PAEDIATRICS

MIS-C: how do patients fare 6 months on?

> the 6-month outcomes of the patients were largely reassuring

Multisystem inflammatory syndrome in children (MIS-C; also known as PIMS-TS) is a rare post-infectious manifestation of SARS-CoV-2 infection in children. Most studies of MIS-C have been limited to the acute phase of illness, whereas less is known about the medium and long-term outcomes and sequelae of this condition. Newly published results in *The Lancet Child & Adolescent Health* provide insight into the 6-month outcomes of MIS-C.

The retrospective cohort consisted of 46 children who were admitted to Great Ormond Street Hospital (a tertiary paediatric hospital in the UK) between 4 April and 1 September 2020, and followed up 6 weeks and 6 months after discharge.

"As this was a completely new disease, we did not know what the consequences of this disease would be. Therefore, we set up a multidisciplinary team clinic, which involved all the professionals who had been involved in the patients' care as inpatients, so we could provide comprehensive holistic follow-up for them as outpatients," explains Karyn Moshal, corresponding author on the study.

Credit: S.Harris/Springer Nature Limited

"In seeing the patients as a group, we could learn from each other, and in meticulously documenting the course of each patient, we could pick up patterns of sequelae across the group and best determine the interventions they required."

"Not only was this new disease process a black box for clinicians but also for parents and children. As MIS-C generally affects otherwise well children who have had no or very little contact with healthcare services prior, this created lots of anxiety for parents and children alike," says Justin Penner, first author on the study. "Our approach allowed us to alleviate some of these anxieties whilst providing appropriate specialist psychological support to children and their families throughout. It has also allowed us to recognise and address important psychological and psychosocial trends such as stigma, shame, and medical vulnerability and discuss important issues such as vaccine hesitancy in our patient cohort."

The 6-month outcomes of the patients were largely reassuring. Systemic inflammation had resolved in all but one patient. Similarly, many of the severe organ-specific manifestations seen at 6 weeks, including various cardiac, gastrointestinal, renal, haematologic and otolaryngologic symptoms, had mostly resolved by 6 months. Nevertheless, some issues persisted in a subset of patients, including muscle weakness and fatigue, subtle neurological findings and psychological issues (such as emotional lability and anxiety).

As acknowledged by the authors, the retrospective design of the study had a number of limitations. "Of most important note was that there were no controls and much of what was observed for the central nervous system and muscle findings may be due to severe illness of any sort and the requirement for intensive care management and not specific for MIS-C," says Rae Yeung, a researcher with a strong interest in MIS-C who was not involved in the study. "It is difficult to tease out the contributions from the inflammatory components unique to children with MIS-C from general severe disease requiring ICU admission."

These limitations also applied to the findings about anxiety and emotional lability. "Because there was no comparison group of children with other serious illness requiring intensive care or prolonged hospitalization, it is difficult to know what to make of the psychological impact of the disorder," remarks Anne Rowley, another researcher independent of the study with an interest in MIS-C. "We know that psychological disorders in children are greatly increased in the pandemic era compared with the pre-pandemic era, so the pandemic itself could have had a psychological impact. Because there were no pre-illness data on some assessed parameters it is possible that some impairments were pre-existing."

Nevertheless, the information gained over the course of the study enabled the clinicians to adjust their own practices and highlighted the importance of physical and mental rehabilitation. The researchers plan to continue to provide comprehensive holistic care for this group of patients and monitor them to identify patterns of persistent or new symptoms over time.

Jessica McHugh

ORIGINAL ARTICLE Penner, J. et al. 6-month multidisciplinary follow-up and outcomes of patients with paediatric inflammatory multisystem syndrome (PIMS-TS) at a UK tertiary paediatric hospital: a retrospective cohort study. *Lancet Child Adolesc. Health* https://doi.org/10.1016/S2352-4642(21)00138-3 (2021)

IN BRIEF

OSTEOARTHRITIS

Sprifermin benefits maintained at 5 years

Structural benefits of treatment with intra-articular sprifermin for knee osteoarthritis were maintained 3.5–4 years post-treatment in the FORWARD trial, in which 378 (69%) patients completed 5-year follow-up. In those who had received sprifermin 100 µg every 6 months, the change from baseline in total femorotibial joint cartilage thickness seen at year 2 was sustained at year 5 with no new safety signals. Clinically relevant improvements in pain in the subgroup at risk of progression ($n = 161$) seen at year 3 were also maintained.

ORIGINAL ARTICLE Eckstein, F. et al. Long-term structural and symptomatic effects of intra-articular sprifermin in patients with knee osteoarthritis: 5-year results from the FORWARD study. *Ann. Rheum. Dis.* https://doi.org/10.1136/annrheumdis-2020-219181 (2021)

RHEUMATOID ARTHRITIS

Tapering csDMARDs leads to more RA flares

In a randomized, open-label study assessing tapering of conventional synthetic DMARDs (csDMARDs) in patients with rheumatoid arthritis (RA) in sustained clinical remission, 19 of 77 patients (25%) whose csDMARD treatment was reduced to half-dose had at least one disease flare, compared with 5 of 78 patients (6%) who continued stable-dose csDMARD therapy without tapering (risk difference 18%; 95% CI 7–29%). Significantly fewer flares occurred in the stable-dose group.

ORIGINAL ARTICLE Lillegraven, S. et al. Effect of half-dose vs stable-dose conventional synthetic disease-modifying antirheumatic drugs on disease flares in patients with rheumatoid arthritis in remission: the ARCTIC REWIND randomized clinical trial. *JAMA* **325**, 1755–1764 (2021)

THERAPY

NSAIDs not linked to worse COVID-19 outcomes

In contrast to anecdotal reports that pre-existing use of NSAIDs is linked to poor outcomes in patients with COVID-19, the findings of a prospective, multicentre cohort study found no increase in mortality or COVID-19 severity among NSAID users. The study included 72,179 patients across 255 health-care facilities in England, 4,211 (5.8%) of whom were taking systemic NSAIDs before they were admitted to hospital.

ORIGINAL ARTICLE Drake, T. M. et al. Non-steroidal anti-inflammatory drug use and outcomes of COVID-19 in the ISARIC Clinical Characterisation Protocol UK cohort: a matched, prospective cohort study. *Lancet* https://doi.org/10.1016/S2665-9913(21)00104-1 (2021)

PSORIATIC ARTHRITIS

Tildrakizumab shows promise in phase IIb study

The anti-IL-23p19 monoclonal antibody tildrakizumab improved skin and joint manifestations of psoriatic arthritis (PsA), but not dactylitis or enthesitis, in a 52-week phase IIb study. 391 patients with active PsA were randomly allocated to receive tildrakizumab 200 mg every 4 weeks, tildrakizumab 200 mg, 100 mg or 20 mg every 12 weeks or placebo; at week 24, those in the 20 mg or placebo groups were switched to tildrakizumab 200 mg every 12 weeks. Compared with the placebo group, more patients in the tildrakizumab groups achieved an ACR20 or ACR50 response, minimal disease activity and PASI 75, PASI 90 and PASI 100 responses at week 24; responses were maintained through week 52, and tildrakizumab was generally well tolerated.

ORIGINAL ARTICLE Mease, P. J. et al. Efficacy and safety of tildrakizumab in patients with active psoriatic arthritis: results of a randomised, double-blind, placebo-controlled, multiple-dose, 52-week phase IIb study. *Ann. Rheum. Dis.* https://doi.org/10.1136/annrheumdis-2020-219014 (2021)

Credit: Getty Images/molotovcoketail

LUPUS NEPHRITIS

Voclosporin improves outcomes in lupus nephritis

Lupus nephritis, a serious manifestation of systemic lupus erythematosus that can result in renal failure and the need for kidney transplantation, has historically been difficult to treat. The AURORA 1 study, in which the new-generation calcineurin inhibitor voclosporin was investigated in addition to standard care (mycophenolate mofetil and low-dose glucocorticoids) in lupus nephritis, has produced positive results, suggesting that this drug could be a useful addition to current lupus nephritis treatments.

"Early intervention and kidney response are linked to better long-term outcomes for lupus nephritis and prevent irreversible kidney damage," explains Robert Huizinga, corresponding author on the AURORA 1 study. "This study reports the phase III data on voclosporin and demonstrates its ability to achieve superior and faster complete renal response rates compared to standard of care alone — a significant advance in addressing this condition."

In AURORA 1, one of the largest lupus nephritis trials to date, 357 patients with active lupus nephritis were randomly assigned to receive either oral voclosporin or placebo in addition to standard care. The primary end point of complete renal response at 52 weeks was met in more of the patients treated with voclosporin (73 out of 179; 41%) than in those treated with placebo (40 out of 178; 23%). The time taken to achieve a 50% reduction in proteinuria was also shorter in those receiving voclosporin than in those receiving placebo.

Calcineurin inhibitors have been used to treat lupus nephritis for a long time, but concerns have been raised about infection risks associated with the use of these drugs. In contrast to the increased risk of serious infection reported in phase II studies of voclosporin, in the phase III AURORA 1 study, the safety profile was similar between those treated with voclosporin and those treated with placebo, allaying safety concerns for this new-generation calcineurin inhibitor.

Many new therapies for lupus nephritis are currently in development, but only voclosporin and belimumab have so far been approved by the FDA. "Our current drugs for lupus nephritis don't do a good job, so we have been excited to see the emergence of voclosporin and belimumab as add-on agents for lupus nephritis with similar efficacy profiles," comments Anne Davidson, an expert in lupus nephritis who was not involved in this study. "Issues of long-term safety, differences in responses among patients of different ethnicities, introducing voclosporin earlier or only in those with initial treatment failures, and overall long-term outcomes will need to be determined in further trials and observational studies to help determine the place for voclosporin in a treatment regime," she suggests.

Follow-up studies are underway that will hopefully address some of these unknown factors. "We are in the process of conducting an AURORA continuation study evaluating patients with up to 104 weeks of total treatment and we plan to share those results over the coming months," says Huizinga.

Joanna Clarke

ORIGINAL ARTICLE Rovin, B. H. et al. Efficacy and safety of voclosporin versus placebo for lupus nephritis (AURORA 1): a double-blind, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet* https://doi.org/10.1016/S0140-6736(21)00578-X (2021)

SPONDYLOARTHRITIS

Tenascin C promotes pathological bone formation in AS

Entheseal new bone formation is a hallmark of ankylosing spondylitis (AS), but the underlying pathogenesis is poorly understood and effective targeted treatments are lacking. New research implicates the extracellular matrix (ECM) protein tenascin C in pathological bone formation in AS and suggests that suppressing aberrant tenascin C expression might present a novel therapeutic strategy.

The authors of the new study first established that tenascin C expression was upregulated in the entheses and spinal ligament tissues of patients with AS as well as in two animal models that mimic the pathological bone formation in AS, namely DBA/1 mice that develop spontaneous arthritis and C57BL/6J mice with collagen-antibody-induced arthritis (CAIA). In both animal models, inhibition of tenascin C with a neutralizing antibody suppressed pathological new bone formation, as did knockout of *Tnc* (the gene encoding tenascin C) in the CAIA model.

In entheseal tissue from mice with CAIA, tenascin C was primarily secreted by $FSP1^+$ fibroblasts; in vitro, several AS-related pro-inflammatory cytokines, including TNF, IL-17A and IL-22, could induce aberrant expression of tenascin C by these cells. Further investigations revealed that tenascin C promotes entheseal new bone formation by decreasing the adhesion force of the ECM, resulting in activation of the Hippo–YAP signalling pathway and increased expression of chondrogenic genes.

> “inhibition of tenascin C with a neutralizing antibody suppressed pathological new bone formation”

“These findings suggest that inflammation-induced modification of ECM biomechanic properties has a substantial effect on the pathological process of new bone formation in AS,” reports corresponding author Hui Liu.

As well as further studies targeting tenascin C to potentially prevent pathological bone formation in AS, the researchers also plan to investigate whether tenascin C contributes to the pathogenesis of the disease in other ways. “Given that tenascin C has multifaceted roles in immunomodulation and inflammation, it might also have other critical roles in the regulation of the entheseal and ligamentous microenvironment or the maintenance of chronic inflammation in AS,” Liu suggests.

Sarah Onuora

ORIGINAL ARTICLE Li, Z. et al. Tenascin-C-mediated suppression of extracellular matrix adhesion force promotes entheseal new bone formation through activation of Hippo signalling in ankylosing spondylitis. *Ann. Rheum. Dis.* https://doi.org/10.1136/annrheumdis-2021-220002 (2021)
RELATED ARTICLE Gracey, E. et al. Tendon and ligament mechanical loading in the pathogenesis of inflammatory arthritis. *Nat. Rev. Rheumatol.* **16**, 193–207 (2021)

PHARMACOTHERAPY

DNA methylation inhibitor resets tolerance in autoimmune arthritis

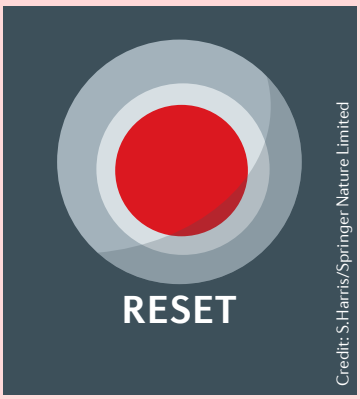
In autoimmune diseases such as rheumatoid arthritis, a loss of immune tolerance occurs that is associated with reduced activity of regulatory T (T_{reg}) cells and increased numbers of pro-inflammatory T helper 1 (T_H1) and T_H17 cells. The results of a new study suggest that the balance between T_{reg} cells and T_H1 and T_H17 cells could be restored in autoimmune arthritis by modulating the epigenetic landscape of these cells.

“The synthesis of proteins normally involved in controlling autoimmunity is inhibited in chronic inflammation due to methylation of CpG motifs within the promoter regions of their respective genes. In addition, previous reports suggest that there is an increase in numbers of T_{reg} cells in patients with cancer treated with azacytidine, a DNA methylation inhibitor,” explains corresponding author Richard Williams. “These findings led us to question whether short-term treatment of autoimmune arthritis with DNA methylation inhibitors could restore immunoregulatory gene expression and activity of T_{reg} cells, leading to long-term suppression of disease.”

> “Decitabine … increased the number of T_{reg} cells in the affected joints and decreased the number of T_H1 and T_H17 cells”

After testing several DNA methylation inhibitors for their ability to induce T_{reg} cells in vitro and for their effects on mice with collagen-induced arthritis, the researchers settled on decitabine, a drug already approved for use in cancer, as a candidate for further investigation. Decitabine not only ameliorated arthritis, it also increased the number of T_{reg} cells in the affected joints and decreased the number of T_H1 and T_H17 cells.

“The decrease in numbers of T_H1 and T_H17 cells was unexpected,” states Williams. “We found that decitabine caused the death of activated effector T cells whilst sparing naive T cells. The molecular basis for the selective action of decitabine was due to expression of ENT1, a nucleoside transporter, which enables the drug to enter the cell and kill it via apoptosis.”



Although the expression of ENT1 on effector T cells helped to produce some specificity for decitabine, off-target effects are a concern when using DNA methylation inhibitors. As more selective epigenetic-modulating drugs emerge, the researchers hope that this approach can move towards trials in humans.

Joanna Clarke

ORIGINAL ARTICLE Huang, Y.-S. et al. Pharmacological modulation of T cell immunity results in long-term remission of autoimmune arthritis. *Proc. Natl Acad. Sci. USA* **118**, e2100939118 (2021)

SYSTEMIC SCLEROSIS

Linking NAD metabolism and DNA repair to inflammation in SSc

Enrico Vittorio Avvedimento and Armando Gabrielli

According to new data, overexpression of the nicotinamide adenine dinucleotide (NAD) hydrolase, CD38, in systemic sclerosis (SSc) leads to NAD depletion and fibrosis. These intriguing findings link inflammation, NAD metabolism and fibrosis and bare striking resemblance to age-related changes in SSc. Could DNA damage also connect these seemingly unrelated pathways?

Refers to Shi, B. et al. Targeting CD38-dependent NAD^+ metabolism to mitigate multiple organ fibrosis. *iScience* **24**, 101902 (2021).

Nicotinamide adenine dinucleotide (NAD) is one of the few molecules shaped by evolution to carry out multiple and diverse redox reactions in various conditions and cell compartments[1]. This molecule is under the control of various factors, including the enzyme CD38. New data from Shi and colleagues suggest that CD38 is an important promoter of fibrosis in systemic sclerosis (SSc) via NAD depletion[2], and provides an interesting link between NAD metabolism and fibrosis in SSc.

NAD^+ (the oxidized form of NAD) is extensively used by different enzymes in essential metabolic pathways and can be considered a universal energy currency for various cellular processes. NAD^+ is consumed by ADP ribosylation in an ancient type of reaction that involves the transfer of ADP ribose from NAD^+ to different substrates, including poly (ADP-ribose) polymerases (PARPs) and sirtuins. PARPs are actively involved in DNA repair, whereas sirtuins are a highly conserved family of proteins involved in the regulation of various processes including mitochondrial stability and metabolism[3,4]. This family of proteins inhibit senescence and dampen inflammation by altering the stability of a number of transcription factors that amplify cytokine signals[4]. Similar to PARPs, sirtuins require NAD^+ as co factor to support their function, and sirtuins are also inhibited by the NAD degradation product nicotinamide[1]; hence, both PARPs and sirtuins are highly sensitive to variations in NAD levels[1]. As a result, NAD controls cell function and survival in response to various environmental changes, such as nutrient availability and DNA repair. More importantly, NAD^+ levels are associated with longevity and decline with age because of increased NAD degradation[5]. The ecto-enzyme CD38, a NAD hydrolase that cleaves NAD^+ to generate nicotinamide and ADP ribose, is expressed in immune and non-immune cells and has been identified by several groups as a major regulator of extracellular and intracellular NAD^+ concentrations[5].

> “these data support a leading role for CD38 in NAD depletion and fibrosis in SSc”

In their new study, Shi and colleagues have linked CD38 expression with SSc. The researchers found that the expression of CD38 was much higher in the skin of patients with diffuse cutaneous SSc than in the skin of healthy individuals, but that the expression levels in the skin of patients with limited cutaneous SSc was similar to that in healthy skin[2]. Indeed, the expression levels of CD38 in the skin was associated with molecular markers of fibrosis and clinical disease scores. Notably, this high expression in the skin was associated with an increased expression of two other enzymes involved in NAD regulation and induced by inflammation: nicotinamide N-methyltransferase (NNMT) and indoleamine 2,3-dioxygenase (IDO1). Overexpression of CD38 is induced by various cytokines (IL-13, TGFβ, TNF and IL-6) in skin fibroblasts in vitro, and induces the production of IL-6 and IL-1β by macrophages. In fact, the production of IL-6 and IL-1β is impaired in CD38-null macrophages, suggesting that an autocrine loop exists between CD38 and inflammatory cytokines, leading to deregulation of NAD metabolism (FIG. 1).

Looking further into the therapeutic implications of these findings, Shi et al. found that in mice with bleomycin-induced fibrosis, treatment with a CD38 inhibitor, dietary supplementation with nicotinamide riboside (a precursor of NAD^+), or both interventions combined boosted the concentrations of NAD^+ in the skin and reduced the accumulation of infiltrating macrophages in the lungs, while having no notably effects on resident macrophages. Ultimately, boosting the expression of NAD^+ protected the mice from the development of fibrosis in the skin and lungs. By contrast, pharmacological reduction of cellular NAD^+ levels using FK866 (an inhibitor of the rate limiting enzyme in the NAD salvage pathway) enhanced the expression of collagen, promoted fibrotic gene expression and increased the amount of acetylation of lysines and manganese superoxide dismutase in skin fibroblasts (indicating reduced activity of sirtuins). Moreover, nicotinamide riboside supplementation combined with CD38 inhibition attenuated, but did not completely prevent, the differentiation of fibroblasts into myofibroblasts.

Altogether, these data support a leading role for CD38 in NAD depletion and fibrosis in SSc. The findings suggest that CD38 reduces NAD levels and, as a consequence, reduces the activities of sirtuins, promoting the production of cytokines and the inflammatory response and leading to fibrosis. However, might the results from Shi and colleagues also be explained by inflammation induced by DNA damage and the DNA damage response (DDR)? The DDR is a complex network of pathways that sense and respond to DNA lesions. DNA damage and/or the DDR elicit a

strong inflammatory response known as the senescence-associated secretory phenotype (SASP), which induces chronic inflammation in the surrounding tissues[6]. These age and DNA damage associated pro-inflammatory changes (known as inflammageing) are characterized by the accumulation of growth-arrested cells and the subsequent production of cytokines, which further amplify the levels of oxidative stress and the DDR[6]. The relevant role of DNA damage and DDR as promoters of fibrosis is exemplified by the effects of bleomycin, which is a potent inducer of fibrosis and a DNA-damaging agent.

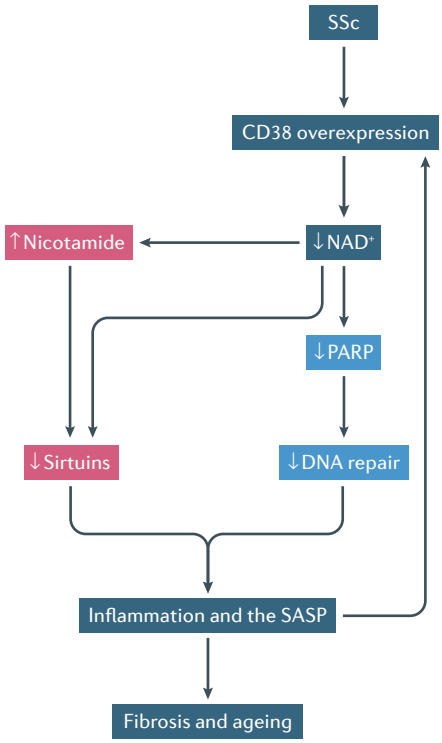


Fig. 1 | **CD38 links NAD, metabolism and DNA repair to inflammation in SSc.** A schematic illustrating the potential link between CD38 overexpression, nicotinamide adenine dinucleotide (NAD) metabolism and fibrosis in systemic sclerosis (SSc) (shown in green), which might occur via the regulation of sirtuin activity (shown in red) and/or the DNA damage response (shown in blue). CD38 is overexpressed in the skin of patients with SSc. This enzyme hydrolyses NAD to nicotamide and ADP ribose, inhibiting the activity of sirtuins and poly (ADP-ribose) polymerases (PARPs) and limiting DNA repair. Low activity of sirtuins and increased activity of the DNA damage response promote inflammation and a senescence-associated secretory phenotype (SASP), leading to fibrosis and ageing. The production of cytokines promotes CD38 expression, exacerbating this process.

Bleomycin evokes a strong DDR, which results in activation of PARP1 and ATM, the master kinase regulator of the DDR and SASP[7]. Hyperactivation of PARP1 in response to persistent DNA damage consumes NAD^+ and results in energy depletion (reviewed elsewhere[1,3]). This effect explains why ageing at the cellular level is associated with a decline in NAD levels and a persistent DDR[7]. Simultaneously, ATM can phosphorylate the transcription factor Jun, which, depending on its binding partner at the chromatin sites, induces the expression of collagen or silences the expression of a soluble inhibitor (WIF-1) of pro-fibrotic Wnt proteins[8]. Knockdown of ATM inhibits collagen expression in fibroblasts from patients with SSc and in healthy fibroblasts exposed to bleomycin[7]. In addition to bleomycin, other pathways can result in upregulation of the DDR and fibrosis. For example, constitutive production of reactive oxygen species (ROS) or an imbalance in the amount of ROS production following chronic PDGF stimulation promote ATM activity and fibrosis[7]. As PARP activity and DNA repair are dependent on NAD^+ levels, depletion of NAD^+ by CD38 might impair the ability of PARP to repair DNA lesions, promoting DNA damage and the DDR and resulting in an pro-inflammatory and pro-fibrotic phenotype (FIG. 1).

The link between NAD decline induced by CD38 overexpression and inflammation adds a new window of opportunity for inhibiting chronic inflammation and fibrosis. In addition to chronic inflammatory diseases such as SSc, replenishing the NAD pool might also represent a viable tool to treat diseases associated with ageing and senescence-induced inflammation. For example, in a mouse model of dilated cardiomyopathy, associated with persistent PARP activation and substantial NAD^+ depletion, supplementation with nicotinamide riboside can preserve cardiac function[8]. Furthermore, blocking TNF signalling or boosting NAD^+ levels with nicotinamide riboside partially rescues premature ageing in mice with T cells containing dysfunctional mitochondria[9].

In conclusion, the data reported by Shi et al. add a new pathogenic mediator (CD38) and mechanism (NAD depletion) to the pathogenesis of SSc and other inflammatory diseases. The decline in NAD associated with CD38 overexpression, a process that is further exacerbated by inflammation, links DNA damage, senescence and fibrosis. Future studies should aim to molecularly dissect the link between inflammation, metabolism and DNA damage and identify the key factors involved. We believe that such analysis will pave the way to novel therapeutic approaches and tools to control not only inflammation-driven diseases, such as systemic sclerosis, but also degenerative diseases associated with inflammageing.

> “Future studies should … molecularly dissect the link between inflammation, metabolism and DNA damage”

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Acknowledgements
The work of E.V.A. is supported by the POR Campania FESR 2014-2020 “SATIN” grant, and the work of A.G. is supported by Fondazione di Medicina Molecolare e Terapia Cellulare (Ancona-Italy).

Competing interests
The authors declare no competing interests.

DEVELOPMENTAL BIOLOGY

Keep your Sox on, chondrocytes!

Andrew A. Pitsillides and Frank Beier

The transcription factor Sox9 is important for cartilage formation during development, but its role in postnatal growth plates and adult articular cartilage has been uncertain. New research is revealing essential roles for Sox9 in postnatal cartilage homeostasis and in preventing post-traumatic osteoarthritis, along with new mechanisms for chondrocyte-to-osteoblast transition.

Refers to Haseeb, A. et al. SOX9 keeps growth plates and articular cartilage healthy by inhibiting chondrocyte dedifferentiation/osteoblastic redifferentiation. *Proc. Natl Acad. Sci. USA* **118**, e2019152118 (2021).

The transcription factor Sox9 has long been known to be essential for cartilage formation[1], with heterozygote mutations in *SOX9* in humans resulting in campomelic dysplasia, a serious, often lethal, chondrodysplasia. What has remained surprisingly vague, however, is the role of Sox9 in adult cartilage during homeostasis and in response to injury or degeneration, such as occurs in osteoarthritis (OA). In fact, a 2012 study using inducible *Sox9* deletion in mice showed unexpectedly mild phenotypes in articular cartilage, characterized by a loss of proteoglycans but no obvious degenerative changes[2]. A new study by Haseeb et al.[3] has addressed this gap in our understanding of adult cartilage biology by using conditional postnatal *Sox9* deletion in the cartilage of mice (Aggrecan-CreER^{T2} mice), accompanied by bulk and single-cell RNA sequencing, to probe the role of Sox9 and the mechanisms by which it operates.

Haseeb et al. describe essential roles for Sox9 in maintaining postnatal growth plate activity and in articular cartilage homeostasis, particularly after joint injury. They also document accelerated chondrocyte conversion to the osteogenic lineage in the absence of Sox9, as well as providing evidence for the reversion of chondrocytes to a progenitor cell type before differentiation along the osteoblast lineage (FIG. 1). The essential role of Sox9 in maintaining growth plate activity is demonstrated by the rapid closure of the growth plate after inactivation of Sox9 in 4-week old mice, which resulted in the anticipated dwarfism but remarkably did not affect animal viability. These results contrast with another study from 2021, in which postnatal co-deletion of *Gsk3a* and *Gsk3b* in cartilage resulted in similar growth plate demise, rapidly followed by death[4]. On the basis of the results from Haseeb et al.[3], premature closure of the growth plate itself seems not to be detrimental to survival. Moreover, the fact that rodent growth plates do not fully fuse during their lifetime has long been quoted as a limitation of the use of mouse models for studying skeletal biology. However, the data shown by Haseeb et al., with their careful description of growth plate thickness during ageing, align with results from a study that characterized the formation of bony fusions across the murine growth plate[5] and suggest that differences between mice and humans are of limited functional consequence.

Haseeb et al. build on their previous work and the strong evidence provided by others[6], which implies accelerated chondrocyte-to-osteoblast 'trans-differentiation' in the absence of Sox9. In the new study[3], the authors show that chondrocytes initiate an osteoblastic marker expression programme while still embedded in their cartilage extracellular matrix. Careful single-cell RNA sequencing suggests that, rather than direct chondrocyte-to-osteoblast conversion, this process instead involves reversion to a progenitor cell type prior to maturation along the osteoblastic pathway. This switching of *Sox9*-deficient chondrocytes to an osteoblastic pathway seems to mostly skip the pre-hypertrophic or hypertrophic stages, which is somewhat different from what occurs in wild-type mice, in which hypertrophic chondrocytes are thought to trans-differentiate into osteoblasts[7].

One puzzling finding from previous attempts to elucidate Sox9 function in the postnatal skeleton was the very subtle phenotype in cartilage in mature mice[2]. In the new study[3], the authors show that Sox9 is indeed required for mature articular cartilage homeostasis. In the absence of challenges, changes were still subtle in *Sox9*-deficient cartilage, as previously shown, and were confined to load-bearing regions. However, after surgery to induce post-traumatic OA in the knee, disease progression was much more rapid in the absence of Sox9 than in wild-type mice. These data confirm that Sox9 is required to keep adult articular cartilage healthy, particularly in response to mechanical stress. The extension of these studies to additional models of OA would be of interest.

Similar to growth plate chondrocytes, loss of Sox9 seems also to promote the differentiation of articular chondrocytes into the osteoblastic lineage[3]. Thus, Sox9 is required in both growth plate and articular chondrocytes to prevent osteogenic differentiation. Nevertheless, substantial differences seem to exist between these two types of chondrocytes.

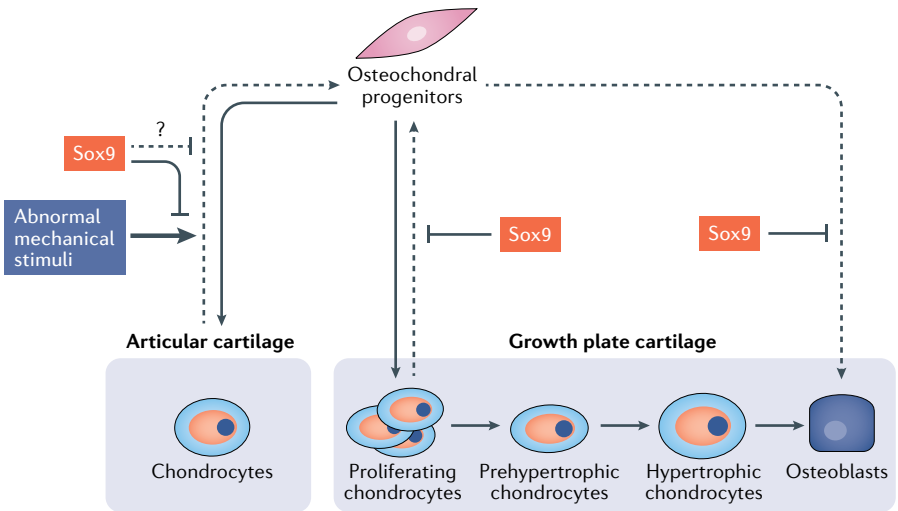

Fig. 1 | **Role of Sox9 in determining chondrocyte fate.** The transcription factor Sox9 serves essential homeostatic postnatal roles in both growth plate cartilage and articular cartilage. By stabilizing the function of chondrocytes, Sox9 influences chondrocyte-to-osteoblast transition by restricting the initial reversion to a progenitor status prior to osteogenic lineage commitment. Likewise, Sox9 is required to keep adult articular cartilage healthy, particularly in response to abnormal mechanical stress.

> Sox9 is required to keep adult articular cartilage healthy, particularly in response to mechanical stress

For example, loss of Sox9 induces more pronounced effects on expression of *Col2a1* and *Acan* transcripts in growth plate versus articular chondrocytes[3], suggesting additional transcriptional regulators controlling expression of these genes in the latter; the identification of these regulators would be compelling as a means of controlling chondrocyte fate.

The existence of active Sox9-mediated restriction of the osteogenic programme in chondrocytes is intriguing and raises many questions. For example, did cartilage evolve hard-wired with its own osteogenic off-switch? Cloning analyses in cartilaginous fish suggest that Runt-related transcription factor (RUNX) genes (*RUNX2* is important for osteoblast differentiation) indeed contribute to a core gene network for cartilage formation[8]. This finding is supported by molecular fingerprinting in zebrafish and spotted gar showing that chondrocytes and osteoblasts both express *sox9*, *col2a1* and *col10a1* (which tetrapod osteoblasts do not)[9], and by data showing that SOX9 directly interacts with RUNX2 to repress its activity at evolutionarily conserved domains[10]. Functions for osteoblast programmes in early cartilages, before co-option for osteogenesis, add to the view that Sox9 (which is not expressed in vertebrate bone) has retained its ancient roles in cartilage. These roles include Sox9-mediated restriction of osteoblastic differentiation, as revealed by Haseeb et al.[3], which seems to be more pronounced in growth plate cartilage than in articular cartilage, unless the latter is mechanically challenged.

Another question is why the study by Haseeb et al. revealed functions of Sox9 that had not been described in previous studies. This discrepancy seems to be partially due to the timing of Sox9 ablation in different studies; in the 2012 study[2], Sox9 expression was switched off in 3-week old mice, whereas in the new study[3], Sox9 was switched off at 4 weeks or at 3 months. When Sox9 ablation was triggered at 3 weeks, the authors found compression but not complete closure of the growth plate 3 weeks later[2]. By contrast, when triggered at either 4 weeks or 3 months of age (both in Aggrecan-CreER^{T2} mice), complete growth plate closure occurred within a few weeks[3]. Thus, the described differences between the models are minor and are potentially related to the specific maturity window in which Sox9 is inactivated. Importantly, previous studies had examined articular cartilage during homeostasis and ageing without additional challenges such as the knee destabilization surgery that provided a basis for the profoundly more severe articular cartilage phenotype reported in the new study.

As with every study, there are limitations to the study by Haseeb et al.[3] that need consideration; for example, by the nature of single-cell RNA sequencing, cells had to be enzymatically released from their authentic extracellular matrix for analyses, which could have altered gene expression patterns. Whether the growth plate and articular cartilage phenotypes are completely independent or sequentially linked (for example, through altered mechanical loads) is also unclear. However, these are minor concerns in our view; they are far outweighed by the exciting new insights into Sox9 function in postnatal growth plate and articular cartilage that the study provides. Haseeb et al. also provide a multitude of bulk and single-cell RNA sequencing datasets that should provide a rich resource to direct future work, such as further elucidating fundamental differences between articular and growth plate chondrocytes.

The study by Haseeb et al.[3] reveals essential postnatal roles for Sox9 in growth plate and articular joint cartilage; exposure of the latter to mechanical joint trauma has intriguing consequences in our search for mechanisms underpinning OA. A homeostatic role for Sox9 in suppressing potential chondrocyte reversion to a progenitor state en route to an osteoblastic fate within cartilaginous environments is both fascinating and yet consistent with conserved evolutionarily relationships between cartilage and bone.

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https://doi.org/10.1038/s41584-021-00628-9

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Competing interests
The authors declare no competing interests.

CLINICAL TRIALS

Striking a balance in rheumatoid arthritis prevention trials

Liam J. O'Neil and Kevin D. Deane

The discovery that autoantibodies and other factors can predict the future onset of rheumatoid arthritis (RA) has encouraged the development of clinical trials looking at RA prevention. Although an exciting area of research, finding an approach that results in the successful completion of an RA prevention trial is challenging.

Refers to van Boheemen, L. et al. Atorvastatin is unlikely to prevent rheumatoid arthritis in high risk individuals: results from the prematurely stopped STAtins to Prevent Rheumatoid Arthritis (STAPRA) trial. *RMD Open* **7**, e001591 (2021) | van Boheemen, L. et al. How to enhance recruitment of individuals at risk of rheumatoid arthritis into trials aimed at prevention: understanding the barriers and facilitators. *RMD Open* **7**, e001592 (2021).

Rheumatoid arthritis (RA) is usually diagnosed and treated when an individual develops clinical signs of inflammatory arthritis; however, a period exists before the onset of clinically apparent disease in which circulating autoantibodies that are highly predictive of future RA are detectable. On the basis of this predictive capacity, multiple trials are either underway or have been completed in which autoantibody-positive individuals (with or without arthralgia) receive interventions to prevent or delay the onset of clinically apparent inflammatory arthritis[1]. However, researchers conducting RA prevention trials face many challenges related to study design and participation, several of which are highlighted in two articles published by van Boheemen et al.[2,3].

The first article describes the results of the STAPRA trial[2], which investigated the use of statins as preventive medication for RA. In this trial, individuals who had arthralgia in the absence of clinically apparent inflammatory arthritis and either had high amounts of anti-citrullinated protein antibodies (ACPAs; greater than three times the upper limit of normal) or had any amount of APCAs and were positive for rheumatoid factor at baseline were randomly allocated to receive either daily atorvastatin or placebo for 3 years. Atorvastatin was selected because its immunomodulatory effect might be beneficial in the early stages of RA and because it is already widely used to prevent cardiovascular disease and has an established safety profile. The sample size for the trial was set at 220 participants on the basis of an expected transition rate to future inflammatory arthritis of 55%, and the expected effect of the study drug (21% risk reduction); however, only 62 participants were randomly allocated and followed for a median of 14 months. Overall, 15 individuals (24%) developed inflammatory arthritis (9 (29%) in the treatment group and 6 (19%) in the placebo group) — a difference that was not statistically significant[2]. The authors concluded that atorvastatin was unlikely to prevent or delay progression to inflammatory arthritis, perhaps because its biological effect was inappropriate or insufficient to interrupt the early stage of RA development. However, another important finding from this study was the difficulty in recruiting study participants. Indeed, of the 175 eligible individuals recruited, 62% were unwilling to participate[2], primarily owing to unwillingness to use the trial medication or a perceived high study burden.

This latter issue was further addressed in the second article by van Boheemen and colleagues, which focused on understanding the barriers and facilitators to recruitment in RA prevention trials[3]. In this study, 18 individuals who were eligible for inclusion in two RA prevention studies (STAPRA[2] and APIPPRA[4]) were recruited to participate in focus group discussions, half of whom had participated in the prevention studies and half of whom had declined. The two studies had similar inclusion criteria but differed in the intervention used; APIPPRA used subcutaneous injection of abatacept, whereas STAPRA used an oral statin. Several major themes were identified by van Boheemen et al.[3] as being important to an individual's decision to participate in a prevention trial. In particular, barriers to participation included a lower personal perceived risk of RA, fear of adverse effects from the study drug, minor joint symptoms and the time investment required. By contrast, facilitators included a greater knowledge of RA, perception that the study drug would improve existing joint symptoms, the ability to have a close medical follow-up, a feeling that an individual's symptoms and concerns were being taken seriously, and altruism and an opportunity to contribute to society.

Multiple factors can affect the 'balance' of developing and completing successful clinical trials (FIG. 1); however, these two articles[2,3] highlight two important inter-related factors — identifying an effective intervention and encouraging participation in trials. The STAPRA investigators discovered that atorvastatin is not an ideal preventive agent for RA[2]. Similarly, in 2019 the PRAIRI study demonstrated that rituximab delayed but did not prevent future RA[5]. As such, the quest will continue for the right intervention to prevent RA. Notably, the results of other RA prevention trials that are using agents known to work in established RA, including methotrexate, hydroxychloroquine and abatacept, are eagerly awaited. However, new targets and new interventions are also likely to be needed[3]; promising candidates include targeting mucosal processes that might have a role in RA development[6] and non-pharmacological interventions such as dietary interventions, nutritional supplements and lifestyle modifications.

> "the expected efficacy of an intervention is critical to the willingness of participants to join a trial"

In addition to safety and tolerability, the expected efficacy of an intervention is critical to the willingness of participants to join a trial. Such issues are influenced by the accuracy and strength of prediction of future RA, as well as how an individual perceives their personal risk of RA. In the focus group study by van Boheemen et al.[3], a perceived risk of future RA of less than 60% was a major barrier to participation for individuals who were eligible for inclusion in prevention trials. This finding aligns with other studies that also demonstrated that a higher perceived risk of RA was associated with increased willingness to participate in trials, and potentially also with increased willingness to take an intervention with greater adverse effects[7]. Importantly, there might be additional cultural considerations to account for when planning preventive efforts in RA, particularly in individuals who are predisposed to RA such as Indigenous populations in North America[8]. Many Indigenous communities in North America are located in remote areas that are far away from tertiary health centres, and individuals in such communities might desire to integrate

Indigenous healing practices into prevention strategies[9].

Another factor that potentially affects the recruitment of individuals into prevention trials is the nomenclature used to describe the risk of future RA. The terms 'pre-clinical RA' and 'pre-RA' have been widely used in the literature and are similar to terms used in other diseases (such as pre-diabetes); however, it has been suggested that these terms should only be used to describe an at-risk state if classifiable RA develops in the future[10]. The term 'at-risk' is also widely used (and was used in the STAPRA study[2]), although it is important to remember that risk exists on a spectrum, with clinical relevance depending to some extent on an individual's perception and values. Moreover, terms such as pre-RA and at-risk might not adequately capture symptomatology that is important to how individuals perceive their current condition and their risk of future RA. Thus, a clear need exists for consensus definitions of nomenclature to ensure that prevention studies are both generalizable and reproducible, as well as to improve communication with individuals who could participate in such studies.

In summary, as highlighted in the studies by van Boheemen et al.[2,3], researchers conducting RA prevention trials face challenges in finding the right balance between study design (selecting plausible biologic interventions and the accuracy of RA prediction) and participation (educating individuals about their personal risks and benefits to inform their decision to participate). However, there is hope that participation in prevention trials can be acceptable to at-risk individuals; the completed PRAIRI study enrolled 81 participants[5], and the ongoing APIPPRA study has fully enrolled at 213 participants (A. Cope, personal communication), even though these two studies have the potential barrier of the use of a biologic therapy (rituximab and abatacept, respectively). Such ongoing trials and additional research should soon provide important insights that will be clinically actionable in this exciting, emerging area of RA prevention.

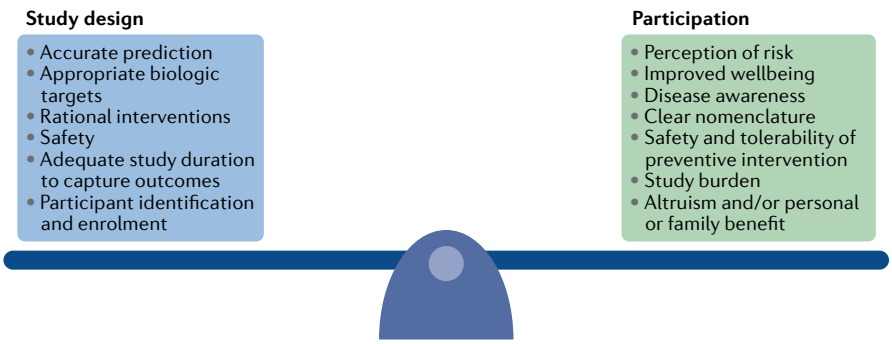

Fig. 1 | **Balancing factors related to study design and participation in RA prevention trials.** Factors that influence clinical trial design in rheumatoid arthritis (RA) prevention trials need to be balanced against factors that affect the participation of individuals in these trials.

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Competing interests
The authors declare no competing interests.

Ankylosing spondylitis: an autoimmune or autoinflammatory disease?

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Abstract | Ankylosing spondylitis (AS) is a chronic inflammatory disorder of unknown aetiology. Unlike other systemic autoimmune diseases, in AS, the innate immune system has a dominant role characterized by aberrant activity of innate and innate-like immune cells, including γδ T cells, group 3 innate lymphoid cells, neutrophils, mucosal-associated invariant T cells and mast cells, at sites predisposed to the disease. The intestine is involved in disease manifestations, as it is at the forefront of the interaction between the mucosal-associated immune cells and the intestinal microbiota. Similarly, biomechanical factors, such as entheseal micro-trauma, might also be involved in the pathogenesis of the articular manifestation of AS, and sentinel immune cells located in the entheses could provide links between local damage, genetic predisposition and the development of chronic inflammation. Although these elements might support the autoinflammatory nature of AS, studies demonstrating the presence of autoantibodies (such as anti-CD74, anti-sclerostin and anti-noggin antibodies) and evidence of activation and clonal expansion of T cell populations support an autoimmune component to the disease. This Review presents the evidence for autoinflammation and the evidence for autoimmunity in AS and, by discussing the pathophysiological factors associated with each, aims to reconcile the two hypotheses.

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https://doi.org/10.1038/s41584-021-00625-y

Spondyloarthritis (SpA) is a group of inflammatory diseases that involve the spine, peripheral joints and entheses. Owing to the high degree of clinical variability in SpA, this group of conditions is currently sub-classified according to the clinical manifestations present into two main groups: peripheral SpA (which includes psoriatic arthritis (PsA)) and axial SpA (axSpA), which includes radiographic axSpA (also known as ankylosing spondylitis (AS)) and non-radiographic axSpA. AS, the second most common entity in the SpA group, is a chronic inflammatory disorder for which the pathogenesis is incompletely understood. The main feature of axSpA is inflammation of the spine and of the sacroiliac joints, leading to aberrant bone remodelling and ankylosis. Peripheral involvement includes enthesitis, dactylitis and arthritis. The most common extra-articular manifestations are uveitis, psoriasis and inflammatory bowel disease (IBD)[1]. Data from genetic studies and experimental models indicate the involvement of the IL-23–IL-17 axis in the pathogenesis of SpA, and IL-17 blocking agents are the cornerstone of the current treatment of this disease[2].

Autoinflammatory diseases are mostly characterized by unprovoked inflammation induced by activation of the innate immune system in the absence of high-titre autoantibodies or autoantigen-specific T cells[3]. In particular, autoinflammatory diseases are defined by the presence of genetically perturbed alterations of innate immunity, local tissue factors having effects at disease prone-sites and predominant innate immune activation[4]. By contrast, autoimmune diseases are pathological conditions caused by an aberrant and prolonged adaptive immune response to a self-antigen, mediated by T cells and B cells[5]. Unlike autoantibody-positive autoimmune diseases, in which antibodies and autoantigen-specific T cells have essential roles, in AS, these features have been difficult to demonstrate, whereas substantial evidence exists for involvement of the innate immune system, suggesting a more autoinflammatory type of disease.

The hypothesis of an autoinflammatory genesis of AS is not entirely new; several reviews have analysed the preponderance of the autoinflammatory component over the autoimmune in this disease[6,7]. However, since the idea was first suggested, new evidence has emerged that provides more robust support for the hypothesis. A critical analysis of the factors supporting an autoinflammatory versus an autoimmune origin for AS offers the opportunity to dissect the genetic, immunological and environmental contributors to the genesis of this

Key points

- The pathogenesis of ankylosing spondylitis (AS) is not fully understood, despite advances in understanding some of the underlying mechanisms.
- Genetic studies and the effects of local tissue factors, such as biomechanical stress and bacterial products, support the importance of a chronic innate immune response in AS.
- Innate and innate-like immune cells can be found at sites of disease and probably represent the major source of IL-17 production in AS.
- Immune pathways such as inflammasome activation, autophagy and ubiquitination are involved in both innate and adaptive immunity in AS.
- The presence of an autoimmune response accompanied by the production of specific autoantibodies is a growing concept in AS.
- Both autoinflammatory and autoimmune factors participate in the pathogenesis of AS in a probable continuum.

disease and to the clinical variability observed every day in rheumatology clinics. However, one should not consider the two entities — autoinflammation and autoimmunity — as dichotomic, but rather as two extremities of a continuum that, in different measures and combinations, produces the clinical whole. As such, the current classification system for SpA, which is based on clinical and non-pathophysiological elements, could represent a conceptual error in the cognitive approach to the nature of the disease.

In this Review, we discuss the pathophysiological factors commonly associated with autoinflammatory and autoimmune diseases (including genetic associations with innate and adaptive immune pathways, local tissue factors at disease-prone sites, and innate and adaptive immune activation), and present evidence to try to reconcile the two hypotheses in relation to the pathogenesis of AS.

Genetics of AS

Host predisposition, either monogenic or polygenic, is an essential factor in the pathogenesis of autoinflammatory diseases[4]. Conversely, monogenic autoimmune diseases are considerably less common than monogenic autoinflammatory diseases, and genetics seem to instead have an additive role in influencing the risk of developing autoimmune diseases. Substantial progress has been made in the past decade in mapping genetic loci associated with AS. To date, at least 114 independent genetic associations have been definitively associated with AS, including 39 that achieve genome-wide significance in analyses of AS on its own[8]; the remainder were identified through analysis of pleiotropic genetic effects associated with AS, psoriasis, IBD and primary sclerosing cholangitis. The genetic heritability of AS is, however, strongly influenced by variation in the MHC region, which accounts for around 40–50% of the total genetic risk of developing the disease, and specifically with positivity for HLA-B27, which explains around 20–30% of the genetic risk[9]. Crucial findings from these associations include the discovery of the important roles of the IL-23 pathway and of aminopeptidase enzymes in AS predisposition, the striking involvement of genes encoding factors involved in gut immunopathology, the overlap of genetic associations with clinically related seronegative diseases (indicating shared aetiopathogenic mechanisms), and the multiple potential therapeutic targets that genetic studies have put forward[10]. Collectively, the genetically related perturbations of innate and adaptive immune function that occur in patients with AS support the view that AS is a polygenic innate immune disease.

IL-23 pathway

In 2007, the identification of *IL23R* as a risk locus in AS[11] shed light on the previously unknown involvement of the IL-23 pathway in the pathogenesis of this disease. Several pieces of evidence have identified a role for the IL-23 signalling axis in both autoinflammatory and autoimmune diseases[12,13]. The discovery that the same variants are also associated with psoriasis and IBD, as well as the previously identified role for the IL-23 pathway in experimental models of IBD[14], provided support for the repositioning of agents that target this pathway for IBD and other forms of SpA. However, although genetic discoveries can, in some instances, provide information about the possible involvement of certain factors in pathogenesis, they cannot predict with absolute confidence the outcome of clinical trials. Hence, IL-17 blockade is efficacious in AS and PsA[15–18], but not in IBD[19], whereas IL-23 blockade shows promise in IBD[20] but not in AS[21], although the latter has a potential genetic explanation given that *IL23A* is associated with IBD but not with AS[22]. Another potential explanation for these disease-specific differences is that the risk alleles that contribute to the development of the disease are not necessarily involved in its perpetuation and, therefore, might not be useful therapeutic targets.

In addition to *IL23R*, several risk genes identified in AS are either directly involved in the IL-23–IL-17 pathway or interact with it, including *IL12B, RUNX3, EOMES, TBX21, TYK2, CARD9, IL1R1, IL1R2, IL6R, IL7R, IL12B, IL27, NKX2* and *PTGER4* (REFS[23–26]). Given its role as an innate immune receptor for lipopolysaccharide (LPS), the association of *TLR4* genetic variants and *TLR4* expression levels with AS implicates innate immunity in AS pathogenesis[27,28]. This association is further supported by evidence that serum concentrations of LPS are increased in patients with AS and correlate with disease activity[29].

MEFV variants

Genetic findings support a substantial role for autoinflammation in AS. A 2019 study in Turkish and Iranian individuals demonstrated a significant association between variants of *MEFV* and the risk of AS; odds ratios for the risk of AS with the M694V variant were 4.3 and 7.8 in Turkish HLA-B27-positive patients with AS and HLA-B27-negative patients with AS, respectively[30]. *MEFV* encodes pyrin, an important promoter of autoinflammation, and is associated with the classic autoinflammatory disease familial Mediterranean fever (FMF); however, the association between AS and *MEFV* variants is not sufficient to prove an autoinflammatory aetiology of AS. Interestingly, axSpA is often reported as a comorbidity in patients with FMF (both HLA-B27-positive and HLA-B27-negative). In a study of 201 patients with FMF, back pain was reported by 157 individuals (78%), and 15 (7.5%) satisfied the modified New York diagnostic criteria for AS[31]. These observations could suggest a common genetic substrate for FMF and AS,

Entheses
The sites at which ligaments and tendons attach to the bones.

Epistatic interaction
Interaction among gene alleles at multiple locations that influence the phenotype.

Ectopic lymphoid neogenesis
The formation of lymphoid structures in the target tissues of chronic inflammation.

as well as a possible common autoinflammatory aetiology. Consistently, the M694V variant of *MEFV* was the most common variation in patients with FMF who had radiographic and MRI evidence of sacroiliitis[31–33]. In line with this consideration, anecdotal evidence from case reports suggests that AS manifestations respond well to treatment with the IL-1 receptor antagonist anakinra in patients with FMF[34,35].

Endoplasmic reticulum aminopeptidases

The discovery of the association between AS risk and the chromosome 5q15 locus harbouring *ERAP1* and *ERAP2*, which encode endoplasmic reticulum aminopeptidase 1 (ERAP1) and ERAP2, demonstrated clearly that altered antigenic peptide processing could be a relevant mechanism in the pathogenesis of AS[36]; ERAP1 and ERAP2 trim peptides in the endoplasmic reticulum (ER) that are required for MHC class I presentation[37]. This assumption was further supported by the association between AS risk and *NPEPPS*, which encodes another aminopeptidase that is important for the processing of MHC class I-restricted antigens[38]. Strikingly, the association of *ERAP1* with AS is restricted to individuals who are positive for either *HLA-B27* or *HLA-B40* (if they are *HLA-B27* negative)[22,39]. *ERAP1* is also associated with *HLA-Cw6* in patients with psoriasis[40], *HLA-B51* in patients with Behçet syndrome[41] and *HLA-A29* in patients with birdshot retinopathy[42]. The strong epistatic interaction that links *HLA-B27* and *ERAP1* (but not *ERAP2*) in AS and other MHC class I-related diseases strongly suggests common genetically determined pathogenic pathways in these diseases. For all MHC class I-associated diseases (with the exception of Behçet syndrome), loss-of-function genetic variants of either *ERAP1* or *ERAP2* are associated with disease protection[43]. These findings implicate peptide handling abnormalities as important underlying factors in MHC class I-associated diseases. *ERAP* genetic variation also has a marked effect on peptide presentation by disease-associated HLA alleles[44]. Given the role of MHC class I molecules in presenting potentially antigenic peptides to $CD8^+$ T cells, these genetic associations strongly suggest the involvement of the adaptive immune system in not just AS but also in psoriasis, Behçet syndrome and birdshot retinopathy[43,45].

Multi-omics studies

Analysis of the intersection between the most strongly disease-associated single nucleotide polymorphisms (SNPs) and epigenetic markers of gene 'activity' provides a hypothesis-free approach to identifying the types of cells and tissues involved in disease. A prediction model performed on SNPs identified in genome-wide association studies and epigenetic marks that was developed to identify causal SNPs and the immune cells that were most likely to be involved in AS pointed mainly to $CD4^+$ T cells (including T helper 17 (T_H17) cells), B cells and other cell types including monocytes and gut mucosal cells[46,47]. These results suggest that disease in AS is not promoted purely by one arm of the immune system, and that diverse factors contribute to pathogenesis, including adaptive and innate immune cells and barrier function components. Only a limited amount of data are currently available on transcriptomic profiles of either immune cells or gut mucosal cells in AS, including expression quantitative trait loci studies. Data obtained from healthy individuals can miss important mechanistic effects; for example, *TBX21* genotypes influence T-bet expression in patients with AS but not in healthy individuals[48]. Further transcriptomic studies in cells from patients with AS will be critical in determining the functional genomic mechanisms that underpin the genetic associations.

Local tissue factors

The propensity to develop disease in a specific location as a function of local tissue factors, such as trauma and/or biomechanical stress or the interaction between bacteria or bacterial products and the innate immune system, is important in the pathogenesis of autoinflammatory diseases[4]. Conversely, in autoimmune diseases, clinical disease manifestations are determined by events taking place in the target tissues; ectopic lymphoid neogenesis often occurs at these locations, potentially contributing to the maintenance of autoimmunity through the production of disease-specific autoantibodies[49]. Although ectopic lymphoid structures have been found in the inflamed intestines of patients with AS[50], specific studies are required to demonstrate the occurrence of ectopic lymphoid neogenesis phenomena in AS at other disease sites. In AS, biomechanical factors and complex interactions between bacteria and bacterial products seem to be important underlying factors for disease, suggesting a predominant autoinflammatory basis of inflammation in AS.

Biomechanical stress

Just as symptoms in patients with axSpA cannot be fully uncoupled from the mechanical load imposed on the joints or spine, so the pathophysiology of the disease is also connected to biomechanical stress[51]. Current evidence and hypotheses suggest that biomechanical factors have an essential role in both the initiation and structural progression of the disease[52,53].

The onset of SpA has been strongly linked to inflammation at the entheses[54–56], which are prone to microdamage under repetitive or increased strain. A diagnostic marker of the early phase of SpA is bone marrow oedema; interestingly, entheses insert into areas of bone in close proximity to bone marrow. In a 2018 study, bone marrow oedema was identified in an average of three to four sacroiliac joint quadrants in 30–41% of recreational and elite athletes, meeting the Assessment of Spondyloarthritis International Society (ASAS) definition of active sacroiliitis[57]; consistent data were reported in a study of military recruits in the Netherlands[58]. Inflammatory changes also occur in the sacroiliac joints of women who have given birth from post-partum to up to 6 months after delivery[59], providing additional evidence that mechanical strain can contribute to typical features of sacroiliitis. These surprising findings implicate entheseal biomechanical stress in inducing bone marrow inflammatory responses in healthy individuals[57] and suggest the hypothesis that entheseal biomechanical stress could promote chronic self-perpetuating

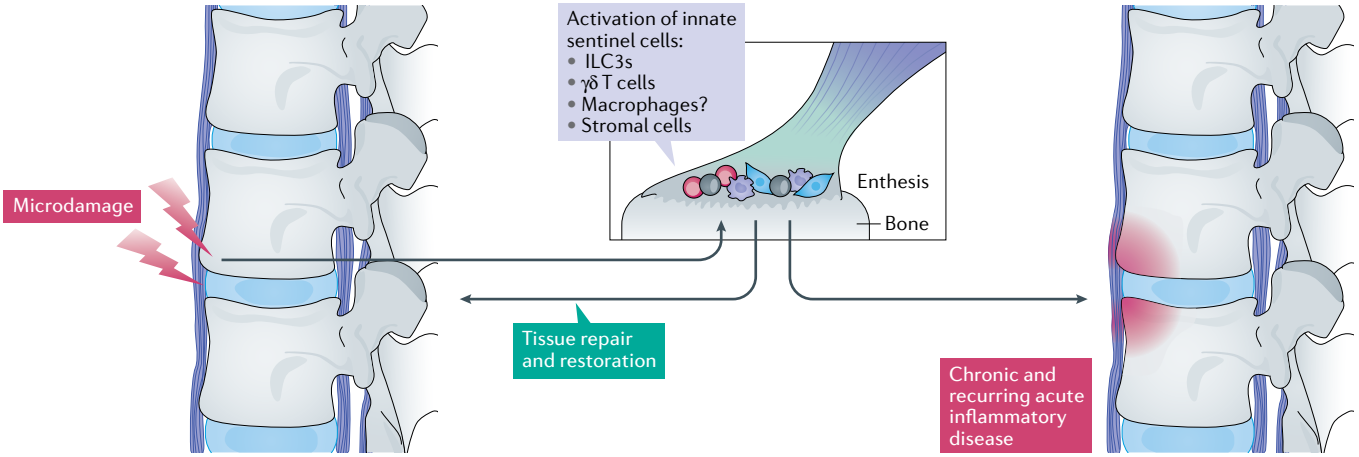

Fig. 1 | **Contribution of biomechanical stress to the onset of axial disease in ankylosing spondylitis.** The entheses are sites of biomechanical stress, which can induce microdamage. Innate immune cells (such as group 3 innate lymphoid cells (ILC3s), γδ T cells and, possibly, macrophages) and stromal cells are hypothesized to be part of the repair response to microdamage, potentially functioning as sentinel cells that detect damage. The successfully orchestrated repair response results in tissue restoration; however, in the presence of certain genetic or environmental factors, such as HLA-B27 positivity, the repair response is hypothesized to become too strong and thereby trigger clinically relevant inflammation. Bidirectional communication between the entheses and osteitis that develops in the underlying bone marrow seems to be critically important in this process.

inflammation in genetically predisposed individuals. More in-depth studies are clearly needed to understand whether bone marrow oedema is the consequence or the cause of entheseal inflammation; if it is the cause, then local bone marrow-derived cells such as monocytes could have a greater role in the pathogenesis of AS than previously appreciated.

These observations also point towards a potential role for local factors rather than systemic factors in disease initiation. In particular, the presence of resident immune cell populations in the enthesis[60–62] could provide a link between microdamage, interactions with myeloid cells and subsequent inflammation in AS (FIG. 1). Whether these cells are actually resident in entheses or derive from the adjacent bone marrow or distant inflamed sites, such as the gut in AS or the skin in PsA, remains to be clarified. Nevertheless, the concept of tissue-resident immune cells acting as sentinels to orchestrate repair responses to different degrees of damage (ranging from micro-trauma to large-scale trauma), involving both the activation of local cells and the recruitment of distant populations, is an attractive explanation for SpA features.

Importantly, the onset and progression of disease are likely to differ in nature. Whereas biomechanical factors in the initiation of disease have been directly studied in mouse models of AS[63], data around their role in disease progression are scarcer. Nevertheless, in humans and in mouse models of AS, mechanical factors (particularly instability) are thought to contribute to radiographic disease progression[64,65]. Once initiated, spinal inflammation is associated with trabecular bone loss, thereby changing the biomechanical properties of the spine. The presence of inflammation in the vertebral bodies probably interferes with the normal bone remodelling processes, thus inhibiting the osteoblastic response towards bone loss[66]. However, the exact role of bone cells in disease progression in AS and the mechanisms by which bone loss and new bone formation occur at sites of inflammation requires further study (BOX 1).

Microbiome

Although the aetiology of AS is unknown, data from experimental models and from humans suggest that bacteria have a role in the initiation and propagation of AS (and other forms of SpA) in genetically predisposed individuals. The composition of the caecal microbiome of Lewis rats transgenic for human HLA-B27 and β_2-microglobulin was dysbiotic compared with the microbiomes of wild-type Lewis rats[67]. Similarly, under controlled specific pathogen-free conditions, BALB/c ZAP70^{W163C} SKG mice have an IL-23-dependent faecal dysbiosis but remain healthy[68,69]; however, SpA develops in these mice after the administration of systemic microbial β-glucan, which causes increased IL-23 production and the promotion of T_H17 cells[70]. SKG.ZAP70-deficient heterozygotes, which have more severe immunodeficiency, develop spontaneous arthritis even under specific pathogen-free conditions[71], demonstrating that adaptive immunity is critical for the host–microbe interface and disease susceptibility.

A growing number of studies have also demonstrated the presence of intestinal dysbiosis in patients with AS, which correlates with disease activity and immune cell activation[72–76]. The main findings of microbiome studies in patients with SpA are listed in TABLE 1. Fundamental problems arise in the interpretation of these studies owing to the methodological variability in microbiome sequencing and analysis, the heterogeneity of the samples analysed and the different ethnicities and diets of the patients enrolled. Intuitively, dysbiosis is not a static process, and the effects of biologic agents can potently influence the gut microbiome composition in patients with AS. In particular, the effect of treatment on AS intestinal dysbiosis has been assessed in two studies

SKG mice
Mice with attenuated T cell receptor signalling that develop spontaneous inflammatory arthritis with extra-articular manifestations, including inflammatory bowel disease, under conventional conditions.

Mechanostat
A cellular and molecular system that senses the mechanical strain exerted on bones.

Paneth cells
Specialized epithelial cells located at the bottom of intestinal crypts that contribute to the maintenance of sterility in the crypts.

Zonulin
A molecule that modulates the permeability of tight junctions between intestinal epithelial cells.

from 2019. In a study of a case–control cohort of 250 Han-Chinese individuals, therapy with TNF inhibitors was accompanied by a normalization of the altered microbiome that was observed in untreated patients with AS compared with that of healthy individuals[77]. TNF inhibitor therapy in patients with AS was also associated with a reduction in the concentration of potentially arthritogenic bacterial peptides compared with untreated patients. In a second study of 15 patients with PsA or SpA and 15 healthy individuals from the USA, IL-17 inhibitor treatment resulted in statistically significant changes in the concentrations of specific taxa in patients with SpA compared with those treated with TNF inhibitors, particularly in Clostridiales spp. and *Candida albicans*[78]. These changes were related to changes in the composition of the bacterial community, the metabolic pathways involved and related cytokines. Ileal tissue samples showed that, in patients with SpA who developed clinically manifest Crohn's disease during treatment with IL-17 inhibitors, specific expansion of IL-25-producing group 2 innate lymphoid cells (ILC2s) occurred[78].

Although the association between AS and intestinal dysbiosis seems to be genuine, mechanistic and functional data causally linking the dysbiosis to the activation of innate and adaptive immune cells in AS are still sparse. In addition, the study of microbial composition does not provide information about the interaction and effect of microbial species on host mucosal tissues and immune cells. In vitro experiments have demonstrated an increase in IFNγ-producing cells in response to a bacterial peptide produced by species that are enriched in the guts of patients with AS, which mimics type II collagen[79]. Bacterial peptides with homology to HLA-B27-presented epitopes are enriched in the stool of patients with AS, suggesting that these peptides could be involved in the induction and maintenance of immune reactions in patients with AS[77]. Notably, HLA-B27 has a major effect on the gut microbiome profile at several locations, even in the absence of disease, supporting the idea that the dysbiosis seen in AS is at least partly influenced by AS-associated genes, rather than being secondary to disease or its treatment[80].

The reactivity of intestinal IgA molecules towards gut-resident bacteria has also been investigated; sequencing of IgA-coated bacteria from patients with Crohn's disease-associated SpA revealed an enrichment in adherent-invasive *Escherichia coli* that could trigger mucosal T_H17 cell-mediated immune reactions upon transfer into germ-free mice[81]. These observations show the ability of SpA-associated microbes to induce a T_H17 cell response and, possibly, a specific adaptive IgA response. Consistently, previous reports had demonstrated an increase in serum IgA concentrations in patients with AS, reactive arthritis or undifferentiated arthritis[82–84]. Interestingly, experiments in a rat model of SpA suggest the involvement of HLA-B27 in determining the IgA response toward intestinal bacteria[85].

Considering the effect of dysbiosis on the intestinal mucosa, ileal Paneth cells are activated in patients with AS and produce increased amounts of antimicrobial peptides[29]. The presence of dysbiosis is in patients with AS is associated with profound alterations of both the gut epithelial barrier (including downregulation of the tight junction proteins claudin 4 and occludin and the upregulation of zonulin) and the gut vascular barrier (including downregulation of the cell–cell adhesion molecules VE-cadherin and JAMA and the upregulation of plasmalemma vesicle-associated protein, a marker of endothelial cell permeability). In turn, these alterations are associated with the translocation of intestinal bacterial products into the blood in patients with AS, which substantially modulates the phenotype and function of circulating monocytes[29].

Taken together, these findings indicate that dysbiosis occurring in the gut in AS profoundly modulates the integrity of the intestinal barrier and local and systemic innate immune responses. However, although alterations in intestinal microbiota composition and increased intestinal permeability have been mainly linked to the activation of innate cells, innate-like cells and subsets of T cells in SpA, changes in the microbiome also occur in diseases characterized by a prominent autoimmune signature, such as systemic lupus erythematous (SLE) and type 1 diabetes[86–88], suggesting that dysbiosis per se is not exclusive to autoinflammation.

Innate immunity in AS

A large body of evidence has accumulated in the past 10 years indicating a predominant activation of immune pathways in patients with AS. Although the activation

Box 1 | Radiographic progression in ankylosing spondylitis

Ankylosing spondylitis (AS) is characterized by bone loss at sites of inflammation occurring in close proximity to new bone formation. The genes robustly identified to date as important in AS do not implicate bone cell types, although the bone-formation phenotype of AS is striking. This discrepancy could be partially explained by considering the bone formation process as a secondary phenomenon that is not genetically detected by comparing patients with healthy individuals. Limited data are available on the progression of new bone formation from a genetic point of view. The AS-associated gene *PTGER4* is an important component of the mechanostat, contributing to the effect of physical stress on promoting IL-23 production[224,225]. Genetic studies of the extent of radiographic disease in AS have also revealed associations with *PTGS1* (involved in pro-inflammatory prostaglandin production) and *RANK* (involved in osteoclastogenesis and in the interaction between T cells and dendritic cells)[226]. To date, these two genes have not been associated with susceptibility to AS, nor is the major gene for AS susceptibility, *HLA-B27*, associated with the extent of radiographic change[226]. These findings indicate that although some overlap exists between the pathogenic mechanisms that influence susceptibility to AS, there are also clearly major differences, suggesting that pathways not involving HLA-B27 are the main determinants of radiographic change in the disease.

A hypothetical model for the bone phenotype in AS is that progenitor cell populations residing in the entheses and periosteum participate in the growth of syndesmophytes and enthesophytes in an aberrant attempt to stabilize the spine, with the consequence of structural damage in the form of ankylosis. This model might explain how bone can be both lost and formed close to sites of inflammation, and also provides a basis for understanding the time-frame required to control and measure new bone formation. Indeed, long-term follow-up data of cohorts of patients with AS treated with TNF inhibitors indicate that an effect on structural disease progression only becomes apparent after several years[227,228]. In the initial phase of treatment, instability caused by inflammation-induced bone loss might be the primary factor underlying structural disease progression. Over time, as inflammation subsides and the normal bone remodelling cycle in the spine resumes, this promoter of disease is no longer active. Data from patients with axial spondyloarthritis corroborate this hypothesis, as trabecular bone loss is longitudinally associated with spinal radiographic progression of disease[229]; the more severe the trabecular bone loss, the stronger the effect on the progression of spinal disease.

Table 1 | **Changes in gut microbiota composition in patients with spondyloarthritis**

Study (year)	Cohort	Methodology[a]	Increased prevalence	Decreased prevalence	Ref.
Stoll et al. (2014)	American cohort of children with enthesitis-related arthritis ($n=25$) and HC ($n=13$)	16S rRNA sequencing of stool samples	Bifidobacterium and *Akkermansia muciniphila*	*Faecalibacterium prausnitzii* and Lachnospiraceae	82
Costello et al. (2015)	Italian and Australian cohort of bDMARD-naive HLA-B27$^+$ patients with AS ($n=10$) and HC ($n=9$)	16S rRNA sequencing of terminal ileum tissue samples	Lachnospiraceae, Ruminococcaceae, Rikenellaceae, Porphyromonadaceae, Bacteroidaceae and a general increase in microbial diversity	Veillonellaceae and Prevotellaceae	72
Wen et al. (2017)	Chinese cohort of patients with AS ($n=97$) and HC ($n=114$)	Deep shotgun sequencing of stool samples	*Prevotella melaninogenica*, *Prevotella copri*, *Prevotella* sp. C561 and *Bifidobacterium*	*Bacteroides* spp.	75
Breban et al. (2017)	French cohort of patients with SpA ($n=96$), patients with RA ($n=32$) and HC ($n=71$)	16S rRNA sequencing of stool samples	*Ruminococcus gnavus*	A general reduction in microbial diversity	74
Tito et al. (2017)	Belgian cohort of bDMARD-naive patients with SpA ($n=27$) and HC ($n=15$)	16S rRNA sequencing of ileum and colon tissue samples	*Dialister* spp. and a general increase in microbial diversity	None reported	73
Zhang et al. (2019)	Chinese cohort of patients with AS ($n=103$) and HC ($n=105$)	16S rRNA sequencing of stool samples	*Megamonas* spp., *Dorea* spp. and *Blautia* spp.	*Lachnospira* spp., *Ruminococcus* spp. and *Clostridium* spp.	76
Zhou et al. (2019)	Chinese cohort of bDMARD-naive patients with AS ($n=85$) and HC ($n=62$)	Metagenomic shotgun sequencing of stool samples	*Bacteroides coprophilus*, *Parabacteroides distasonis*, *Eubacterium siraeum*, *Acidaminococcus fermentans* and *Prevotella copri*	*Eubacterium hallii*, *Coprococcus catus*, *Faecalibacterium prausnitzii* and *Coprococcus eutactus*	79
Klingberg et al. (2019)	Swedish cohort of patients with AS ($n=150$), patients with UC ($n=18$) and HC ($n=17$)	Stool samples were analysed with 54 DNA probes targeting ≥300 bacteria	Proteobacteria, Enterobacteriaceae, Bacilli, *Streptococcus* spp. and Actinobacteria	*Bacteroides* spp., Lachnospiraceae, *Faecalibacterium prausnitzii* and *Clostridium* spp.	223
Yin et al. (2020)	Han Chinese cohort of patients with AS ($n=127$) and HC ($n=123$)	Metagenomic shotgun sequencing of stool samples	*Clostridiales* bacterium 1 7 47FAA, *Clostridium bolteae*, *Clostridium hathewayi*, *Prevotella copri* and *Dialister invisus*	*Bifidobacterium adolescentis*, *Coprococcus comes*, *Lachnospiraceae* bacterium 5 1 63FAA and *Roseburia inulinivorans*	77

AS, ankylosing spondylitis; bDMARD, biologic DMARD; HC, healthy controls; RA, rheumatoid arthritis; SpA, spondyloarthritis; UC, ulcerative colitis. [a]The sequencing and analytical methods differ between studies, making direct comparisons of findings difficult.

and expansion of innate immune cells in patients with AS does not imply the absolutely autoinflammatory nature of this disease, it does underline the predominance of autoinflammation over autoimmunity. Supporting this hypothesis is evidence indicating that immune cells with an innate-like behaviour, such as ILCs and innate-like T cells (some of which are de facto mucosal-derived cells), are a major source of IL-17 in patients with AS (discussed in detail in this section). Such evidence lends support to the idea of a complex interaction between dysbiosis and innate immunity in the intestines that would result in aberrant activation and expansion of cells capable of migrating from the intestine to extra-intestinal sites (FIG. 2). The demonstration that tissue-resident or gut-derived myeloid cells might sense biomechanical stress at disease-prone sites and produce large amounts of IL-23 reinforces this hypothesis[89].

Innate cells and innate lymphoid cells

Evidence indicates a predominant activation of the innate immune system in patients with AS. In this section we discuss the potential role of granulocytes (such as mast cells and neutrophils), myeloid cells (such as macrophages) and ILCs in AS.

Mast cells and neutrophils. Granulocytes such as neutrophils and mast cells are at the forefront of the innate immune response. Synovial infiltration by neutrophils and mast cells has been described in several types of inflammatory arthritis; however, mast cell infiltration seems to be more abundant in SpA than in RA[90,91]. In 2012, a study showed that c-Kit$^+$ mast cells were considerably increased in synovial infiltrates in the peripheral joints of patients with psoriatic and non-psoriatic SpA, independent of the presence of psoriasis[90]. Immunohistochemical analysis showed that mast cells constituted 63% of the IL-17$^+$ synovial cells in these patients. Although the increase in IL-17$^+$ cells was not influenced by patients being treated with etanercept, the c-Kit tyrosine kinase inhibitor imatinib was able to induce mast cell apoptosis in synovial tissue culture experiments and reduce IL-17 production[90]. A similar analysis performed on facet joints obtained from patients with late-stage AS confirmed the high degree of infiltration by IL-17$^+$ cells; although, in this study, neutrophils were the most abundant IL-17$^+$ cell population[92]. Joint and enthesis data from patients with AS and peripheral blood data from patients with SpA suggest that innate immune cells might be of greater relevance than T_H17 cells for IL-17 production[92].

Notably, neither functional experiments with human cells nor reporter mice experiments have confirmed active mast cell production of IL-17 (REFS[93,94]). The reason for these results could be that although human mast cells contain IL-17A, they lack the transcriptional machinery to produce it, so could potentially simply be acting as a sponge for IL-17 that is produced by other cell types (reviewed elsewhere[95]). Mast cells actively engulf IL-17A from their extracellular environment, store it within granules and release it as a biologically active cytokine[94], a mechanism that could potentially also occur in neutrophils[96,97]. What triggers the release of IL-17 from mast cells and neutrophils is still not completely understood. However, data from a 2019 study in peripheral SpA suggest a dynamic accumulation of IL-17 in non-inflamed tissue, rather than in tissue with ongoing inflammation, and that treatment with an IL-17 inhibitor increases the number of IL-17$^+$ human synovial mast cells[98]. Thus, mast cells and neutrophils might have a buffering function in SpA, releasing pre-stored pro-inflammatory cytokines into already inflamed tissue. Therapeutic targeting of mast cells in SpA has been attempted in a small proof of concept, randomized, double-blind, placebo-controlled study[99]. Nilotinib, a tyrosine kinase inhibitor, was administered for up to 24 weeks in patients with either axSpA or peripheral SpA and led to a statistically significant reduction in mast cell and CD68$^+$ macrophage infiltration in peripheral synovial tissues. Nevertheless, clinical improvement was only observed in patients with peripheral SpA and not in those with axSpA[99]. Despite emerging data suggesting the involvement of mast cells in SpA, further studies are required to establish the clinical relevance of these cells in axial manifestations.

Macrophages. Macrophages, innate immune cells that are actively involved in the first line of defence against pathogens, are implicated in the pathogenesis of AS by multiple lines of evidence. In patients with AS, the number of macrophages is closely related to disease severity, and perturbations of monocytes and macrophages have been described in the inflamed tissues, such as the gut, synovia and entheses, as well as in peripheral blood. Large numbers of CD68$^+$ macrophages have been reported in entheseal fibrocartilage[100] and in the synovial portion of sacroiliac joint lesions in patients

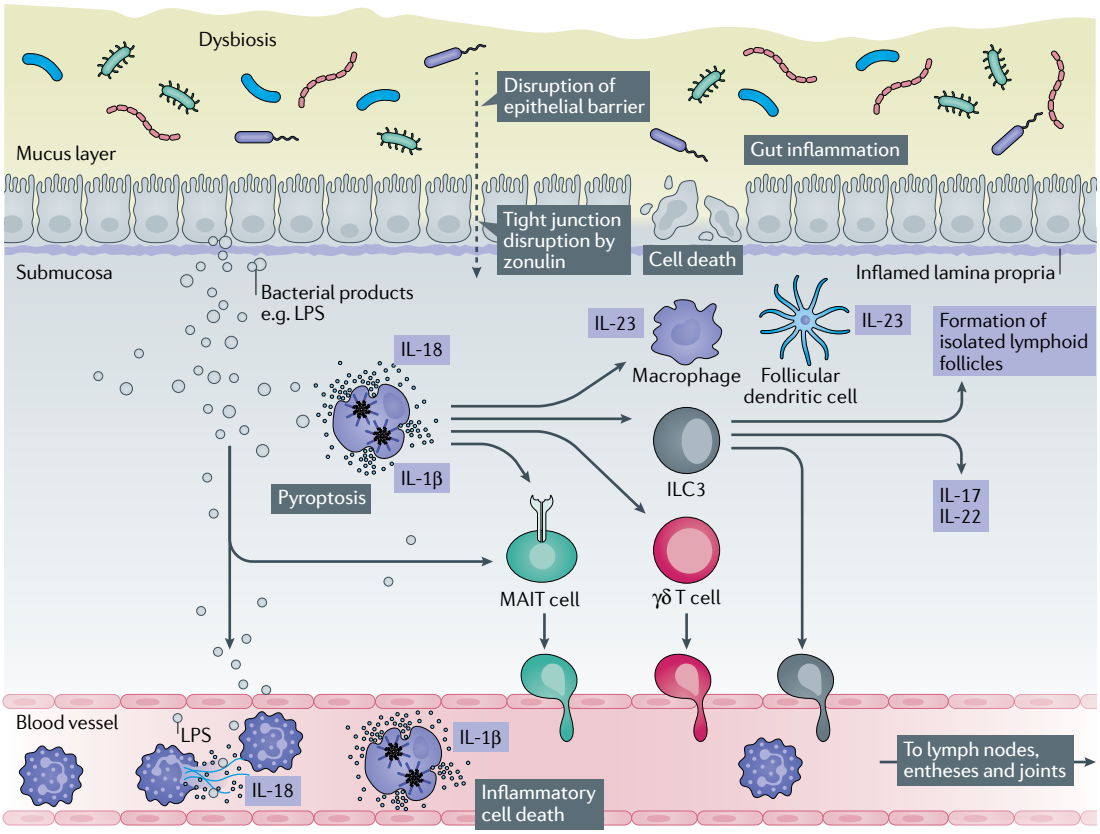

Fig. 2 | **Immune mechanisms linking intestinal dysbiosis to disease in ankylosing spondylitis.** In patients with ankylosing spondylitis, altered intestinal permeability, at least partially mediated by zonulin, allows bacterial products such as lipopolysaccharide (LPS) to penetrate the intestinal wall and interact with gut-resident and circulating peripheral blood mononuclear cells, triggering inflammasome activation and inflammatory cell death, known as pyroptosis, in gut-resident cells and peripheral blood mononuclear cells. The inflammasome dependent cytokines IL-1β and IL-18 can perpetuate gut inflammation and activate local and systemic mucosal-associated invariant T (MAIT) cells, group 3 innate lymphoid cells (ILC3s), γδ T cells, dendritic cells and macrophages, triggering IL-23, IL-17 and IL-22 secretion and epithelial cell death. The activated innate immune cells contribute to the formation of isolated lymphoid follicles and enter the bloodstream and circulate into peripheral tissues, including lymph nodes, entheses and joints.

with AS, particularly in those with early and active sacroiliitis, in whom the number of macrophages correlates with the degree of enhancement detected by MRI[101]. These inflammatory infiltrates contain a high concentration of *TNF* mRNA and a low concentration of *TGFB1* or *TGFB2* mRNA[102]. The absence of transforming growth factor-β (TGFβ) in the inflammatory infiltrate could suggest that TGFβ does not have immunomodulatory effects on the infiltrating immune cells, whereas TGFβ is known to contribute to aberrant new bone formation that is typical of SpA[102]. A 2019 study found a $CD45^+HLA\text{-}DR^+CD14^+CD11c^+$ myeloid cell population that was expanded in both healthy human enthesis soft tissue and in the adjacent peri-entheseal bone[62]. The dominant source of IL-23 in the entheseal cells was the $CD14^+$ myeloid cell population, which could be downregulated by agents that increase intracellular cAMP, such as phosphodiesterase 4 inhibitors, histamine and 8-bromo-cAMP. Entheseal $CD14^+$ cells showed a similar gene expression profile to a corresponding matched circulating $CD14^+$ cell population, except for substantially decreased *CCR2* expression[62]. Analysis of circulating monocytes from patients with AS also confirms an altered profile; upon treatment with LPS and granulocyte-macrophage colony-stimulating factor, monocytes from patients with AS had a 'reverse' interferon signature compared with monocytes from healthy individuals, in which genes normally upregulated by IFNγ were under-expressed, and normally downregulated genes were overexpressed[103].

Accordingly, a predominance of $CD163^+$ macrophages (essentially M2-polarized macrophages) has been demonstrated in both synovial tissue and intestinal tissue in patients with AS[104,105]. In healthy gut, CX_3CR1^+ mononuclear phagocytes (MNPs) produce large amounts of IL-23 and TNF ligand superfamily member 15 (TNFSF15), efficiently supporting the production of IL-22 by ILC3s[106]. Although considered to be a type of tissue-resident macrophage, during intestinal inflammation, CX_3CR1^+ MNPs can acquire migratory potential and move to secondary lymphoid organs, thereby initiating immune responses[107]. In patients with AS, $CX_3CR1^+CD59^+$ MNPs are expanded in inflamed gut tissue in correlation with the presence of bacteria[108]. These cells are mainly located close to intestinal crypts, produce IL-23 and TNFSF15 and support the expansion of ILC3s, but are also expanded in the peripheral blood, synovial fluid, synovial tissue and bone marrow of patients with AS. Interestingly, the majority of the $CX_3CR1^+CD59^+$ cells in non-intestinal tissue express CCR9, a marker of intestinal homing, possibly indicating their intestinal origin, and have a specific pro-inflammatory transcriptomic profile[108].

Macrophage migration inhibitory factor (MIF), a 'chemokine-like' molecule that is involved in the differentiation of M2-polarized macrophages[109], is also potentially involved in the pathogenesis of AS. MIF has pleiotropic functions, including involvement in phagocytosis, cell spreading and tumoricidal activity[110]. Concentrations of MIF are increased in the serum of patients with AS compared with healthy individuals and in synovial fluid compared with patients with osteoarthritis[111]. Higher serum concentrations of MIF are independently predictive of disease progression in patients with AS, and increased numbers of MIF-producing macrophages are present in gut tissue from patients with AS compared with healthy individuals. In vitro, MIF promotes TNF production by monocytes, activates β-catenin in osteoblasts and promotes osteoblast mineralization[111]. All things considered, MIF seems to contribute to both inflammation and new bone formation in AS, and concentrations of circulating MIF contribute to the prediction of radiological progression[111]. These data contribute to the identification of MIF as an important molecule in the pathogenesis of AS and could also lead to the identification of novel therapeutic targets, as MIF inhibitors are emerging in cancer research and might also be beneficial in inflammatory conditions such AS[112].

Innate lymphoid cells. ILCs can be divided into three main groups of cells (ILC1s, ILC2s and ILC3s) on the basis of the transcription factors that regulate their development, function and cytokine production[113]. ILC3s are a mucosal-restricted lymphoid cell population that require the transcription factors RORγt and T-bet for their differentiation, and which lack cytotoxic effector mechanisms such as perforin, granzymes and death receptors[114]. Specifically, human ILC3s can be further characterized by their expression of the natural cytotoxicity receptor NKp44; similar to T_H17 cells, $NKp44^-$ ILC3s produce IL-17, whereas $NKp44^+$ ILC3s are a source of IL-22 and have a homeostatic function in the gut[115]. Despite the strict bi-directional relationship between intestinal microbiota and ILC3s, ILC3s cannot directly detect bacterial products. Therefore, ILC3 activation relies on an intricate cellular network involving macrophages, dendritic cells (DCs) and cytokines, which function as an immunological bridge between the intestinal lumen and the immune system[106].

ILC3s are expanded in the blood, bone marrow and synovial fluid in patients with AS, and this expansion correlates with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores[116]. However, as entheseal tissue from patients with AS is not easily accessible for research purposes, direct evidence of an inflammatory role for ILC3s within entheses is still lacking. A 2017 study reported that ILC3s are enriched in normal human entheses compared with blood[61]. Although this study was ground-breaking in demonstrating the physical presence of these cells in the target organ of AS, the origin of the entheseal ILC3s is unclear. ILC3s are mucosal-derived, mainly intestinal, cells, thus raising the question of whether entheseal ILC3s originated in the gut. The subclinical gut inflammation that is a feature of AS could be considered a cause or a consequence of the expansion of ILC3s in these individuals. Circulating, synovial and bone marrow ILC3 populations express the intestinal homing molecule α4β7 integrin, and the recirculation of $\alpha4\beta7^+$ ILC3s in patients with AS is associated with overexpression of MADCAM1 (the specific ligand for α4β7 integrin) in the inflamed bone marrow[116]. Thus, ILC3s could potentially migrate from the ILC3-rich cryptopatches in the intestinal submucosa

to the bone marrow in patients with AS, thereby actively participating in the induction and/or perpetuation of tissue inflammation at both sites[29,117]. ILC3s are normally in close proximity to gut microbiota and contribute to their compartmentalization in the intestinal lumen, suggesting that aberrant autoinflammatory activation and reduced production or increased death of the homeostatic IL-22$^+$ intestinal ILC3s could contribute to the derangement of the gut–endothelial barrier that occurs in AS.

Innate-like T cells

The behaviour of innate-like T cells, such as γδ T cells, mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells, challenges the traditional classification of immune responses into adaptive and innate (reviewed elsewhere[118]). Despite formally and ontogenically being T cells, innate-like T cells cross the boundaries of adaptive immune cells as they normally do not require clonal expansion and can respond promptly upon stimulation in an innate-like fashion. Innate-like T cells participate in the IL-17-mediated immune response that is mostly orchestrated by the transcription factor RORγt, which has been proposed as a therapeutic target in SpA[119].

MAIT cells. In keeping with the link between the gut and the joints, MAIT cells have been identified as a major source of IL-17 in patients with AS[120]. MAIT cells are RORγt$^+$CD3$^+$CD4$^{+/-}$CD8$^{+/-}$ T cells that reside mainly in the gut mucosa and represent up to 10% of T cells in the blood and up to 45% in the liver[121]. These predominantly CD8$^-$ T cells have a restricted T cell receptor (TCR) repertoire with specificity for the MHC class I-like molecule MR1 (REF.[122]). Through antigen presentation by MR1, MAIT cells recognize small molecule vitamin B metabolites of bacterial origin[123].

A 2016 study reported that circulating MAIT cells are reduced in number in patients with AS compared with healthy individuals but are skewed towards an IL-17A$^+$ phenotype, and that IL-17A$^+$ MAIT cells are expanded in the synovial fluid of patients with AS[120]. Interestingly, the skewing towards IL-17$^+$ cells noted in this study was dependent on IL-7 receptor activation rather than the TCR, suggesting a possible functional link between the aberrant activation of MAIT cells and an allele of *IL7R* associated with the risk of developing AS. Although the authors of this study did not find any genetic influence of the rs11742270 SNP on *IL7R* expression in total peripheral blood mononuclear cells (PBMCs) or in stimulated MAIT cells[120], the rs11742270 SNP is in strong linkage disequilibrium (R^2 0.0928, $P < 0.001$) with other quantitative trait loci that strongly influence *IL7R* expression in at least some types of cell upon stimulation[124].

MAIT cells have also been identified as a source of IL-22 in patients with AS, reinforcing the relevance of innate cells as a source of IL-17 and IL-22 in the pathogenesis of AS[125]. Another study also reported a reduction in circulating MAIT cells in patients with AS, and demonstrated that activated MAIT cells from patients with AS produce IL-17 more abundantly than MAIT cells from healthy individuals, suggesting a pre-activation status for MAIT cells in AS or the contribution of genetics in influencing MAIT cell responses, or both[126]. Interestingly, expression of the activation marker CD69 on MAIT cells correlated with the Ankylosing Spondylitis Disease Activity Score (ASDAS)[126], suggesting that activation of MAIT cells contributes to, or at least mirrors, disease activity in AS. Preliminary data also suggest that MAIT cells (and γδ T cells) could be the most important producers of IL-17 in AS[127], reinforcing the importance of the gut mucosa in AS pathogenesis.

γδ T cells. γδ T cells are a CD3$^+$ T cell subset that express a particular oligoclonal TCR formed by gamma and delta chains. The identification of γδ T cells as an innate source of IL-22 and IL-17 (REF.[128]) has elevated their importance in the genesis of type 3 immune responses in AS. IL-23R$^+$ IL-17-producing γδ T cells are expanded in the blood of patients with AS, but not in those with rheumatoid arthritis (RA), and are hyper-responsive to IL-23 stimulation[129,130]. Similarly, increases in IL-17-producing γδ T cells have been reported in synovial fluid and blood of patients with seronegative arthritis (reactive arthritis and undifferentiated SpA)[131]. Overexpression of IL-23 by minicircle technology in mice induces enthesitis, aortic root inflammation and psoriasis, and triggers the activation of an entheseal resident IL-23R$^+$RORγt$^+$CD3$^+$CD4$^-$CD8$^-$ population, largely composed of γδ T cells that have been activated by IL-9 (REFS[60,89,132]). A 2019 study demonstrated that γδ T cells are actually tissue resident cells in the normal spinal entheseal tissue, and that IL-17 production was inducible in this tissue in a IL-23-independent fashion[132]. The existence of mechanisms of IL-23-independent IL-17 production in spinal entheses could be one of the possible explanations for the lack of efficacy of IL-12–IL-23 inhibition in axSpA; however, similar data from patients with AS are still awaited.

Although it is difficult to conclusively demonstrate direct activation of IL-17 production in γδ T cells by IL-23 at the entheses in humans or mice, another possibility is that γδ T cells could be activated by IL-1, IL-6 or TGFβ at the source of bacterial pathogen-associated molecular patterns (PAMPs) and by IL-23 produced in the gut, and could then traffic via the blood to the entheses. A 2019 study reported the presence of two populations of γδ T cells in patients with SpA, a more abundant population that had intermediate TCR γδ levels and a smaller population that had high expression of TCR γδ (TCRγδ-hi cells)[119]. RORγt$^+$T-bet^{lo} cells were substantially enriched within the TCRγδ-hi population, which also expressed abundant *IL23R* and were particularly responsive to IL-23 stimulation in the presence of TCR engagement, leading to the production of IL-17 and IL-22. The TCRγδ-hi subset was expanded in synovial fluid from patients with SpA, including axSpA, and specifically enriched for IL-23R$^+$ and IL-17$^+$ cells. Despite not being the most numerically abundant cell types, γδ T cells (and iNKT cells) seemed to be the most relevant source of IL-17 in the joints of patients with SpA. In addition, given that IL-17 production relies on RORγt, RORγt inhibition was tested therapeutically in an animal model and in human cells ex vivo, demonstrating a selective suppression of IL-17 (REF.[119]).

Minicircle technology
The use of small circular DNA elements to induce the expression of genes in vivo or in vitro.

TNF$^{\Delta ARE}$ mice
Mice with a deletion of the AU-rich element (ARE) from the *TNF* gene; ARE controls the stability of the *TNF* mRNA; therefore these mice have increased production of TNF.

Pyroptosis
An inflammatory form of lytic programmed cell death that occurs following inflammasome activation.

Invariant NKT cells. iNKT cells express a semi-invariant TCR formed by an invariant α-chain and a restricted repertoire of β-chains[118]. In TNF$^{\Delta ARE}$ mice, which lack iNKT cells, gut and joint inflammation is exacerbated[133]; however, the functional role of iNKT cells in the pathogenesis of SpA has not been entirely clear. A 2019 study demonstrated the presence of a rare subset of RORγt^{+}T-bet^{lo} iNKT cells that were able to secrete IL-17 and, to a lesser extent, IL-22 in patients with SpA[119]. The transcriptomic signature of the IL-17-producing iNKT cells was more similar to ILC3s or γδ T cells than to T_H17 cells and, similar to γδ T cells, human circulating iNKT cells express *IL23R* and promptly respond to stimulation to mediate a T_H17 cell-like response. iNKT cells from patients with SpA also exhibit a disease-specific signature that is different to iNKT cells from patients with RA and, together with γδ T cells, accumulate in the joints and contribute to IL-17 production[119].

Shared immune pathways

Although belonging to a greater extent to autoinflammatory responses, several immune pathways that are altered in AS, such as inflammasome activity, autophagy and ubiquitination, can also contribute in various ways to adaptive responses.

Inflammasomes

Inflammasomes are multi-protein complexes activated by PAMPs and danger-associated molecular patterns that trigger a caspase 1-mediated cascade, inducing pyroptosis and resulting in inflammation[134]. Dysregulated inflammasome activity has been reported in patients with AS, and growing evidence is highlighting direct roles for components of the inflammasome or inflammasome-related cytokines in inducing adaptive immune responses (FIG. 2); for example, IL-1β can support T_H17 cell responses, and IL-18 can induce the secretion of IFNγ in an antigen-independent manner by memory CD8^{+} T cells[135–137].

Genes encoding several inflammasome components are associated with the risk of AS, including *MEFV* M694V, *CARD9*, *CARD15*, *IRGM*, *IL1R1* and *IL1R2* (REFS[36,138–140]), suggesting possible dysfunctional inflammasome activation in AS. PBMCs from patients with AS have higher mRNA expression of *NLRP3*, *ASC*, *IL1B*, *IL17A* and *IL23* than PBMCs from healthy individuals, and a statistically significant correlation exists among the expression of NACHT, LRR and PYD domains-containing protein 3 (NLRP3), caspase 1, apoptosis-associated speck-like protein containing a CARD (ASC), IL-1β, IL-17A and IL-23 (REF.[141]). Data suggest that the expression of components of the inflammasome is increased in the gut of HLA-B27 transgenic rats, and can be reduced by treatment with antibiotics[141], supporting a role for dysbiosis in regulating the expression of inflammasome components. Inflammasome activation also seems to occur in the inflamed gut of patients with AS and is accompanied by the activation of caspase 1 and overexpression of IL-1β and IL-18. Interestingly, preliminary data indicate that only bacteria isolated from the ileum of patients with AS can increase the expression of inflammasome genes in isolated lamina propria mononuclear cells from healthy individuals, suggesting the possibility of bacteria-dependent activation of the inflammasome in AS[141].

IL-1β blockade with anakinra has been somewhat effective as a treatment in patients with AS. In a 3-month open label study in which nine patients with AS were treated with anakinra, six achieved a 20% improvement in ASAS criteria (an ASAS20 response)[142]. Improvement or regression was reported in 23 of the 38 regions of enthesitis or osteitis that were determined by MRI at the outset of the study[142]. Conversely, in another open label trial of anakinra, this time for 24 weeks in 13 patients with active AS, only 30% achieved an ASAS20 response, and no changes were reported in MRI scores[143]. Obviously, no definitive conclusion regarding the potential benefit of IL-1 inhibition in AS can be drawn from these studies given the low number of patients enrolled and the short half-life of anakinra. However, it seems conceivable that some patients, especially those who have high inflammation indexes or carry genetic variants known to lead to increased IL-1 production (such as the *MEFV* M694V variant) and have an inadequate response to TNF inhibition or IL-17 inhibition, could be empirically treated with anakinra or with other, longer-acting, IL-1 antagonists.

Autophagy and the UPR

Autophagy works in close connection with the cellular response to ER stress, known as the unfolded protein response (UPR)[144]. Both autophagy and the UPR are critical pathways that control the activity of innate immune cells[145]. Uncontrolled autophagy is linked to the UPR and inflammasome activation, pathways that are involved in autoinflammatory diseases such as TNF receptor-associated periodic syndrome, FMF and mevalonate kinase deficiency[145,146]. However, data indicate that in addition to positively or negatively regulating the innate immune response, autophagy can also act as a bridge between innate and adaptive immunity via its involvement in antigen presentation[147,148]. One striking example is the requirement of autophagy for the survival of autoreactive B cells and for plasmablast differentiation in the prototypical autoimmune disease SLE[149]. The UPR also intervenes in adaptive immunity by affecting the differentiation, activation and effector function of T cells[150] and plasma cells[151], which activate the UPR to adapt to antibody synthesis.

In patients with AS, the HLA-B27 molecule has a high propensity to oligomerize, forming complexes in the ER with the chaperone protein BiP[152]. In HLA-B27 transgenic rats, the misfolding of HLA-B27 synergizes with LPS to induce hyper-expression of IL-23 in macrophages undergoing a UPR induced by pharmacological agents[153]. Furthermore, increased expression of IL-23 and IL-17 has been documented in the colon of HLA-B27 transgenic rats, in conjunction with the development of intestinal inflammation[153]. However, unlike in transgenic rats, the accumulation of incorrectly folded HLA-B27 heavy chains in patients with AS does not seem to be able to activate the UPR[154]. The close connection between autophagy and the UPR has also been demonstrated by in vitro studies, in which the

Necroptosis
A form of inflammatory cell death similar to necrosis that is regulated in a caspase-dependent manner and that can be induced by extracellular stimuli such as TNF.

TNF receptor complex I
A receptor complex of TNF that contains a death domain that mediates the induction of apoptosis and necroptosis.

suppression of autophagy induced a substantial increase in the expression of free HLA-B27 heavy chain, and consequently a substantial increase in markers of the UPR[155]. These findings could indicate that the absence of substantial upregulation of the UPR in ex vivo studies using cells from patients with AS is due to a compensation mechanism caused by excess autophagy. Altogether, these findings suggest that although HLA-B27 has a propensity to misfold and can induce ER stress when overexpressed, this inclination might be compensated for by an increase in autophagy.

Genes involved in the autophagy pathway (*ATG16L1*, *IRGM* and *MAP1LC3A*) are upregulated in inflamed ileal tissue from patients with AS, as is LC3-II, a global marker of autophagy, and autophagy protein 5 (ATG5) and ATG12, which are involved in autophagosome formation[156]. Interestingly, the expression of autophagy-related genes and that of *IL23A* are correlated, and inhibition of autophagy in the presence of LPS reduces the percentage of IL-23-expressing cells while increasing the amount of *IL23A* mRNA in lamina propria mononuclear cells from patients with AS and from healthy individuals. One explanation for these findings could be that although LPS and ER stress promote *IL23A* transcription[157], autophagy suppresses the active cellular release of IL-23, suggesting a homeostatic role in AS. The particular increase in autophagy in the gut in AS also suggests that factors other than HLA-B27 could be involved. For example, autophagy might be stimulated within phagocytic cells by the uptake of bacteria that have crossed the epithelial barrier as a mechanism to control intracellular replication[158]. In contrast to these findings in gut tissues, another study reported no differences in the expression of autophagy-related genes in the synovial tissues of patients with SpA, and a reduced expression of *ATG16L1*, *IRGM* and *HSP90AA1* in PBMCs from patients with SpA compared with healthy individuals[159]. Expression of *MAP1LC3A*, *ATG5* and *HSPA8* did not differ. These findings support the idea that activation of autophagy in patients with AS might be a tissue-specific process that is predominantly involved in the modulation of gut innate immune responses.

Overall, even if the involvement of autophagy in AS could favour the idea of an autoinflammatory aetiology, no definitive conclusion can be drawn on the basis of its involvement alone, and further studies are required to address its role in modulating adaptive immunity.

A20 and ubiquitination

A20, encoded by *TNFAIP3*, is a ubiquitin-editing enzyme involved in modulating NF-κB activation, inflammasome activity, Toll-like receptor (TLR) signalling and receptor interacting protein kinase 1 (RIPK1) activity[160–162]. This highly conserved protein contains an ovarian tumour domain and a series of seven zinc finger (ZnF) domains; whereas the ZnF domains, mainly ZnF4 and ZnF7, mediate ubiquitin chain binding (mostly K63-linked and linear ubiquitin), the ovarian tumour domain exerts the enzymatic activity by deubiquitinating the target proteins[163]. Linear ubiquitination and A20 are involved in modulating the balance between inflammatory cell death and cell proliferation by stabilizing linear ubiquitin chains and removing K63-linked ubiquitin chains from target proteins[164,165].

Insights into the biological activity of A20 are providing logical explanations for the phenotypes observed in mice in which *TNFAIP3* has been genetically manipulated. *TNFAIP3*$^{-/-}$ mice are cachectic and die from severe inflammation and exaggerated cell death[164]. Consistently, A20-deficient cells are susceptible to TNF-induced or TLR-induced necroptosis[166,167]. DC-specific *TNFAIP3* deletion causes the activation of DCs and T cells and is associated with an SpA-like phenotype characterized by seronegative ankylosing arthritis, enthesitis and lymphocyte-dependent colitis[168]. One of the reasons behind the T cell expansion seems to be the aberrant presentation of apoptosis-derived material in an immunogenic manner by *TNFAIP3*$^{-/-}$ DCs[169]. Other experimental strategies have mostly confirmed this observation; deficiency of *TNFAIP3* in macrophages, neutrophils and a proportion of DCs in mice led to enthesitis, polyarthritis, T_H17 cell expansion and break of tolerance[170,171]. However, T cells and B cells were dispensable for the induction of arthritis[171], suggesting an IL-1β-mediated autoinflammatory phenotype[38] (reviewed elsewhere[172]). However, the abrogation of A20 deubiquitination or ubiquitin ligase activities by site-directed mutagenesis did not recapitulate the severe phenotype observed in *TNFAIP3*$^{-/-}$ mice, pointing to the non-enzymatic activity of the protein in controlling necroptosis[173,174].

In 2020, two independent studies demonstrated that ZnF7 and ZnF4 are essential for the anti-inflammatory and cytoprotective activity of A20 and that the inactivation of both domains phenocopies the postnatal lethality and multiorgan inflammation of *TNFAIP3*$^{-/-}$ mice[175,176]. Although A20$^{ZnF7/ZnF7}$ mice develop a spontaneous inflammatory phenotype, they do not fully recapitulate the phenotype of *TNFAIP3*$^{-/-}$ mice, instead developing polyarthritis, bone erosions and intestinal and skin inflammation[175]. Specifically, A20$^{ZnF7/ZnF7}$ mice develop distal digit swelling, arthritis of the distal interphalangeal joints and nail loss, mimicking PsA[176]. Consistently, psoriasis features such as epidermal hyperplasia and hyperkeratosis were observed in the skin of these mice[176]. The arthritis in A20$^{ZnF7/ZnF7}$ mice was dependent on T cells, TNF, TLRs and IL-17, but independent of B cells and commensal flora[176].

At a cellular level, ZnF7 seems to be necessary to restrict late-phase NF-κB activation[176]. A20, via ZnF7, binds the linear ubiquitin chains that start to form on NF-κB essential modulator (NEMO) following TNF signalling via TNF receptor complex I, and non-catalytically blunts the activation of NF-κB[177]. Thus, ZnF7 is crucial in suppressing inflammatory cell death by favouring the stabilization of TNF receptor complex I and its effects on linear ubiquitin chain formation on NEMO (an important step in blocking NF-κB activation)[175]. The lack of ZnF7 in A20$^{ZnF7/ZnF7}$ mice reduced the linear ubiquitin chains that formed on NEMO and instead enhanced the cell death-inducing activity of the alternative RIPK1-containing complex II signalling pathway[175]. These findings[175,176] are in line with the increased inflammatory cell death and inflammation that occur in mice

Linear ubiquitin assembly complex
A three-protein complex with ubiquitin ligase activity that forms ubiquitin chains linked to the first lysine and is involved in intracellular signalling.

and humans carrying mutations in components of the linear ubiquitin assembly complex[178–182]. A rare *TNFAIP3* haploinsufficiency can also occur in humans, and leads to an autoinflammatory phenotype via uncontrolled NF-κB signalling and exaggerated caspase 8 and inflammasome activity[183–185].

Altogether, these data point to the possible involvement of linear ubiquitination in the mechanisms that underlie some of the inflammatory processes that occur in AS, particularly given that *TNFAIP3* and *UBE2L3* are known risk genes for the development of AS[36,186]. The enzyme UBE2L3 is rate-limiting for linear ubiquitin assembly complex activity and, in turn, for linear ubiquitination downstream of TNF or CD40 ligand[187]. Overall, the involvement of A20 in the regulation of necroptosis and inflammasome activation could suggest autoinflammatory processes. However, it should be noted that A20 also has a critical role in the function of adaptive immune cells, including B cells, and seems to be involved in the pathogenesis of primarily autoimmune diseases such as SLE[188].

Adaptive immunity in AS

In contrast to the vast body of evidence that has accumulated for the involvement of the innate immune system in the pathogenesis of AS, evidence for the involvement of the adaptive immune system is limited, but does suggest some contribution towards the pathogenesis of this disease.

T cells

The identification of *HLA-B27* as a major contributor in the heritability of AS led to speculation regarding the causative role of MHC class I in this disease. The highly polymorphic sites associated with AS risk encode the amino acid residues at positions 67 and 97 of the peptide-binding domain of HLA-B27 (REFS[39,189]). According to the arthritogenic peptide theory, risk alleles of *HLA-B27* could contribute to the aberrant presentation of an arthritogenic peptide (possibly of bacterial origin) to T cells, thereby triggering the differentiation of cytotoxic $CD8^+$ T cells[190]. Activated $CD8^+$ T cells could then perpetuate inflammatory responses if the peptide they recognize showed molecular mimicry with a self-antigen[190]. Surprisingly, functional experiments in HLA-B27 transgenic mouse models of AS demonstrated that $CD8^+$ T cells are dispensable for the development of clinical features. Specifically, no differences were observed in mice transgenic for human HLA-B27 and β_2-microglobulin that lacked CD8a compared with those that did not[191].

Conversely, SKG mice illustrate that multiple mechanisms that involve both innate and adaptive immune responses can promote SpA. In response to β-glucan, arthritic SKG mice develop autoantibodies that recognize multiple joint cartilage proteins (including type II collagen and proteoglycan) and, in some environments, rheumatoid factor, which could potentially perpetuate synovitis in these animals[192]. Arthritis (but not ileitis) can be transferred to T cell-deficient hosts by adoptive transfer of $CD4^+$ T cells from SKG mice in the absence of any further trigger, demonstrating that autoreactivity and poor immune regulation are sufficient for the development of arthritis[70]. In contrast to synovitis, enthesitis in SKG mice is specifically mediated by IL-17 (REF[193]). In SKG mice in which disease has been triggered with β-glucan or in the SKG T cell transfer model, homeostatic T cell proliferation is potentially rendered pathogenic by the adjuvant effect of PAMPs that translocate across a leaky gut epithelial barrier, or by persistent TNF secretion by macrophages exposed to bacterial PAMPs[68,194]. Furthermore, $FOXP3^+$ regulatory T (T_{reg}) cells from SKG mice suppress autoreactive T cells less efficiently than T_{reg} cells from BALB/c mice, and the number of IL-22^+ ILCs is reduced in SKG mice relative to BALB/c mice[68,71]. Besides the effect of thymic selection, the dysbiotic microbiota and hostile host mucosal environment potentially also adversely affect the development of peripheral T_{reg} cells. Consistent with impaired T_{reg} cell function, immunodeficient mice receiving T cells from SKG mice develop colitis[70,192]. Thus, the balance between effector cells and T_{reg} cells in the lymphopenic environment of SKG mice can be easily shifted towards autoimmunity. Together, the evidence supports the concept that SpA-like disease in SKG mice results from a genetic predisposition to autoreactivity, dysregulation of T_{reg} cells and an immune deficiency that increases the exposure of host autoreactive T cells to the innate immune system-triggering effects of bacterial adjuvant.

In humans, the involvement of $CD8^+$ T cells is substantiated by the fact that $CD8^+$ T cells reactive towards *Chlamydia trachomatis* are present in the joints of patients with reactive arthritis[195], and many studies have investigated HLA-B27-restricted $CD8^+$ T cells in patients with AS[196–198]. $CD8^+$ T cells from patients with AS are reactive towards HLA-B27-restricted self-epitopes and non-self-epitopes[196,199,200], and activated cytotoxic $CD8^+$ T cells have been found in synovial fluid from patients with AS[201]. Interestingly, an unusual population of IL-4-producing $CD8^+$ T_{reg} cells seems to be expanded in both patients with AS (mostly HLA-$B27^+$) and in HLA-$B27^+$ healthy individuals[202]. The maturation of IL-4-producing $CD8^+$ T_{reg} cells relies on their interaction with DCs, which confers on them immunomodulatory properties mediated by cell-to-cell contact. To date, the role of this population in the pathogenesis of AS is still unclear, as is whether they are pathogenic or represent a compensatory mechanism; however, the strict correlation with HLA-B27 could suggest a role for genetics in determining the expansion of these cells[202]. Immuno-sequencing the TCR repertoires of HLA-$B27^+$ patients with AS has revealed an increased TCR diversity, an expansion of $CD8^+$ TCR clonotypes reactive to Epstein–Barr virus and cytomegalovirus, and an increased incidence of 'public' TCRs compared with HLA-$B27^+$ healthy individuals[203]. Interestingly, the public TCRs included clonotypes that matched those previously found in patients with bacteria-induced reactive arthritis[203]. Furthermore, within the $CD8^+$ T cell population, a unique subset of mature $CD103^+CD49a^+$ T cells seems to be expanded in the synovial fluid of patients with AS[204]. These synovial $CD103^+CD49a^+$ T cells exhibited a distinct pattern of integrin expression (β7 integrin,

CD103, CD29 and CD49a) and substantial overexpression of *TNFAIP3*, *GZMB*, *PRF1* and *IL10*, and are reminiscent of human tissue-resident memory T (T_{RM}) cells, which are a critical component of the mucosal immune response. T_{RM} cells are expanded in the gut, peripheral blood and synovial fluid of patients with AS, and the majority of $CD8^+CD69^+CD103^+$ T_{RM} cells in synovial fluid express the intestinal homing receptor α4β7 integrin, suggesting a gut origin[205].

Although it has not been universally decided if other T helper cells act synergistically with T_H17 cells to trigger inflammation in AS, some studies do suggest a role for T_H1 cells in perpetuating inflammation[206]. Circulating T_H17 and T_H1 cells are both expanded in the blood of patients with AS and are reduced by treatment with TNF inhibitors[207]. In conclusion, the underlying role of IL-17 and the expansion of the T_H17 cell population in AS is widely accepted, and IL-17 is a valid therapeutic target in SpA. However, T_H17 cells are not the sole source of IL-17 in AS, and multiple types of innate cells, including ILC3s, could also be relevant sources of IL-17 (REFS[96,208]). Similarly, other subsets of T cells (such as T_{RM} cells) and the impaired function of T_{reg} cells contribute to the disease manifestation.

B cells and autoantibodies

To date, the contribution of B cells to the pathogenesis of AS seems rather marginal, and studies in experimental models of SpA have established that B cells and T cells are not necessary for the development of enthesitis[63]. Nevertheless, alterations in B cell composition and the presence of some autoantibodies have been described in patients with AS. A small study involving 18 patients with AS showed a reduction in the number of circulating $CD27^+$ B cells and an increase in $CD86^+$ B cells and $CD27^-CD95^+$ B cells compared with healthy individuals; the number of $CD38^+$ B cells and $CD95^+$ B cells correlated positively with BASDAI scores[209]. Conversely, the activity of regulatory B cells and the secretion of IL-10 seems to be impaired in AS[210], despite conflicting data on the number of regulatory B cells in patients with AS[210,211]. The marginal role of B cells in promoting AS is also suggested by the limited benefit gained when treating patients with AS with B cell-targeting therapies[212]. Although the accumulation of B cells and the formation of ectopic germinal centres similar to those seen in RA have been reported in patients with AS, their role in axial disease is unclear[213,214].

Increasingly, autoantibodies are being identified in patients with AS, challenging the traditional definition of AS as a seronegative disease and raising the possibility of an autoimmune aetiology. Screening serum from patients with AS for reactivity against 3,498 proteins using a high-density nucleic acid programmable protein array showed multiple reactivities and a skewing towards proteins expressed in musculoskeletal and connective tissues[215]. Among the target proteins identified, the MHC class II histocompatibility antigen gamma chain CD74 (which is also the receptor for MIF) seems to be the most promising; anti-CD74 autoantibodies were detected in 56% of patients with AS and 5% of healthy individuals in one cohort, findings that were reproduced in 69% of patients with axSpA and 65% of patients with peripheral SpA in a second cohort[216]. Antibodies that specifically target the extracellular domain of CD74 (known as CLIP) were found in 97% of patients with axSpA, 45% of patients with PsA and 11% of patients with RA[216]. Further confirmation studies showed a preferential increase of anti-CILP antibodies in patients with axSpA with a sensitivity of 85% and specificity of 92% compared with individuals who did not have SpA. However, no correlation between autoantibodies and disease activity has been observed[217]. Furthermore, anti-CD74 antibodies were not useful in the identification of patients with early axSpA[218]. Whether these autoantibodies might aberrantly activate CD74, increase the concentration of circulating MIF by inhibiting its receptor binding, or prevent the exchange of CLIP for processed antigen within MHC class II molecules is not yet known.

Immune complexes of autoantibodies that recognize sclerostin or noggin have also been found in patients with AS and are associated with lower amounts of sclerostin and noggin[219]. Sclerostin is an osteocyte protein that negatively controls bone formation by interfering with Wnt signalling, and is involved in syndesmophyte formation[220]. A lack of sclerostin is associated with enhanced bone formation, and low serum sclerostin concentrations have been reported in AS[220]. Similarly, noggin modulates bone morphogenetic protein signalling and its overexpression in experimental models of arthritis protects against the development of ankylosing enthesitis[221]. The serum concentrations of sclerostin and anti-sclerostin antibodies perform well in the prediction of axial involvement in patients with IBD (area under the curve = 0.88 and 0.84, respectively)[222]. Altogether, although several lines of evidence suggest the activation of B cells and some degree of breach of tolerance in axSpA, the contribution of B cells and humoral immunity seems not to be one of the main pathogenic pathways.

Autoinflammation or autoimmunity in AS

Overall, although much of the evidence discussed in this Review highlights the important role of autoinflammatory mechanisms in the pathogenesis of AS, in reality, the innate and adaptive immune systems seem to closely intersect in the pathogenesis of this disease (FIG. 3). Evidence supporting the involvement of adaptive immunity in the pathogenesis of AS and data on the presence of autoantibodies prevent AS from being defined as a purely autoinflammatory disease. In some patients, autoinflammatory and autoimmune components could conceivably coexist or exist pathophysiologically at different moments, and could therefore contribute to the clinical variability seen in terms of disease manifestation, disease evolution and response to therapy in patients with AS. However, it also seems possible that, in some patients, either the autoinflammatory component or the autoimmune component could prevail, thereby determining the specific disease phenotype that manifests. At present, the most plausible hypothesis is that AS can be considered as being a continuum between autoimmunity and autoinflammation, in which the innate immune

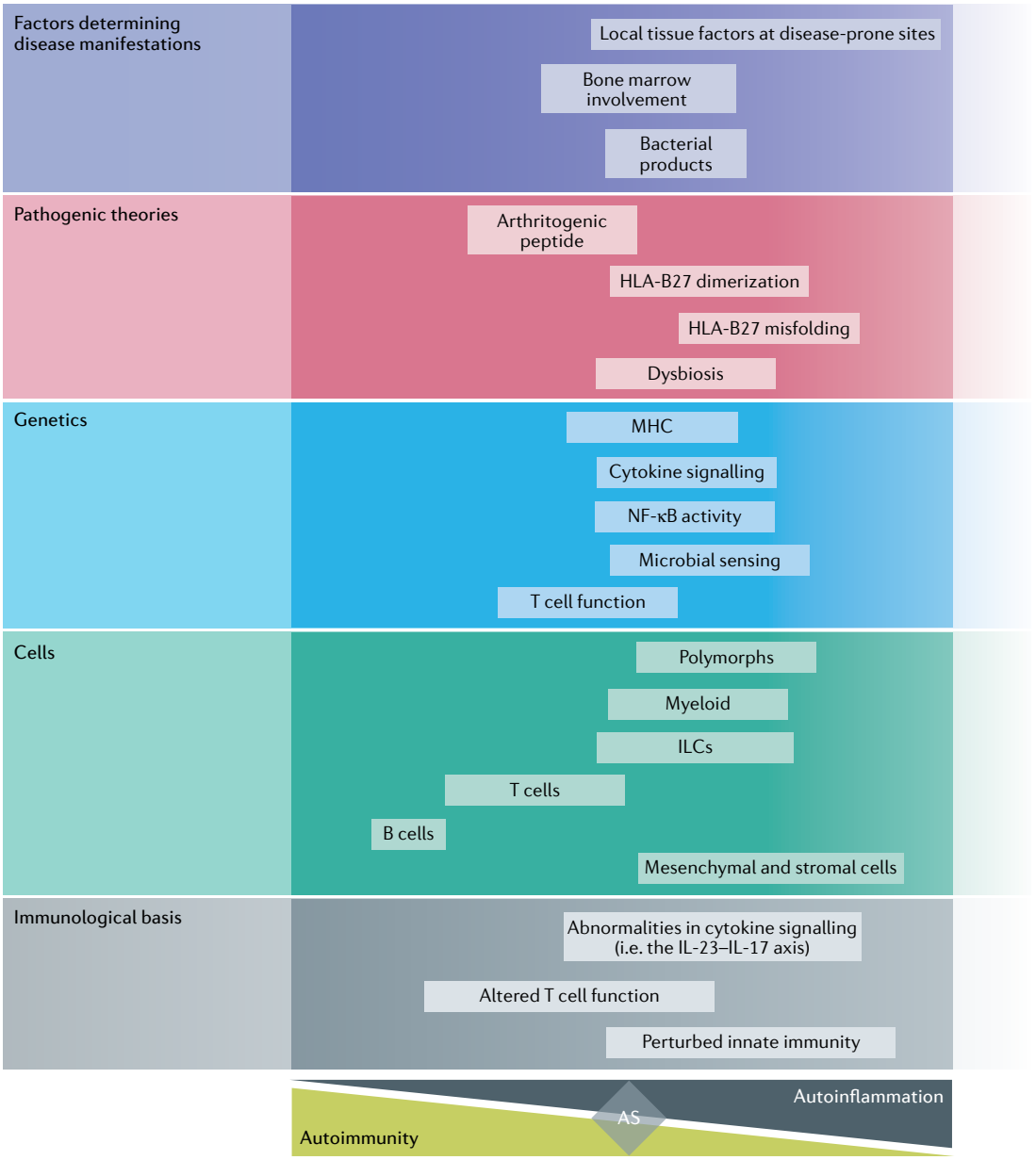

Fig. 3 | **Autoimmunity versus autoinflammation in the pathogenesis of ankylosing spondylitis.** The factors that contribute towards the pathogenesis of ankylosing spondylitis (AS) are summarized according to the classification of immunological disease proposed by McGonagle & McDermott[4], which enables the differentiation of 'pure autoinflammatory diseases' from 'pure autoimmune diseases'. Features of AS that support an autoinflammatory origin are towards the right and features that support an autoimmune origin towards the left. Currently, it is difficult to clearly place AS in one of the two categories; rather, AS seems to contain a mosaic of both types of disease. ILCs, innate lymphoid cells.

component promotes the onset of the disease and the adaptive component is responsible for the perpetuation of the inflammatory process.

The precise identification of patients in whom the autoinflammatory or autoimmune component is predominant will contribute to a better understanding of the disease and to the potential stratification of patients for treatment. To achieve this goal, a more precise stratification of patients from a pathophysiological point of view according to the time of disease onset (early versus late), the predominant clinical manifestations (axial versus peripheral) and the characteristics of the patients (for example, men versus women) is needed. Only in this way will we perhaps be able to answer the question posed in the current Review of whether AS is predominantly autoinflammatory or autoimmune in nature.

Conclusions

Research in the field of SpA is advancing quickly and constantly, offering new insights into the pathogenesis of AS. However, we still lack a unifying explanation that puts together all of the pieces of the pathogenesis puzzle — genetic predisposition, mechanical stress, microbiome, innate immune response, autoreactivity and the spectrum of manifestations associated with SpA. One way that science can find order among a large number of

Procrustean
Forced classification into an arbitrary standard, deriving from the Procrustean bed ancient Greek myth.

facts is by using classification; however, one of the basic rules of classification is that each principle of division should produce mutually exclusive classes. In the light of the complexity of the pathogenesis of AS, its classification as either an autoinflammatory or an autoimmune disease would require a procrustean approach. Perhaps it would be more appropriate to move towards a new taxonomy based on the pathophysiological mechanisms of the disease by imagining SpA, and AS in particular, as a group of diseases that straddle autoinflammation and autoimmunity in most patients, although in some patients one mechanism or the other might prevail. The role of autoimmunity, and specifically of autoantibodies, seems to be worthy of further study, as the data currently available do not clarify if autoantibodies are an epiphenomenon of the systemic inflammatory process, or if they could be used as diagnostic markers. Further research that finally enables us to determine whether AS is promoted by a prevalent autoinflammatory or autoimmune mechanism could have real-world implications, guiding the research agenda and contributing to understanding patient heterogeneity.

Published online 10 June 2021

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Author contributions
All authors researched data for the article. D.M., R.T., M.A.B. and F.C. provided substantial contributions to discussions of content. All authors wrote the article. D.M., R.T., R.L., M.A.B. and F.C. reviewed and/or edited the manuscript before submission.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Rheumatology thanks R. Inman and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Molecular mechanisms of phenotypic variability in monogenic autoinflammatory diseases

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Abstract | Monogenic autoinflammatory diseases are a group of rheumatologic disorders caused by dysregulation in the innate immune system. The molecular mechanisms of these disorders are linked to defects in inflammasome-mediated, NF-κB-mediated or interferon-mediated inflammatory signalling pathways, cytokine receptors, the actin cytoskeleton, proteasome complexes and various enzymes. As with other human disorders, disease-causing variants in a single gene can present with variable expressivity and incomplete penetrance. In some cases, pathogenic variants in the same gene can be inherited either in a recessive or dominant manner and can cause distinct and seemingly unrelated phenotypes, although they have a unifying biochemical mechanism. With an enhanced understanding of protein structure and functionality of protein domains, genotype–phenotype correlations are beginning to be unravelled. Many of the mutated proteins are primarily expressed in haematopoietic cells, and their malfunction leads to systemic inflammation. Disease presentation is also defined by a specific effect of the mutant protein in a particular cell type and, therefore, the resulting phenotype might be more deleterious in one tissue than in another. Many patients present with the expanded immunological disease continuum that includes autoinflammation, immunodeficiency, autoimmunity and atopy, which necessitate genetic testing.

Monogenic
A phenotype or disease that is caused by variation in a single gene and has well-defined inheritance pattern.

Phenotype
An organism's observable traits such as height, hair colour or blood type.

Null alleles
Genetic changes that cause a complete lack of protein expression or can notably alter protein function.

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https://doi.org/10.1038/s41584-021-00614-1

Monogenic autoinflammatory disorders have been linked to germline and somatic pathogenic variants in over 40 genes[1,2]. As these disease-causing variants have a major effect on protein function, one might expect that phenotypes associated with each of these mutated genes have a high degree of clinical similarity, yet there are numerous examples of variability in disease severity and expression that are still poorly understood.

Various molecular mechanisms can lead to phenotypic variability in autoinflammatory conditions (Supplementary Table 1). For some of these disorders, the explanation is more straightforward than for others, as is generally the case with diseases linked to enzyme deficiencies. Null alleles tend to cause severe congenital syndromes, whereas hypomorphic or somatic variants lead to milder and late-onset forms of the disease, and this difference could translate into different clinical diagnoses[3]. Pathogenic variants that affect residues critical for protein function, such as phosphorylation, protease cleavage or glycosylation, will have more deleterious effects than other less-critical genetic alterations in the same gene. The magnitude of phenotypic effects of a pathogenic variant is also closely related to its location in distinct structural protein domains as the genetic change might interfere with intramolecular and intermolecular interactions. As more disease-associated variants are discovered and mapped onto protein domains, a better understanding of genotype–phenotype correlations is likely to emerge (BOXES 1,2).

In this Review, we discuss the mechanisms underlying the phenotypic variability of a subset of monogenic autoinflammatory disorders that present with remarkable phenotypic variability and, in some cases, different inheritance patterns in the same gene. The diseases covered include various inflammasome-mediated disorders, nucleotide-binding oligomerization domain 2 (NOD2)-associated diseases, caspase recruiting domain 14 (CARD14)-associated diseases, inflammatory actinopathies, interferonopathies and diseases caused by enzyme dysregulation.

Pyrin inflammasome-mediated diseases

Inflammasomes are multiprotein complexes that function as intracellular sensors for the recognition of pathogens (pathogen-associated molecular patterns) and endogenous danger-associated molecular patterns[4]. Inflammasomes are nucleated by proteins that belong to the family of NOD-like receptor (NLR)

Key points

- Mendelian-inherited pathogenic variants in a given gene can have different inheritance patterns and can cause distinct and sometimes opposing clinical phenotypes.
- Pathogenic variants in the same gene can have variable disease expressivity depending on their effect on protein function.
- Pathogenic variants in different genes can result in a similar phenotype by virtue of converging on the same signalling pathway (as exemplified by the spectrum of phenotypes denoted as familial cold autoinflammatory syndromes (FCAS).
- A growing number of autoinflammatory diseases can be explained by non-Mendelian inheritance of somatic variants.
- Although systemic inflammation and fevers are common features of autoinflammatory diseases, specific organ manifestations are determined by the tissue expression of mutated proteins.
- Other genetic alleles and risk factors, such as infections and stress, also contribute to the phenotypic variability of autoinflammatory diseases.

Somatic variants
Genetic alterations that occur post-zygotic in somatic cells (for example, leukocytes and keratinocytes) and are not passed on to children.

Genotype
An organism's complete set of genetic material; this term can be used to refer to the variants of a single gene or a set of variants in multiple genes.

Mendelian
The manner by which genes and traits are passed from parents to their offspring, described by Gregor Mendel.

Oligogenic
A phenotype or disease that is dependent on a few genes and is an intermediate between monogenic and polygenic inheritance.

proteins (also known as nucleotide-binding leucine-rich repeat receptors; discussed in a latter section on NLR-like receptor-associated diseases), the protein absent in melanoma 2 (AIM2) or pyrin (as discussed in this section). The aberrant activation of inflammasomes leads to the caspase 1-mediated production of pro-inflammatory cytokines of the IL-1 family. The activity of caspase 1 also induces a specific type of cell death known as pyroptosis, which results in the release of pro-inflammatory intracellular components and cytokines from dying cells[5,6]. Disease-causing variants have gain-of-function effects on the inflammasome pathway; however, these variants have different thresholds for the autoactivation of inflammasomes, which ultimately determines whether one or two pathogenic variants are necessary to trigger inflammation. Although high-penetrance variants lead to the constitutive activation of inflammasomes, by triggering ligand-independent protein oligomerization, milder variants might instead reduce the threshold for activation and enable inflammasome assembly (FIG. 1). The clinical presentation of inflammasomopathies is dependent not only on the protein domain affected by the pathogenic variant but also on the cell-specific expression of these proteins.

Box 1 | **Genotype–phenotype relationships**

Advances in high-throughput sequencing technologies have provided a vast amount of data on genetic variations in humans and thus have revolutionized the conceptual thinking from human monogenic disease towards a continuum of Mendelian–digenic–oligogenic inheritance and the occurrence of somatic variants. The assumption of one gene–one phenotype is overly simplified as heritability is more complex and might change over a lifetime with acquired genetic variations. Pathogenic variants in the same gene can have different inheritance patterns and can lead to distinct conditions and sometimes even contrasting phenotypes depending on whether the variants exert a gain-of-function or a loss-of-function effect on protein function. In the same gene, severe high-penetrance variants can cause the disease phenotype in a monoallelic state, whereas milder variants might need to be doubled in '*cis*' or '*trans*' to attain a deleterious effect. The effect of somatic pathogenic variants is influenced by the time of occurrence during development and the affected cell types. Genetic heterogeneity and digenic inheritance are other factors to consider for autoinflammatory disorders, exemplified by proteasome-associated diseases. Genetic testing has become instrumental in the diagnostics of most immunological diseases owing to an increasing number of patients who present with a continuum of features, including autoinflammation, autoimmunity and immunodeficiency.

Pyrin-associated autoinflammatory disease

Pyrin, encoded by *MEFV*, forms an inflammasome in response to bacterial toxin-induced inactivation of the host GTPase protein Ras homologue gene family member A (RhoA) and changes in the actin cytoskeleton[7]. GTPases function as 'molecular switches' by cycling between inactive (GDP-bound) and active (GTP-bound) conformations to regulate many effector proteins and a variety of signalling pathways[8]. Pathogens modulate RhoA GTPase activity to suppress host immune responses, and these changes are sensed by pyrin. The activation of RhoA results in pyrin inhibition through the phosphorylation of pyrin at residues Ser208 and Ser242 mediated by protein kinase N (PKN1 and PKN2)[9,10]. Pyrin is highly expressed in myeloid cells[11], and pathogenic variants activate pyrin in different ways but ultimately cause proteolytic cleavage of caspase 1 and the release of the pro-inflammatory cytokines IL-1β and IL-18 (FIG. 1). Activating variants result in a variety of phenotypes owing to alleles inherited in a dominant or recessive manner[12].

Familial Mediterranean fever. The most common pyrin-associated recessively inherited disease is familial Mediterranean fever (FMF). FMF is characterized by recurrent episodes of short-lasting fever accompanied by serositis, erysipelas-like erythematous rash and mono-articular arthritis, which can lead to potentially fatal amyloidosis[13]. Inflammatory attacks in FMF are often precipitated by stress[14]. Classical FMF is caused by biallelic missense variants that reside almost exclusively in exon 10, which encodes the C terminal B30.2/SPRY domain[15]. The function of the B30.2 domain is still unclear, and it remains to be seen whether this domain has a pro-inflammatory or autoinhibitory function. The most common pathogenic variants affect the amino acid residues Met680 and Met694[12] (FIG. 2a). Homozygosity for Met694Val is associated with the severe form of FMF and susceptibility to serum amyloid A (SAA) amyloidosis[16]. Although recessively inherited diseases typically result from loss-of-function variants, FMF-associated variants are considered gain-of-function and have a gene dosage effect[17]. Notably, approximately one-third of patients with clinical symptoms carry a single pathogenic variant in *MEFV*[18]. Additionally, asymptomatic individuals who are heterozygote carriers for FMF-associated variants have increased serum levels of acute phase reactants compared with individuals without an FMF-associated variant[19]. A study in primary human monocytes showed that FMF hypermorphic variants have an increased ability to sense bacterial toxin-mediated RhoA inhibition and that these variants lower the threshold for activation of the pyrin inflammasome[20]. Given the existence of an intermediate trait in heterozygote carriers, FMF might be considered a disease with an incomplete dominance inheritance pattern.

The carrier frequency of FMF-associated variants is as high as 10% in multiple Mediterranean populations[21], which has raised the question as to whether these variants might be under some form of positive evolutionary selection. Notably, one study has shown that FMF-associated mutations confer a heightened

Box 2 | **Effects of genotype on protein function**

Aside from enzyme deficiencies, there are common themes in the molecular mechanisms underlying autoinflammatory disorders. Disease-causing variants might follow a dominant or recessive inheritance pattern but fundamentally activate inflammatory pathways either by an upregulation of innate immune sensing pathways or by a lack of proteins that downregulate inflammatory responses. Other mechanisms that can cause unrestrained inflammatory responses are related to defects in protein degradation pathways that lead to upregulation in endoplasmic reticulum stress and the unfolded protein response. Nearly all disease-associated variants identified in 'immune sensors' (such as inflammasomes, nodosomes and signalosomes) are missense substitutions with a gain-of-function effect. High-impact pathogenic variants in highly conserved domains lead to the constitutive activation of proteins and result in severe early-onset phenotypes. Milder variants facilitate the autoactivation of inflammasomes or other immune sensors and, in some instances, necessitate additional factors such as stress, cold or heat to reach the threshold for autoactivation. The deficiency of proteins that negatively regulate inflammatory pathways, including NF-κB, type I interferon and IL-1 signalling pathways, leads to uncontrolled cytokine production manifesting with a variable degree of inflammation. With increasing knowledge about proteome function, regulation and interactions, we are beginning to elucidate the mechanisms of phenotypic variability in human traits and diseases. Cryogenic electron microscopy will ultimately help us better understand the functional consequences of various pathogenic variants and the molecular basis of diseases.

Dominant
The type of inheritance pattern referring to when a single copy of the altered gene is sufficient to cause disease or express the trait.

Recessive
A type of inheritance pattern referring to when both copies of a gene are required for the phenotype or disease expressivity.

Biallellic
A term used to refer to both alleles of a single gene or gene locus (both paternal and maternal).

Missense variants
Genetic changes in a single nucleotide that might or might not result in the substitution of one amino acid for another in the protein.

Hypermorphic variants
A type of genetic change (also known as a gain-of-function mutation) where the altered gene product has an increased level of activity or is expressed at higher levels. These mutations are typically dominantly inherited.

Monoallelic
A term used to refer to when only one of the two copies of a particular gene (alleles) is actively expressed and the other allele is silent.

Heterozygous
An individual who has two different alleles of a particular gene.

resistance to *Yersinia pestis*, the causative pathogen for plague pandemics[22]. Leukocytes from asymptomatic heterozygote carriers release heightened levels of IL-1β in response to *Y. pestis* compared with cells from non-carriers[22]. Thus, although monoallelic hypermorphic *MEFV* variants have been positively selected to confer heightened resistance to an endemic pathogen, biallelic hypermorphic variants cause a hyperinflammatory disease, that is, FMF.

Pyrin-associated dominant diseases. In addition to the recessively inherited FMF variants, other phenotypes exist that are associated with dominant pathogenic variants in *MEFV*. Booth et al. were the first to report patients with typical FMF and SAA amyloidosis carrying the single Met694del variant[23]. The clinical phenotype associated with this monoallelic pathogenic variant is similar to classical FMF but seems to occur at a later age of onset[24]. Another single amino acid deletion, Ile692del, has been linked to a dominantly inherited disease in some families. Interestingly, the Ile692del is also found in patients with the classical recessive FMF genotype, owing to the high carrier frequency of missense pathogenic mutations in affected populations[25]. The importance of this mutation hotspot is unclear and awaits the solving of the protein crystal structure.

A very different phenotype is observed in patients with the heterozygous variants Ser242Arg or Glu244Lys, which affect the residues critical for pyrin inhibition. Variations at these residues prevent pyrin binding to inhibitory 14-3-3 proteins and cause constitutive activation of the pyrin inflammasome[26,27]. Individuals with these variants present with a specific condition, named pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), that presents with severe skin inflammation, myalgia, polyarthralgia, longer-lasting episodes of fever and, unlike in FMF, no evidence of serositis. Monocytes from patients with PAAND have a higher spontaneous production of IL-1β and IL-18 than monocytes from patients with FMF[26]. Although earlier reports have suggested that the Ser208 residue is also critical for pyrin inhibition[28], the inflammatory phenotype associated with variants at this location is present only in patients carrying biallelic pathogenic alleles, either Ser208Thr or Ser208Cys, at this residue. The disease is different from PAAND or FMF and manifests with fevers, oral ulcers, purpuric rash, lymphadenopathy and hypereosinophilia, without elevated secretion of IL-18 (REF.[29]).

Dominantly inherited pathogenic variants in a central domain of pyrin, which is composed of B-box and coiled-coil (CC) subdomains, have been linked to severe inflammatory phenotypes that often progress to SAA amyloidosis. Examples include the His478Tyr variant, found in a family presenting with colchicine-resistant long episodes of fever, peritonitis/pleuritis, severe polyarticular arthritis and renal SAA amyloidosis, and various heterozygous substitutions in residue Thr577 reported in several families[30,31]. Another dominantly inherited variant, Pro373Leu, has been reported in four generations of one family, and was associated with severe inflammation and a high incidence of SAA amyloidosis[32]. The molecular effect of these heterozygous variants on protein function is still unknown; however, they cause higher pyrin activation and enhanced cell death in in vitro conditions[33]. The CC domains play a role in protein oligomerization; thus, these monoallelic variants might trigger the assembly of the pyrin inflammasome.

The clinical relevance of low-penetrance variants, such as Glu148Gln (rs3743930) and Pro369Ser (rs11466023), is still debated, although recent functional studies have disputed their pathogenicity[33,34]. These variants have been linked to typical and atypical FMF as well as to many other polygenic, complex inflammatory diseases[35,36].

In summary, *MEFV*-associated disorders could be considered a continuous disease spectrum owing to the different activation levels of the pyrin inflammasome, which translates into variable responses to therapies. Most patients with FMF respond well to colchicine treatment, whereas patients with a severe phenotype and/or amyloidosis require targeted therapy with IL-1 inhibitors[37].

PSTPIP1-associated diseases

Proline serine threonine phosphatase-interacting protein 1 (PSTPIP1; also known as CD2-binding protein 1 (CD2BP1)) is a cytoskeleton-associated adaptor protein that regulates F-actin remodelling and cell migration and is predominantly expressed in leukocytes, including T cells[38]. PSTPIP1 interacting proteins include pyrin, the Wiskott–Aldrich syndrome protein (WASP), the protein-tyrosine phosphatase PTP-PEST and tyrosine-protein kinase ABL1. PSTPIP1 serves as a scaffold protein for guiding PTP-PEST for the dephosphorylation of WASP, a critical mediator of actin-cytoskeletal polymerization in haematopoietic cells (as discussed in more detail in the later section on actinopathies). WASP is of haematopoietic cells[39], and deficiency in WASP gives rise to an X-linked primary

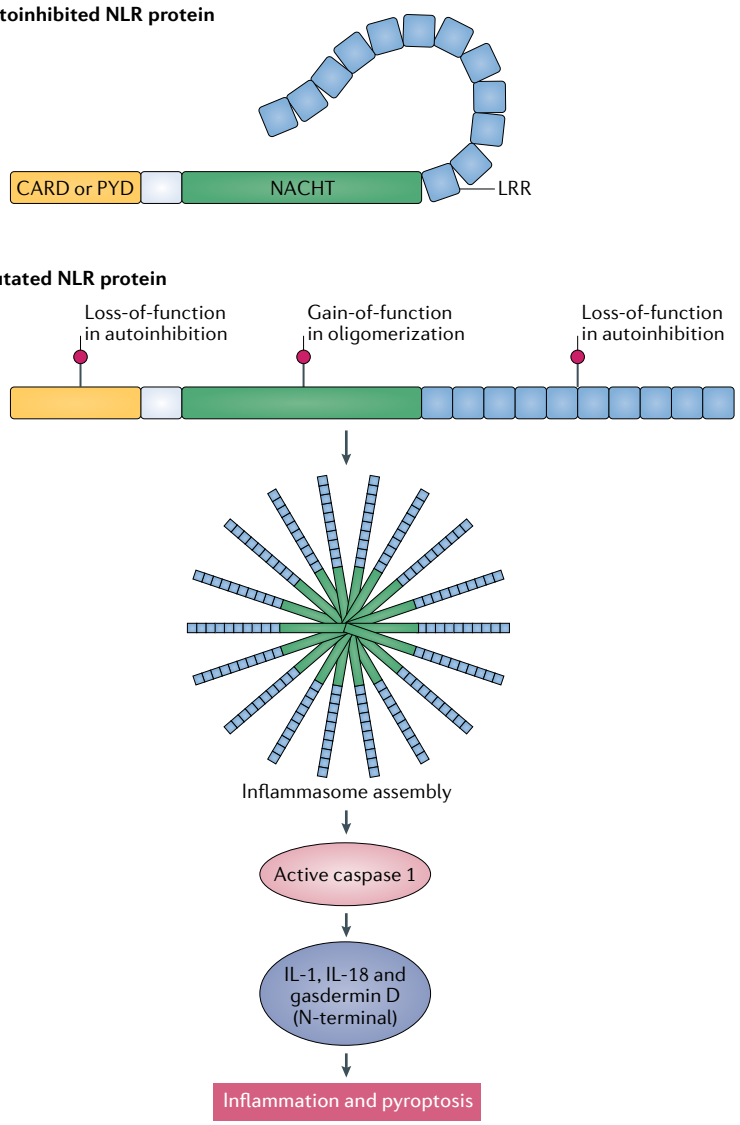

Fig. 1 | **Inflammasome-mediated autoinflammatory disorders. a** | Under physiological conditions, nucleotide-binding domain leucine-rich repeat (NLR) proteins are in a closed form owing to intramolecular domain interactions. **b** | Upon ligand binding (not shown) or in the presence of pathogenic variants, NLR proteins interact with apoptosis-associated speck-like protein containing a CARD (ASC) and caspase 1 to form canonical inflammasomes that process pro-IL-1β and pro-IL-18 into their active forms IL-1 and IL-18, resulting in inflammation and cell death by pyroptosis. The oligomerization of ASC is mediated through the pyrin domain (PYD) of NLRP1 and NLRP3 and the CARD of NLRC4. Gain-of-function variants in the NACHT domain cause inflammasome oligomerization and might result in constitutive activation. Loss-of-function variants in a putative autoinhibitory leucine-rich repeat (LRR) domain or PYD reduce the threshold for activation. The pyrin inflammasome has a different protein structure but is activated in a similar way (not shown).

immunodeficiency, Wiskott–Aldrich syndrome (WAS). Pyrin interacts with PSTPIP1 via its B-box–CC domain; however, the molecular mechanism of this interaction is unclear. The findings of one study suggested that the binding of PSTPIP1 activates pyrin inflammasome by releasing the effects of intramolecular autoinhibition[40].

PSTPIP1-associated arthritis, pyoderma gangrenosum and acne (PAPA) syndrome is a dominantly inherited disorder caused by heterozygous missense pathogenic variants in PSTPIP1 (FIG. 2b). Patients with PAPA present with a variable degree of skin inflammation from severe cystic acne to pyoderma gangrenosum and/or sterile joint inflammation[41]. The most common causal variants, Ala230Thr and Glu250Gln, are located in exons 10 and 11 of *PSTPIP*, which encodes the F-BAR domain[42]. Other pathogenic variants associated with PAPA syndrome are Asp246Asn and Glu256Gly[43,44]. Patients with the Glu250Lys pathogenic variant, which affects the same amino acid residue as in classical PAPA syndrome, or with the Glu257Lys variant present with a distinct phenotype named PSTPIP1-associated myeloid-related proteinemia inflammatory (PAMI) syndrome, also known as hyperzincaemia and hypercalprotectinaemia[45]. This phenotype is far more severe than PAPA syndrome and is characterized by very high serum levels of the pro-inflammatory alarmins S100A8 and S100A9 (REF.[46]). In addition to skin and joint inflammation, patients with PAMI have bone marrow abnormalities that manifest as recurrent infections, bleeding diathesis and autoimmunity similar to WAS[47,48].

Classical PAPA-associated variants are activating mutations that cause the increased production of various pro-inflammatory cytokines, including IL-1β[49]; however, the mechanism by which this effect occurs is not yet fully understood. The PAPA-associated form of PSTPIP1 is hyper-phosphorylated owing to a reduced interaction with PTP-PEST and has a stronger affinity for pyrin[50]. The PAMI-associated Glu250Lys variant has a stronger avidity to pyrin than the Glu250Gln variant, which is attributed to an altered electrostatic potential of PSTPIP1 rather than to an altered level of protein phosphorylation[45]. PSTPIP1 interacts with the B-Box–CC domains of pyrin via its CC–SRC homology domain 3 (SH3) domain, and both the F-BAR and CC domains are known to play a role in protein oligomerization. Fundamentally, all four pathogenic variants lead to the increased activity of the pyrin inflammasome through variable degrees of binding to pyrin; however, the strength of these interactions influences the activity of pyrin and the severity of the inflammatory phenotype.

The heterozygous variants Arg228Cys (rs781341816) and a novel Thr274Met mutation in PSTPIP1 have been identified in two patients with severe T cell deficiency but without signs of autoinflammation[51]. These variants cause an impairment in T cell differentiation by a reduction in F-actin polymerization that is essential for immune synapse formation. LPS-induced stimulation of peripheral blood mononuclear cells (PBMCs) from these patients did not result in increased levels of IL-1β compared with PAPA-associated variants, which might explain the lack of inflammation in these patients.

The C-terminal SH3 of PSTPIP1 is essential for the interaction with WASP and ABL1. The PSTPIP1 variant Arg405Cys (rs201253322) was identified in a boy with aggressive pyoderma gangrenosum and in his father, who had a history of severe acne[52]. This variant impairs PSTPIP1 binding to WASP and does not affect its interaction with PTP-PEST. Another missense variant in the SH3 domain (Gly403Arg) was identified in a patient with pyoderma gangrenosum, acne and ulcerative colitis[53]. Together, these data suggest that PSTPIP1,

Polygenic
A phenotype or disease that is influenced by several genes and often by environmental factors.

via its interaction with WASP, might negatively regulate macrophage migration and function. However, why some patients with pathogenic mutations in PSTPIP1 present only with systemic inflammation and others present with T cell dysfunction is unclear.

Pyoderma gangrenosum, acne and hidradenitis suppurativa (PASH) syndrome is a genetically heterogeneous disease linked to pathogenic variants in *PSTPIP1* and other genes, including *NCSTN*, *NOD2* and *MEFV*[54]. PASH syndrome can be distinguished from PAPA syndrome by the absence of pyogenic arthritis; however, there is a continuum of features common to both disease entities. An increased number of CCTG repeats in the 5′-untranslated region (UTR) of *PSTPIP* as well as Tyr345Cys and Arg405Cys variants in *PSTPIP1* have been identified in patients with PASH syndrome[55–57].

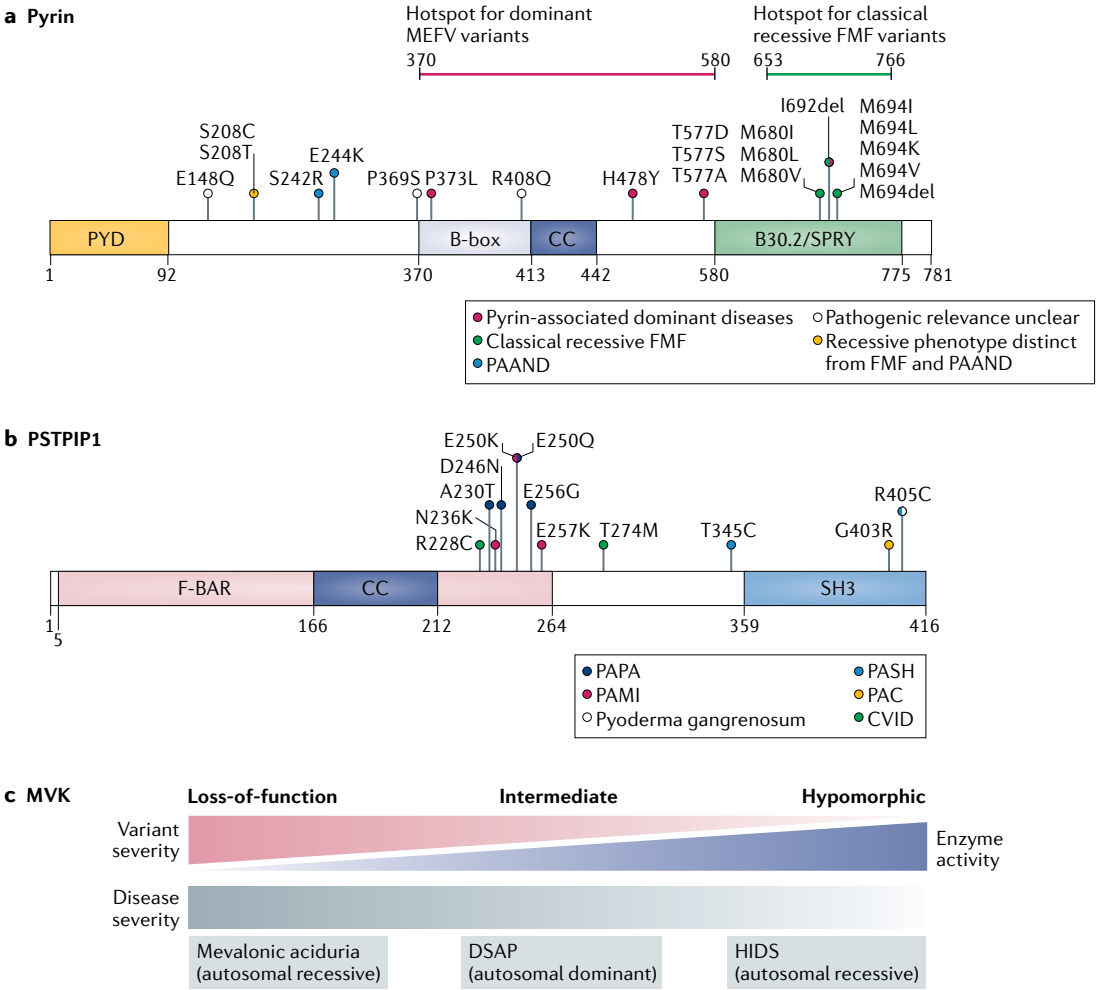

Fig. 2 | **Disease-causing variants of pyrin-associated autoinflammatory diseases.** Variants in pyrin, proline serine threonine phosphatase-interacting protein 1 (PSTPIP1) and mevalonate kinase (MVK) are associated with a spectrum of phenotypes, depending on its effect on protein function. The location and inheritance pattern of various disease-causing variants and their resulting clinical phenotypes are shown. Protein domains are annotated based on the UniProt database[253]. **a** | Dominantly inherited (or de novo) pathogenic variants can cause a severe inflammatory phenotype (shown in red) or the specific condition pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND; shown in light blue), whereas other recessively inherited variants are either associated with classical familial Mediterranean fever (FMF; shown in green) or with a recessive phenotype distinct from FMF and PAAND (shown in yellow). Additional low-penetrance variants have been linked to typical and atypical FMF (shown in white) but their pathogenic relevance is unclear. **b** | Pathogenic variants in PSTPIP1 are either de novo or dominantly inherited and affect various protein interactions. Common variants associated with pyogenic arthritis with pyoderma gangrenosum and acne (PAPA) syndrome are shown in dark blue. The most severe pathogenic variants associated with PSTPIP1-associated myeloid-related proteinemia inflammatory (PAMI) syndrome are shown in red. Other disease-causing variants have been reported in single patients with variable clinical manifestations, including pyoderma gangrenosum (shown in white), pyoderma gangrenosum, acne and hidradenitis suppurativa (PASH) syndrome (shown in light blue), pyoderma gangrenosum, acne and ulcerative colitis (PAC; shown in yellow), or combined variable immunodeficiency (CVID; shown in green). **c** | Recessively inherited loss-of-function (null) variants in *MVK* cause the severe mevalonic aciduria phenotype. By contrast, recessively inherited hypomorphic variants exhibit some residual enzyme activity and are associated to the milder hyper IgD syndrome (HIDS) phenotype. Disseminated superficial actinic porokeratosis (DSAP) is within the MVK deficiency MKD disease spectrum and is caused by one dominantly inherited germline variant together with one skin-specific somatic variant in *MVK*.

Prenylation
A post-translational modification that involves covalent attachment of a lipid consisting of either three (farnesyl) or four (geranylgeranyl) isoprene units to a cysteine residue at or near the C terminus of a protein.

Hypomorphic variants
A type of genetic change (also known as loss-of-function mutation) where the altered gene product has decreased activity or expression. These mutations are typically recessively inherited.

Nonsense mutation
A genetic change that causes the translation of a protein to terminate earlier than would occur with the wild-type gene.

Tyr345 is critical for the dephosphorylation of PSTPIP1 by PTP-PEST[58]. The clinical relevance of the CCTG repeat remains elusive, although it might function as a gene expression modifier.

Thus, pathogenic variants in *PSTPIP1* are linked to a spectrum of diseases manifesting with neutrophilic dermatosis and variable degrees of immunodeficiency. Patients with PAPA require a high dose of anti-cytokine therapies to control inflammation[59], whereas patients with PAMI can be treated effectively with haematopoietic stem cell transplantation (HSCT)[60].

MVK-associated diseases

Mevalonate kinase (MVK) is an ubiquitously expressed enzyme with an important function in cholesterol and isoprenoid biosynthesis[61]. Defects in MVK activity lead to depleted levels of geranylgeranyl pyrophosphate, which is an important metabolic intermediate required for the prenylation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and the small GTPases KRas and RhoA[10,62,63]. GTPases play a role in the regulation of the pyrin inflammasome by activating protein kinases (PKN1/PKN2) and phosphatidylinositol-3-OH kinase (PI3K), which keep pyrin in an inactive state. Thus, a dysregulation in protein prenylation in haematopoietic cells results in increased activity of the pyrin and NLRP3 inflammasomes.

The term MVK deficiency encompasses a continuum of mild to severe diseases. Patients with nearly absent enzymatic activity of MVK exhibit recurrent fevers and severe developmental disabilities, and this phenotype is named mevalonic aciduria. Biallelic hypomorphic variants in *MVK* are associated with a milder phenotype historically referred to as hyper IgD syndrome (HIDS) (FIG. 2c). In infancy, patients with HIDS present with episodes of high fever, erythematous rash, pharyngitis, swollen lymph nodes, abdominal pain, diarrhoea, aphthous ulcers, and arthralgia and/or arthritis and symptoms are often triggered by immunization[64]. Patients who carry one null variant and a second hypomorphic pathogenic variant have an intermediate phenotype, less severe than classical mevalonic aciduria and with chronic inflammation. Stimulated PBMCs from patients with MVK deficiency produce higher levels of IL-1β compared with PBMCs from healthy individuals[65].

Most variants causing mevalonic aciduria are nonsense mutations or create truncated proteins, whereas nearly all HIDS-associated variants are missense substitutions and are thought to impair protein stability in a temperature-sensitive manner[66]. Patients with biallelic HIDS-associated variants maintain residual enzyme activity in the range of 1–10%[66]. Two pathogenic variants (Ile268Thr and Val377Ile) account for more than 50% of patients with HIDS in multiple populations. The carrier frequency of Val377Ile in the founder Dutch population is high; however, the disease incidence is lower than predicted, which implies that Val377Ile has a milder effect on protein stability compared with other disease variants[67]. The severity of phenotype in HIDS correlates with the effect of the variant on the expression, folding or stability of MVK, whereas the catalytic properties of the enzyme are not affected[68].

A genome-wide association study identified novel heterozygous loss-of-function variants in *MVK* in Chinese patients with a distinct phenotype named disseminated superficial actinic porokeratosis (DSAP), which presents with potentially malignant keratotic skin lesions and without inflammatory features[69]. DSAP-associated variants were described as dominantly inherited with variable penetrance. The pathogenesis of DSAP was puzzling until a study in 2019 showed that DSAP was caused by a skin-specific deficiency of MVK[70]. Essentially, patients with DSAP carry one germline *MVK* pathogenic variant, which explains the familial inheritance of DSAP, and then acquire a second somatic *MVK* variant in their epidermis.

Overall, patients with nearly absent enzymatic activity have severe developmental disabilities, whereas a partial deficiency of MVK causes the HIDS phenotype. The inflammatory features in patients with HIDS are ameliorated with anti-IL-1 therapy whereas mevalonic aciduria can be effectively treated with HSCT[37,71].

NLR-associated diseases

NLRs are cytosolic innate immune sensors that regulate inflammatory responses and cell death by forming inflammasome complexes[72,73]. Their protein structure is highly conserved and includes a centrally located NACHT domain with ATPase activity, a C-terminal leucine-rich repeat (LRR) domain and a variable N-terminal domain that consists of either a CARD or a pyrin domain (PYD) (FIG. 1). The NACHT domain itself is composed of several subdomains: a nucleotide-binding domain (NBD), two helical domains (helical domain 1 (HD1) and HD2) and a winged-helix domain (WHD). Within the NACHT, several highly conserved motifs are essential for nucleotide-binding and/or hydrolysis, including a P-loop that is specific for ATPase and GTPase and the Mg^{2+}-binding site (known as Walker A and Walker B motifs, respectively)[74]. Nucleotide exchange of ATP for ADP is required for protein activation in response to ligand binding. The LRR domain is thought to serve as an autoinhibitory domain and to function as a ligand sensor. Through intramolecular interactions, the LRR domain keeps the protein in an autoinhibitory state. The ADP-bound 'closed' protein conformation is released upon the binding of specific ligands to the LRR domain and results in conformational changes and the exposure of the N-terminal effector domains (CARD or PYD). This change enables homotypic interactions of NLRs with other CARD-containing or PYD-containing proteins and subsequent inflammasome formation. Pathogenic variants in NLRs are activating variants and lead to the ligand-independent activation of the respective proteins that result in the overproduction of IL-1β and IL-18 (REF.[75]). Although NOD2 belongs to the family of NLR proteins, disease-associated variants in this protein lead to the ligand-independent activation of the NF-κB pathway.

IL-1-mediated or IL-18-mediated diseases

NLRP1-associated diseases. NLRP1 functions as a cytosolic sensor for bacterial toxins and viral dsRNA, including *Bacillus anthracis* lethal toxin[76], and regulates caspase

1-dependent cell death (pyroptosis)[77–79]. This protein is highly expressed in keratinocytes and haematopoietic cells[80]. NLRP1 is unique among inflammasomes in that its C-terminal end is composed of a 'function-to-find' domain (FIIND) and a CARD (FIG. 3a). The PYD and LRR domains of NLRP1 are assumed to suppress its activation. The autoproteolytic cleavage of FIIND leads to the release of a C-terminal CARD domain, which is sufficient for inflammasome activation[81]. The activity of the NLRP1 inflammasome is further regulated by the sequestration of its C terminus[82].

Activating variants in the NLRP1 inflammasome are linked to a spectrum of phenotypes, ranging from a hyperplastic skin disorder to autoinflammation. These pathogenic variants reside in different protein domains and can be inherited in a dominant or a recessive manner but eventually lead to increased NLRP1 self-oligomerization by disrupting autoinhibitory domains (FIG. 3a). For example, heterozygous missense variants in the PYD (Ala54Thr, Ala59Pro, Ala66Val and Met77Thr) are linked to multiple self-healing palmoplantar carcinoma (MSPC) and corneal dyskeratosis[83–85]. These patients have no features of systemic inflammation and instead present with epidermal hyperplasia and susceptibility to malignant squamous cell carcinoma. Biallelic loss-of-function variants in other domains of NLRP1 have been associated with an inflammatory skin phenotype denoted as familial keratosis lichenoides chronica (FKLC) or the phenotypically distinct disorder NLRP1-associated autoinflammation with arthritis and dyskeratosis (NAIAD)[84,86]. Two siblings with FKLC were found to be homozygous for the in-frame deletion Phe787–Arg843del in the first LRR domain of NLRP1. Heterozygous carriers for this variant have a mild phenotype, suggesting that two mutant alleles are necessary for a fully penetrant phenotype[84]. Patients with NAIAD carry the homozygous pathogenic variant Arg726Trp in the linker region between the NACHT and LRR domains and present with a spectrum of inflammatory features, which is in contrast to patients with MSPC or FKLC[86]. Another homozygous variant in the linker domain (Thr775Asn) is reported to cause a severe phenotype called juvenile-onset recurrent respiratory papillomatosis (JRRP), a rare disease that manifests with keratotic skin lesions and recurrent respiratory papillomas that can cause potentially life-threatening airway obstructions[87]. Finally, one patient has been found to carry a de novo heterozygous variant (Pro1214Arg) close to the cleavage site in the FIIND domain, which is notable as NLRP1 activity is dependent on autolytic cleavage within this domain. This patient presented with a more severe inflammatory phenotype and much higher serum levels of IL-1β and IL-18 than two patients with NAIAD[86].

Low-penetrance common single nucleotide polymorphism (SNP) variants in the promoter and non-coding regions of *NLRP1* are associated with susceptibility to vitiligo and other autoimmune and autoinflammatory diseases[88–90]. These variants might augment the transcription or translation of NLRP1 (REF.[89]). The inflammatory phenotype in both monogenic and polygenic NLRP1-associated disorders is most prominent in the skin; however, the disease expression is variable[91].

Overall, irrespective of the mode of inheritance, NLRP1 pathogenic variants activate the pro-inflammatory milieu in the skin, especially IL-1 and IL-18, leading to epidermal hyperplasia.

***NLRP3*-associated diseases.** The NLRP3 inflammasome functions as a cytosolic sensor for a number of pathogen-associated molecular patterns and danger-associated molecular patterns and is critical for host defences[92,93]. NLRP3 is highly expressed in haematopoietic cells, primarily in myeloid cells[94], and its regulation involves various multiple positive and negative regulators, including reactive oxygen species, calcium signalling, ubiquitylation, prenylation, SUMOylation and microRNAs. NLRP3 is further regulated through phosphorylation at residue Tyr861 in the LRR domain and through its interaction with the Ser/Thr protein kinase NEK7 (REF.[95]).

Heterozygous gain-of-function variants in *NLRP3* cause a continuum of phenotypes, denoted as cryopyrin-associated periodic syndrome (CAPS), that ranges from familial cold autoinflammatory syndrome (FCAS) to Muckle–Wells syndrome (MWS) to the most severe phenotype denoted as neonatal-onset multisystem inflammatory disease (NOMID; also known as chronic infantile neurological, cutaneous and articular syndrome (CINCA))[96] (FIG. 3b). Clinical features span from recurrent fevers, urticaria-like rash and arthralgia in patients with FCAS, to early-onset severe systemic inflammation, epiphyseal bone overgrowth, sensorineural hearing loss, vision loss, aseptic meningitis and cognitive disability in patients with NOMID. NOMID is associated with systemic inflammation that is chronic and persistent, whereas flares in FCAS are triggered by cold temperature and humidity. Patients with severe chronic inflammation in the MWS–NOMID spectrum can develop SAA amyloidosis[96].

Most CAPS-associated variants are missense substitutions that reside in the NACHT domain (FIG. 3b). However, the severity of the clinical presentation depends on the position and biochemical properties of these substitutions[93]. Disease-causing variants located around the ATP-binding pocket are usually inherited de novo and cause constitutive inflammasome activation[95]. Other 'milder' variants probably destabilize the interdomain interaction and facilitate the active state of NLRP3.

The only nonsense pathogenic variant in *NLRP3*, Arg554Ter (also known as Arg556Ter), has been described in a patient with FCAS and causes a complete loss of the autoinhibitory LRR domain[97]. Several missense pathogenic variants in the LRR domains have been reported in patients with late-onset symptoms, hearing loss and atypical presentation[98–101] (FIG. 3b). The hearing loss is not always accompanied by clinical evidence of inflammation, indicating a milder impact of these variants.

Thus far, only two pathogenic variants have been identified in the PYD of NLRP3. A novel substitution, Asp31Val, was found in a patient with MWS, and this mutation increases the interaction of NLRP3 with apoptosis-associated speck-like protein containing a CARD (ASC)[102]. A second rare variant, Asp21His

Homozygous
An individual who has inherited the identical alleles of a particular gene from both parents.

De novo
A genetic change that arises in a germ cell or fertilized egg and is not inherited from the parents.

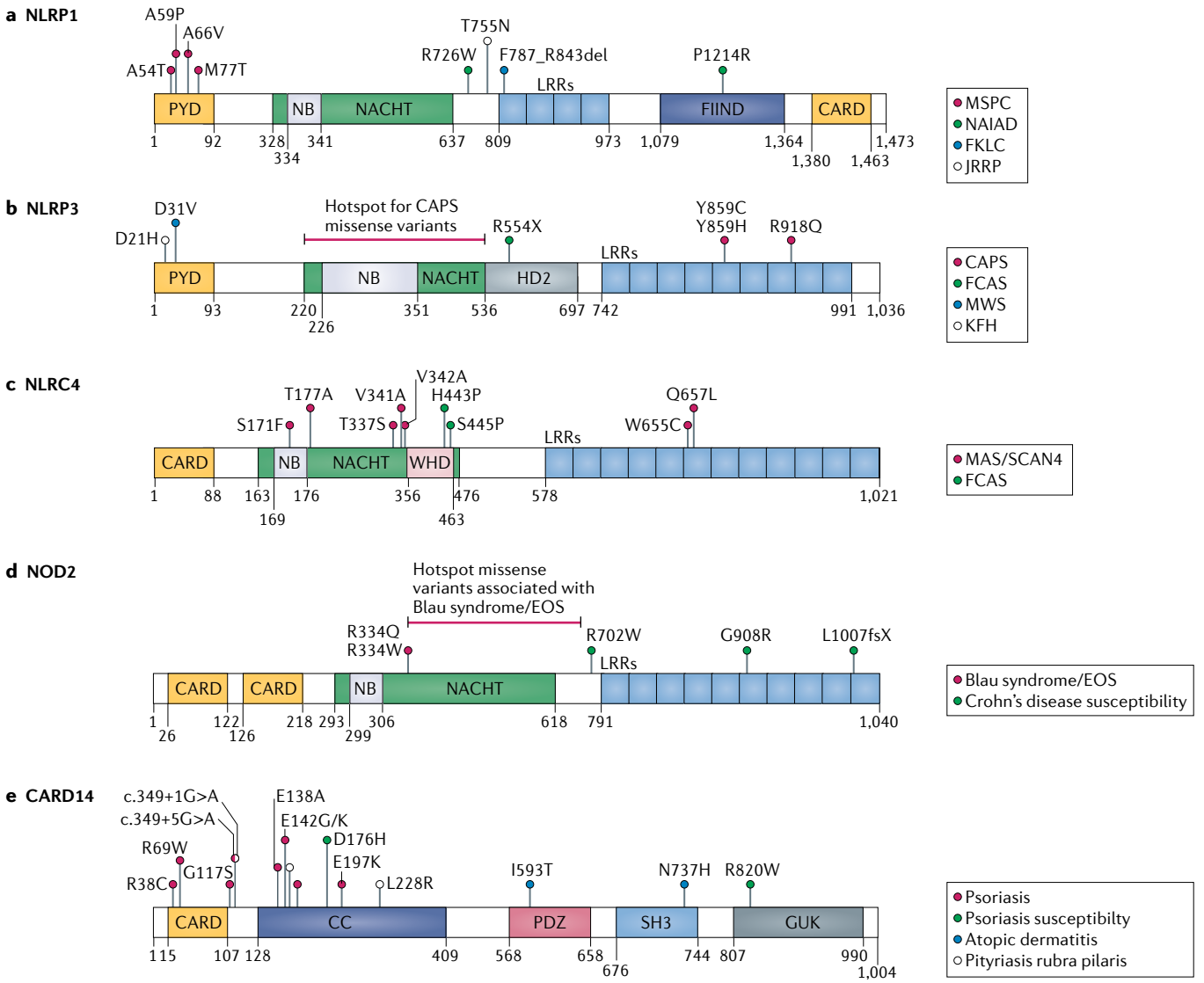

Fig. 3 | **Disease-causing variants of NOD-like receptor-associated diseases.** Numerous variants in the proteins NACHT, LRR and PYD domains-containing protein 1 (NLRP1), NLRP3, NLR family caspase activation and recruitment domain (CARD) domain-containing protein 4 (NLRC4), NACHT domain of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and CARD14 can cause a variety of autoinflammatory diseases. The inheritance pattern of NOD-like receptor-associated autoinflammatory diseases can be autosomal dominant (or occur de novo) or autosomal recessive. Pathogenic variants are shown in various colours to depict different associated phenotypes. Protein domains are annotated based on the UniProt database[253]. **a** | Pathogenic variants in the pyrin domain (PYD) of NLRP1 that are linked to autosomal dominant multiple self-healing palmoplantar carcinoma (MSPC) are highlighted in red . Recessively inherited variants (or dominantly inherited variants in the case of P1214R) in other domains that cause either juvenile-onset recurrent respiratory papillomatosis (JRRP), NLRP1-associated autoinflammation with arthritis and dyskeratosis (NAIAD) or familial keratosis lichenoides chronica (FKLC) are shown in white, green or blue, respectively. **b** | The NACHT domain of NLRP3 can contain various missense gain-of-function variants (shown across as a red line) that are associated with severe cryopyrin-associated periodic syndrome (CAPS) phenotypes (including Muckle–Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID)), as well as other missense variants associated with the milder familial cold autoinflammatory syndrome (FCAS) phenotype. The only described nonsense variant in *NLRP3* that can cause FCAS is shown in green, whereas the D21H variant (shown in white) is associated with the eye disease keratoendotheliitis fugax hereditaria (KFH). A pathogenic variant D31V (shown in blue) in the pyrin domain has also been reported in patients with MWS. Pathogenic variants in the leucine-rich repeat (LRR) domain are milder but can be associated with neurological phenotypes, for example, sensorineural deafness. **c** | Heterozygous gain-of-function variants in NLRC4 that cause the severe life-threatening condition syndrome of enterocolitis and autoinflammation associated with mutation in NLRC4 (SCAN4) or macrophage-activation syndrome (MAS) are shown in red and milder variants, associated with FCAS, are highlighted in green. **d** | Missense high-penetrance variants in the NOD2 that are associated with Blau syndrome are depicted in red (only the two most common variants are shown). Most common low-penetrance single nucleotide polymorphisms that are associated with susceptibility to Crohn's disease are shown in green. **e** | CARD14 can contain various dominantly inherited novel or rare pathogenic variants that are associated with psoriasis (shown in red), pityriasis rubra pilaris (shown in white) or atopic dermatitis (shown in blue) as well as low-penetrance common variants linked to susceptibility to psoriasis (shown in green). This diagram only shows variants for which there is a sufficient functional evidence that they cause an increase in NF-κB activity. EOS, early-onset sarcoidosis; FIIND, 'function-to-find' domain; GUK, guanylate kinase domain; NB, nucleotide binding core residues; PDZ, PDZ domain; SH3, SRC homology domain 3; WHD, winged-helix domain.

Disease expressivity
The extent to which a genotype shows its phenotypic expression in different people with the same genetic disease.

(rs200154873), has been identified in seven Finnish families diagnosed with keratoendotheliitis fugax hereditaria; however, the effect of the variant on inflammasome activation is unclear[103]. NLRP3 is expressed in the human cornea and eye inflammation is a known feature of CAPS. Indeed, patients with these variants present with conjunctival injection, corneal opacities, pain and photophobia albeit with no evidence of systemic inflammation.

In addition to germline inheritance, somatic missense variants have been reported in about 30% of patients with classical and late-onset CAPS[104–108]. The mutant alleles are found predominantly in myeloid cells with a variable allele frequency as low as 5%[108,109]. These somatic variants often arise at residues critical for protein function, which suggests that germline mutations at these residues are likely incompatible with life[110]. Specifically, two highly conserved subdomains, NBD and HD2, harbour many CAPS-associated somatic mutations. A somatic in-frame deletion of three amino acids (Gly309, Ala310 and Phe311) in close proximity to the Walker B motif was identified in a patient with adult-onset urticaria and systemic inflammation[109].

A low-penetrance variant, Val198Met (rs121908147; also known as Val200Met), has been linked to Schnitzler syndrome, a late-onset disease manifesting with neutrophilic urticaria and monoclonal gammopathy[111]. This variant was germline inherited in two patients with classical (IgM) Schnitzler and was found as a somatic variant in two patients with variant (IgG) Schnitzler[112,113]. The same Val198Met and the rare variant Lys488Arg (rs145268073; also known as Lys490Arg) have been reported in patients with mild non-specific inflammatory phenotypes[114]. Whether these variants influence inflammasome function is still debated.

In summary, pathogenic variants in *NLRP3* enhance inflammasome activity; however, although some variants maintain the protein in the constitutively active state, milder variants might require environmental factors such as cold or stress to trigger inflammasome activation. Mutant cells spontaneously secrete high levels of IL-1β and treatment with IL-1 inhibitors is highly effective[37,115,116].

NLRC4-associated diseases. The NLR family CARD domain-containing protein 4 (NLRC4, also known as IPAF) inflammasome functions as a cytosolic sensor in innate immune and intestinal epithelial cells[117–119]. Through its interaction with the NLR family apoptosis inhibitory protein (NAIP), NLRC4 senses and restricts the intraepithelial replication of Gram-negative bacteria. NLRC4 is a unique NLR protein in that NLRC4 does not bind to bacterial ligands but rather this protein co-assembles with the NAIP receptor to form a functional inflammasome. In contrast to other inflammasomes, the NLRC4 protein contains an N-terminal CARD, instead of a PYD, and can activate caspase 1 independent of ASC[120]. As with other inflammasomes, NLRC4 autoinhibition relies on complex interdomain interactions that stabilize a closed and inactive conformation[121]. Phosphorylation at residue Ser533 is critical for protein function[122]. The NLRC4 inflammasome uses two functions to eliminate bacteria from epithelial cells: the release of pro-inflammatory cytokines (IL-1β and IL-18) and pyroptosis.

Heterozygous gain-of-function missense variants in *NLRC4* result in a spectrum of autoinflammatory phenotypes ranging from milder FCAS to severe life-threatening enterocolitis or macrophage-activation syndrome (MAS)[123,124]. In patients who survive the early-onset severe disease, intestinal inflammation can subside[123], suggesting that host–microbiome interactions probably regulate the disease expressivity.

Various severe MAS-associated variants (Thr337Ser, Val341Ala and Val342Ala) reside in a highly conserved subdomain of NACHT, the HD1, and cause constitutive protein activation (FIG. 3c). A second cluster of pathogenic variants includes the germline variant Ser171Phe and the somatic variant Thr177Ala[125,126]. The Ser171Phe substitution was identified in an infant with congenital anaemia, systemic inflammation, ascites, hepatosplenomegaly and haemophagocytosis, who died at the age of 2 months[125]. The somatic variant Thr177Ala was identified at a high variant frequency in induced pluripotent stem cell clones from a child with NOMID-like features, including severe central nervous system inflammation[126]. These pathogenic variants reside in the vicinity of the ATP-binding pocket and result in a highly active protein. Two other MAS-associated variants, Trp655Cys and Gln657Leu, are thought to increase protein activation by creating an LRR–LRR interface important for NLRC4 oligomerization[127,128].

FCAS-associated *NLRC4* variants are inherited in an autosomal-dominant manner and reside in the WHD of NACHT. The His443Pro pathogenic variant was identified in a three-generation family with cold-induced rash[129]. This variant could trigger constitutive caspase 8-mediated Fas-associated protein with death domain (FAAD)-dependent cell death, independent of Ser533 phosphorylation[130]. A second family with 13 affected members was found to carry the Ser445Pro variant[131]. In this family, all patients presented with cold-induced and/or stress-induced skin lesions, arthralgia and conjunctivitis, and only 2 of the 13 patients had enterocolitis[131]. Thus, similar to CAPS, pathogenic variants in NLRC4 lead to enhanced inflammasome activity; however, although variants affecting the highly conserved motifs spontaneously activate the protein, milder variants increase the propensity for inflammasome activation. Cultured myeloid cells of patients with NLRC4-MAS spontaneously secrete IL-18 and patients respond well to blockade with IL-18 binding protein[132].

NF-κB mediated diseases

NOD2-associated granulomatous disease. NOD2 (also known as CARD15) is a cytosolic sensor for bacterial muramyl dipeptides derived from the cell wall of bacteria[133]. NOD2 is expressed in myeloid and lymphoid cells as well as in intestinal epithelial cells[134]. Ligand binding to the LRR domain triggers conformational changes that lead to self-oligomerization (to form a signalling complex known as the nodosome[135]), recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIPK2), and activation of the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways.

NOD2 was first linked to human disease as one of the strongest Crohn's disease susceptibility genes. Common low-penetrance SNP variants have been linked to Crohn's disease in multiple, predominantly Caucasian populations (FIG. 3d). Although Crohn's disease-associated SNPs are found throughout the gene, the most common risk alleles are Arg702Trp, Gly908Arg and Leu1007fsX, and it is estimated that up to 30% of patients with Crohn's disease carry one or two copies of these SNPs. All three variants are located in the LRR domain and are thought to affect the sensing activity of LRRs or its function as a putative autoinhibitory domain. The detailed mechanism of intestinal inflammation in Crohn's disease is still unclear; however, Crohn's disease susceptibility alleles are considered functionally hypomorphic as they impair mucosal barrier function and bacterial clearance. Other possible contributing mechanisms include alterations in the immunomodulatory function of NOD2 in regulating TLR responses and autophagy[136,137].

By contrast, high-penetrance novel or rare pathogenic variants in the NACHT domain are associated with a severe dominantly inherited disorder, Blau syndrome (also known as early-onset sarcoidosis), which is characterized by granulomatous arthritis, skin lesions and uveitis[138,139]. In addition to germline-inherited pathogenic variants, somatic de novo and gonosomal variants have been reported in some patients with variable frequencies of a mutant allele in different cells[140,141]. The Blau syndrome-associated missense gain-of-function variants facilitate the formation of the constitutively active NOD2 nodosome and result in increased NF-κB basal activity and IFNγ-mediated inflammatory response[142–144].

A common missense variant in the non-coding region of *NOD2*, c.2798 +158C>T (rs5743289), has been linked to a non-specific inflammatory phenotype termed NOD2-associated autoinflammatory disease[145]. Patients with NOD2-associated autoinflammatory disease typically present in their mid-30s with fevers, arthralgia and erythematous patches or plaques on the trunk. This variant is associated with increased mRNA levels of NOD2 and increased basal MAPK pathway activity in PBMCs but to a much lesser extent than that seen with Blau syndrome-associated mutations[146]. Additional risk factors, such as age-dependent changes in commensal microbiota, might contribute to the disease expression.

In general, high-impact gain-of-function mutations associated with Blau syndrome result in constitutive activation of the NF-κB pathway, whereas common hypomorphic variants in the LRR domain are associated with a susceptibility to Crohn's disease. In contrast to the other NLR-associated diseases, Crohn's disease and Blau syndrome are not mediated by increased IL-1β secretion[147], and anti-TNF therapy has been highly efficacious in these patients[148].

CARD14-associated psoriasis. CARD14 is a scaffold protein, highly expressed in keratinocytes and endothelial cells, that functions as an epidermal regulator of NF-κB signalling[149]. CARD14 exists in a closed autoinhibited form; upon stimulation, CARD14 is phosphorylated by protein kinase C and binds to MALT1/BCL10 to activate the NF-κB signalling pathway[150].

Pathogenic variants in CARD14 have been linked to a spectrum of skin inflammatory phenotypes, including psoriasis vulgaris, familial pityriasis rubra pilaris and pustular psoriasis, with variable disease expressivity and penetrance. Fever and other inflammatory manifestations are not generally present in these patients. Collectively, these diseases are described as CARD14-mediated pustular psoriasis (CAMPS) (also denoted as psoriasis susceptibility 2 locus; PSOR2).

Pathogenic CAMPS-associated variants in *CARD14* are dominant gain-of-function missense substitutions that lead to a range of amplified NF-κB activities[151]. These variants are found in all protein domains, although there is enrichment of pathogenic mutations in exon 4 that encodes the CC domain, which suggests that the CC domain is important for the regulation of CARD14 activity[152–156]. The CC domain is known to mediate protein oligomerization upon its activation. Three mutations, Gly117Ser, and intronic variants, c.349+5G>A and c.349+1G>A, create a cryptic splice site that results in a 22 amino acid insertion, disrupting the CARD domain (FIG. 3e). The most severe phenotype has been observed in a patient with early-onset general pustular psoriasis who had a de novo variant, Glu138Ala[153]. The mutant protein induced the highest level of NF-κB activity relative to the other CAMPS-associated rare mutations and CAMPS-associated SNPs as shown by in vitro experiments. This missense substitution disrupts the autoinhibitory linker domain of CARD14 and causes constitutive activation of the protein. The mutant protein facilitates the formation of the B cell lymphoma/leukaemia-10 (BCL-10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) signalosome and promotes pro-inflammatory gene expression in keratinocytes[151]. Most other CAMPS-associated variants result in moderately increased NF-κB activity in overexpression experiments[157].

Low-penetrance rare and common variants in *CARD14*, including Arg820Trp (rs11652075) and Asp176His (rs144475004), have been associated with the susceptibility to psoriasis and general pustular psoriasis in large cohort studies[152,149,158]. These missense variants probably destabilize the inactive form of the protein but require additional risk factors for disease expressivity. Interestingly, dominant-negative variants that impair downstream NF-κB signalling in keratinocytes have been associated with a severe form of atopic dermatitis[159].

Thus, on the whole, activating variants in *CARD14* increase the transcription of pro-inflammatory chemokines and cytokines in the skin[152,153]. A range of phenotypes has been observed even in patients with the same pathogenic variants, suggesting a role for environmental and genetic modifiers. Patients with CARD14-associated diseases have a favourable response to treatment with ustekinumab[160].

Inflammatory actinopathies

The actin cytoskeleton is critical for the regulation of many cellular functions, including inflammatory and immune responses[161,162]. The maintenance of cytoskeleton dynamics, including its assembly and

depolymerization, requires the highly coordinated action of many effector and regulatory proteins. In immune cells, the actin cytoskeleton is required for functions such as immune synapse stability, signalling, migration and cytokine release. Pathogenic variants in proteins involved in cytoskeleton organization have been mainly reported in patients with early-onset primary immunodeficiencies and thrombocytopenia[162]. However, variable degrees of autoinflammatory features, including a predisposition to haemophagocytic lymphohistiocytosis (HLH), have been described in all these diseases.

The actin-related protein 2/3 (ARP2/3) complex is pivotal for assembling branched actin filaments by bringing actin monomers into the complex to initiate actin polymerization (FIG. 4). The small GTPases cell division control protein 42 (CDC42) and RAC1 are essential for the activation of the ARP2/3 complex via WASP or the WASP-family verprolin-homologous protein (WAVE) regulatory complex (WRC), respectively[163]. The Nck-associated protein 1-like (NCKAP1L) functions as a haematopoietic lineage-specific regulator of the actin cytoskeleton in response to the engagement of immune receptors in lymphoid and myeloid cells. NCKAP1L is required for the RAC1-mediated stabilization and activation of the WRC[164]. The function of the WRC complex is similar to WASP in terms of enabling the ARP2/3 complex to initiate and coordinate

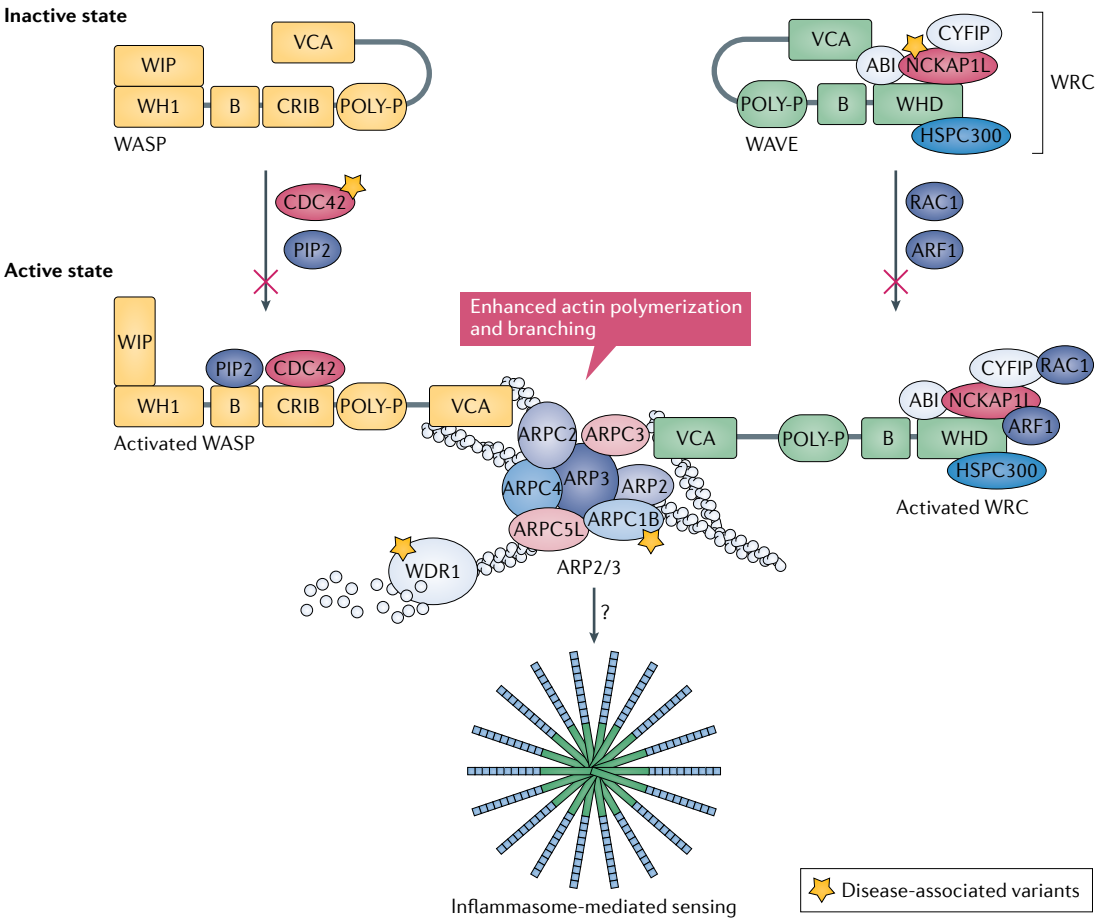

Fig. 4 | **Pathogenesis of inflammatory actinopathies.** Wiskott–Aldrich syndrome protein (WASP, shown on the left) and the WASP-family verprolin-homologous protein (WAVE) regulatory complex (WRC, shown on the right) can both promote actin polymerization via the actin-related proteins 2/3 (ARP2/3) complex, which is composed of seven subunits (shown in the centre). In the inactive state, WASP is inhibited by an interaction between the CDC42/RAC interactive binding motif (CRIB) domain and the verprolin homology/central/acidic (VCA) region. Cooperative binding of active CDC42 to the CRIB domain and phosphatidylinositol 4,5-bisphosphate (PIP2) to the B domain exposes the VCA domain and results in the activation of the ARP2/3 complex. Disease-associated forms of CDC42 are unable to interact with various effectors and regulators and fail to activate WASP. In its inactive state, WRC contains WAVE, ABI (Abl interactor protein), CYFIP (cytoplasmic FMR1-interacting protein), HSPC3000 (haematopoietic stem/progenitor cell protein 300) and NCK associated protein 1-like (NCKAP1L). The binding of active RAC1 to CYFIP, and ARF1 to NCKAP1L, shifts the WRC from an inactive to an active state and enables ARP2/3 complex activation. Disease-associated forms of NCKAP1L impair ARF1 binding and cause the destabilization of the WRC. Activated ARP2/3 triggers actin polymerization and branching. Pathogenic variants in the ARP2/3 subunit ARPC1B cause impaired actin polymerization, whereas biallelic loss-of-function variants in WDR1 lead to defective actin depolymerization. Changes in the cytoskeleton dynamics might be sensed by the inflammasome or perhaps by other cytosolic sensors and can result in an inflammatory phenotype. POLY-P, poly-proline; WH1, WASP homology region 1; WHD, winged-helix domain; WIP, WAS/WASL-interacting protein family member 1.

actin polymerization. Conversely, WD-repeat protein 1 (WDR1, also known as AIP1) and actin-depolymerizing factor 1 (ADF1) are critical for actin depolymerization and thus for the regulation of the dynamics of the actin cytoskeleton. Together, all these proteins play a critical role in the maintenance and turnover of the actin filament network. Studies of the pyrin inflammasome have provided insights on how cells might sense changes in the cytoskeleton dynamics. In this section, we discuss the phenotypes associated with deficiencies in CDC42, NCKAP1L, ARP2/3 complex subunit 1B (ARPC1B; a component of the ARP2/3 complex) and WDR1.

CDC42 deficiency

Dysregulation of the function of CDC42 has been linked to various immune and non-immune phenotypes, depending on the effect of the mutation on cell-specific transcript isoforms. Heterozygous dominant loss-of-function variants in the brain-restricted isoform of CDC42 (isoform 2) cause diverse neurodevelopmental phenotypes that include growth failure, facial dysmorphism, brain malformations, intellectual disability and cardiac defects[165]. These variants disrupt the switch between the active and inactive state of the protein and its interaction with various effector proteins and consequently affect multiple cellular functions. Heterozygous loss-of-function de novo variants in the ubiquitously expressed transcript of CDC42 (isoform 1) have been identified in patients with a severe and potentially fatal haematological disease named neonatal onset of pancytopenia, autoinflammation, rash and haemophagocytosis (NOCARH)[166,167]; some of these patients have mild dysmorphic features. The recurrent NOCARH-associated Arg186Cys variant affects a highly conserved di-arginine motif (Arg186 and Arg187) critical for the binding of CDC42 to the interacting proteins such as WASP and Ras GTPase-activating-like protein IQGAP1. This genetic alteration results in protein mislocalization, disruption of the actin architecture and defects in cell differentiation, polarity and migration[166]. Other pathogenic variants in the C-terminal domain reside in close spatial proximity to the di-arginine motif[167]. Fundamentally, all the NOCARH-associated variants affect the last five amino acids of the protein that are required for post-translational processing and its proteolytic cleavage[168]. Bone marrow-derived mononuclear cells from these patients spontaneously release IL-1 and IL-18 and, during HLH episodes, the plasma levels of CXCL9 and IFNγ are very high[166]. Most patients improve considerably with IL-1 inhibitor therapy[167], and the targeting of IL-18 or IFNγ and HSCT are other promising therapeutic options[166]. How exactly defects in actin assembly induce the high production of IL-1 and IL-18 and predispose to HLH remains to be investigated.

NCKAP1L deficiency

NCKAP1L deficiency in humans leads to profound immune dysregulation with features of immunodeficiency, lymphoproliferation, atopy and severe inflammation[169]. Pathogenic biallelic loss-of-function variants in NCKAP1L (also known as HEM1) dysregulate the activation of the WRC, which leads to defective F-actin polymerization, abnormal immune cell activation, differentiation and migration[170]. Two patients with NCKAP1L deficiency had flares of disease consistent with HLH that are thought to result from prolonged immune stimulations owing to impaired T cell activation and pathogen control[169]. The respective pathogenic variants include a missense substitution (Val141Phe) and a splice site variant (c.2862+1G>A) that leads to skipping of exon 26. Data from structural modelling predict that these variants affect the binding of NCKAP1L to the Abl interactor 2 (ABI2; a regulator of actin cytoskeleton dynamics). Additional missense variants (Arg258Leu, Pro359Leu, Met371Val and Val519Leu) have been reported in five patients with a complex immune dysregulation but without features of HLH. These pathogenic variants affect the stability of WRC or the binding of NCKAP1L to ARF1 (REF.[170]), which is notable as ARF1 is critical for the activation of the WAVE complex[171]. T cells and neutrophils containing this variant exhibit abnormal F-actin formation, loss of lamellipodia extensions and migratory dysfunction. In addition, independently of its function as a WAVE regulator, mutated forms of the NCKAP1L protein can abrogate the mTORC2-mediated activation of protein kinase B (AKT) signalling, which might explain the abnormalities in T cell activation and function[170].

ARPC1B deficiency

ARPC1B is one of the seven subunits of the human ARP2/3 protein complex, and its expression is restricted to blood cells[172]. Given the central role that ARPC1B plays in the development and function of haematopoietic cells, patients with recessive loss-of-function variants in *ARPC1B* present with a broad spectrum of immune dysregulations[172–174]. These symptoms include combined immunodeficiency, bleeding disorder, eczema and autoimmunity similar to that seen in patients with WAS. A deficiency of ARPC1B leads to microthrombocytopenia and defective platelet function, whereas persistent thrombocytopenia is observed in patients with null mutations in *ARPC1B*. Immunological features in patients with ARPC1B deficiency include impaired TCR-mediated proliferation, T cell lymphopenia, loss of cytotoxic T lymphocyte function, eosinophilia and elevated IgE antibody levels[175], whereas inflammatory manifestations can include cutaneous vasculitis and inflammatory bowel disease.

WDR1 deficiency

Recessive loss-of-function variants in the *WDR1* gene are linked to the disease named periodic fevers, immunodeficiency and intermittent thrombocytopenia[176,177]. WDR1 deficiency leads to a defect in actin depolymerization, which affects neutrophil morphology and function. WDR1 also plays a crucial role in lymphocyte development and activation, particularly in the B cell department. Consequently, neutropenia, thrombocytopenia and B cell lymphopenia are features of this phenotype. Affected individuals suffer from severe recurrent respiratory infections, impaired wound healing and autoinflammatory features, including severe stomatitis, perianal ulceration and periodic fever. LPS-stimulated myeloid cells from these patients have increased

caspase 1 activity and produce very high levels of IL-18, although IL-1 secretion is not upregulated. In transfected HEK293T cells, overexpressed mutated forms of WDR1 colocalize with pyrin, suggesting that the pyrin inflammasome activation probably contributes to these patients' inflammatory manifestations.

In summary, pathogenic variants in proteins involved in cytoskeleton organization affect the function of all haematopoietic cells, leading to defects in innate and adaptive immune responses. Dysregulation in actin polymerization also results in a spectrum of inflammatory manifestations. Further studies are necessary to elucidate the molecular abnormalities causing the overproduction of IL-18 in these diseases.

Interferonopathies

The interferon pathway is critical for the recognition of pathogen-generated nucleic acids and the generation of antiviral responses. Monogenic diseases caused by constitutive upregulation in type I interferon signalling are known as primary interferonopathies. The inheritance pattern of pathogenic variants correlates with the function of the mutated proteins. Interferonopathies can arise from a heterozygous gain-of-function variant in a sensor protein, for example, in melanoma differentiation-associated protein 5 (MDA5), stimulator of interferon signalling (STING) or retinoic acid inducible gene 1 (RIGI), or from biallelic loss-of-function variants in a protein with nuclease activity, for example, in three-prime repair exonuclease 1 (TREX1) or deoxynucleoside triphosphate triphosphohydrolase (SAMHD1), or from biallelic loss-of-function variants in a protein that downregulates type I interferon responses, for example, in ubiquitin-like protein ISG1 (ISG15) or in Ubl carboxyl-terminal hydrolase 18 (USP18)[178,179]. The binding of type I interferons to heterodimeric IFNAR1–IFNAR2 receptors induces the activation of Janus tyrosine kinases (JAK) and the dimerization of activator of transcription 1 (STAT1) and STAT2, which then bind to interferon regulatory factor 9 (IRF9) to form the phosphorylated transcription factor complex interferon-stimulated gene factor 3 (ISGF3). The activated transcription complex translocates to the nucleus and upregulates the gene expression of interferon-stimulated genes (ISGs). The clinical presentation of patients with primary interferonopathies is in the autoinflammatory–autoimmune disease spectrum. In this section, we focus on two diseases that have complex inheritance patterns and present with notable disease variability: STING-associated vasculopathy with onset in infancy (SAVI) and proteasome-associated autoinflammatory syndromes (PRAAS).

STING-associated diseases

STING functions as a cytosolic DNA-sensing adaptor protein. STING is bound and activated by cyclic GMP-AMP (cGAMP), which is produced by the cyclic GMP-AMP synthase (cGAS) in response to pathogen-derived and mitochondrial DNA[180] and induces both interferon type I gene expression and NF-κB-mediated cytokine production[181,182]. cGAMP binding causes the release of the STING inhibitory C-terminal tail and its polymerization[183]. Residue 263 on STING is essential for ligand binding, whereas phosphorylation at residue 366 is necessary for TBK1–IRF3-mediated interferon type I production and antiviral activity[184]. STING is encoded by *TMEM173* and is highly expressed in myeloid cells, natural killer (NK) cells, T cells, vascular endothelial cells, alveolar pneumocytes and the bronchial epithelium[185].

Heterozygous gain-of-function variants in *TMEM173* are linked to a systemic inflammatory disease named SAVI[186] (FIG. 5a). SAVI manifests with fevers, erythematous rash, acrocyanosis, telangiectasia, small vessel vasculitis, peripheral amputations, interstitial lung disease and failure to thrive[187]. The activity of the mutated form of STING in SAVI is particularly high in dermal vascular endothelial cells, which explains the severity of cutaneous vasculitis in patients with this disease. The first reported disease-causing variants (Val147Leu/Met, Phe153Val, Asn154Ser and Val155Met) were identified as de novo variants in patients with severe early-onset vasculitis[186]. In addition to these germline variants, Val147Leu has also been found to be a somatic mutation in haematopoietic cells, dermal fibroblasts and other cell types[186]. Collectively, these pathogenic variants are known as class 1 mutations and they reside in a highly conserved domain, the connector helix loop, which controls the ligand-induced rotation of the dimers required for the activation of STING. These hyperactive variants reside in close proximity to the cysteine residue 148, which is critical for the stabilization of STING dimers[183]. Essentially, class 1 mutations cause ligand-independent activation of STING. The Val155Met variant is associated with a variable disease expressivity, and patients who carry this variant present with a spectrum of phenotypes, including chilblain lupus, severe vasculopathy or severe pulmonary fibrosis, that could be the first manifestation of the disease[188,189]. In addition to these class 1 mutations, a Gly166Glu variant has been identified in four generations of one family with chilblain lupus and without fever episodes or lung disease[190]. This variant is postulated to increase interactions at the dimer interface by inducing stronger hydrogen bonds between monomers. Furthermore, variants that reside in the polymerization interface (Cys206Tyr, Gly207Glu, Arg281Gln and Arg284Gly/Ser) cause a constitutive activation of STING either by making the polymerization interface available or by preventing the binding of a putative inhibitor[191–193]. Specifically, Arg281Gln and Arg284Gly/Ser are proposed to affect the binding of a C-terminal tail that keeps STING in an inactive state[183]. Patients who carry these mutations present with early-onset symptoms, failure to thrive, and variable degrees of skin and lung disease.

Pathogenic variants in STING can have additive effects. Two de novo variants, Ser102Pro and Phe279Leu, were inherited on the same chromosome (in *cis*) in a patient with systemic inflammation, telangiectatic skin lesions, brain infarctions, pulmonary dysfunction and recurrent infections[194]. This finding suggests that mutations with a weaker effect on STING function might not be sufficient as a single variant or in a monoallelic state to induce protein activation. In support of this hypothesis, six patients with a severe, potentially lethal

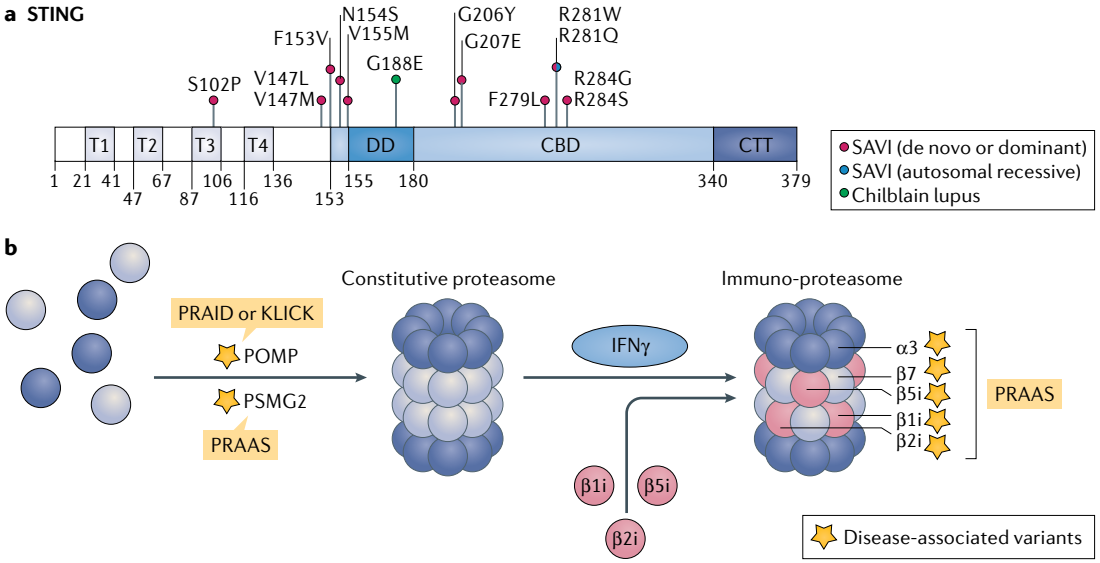

Fig. 5 | **Interferonopathies and disease-causing variants.** Various pathogenic variants in stimulator of interferon signalling (STING) and proteasome or immunoproteasome components can cause type I interferon-mediated diseases. **a** | A number of de novo or dominantly inherited gain-of-function variants in STING can cause STING-associated vasculopathy with onset in infancy (SAVI; shown in red), whereas another variant Arg281Gln (shown in blue) causes autosomal recessive SAVI. The variant shown in green is associated with chilblain lupus. **b** | The constitutive proteasome complex is expressed in all cell types and is composed of four stacked rings, each consisting of either 7α or 7β subunits. The immunoproteasome differs from the proteasome in that three of the proteasome subunits are replaced by different subunits, β1i, β2i and β5i, which are encoded by *PSMB9*, *PSBM10* and *PSMB8*, respectively. IFNγ can also induce immunoproteasome assembly. Heterozygous de novo variants in the proteasome maturation protein (POMP), which serves as a chaperone for proteasome assembly, function as dominant-negative variants and cause POMP-related autoinflammation and immune dysregulation disease (PRAID). The recessively inherited single nucleotide deletion in the 5′ untranslated region of POMP (rs112368783) is associated with the distinct syndrome keratosis linearis with ichthyosis congenita and sclerosing keratoderma (KLICK). Biallelic compound heterozygous variants in the chaperone protein PSMG2 cause a proteasome-associated autoinflammatory syndrome (PRAAS)-like phenotype with autoimmune haemolytic anaemia. Recessively inherited loss-of-function variants in α3 (encoded by *PSMA3*), β7 (encoded by *PSMB4*), β5i, β1i and/or β2i are associated with PRAAS. In cells that contain mutated components of the proteasome or immunoproteasome, ubiquitylated proteins accumulate and might trigger cellular stress and the upregulation of the type I interferon response (not shown). CBD, cGAMP binding domain; CTT, C-terminal tail; DD, dimerization domain.

SAVI phenotype were found to carry the homozygous Arg281Trp variant[195]. A missense variant at the same amino acid residue, Arg281Gln, was reported in another patient to be dominantly inherited[196]. The nature of the amino acid change from a positively charged arginine (Arg) to an uncharged glutamine (Gln) probably explains the high impact of this mutation and the difference in the inheritance pattern compared with the homozygous Arg281Trp variant. Common nucleotide variants (SNPs) in *TMEM173* might further contribute to the varying phenotype spectrum in SAVI[193].

Hence, in general, pathogenic STING variants are activating mutations that have a variable effect on protein activation and different inheritance patterns. The cells of patients with STING have a highly elevated ISG expression signature and produce high levels of interferon-induced cytokines[186]. Treatment with JAK inhibitors is beneficial but does not entirely ameliorate pulmonary involvement and skin disease[197].

Digenic
A phenotype or disorder that is expressed only when two non-allelic controlling genes interact.

PRAAS

Proteasomes are multiprotein complexes responsible for the K48 ubiquitin-dependent degradation of proteins and the maintenance of cellular homeostasis[198]. The constitutive proteasome is ubiquitously expressed, whereas the immunoproteasome is highly expressed in immune cells (FIG. 5b). A deficiency in proteasome activity leads to a build-up of undegraded proteins in cells, resulting in endoplasmic reticulum (ER) stress, the unfolded protein response and activation of type I interferon signalling[199].

Biallelic pathogenic variants in the immunoproteasome catalytic subunits or in the constitutive proteasome subunits are associated with a spectrum of autoinflammatory diseases collectively described as PRAAS (also known as chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature)[200–203]. Patients with PRAAS present with an early-onset recurrent fever, skin lesions, lipodystrophy, progressive joint contractures, multi-organ failure and neurological impairments[204]. PRAAS-associated variants are typically recessively inherited loss-of-function variants, and they affect proteasome assembly, maturation and, ultimately, proteasome activity. However, the inheritance pattern of PRAAS can be monogenic or digenic. Most patients are homozygous for the Thr75Met variant in *PSMB8*, which encodes the catalytic subunit β5i of the immunoproteasome[204]. Biallelic pathogenic variants have

also been identified in the immunoproteasome-specific gene *PSMB10* and in the *PSMB4* gene, which encodes the constitutive proteasome β7 subunit[205,206]. A subset of patients carry pathogenic variants at two separate genetic loci (*PSMB8* and *PSMA3*, *PSMB8* and *PSMB4*, or *PSMB9* and *PSMB4*)[205]. These patients tend to present with a severe phenotype, presumably because they have a defect in a constitutively expressed proteasome subunit. Recently, a de novo monoallelic mutation Gly156Asp in *PSMB9*, which suppresses protein expression, was reported in a patient who was subsequently successfully treated with HSCT[207].

Further expanding on what is known on the phenotype and inheritance pattern of PRAAS, three unrelated patients have been reported to carry de novo heterozygous variants in the proteasome maturation protein (POMP) that serves as a chaperone for proteasome assembly[208]. Two of these pathogenic variants are frameshift deletions in the penultimate exon 15 of *POMP* and produce truncated proteins with a dominant-negative effect. This distinct phenotype, termed POMP-related autoinflammation and immune dysregulation disease (PRAID), is characterized by severe neonatal-onset neutrophilic dermatosis, susceptibility to infections and autoimmunity features[208]. Interestingly, a homozygous 1bp-deletion in the 5′-UTR of another POMP transcript isoform causes a very different phenotype: keratosis linearis with ichthyosis congenita and sclerosing keratoderma syndrome (KLICK)[209], which is hypothesized to arise from a deficiency of a skin-specific isoform of POMP. The genetic heterogeneity of PRAAS is further emphasized by a report of compound heterozygous pathogenic variants in another chaperone protein (PSMG2) in a patient with features of PRAAS and autoimmune haemolytic anaemia[210].

Therefore, patients with PRAAS have complex inheritance patterns that include genetic heterogeneity, digenic inheritance, and a dominant or recessive inheritance pattern. Nonetheless, all PRAAS-associated variants are loss-of-function mutations that cause a defect in the proteasome function. Mutant cells have a strong type I interferon gene expression signature and JAK inhibitors ameliorate disease activity[197].

Diseases caused by enzyme dysregulation

PLCγ$_2$-associated diseases

Phosphoinositide-specific phospholipase Cγ2 (PLCγ2) is an enzyme that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the secondary messengers inositol triphosphate (IP3) and diacylglycerol (DAG) and is an important component of the B cell receptor signalosome[211]. In activated immune cells, IP3 induces the release of Ca^{2+} from the ER. High intracellular levels of Ca^{2+} activate various signalling pathways, including the protein kinase C (PKC) and the extracellular signal-regulated kinase (ERK)–MAPK pathway[212].

Monogenic *PLCG2*-associated autoinflammatory diseases include PLCγ2-associated antibody deficiency and immune dysregulation (PLAID)[213] and autoinflammation and PLAID (APLAID)[214] (FIG. 6a). Patients with PLAID or APLAID present with distinct skin and organ-specific disease manifestations. Features common to both diseases include low serum titres of IgA and IgM antibodies and decreased numbers of class-switched memory B cells, circulating $CD19^+$ B cells and NK cells. PLAID is characterized by cold-induced urticarial rash, skin granuloma, soft tissue destruction, and variable degrees of immunodeficiency and autoimmunity[213], whereas patients with APLAID present early in life with blistering skin lesions on exposure to heat or the sun, cutis laxa, arthralgia, ulcerative colitis, central nervous system inflammation, interstitial lung disease, and recurrent skin and sinopulmonary infections[214–217]. Both conditions result from activating, dominantly inherited pathogenic variants in PLCγ2 but differ in the nature of the causal variants. Patients with PLAID carry heterozygous in-frame genomic deletions of exon 19 or exons 20–22 spanning the C-terminal SRC homology 2 (SH2) autoinhibitory domain of PLCγ2, whereas APLAID is linked to the de novo missense variants Ser707Tyr, Ala708Pro, Leu848Pro and Leu845_Leu848del (FIG. 6a).

The pathophysiology of PLAID and APLAID is caused by a combination of gain-of-function and loss-of-function cell-specific effects on the PLCγ2 signalling pathway[218]. PLAID-associated deletions render the protein constitutively active; however, B cells and NK cells from patients with PLAID have an anergic phenotype at physiological temperatures owing to feedback inhibition secondary to chronic activation. T cell function is not affected, whereas mast cells spontaneously activate following exposure to temperatures lower than 37 °C, which explains the occurrence of cold urticaria in this disease. The APLAID missense variants Ser707Tyr and Ala708Pro reside in the same SH2 domain and have a gain-of-function effect that occurs in a temperature-independent manner but requires upstream activation by receptor tyrosine kinases. Two other APLAID causal mutations, Leu848Pro and Leu845_Leu848del, are located in the split pleckstrin homology (spPH) autoinhibitory domain. The most severe phenotype has been observed in a patient with the Leu845_Leu848del variant who presented at birth with a complete absence of B cells[217]. APLAID-associated missense substitutions enhance the basal activity of PLCγ2 and are hypersensitive to stimulation by GTPase Rac2 (REF.[219]). The net effect of these missense substitutions is an elevated production of IP3 and DAG and the calcium-dependent activation of the ERK–MAPK pathway.

In addition to PLAID and APLAID, rare missense variants in PLCγ2 have also been reported in patients with FCAS[220]. Finally, somatic variants that arise at the same residues as APLAID causal mutations (Ser707Tyr and Leu845Phe) give rise to Bruton tyrosine kinase inhibitor (ibrutinib)-resistant chronic lymphocytic leukaemia[221].

Overall, pathogenic variants in PLCγ2 activate the protein by altering the protein structure, resulting in the upregulation of various downstream signalling cascades and inflammatory features that are non-responsive to targeted cytokine therapies. As PLCγ2 is an essential regulator for B cell differentiation and proliferation, hypogammaglobulinemia is a prominent feature of the disease.

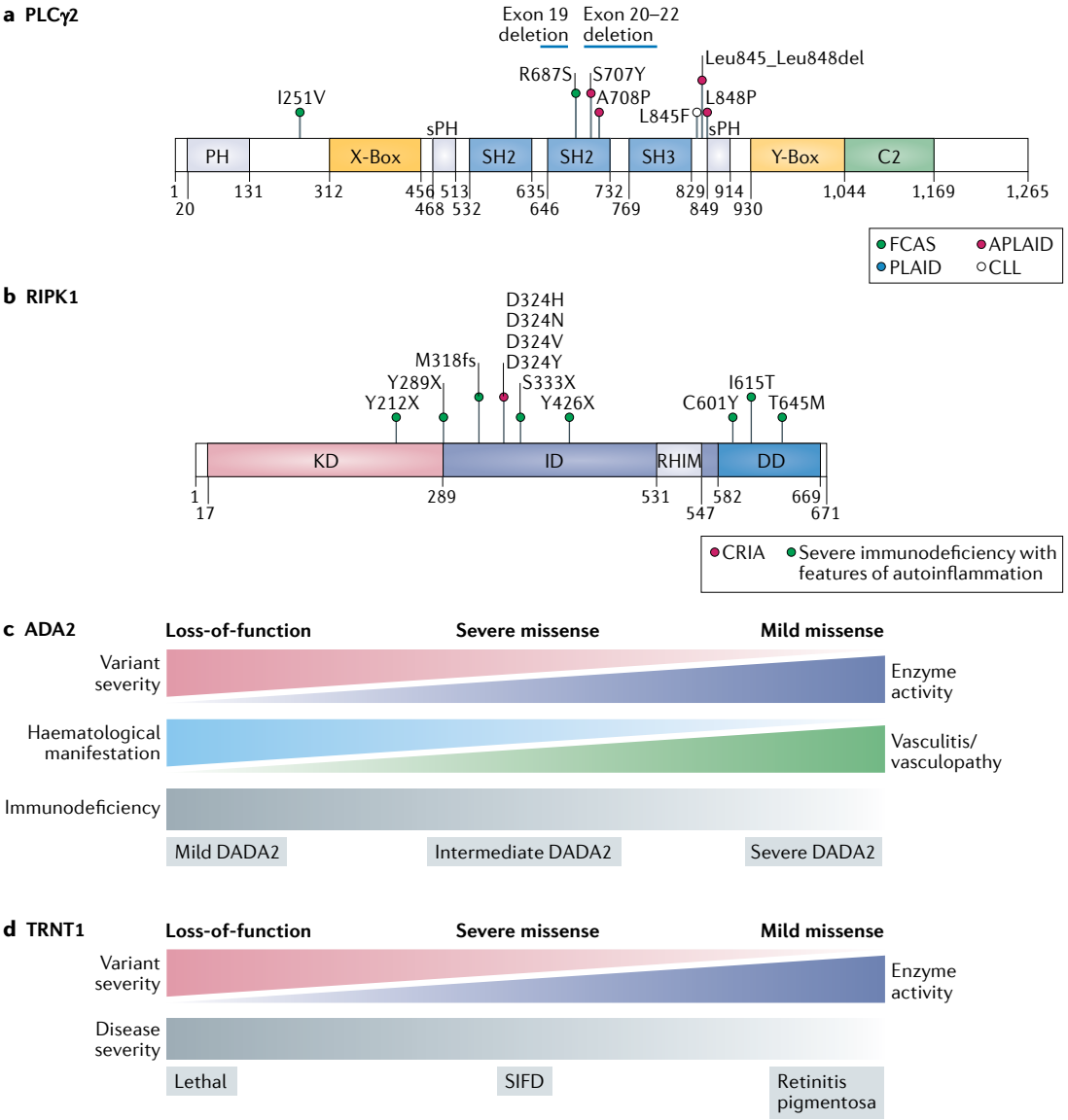

Fig. 6 | **Genotype–phenotype correlations in autoinflammatory diseases associated with enzyme deficiencies.** The dysregulation of various enzymes (ranging from missense to loss-of-function mutations) can cause a variety of autoinflammatory phenotypes, depending on the location and severity of the mutation. Pathogenic variants are colour-coded according to their associated phenotype. **a** | Rare variants in phosphoinositide-specific phospholipase Cγ2 (PLCγ2) have been identified in patients presenting with familial cold autoinflammatory syndrome (FCAS; shown in green). Other variants include in-frame genomic deletions that are associated with PLCγ2-associated antibody deficiency and immune dysregulation (PLAID; shown in blue); variants that are associated with autoinflammation, PLCγ2-associated antibody deficiency and immune dysregulation (APLAID, shown in red); and somatic variants that can cause chronic lymphocytic leukaemia (CLL; shown in white). **b** | Various recessively inherited loss-of-function pathogenic variants in receptor-interacting serine/threonine-protein kinase 1 (RIPK1) can cause severe immunodeficiency with features of autoinflammation (shown in green). Other dominant variants that affect the cleavage site of RIPK1 (residue 324) are associated with cleavage-resistant RIPK1-induced autoinflammatory (CRIA) syndrome (shown in red). **c** | Variants in adenosine deaminase 2 (ADA2) can lead to a spectrum of disease referred to as deficiency of adenosine deaminase 2 (DADA2). Variants leading to complete loss of ADA2 activity are associated with a higher incidence of severe haematological manifestations. By comparison, milder missense variants that exhibit some residual enzyme activity (>3%) are more often observed in patients with vasculitis or vasculopathy. Immunodeficiency can occur in patients carrying pathogenic variants across the whole mutational spectrum. **d** | The enzyme function of tRNA nucleotidyltransferase, CCA-adding 1 (TRNT1) correlates with disease severity. Complete TRNT1 enzyme deficiency is lethal early in development, whereas severe missense variants that exhibit some residual enzyme activity are associated with early-onset congenital sideroblastic anaemia, immunodeficiency, fevers and developmental delay (SIFD). Milder missense variants often lead to non-syndromic retinitis pigmentosa. DD, death domain; ID, intermediate domain; KD, kinase domain; PH, pleckstrin homology domain; RHIM, RIP homotypic interaction motif; SH, SRC homology domains.

RIPK1-associated diseases

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is an important regulator of TNF-induced activation of the canonical NF-κB signalling pathway and cell death[222], and this kinase is expressed in many tissues. The function of RIPK1 is regulated by changes in post-translational modifications, including phosphorylation, ubiquitylation and caspase 8-mediated cleavage[223]. The disruption of TNF-mediated ubiquitylation of RIPK1 induces the activation of cell-death pathways (apoptosis or necroptosis).

Biallelic loss-of-function variants in *RIPK1* are linked to a disease consisting of primary immunodeficiency, early-onset inflammatory bowel disease and polyarthritis[224,225] (FIG. 6b). Cells from patients with these variants have reduced levels of NF-κB and MAPK signalling and defective B cell and T cell differentiation, which explains the susceptibility of these patients to infections. The inflammatory component in this disease might result from increased inflammasome activity or the dysregulated response of intestinal epithelial cells to TNF-induced apoptosis[225].

In addition to RIPK1 deficiency, pathogenic variants in this gene can lead to a dominantly inherited inflammatory phenotype named cleavage-resistant RIPK1-induced autoinflammatory (CRIA) syndrome. Patients with CRIA syndrome carry heterozygous missense variants at residue Asp342 that function as gain-of-function variants by inhibiting the cleavage and inactivation of RIPK1. The associated disease manifests with early-onset recurrent periodic fevers and severe intermittent lymphadenopathy[226,227]. Studies in primary cells have shown that the activating *RIPK1* variants sensitize PBMCs but not fibroblasts to TNF-induced cell death by apoptosis, necroptosis and ferroptosis[226].

In summary, pathogenic variants in RIPK1 are either recessively inherited hypomorphic variants or dominantly inherited activating variants and they cause opposing clinical phenotypes. Patients with CRIA syndrome respond to therapy with a cytokine inhibitor[227], whereas HSCT might be a curative option for patients with RIPK1 deficiency[224].

ADA2-associated diseases

The deficiency of adenosine deaminase 2 (DADA2) is caused by biallelic hypomorphic variants in the gene encoding adenosine deaminase 2 (ADA2)[228,229]. ADA2 is a secreted enzyme that regulates purine metabolism by breaking down adenosine and 2′-deoxyadenosine at sites of inflammation and has growth factor-like properties[230]. ADA2 is highly expressed in myeloid cells and is produced by activated monocytes, macrophages and dendritic cells[231].

Patients with DADA2 present with early-onset recurrent fever, hepatosplenomegaly and variable degrees of vasculopathy ranging from livedo racemosa and reticularis to polyarteritis nodosa. Severe disease manifestations consist of recurrent life-threatening ischaemic and/or haemorrhagic strokes and haematological manifestations, including pure red cell aplasia, immune thrombocytopenia and neutropenia, bone marrow failure, combined variable immunodeficiency and lymphoproliferation[232,233].

DADA2-associated variants are located over the entire gene, with no predilection to cluster in specific protein domains. Ultimately, pathogenic variants affect various protein functions of ADA2 such as its catalytic activity, dimerization, glycosylation and interactions with other proteins[234]. Disease severity correlates with the effect of the variants on ADA2 enzyme activity. Low or absent ADA2 activity is associated with severe haematological manifestations, whereas a higher residual activity is linked to vascular phenotypes[235] (FIG. 6c).

ADA2-deficient myeloid cells are prone to activation and produce high amounts of TNF, which causes tissue inflammation and damage to endothelial cells[229]. The underlying mechanism of bone marrow dysregulation is unclear; it can result either from a lack of ADA2 growth factor activity or from the infiltration of bone marrow with activated immune cells. The establishment of genotype–phenotype correlations in DADA2 has important implications for the therapy of these patients. Treatment with TNF inhibitors reduces inflammation and restores vascular integrity[236]. Bone marrow transplantation is an option for patients presenting with severe haematological disease and not responding to TNF inhibitors[237].

TRNT1 deficiency

TRNT1 encodes the ubiquitously expressed transfer RNA nucleotidyltransferase 1 (TRNT1), an enzyme that is responsible for transferring CCA trinucleotides to the 3′ end of all precursor transfer tRNAs. This post-transcriptional modification is essential for the accurate attachment of amino acids and proper protein translation[238]. TRNT1 also plays a critical role in maintaining the homeostasis of cellular tRNAs by selectively marking structurally defective or unstable tRNAs for degradation[239].

Biallelic loss-of-function variants in *TRNT1* were first reported in patients with early-onset congenital sideroblastic anaemia, immunodeficiency, fevers and developmental delay (SIFD)[240,241] (FIG. 6d). Disease-linked variants affect the protein stability and catalytic activity[242]. Patients at the severe end of the disease spectrum present with neonatal-onset anaemia and prominent extramedullary erythropoiesis, profound immunodeficiency, and metabolic and neurological abnormalities[243]. Disease mortality is high in these patients owing to multi-organ or cardiac failure. Immunodeficiency can be the first disease manifestation and is caused by defects in B cell differentiation[244]. SIFD-associated mutations include missense, nonsense, frameshift and splice site pathogenic variants, whereas biallelic nonsense or truncating variants are non-viable[245–248]. Unprocessed and misfolded proteins accumulate in TRNT1-deficient cells, which can exhaust the protein degradation machinery and the autophagy pathway and induce ER stress and cell death[246]. At the milder end of the TRNT1 deficiency spectrum are patients with a very different phenotype that includes non-syndromic retinitis pigmentosa and subtle haematological features[249,250]. Retinitis pigmentosa is characterized by the progressive degeneration of photoreceptors leading to low night vision and visual field defects. Although vision loss is observed in patients with severe

SIFD, the haematological features of these patients often require immediate medical interventions.

Hence, the severity of TRNT1-associated diseases correlates with the levels of residual enzyme activity. Treatment modalities include blood transfusions, intravenous immunoglobulin and anti-TNF therapy to alleviate inflammatory symptoms[246]. HSCT performed early in life can effectively treat the haematological phenotype[240].

Conclusion

The classic concept of one gene–one phenotype is overly simplified as different disease-causing variants within a gene might affect various aspects of protein function and lead to clinically distinct conditions. High-impact, mostly de novo, pathogenic variants that cluster in specific protein domains often result in a similar phenotype. By comparison, milder variants tend to present with variable disease expressivity and are more influenced by other genetic and non-genetic modifying factors. Furthermore, pathogenic variants in the same gene can have different inheritance patterns that translate into different effects on protein function. Adding to the complexity of genetics in autoinflammatory diseases, various reports have emerged of several diseases caused by somatic variants primarily in myeloid cells. These patients typically present with late-onset symptoms, and their disease severity might be related to the type and proportion of cells carrying a mutant allele[251]. By contrast, somatic variants that occur in pluripotent cells during early embryogenesis might result in a clinical phenotype similar to those observed in patients with germline pathogenic variants[252]. Understanding the factors contributing to the variable disease penetrance and expressivity of monogenic autoinflammatory and other human diseases will ultimately require large-scale genomic, epigenomic, transcriptomic and proteomic studies. Understanding these aspects will also help to understand variabilities in treatment response and enable patient-tailored therapy as we approach the era of precision medicine.

Published online 25 May 2021

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Acknowledgements
The authors would like to thank David Beck and Christina Kozycki for the critical reading of the manuscript.

Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Rheumatology thanks M. Gattorno, S. Ozen and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41584-021-00614-1.

The role of HIF proteins in maintaining the metabolic health of the intervertebral disc

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Abstract | The physiologically hypoxic intervertebral disc and cartilage rely on the hypoxia-inducible factor (HIF) family of transcription factors to mediate cellular responses to changes in oxygen tension. During homeostatic development, oxygen-dependent prolyl hydroxylases, circadian clock proteins and metabolic intermediates control the activities of HIF1 and HIF2 in these tissues. Mechanistically, HIF1 is the master regulator of glycolytic metabolism and cytosolic lactate levels. In addition, HIF1 regulates mitochondrial metabolism by promoting flux through the tricarboxylic acid cycle, inhibiting downsteam oxidative phosphorylation and controlling mitochondrial health through modulation of the mitophagic pathway. Accumulation of metabolic intermediates from HIF-dependent processes contribute to intracellular pH regulation in the disc and cartilage. Namely, to prevent changes in intracellular pH that could lead to cell death, HIF1 orchestrates a bicarbonate buffering system in the disc, controlled by carbonic anhydrase 9 (CA9) and CA12, sodium bicarbonate cotransporters and an intracellular H^+/lactate efflux mechanism. In contrast to HIF1, the role of HIF2 remains elusive; in disorders of the disc and cartilage, its function has been linked to both anabolic and catabolic pathways. The current knowledge of hypoxic cell metabolism and regulation of HIF1 activity provides a strong basis for the development of future therapies designed to repair the degenerative disc.

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https://doi.org/10.1038/s41584-021-00621-2

In the past century, Nobel Prizes have been awarded for discoveries underlying oxygen-sensing and cellular respiration. The 2019 Nobel Prize in Physiology and Medicine, awarded to William Kaelin, Peter Ratcliffe and Gregg Semenza, recognized the importance of understanding how oxygen regulates cellular metabolism in various tissue types and organisms throughout their lifespans. The foundation for this work was based on studies by the 1931 Nobel laureate, Otto Warburg, who discovered the enzymatic basis of cellular respiration, from which has emerged an appreciation of the role of oxidative metabolism in health and disease. The combined efforts of these Nobel laureates and other scientists delineated how intracellular oxygen levels are directly coupled to changes in gene expression through the regulation of hypoxia-inducible factor (HIF) and its degradation by the von Hippel–Lindau (VHL) axis[1–4].

Mounting evidence demonstrates that HIF function is required for intervertebral disc (IVD) integrity[5–9], fetal growth plate development[10] and endochondral ossification[11,12], whereas dysregulation of HIF signalling is an early impetus for degenerative cascades (BOX 1). To date, there are no cures for disc degeneration, osteoarthritis (OA), or rheumatoid arthritis, despite being the most common joint diseases. Low back pain (LBP) and neck pain, specifically, are the first and fourth leading causes of chronic disability in the USA, respectively, and contribute considerably to the global disease burden[13]. The common risk factor for LBP is IVD degeneration, a multifactorial pathology characterized by progressive loss of extracellular matrix (ECM) components and increased intradiscal acidosis, both of which increase susceptibility to herniation, fissuring of the endplate cartilage, a raised immune response and, thus, discogenic pain[14–19]. Genetic evidence from various studies links HIF family members to disc degeneration and OA. Firstly, single nucleotide polymorphisms in genes encoding HIF1 and HIF2 correlate with the susceptibility and severity of lumbar disc degeneration and OA in single-ethnicity association studies[12,20]. Secondly, the regulation of a myriad of hypoxia-responsive signalling pathways, including those required for matrix remodelling and cellular metabolism, is under disarray during disc degeneration and arthritis[5,7,21,22].

Key points

- Loss of control of hypoxia-inducible factor 1 (HIF1) and HIF-dependent metabolic pathways can lead to intervertebral disc degeneration, whereas loss of HIF2 function is implicated in osteoarthritis.
- In nucleus pulposus cells, HIF1 and HIF2 are uniquely regulated by both oxygen-dependent and oxygen-independent mechanisms involving prolyl hydroxylase domain-containing proteins (PHDs) and circadian clock genes.
- Cells of the intervertebral disc possess functional mitochondria and, in nucleus pulposus cells, mitochondria undergo HIF-dependent mitophagy and fragmentation.
- HIF1 maintains glycolytic and tricarboxylic acid cycle flux while simultaneously inhibiting oxidative phosphorylation in nucleus pulposus cells.
- HIF1 controls intracellular H^+/lactate levels via monocarboxylate transporter 4 (MCT4); conversely, the accumulated lactate is capable of stabilizing HIF proteins by inhibiting PHD function as well as controlling transcriptional programmes.
- In addition to the well-studied proton extrusion mechanisms, the intracellular pH in nucleus pulposus cells is maintained by a HIF-dependent bicarbonate buffering mechanism controlled by various components including carbonic anhydrases.

Tricarboxylic acid (TCA) cycle
A series of chemical reactions following the oxidation of acetyl-CoA. This cycle generates biosynthetic intermediates, reducing agents and CO_2, which support multiple cellular reactions. In aerobic cells, NADH generated by the TCA cycle is oxidized in the electron transport chain via a set of reactions that generate ATP.

In this Review, we discuss how HIF family members are regulated and their role in maintaining the metabolic health of the intervertebral joint, with particular focus on nucleus pulposus cells. We present the argument that HIF1 and HIF2 are non-canonically regulated in the hypoxic IVD and that they are distinctly required for the regulation of cellular metabolism, pH regulation and cell survival pathways. We also discuss how loss of regulation of these pathways causes disc degeneration, suggesting a possible link between HIF dysregulation and IVD degeneration. From this perspective, HIF transcription factors provide an important opportunity for the development of therapeutic targets for degenerative skeletal diseases.

Box 1 | **Diverging roles of HIF1 and HIF2 in disc and cartilage**

Hypoxia-inducible factor 1α (HIF1α) is considered the guardian of hypoxic cells. Functionally, HIF1α signalling mediates nucleus pulposus cell survival[62] and chondrocyte growth and development[125], whereas HIF1α expression prevents the loss of articular cartilage in osteoarthritis (OA) by inhibiting the pathological effects of β-catenin signalling on matrix degeneration[126]. By contrast, the role of HIF2α in the intervertebral disc has not been characterized in vivo; yet, HIF2α expression can promote both matrix anabolism and catabolism in articular chondrocytes[11,12,127]. Two studies in 2010 found that HIF2α is a central mediator of endochondral ossification and might induce development of an OA phenotype[11,12]. Specifically, HIF2α is elevated in human OA tissue and upregulates the expression of *Col10a1*, *Mmp13* and *Vegfa* in mouse models of OA. Further analysis showed that HIF2α controls the IL-1β-induced expression of matrix metalloproteinases, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), type 2 nitric oxide synthase (NOS2) and prostaglandin G/H synthase 2 (PTGS2); notably, exogenous introduction of HIF2α into mouse and rabbit knees induced cartilage destruction. Collectively, these studies showed that HIF2α is an important participant in the induction of OA. However, this pathogenic role of HIF2α was confounded by findings that HIF2, through the regulation of the transcription factor SOX9, promotes extracellular matrix production and cartilage repair[127]. HIF1α and HIF2α also have diverging roles in the fetal growth plate and limb bud. HIF1α is indispensable for chondrocyte survival and differentiation in the hypoxic regions of the fetal growth plate[125] and the limb bud mesenchymal cells[128,129]. By contrast, the contribution of HIF2α to growth plate development is minor[130]. Loss of HIF2α in the limb bud mesenchyme causes a modest delay in endochondral bone development, which is linked to impaired differentiation of chondroprogenitor cells into hypertrophic chondrocytes[130]. These studies underscore the divergent functions of HIF1α and HIF2α in skeletal tissues, and demonstrate that HIF2α function differs between articular and growth plate chondrocytes.

The hypoxic intervertebral disc niche

The IVD and adjoining superior and inferior vertebrae form the functional spinal motion segment of the spine that is capable of polyaxial movements and withstanding compressive and tensile loads[23]. It can be argued that the motion segment of the spine is a diarthrodial joint with two articulating hyaline cartilaginous endplates, surrounding an inner nucleus pulposus, rich in chondroitin sulfate proteoglycans that give the disc its characteristic swelling properties[15,24]. The nucleus pulposus is encapsulated by the annulus fibrosus, a fibrocartilaginous structure composed of concentric collagen I lamellae. The local blood supply in the subchondral bone reaches the bony endplate and outer layers of the cartilaginous endplates and annulus fibrosus, but does not infiltrate the inner annulus fibrosus nor the innermost nucleus pulposus[25]. Because of these anatomical constraints, the IVD is considered the largest avascular organ in vertebrates. As a consequence of avascularity, nucleus pulposus cells experience hypoxia[26–29]. Understanding how these cells adapt to this hypoxic niche and are regulated under hypoxic conditions could aid our understanding of the pathological mechanisms that occur during IVD degeneration.

Hypoxia and the HIF family

Although most cell types utilize local oxygen in the mitochondrial electron transport chain (ETC) to generate ATP, hypoxic cells adapt to the decreased oxygen availability by inducing HIF-dependent transcription of genes necessary for survival[30]. The HIF family comprises three heterodimeric transcription factors composed of an oxygen-regulated subunit (HIF1α, HIF2α or HIF3α) dimerized with a constitutively expressed subunit (HIF1β)[4], forming a HIF1α–HIF1β heterodimer (referred to as HIF1), a HIF2α–HIF1β heterodimer (referred to as HIF2) or a HIF3α–HIF1β heterodimer. The α-subunits contain a basic helix–loop–helix (bHLH) domain, two PER–ARNT–SIM (PAS) homology domains, a PAS-associated COOH-terminal (PAC) domain, an oxygen-dependent domain (ODD) with an associated NH_2-terminal transactivation domain (N-TAD) and a carboxy terminus transactivation domain (C-TAD). In normoxic cell types, prolyl hydroxylase domain-containing proteins (PHDs) utilize oxygen, α-ketoglutarate and the co-substrate l-ascorbate to hydroxylate proline residues in the ODD domain of the HIFα subunit. Proline hydroxylation results in interactions of the HIFα subunit with the VHL–E3-ubiquitin ligase protein complex, which targets HIFα for proteasomal degradation[1,31]. However, under hypoxic conditions, the limited availability of oxygen and a tricarboxylic acid (TCA) cycle-derived substrate, α-ketoglutarate, impedes PHD-mediated hydroxylation of HIFα, which results in HIFα accumulation, subsequent heterodimerization and transcriptional activation of target genes.

Much of our understanding of HIF function stems from studies of HIF1α and HIF2α. These isoforms are structurally similar and regulate transcription through hypoxia-response elements (HRE) contained within target gene loci[32]. Hypoxia-responsive genes

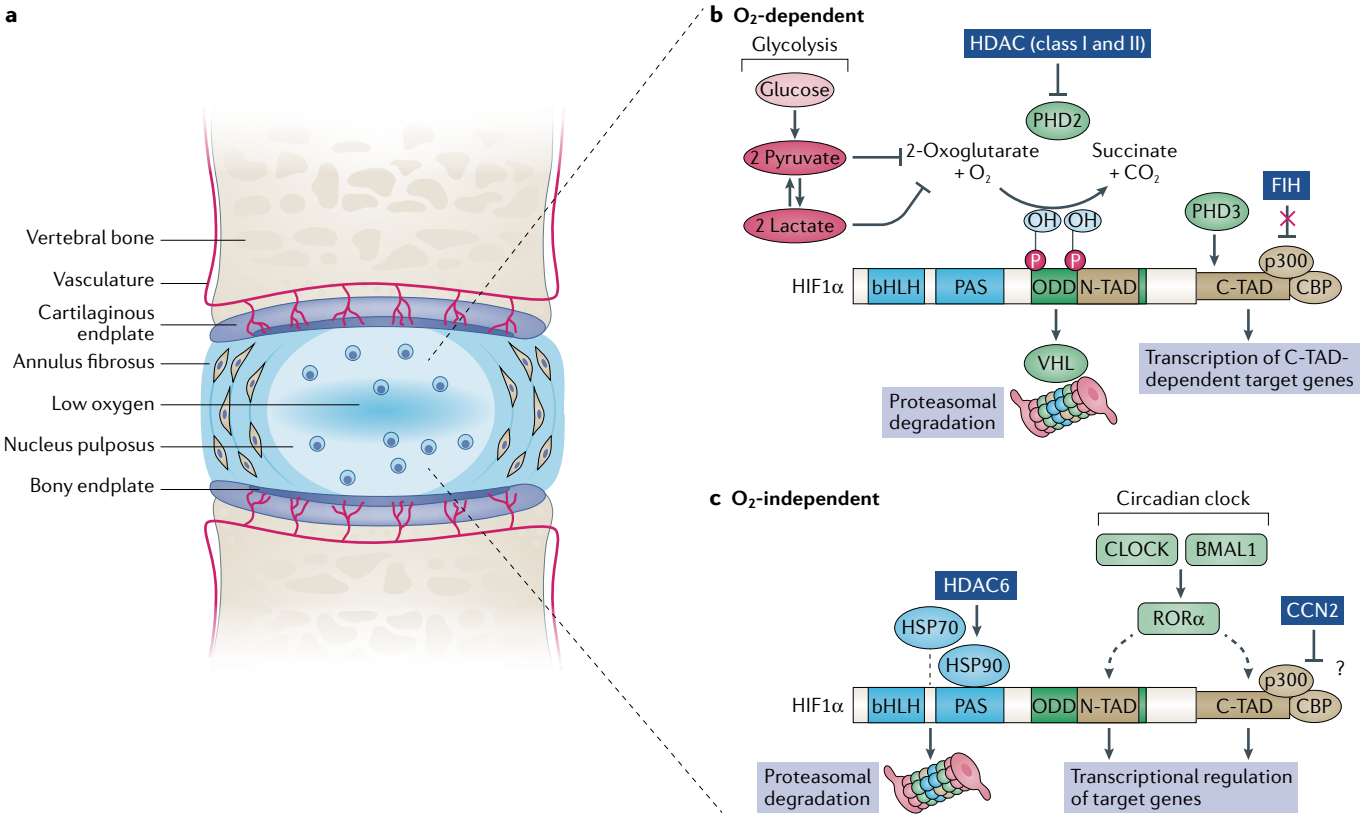


Fig. 1 | **Regulation of HIF1α in hypoxic nucleus pulposus cells.** **a** | A schematic of the intervertebral disc tissue compartments and vasculature. The avascular nature of the disc compartments makes the nucleus pulposus tissue physiologically hypoxic, resulting in robust hypoxia-inducible factor 1α (HIF1α) expression. **b** | Various oxygen-dependent mechanisms regulate HIF1α expression and function. In the presence of sufficient oxygen, prolyl residues in the oxygen-dependent domain (ODD) of HIF1α target this protein for von Hippel–Lindau (VHL)-mediated polyubiquitination and 26S proteasomal degradation via prolyl hydroxylase domain-containing protein 2 (PHD2). The function of PHD2 can be blocked by two mechanisms. First, lactate accumulation generates metabolic intermediates, including pyruvate and succinate, which compete with the PHD2 substrate, 2-oxoglutarate, and inhibit PHD activity. Second, class I and II histone deacetylases (HDACs) directly inhibit the PHD2–HIF1α axis. Unlike PHD2, PHD3 serves as a cofactor for transcriptional activation of C terminus transactivation domain (C-TAD)-dependent target genes. In nucleus pulposus cells, HIF1 function is refractory to inhibition mediated by factor inhibiting HIF1 (FIH1). **c** | Various oxygen-independent mechanisms also regulate HIF1α expression and function. HIF1α can be targeted for 26S proteasomal degradation by heat shock protein 70 (HSP70), possibly through displacement of HSP90. In nucleus pulposus cells, HIF1α is a circadian clock-regulated gene. The circadian clock transcription factors CLOCK, brain and muscle ARNT-like protein 1 (BMAL1) and retinoic acid-related orphan receptor-α (RORα) synergize to upregulate NH_2-terminal transactivation domain (N-TAD)-dependent and C-TAD-dependent target genes, without evidence of direct binding to HIFα. HDAC6 can recruit HSP90 as a cofactor to upregulate HIF target gene expression, whereas the connective tissue growth factor CCN2 can block HIF1α cofactor binding and diminish its activity, although the mechanism of action is unknown. bHLH, basic helix–loop–helix; CBP, CREB-binding protein; PAS, PER–ARNT–SIM.

are implicated in the physiological regulation of the developing embryo and adult tissues, as well as in the pathogenesis of arthritis, inflammation and cancer[11,12,33]. Numerous studies evaluating tissue-specific expression, temporal induction and phenotypes following gene deletion or mutation confirm that the isoforms are functionally non-redundant[34]. HIF1α is ubiquitously expressed and considered a master regulator of metabolic reprogramming, cell cycle regulation, angiogenesis and tumorigenesis[35], whereas HIF2α is notably expressed in endothelial tissues and bone marrow macrophages where it exerts a larger role in angiogenic signalling, guidance cues and ECM remodelling[33,35].

Regulation of HIF proteins in the IVD

Over the past two decades, several studies have highlighted the unique regulation of HIF activity in nucleus pulposus cells. Unlike in other cell types, robust expression of HIF1 and HIF2 isoforms is detected under normoxic conditions in nucleus pulposus cells, and their levels are mostly insensitive to oxygen tension[26,27]. Furthermore, HIF proteins and their target genes in the disc are regulated by the peripheral circadian clock[36,37]. Within this section, we discuss how the unique HIF signature in nucleus pulposus cells is attributed to three main types of regulation: oxygen-dependent regulation, oxygen-independent regulation and the circadian rhythm (FIG. 1).

Oxygen-dependent regulation

The atypical regulation of HIF1α and HIF2α stability and signalling in nucleus pulposus cells is largely due to non-canonical regulation by PHDs. In contrast to HIF regulation in other cell types, 26S proteasomal degradation of HIF1α in nucleus pulposus cells is mediated by PHD2, but not by PHD1 or PHD3 (REFS[28,38]).

PHD activity in all cell types is dependent on the availability of substrates derived from the TCA cycle (an aerobic process), including α-ketoglutarate and l -ascorbate[31]. Although nucleus pulposus cells rely on glycolysis (an anaerobic process) for ATP production, it has been shown that α-ketoglutarate, and other TCA cycle-generated intermediates, are present at sufficient concentrations to facilitate PHD2 activity[5,28,38]. Therefore, PHD2, the predominant PHD homologue, is active and not substrate-limited in nucleus pulposus cells, despite the hypoxic niche of these cells. However, HIF1α expression is still maintained at robust levels in nucleus pulposus cells under both normoxic and hypoxic conditions[27], suggesting that the rate of HIF1α synthesis is greater than its rate of degradation. Surprisingly, proteasomal degradation of HIF2α is independent of PHD activity in nucleus pulposus cells[28,38], which warrants future investigations. In contrast to nucleus pulposus cells, both HIF1α and HIF2α degradation in chondrocytes proceeds through PHD-mediated hydroxylation, suggesting that the regulation of these proteins by PHDs is cell type specific[28,39].

In addition to regulating proteasomal degradation of HIFα proteins in various cell types, PHDs are known transcriptional targets of HIF proteins[31]. In nucleus pulposus cells, HIF1α and PHDs are involved in a reciprocally dependent regulatory loop[38,40]. In these cells, PHD3 expression is increased under hypoxia and the activity of its gene promoter and enhancer is regulated by HIF1α and HIF2α[38]. Although PHD3 modulation has little effect on HIF1α protein levels, PHD3 enhances the transcriptional activity of HIF1α in nucleus pulposus cells[28,38]; that is, PHD3 functions as a HIF1α cofactor and is required for the transcriptional activation of a subset of HIF1α C-TAD-dependent genes[40]. The expression of various C-TAD-dependent HIF1α targets, including *Vegfa*, *Glut1* (also known as *Slc2a1*) and *Ldha*, are decreased in 12.5-month-old PHD3-null mice, which is notably accompanied by disc degeneration[40]. Unlike other cells, in which PHD3 functions as a HIF1α co-activator through a PKM2–JMJD5 axis[41], manipulation of PKM2 and JMJD5 levels in nucleus pulposus cells has no effect on PHD3-dependent HIF1α activity or target gene activation[40,42]. This finding implies that in nucleus pulposus cells, PHD3-dependent HIF1α activity functions through a yet-unknown axis.

Oxygen-independent regulation

The available literature on HIF and PHDs suggests that the degradation of HIF1α and HIF2α in nucleus pulposus cells is also prominently controlled by oxygen-independent pathways, for example, lysosomal autophagic degradation[28]. Heat shock proteins (HSPs) are specifically implicated in oxygen-independent mechanisms that control cell adaptations to hypoxic conditions[43]. The involvement of HSPs is particularly apparent in nucleus pulposus cells; in these cells, HSP70 promotes the proteasomal degradation of HIF1α, whereas HIF proteins reciprocally suppress the transcription of the HSP70 gene[44]. Histone deacetylase 6 (HDAC6) is required for the recruitment of HSP90, a cofactor necessary for HIF1α-mediated transcription, and thereby functions as a positive regulator of HIF1α[45]. Moreover, class I and IIa HDACs are involved in HIF1α stabilization through modulation of the HIF–PHD2 axis[45]. A second oxygen-independent regulatory loop in nucleus pulposus cells links HIF1α and the connective tissue growth factor CCN2 (REF.[46]). Unlike HSPs, CCN2 diminishes HIF1 target gene expression and blocks HIF1α from recruiting additional co-activators. The detailed mechanism of actions by which CCN2 controls HIF function remain to be elucidated, but is unlikely to involve the regulation of HIF degradation. Finally, it is important to note that in many cell types, HIF1α activity is regulated by factor inhibiting HIF1 (FIH1, an asparaginyl hydroxylase), which modulates the interaction of HIF1α with the co-activator proteins p300 and CREB-binding protein (CBP) through hydroxylation of a conserved asparagine residue in the C-TAD domain of HIF1α[47]. However, data suggest that FIH1 is dispensable in nucleus pulposus cells and does not regulate canonical HIF1 targets[48]. Interestingly, FIH1-null mice lack any HIF-related phenotypes, but studies of these mice instead implicate FIH1 in processes related to glucose and fatty acid metabolism. Hence, this observation raised questions about the in vivo relevance of FIH1-mediated post-translational modifications on HIF activity[49].

Regulation by the circadian rhythm

Given that the IVD is hypoxic, it is logical to focus on how HIF is regulated in response to oxygen availability. However, from an anatomical and functional perspective, the disc is also characterized by kinesiological factors; that is, diurnal patterns of cyclical loading during active hours and unloading during inactive hours. Joint tissues in general are influenced by daily rest–active cycles, which are indirectly controlled by the peripheral circadian clock[50]; conversely, the expression of *CLOCK* (encoding circadian locomotor output cycles protein kaput (CLOCK), a core component of the circadian clock) is mechanosensitive, implying that daily activity could manipulate the joint circadian rhythm[51]. Intriguingly, several studies have shown that the cellular hypoxic response and circadian clock are linked by a synergistic cross-talk, utilizing HIF1 as the central mediator[52–54]. Briefly, *HIF1A* (encoding HIF1α) is an E-box-regulated gene under the transcriptional control of two circadian clock transcription factors, CLOCK and brain and muscle Arnt-like protein 1 (BMAL1; also known as ARNTL), whereas period circadian clock 2 (PER2) recruits HIF1α to HRE motifs on target genes[52,53]. Downstream, HIF1α reciprocally modifies the expression of circadian clock components, including PER2, effectively dampening the circadian rhythm[53]. As such, the hypoxic induction of HIF1α signalling can cause tissue-specific misalignments in the circadian rhythm[54].

Importantly, both HIF1 and canonical clock components are dysregulated during cartilage degeneration in human OA[55,56]. Furthermore, the master clock gene, BMAL1, has been implicated in the maintenance of articular cartilage integrity. Conditional loss of BMAL1 in chondrocytes leads to progressive degeneration of the articular cartilage in mouse knee joints, and

correlates with loss of circadian rhythm in the cartilage[57]. It is therefore not surprising that the transcriptome of nucleus pulposus cells is dependent on circadian rhythm components CLOCK, BMAL1 and retinoic acid-related orphan receptor-α (RORα)[36,37]. In fact, researchers have identified the expression of 607 rhythmic genes in the disc, which account for approximately 3.5% of the disc transcriptome[37]. The importance of rhythmic gene regulation in the disc was confirmed using BMAL1 knockout mice, which present with diverse degenerative phenotypes in the IVD. This study also showed the existence of a BMAL1–RORα–HIF1α regulatory loop in nucleus pulposus cells in vitro, whereby HIF1α and HIF target gene expression are regulated by BMAL1 and RORα[36]. Hence, it is plausible that downregulation of HIF1α signalling in the BMAL1 knockout mice might account for some of the degenerative changes observed in the IVD. Furthermore, individuals who experience disruptions of circadian rhythms, such as shift workers, are at higher risk of LBP than other individuals[58]. Additional studies linking circadian disruptions to IVD degeneration would therefore be highly relevant to human pathologies; that is, desynchronization of IVD loading and the circadian rhythm might have dire consequences for disc health.

Regulation of the IVD by HIF proteins

Hypoxic regulation of cell survival

HIF1α regulates the expression of many genes critical to the survival of nucleus pulposus cells (for example, plasma membrane glucose transporters and glycolytic enzymes)[27]. For glycolysis to occur, glucose must passively diffuse from the vertebral capillaries through the cartilaginous endplates and dense proteoglycan matrix of the nucleus pulposus compartment to reach resident nucleus pulposus cells at the centre of the disc[59]. In an environment where glucose and oxygen concentrations are limiting and H^+/lactate concentrations are relatively high[60], maintaining glycolytic flux and the nutrient–metabolite balance is critical for nucleus pulposus survival[27,61]. Importantly, two mouse models of notochord-specific HIF1α conditional deletion under the control of *Foxa2*-Cre or *Shh*-Cre exhibit severe disc degeneration that is probably instigated by metabolic failure of nucleus pulposus cells[62,63]. In the first model (*Foxa2*-Cre-mediated *Hif1a* deletion), the null mice show a reduced size of the nucleus pulposus compartment at embryonic day 15.5, followed by a massive amount of cell death in this compartment at birth and subsequent postnatal disappearance and remodelling[62]. One hypothesis is that the *Hif1a*-null nucleus pulposus cells die owing to metabolic failure. In support of this hypothesis, *Hif1a* deletion results in a notable reduction in the expression of the HIF1α target gene, *Pgk1*, which encodes an enzyme that catalyses a reversible conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and phosphorylates ADP, at the substrate level, to ATP. Accordingly, loss of PGK function would result in blockage of glycolytic flux and contribute to cell death in the nucleus pulposus. In the second mouse model of nucleus pulposus-specific HIF1α deletion, involving *Shh*-Cre-mediated deletion of *Hif1a*, the amount of cell death and disc degeneration increase by 6 and 12 weeks of age, respectively[63]. The discs of mutant mice have lower levels of *Acan*, *Col2a1* and *Vegfa* than wild-type mice, which might also contribute to cell death. An additional mouse model of spontaneous, early-onset disc degeneration has also been characterized[14,64]. The inbred SM/J mouse strain, that is known to have poor cartilage regenerative properties, shows early signs of disc degeneration characterized by loss of nucleus pulposus cells and the presence of cells with hypertrophic chondrocyte-like characteristics. In this model, diminished expression of the HIF1α target gene, *Vegfa*, correlates with increased amounts of cell death in the nucleus pulposus compartment of the disc. Related to this observation, emerging data suggest that vascular endothelial growth factor A (VEGFA) has a pro-survival role in nucleus pulposus cells[65].

It is also known that hypoxia and HIF1α modulate important survival and adaptive pathways, including autophagy, endoplasmic reticulum (ER) stress and mitophagy[66,67]. The hypoxic induction of autophagy is often mediated by HIF1α through BCL-2/E1B 19 kDa protein-interacting protein 3 (BNIP3)[68], whereas the HIF-independent autophagic response is signalled through a nutritional stress response via the AMP-activated protein kinase (AMPK)–mammalian target of rapamycin (mTOR) pathway and the unfolded protein response (UPR). Nucleus pulposus cells can adapt to their hypoxic niche through the modulation of autophagy and ER stress, whereby hypoxia increases the rate of autophagic degradation and lowers the ER stress burden[9,66,69]. However, the hypoxic induction of autophagy in nucleus pulposus cells is also regulated in a non-canonical manner that is independent of both HIF1α and mTOR signalling. Although inhibition of non-canonical autophagic degradation has no effect on glycolytic metabolism, long-term inhibition compromises nucleus pulposus cell survival[9]. This finding suggests that autophagy has a unique, non-metabolic role in hypoxic nucleus pulposus cells that should be further investigated. By contrast, HIF1α activity in nucleus pulposus cells has been directly linked to attenuation of ER stress and modulation of the nucleus pulposus cell secretome[69]. Hence, dysregulation of autophagic and ER stress-related pathways might explain the increased UPR and decreased ECM integrity of aged and degenerated discs[66,69].

As nucleus pulposus cells reside in a physiologically hypoxic environment and utilize glycolysis for energy production, a prevalent notion was that mitochondria only have a minor biological role in disc physiology. In 2020, it was demonstrated using a *mito*-QC mouse model that, in fact, nucleus pulposus cells contain numerous well-networked, tubular and hypoxia-responsive mitochondria[6]. Specifically, hypoxia and HIF1α govern mitochondrial morphology, composition and mass in nucleus pulposus cells. The researchers leveraged the finding that nucleus pulposus cells have functional, HIF-dependent mitochondria to study mitochondrial dynamics. They noted that mitophagy and mitochondrial fragmentation are regulated by HIF1α through modulation of BNIP3 and a dynamin

Redox homeostasis
A balance of reduction and oxidation enzymatic reactions (redox) within a cell. Among many redox systems, the NAD^+ to NADH ratio is essential for the redox homeostasis required for glycolysis and mitochondrial function.

1-like protein (DRP1)–optic atrophy protein 1 (OPA1) axis, respectively. Although the hypoxic induction of mitophagy normally requires the HIF1α–BNIP3 axis, NIP3-like protein X (NIX; also known as BNIP3L) and non-receptor-mediated pathways could compensate for HIF1α when HIF1α expression is silenced. Although research into mitochondrial dynamics in nucleus pulposus cells is at an early stage, each discovery has the potential to change how researchers in the field consider the role of mitochondria within a hypoxic niche. In the following section that deals with the functional importance of the mitochondrial TCA cycle in nucleus pulposus cell metabolism, we consider the hypothesis that dysregulation of mitochondrial function compromises nucleus pulposus cell survival.

Hypoxic regulation of cell metabolism

For decades, IVD researchers have primarily focused on delineating the mechanisms of disc cell survival and function in their physiologically hypoxic and acidic milieu. Consequently, until recently, research on disc cell metabolism was limited to understanding basal nutrient and metabolite concentrations in the disc, solute transport dynamics through the disc matrix and the effects of dynamic niche conditions on cell viability in culture systems[60,61,70,71]. More recently, studies have linked general metabolic changes in the IVD to both ageing and disc degeneration (BOX 2). Considering the importance of disc cell metabolism for IVD health and integrity, this Review highlights a series of publications within the last 5 years that show that hypoxic signalling and HIF1α can control the complex metabolic systems in the disc. In the following sections, we summarize how HIF1α regulates the overall biosynthetic capacity of nucleus pulposus cells by modulating both glycolytic and mitochondrial metabolism (FIG. 2).

Regulation of glycolysis and the TCA cycle. Nucleus pulposus cells generate ~75% of their ATP through glycolysis[27]. HIF1α regulates glycolytic flux through the transcriptional regulation of glucose transporters and glycolytic enzymes[26,27,72,73]. By contrast, the mechanisms by which flux through the TCA cycle is controlled in the hypoxic nucleus pulposus, or why this cycle is important for cell survival, are still unresolved.

Box 2 | **Metabolism in the ageing disc**

Ageing is one of the important risk factors for disc degeneration and affects nucleus pulposus and annulus fibrosus cell bioenergetics[131]. Nucleus pulposus cells lose both glycolytic and mitochondrial function with age, as evidenced by a decrease in the glycolytic and mitochondrial reserve capacity and the maximum aerobic capacity[131]. These decreases correlate with loss of matrix synthesis, which is otherwise an energy demanding process. By contrast, ageing does not change mitochondrial respiration in annulus fibrosus cells; however, ageing does cause an increase in glycolytic flux.
In addition to these mechanistic insights, findings from mouse models have provided evidence of a strong clinical link between cellular metabolism and disc degeneration[123]; data from two separate mouse models of ageing have shown that many aspects of metabolism, including glucose homeostasis, carbohydrate homeostasis, lipid metabolism and phosphate metabolic processes, are modulated during ageing.
Taken together, these studies suggest that, HIF1α aside, the straightforward study of metabolic pathways and processes might further illuminate our understanding of the ageing disc.

Two studies have explored the complex interplay between oxygen availability, HIF1 function and metabolic flux in nucleus pulposus cells[6,7]. Hypoxia increases the overall concentrations of glycolytic pathway intermediates and decreases the concentrations of TCA cycle intermediates in nucleus pulposus cells[6]. These studies suggest that HIF1α regulates these specific reactions in novel ways. For example, loss of HIF1α in hypoxia leads to an increase in the concentration of initial glycolytic intermediates, glucose and glucose-6-phosphate, but also leads to a decrease in middle-stage and late-stage glycolytic intermediates, dihydroxyacetone phosphate and pyruvate[6]. Surprisingly, the concentration of the TCA intermediates citrate, succinate, fumarate, malate and oxaloacetate are also reduced, suggesting that although hypoxia downregulates the TCA cycle, HIF1α either directly or indirectly maintains flux through the TCA cycle. This finding raises the question of whether the decreased flux through glycolysis in HIF1α-deficient cells reduces TCA cycle flux, or if HIF1α regulates specific reactions within these linked pathways. Emerging data suggest that loss of HIF1α promotes the redirection of TCA flux towards glutamate production, through glutamate dehydrogenase (GDH)[6]. When reconciled with metabolic profiling data, the evidence suggests that increased flux to glutamate maintains the metabolic activity in HIF1α-deficient cells, by balancing the NAD^+ to NADH redox ratio. As a result, the TCA cycle flux to succinate is reduced, effectively slowing the generation of 4-carbon intermediates of the TCA cycle[6]. Accordingly, HIF1α regulates TCA cycle flux, in addition to glycolytic flux, to maintain redox homeostasis in nucleus pulposus cells.

How is it possible to reconcile the observation that HIF1α positively regulates pyruvate entry into the TCA cycle in hypoxic nucleus pulposus cells with the observation that HIF1α transactivates pyruvate dehydrogenase kinase 1 (PDK1) in other cell types[74]? In general, PDK1 promotes the reduction of pyruvate to lactate rather than its oxidation to acetyl-CoA in the TCA cycle by inactivating pyruvate dehydrogenase (PDH). Indeed, HIF regulation of PDK1 has been considered a 'metabolic switch' that shifts cells towards glycolytic metabolism, a process that is unique to hypoxic cells. Perhaps, in nucleus pulposus cells, glycolytic metabolism is only suitable for the generation of ATP, whereas the TCA cycle might be required for the generation of anabolic intermediates needed for critical protein, lipoprotein and proteoglycan synthesis[15,75,76]. If this scenario is the case, then a strict metabolic switch that blocks pyruvate entry into the TCA cycle would be detrimental to nucleus pulposus cell survival.

Regulation of the mitochondrial ETC. As discussed in the previous section, the contribution of mitochondria to biosynthetic flux and ATP production in nucleus pulposus cells has been a subject of controversy. Although HIF1α was found to regulate TCA flux, its role in mitochondrial respiration and ETC function remained an open question. Studies of mitochondrial activity have been performed under normoxic conditions to assess whether nucleus pulposus cell

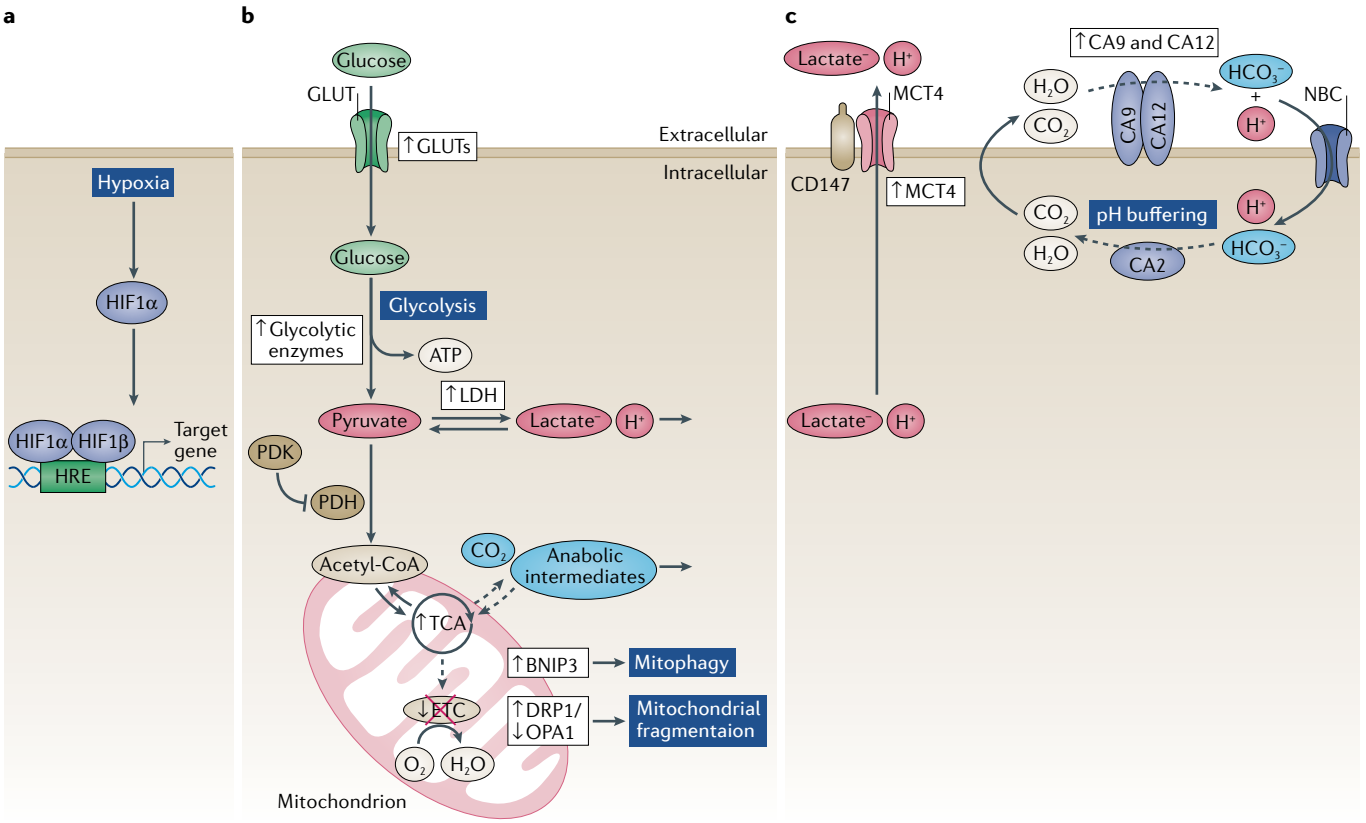


Fig. 2 | **HIF1α-dependent metabolic and pH regulatory pathways in nucleus pulposus cells. a** | In the hypoxic nucleus pulposus cell, hypoxia-inducible factor 1α (HIF1α) transcriptionally regulates many genes involved in glycolysis and pH regulation. **b** | HIF1α targets are shown in white boxes, with arrows denoting upregulation or downregulation by HIF1α. HIF1α promotes glycolytic flux and lactate generation by controlling glucose import through glucose transporter 1 (GLUT1) and upregulating glycolytic enzymes, including lactate dehydrogenase (LDH), which catalyses the reduction of pyruvate to lactate in the final step of glycolysis. HIF1α also modulates pyruvate entry into the mitochondrial tricarboxylic acid (TCA) cycle, although it is unclear how HIF regulates the pyruvate dehydrogenase (PDH)–pyruvate dehydrogenase kinase 1 (PDK1) axis in nucleus pulposus cells. Although the function of the TCA cycle is preserved in the nucleus pulposus, the mitochondrial electron transport chain (ETC) is inhibited by hypoxia. To maintain healthy mitochondrial activity, hypoxia and HIF1α modulate autophagic and mitophagic pathways. **c** | To maintain intracellular pH and the perpetuation of pyruvate reduction, the HIF1α target monocarboxylate transporter 4 (MCT4), aided by its chaperone protein CD147, facilitates the export of H^+ and lactate. Furthermore, to tightly control the intracellular pH in glycolytic nucleus pulposus cells, HIF1α orchestrates a HCO_3^- buffering system, governed by carbonic anhydrase 9 (CA9) and CA12 and sodium bicarbonate cotransporters (NBCs), and fuelled by recycled and TCA cycle-derived CO_2. BNIP3, BCL-2/E1B 19 kDa protein-interacting protein 3; DRP1, dynamin 1-like protein; HRE, hypoxia-response element.

mitochondria have a functional ETC in the presence of available oxygen[7]. Silencing HIF1α in nucleus pulposus cells under normoxic culture conditions decreased the extracellular acidification rate; however, this effect was reversed by blocking the function of the mitochondrial ETC with antimycin A[7]. This finding suggests that nucleus pulposus cells possess a functional ETC, as loss of HIF1α under normal oxygen tension caused a metabolic switch from glycolytic to oxidative metabolism. HIF1α silencing also increases the mitochondrial oxygen consumption rate (OCR) in culture conditions where oxygen is readily available. Under these conditions, mitochondrial OCR increases in a dose-dependent manner when treated with the mitochondrial uncoupler, trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP). Normoxic culture conditions are not physiological; nonetheless, these results show that nucleus pulposus cells possess sufficient metabolic plasticity, are capable of oxidative metabolism and have reserve mitochondrial ETC capacity that can be utilized under specific circumstances, such as when HIF1α signalling is compromised. It is important to note that neither sustained oxygen availability nor re-oxygenation after hypoxic culture are sufficient to upregulate the OCR in nucleus pulposus cells when HIF1α is expressed[6,7].

How does HIF1α simultaneously upregulate TCA cycle flux and downregulate ETC function in nucleus pulposus cell mitochondria? Some evidence suggests that hypoxia decreases the levels of ETC complexes and cytochrome *c* in nucleus pulposus cells[6]. It is also conceivable that although the TCA cycle is required to generate metabolic intermediates, including CO_2, and to maintain the redox balance in hypoxic nucleus pulposus cells, the oxygen-dependent flow of electrons through the cytochromes might be redundant if ATP generation through glycolysis is sufficient.

We can learn about nucleus pulposus cell metabolism from insights from other glycolytic cells. For example, mitochondria are uncoupled in hypoxic growth plate chondrocytes[77]. This uncoupled state is mediated by a

Extracellular acidification rate
The rate of change of pericellular proton (H^+) production by cells as measured in vitro by a Seahorse Flux analyser.

Oxygen consumption rate
(OCR). The rate of change of pericellular oxygen (O_2) consumption by cells as measured in vitro by a Seahorse Flux analyser.

HIF1α-dependent protonophore, mitochondrial uncoupling protein 3 (UCP3), that modulates ATP synthesis by facilitating H^+ transport across the inner mitochondrial membrane[77]. In chondrocytes, mitochondrial uncoupling is necessary to limit oxygen utilization and maintain the mitochondrial membrane potential and autophagic flux, rather than for the common physiological role of thermogenesis. Supporting these ideas, HIF1α can suppress mitochondrial respiration in the developing growth plate to prevent anoxia[10]. The findings support the conclusion that mitochondrial respiration is detrimental to fetal chondrocytes and HIF1α signalling is protective during development. Accordingly, future studies into the relationship between the TCA cycle and ETC are warranted in nucleus pulposus cells, as the two mitochondrial pathways might be similarly uncoupled.

Regulation of lactate/H^+ efflux. HIF1α controls glycolytic flux through the transcriptional activation of various enzymes at the beginning, middle and late stages of glycolysis. However, some observations have shown that HIF1α regulates glycolytic flux, in part, by maintaining H^+/lactate efflux from nucleus pulposus cells at the final stage of glycolysis[5]. Specifically, HIF1α upregulates *Slc16a3*, which encodes the coupled H^+/lactate transporter, monocarboxylate transporter 4 (MCT4). The MCTs are a family of 14 lactate, pyruvate and ketone body transporters, each with a distinct tissue-specific function and localization[78,79]. MCT4 is thought to be adapted for lactate efflux in glycolytic cells because of its relatively high affinity for l -lactate (as indicated by the low K_M value of 28 mM) compared with pyruvate (K_M value of 150 mM), preferentially exporting l -lactate over pyruvate from the cell and maintaining the cytosolic redox balance[80,81]. The hypoxic induction of *Slc16a3* transcription is mediated by HIF1 binding to an intronic enhancer in nucleus pulposus cells[5], rather than to a previously reported HRE in the proximal promoter[82]. Strikingly, acute inhibition of MCT4 downregulates glycolysis and increases TCA cycle flux, essentially rewiring nucleus pulposus cell metabolism[5]. This metabolic switch is particularly notable as MCT4, unlike HIF1α, is not a transcription factor or a major regulator of glycolytic metabolism. Instead, the metabolic changes following acute MCT4 inhibition might be explained by the downstream effects of increased intracellular H^+/lactate concentrations caused by blockage of H^+/lactate export. To prevent intracellular acidification and cytotoxic acidosis, nucleus pulposus cells might oxidize the excess lactate into pyruvate, which then enters the TCA cycle and increases the TCA cycle flux. In addition, accumulation of intracellular H^+/lactate could inhibit the activity of lactate dehydrogenase (LDH) in a negative feedback loop. As LDH catalyses the reduction of pyruvate to lactate in the final step of glycolysis, feedback inhibition of LDH by intracellular H^+/lactate might explain the downregulation of glycolysis following MCT4 inhibition. Overall, upregulation of TCA cycle flux and glutamate production following MCT4 inhibition might serve to regenerate NAD^+ reducing equivalents, to ensure a balanced redox ratio. The fact that acute MCT inhibition does not affect the NAD^+ to NADH ratio supports this idea and shows that nucleus pulposus cells can still produce NAD^+ under these conditions.

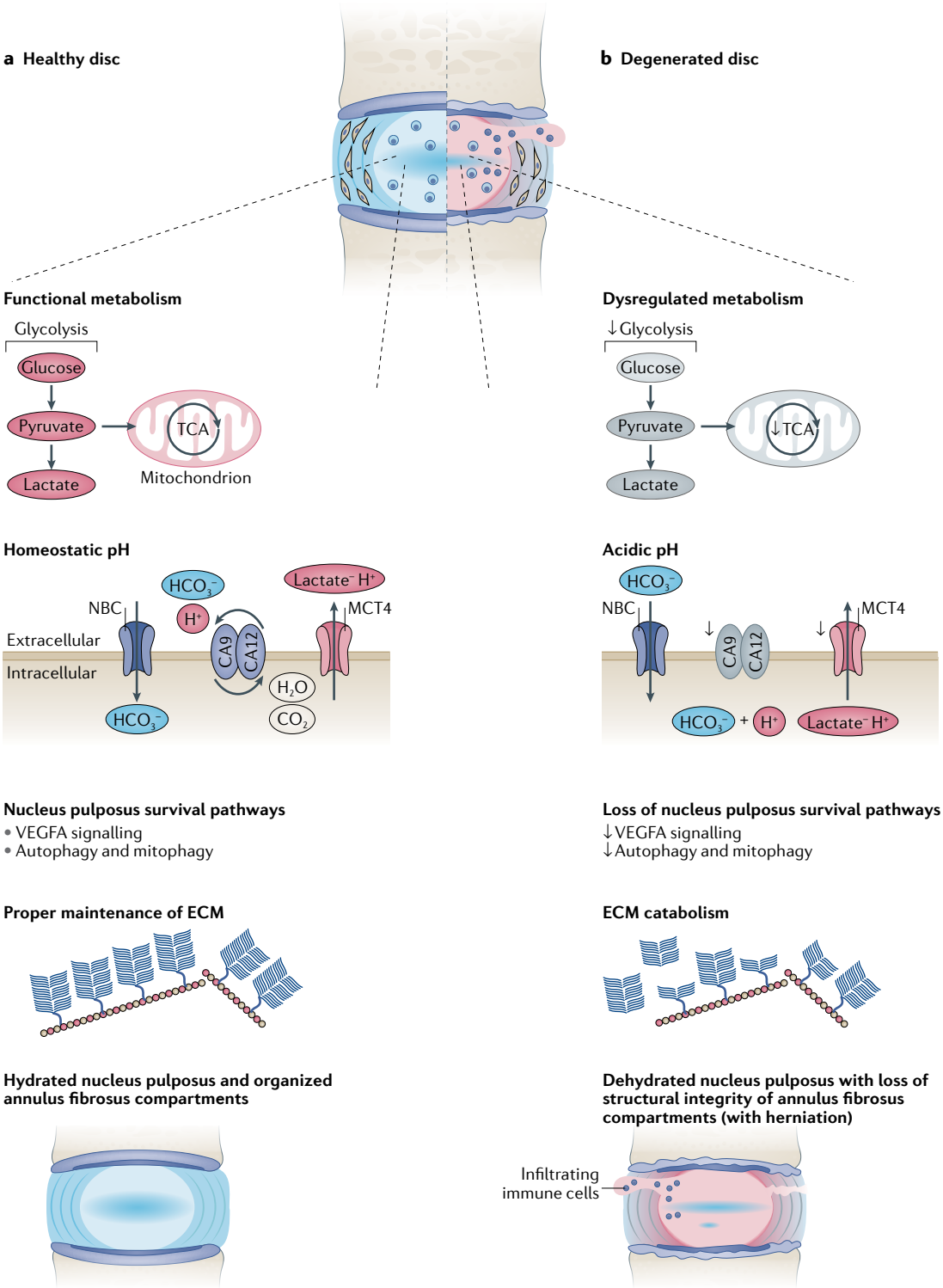
In the short term, the metabolic plasticity of nucleus pulposus cells enables them to withstand the consequences of intracellular H^+/lactate accumulation on intracellular pH and redox homeostasis. However, long-term inhibition of MCT4 in vivo can compromise nucleus pulposus cell viability, likely because of cytosolic acidification and failure to maintain a high TCA flux. MCT4 silencing in vivo correlates with decreased nucleosome assembly and epigenetic programming in degenerated nucleus pulposus tissue (as discussed in a later section). Notably, the loss of MCT4 in mice recapitulates the major pathoanatomical hallmarks of human disc degeneration, including loss of cellular phenotypic markers and matrix integrity[83,84]. The results from these in vivo studies suggest that loss of MCT4 expression contributes to the cascade of events directly linked to human disc degeneration.

Lactate as a signalling molecule. In addition to controlling metabolic flux and intracellular pH levels, high lactate levels function as a hypoxia mimetic factor by instigating the biosynthesis of TCA cycle intermediates. These intermediates functionally compete with the TCA cycle intermediate and cofactor, α-ketoglutarate, that is necessary for HIF1α hydroxylation and degradation by PHDs[85,86]. Mechanistically, hydroxylation of HIF1α is catalysed by Fe^{2+}-dependent PHD dioxygenases, which use oxygen and α-ketoglutarate as substrates for HIF hydroxylation in a reaction that produces CO_2 and succinate[31]. The PHDs have a tight affinity for α-ketoglutarate, with a K_M of 1–2 μM for PHD1 and PHD2 and a K_M of 12 μM for PHD3 (REF.[87]). However, the hydroxylase function of PHDs can be competitively inhibited by the TCA cycle intermediates succinate and fumarate (K_i values of 50–80 μM and 350–460 μM, respectively)[87]. Data from crystallographic studies show that succinate and fumarate competitively inhibit α-ketoglutarate-dependent dioxygenases by directly binding to Lys214 and Tyr145 residues in the substrate binding pocket of PHDs[88]. Binding to this site blocks the necessary ligation of the 2-oxo group of α-ketoglutarate to the Asp201 residue in the active site, which results in PHD inactivation[88]. Other studies have shown that both lactate and pyruvate are also capable of inhibiting PHD activity. Some reports suggest that the conversion of lactate to pyruvate by LDH increases the concentration of TCA cycle intermediates, succinate and fumarate, which in turn inhibit PHD activity[86]. It is also possible that pyruvate might function as a competitive structural mimic of α-ketoglutarate and is capable of blocking PHD function independent of its downstream TCA cycle intermediate metabolites[89].

Lactate as a regulator of transcription. Growing evidence suggests that metabolic intermediates can directly link cellular metabolism to physiological functions, such as cell proliferation and survival, through alterations in the epigenetic landscape. The enriched biological processes in the nucleus pulposus cells of MCT4-null mice are in fact driven by changes in histone genes and

K_M
A measure of the 'affinity' of an enzyme or transporter for its substrate. More precisely, K_M is the concentration of a substrate that is needed for an enzyme or transporter to reach its half-maximum velocity (for enzymes) or binding site occupancy (for transporters); therefore, a lower K_M signifies a higher affinity.

Intracellular acidification
Cytosolic pH of cells is tightly regulated within a physiological range. When the H^+ concentration exceeds this range, due to dysregulation of H^+ export and cytosolic pH buffering systems, intracellular acidification occurs.



DNA-binding proteins[5]. The major pathways associated with these genes are those involved in nucleosome assembly, regulation of epigenetic gene expression and negative regulation of cell proliferation. These findings suggest that a build-up of H^+/lactate directly affects gene transcription in nucleus pulposus cells[5]. In fact, many essential glycolytic and mitochondrial enzymes also function in the nucleus where they are involved in DNA binding and the generation of intermediates that regulate gene transcription[90–93]. Phosphorylation of Tyr238 on LDH promotes nuclear translocation of lactate, where lactate inhibits HDACs and increases gene transcription[94,95]. Most encouragingly, a process called 'lactylation' has been described, in which the lactate-derived epigenetic modification of 28 distinct histone lysine residues directly stimulates gene transcription according to a 'lactate clock'[96]. Hence, elevated intradiscal lactate levels might contribute to disc

Fig. 3 | **Pathological link between loss of HIF1α function and intervertebral disc degeneration.** Schematics of a healthy intervertebral disc and a degenerated intervertebral disc, and the potential link between loss of HIF1α function and intervertebral disc degeneration. **a** | Glycolytic pathways and the tricarboxylic acid (TCA) cycle function normally in healthy nucleus pulposus cells. These cells possess multiple pathways to buffer intracellular H^+ production and maintain homeostatic intracellular pH, including H^+/lactate extrusion by monocarboxylate transporter 4 (MCT4) and HCO_3^- buffering by the carbonic anhydrase CA9/CA12–sodium bicarbonate cotransporter (NBC) axis. Functional nucleus pulposus tissue compartments are maintained by hypoxia and hypoxia-inducible factor (HIF)-dependent survival pathways (such as vascular endothelial growth factor A (VEGFA) signalling, autophagy and mitophagy). Healthy nucleus pulposus tissue possess a chondroitin sulfate proteoglycan-rich extracellular matrix (ECM), which is responsible for the biomechanical function of the disc. **b** | In degenerated intervertebral discs, HIF function and activity is reduced. Loss of HIF1α signalling diminishes target gene expression required for cell metabolism and intracellular pH buffering. Dysregulation of the critical nucleus pulposus cell survival pathways and acidosis results in nucleus pulposus cell death and increased matrix breakdown. A compromised ECM and diminished biomechanical function makes the tissue susceptible to herniations and pain.

degeneration through a direct and cumulative effect on the transcriptional and epigenetic regulation in nucleus pulposus cells. Likewise, the metabolic regulation of epigenetics in chondrocytes is an interesting area for future study.

Overall, studies in nucleus pulposus cells show a dynamic relationship between metabolic flux and HIF1α activity that is mediated by intracellular lactate levels[5]. On the one hand, intracellular lactate accumulation increases HIF1α activity; on the other hand, MCT4 transcriptional activation is regulated through a newly discovered HIF1-sensitive intronic enhancer. This observation suggests that a positive feedback loop exists between hypoxia-inducible MCT4 function and HIF1α stability in nucleus pulposus cells, and that this loop is modulated by intracellular lactate levels.

Hypoxic regulation of intracellular pH

A consequence of glycolysis in hypoxic tissues is H^+/lactate accumulation and intracellular acidification, unless a homeostatic pH is properly maintained. In nucleus pulposus cells, an acidic pH (~6.5) exacerbates the breakdown of ECM proteins and decreases glycolytic flux[61,76,97,98]. As a result, glycolytic cells recruit a robust network of enzymes for intracellular pH regulation, many of which are controlled by HIF1α[99]. One mechanism to regulate intracellular pH is mediated by proton (H^+) extrusion; indeed, nucleus pulposus cells express both Na^+/H^+ exchangers (NHEs) and H^+-ATPases (V-ATPases). Emerging data from the past few years has expanded our knowledge of pH control by nucleus pulposus cells to include HIF1α-dependent CO_2 and HCO_3^- recycling by carbonic anhydrase 9 (CA9) and CA12 (REF.[7]) (FIG. 2).

Carbonic anhydrases are a group of ubiquitously expressed metalloenzymes that function as efficient catalysts for the reversible hydration of CO_2 to HCO_3^- and H^+, which occurs at a rate of up to 1×10^6 units per second at 37 °C (REF.[100]). Given that carbonic anhydrases rapidly produce and consume HCO_3^- and H^+, these enzymes are well adapted to control various physiological and pathological processes including pH regulation and the balance and secretion of fluid in most cell types[100]. The 16 carbonic anhydrase isoforms have versatile and diverse functions. Of the four isoforms known to be expressed in nucleus pulposus cells, CA9 and CA12 are localized to the plasma membrane and favour the production of extracellular HCO_3^- and H^+. CA2 is expressed in the cytosol and favours the production of H_2O and CO_2, whereas the CA3 isoform has a high cytosolic expression but possesses negligible carbonic anhydrase enzymatic activity[7,101]. In this section, we discuss the role of the extracellularly-facing and plasma membrane-bound CA9 and CA12 isoforms that contribute to the majority of extracellular H^+ production by nucleus pulposus cells[7], which is often erroneously attributed to intracellular glycolytic H^+/lactate production and export by these cells[102].

In nucleus pulposus cells, HIF1α binds to the conserved HREs closest to the transcriptional start sites of the promoters of the genes encoding CA9 and CA12 and induces their expression under hypoxia[7,103]. Mechanistically, CA9 and CA12 catalyse the hydration of CO_2 (recycled and/or generated by the TCA cycle) to HCO_3^- and H^+ ions in the pericellular space of nucleus pulposus cells[7]. The extracellularly produced HCO_3^- ions are shuttled into the cytosol by sodium bicarbonate cotransporters (NBCs) to buffer the intracellular pH in a reaction that regenerates CO_2 and H_2O inside the nucleus pulposus cell via the ubiquitously expressed CA2 isoform. This mechanism of pH regulation is dubbed the 'bicarbonate transport metabolon'[104]. In most cells, a good proportion of extracellular H^+ production occurs as a result of intracellular H^+/lactate generation via glycolysis and subsequent export by MCTs. However, in nucleus pulposus cells, inhibition of CA9 and CA12 results in a striking decrease in extracellular H^+ production and an increase in intracellular acidification, despite the fact that this inhibition has no effect on pathways that regulate or are regulated by glycolytic flux (that is, extracellular lactate concentrations, HIF1α activity or MCT4 levels). In other words, the measured decrease in extracellular H^+ production following acute inhibition of CA9 and CA12 in nucleus pulposus cells is directly caused by inhibition of CA9 and CA12 enzymatic activity on the extracellular surface of the cells. If such a high concentration of extracellular H^+ units is directly generated by reactions mediated by CA9 and CA12, then what is the fate of these H^+ units? It could be that the protons transit the pericellular space and diffuse out of the disc; alternatively it could be that protons are used by nearby membrane-associated importers as an energy currency; or both[105,106].

An additional nuance was added to this system following the discovery that CA12 expression is simultaneously controlled by the RNA-binding protein, ELAV-like protein 1 (ELAVL1; also known as HuR). This discovery established an additional mechanism by which CA12 is regulated to maintain intracellular pH levels in nucleus pulposus cells. Unlike in other cell types, HIF1α is not a functional target of ELAVL1 in nucleus pulposus cells, suggesting that CA12 regulation by ELAVL1 occurs independently of HIF1α activity[8]. Notably, CA12 expression is increased in degenerated human discs[103], which could be to compensate for a loss of enzymatic activity of CA12 or could be a way to resist further acidification in degenerated discs.

In addition to carbonic anhydrases, other evidence shows that MCT4 is responsible for the facilitated cotransport of H^+/lactate out of the nucleus pulposus cell to maintain intracellular pH[5]. Although carbonic anhydrases buffer the pH of cells via a HCO_3^- transport metabolon that involves multiple proteins and available CO_2 and HCO_3^- stores, MCT4 simply expedites H^+ extrusion. This mechanism of H^+ extrusion is similar to the method of pH regulation mediated by other transporters found in nucleus pulposus cells (that is, the Na^+/H^+ exchangers and H^+-ATPases)[107]. Acute inhibition of MCT4 function does not affect intracellular pH in nucleus pulposus cells to the same extent as inhibition of CA9 and CA12, possibly owing to rapid utilization of lactate by the TCA cycle[5]. Accordingly, as MCT4 regulates intracellular pH in a manner that involves a critical metabolite, lactate, its expression alters nucleus pulposus cell metabolism in a way that is not observed with CA9 and CA12.

HIF and hypoxia in tissue regeneration

Various data lend strong support to the longstanding hypothesis that cells of the nucleus pulposus and cartilage have adapted to their adverse hypoxic, acidic and nutrient-limiting environments. In vitro studies confirm that the nucleus pulposus niche conditions are inhospitable for stem and progenitor cell proliferation, promote chondrogenic differentiation and decrease matrix biosynthesis[108,109]. The limited nutrient supply in the nucleus pulposus compartment is a result of tissue avascularity; thus, the sole reliance of this compartment on diffusion for metabolite transport and maintenance of cellular metabolism affects cell survival in degenerating discs[60]. With these aspects in mind, researchers must acknowledge the importance of survival factors when developing strategies for disc and cartilage regeneration using biological therapies. Three commonly investigated regenerative therapies include the implantation of differentiated cells or stem cells to produce healthy matrix molecules[110], the implantation of whole, tissue-engineered scaffolds seeded with cells[111,112] or the alteration of activity of degenerated cells using gene therapy or intradiscal injection of therapeutic growth factors[113,114]. Cumulatively, these strategies rely on the fact that the implanted or regenerated cells survive and remain biosynthetically active despite diminished solute uptake through degenerated cartilaginous endplates[115]. One study has demonstrated the utility of improving nutrient diffusion into human discs by treating the cartilaginous endplates with matrix metalloproteinase 8 (MMP8)[116]. However, the concentration of advanced glycation end-products in the cartilaginous endplate notably affects the efficacy of matrix perturbation with MMPs and the subsequent capacity for nutrient uptake into the disc.

Considering that native progenitor cells are scarce in the disc and cartilage and decline with age[117], researchers have differentiated human pluripotent stem cells into notochord-like and nucleus pulposus-like cells, often leveraging hypoxic culture conditions to prime cell metabolism or push them towards a desired lineage[112,118,119]. However, survival of these new cells is dependent on maintenance of the local nutrient supply. For example, implantation of highly active stem cells or growth factors that increase rates of biosynthesis and proliferation might be counterproductive in that they alter the delicate nutrient–metabolite balance in the already degenerated tissues[112]. Therefore, pairing therapeutic approaches with inhibitors of cytokine production, given that cytokines are known to increase nutrient consumption, might be useful[18,120]. Furthermore, loss of HIF1α function might result in a cascade of degenerative changes in the IVD (FIG. 3). Therefore, in addition to biological therapies, the use of pharmacological PHD inhibitors, or HIF activators, evaluated in clinical trials for the treatment of anaemia[121,122] might be of value for the regeneration of both IVD and cartilage tissue (BOX 3).

Box 3 | Clinical development of HIF and/or PHD inhibitors

Pharmacological prolyl hydroxylase inhibitors (PHIs) and hypoxia-inducible factor (HIF) inhibitors were initially developed for the treatment of renal anaemia and cancer therapy[124,132,133]. The question remains as to which of these inhibitors would be most suitable for the regeneration of hypoxic skeletal tissues where HIF1α signalling is considered a hallmark of healthy tissue, and degeneration correlates with a loss of HIF function[4,5,14,36,62] (FIG. 3). PHIs, also known as HIF activators, might be the preferred therapeutic candidate for the following reasons: PHIs induce a transient increase in HIF-regulated gene expression[134] and various clinical studies have demonstrated the efficacy of PHIs in the treatment of renal anaemia[124,133]. Promising results from completed clinical trials evaluating vadadustat and daprodustat make a strong argument for the future evaluation of PHIs in the treatment of disc and cartilage regeneration[121,122,124]. Inhibition of prolyl hydroxylase domain-containing protein 2 (PHD2), specifically, might be capable of elevating and/or stabilizing the expression of HIF1α in nucleus pulposus cells and articular chondrocytes[28,45] and thus upregulate the expression of HIF target genes (including *Vegfa*, *Car12* (encoding CA12) and *Slc16a3* (encoding MCT4)) in degenerated discs and osteoarthritis (OA).

Unlike in the intervertebral disc, HIF2α has been associated with both agonistic and antagonistic pathways in cartilage In some instances, inhibitors that block HIF2 dimerization[135] might be useful to reduce the negative effects of HIF2 on matrix catabolism and the development of OA. Several highly selective HIF2 inhibitors are currently in phase II clinical trials for the treatment of von Hippel–Lindau (VHL)-associated renal cell carcinoma (RCC) and other cancers; for example, novel compounds, PT2385 (REF.[136]) and PT2977 (REF.[137]), inhibit HIF2 dimerization and DNA binding[132,138]. A similar compound, PT2399, has shown even greater effectiveness in the treatment of RCC in a xenograft platform[139]. Investigating if such HIF2α inhibitors exert positive effects on the fate of chondrocytes in OA is important.

Conclusion

HIF transcription factors are known to have a role in the physiological maintenance of hypoxic tissues as well as in related pathologies. Although most of what is known on the role of HIF1α comes from decades of research on tumorigenesis, emerging data are helping to explain how HIF1α enables IVD cells to adapt to their avascular and hypoxic tissue environment[4,29]. Briefly, the regulation of HIF1α itself is uniquely tuned to the nucleus pulposus cell microenvironment through oxygen-dependent mechanisms, governed by PHD2 and PHD3, as well as by oxygen-independent mechanisms, including regulation by HSPs and the nucleus pulposus cell-intrinsic circadian rhythm. By adjusting nucleus pulposus cellular metabolism to function within a low oxygen tension range, HIF1α maintains energy production, intracellular pH and redox homeostasis while indirectly controlling critical cellular processes such as matrix biosynthesis[5–7]. Furthermore, emerging data suggest a role for lactate as a regulator of HIF1α activity and in epigenetic

programming in nucleus pulposus cells, as well as in other cell types[5]. In fact, some data suggest that lactate has a function in the nucleus of cells where it facilitates lactate-derived histone modifications[96]. This finding has major implications for the link between epigenetics and degenerative disc disease, which is an area that has not yet been thoroughly explored.

An important consideration is understanding how dysregulation of HIF signalling contributes to the pathogenesis of skeletal diseases and disorders, including but not limited to degenerative disc disease and OA. Although deletion of HIF1α leads to total loss of the nucleus pulposus cell compartment, additional mouse models of spontaneous IVD degeneration, such as SM/J and LG/J inbred strains of mice[14,123], and models with alterations to genes controlling PHD function[40], circadian rhythm[36] and H^+/lactate export[5] have all demonstrated dysregulation of HIF target genes. These findings imply that downregulation of HIF1α target genes is commonly associated with degenerative changes in the disc; therefore, HIF activators with clinical efficacy might have potential for the treatment of disc degeneration[124]. Cumulatively, the studies discussed in this Review add nuance to what is currently known about disc cell metabolism and provide evidence that dysregulation of HIF1α signalling causes IVD degeneration through metabolic disruption. Advances in technology and animal models have contributed to the reinvigoration of metabolism research in the broader field of skeletal biology, and should continue to broaden our knowledge and provide potential therapeutic avenues in future.

Published online 3 June 2021

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Acknowledgements
The work of M.V.R. and E.S. is supported by grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS): R01-AR055655 and AR074813 (M.V.R.) and R01-AR074079 and AR073022 (E.S.). The work of E.S.S. is supported by grant T32-AR052273. The authors would like to thank all scientists who contributed to the data and discoveries described in this Review.

Author contributions
All authors researched data for the article, wrote the article, made substantial contribution to discussion of content and reviewed and/or edited the manuscript before submission.

Competing interests
The authors declare no competing interests

Peer review information
Nature Reviews Rheumatology thanks S. Grad, Q.-J. Meng, W.K. Tam and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.