nature, the authors suggest an unbiased stratification of SLE patients that identifies distinct molecular pathways underpinning disease but do not validate their cell type-specific patient clusters in another dataset.

Nehar-Belaid *et al.* [16▪▪] in 2020 dissected the heterogeneity of cell subpopulation abundance and immune cell gene expression from scRNA-seq data from childhood SLE (cSLE) patients and healthy controls. They applied hierarchical clustering to the cSLE patients and healthy controls based on the frequency of identified scRNA-seq subclusters. Samples were resolved into six clusters, although the precise mechanism or definition of cluster identification was not identified. The resultant subgroups capture some clinical heterogeneity in addition to transcriptomic heterogeneity, and importantly, identify a subgroup of lupus patients that cluster with healthy controls. The authors claim that leukocyte blood gene expression profiles were similar between cSLE and adult SLE (aSLE) patients that were also analyzed by scRNA-seq but only a small number of adult participants were examined, and the data showed differentially expanded immune cell subsets between adults and children, and primarily, shared expression of the IGS. Nonetheless, they repeated hierarchical clustering with the adult and cSLE combined cohort and found four subgroups of patients, in which different scRNA-seq immune cell subclusters were enriched. Three out of the four groups were constituted strictly of SLE patients, with cSLE patients broadly clustering together and one group containing healthy controls, cSLE patients, and aSLE patients, once again indicating a subset of SLE patients with transcriptomic profiles similar to those of controls. Overall, patient stratification was accomplished and clinical variables including medication, disease activity, and ancestry were annotated accordingly but further deconvolution and validation of these patient groupings are required.

Lopez-Dominguez *et al.* [17▪▪] in 2021 also revealed a subset of SLE patients that appear to have features of healthy controls. In independent adult and pediatric cohorts, they stratified SLE patients into two subgroups each based on inferred enrichment of transcription factor activity. In each case, the group that clustered with healthy controls contained samples with increased percentages of lymphocytes and decreased percentages of neutrophils compared with the other cluster. This group also had similar neutrophil-to-lymphocyte ratios (NLRs) to those of healthy controls, whereas the other subgroup of SLE patients they identified had higher NLRs. This work largely corroborated the authors' previous studies suggesting NLR as a biomarker for patient stratification and improving drug selection and responsiveness [18–19].

Building upon previously published blood transcriptional modules to characterize and stratify cSLE [20], Guthridge *et al.* [21▪▪] in 2020 carried out subclassification of aSLE patients by analyzing blood gene expression profiles obtained by RNA-seq. As previously reported [20,22–24], immune pathway-related modular scores were calculated from their gene expression data of a diverse, multiethnic adult lupus cohort and were correlated to one another and to measured soluble factors. Using the co-expression scores and soluble mediator levels, the authors applied unsupervised random forest and k-means clustering to arrive at seven subgroups. Despite the unconventional bioinformatic pipeline, stratification of SLE patients into subsets appeared to be accomplished. However, the end goal of arriving at clinically actionable groups was only modestly achieved. Only a few subsets were distinct in their transcriptomic profiles and the clinical variables of each cluster were statistically similar, apart from antidsDNA and complement status. However, when patients were stratified by these two variables, there were no notable distinctions in molecular phenotypes. Different ancestries, involvement of specific

organ systems, and history of involvement of specific organ systems were represented in patients from multiple subsets, indicating intracluster and intercluster heterogeneity. Rather than dissect a diverse group of SLE patients into homogeneous subgroups, Guthridge *et al.* [21▪▪] appear to have mapped lupus heterogeneity into seven patient clusters. Given that medication use differed somewhat between these clusters, some of the different molecular profiles observed could be related to medication effects. The authors discuss these limitations and suggest additional studies to elucidate such modulators of gene expression. Comparing these results with those of Nehar-Belaid *et al.* [16▪▪], who demonstrated multiple immune cell phenotypes with different transcription profiles, with distinct subsets contributing to the IGS, it seems very likely that some of the heterogeneity of lupus patients can be attributed to several of the factors mentioned by Guthridge *et al.* [21▪▪] – differences in distributions of immune cell populations, differences in active immune pathways and levels of activation of these pathways, and altered transcriptional regulation within cells.

Applying the same bioinformatic/machine learning pipeline as Guthridge *et al.* [21▪▪], Zhu *et al.* [25▪▪] analyzed whole blood transcriptomes of patients with cutaneous lupus erythematosus (CLE) and observed six patient clusters. Differences in ancestry, CLE subtype, and cutaneous lupus activity severity index damage score were notably different between clusters; however, few differences could be observed in transcriptional modular scores. One cluster marked by patients with lymphopenia was increased in the IGS, cell death, and apoptosis signatures, as well as neutrophil and LDG signatures. Another cluster was enriched in inflammation modules and cell death. Likely there were too few patients analyzed in this study ($n = 62$) for a total of six clusters to be more than suggestive.

CHALLENGES TO SYSTEMIC LUPUS ERYTHEMATOSUS PATIENT STRATIFICATION

Although many studies discussed herein have employed conventional and unconventional bioinformatic techniques to uncover hidden patterns and molecular signatures contributing to stratification of SLE patients, lack of understanding of the influence of background gene expression signatures may limit the utility of these approaches. A study by Catalina *et al.* [6▪] provided a thorough analysis of gene expression and clinical metadata and how the two interrelate, suggesting that deconvolution of transcriptome data may improve SLE patient stratification. Specifically, this study revealed that patient ancestry, standard of care (SoC) therapy, and serologic profiles contribute to gene expression heterogeneity in SLE (Fig. 1). The authors delineated patient ancestry-specific gene expression signatures in SLE based on 34 transcript modules largely characteristic of immune cells and processes and found significant changes in 23 of the 34 modules relating

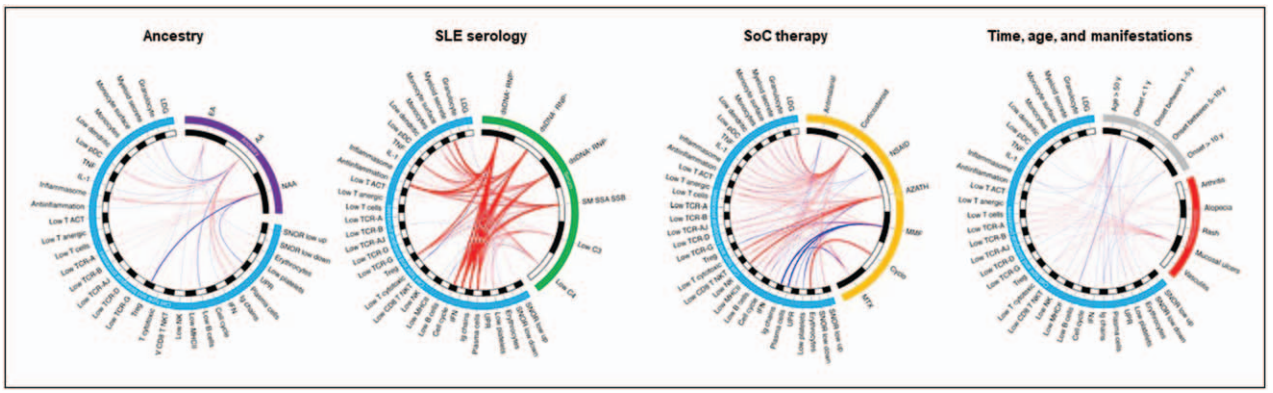

FIGURE 1. Stepwise logistic regression analysis determined the importance of ancestry, standard-of-care drugs, and Systemic Lupus Erythematosus Disease Activity Index components to the gene expression profile. Circos visualizations of odds ratios (OR) using stepwise logistic regression analysis for ancestry, serology, SoC drug, and time from onset of disease, age older than 50 years, and SLE manifestation to GSVA categories with *P* less than 0.05. The thickness of the lines from the 26 variables to the GSVA categories represent the magnitude of the ORs. An interval graph was used to assign thickness of the lines where OR less than 2, 1 pt; $2 \geq OR < 3$, 5 pt; $3 \geq OR < 10$; 10 pt; OR at least 10, 20 pt. Red lines indicate OR above 1, and blue lines indicate OR below 1. OR between 0 and 1 are represented as 1/odds ratio to accurately reflect the magnitude of the negative relationship to the GSVA enrichment score. Reproduced with permission from Catalina *et al.* [6▪]. GSVA, gene set variation analysis; SoC, standard-of-care.

to ancestry among African Ancestry, European Ancestry, and Native American Ancestry SLE patients. Logistic regression and machine learning approaches were utilized to distinguish an African Ancestry-unique SLE gene signature, and characterization of this signature revealed perturbations in B-cell signaling. Importantly, the abnormalities in African Ancestry SLE gene expression reflected those in normal African Ancestry not only emphasizing the importance of ancestry-matched controls in studies of SLE but also indicating that ancestry-specific genetic influences may contribute not only to the risk of lupus but also to the specific molecular pathways perturbed. It is notable that the enhanced B-cell activity in African Ancestry lupus was associated with the tendency to produce multiple autoantibodies, including anti-RNP and anti-Smith. It is also of interest that time since disease onset, age, sex, and most clinical manifestations were the variables with the fewest associations with gene expression. Notably, this study also revealed that individual clinical manifestations used to calculate SLEDAI did not confer strong relationships to gene expression profiles. This suggests that clinical assessment of SLE may be less informative than gene expression-based tools to subset lupus patients into meaningful groups.

Another challenge that arises from efforts to stratify SLE patients and translate the results into clinical utility is the heterogeneity of the studies themselves as emphasized in Fig. 2, impeding arrival at global consensuses. The majority of recent transcriptomic-based SLE stratification studies employ machine learning algorithms and focus on delineating specific cell types implicated in disease pathogenesis, followed by studies focusing on patient ancestry. Many studies have characterized resultant subgroups based on SLEDAI or implemented a SLEDAI cutoff as inclusion/exclusion criteria for downstream analyses. Interstudy subgroup comparison is, therefore, challenged by definitions of low or high disease activity, where some studies use SLEDAI = 4 as the cutoff [16▪▪,21▪▪] and others use SLEDAI = 6 [6▪,15▪▪], yet even others create their own definitions for 'high' versus 'low' disease activity [14▪,17▪▪].

Other differences include the use of control participants. Several studies have used normal, healthy participants as comparators to arrive at molecular profiles as the basis for patient subclassification. In other cases, non-SLE disease samples are used. Guthridge *et al.* [21▪▪] compared lupus transcriptomes to nonautoimmune, rheumatic disease patients before calculating gene co-expression modular scores. Differences in data availability and study design, therefore, impose challenges to SLE patient stratification efforts and comparison of results.

Finally, when patient stratification is achieved, there is difficulty in appropriate interpretation of the features of these subgroups and comparison to subgroups in a validation cohort. Some studies rely on qualitative observations [16▪▪,21▪▪] and/or fail to validate patient clusters in an independent cohort [15▪▪,17▪▪,21▪▪].

TOWARDS PREVENTIVE MEDICINE

Efforts have also been made to uncover transcriptomic signatures that may protect against the development of autoimmunity in antinuclear antibody (ANA)+ healthy participants. Slight-Webb *et al.* [26▪▪] immunoprofiled European Ancestry and African Ancestry participants with SLE, ANA+ without diagnosis of an autoimmune, rheumatic disease, and ANA− healthy controls through RNA-seq of

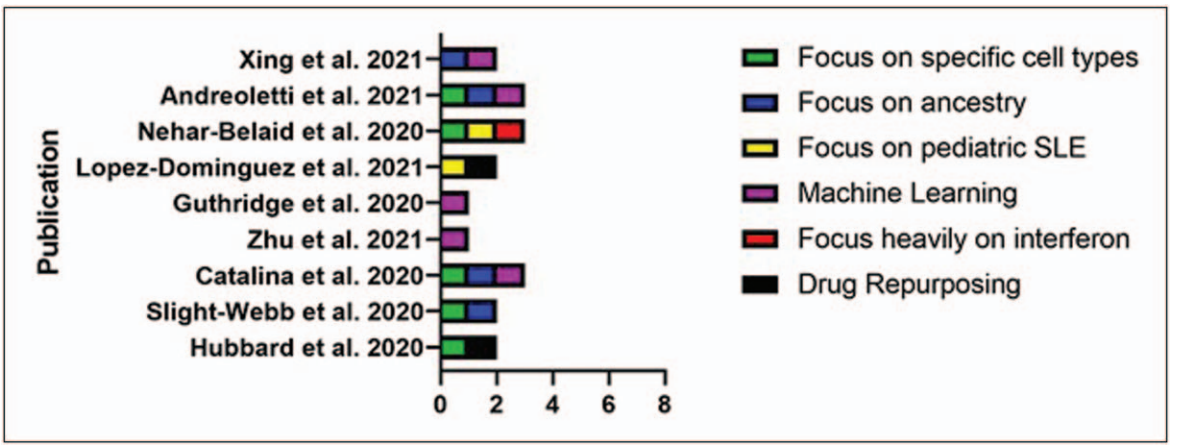

FIGURE 2. Summary of featured content of articles reviewed. A categorical breakdown of the major focuses of the transcriptomic SLE studies reviewed herein. SLE, systemic lupus erythematosus.

sorted immune cells from the blood, mass cytometry, and serology. Under the presupposition that European Ancestry participants are less at risk of developing SLE than African Ancestry participants, the authors describe an 'immune suppressed' phenotype in European Ancestry ANA+ healthy people characterized by lower numbers of T cells, NK cells, autoimmunity-associated B cells, dysregulation of T-cell signaling, decreased expression of the IGS and HLA genes, and increased expression of inhibitory and regulatory cell surface markers that may confer protection against SLE development. Although the incidence of SLE is higher in African Ancestry than European Ancestry populations, there are a greater number of variables that likely play a role in SLE development, such as genetics, and ancestry alone may not be relied upon to imply increased risk.

ADVANCES IN DRUG REPURPOSING

Personalized and precision medicine in lupus hinges upon the ability to treat individuals with the right therapy at the right time. Given the last three decades of many failed clinical trials in SLE and its organ-specific manifestations, coupled with the Food and Drug Administration (FDA) approval of only one biologic in over 60 years [27], some scientists and clinicians have turned to drug repurposing. Hubbard *et al.* [28▪] compared gene expression profiles from synovial biopsies of SLE patients with arthritis to the transcriptomes of rheumatoid arthritis and osteoarthritis patients. By upstream regulator analysis and query of the Library of Integrated Network-Based Cellular signatures (LINCS) database with SLE differentially expressed genes, the authors suggested several compounds that may prove beneficial to SLE patients with synovitis, including inhibitors of TNF, type I IFN, JAK signaling, and the NFκB pathway.

Lopez-Dominguez *et al.* [17▪▪] similarly sought drug repurposing possibilities using the significant transcription factors identified in their analyses. Using the cMAP-Linked User Environment (CLUE) database (previously LINCS), these authors matched their list of significant transcription factors to the drug targets of compounds in the database and identified drugs targeting PPARD, NFATC1, IRF3, and STAT1. These targets all had higher inferred transcription factor activity in SLE patients compared with controls.

CONCLUSION

The recent studies reviewed herein demonstrate the effect that, given transcriptomics data, numerous algorithms and computational methods are capable of stratifying SLE patients into subgroups. We highlight the caveat that the resultant subclassifications of SLE patients can only be as good as the data that was inputted into, and the design of, the classification pipeline, and that the robustness and reproducibility of the proposed SLE subclusters need to be evaluated and will likely depend on proper deconvolution of input transcriptome data to account for effects of ancestry and medications. Several groups have suggested different stratifications of SLE patients into endotypes with distinct clinical and demographic features; whether these purported subtypes are truly indicative of differences in disease mechanisms or underlying pathological processes remains to be seen, along with the translation of these results into clinically useful tools, a major challenge to precision medicine in lupus.

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:
- ▪ of special interest
- ▪▪ of outstanding interest

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This study is the first deconvolution of transcriptomic data of healthy and European Ancestry and African Ancestry SLE participants based on ancestry, serology, SoC medications, and other clinical variables including SLEDAI components. This study examines how these variables affect gene expression profiles and raises important considerations for future transcriptomic studies. Furthermore, it distinguishes an African Ancestry-specific SLE gene expression signature revealing perturbations in B-cell signaling.

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This work enabled a novel prioritization of gene targets in SLE via development of a novel bioinformatic method, an extension of weighted gene co-expression network analysis (WGCNA) termed multicell WGCNA, to interrogate RNA-seq data from 6 sorted blood immune cell subsets. This allowed identification of a co-expressed module of genes characterized by the IGS contributed by all six cell types, a cell type correlation associating T helper cell gene expression with B-cell response and myeloid cell expression of *TNFSF13B*.

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This work describes a patient stratification approach for AsA and European Ancestry lupus based on specific immune cell subsets from the blood, which could allow for precision medicine if these patient groupings are validated. Identified transcriptomic signatures that may contribute to SLE pathogenesis specific to ethnicity and disease activity status, suggesting pathways and candidate genes that may be of therapeutic interest.

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Examined transcriptionally distinct immune cell subpopulations that exhibit the IGS in both childhood and adult SLE. This work also stratified adult SLE, childhood SLE, and healthy controls into subgroups with heterogeneous clinical and transcriptomic profiles. It developed the groundwork for patient stratification efforts for further validation.

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This study describes immune and inflammatory mechanisms involved in the pathogenesis of lupus synovitis on an individual patient level and suggests drug repurposing candidates to alleviate synovial inflammation in SLE.

The role of CD8$^+$ T-cell systemic lupus erythematosus pathogenesis: an update

Ping-Min Chen and George C. Tsokos

Purpose of review
Systemic lupus erythematosus (SLE) is a serious autoimmune disease with a wide range of organ involvement. In addition to aberrant B-cell responses leading to autoantibody production, T-cell abnormalities are important in the induction of autoimmunity and the ensuing downstream organ damage. In this article, we present an update on how subsets of CD8$^+$ T cells contribute to SLE pathogenesis.

Recent findings
Reduced cytolytic function of CD8$^+$ T cells not only promotes systemic autoimmunity but also accounts for the increased risk of infections. Additional information suggests that effector functions of tissue CD8$^+$ T cells contribute to organ damage. The phenotypic changes in tissue CD8$^+$ T cells likely arise from exposure to tissue microenvironment and crosstalk with tissue resident cells. Research on pathogenic IL-17-producing double negative T cells also suggests their origin from autoreactive CD8$^+$ T cells, which also contribute to the induction and maintenance of systemic autoimmunity.

Summary
Reduced CD8$^+$ T-cell effector function illustrates their role in peripheral tolerance in the control of autoimmunity and to the increased risk of infections. Inflammatory cytokine producing double negative T cells and functional defects of regulatory CD8$^+$ T cell both contribute to SLE pathogenesis. Further in depth research on these phenotypic changes are warranted for the development of new therapeutics for people with SLE.

Keywords
CD8T cells, lupus, systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production with the presentation of inflammation and damage in multiple organs with serious life-threatening complications [1]. The cause of autoimmunity is multifactorial and various immune cell type abnormalities are involved in the pathogenesis of the disease (reviewed in [2]). Besides the aberrant expansion of autoreactive B cells, abnormalities in numerous T-cell subsets also contribute to the development of autoimmunity and systemic inflammation through direct action or cytokine production (reviewed in [3]). In this article, we focus on the role of CD8$^+$ T cells in SLE pathogenesis and review how each of the various subsets contributes to the progression of autoimmunity (Fig. 1).

CYTOTOXIC CD8$^+$ T CELL

Cytotoxic T lymphocytes (CTL), the most abundant type of CD8$^+$ T cells, are characterized by their cytolytic activity through the expression of perforin and granzymes, as well as cytokine production, including IFN-γ and TNF-α. With specific T-cell receptors recognizing foreign antigens presented by antigen presenting cells, such as dendritic cells or macrophages, naïve CD8$^+$ T cells differentiate into effector cells and expand exponentially. These expanded activated effector CTLs are cytotoxic against cells expressing nonself antigens – either virus-infected cells or cancer cells. After controlling the infection, the majority of these effector T cells undergo apoptosis, while a small portion of activated CD8+ T cells differentiate into memory T

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KEY POINTS

- Reduction in systemic CD8$^+$ T-cell effector function contributes to the induction of autoimmunity as a result of failure to remove autoreactive B cells, and the control of infections.
- IL-17-producing double negative T cells originate likely from autoreactive CD8$^+$ T-cell and promote systemic autoimmunity.
- Functional impairment in regulatory CD8$^+$ T cells contributes to disease pathogenesis.

cells, a resting state that can be promptly reactivated upon demand.

CD8$^+$ T cells from the peripheral blood of SLE patients frequently display a reduction in effector function, including attenuated granzyme B and perforin production [4]. The impaired cytolytic defect in CD8$^+$ T cells likely contributes to the pathogenesis of autoimmunity [5]. Genetic elimination of perforin production in lupus-prone mice results in accelerated disease progression and confirms the role of CTLs in halting autoimmunity [6]. These data likely suggest the role of CD8$^+$ T cells in the establishment and maintenance of peripheral tolerance, and defects in their cytolytic function results in failure to remove autoreactive B cells. In graft-vs.-host murine lupus models, both perforin-mediated and Fas-ligand-mediated cytotoxicity are required for optimal control of autoreactive B cells [7,8]. Enhancing cytolytic function of CTL suppresses B-cell autoreactivity and limits disease progression [9,10], but whether such treatment could apply to help reduce autoreactive B cells in patients remains to be investigated.

Reduced cytolytic function of CTLs in lupus patients also poses another unfavorable outcome in lupus patients, because this reduction of T-cell effector function correlates with higher risk of infection [11] including a defect in controlling latent Epstein–Barr virus (EBV) [12,13]. Prescription of immunosuppressive medications could explain part of these defects, but T-cell function is still greatly diminished even in those taking low doses of immunosuppressive drugs [14]. Further, lupus-prone mice are highly susceptible to infections [15]. Poor latent

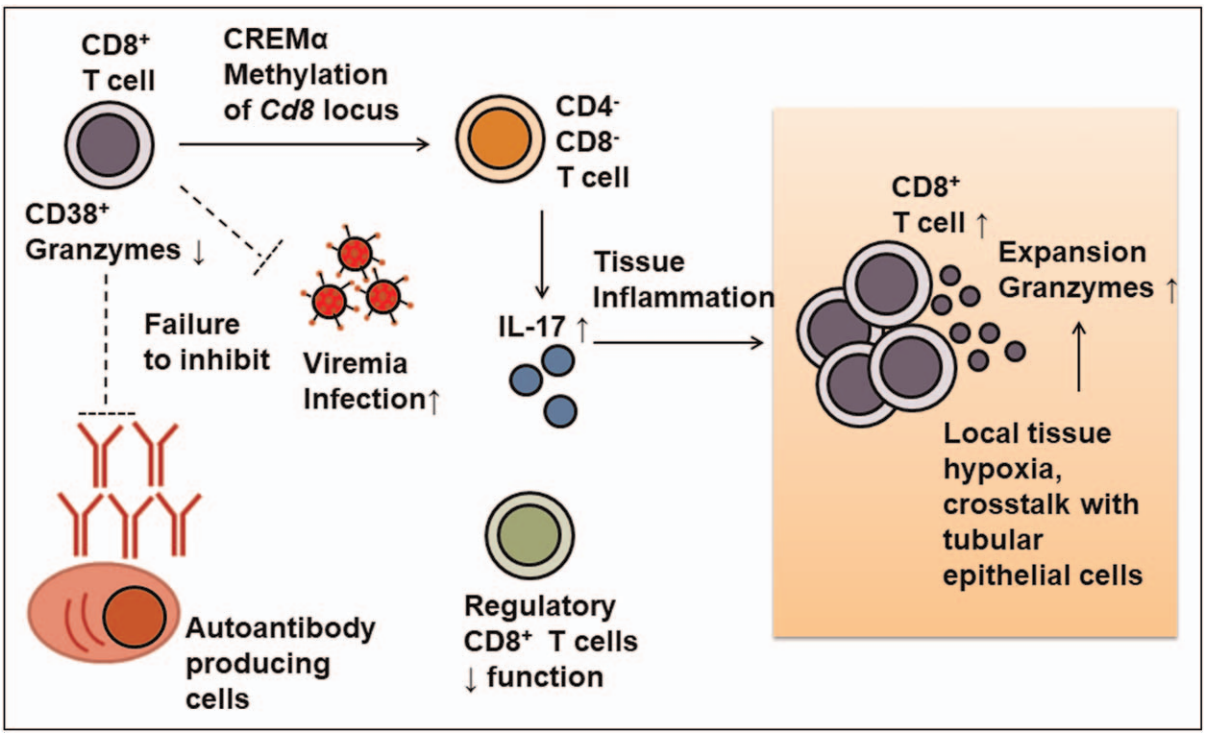

FIGURE 1. Immunopathology of various CD8$^+$ T-cell subtypes and contribution to pathogenesis. Reduced cytolytic function of systemic CD8$^+$ is related to the expanded CD38$^+$ subpopulation and correlates with the increased risk of infection. Functional defects of cytotoxic T cells also result in failure to remove autoreactive B cells and thus increase autoantibody producing cells. Functional defects in regulatory CD8$^+$ T cells also contribute to the loss of peripheral tolerance. Meanwhile, methylation of *Cd8* locus through cAMP-responsive element modulator α and the inflammatory milieu lead to generation and expansion of IL-17-producing CD4$^-$ CD8$^-$ double negative T cells. Tissue infiltrating CD8$^+$ T-cell cause tissue damage as a result of exposure to changes in tissue metabolic factors, such as hypoxia and arginine levels.

EBV control also poses another unfavorable effect on lupus pathogenesis, since molecular mimicry of latent viral protein EBV nuclear antigen-1 (EBNA-1) progressively contributes to the production of autoantibodies [16]. Consistent with the above findings, reduced EBNA-1 specific T-cell response correlates with higher disease activity measured by SLEDAI score in SLE patients [17]. The functional defects in lupus $CD8^+$ T cells are also linked to changes in the expression of several surface proteins. Signaling lymphocytic activation molecule family member 4 (CD244), also known as natural killer (NK) cell receptor 2B4 and thought to modulate cytolytic activity, is downregulated in $CD8^+$ T cells from SLE patients [18]. CD38, cell surface expressing cyclic ADP ribose hydrolase, is a major regulator of cellular NAD^+ levels through its catalytic function of NAD^+ to synthesize ADP ribose and cyclic ADP-ribose. Studies of tumor infiltrating lymphocytes reveal that the expression of CD38 represents an irreversibly dysfunctional group of $CD8^+$ T cells, which fail to recover cytokine production after ex vivo stimulation, and such fixed dysfunctional state of the $CD38^+$ $CD8^+$ T cell is strongly associated with a discrete chromatin landscape [19]. Similarly, dysfunctional $CD38^+$ $CD8^+$ T cells are also found expanded in the peripheral blood of SLE patients, with features of reduced granzyme and perforin production, and this functional defective state represents the result of CD38-dependent activation of histone methyl-transferase EZH2 in limiting chromatin accessibility of several key gene loci responsible for the regulation of T-cell effector function including RUNX3, EOMES and TBX21 [20▪▪]. Increased frequencies of these dysfunctional $CD38^+$ $CD8^+$ T cells correlate with a high risk of infection in SLE patients. Inhibition of CD38 could potentially reverse these adverse effects on CTL function. Administration of daratumumab, an anti-CD38 antibody, not only suppresses autoreactive plasma cells and reduces autoantibody titers, but also restored cytotoxicity of peripheral $CD8^+$ T cells in two SLE patients [21].

$CD8^+$ T CELL IN DAMAGED ORGAN

In SLE patients with class III or IV nephritis, $CD8^+$ T cells are one of the predominant infiltrating immune cells, and their accumulation in periglomerular areas correlates with the degree of disease activity [22]. These $CD8^+$ T cells presenting with effector memory phenotype are also found in the urine sediment, and are thought to contribute to tissue damage [23]. T cell receptor sequencing of infiltrating T cells shows clonal expansion and, interestingly, the same clonotype persists in the kidney years later in the repeat kidney biopsy tissue [24]. Taken together, these data suggest that the infiltrating $CD8^+$ T cells in nephritic kidney are tissue-resident and likely expand locally as disease exacerbates. However, whether this whole process involves antigen-specific T-cell response and the nature of the local autoantigen remains to be investigated. Several therapeutic approaches have been designed to target these tissue resident memory cells. The Janus kinase inhibitor tofacitinib not only blocks cytokine receptors necessary for aberrant T-cell activation, but also effectively prevents the expansion of tissue-resident memory T cells in murine lupus nephritis [25]. These memory $CD8^+$ T cells in nephritic kidneys also express high voltage-dependent Kv1.3 potassium channels, and targeted knockdown of Kv1.3 suppress CD40L expression and IFNγ production in a humanized mouse model of lupus nephritis [26▪]. These tissue resident memory suppressing therapeutics also improve the outcome of kidney damage in lupus-prone mice, suggesting the role of tissue-resident memory $CD8^+$ T cells in organ damage pathogenesis.

The nature of kidney infiltrating T cells is long thought to be functionally active and through their cytolytic function contribute to tissue damage. However, contrasting evidence by Tilstra *et al.* [27] surprisingly demonstrated near complete abolishment of the effector function in kidney-infiltrating of both $CD4^+$ and $CD8^+$ T cells. These data raises the questions about the functionality of kidney infiltrating T cells, and whether chronic antigen exposure can lead to exhaustion locally at the site of inflammation. However, single-cell RNA-sequencing of lupus nephritis biopsy samples did not reveal features of exhausted cell subsets, and the clusters of NK and $CD8^+$ T cells were noted to express high numbers of *GZMB* and *GZMK* transcripts [28▪]. These seemingly discrepant data likely suggest the complex nature of lupus, with the result of heterogeneous features of kidney infiltrating T cells. Another intriguing cause of T-cell functional changes may stem from tissue microenvironment as the result of tissue inflammation and damage. These alterations of in situ cues instruct phenotypic changes of infiltrating T cells, and could be used to selectively suppress the local inflammatory response. Local tissue hypoxia was found to result from organ damage in lupus nephritis followed by the subsequent activation of the transcription factor hypoxia-inducible factor-1 (HIF-1) which controls cell survival and adequate glycolytic metabolism required for effector function in kidney-infiltrating T cells [29▪]. These data indicate the therapeutic potential of HIF-1 inhibition to block microenvironmental cues to restore tissue infiltrating T-cell

functionality and reverse organ damage. Changes in local metabolite concentrations could also serve as an important route to control the crosstalk between resident tissue cells and infiltrating T cells. Renal tubular epithelial cells downregulate arginine degrading enzyme arginase 1 through the response of IL-23 receptor and calcium/calmodulin kinase IV (CaMK4). The resulting increase in free L-arginine promotes proliferation of infiltrating T cells, and targeted CaMK4 inhibition in renal tubular epithelial cells prevents the expansion of T cells locally [30▪]. These data suggest phenotypic difference between systemic and local $CD8^+$ T cells could arise from metabolic changes in the damaged tissue, and these phenotypic alterations could serve as potential therapeutic targets.

DOUBLE NEGATIVE T CELLS

Double negative T cells, a particular group of αβ T cells without surface expression of either CD4 or CD8, are thought to contribute to SLE pathogenesis as one of the major sources of IL-17 production [31]. Despite our limited knowledge about this population, current information suggests its derivation from self-reactive $CD8^+$ T cells in tissues expressing autoantigens [32]. Transcriptome analysis has confirmed gene expression profile similarities between double negative T cells and $CD8^+$ T cells [33], and double negative T cells share a skewed oligoclonal T-cell receptor repertoire with $CD8^+$ T cells in patients with autoimmune lymphoproliferative syndrome [34]. These data strongly suggest the possibility of lineage transition from $CD8^+$ T cells to double negative T cells, and this process involves downregulation of CD8 surface expression through cAMP-responsive element modulator α (CREMα)-dependent transcriptional silencing of *CD8A* and *CD8B* [35]. CREMα suppression of CD8 is mediated through histone methylation of the *Cd8* locus mediated by the recruitment of DNA methyltransferase 3a and histone methyltransferase G9a [36]. Double negative T cells are expanded in lupus mice carrying *lpr* or *gld* variants as disease progresses [37,38]. These murine lupus models have defects in T-cell apoptosis due to loss of function of the Fas mutation, and prevent activation induced cell death despite repeated T-cell receptor stimulation. These murine models are especially useful to depict the pathogenic role of double negative T cells, as T-cell receptor stimulation of $CD8^+$ T cells also promotes loss of surface CD8 expression to form double negative T cells [39]. Taken together, these double negative T cells in lupus arise likely from autoreactive $CD8^+$ T cells as a result of chronic stimulation by autoantigen presented from apoptotic cells [40▪▪]. Furthermore, changes of cytokines in the inflammatory milieu, including reduction of TGF-β and elevation of IL-23 also contribute to the loss of tolerance and expansion of IL-17 producing double negative T cells [40▪▪]. Double negative T cells can also provide aberrant B cell help to augment anti-DNA autoantibody production [41]. In addition to the murine models, these double negative T cells are highly expanded in the peripheral blood and inflamed kidneys of lupus patients, further indicating their pathogenic role in the development of systemic autoimmunity and organ damage [42]. In summary, unlike the regulatory double negative T cell that can inhibit activation and proliferation of antigen-specific $CD8^+$ T cells [43], double negative T cells in autoimmune diseases predominantly present with pathogenic and proinflammatory phenotypes. These phenotypic differences likely stem from the presence of autoantigens from apoptotic cells and cytokines that promote the inflammatory phenotype.

$CD8^+$ REGULATORY T CELLS

Numerous subsets of $CD8^+$ T cells, either natural or inducible, possess a cell suppressive activity similar to that of regulatory $CD4^+$ T cells. The natural $CD8^+$ $CD25^+$ T regulatory cells express high levels of Foxp3 and exert their inhibitory function mainly through the secretion of transforming growth factor β1 (TGF-β1) and contact-dependent suppression through the cytotoxic T-lymphocyte-associated antigen 4 [44]. $CD8^+$ T regulatory T cells can also be induced by continuous antigen stimulation with $CD14^+$ monocytes [45], or coculture with CD40 ligand-activated plasmacytoid dendritic cells [46], or CD40-activated B cells [47]. The suppressive activity of these inducible $CD8^+$ T regulatory T cells depends on IL-10 production [46,48] and TGF-β secretion [49].

The immune suppressive function of regulatory $CD8^+$ T cells can also alleviate autoimmunity in lupus. In lupus prone NZB × NZW F1 mice, the immune tolerance induced by administering artificial peptide pConsensus depends on the activity of TGF-β secreting $CD8^+$ T cells [49], and the inhibitory activity of these $CD8^+$ suppressor cells depends on the expression of the transcription factor Foxp3 [50]. $CD8^+$ regulatory T cells can have been reported during the induction of tolerance by nucleosomal histone peptides in lupus prone SWR × NZB F1 mice, and their suppressive function depended upon TGF-β secretion [51]. In another tolerogenic model induced by complementarity-determining region-1 peptide for NZB × NZW F1 lupus mice, Foxp3-expressing $CD8^+$ cells promoted the expansion

and suppressive function of $CD4^+$ $CD25^+$ regulatory T cells [52]. In addition to these tolerogenic models, ex-vivo-generated autologous $CD4^+$ and $CD8^+$ regulatory T cells help ameliorate autoimmunity in a graft-vs.-host model presenting with lupus-like syndrome [53]. Studies of peripheral blood mononuclear cells from SLE patients have reported that $CD8^+$ suppressive cells are functionally impaired with reduced IL-10 and TGF-β production [54,55]. In patients with refractory disease who receive autologous hemopoietic stem cell transplantation, immunological remission depends on the suppressive activity of TGF-β producing $CD8^+$ regulatory T cells [56]. These data imply the potential therapeutic exploitation of $CD8^+$ regulatory T cells in SLE patients, but the key to bolster their suppressive activity to induce immune tolerance remains to be investigated.

CONCLUSION

Reduced effector function of peripheral blood $CD8^+$ T cells promotes autoimmunity by failing to induce peripheral tolerance by removing autoreactive B cells, and by controlling the risk of infection. In contrast, at least part of the effector function of tissue $CD8^+$ T cells remain intact, and correlates with the extent of organ damage. The difference in effector phenotype likely stems from metabolic changes in the tissue microenvironment and from the crosstalk with tissue resident cells. IL-17-producing double negative T cells and dysfunctional regulatory $CD8^+$ T cell also contribute to disease pathogenesis. Recent studies have helped us understand better the immunopathology of $CD8^+$ T cell and their subsets but more information is needed for the development of new therapeutic strategies.

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There are no conflicts of interest.

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Immune checkpoints and the multiple faces of B cells in systemic lupus erythematosus

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Purpose of review
B-lymphocytes are crucial in the pathogenesis of systemic lupus erythematosus (SLE), including autoantibody production, antigen presentation, co-stimulation, and cytokine secretion. Co-stimulatory and co-inhibitory molecules control interactions between B and T cells during an inflammatory response, which is essential for an appropriate host protection and maintenance of self-tolerance. Here, we review recent findings about checkpoint molecules and SLE B cells including their potential therapeutic implications and experiences from clinical trials.

Recent findings
Most prominent checkpoint molecules involved in pathologic B and T cell interaction in SLE are CD40/CD40L and inducible co-stimulator/ICOSL, both also intimately involved in the formation of germinal centers and ectopic lymphoid tissue. Dysregulations of inhibitory checkpoint molecules, like programmed death-1/programmed death-ligand 1 and B- and T-lymphocyte attenuator have been suggested to impair B cell functions in SLE recently.

Summary
Accumulating evidence indicates that dampening immune responses by either blocking co-activating signals or enhancing co-inhibitory signals in different cell types is a promising approach to treat autoimmune diseases to better control active disease but may also allow resolution of chronic autoimmunity.

Keywords
B cells, CD40, checkpoint molecules, systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) patients have a characteristic peripheral B cell lymphopenia including decreased numbers of naive B cells but expanded plasmablasts, memory and transitional B cells (reviewed in [1]). Autoantibodies represent hallmarks of the disease which resulted in the consideration of antinuclear antibodies as entrance criterion of the recent classification [2] and participate in the induction and maintenance of tissue damage (reviewed in [3]). Consistent with the important role for B cells in SLE pathogenesis, the only new drug approved to treat SLE in decades, belimumab, targets B cells by blocking BLyS/BAFF, a survival cytokine with impact on transitional and plasma cells [4]. Nevertheless, independent of plasma cells producing autoantibodies, B cells contribute to the pathogenesis of SLE also through antigen presentation to T cells, formation of germinal centers (GC) and ectopic lymphoid tissue [5], production of pro- and anti-inflammatory cytokines [6] and co-stimulation [7]. In this context, B cell tolerance to autoantigens appears to be controlled (at least in part) by a balance of TLR7 and TLR9 signaling (reviewed recently [8]) clearly indicating their dual role in autoimmunity. In addition, certain checkpoint molecule interactions appear to provide distinct roles for B cells during acute immune reactions but may also control chronic maintenance of autoimmune memory. Thus, better understanding of these B cell functions may hold promise to resolve chronic autoimmunity [9].

Co-stimulatory and co-inhibitory signals regulate the interaction between B and T cells during an

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KEY POINTS

- B and plasma cells can also regulate the immune responses by engaging checkpoint molecules.
- Naïve SLE B cells are able to shape the immune responses via ICOS-L by differentiation of inflammatory Th subsets.
- Dysregulation of co-inhibitory molecules (PD-1/PD-L1 and BTLA) have been recently described in SLE B cells suggesting a possible role in breakdown of B cell tolerance.
- Blocking co-activating signals or enhancing co-inhibitory signals in different cell types is a promising approach to treat autoimmune diseases.

inflammatory response, which is crucial for an appropriate host defense and maintenance of self-tolerance. In this context, the two-signal model proposes that full activation of naïve B cells requires for their activation, maturation and function engagement of the B cell receptor (BCR, Signal 1) and also second signals, such as ligand–ligand interaction or cytokines for B cell survival [10,11]. The complex multifactorial etiology in SLE results in breakdown of immune homeostasis and self-tolerance with activation of autoreactive T and B cells and impaired function of co-stimulation and co-inhibition [12]. The main immune checkpoint molecules with impact on SLE pathology and the corresponding pathways are summarized in Fig. 1. Co-stimulatory and co-inhibitory pathways that affect formation of GC and ectopic lymphoid tissue, such as the CD40 and inducible co-stimulator (ICOS) pathways, are relevant for acute inflammation in SLE although their role during chronic autoimmune phases remain to be delineated (as reviewed elsewhere [13]). Thus, these pathways have been recognized as potential therapeutic targets in the treatment of SLE, including proof of concept studies blocking CD40/CD40L in other autoimmune diseases [14–19].

Although aberrant co-stimulation appears to promote induction and acute inflammation of SLE, co-inhibitory pathways are possibly required to limit and control chronic autoimmune reaction, although less clinical proof is available with regard to inhibitory signals. Lessons from immune checkpoint blockade in cancer patients reveal multiple immune-related adverse events (IRAEs), mimicking autoimmune diseases along with changes in the B cell compartment and autoantibody formation [20]. Observations in cancer treatment blocking PD-1 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) suggest their role in breakdown of B cell tolerance. In line with this, recent data about dysfunctional inhibitory molecules, PD-L1 and B- and T-lymphocyte attenuator (BTLA) in SLE B cells underline defective inhibitory checkpoint and immune balance as part of autoimmune pathogenesis. In contrast to enhanced co-stimulatory pathways signaling employing NF-κB and TNF receptor-associated factors (TRAF), most inhibitory molecules function via phosphorylation of cytoplasmic immunoreceptor tyrosine-based

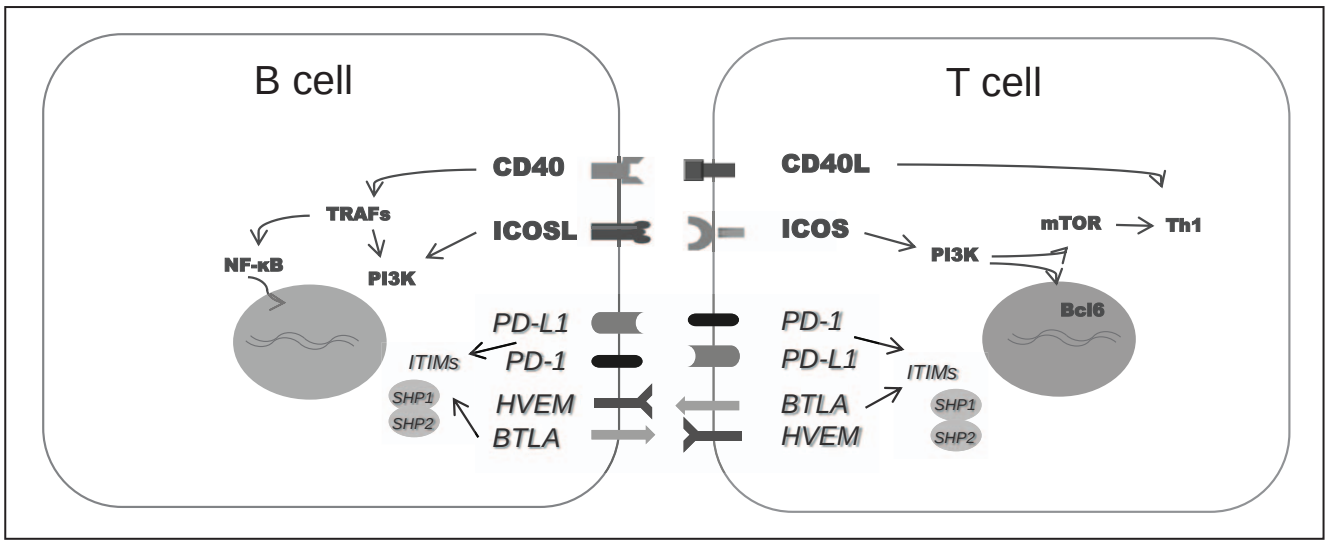

FIGURE 1. Main immune checkpoints on B and T cells with their key intracellular signaling pathways with implication in SLE pathology. Co-stimulatory axes (bold) promote B and T cell activation, while co-inhibitory molecules (italic) limit inflammatory responses. TRAF, TNF receptor associated factors; PI3K, phosphoinositide 3-kinase; NK-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SHP, Src homology region 2 domain-containing phosphatase; mTOR, mammalian target of rapamycin; Bcl6, B-cell lymphoma 6 protein; ITIMs, immunoreceptor tyrosine-based inhibitory motif.

inhibition motifs (ITIM) (Fig. 1). Protein tyrosine phosphatases, which include receptor type and non-receptor type intracellular phosphatases, play a significant role in autoimmunity [21] and have been found to be associated to counterbalance the immune response toward an inhibitory or anergic postactivated status (APA). There was no association between the functional and phenotypic characteristics of APA B cells and lupus activity suggesting that the findings reflect chronic or underlying autoimmune findings. A key mechanism to overcome this condition of B cells in autoimmunity is CD40 activation [21].

STIMULATORY CHECKPOINT MOLECULES AND PATHOGENIC SYSTEMIC LUPUS ERYTHEMATOSUS B CELLS

Several co-stimulatory pathways are involved in the development of lupus, with a particular focus on T cells [22]. Regarding B cells, the key co-stimulatory molecules (CD40, ICOS) are involved in GC and ectopic lymphoid tissue formation [23,24] along with aberrant autoantibody formation in SLE. T follicular helper cells (Tfh) have a central role in regulating B-cell selection by sensing the density of major histocompatibility complex peptide antigen complexes and influence costimulatory activity through CD40L and ICOS including appropriate IL-21 production. Whereas CD40/CD40L is important for the initiation of GC response, ICOS/ICOSL interaction appears to provide bi-directional signals: for maintenance and functional maturation of GC B cells [25] but also regulating inflammatory Th differentiation [26▪▪]. Beside GC formation, in SLE we observe increased expansion of so-called atypical memory B cells, like CD27-IgD- CXCR5-CD11+ Tbet+ (DN2) cells, which likely derive from extrafollicular activation and differentiation [5]. The induction of these subsets appears to be less dependent on T cell interaction but rather by TLR signaling, predominantly TLR7 and TLR9 with possibly counterregulatory effects (as reviewed recently [8]). In this context, the relation between these TLR signaling pathways and conventional CD40 and ICOS ligation, respectively, remains to be further delineated.

CD40–CD40L (CD154) axis

The CD40–CD40L (CD154) interaction is crucial for T and B cell interaction, where mutations within these molecules result in hyper-IgM syndrome and typically lack the formation of classical GCs. The latter requires interactions between CD40L highly expressed on TfH cells with CD40 on B cells. In this context, B cell activation, proliferation, and initiation of immunoglobulin isotype switch are critically dependent on proper CD40–CD40L stimulation. In lupus patients, CD40L has been found to be also expressed on B cells [27] together with upregulated expression of CD40 on B cells in lupus kidney biopsies [28].

Most mature SLE B cells regardless of their differentiation stage or disease activity, are in an APA status, characterized by hyporesponsiveness to BCR and TLR9 stimulation [21,29,30] as well as diminished cytokine production [31]. Interestingly, postactivation in SLE B cells can be functionally restored by signaling through CD40, which appears to be a key checkpoint molecule controlling APA B cells [21]. In line with this and responses in immune thrombocytopenic patients [18], initial treatment with an anti-CD40L antibody in lupus nephritis resulted in timely improvements, abrogated anti-DNA antibody production [14] and simultaneously normalized several peripheral B cell abnormalities [32]. However, the occurrence of thromboembolic events prevented further studies at that time, but second-generation anti-CD40L antibodies using an engineered/glycosylated Fc portion (NCT02804763, Dapirolizumab [15,33]) and additional anti-CD40 antibodies (NCT03656562) are currently in clinical trials for SLE and Sjögren's [34].

Inducible co-stimulator/ICOSL pathway

ICOS, which is recognized as a member of the CD28 family serves as a co-stimulatory receptor of T cells that is essential for their activation and can further promote downstream humoral immunity [35]. Noteworthy, ICOS signaling induces Tfh differentiation in GCs [36], facilitating GC formation, B cell maturation and IgG production. The ligand for ICOS, ICOS-L is constitutively expressed on all professional antigen-presenting cells, including B cells [37]. In SLE, there are increased levels of ICOS expressing CD4 and CD8 T cells, whereas downregulation of ICOSL was found particularly on SLE memory B cells, suggesting recent interaction of B cells with ICOS+ T cells [38]. In this regard, plasma cells were identified in the vicinity of ICOS+ T cells in lupus nephritis, indicating the involvement of ICOS–ICOSL interaction within targeted organs.

Interestingly, ICOSL expressing naïve B cells were recently described to trigger inflammatory memory Th subsets [26▪▪]. After interacting directly with ICOSL+ B cells, T cell receptor (TCR)-primed memory Th cells differentiate into pathogenic inflammatory Th subsets via mammalian target of rapamycin and glycolysis, creating conditions similar to chronic inflammation. In SLE consistently, memory T cells spontaneously differentiate into inflammatory Th subsets after contact with ICOSL+ B cells.

Thus, B cells employing certain checkpoint molecules, i.e., ICOSL are able to shape immune responses not only by antigen-experienced subsets, but also via naïve subpopulations. This observation is notable as the instruction of T cells appeared to be independent of B cell memory and as such appear as a potential 'innate' predisposition. Alternatively, they may belong to the group of atypical memory B cells, lacking classical memory phenotype markers. Anyhow, modulation of ICOS/ICOSL interaction may represent a mechanism that controls the inflammatory response (actual in clinical trials NCT00774943) not only via GC but also extrafollicular responses. The nature of these naïve B cells exerting their immune activity via ICOS-L in SLE needs to be further delineated.

A hitherto unanswered question is if targeting the two above discussed activatory checkpoint pathways will not only control active disease but also intervene chronic autoimmunity in the long term.

INHIBITORY CHECKPOINT MOLECULES ARE DYSREGULATED IN SYSTEMIC LUPUS ERYTHEMATOSUS B CELLS

During the last decade, multiple regulatory responses with a particular focus on limiting hyper-activation and inflammatory reactions were found in autoimmune diseases. Regulatory B cells have been initially identified by their production of the anti-inflammatory cytokine IL-10 [6,39–42]. Recently it became evident that regulatory B cells can also act through IL-10-independent mechanisms, such as IL-35 production or other mechanisms, like expression of inhibitory immune checkpoint molecules [6,43,44].

Immune checkpoint blockade in cancer patients improved substantially morbidity and mortality, but carry an increased risk of developing multiple IRAEs, mimicking various autoimmune diseases [45]. Even though the most effects are related to T cell activation, several changes in the B cell compartment along with autoantibody formation have been described in melanoma patients under anti-CTLA-4 and anti-PD-1 treatment alone or in combination [20]. In these instructive cases, there was a decline of total circulating B cells especially under combined therapy. On the other hand, plasmablasts and CD21lo B cells were increased and showed a greater clonality. Moreover, changes in B cells preceded and correlated with both the frequency and timing of IRAEs, suggesting an early breakdown of B cell tolerance due to checkpoint blockade.

Although enhancing the co-inhibitory molecule CTLA4 via abatacept is an approved treatment in rheumatoid arthritis, it failed to induce a robust remission in several randomized controled trials of SLE [46]. Even though additional mechanistic studies of CTLA4 and its ligands during initiation and maintenance may improve our understanding in SLE, the intrinsic activation of this inhibitory pathway may have only limited potential to control established SLE.

Programmed death-1/programmed death-ligand 1 axis

The programmed death 1 (PD-1) pathway is one of the best studied immune checkpoints in SLE, mostly regarding T cells [47]. PD-1, member of the CD28 superfamily, and major inhibitory receptor is expressed mainly by activated lymphocytes. Ligation of PD ligand 1 (PD-L1) or 2 (PD-L2) induces the activation of an ITIM in the PD-1 cytoplasmic tail, which inhibits activation sequences contained in the immunological synapse [48]. After upregulation of PD-1 and PD-L1 on human B cells in vitro via stimulation with CpG and CD40L, activated B cells gain regulatory functions and are able to suppress T cell proliferation [49].

PD-1 polymorphisms have been reported to be associated with susceptibility to SLE [50]. Increased PD-1 expression was characteristic of lupus B and T cells [51,52], particularly on naïve and switched memory B cells [52]. Interestingly, upon stimulation with anti-BCR, CpG, and CD40L the capacity of SLE B cells to up-regulate PD-L1 expression is markedly diminished, along with reduced B cell proliferation. PD-L1+ B cells are supposed to shape the inflammatory response especially by interaction with PD-1+ Tfh cells, reducing T cell recruitment into the follicle and down-regulating humoral immune responses [53]. Moreover, PD-L1/PD-1 interactions increase the stringency of GC affinity maturation [44]. A diminished upregulation of PD-L1 by SLE B cells may be involved in the outgrowth of low-affinity or irrelevant antibodies in SLE, defective selection and consequently initiation and possibly most importantly perpetuation of autoimmunity as increased expression of PD-1 on B and T cells is independent of lupus disease activity.

B cells expressing B- and T-lymphocyte attenuator (BTLA)

BTLA is constitutively expressed in B and T cells and can negatively regulate both, TCR and BCR signaling in vitro [54]. The ligand of BTLA, herpesvirus entry mediator (HVEM) is expressed on resting T cells and on naïve and memory B cells [55]. BTLA is also a member of the CD28 superfamily, similar to PD-1 and CTLA-4 in terms of its structure and function [56]. Upon activation, tyrosine domains of the ITIMs are phosphorylated and lead to the recruitment of the Src-homology domain 2 (SH2)-containing

phosphatases, SHP-1 and SHP-2, mediating immunosuppressive effects [57]. BTLA/HVEM ligation results into reduced activation of BCR downstream signaling molecules [54] and can suppress proliferation, cytokine secretion, and co-stimulatory molecule upregulation in B cells [58].

In SLE, recent studies found reduced BTLA expression on CD27-IgD+ naïve B cells which correlated with antidsDNA antibody titers and type I interferon signature [59▪]. In healthy controls, BTLA engagement was found to control CpG/TLR9 induced B cell memory differentiation and plasmablast formation, but was impaired in SLE B cells. This lack of immune control may contribute to the characteristically enhanced plasmacytosis in SLE patients, followed by autoantibody formation and differentiation of atypical memory B cells via TLR driven extrafollicular activation. In this context, PB precursors seem to originate not only from antigen-experienced memory B cells, but also from naïve SLE B cells with impaired BTLA function. Interestingly, inhibition of SYK could mimic the effects of BTLA activity in vitro, suggesting that SYK inhibition may hold promise to overcome abnormalities of BTLA in SLE. Expression of BTLA on certain B cell subsets did not show any relation to lupus activity [59▪]. This shared characteristic with PD-1 as another co-inhibitory checkpoint molecule permits the conclusion that they are involved in chronic immunity.

CONCLUSION

Even though SLE is considered a B cell disease, their role including the relevance of their certain differentiation stages (naïve, transitional, memory, atypical, plasmablasts, plasma cells) in the induction and maintenance is far from complete. Certain co-inhibitory pathways in SLE remain to be fully delineated but possibly control chronic autoimmunity. Co-stimulatory signals, such as CD40 and ICOS appear to be involved during active phases, including GC and ectopic lymphoid structure formation with corresponding B cell activation. Recent data found that naïve SLE B cells are able to shape the immune responses via ICOS-L by differentiation of inflammatory Th subsets escaping immune selection.

Co-stimulatory pathways employing intracellular signaling through NF-κB and TRAF gained interest as therapeutic targets, whereas inhibitory molecules engage protein tyrosine phosphatases via ITIMs which remain underexplored for their therapeutic value in autoimmunity (Fig. 1). Given the complexity of immune responses in SLE, future studies may consider strategies that target co-stimulatory as well as co-inhibitory pathways to enhance control of active and chronic autoimmunity.

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:
- ▪ of special interest
- ▪▪ of outstanding interest

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First publication describing BTLA dysregulation in SLE B cells which affect naïve as well as memory B cells in SLE. It remains to be shown how these abnormalities affect induction and maintenance of the disease.

Integrating genetic and social factors to understand health disparities in lupus

Paula S. Ramos[a,b]

Purpose of review
Both social and genetic factors are associated with health outcomes in systemic lupus erythematosus (SLE), thus playing a role in its health disparities. Despite the growing list of social and genetic factors associated with SLE outcomes, studies integrating sociocultural and individual determinants of health to understand health disparities in SLE are lacking. We review the contributions of different social and genetic factors to the disparities in SLE, and propose a socioecological model to integrate and examine the complex interactions between individual and social factors in SLE outcomes.

Recent findings
Multiple studies collecting comprehensive social data and biospecimens from diverse populations are underway, which will contribute to the elucidation of the interplay and underlying mechanisms by which positive and negative social determinants of health influence epigenomic variation, and how the resulting biological changes may contribute to the lupus health disparities.

Summary
There is growing awareness of the need to integrate genomic and health disparities research to understand how social exposures affect disease outcomes. Understanding the contributions of these factors to the SLE health disparity will inform the development of interventions to eliminate risk exposures and close the health disparity gap.

Keywords
genetic factors, health disparities, social factors, systemic lupus erythematosus

INTRODUCTION

Health disparities in systemic lupus erythematosus (SLE, or lupus) are well established and supported by decades of evidence. As recently reviewed [1▪▪,2▪], there are marked demographic differences in the incidence, prevalence and disease outcomes of SLE. For example, women are 8–10 times more likely than men to develop lupus; relative to European American, African–Americans are three to four times more likely to develop lupus, suffer from remarkably higher disease severity and death rates, and are more likely to suffer from multiple comorbidities such as depression, cardiovascular disease, diabetes and worse health-related quality of life. SLE is among the leading causes of death in young girls (highest for African–American and Hispanic women) [3], underscoring its impact as an important public health issue.

Despite the disproportional impact of SLE on minority racial and ethnic communities, the factors underlying these health disparities remain elusive. The causal mechanisms underlying SLE risk and outcomes among and within ethnic groups are complex, involving biological, sociocultural, physical and other environmental exposures. However, most SLE research to date has focused on biological mechanisms while ignoring the effects of social exposures. Similarly, health disparities research has focused primarily on the influence of socioeconomic determinants on outcomes without considering the biological mechanisms involved. Furthermore, studies of sociocultural determinants are sparse in SLE. This has resulted in a knowledge gap regarding the interactions between individual and social factors that contribute to disparities in SLE outcomes. We will herein review the contributions of different

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KEY POINTS

- The mechanisms underlying SLE disparities are complex and poorly understood, involving biological, sociocultural, physical and other environmental exposures.
- We propose a socioecological model to examine the complex interactions between individual (including genetic) and social factors that contribute to disparities in SLE outcomes.
- Ongoing mechanistic studies integrating multiple individual with positive and negative social determinants of health will elucidate how protective social factors buffer the effects of risk factors on SLE outcomes, and the contributions of these factors to the SLE health disparity.
- Understanding the effects of positive and negative social environments on SLE through epigenomic changes can inform the development of services or interventions that promote positive and mitigate negative exposures, helping close the health disparity gap.

social factors and genetic factors to the health disparities in SLE. We propose a socioecological model of SLE outcomes that emphasizes the importance of integrating sociocultural and individual determinants to understand and address health disparities in SLE. We will summarize emerging studies poised to elucidate the mechanisms linking physical and social environments with differential gene expression and health disparities. Given our focus on integrating genetics into health disparities research, we start by discussing the importance of acknowledging the broader social context of health disparities.

RACE, ETHNICITY AND ANCESTRY

Defining race, ethnicity and ancestry and using these concepts in biomedical research has wide-ranging implications for how the research is translated into clinical care, reported in the media, incorporated into public understanding and implemented in public policy [4▪▪]. Race and ethnicity are self-ascribed or socially ascribed identities and are often 'assigned' by police, hospital staff or others on the basis of physical characteristics; these concepts have no genetic or biological basis [5]. Ancestry is generally used to imply one's genetic origins. As reviewed elsewhere [4▪▪], often these concepts are conflated in scientific literature, implying that racial groups map to discrete genetic groups, and conveying that health inequities are caused by genetic factors rather than structural racism. These misconceptions can lead to the biological reification of social categories and be used to fuel racism and discrimination [4▪▪]. This conflation can also lead to results with poor scientific validity [4▪▪]. It is thus essential to explicitly distinguish between variables that derive from nongenetic, reported information, versus genetically inferred information.

Although race and ethnicity are often correlated with genetic ancestry, the sociocultural and genetic information the former and later capture, respectively, are different information. The use of race and ethnicity in biomedical research and clinical practice is an imperfect proxy for important epidemiologic information, including social determinants of health such as racism and discrimination, economic stability, healthcare access and quality, education access and environmental exposures. These social and environmental determinants are differentially experienced across racial/ethnic groups due to historical and contemporary discriminatory policies and practice, resulting in health disparities across groups and geography. As for most conditions, the role of social and physical environmental factors on SLE outcomes is poorly understood.

Mixing the concepts of race and genetic ancestry is especially problematic in admixed populations who are often assumed as homogeneous when they are, in fact, extremely heterogeneous [4▪▪]. For example, individuals who self-report as Hispanic/Latino have diverse cultural backgrounds as well as varying proportions of genetic ancestry from Africa, America and Europe. Similarly for individuals who self-report as Black/African–American: their mean sub-Saharan African ancestry varies between 10 and 20% in Central and South America to about 75% in the United States and British Caribbean, but can vary from 2 to 100% among different individuals [6]. The heterogeneity of African–Americans is well exemplified by our genetic studies in Gullah African–Americans, a culturally distinctive group of African–Americans living in the Sea Islands along the coast of the southeastern United States, from North Carolina to Florida. Despite their unique culture that retains deep African features, our results are consistent with historical data [7], confirming that the Gullah have complex African ancestry and reduced European admixture, and are a mixture of numerous people from different genetic, ethnic and linguistic currents who formed their own culture and language [8]. This heterogeneity underscores the need to investigate within-group ethnic differences, which are greatly underexplored.

SOCIAL FACTORS

As recently reviewed [1▪▪,2▪,9▪], history of trauma is associated with an increased risk of incident SLE, and multiple socioeconomic and psychosocial stressors negatively affect SLE outcomes. These include low

household income, poverty, unemployment, food insecurity, housing inability, medical care insecurity, exposure to violence, exposure to adverse childhood experiences, physical victimization, unfair treatment, perceived stress, depression, racial discrimination and vicarious racism [1▪▪,2▪,9▪]. It is noteworthy that those who are poor with SLE are estimated to live 14 fewer years than their nonpoor counterparts [9▪]. Recently, Spears *et al.* [10] added anticipatory racism stress to this list of social stressors associated with poor disease outcomes.

Notably, African–American women are more likely to experience these stressors [11]. African–American women report racial discrimination as a particularly salient and chronic stressor over their life course, distinct from other forms of unfair treatment [12]. The health consequences of racial discrimination, whether structural (e.g. chronic poverty, poor infrastructure), institutional (e.g. educational institutions and employment discrimination) or individual (e.g. interpersonal discriminatory acts), are evidenced by poorer health for African–American women across socioeconomic strata, including higher rates of cardiovascular, metabolic, immune and endocrine chronic conditions [13].

Despite stressors coexisting in areas of concentrated poverty, protective factors may buffer the negative impacts of stressors [14]. For example, protective parenting behavior buffers the impact of racial discrimination on depression among Black Youth [15]. Resilience is traditionally conceptualized based on personal traits that include not only the individual, but also the role of family, community, physical and social ecology [16]. The compensatory model of resilience postulates that resilience resources may neutralize exposure to a social risk factor given a specific outcome [17]. Social support might have a positive impact in SLE [9▪]. For example, several peer support programmes designed to enhance social support and provide health education among African–American and Latino patients with SLE have decreased depression and anxiety, and resulted in improved outcomes [9▪]. Amongst other outcomes, the Georgians Organized Against Lupus (GOAL) research cohort showed that a self-management programme benefited low-income African–American women with SLE, and revealed a significant association between organ damage and depression in African–American women, with social support being protective of depression [2▪]. In addition, exiting poverty can mitigate the strong effect of living in concentrated poverty on SLE damage [9▪]. A large prospective study has shown that a combination of healthy lifestyle behaviors based on alcohol consumption, body mass index, smoking, diet and exercise, could reduce the risk of incident SLE to half [18]. Collectively, these data suggest that peer-support, self-management and programmes to alleviate poverty and support healthy lifestyle behaviours can help improve SLE outcomes.

Most studies to date have focused on social risk factors, and there is a paucity of research investigating protective social factors on SLE. Studies integrating multiple positive and negative social determinants of health will allow a thorough understanding of how protective factors buffer the effects of risk factors on SLE outcomes, and of the contributions of these factors to the SLE health disparity.

GENETIC AND EPIGENETIC FACTORS

Human genetic variation changes gradually according to geographical gradients, so alleles that are common in one population might be rare in another, geographically distant group. Differences in disease risk allele frequency in populations might be underlying some of the health disparities. Many genetic loci are associated with increased risk of SLE; Lanata *et al.* [19▪] have recently reviewed the genetic risk factors for SLE that vary among populations. For example, two *apolipoprotein L1* (*APOL1*) alleles confer a substantially increased risk of kidney disease in African ancestry individuals [20]. Although a large proportion of the ethnic disparity in end-stage renal disease (ESRD) in African–Americans with lupus nephritis is attributed to the *APOL1* risk alleles [21], once these risk alleles are accounted for the ethic disparity in SLE-ESRD is nearly absent. This suggests that nongenetic factors can be leveraged to reduce the development of *APOL1*-associated kidney disease in genetically susceptible individuals [22]. Integrating genetic and nongenetic factors could be a powerful way to reduce health disparities by more sharply identifying residual disparities and leveraging actionable social factors.

Although candidate genes or polygenic scores explain part of the variation in health outcomes, social determinants of health such as economic inequality generally explain considerably more variation [23▪▪]. This suggests that social factors have biological consequences, with epigenetics potentially playing a role in linking individual and contextual factors with health outcomes across the life course [23▪▪]. Despite the role of genetic factors in SLE, health disparities are typically due to social and structural determinants of health. Adverse experiences might influence SLE through epigenetic changes. Epigenetic marks such as DNA methylation impact gene expression and can govern cell function and physiological response to social exposures. Variation in DNA methylation in multiple blood cell subsets is associated with SLE. The role

of genetic and epigenetic factors in health disparities observed in SLE and other rheumatic diseases has been recently summarized [19▪]. Although DNA methylation varies between populations [24–31], and this variation is partially explained by their distinct genetic ancestry, environmental factors not captured by ancestry are significant contributors to variation in DNA methylation [26]. This supports the notion that an interaction between social, genetic and epigenetic factors underlies the health disparity in SLE.

In addition to their association with disease status, DNA methylation levels are also associated with psychosocial factors such as socioeconomic status [32,33], poverty [34], general perceived stress [35] and childhood stress and maltreatment [36,37]. A DNA methylation biomarker for accelerated ageing is associated with adverse environmental exposures, including low socioeconomic status, stress and childhood adversity [38]. A DNA methylation biomarker of mortality risk is associated with neighbourhood disadvantage [39]. The field of social epigenetics aims to elucidate the pathways linking the physical, built and social environments with differential gene expression and health disparities. Most studies to date have focused on socioeconomic status and early-life adversity, followed by social exposures [23▪▪]. Given its relative infancy, the interpretation of results from these social epigenetic studies remains challenging: the majority lacked diversity and included individuals from North America and Western Europe; there was substantial variation in cell and tissue types, in different epigenetic measurements and in the age of the study participants [23▪▪]. Future social epigenetics research including larger, representative groups, and well defined social factors is poised to unravel the biological consequences of social exposures on gene expression, disease cause and health inequities.

EMERGING STUDIES INTEGRATING GENETIC AND SOCIAL FACTORS

The socioecological model of health asserts that health is affected by the interaction between the characteristics of the individual, the community and the environment that includes the physical, social and political components. We propose a conceptual framework based on the socioecological model that emphasizes the importance of integrating societal, community, interpersonal and individual determinants to understand and address health disparities in SLE (Fig. 1) [40,41]. Social determinants of health span the socioeconomic (employment, income, housing and food security), community (family and social support), neighbourhood and physical environment (access to food and housing, crime and violence, safety, transportation, air and water quality) and the healthcare system (access, quality). Individual determinants include genetic (sex chromosomes, DNA, epigenetic and gene expression variation) and behavioural factors (diet, smoking, alcohol use, physical and mental health). As exposures and experiences vary across individuals from different populations, locations and cultures, it is critical to study population differences in lupus health disparities within the sociocultural context. This need is further underscored by both the paucity of disadvantaged communities in research, and the heterogeneity of racial/ethnic groups.

As reviewed above, both socioeconomic and psychosocial factors, as well as genetic factors are associated with poorer health outcomes in African–American and other racial/ethnic minority patients with SLE. However, these groups are underrepresented in research, and the role of both individual and sociocultural determinants of health in heath disparities in SLE are poorly understood. Several research cohorts in the U.S. have been collecting data and biospecimens from racially and ethnically diverse populations to allow investigation of how various risk factors interact to influence SLE. These include the LUMINA (Lupus in Minorities: Nature vs. nurture), the Georgians Organized Against Lupus (GOAL), the Michigan Lupus Epidemiology & Surveillance (MILES) and the California Lupus Epidemiology Study (CLUES) cohorts [42]. The clinical, sociodemographic, psychosocial and health services data collected from the patients from different racial/ethnic communities, together with genetic and other biologic material, is expected to provide a more comprehensive understanding of the reasons why disadvantaged groups experience disparities in SLE burden and outcomes, which will aid in the development of interventions to eliminate or mitigate SLE disparities.

Currently, it is not known how social or environmental experiences influence disease outcomes. Although studies linking specific experiences or behaviors to epigenetic changes in SLE are lacking, mounting evidence across several traits suggests that epigenetic mechanisms may provide a causal link between social adversity and health disparity [1▪▪]. In reponse to the increasing awareness for the need for social epigenomic research (e.g. PAR-19–372), the goal of a recently funded project titled *Social Factors, Epigenomics, and Lupus in African American Women (SELA)* is to identify epigenetic changes by which positive and negative social factors affect gene function, and thereby influence lupus in African–American women. Innovative aspects of this study include the focus on culturally distinct Gullah and

FIGURE 1. Simplified socioecological model of lupus outcomes. Socioecological factors that contribute to lupus disparities at the individual and social levels (including interpersonal, community and broader societal levels) are shown. As denoted by the lateral arrows, factors at each level interact to contribute to lupus disparities.

non-Gullah African–American women, the community partnership and the integrative analysis of multiple individual and social factors, including risk and protective social effects. The identification of epigenetic mechanisms by which adverse and protective factors affect gene function and thereby influence SLE may inform the development of psychosocial interventions that prevent or mitigate risk exposures, and services or interventions that promote positive exposures. Development of these novel treatments and preventive interventions, as informed by the results of this study, is paramount to the closure of the health disparities gap.

Finally, future studies ought to include and analyze the role of metagenomic variation and intestinal barrier permeability on SLE disparities. Associations of microbiota dysbiosis, intestinal permeability and intestinal inflammation with several autoimmune diseases have been reported [43]. Interestingly, social stress is a well described intestinal disrupting factor [43]. Hence, studies are needed to understand the role of intestinal barrier disruption, intestinal inflammation, gut dysbiosis and their interplay with other individual and social factors in SLE disparities.

CONCLUSION

The role for both genetic and social determinants of health on SLE disparities is well documented. However, knowledge of how physical and social exposures influence differential gene expression and disease outcomes is lacking, disadvantaged communities are poorly represented in research, racial/ethnic groups are heterogeneous and their within-group disparities unexplored, many studies mix biological with socially constructed ethnoracial categories, the mechanisms by which adverse and protective social factors synergistically modulate disease outcomes are not understood, and comprehensive studies

integrating multiple individual and social factors have not been published. Nevertheless, emerging studies in SLE have been collecting extensive genetic and social data, and are poised to elucidate how risk and protective factors from multiple levels of the social environment interact and influence SLE outcomes through epigenomic variation.

Results from these studies are expected to elucidate how risk factors affect SLE, how they can be mitigated in patients with SLE, inform the development of targets for interventions to minimize adverse stressors, improve outcomes for vulnerable patients with SLE and minimize SLE disparities.

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Conflicts of interest

There are no conflicts of interest.

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