Insights into peptidylarginine deiminase expression and citrullination pathways

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Abstract

Peptidylarginine deiminases (PAD) are calcium-dependent enzymes that mediate citrullination, an irreversible posttranslational modification. PAD enzymes have received increasing attention in (patho-)physiology since multi-omics analysis accelerated their expression profiling. This article provides a comprehensive overview of PAD expression at the RNA and protein levels, and includes a list of annotated substrates per PAD isozyme. We discuss novel roles of citrullination in cellular growth, epigenetic regulation, tissue remodelling, inflammation and cancer in mouse models and humans. Additionally, we cluster similar effects of protein deimination to offer a different perspective and improve our understanding towards citrullination in health and disease. Citrullination should no longer be considered as a rare posttranslational modification, but as an important regulatory mechanism in physiology and pathology.

A new era of peptidylarginine deiminases

Peptidylarginine deiminases (PAD) are enzymes that **deiminate** (See glossary) **peptidylarginine**, and not free arginine, to **peptidylcitrulline** residues in a **posttranslational modification** named citrullination. In humans, five enzyme isoforms have been identified, namely PAD1-4 and PAD6 [1]. Citrullination causes proteins to gain 0.98 mass units and lose one positive charge per deimination. These modifications profoundly affect human biology but are small at the molecular level, making it difficult to study [2]. The effects of PAD activity were explored in autoimmune diseases i.e. rheumatoid arthritis (RA), multiple sclerosis (MS) and in skin physiology, but poorly in other areas until the emergence of modern technologies. High-resolution mass spectrometry facilitated accurate detection of citrullination and with current 'omics' data, hundreds of putative PAD substrates were annotated [3]. This article offers a comprehensive review of the PAD enzyme family and provides a complete guide to the expression pattern of PAD isoforms, including their substrates (Table 1). In addition, we discuss knowledge on the role of PAD enzymes gathered from human and animal studies to provide novel insights into citrullination pathways in health and disease.

PAD1 maintains epidermal integrity and safeguards embryonal growth

The discovery of deiminated keratins (K1, K10) and profilaggrin revealed the presence of PAD1 in the skin [1]. PAD1 is expressed throughout the epidermis and facilitates terminal differentiation of keratinocytes. Citrullination of profilaggrin allows proteases to cleave the protein into filaggrin, an essential protein that retains moisture in the cutaneous barrier. PAD1 and PAD3, colocalize with profilaggrin, suggesting both enzymes organize keratin cytoskeleton, although the rate of citrullination by PAD1 was much higher *in vivo* compared to PAD3 [4].

PAD1 is highly expressed in male and female reproductive systems [5], though its function is mainly established in female fertility. PAD1 expression in the uterus increases after estradiol injection and upon further female maturation. PAD1 actively citrullinates histone tails during oocyte maturation. Moreover, blocking PAD activity using **Cl-amidine** administration, a pan-PAD inhibitor, caused early mouse embryos to go under cell arrest. More evidence for the critical role of PAD1 induced citrullination during embryonic development was provided using an isozyme-selective inhibitor that blocked histone H3 citrullination in PAD1 expressing HEK293 cells and mouse zygotes [6].

PAD2, ubiquitous in expression and versatile in function

PAD2 is the most prevalent human citrullinating enzyme, thus ensuing its polyvalent nature (Table 1) [5]. PAD2 is highly expressed in neural cells, including astrocytes, microglia, Schwann cells and oligodendrocytes [7]. However, detection of citrullinated myelin extracts was the first indication for a role for PADs in the central nervous system (CNS). Since citrullination lowers protein charge, deiminated myelin basic protein (MBP) binds less to phospholipids, which greatly affects myelin organization [8]. MBP C18, the most abundantly citrullinated MBP isoform, is found in loosely compacted myelin and young children [9]. This suggests that myelin partially loses its structural integrity upon citrullination, providing plasticity in growing children, but compromising **saltatory conduction**, as observed in MS. Other natural cerebral substrates of PAD2 are Glial fibrillary acidic protein (GFAP) and vimentin. Both are cytoskeletal proteins, expressed by mature astrocytes and upregulated upon neural damage [8,10]. Therefore, GFAP and vimentin are target proteins in multiple neurodegenerative diseases and their citrullinated forms are studied mostly in pathology (Table 2) [10,11]. We will discuss this subject more detailed later in the manuscript.

PAD2 is highly expressed in female tissues [5] and is a transcriptional activator of the estrogen receptor [12]. Experimental data from MCF-7 cells revealed that PAD2 modulates transcription through intracellular histone H3 citrullination despite lacking a nuclear localization signal. According to biochemical studies, PAD2 resides in a stable complex with annexin A5 during homeostasis but dissociates from this complex upon calcium influx. Subsequently, calcium-bound PAD2 would use Ran, a cytoplasmic enzyme, as carrier protein to translocate to the nucleus [13]. Its association with the estrogen receptor rendered PAD2 a study target in breast cancer, in which hormonal therapy can be effective to treat patients with estrogen receptor-positive breast cancer. The role of PAD2 was assessed using tamoxifen-resistant MCF7 cell lines, which apparently express high levels of this enzyme [14]. Depletion and Cl-amidine inhibition of PAD2 worked synergistically with docetaxel and gave rise to more responsive and less proliferative breast cancer cells. The proposed molecular mechanism behind these observations is mainly genetic. PAD2 promotes the degradation of p53, an essential tumor suppressor, thereby loosening the brake on cell-cycle arrest and apoptosis. Indeed, PADs are known to regulate carcinogenic genes and are considered promising therapeutic targets in cancer (Box 1).

Although PAD2 is highly expressed in the gastro-intestinal (GIT) tract, its GIT role is considerably less studied compared to other tissues [5]. Literature reports that colonic samples from patients suffering from irritable bowel disease contains citrullinated proteins [15]. In line with these findings, Cl-amidine suppresses colitis in mice by promoting apoptosis of inflammatory cells and subsequently decreasing inflammation [16]. Given these limited findings are inflammation oriented, we speculate PADs have an immunoregulatory role in the GIT.

PAD2 in blood is expressed mainly by myeloid leukocytes that facilitate immune regulation [17]. Healthy neutrophils actively secrete PAD to deiminate extracellular proteins, although its intended function remains unresolved. [18,19]. Extracellular citrullination was originally considered a unique observation in RA synovium. However, the presence of PAD2 in bronchoalveolar lavage fluids of smokers and sepsis patients confirmed otherwise [18, 20, 21]. These results substantiate extracellular citrullination and support the hypothesis claiming **epitope spreading** triggers RA onset [22]. PAD2 expression in mononuclear immune cells is crucial for their function, therefore tightly regulated [23]. Monocytes carry PAD2 mRNA and translate it to protein only after differentiation to macrophages. Macrophage-like cells without the *PADI2* gene failed to produce and secrete proinflammatory cytokines IL-1β, IL-6 and TNF-α upon **lipopolysaccharide (LPS)** stimulation. In addition, macrophage apoptosis and adhesion were disrupted in PAD2 knockout cells due to abrogated caspase activation and phosphokinase downregulation, respectively. Clearly, PAD2 activity sustains inflammation and can tip the intricate balance between adequate and excessive inflammation [24]. Lymphocytes express PAD2, but less compared to myeloid leukocytes [17].

Box 1: PAD2 and PAD4 activity in cancer biology

PAD2 and PAD4 enzymes are expressed in malignant cells and have implications in various solid tumors [33,88]. In this box, we briefly discuss the main citrullination pathways exploited in tumor biology and refer for a detailed review to *AE Yuzhalin* [88].

Citrullination in cancer is mainly an intracellular phenomenon. PAD enzymes target genes involved in tumor growth, cell cycles and protein synthesis to alter cell signalling. PADs citrullinate histones to create a proinflammatory environment. In breast cancer, citrullination has a gene regulatory and inflammatory component. We illustrated that PAD2 is suggested to

degrade p53 in MCF7 cells, thus allowing tumor cells to grow beyond the **G1/S checkpoint**. In parallel, when PAD4 knockout mice failed to form NETs, breast to lung metastasis decreased. The depletion of *PADI4* also reduced primary tumor growth, although the authors considered this reduction unrelated to NET formation. Instead, this might be a complementary effect from PAD2-induced abrogation of p53 function [89]. In line with this, interaction of p53 with PAD4 upon DNA damage is associated with a decreased expression of the tumor suppressor gene OKL38. PAD4 would impede methylation of histones by modifying the promotor region of OKL38, which prevents epigenetic activation [90].

PAD enzymes were also reported with favourable actions in cancer development. For instance, a study with HCT116 cancer cells showed how citrullination of nucleophosmin, a proliferation factor, suppressed tumor growth. PAD4 translocates nucleophosmin to a different subcellular location and disturbs ribosome biogenesis and tumor growth [91]. Furthermore, PAD4 and PAD2 have been described to enhance responsiness to treatment in breast cancer. Overexpression of PAD4 could resensitize multidrug resistant breast cancer cells to Adriamycin, a chemotherapeutic drug by promoting apoptosis and accumulation of glycogen synthase kinase-3β (GSK3β) and p53 [92]. Similarly, histone H3 citrullination by PAD2 restored estrogen receptor availability, which sensitized breast cancer patients to hormonal treatment. A novel mechanism of generating an antitumor effect using citrullinated peptides as immunogens was experimented in mice. α-enolase and vimentin peptides conjugated to **Toll-like receptor (TLR) 1/2 ligands** were injected into carcinogenic tissues to stimulate host immunity [93]. Antitumor effects were achieved by increasing CD4+ T-cell responses that caused rejection of melanoma, lung and pancreas carcinomas. Collectively, one could support the idea of citrullination as therapeutic targets in cancer, whether by modulating PAD activity or further exploration of citrullinated peptides as immune therapy.

PAD3 citrullination offers tissue support and modulates cell death in neural stem cells

PAD3 prefers modification of structural proteins (Figure 1) and is well-described to citrullinate proteins in hair follicle. Modified trichohyalin unfolds and denatures upon hypercitrullination. Although PAD3-induced unfolding of trichohyalin seems drastic, this pivotal physiological process precedes keratin filament crosslinking. The open conformation of citrullinated trichohyalin allows keratin to adhere to the inner root sheath, thereby providing mechanical support to the hair follicle [25]. The skin specific S100A3 protein naturally resides in a dimerized state and forms tetramers upon citrullination. Functionally, the polymerization of S100A3 induced by PAD3 enhances its calcium binding properties and could generate a conformation that stores calcium ions [26,27]. Considering calcium as necessary cofactor for PAD activity, this mechanism might be exploited to sustain citrullination in hair follicles. A similar function for PAD3 was suggested in mammary glands, but only validated in mice [28]. Female mice secrete prolactin and utilize JAK2/STAT5 signalling to promote local citrullination. In the epithelium, citrullinated tubulin was detected in a highly **polymerized** state. The authors reason that PAD3-induced citrullination of tubulin increases structural support to the microtubules, which is probably necessary during lactation [28].

PAD3 is highly expressed in human neural stem cells and was studied using HEK293T cell lines and PAD inhibition experiments. PAD3 modulated apoptosis during neural development in a calcium-dependent manner. PAD3 functions upstream of the apoptotic event, specifically by citrullinating apoptosis-inducing factor (AIF). AIF is a mitochondrial protein that upon a rise in intracellular calcium concentration and subsequent PAD3 interaction translocates to the nucleus [29]. Here, citrullinated AIF triggers chromatin condensation and DNA fragmentation in a caspase 3-independent way. Both citrullination and proteolysis affect AIF activity and

induction of cell death. Further studies are needed to determine which part of the AIF protein is targeted by PAD3 [30].

Intracellular and extracellular functions of PAD4

PAD4, formerly known as PAD5, carries a nuclear localization signal, is widely expressed but abundantly in neutrophils (Table 1). The highest tissue expression of PAD4 is observed in bone marrow, lymphoid tissues and blood [17]. Histones, as most important nuclear PAD4 substrates, have been strongly associated with gene regulation and the formation of neutrophil extracellular traps (NETs) [31,32]. Although the definition of NETosis is a matter of debate, it is the most described PAD4-related function. We discuss histone citrullination and its inflammatory effects in different pathologies in a separate paragraph later in this article.

PAD4 modulates the extracellular matrix (ECM) through citrullination [33]. Myofibroblasts deposit collagen, an ECM protein and substrate of PAD4, to the vascular wall, which stimulates fibrosis in vessels [33]. Since organ fibrosis is dependent on PAD4-induced NET formation, it is plausible that the same mechanism affects **vascular fibrosis** [34]. However, both pharmacological inhibition and knocking out *PADI4* failed to regenerate muscle fibres, despite the decreased NETosis. Abolishing PAD4 activity in mice by Cl-amidine treatment did lower CXCL1, CXCL2 and CCL2 concentrations and dampened inflammation. Therefore, the authors concluded an indirect, inflammatory role for PAD4 in ischemia reperfusion injury. Notably, citrullinated **chemokines** are less chemotactic and elicit a lower T- and B-cell response [35]. Together, we believe that controlled citrullination of chemokines can reduce inflammation, but excessive PAD activity can modify the ECM and trigger inflammation.

PAD4 acts as a **transcriptional corepressor** through modification of histones (Figure 1). The enzyme can either directly affect the chromatin organization or indirectly recruit other

transcriptional mediators with inhibitory functions. Alternatively, PAD4 was suggested to modulate gene expression by converting methylarginine to citrulline with the release of methylamine, a process termed **demethylimination** [36]. Although these data suggest PAD4 to revert histone methylation, hydrolysis of the methyl group by PAD4 is controversial. PAD4 may just replace methylarginine with peptidylcitrulline [37]. The low affinity of PAD4 for methylarginines questions whether PAD4 as 'demethylating' enzyme is truly relevant in physiological conditions. In addition, PAD4 also modulates gene expression by citrullination of non-histone substrates. For instance, inhibitor of growth 4 (ING4) is a tumor suppressor protein that fails to activate p53 transcription upon citrullination. Considering p53 is essential for inhibiting tumor growth, citrullination of ING4 may greatly affect cancer progression (Box 1) [38].

Through NET formation, PAD4 has been proposed as a regulator of **thrombosis** [39]. Mice lacking PAD4 were protected from deep vein thrombosis due to disrupted NET formation that normally stimulates blood clotting. Moreover, the von Willebrand factor (VWF)-cleaving protease and metalloprotease ADAMTS13 are natural substrates of PAD4. Citrullination causes ADAMTS13 to lose enzymatic activity, therefore intact VWF stays longer in circulation and increases the risk for thrombosis [40]. Further exploration in this field led to the discovery that transplants are better tolerated in PAD4-deficient mice and after recombinant human ADAMTS13 treatment [41]. The authors suppose that PAD4 depletion lowers NET formation and subsequently reduces rejection of skin allografts, though increased ADAMTS13 functionality may also assist in reduced skin graft loss.

PAD6 expression is limited, but established in oocytes

PAD6 is the least expressed in the human body and the only member of this enzyme family that is unable to deiminate proteins [5]. Despite the lack of catalytic activity, PAD6 expression correlates to the level of citrullination in female germ cells and appears crucial in early embryos. Mouse studies showed that PAD6 can activate PAD1 and regulates development in early embryos by controlling PAD1-induced citrullination [42]. Consequently, PAD6 deficiency, like PAD1 deficiency, prevents embryos to grow beyond the two-cell stage and severely affects female fertility. In addition, PAD6 is suggested to stabilize oocyte cytoplasmic lattices in immature zygotes, which are ribosomal complexes that are necessary for protein synthesis. Without PAD6, developing embryos experience disrupted *de novo* protein synthesis and go into complete cell arrest [43].

Disease clustering based on shared citrullination pathways

(Hyper)citrullination is commonly observed in disease and featured as abnormal physiology. PAD as such does not cause pathology. Pathological effects only occur upon **aberrant PAD activity**. However, physiological substrates are more susceptible to drive disease upon excessive deimination. For this reason, we believe overlapping patterns can be found between different diseases that follow similar citrullination pathways or involve the same (type of) PAD substrate. In physiology, PAD enzymes predominantly modify cytoskeletal and intracellular proteins [4,8,26]. Thus, hypercitrullination tends to induce structural defects and altered gene expression due to dysregulated histone citrullination results in biological changes. A spectrum of diseases fall into these categories and we clustered those accordingly (Table 2). A distinct feature of hypercitrullination is the generation of autoantigens; a hallmark of RA and typically, though not exclusively, observed in rheumatic diseases [44]. We speculate that citrullinated

neo-epitopes are by-products generated by overactive intracellular, but also extracellular PAD enzymes [19-21] (Figure 1). Although the role of physiological extracellular PAD activity remains unclear, we believe that this pathogenic effect, in analogy with other disease clusters, results from exploited citrullination pathways (Figure 1, dashed arrows). In the following paragraph, we will elaborate on each disease cluster.

Extracellular citrullination potentiates the generation of autoantigens

The discovery of spontaneous PAD2 release and PAD4 cell surface expression by healthy neutrophils supports our hypothesis that extracellular citrullination is not a disease-specific process [18]. That said, uncontrolled extracellular citrullination is a detrimental feature in rheumatic diseases, especially in RA. Autoimmune responses in RA are generated towards citrullinated forms of vimentin, aggrecan, collagen, α-enolase, fibrinogen and histone H3 but also against PAD enzymes, including PAD1 and PAD6 [44-46]. In many ways, the joint synovium is a citrullination-prone environment. Firstly, synovial tissue is ideal to harbour PAD enzymes via local enzyme production by synovial fibroblasts and through constant recruitment of leukocytes during inflammation [47]. Secondly, in absence of inflammation the joint lacks oxidising molecules, which is optimal for PAD activity and could sustain physiological extracellular citrullination [48]. However, the need for reducing agents to keep PAD active in vitro would argue that PAD enzymes become inactive during inflammation due to ROS production. Instead, more citrullinated peptides and active PAD enzymes are reported during joint inflammation. *In vitro* studies suggest that consistent cell death upon inflammatory insult is responsible for supplying active PAD enzymes that leak out the cell [49]. However, additional research is required to confirm these findings *in vivo,* or to find other mechanisms that stabilize PAD activity during oxidative stress.

On a different note, smoking is an established environmental risk factor in RA and promotes extracellular citrullination in the lungs. Alveolar macrophages of smokers express more PAD2 and stimulate the production of citrullinated peptides in the lungs [21]. Moreover, smokers carry higher proinflammatory cytokine levels including TNFα, IL-1β and IL-6, leading to leukocyte recruitment and release of more citrullinating enzymes. Besides extracellular citrullination, smoking increases the susceptibility of developing RA by the generation of peptides presented on **HLA-DRB-1** (Box 2).

Periodontal infection is a well-documented risk factor in RA pathogenesis with potential implications towards loss of tolerance against citrullinated proteins. *Porphyromonas gingivalis* is the only bacterial species to express a peptidylarginine deiminases (PPAD) capable of deiminating proteins [45]. Although this enzyme does not function through host citrullination pathways, it contributes to citrullination burden in tissues. Literature stipulates that PPAD mainly modifies bacterial proteins [50]. These exogenous citrullinated peptides were suspected targets of **anti-cyclic citrullinated protein antibodies (ACPAs)** [50], although sera from early RA patients did react to autocitrullinated proteins from *P. gingivalis* [51]. Alternatively, prokaryotic citrullination may spread the citrulline epitope through **molecular mimicry** and drive the autoimmune reaction against endogenous deiminated proteins that were initially well tolerated [50]. The different substrate preferences of PPADs could explain why only a few host proteins [45,50] are confirmed as PPAD substrates and, except for histone H3 [52,53], were only demonstrated *in vitro.* For example, PPAD citrullinated CXCL8 much slower than human enzymes. Likewise, complement protein C5a undergoes different processing upon PPAD modification as this enzyme prefers C-terminal arginines as opposed to human PAD enzymes. Although the activity of C-terminally modified C5a is altered, it is not immunoreactive in RA patients [54]. Periodontitis associated *Aggregatibacter*

actinomycetemcomitans releases a bacterial toxin during infection that can activate human PADs [55]. This human PAD hyperactivation generates epitopes that are recognized by autoantibodies in RA and may be the missing link between periodontitis and RA.

Novel insights were made in Type 1 diabetes mellitus (T1DM) pathogenesis since the discovery of autoreactivity towards citrullinated GRP78. Although the original findings were made in NOD mice [56,57], patients with T1DM were confirmed more responsive to citrullinated GRP78 compared to healthy subjects. Furthermore, stimulating human pancreatic cells with proinflammatory cytokines relocates GRP-78 from the endoplasmic reticulum to the plasma membrane. This could expose GRP-78 to extracellular PAD enzymes that are released during the inflammatory response from dying cells. Indeed, pancreatic tissue of most T1DM patients are infiltrated with neutrophils that release NETs and stain positive for citrullinated histones thereby validating local PAD activity. Moreover, apoptotic β-cells might also induce citrullination [57]. Promising animal studies have shown that PAD-inhibitors can relieve the pancreas from inflammatory stress and even restore insulin production in diabetic mice [56]. Altogether, citrullination is a promising subject in T1D that could improve our understanding of PAD activity in the pancreas and its role in this debilitating disease.

Box 2: Intolerance against citrullinated peptides and ACPA serology

Although ACPAs are markers for RA [3], only a minority of patients are ACPA-positive in other diseases [94]. What drives the **loss of tolerance** towards citrullinated proteins in RA? Losing tolerance can result from genetic predisposition, environmental exposure or most likely, the combination thereof [44]. Genetically, **HLA-DRB1** polymorphisms are the foremost risk factors in RA pathogenesis. Patients with HLA-DRB1 alleles that code for the **shared epitope (SE)** are typically CCP-positive and predisposed to develop a more severe autoimmunity. The strong

link between the SE motif and enhanced reactivity towards citrullinated peptides was recently clarified on the molecular level. Crystallization studies showed that the preference of SE-positive HLA molecules for citrulline is dependent on the positive nature of the peptide binding pocket, which repulses arginine [95]. Moreover, genome-wide association studies have confirmed that smoking is an environmental risk factor that generate peptides that interact with HLA-DRB1 and smoking accelerates loss of tolerance in predisposed individuals. In fact, children exposed to cigarette smoke during childhood are more likely to develop RA later in life, suggesting passive smoking is sufficient to trigger adult-onset RA [96]. Besides HLA-DRB1, *PADI4* polymorphisms have also been identified as genetic risk factors through association studies. However, it is probably *PADI4* overexpression rather than specific **single nucleotide polymorphisms (SNPs)** that carry a risk for RA development as increased enzyme availability could trigger aberrant citrullination [97].

As for other diseases, ACPAs have been detected in Primary Sjögren Syndrome (PSS), Chronic Obstructive Pulmonary Disease (COPD), Systemic Lupus Erythematosus (SLE) and Mixed Connective Tissue Syndrome. However, except for PSS and in contrast to RA, only a small fraction of the respective patients is ACPA-positive (<20%) [94]. In most cases, serologically positive patients suffer from chronic inflammatory diseases and are likely in a preclinical stage of RA [94]. For instance, many CCP-positive COPD patients have a history of smoking, making them more susceptible to develop RA [98]. Therefore, ACPAs currently have little prognostic value and are considered clinically irrelevant outside RA. Nonetheless, the presence of ACPAs does provide evidence for potential generation of immunoreactive citrullinated epitopes and poses an immunological risk in the disease.

Structural defects caused by excessive and insufficient citrullination

In neurodegenerative diseases citrullination pathways that lead to flexibility during organism growth may in adulthood induce neural defects [8-10]. MS is a demyelinating disease characterized by neurodegeneration and autoimmunity. PAD enzymes could play a role in both by deiminating GFAP, vimentin and particularly MBP [58,59]. MS brains, especially those of progressed patients, have proportionally more citrullinated MBP compared to healthy individuals. Excessive deimination of MBP by PAD2 disturbs its ionic interaction with the lipid bilayer, causing impaired self-assembly of the myelin sheath [9]. The possibility exists that PAD enzymes are needed for remyelination between MS relapses as purposefully done during childhood [9]. However, loosely compacted myelin primarily weakens the CNS rigidity and becomes more prone to protease activity [60]. Proteolytic degradation of MBP generates immunodominant peptides to which autoreactive T and B cells in MS respond and the MBP fragments may persistently contribute to the humoral response [61]. Promising results were obtained in murine MS models using a PAD inhibitor, chloroacetamidine, which was able to suppress T-cell autoreactivity and restore myelination in treated mice [62]. Conflicting data question the relevance of T-cell driven autoreactivity against citrullinated MBP in humans. CD4+ T cells isolated from cerebrospinal fluid of MS patients have limited reactivity towards citrullinated MBP epitopes. Perhaps, PAD inhibitors reduce T-cell responses indirectly by decreasing their recruitment and activation. In mice, intracerebral administration of citrullinated myelin stimulated microglial production of the chemokine CCL5, a chemoattractant for T-cells, and TNF-α, an inflammatory cytokine that promotes T-cell differentiation [63]. Thus, PAD inhibitors could also reduce neuroinflammation by downregulating adaptive immune responses.

Citrullination studies in other neurological diseases are more limited compared to MS. However, AD, Parkinson's disease and retinal injury share the observation of elevated PAD

activity in affected tissues [7,64,65]. AD brains are burdened with increased levels of citrullinated GFAP and amyloid-β proteins [66]. Although there is speculation that citrullination of amyloid plaques facilitates degradation and clearance of these protein deposits [67], it is more plausible that the increase in PAD activity in the hippocampus is related to neuroinflammation and demyelination [10]. In rodent models of Parkinson's disease and retinal injury, the expression of PAD2 and PAD4 were established and revealed the presence of citrullinated histone H3 and citrullinated GFAP respectively [11]. To understand the true effects of citrullination in each disease more research is needed.

In contrast to neurodegenerative diseases, the lack of PAD activity underlies several hair-related diseases. A third of the women suffering from central centrifugal cicatricial alopecia carry a missense mutation in the *PADI3* gene that drastically lowers enzyme activity [68]. Similar mutations in *PADI3* were observed in children with uncombable hair disease and were later confirmed in knockout mice. Mutated *PadI3, transglutaminase and trichohyalin* caused a loss of function in these proteins and disrupted the formation of the hair shaft [69]. More specifically, unmodified trichohyalin failed to adhere keratin filaments because its conformational state was too compact to allow crosslinking with other proteins [25]. These findings underline that the lack of citrullination can cause severe pathological effects and highlight that protein deimination should not be regarded as a general disease marker.

Histone citrullination as a signature of (hyper)inflammation

Histone citrullination is widely used as a marker for NETosis, a form of neutrophil function that sparked much research interest and controversy (Box 3). Many publications attribute the role of PAD in disease to its ability to induce histone-citrullination triggered NETosis, although citrullination also modulates the inflammatory response in alternative ways. To avoid

semantic confusion, we define 'NETosis' as the programmed expulsion of extracellular DNA from the nucleus of neutrophils as part of cellular responses in infection and inflammation. It is important to realize alternative forms of cell death resemble NETosis, making it difficult to distinguish dying cells from netting cells [32]. Other cell types including macrophages, mast cells, basophils and eosinophils are also capable of expulsing DNA while producing citrullinated histones [70,31]. This is an argument not to use citrullinated histones as a single marker for NETosis (Box 3), as histone citrullination is not exclusive for neutrophils. Therefore, we discourage the use of extracellular DNA detection as single proof for intended neutrophil suicide and believe double staining for myeloperoxidase or neutrophil elastase and citrullinated histones is more reliable.

Although there are conflicting results amongst mice studies, PAD is able to induce citrullination of histones in response to LPS [71,72]. Furthermore, PAD activity can potentiate this inflammatory response by modulating cytokine levels. The absence of citrullination drastically reduced the expression of proinflammatory cytokine IL-1β [73]. It seems TLR4 can interact with histones to exacerbate inflammation during bacterial infection. Above all, PAD4 modulates **Nuclear factor-κB (NFκB)** signalling through citrullination of p65. Citrullination increases the affinity of p65 for importin, thus facilitating the translocation of NFκB to the nucleus [74]. Hence, pharmacological inhibition of PAD activity decreases TNF-α and IL-1β expression and reduces overall inflammation. Surprisingly, *in* vitro citrullination of TNF-α itself lowers the production of inflammatory chemokines while retaining its capacity to induce neutrophil chemotaxis and apoptosis [75]. Collectively, we conclude that PAD4 modulates the NFκB pathway in a substrate-dependent way to create initially a pro-inflammatory state. Subsequent citrullination of TNF and chemokines may create a negative feed-back loop to downregulate their activity and control the inflammation [75].

In mice, citrullinated histone H3 is released into circulation upon LPS-induced **endotoxic shock** [76,77]. Interestingly, citrullinated histone H3 is detectable as early as 30 minutes after LPS insult and remains in the blood stream up to 24h. Because this period is vital for treatment and currently no reliable biomarker is available, citrullinated histone H3 could be used for early detection of endotoxic shock. Citrullinated histone H3 serum levels could differentiate between sepsis patients with or without infection, further reinforcing its diagnostic value [20,77]. Moreover, recent mouse studies showed that neutralizing citrullinated histone H3 improved survival, suggesting it as a therapeutic target [78]. PAD2 deficient mice were observed with enhanced bacterial clearance and improved organ functions which coincided with the decrease formation of NETs suggesting that PAD2 may also regulate NET-formation [20]. The lack of PAD2 activity also improved survival in haemorrhagic shock, where the effects of PAD2 expression were assessed by inducing myocardial infarction in respective knockout mice [79]. Similar to endotoxin shock, also severe COVID19 patients contain higher levels of circulating citrullinated H3 compared to non-severe patients and healthy controls [80].

Neutrophils, which highly express PAD2 and PAD4, are key mediators of atherosclerosis and ischemia reperfusion injury [81-83]. In normal physiology, neutrophils act as first line of defence by migrating towards hypoxic and injured areas to clear leaked pathogens and activate platelets. However, in vascular disease proinflammatory chemokine ligands for CXCR1 and CXCR2 (the major chemokine receptors on neutrophils) recruit more, and hyperactivated, neutrophils to infiltrate the perfusion site. This triggers release of reactive oxygen species, proteolytic enzymes and PADs [84]. PAD4 in particular takes an active part in vascular damage by inducing histone citrullination and subsequent NET formation [85]. The resulting necrotic debris attracts more leukocytes and activates the complement cascade, thereby promoting inflammation and preventing resolution [86].

In neonatal hypoxic ischaemic injury, it is thought that neural damage is potentiated by the inflammatory insult and calcium dysregulation through PAD4 activity and reversed upon Cl-amidine treatment. Data obtained from murine hypoxic ischemia injury models suggest that LPS may trigger PAD4-induced histone citrullination while hypoxia would elevate local calcium levels and stimulate enzyme activity. As a result, hypoxic injured areas were characterized with deiminated proteins, apoptotic cells, activated microglia; all of which contributed to substantial neural loss [87].

Conclusively, citrullinated histones and citrullination in general clearly play a role in inflammatory diseases. Although we acknowledge the strong correlation to NET formation, we favour the use of citrullinated histones to indicate hyperinflammation rather than as a single marker for NETosis.

Box 3: Is PAD activity a prerequisite for inducing NETosis?

PAD4-induced histone citrullination is proposed to mediate a crucial step occurring in NETosis, namely decondensation of chromatin. Citrullination reduces the positive charge of histones, which causes them to lose their affinity for the negatively charged phosphate groups of DNA. This would, in turn, promote the extracellular release of chromatin fibres and fit the framework of NETosis. Although histone citrullination is strongly correlated to NET formation, not all types of NETosis completely rely on PAD activity. Moreover, bacteria may induce NETosis in a NADPH-dependent but PAD-independent manner [31]. Citrullination, being an irreversible modification, is strictly controlled by the local environment [48]. PAD enzymes are only active when molar concentrations of calcium increase and a reducing environment is present. However, NADPH-oxidase-dependent release of NETs and ROS production generate an oxidative environment, which directly inhibits PAD activity. As such, PAD-mediated NETosis

would only be feasible in the absence of NADPH-oxidase. However, increased citrullination of histones and other proteins under oxidative stress has been described [99]. It is possible that the suboptimal condition for citrullination is compensated by infiltrating leukocytes that abundantly release PAD enzymes or that their activity is protected by an alternative mechanism. Degranulating neutrophils use natural antioxidants i.e. ascorbate and glutathione to prevent oxidative damage to the cell [100]. A similar mechanism could shield PAD enzymes from oxidation *in vivo*. If so, *in vitro* NETosis assays could be flawed since not all assay buffers contain reducing agents such as DTT necessary to protect PAD against oxidation.

By acknowledging PAD4-induced NETosis cannot occur in the presence of ROS, PAD4 activity would only be relevant in NADPH-oxidase independent NETosis. But also here, citrullination is not essential as myeloperoxidase and neutrophil elastase can promote chromatin unfolding without PAD4 [32]. It is true though that PAD-mediated NETosis *in vitro* does not involve NADPH-oxidase activity. However, we have doubts regarding the biological representation of this assay since neutrophils are stimulated with calcium ionophores that trigger an extreme calcium release. The generated conditions do not truly reflect the *in vivo* process. The same critique is applicable for PMA stimulated NET formation as PMA is a carcinogenic toxin and does not resemble a microbial stimulus. Collectively, we postulate that PAD activity and histone citrullination are not required for NETosis but the concurrence of PAD activity with citrullinated histones could be an indication of (hyper)inflammation.

Concluding remarks and future perspectives

Citrullination is a biologically important posttranslational modification mediated by ubiquitously expressed PADs. Functional citrullination provides flexibility during embryonic and neural development, offers structural strength in epidermal and female tissues and is

critical during host defence and inflammation. Although PAD6 has a clear role in development, we far from understand the molecular mechanisms involved. PAD activity in male tissues, the GI-system and in several neurological diseases also need more detailed investigation (Outstanding questions). Aberrant citrullination can create (neo-)citrullinated epitopes generating autoimmunity, may induce conformational defects that impair tissue structures and enforce hyperinflammation. We postulate that PAD-mediated disease effects mainly result from the exploitation of physiological citrullination pathways and occur upon uncontrolled, mostly enhanced, enzyme activity. Considering PAD enzymes depend heavily on calcium availability and non-oxidative context, future research should characterize the microenvironment of citrulline-rich and -deprived tissues at the molecular level. To achieve this, assays that allow kinetic monitoring of PAD activity in tissues need to be developed. Additionally, we advocate for an alternative use of citrullinated histones, namely to indicate hyperinflammation, potentially coinciding with NETosis. Too many data argue against the prerequisite nature of PADs in NET formation. Furthermore, the similarity to cell death and DNA release by other immune cells complicates unambiguous identification of NETosis in tissues. Lastly, although functional proteins such as enzymes or chemokines can be PAD substrates e.g. ADAMTS13, MMP-9, CXCL8, they are underrepresented in citrullination studies. Obtaining in-depth knowledge on their functional changes will create a better understanding of citrullination in health and disease. Current insights into the role of PAD enzymes demonstrate that citrullination should not be considered a niche posttranslational modification, but rather as an important regulatory mechanism in health and disease.

Table 1: Peptidylarginine deiminase expression profiles and isoform-specific substrates

	Substrates		Tissue		Expression			
					protein	RNA	functional	species
PAD1	keratin 1	[4]	epidermis	[4]	x	x	x	human
	keratin 10	[4]	uterus	[5]	x	x	x	human
	profilaggrin	[4]	kidney	[5]	x	x		human
			proximal digestive tract	[5]	x	x		human
			testis	[5,101]	x	x		human
			bone marrow	[5]		x		human
			prostate	[101]		x		human
			placenta	[101]		x		human
			lung	[101]		x		human
			liver	[101]		x		human
			muscle	[101]		x		human
			spleen	[101]		x		human
			pancreas	[101]		x		human
			thymus	[101]		x		human
			colon	[101]		x		human
			inner ear	[102]		x		mouse
PAD2	MBP	[9]	blood	[17]	x	x	x	human
	vimentin	[59]	brain	[5]	x	x	x	human
	GFAP	[11]	bone marrow	[5]	x	x	x	human
	fibrinogen	[19]	breast	[5]	x	x	x	human
	histone h3	[12]	eye	[107]	x	x	x	human
	enolase 1	[103]	lung	[20,21]	x	x	x	human
	enolase 2	[103]	proximal digestive tract	[3,5]	x	x		human
	aldolase 1	[103]	gastro-intestinal tract	[5]	x	x	x	human
	aldolase 3	[103]	testis	[5]	x	x		human
	malate dehydrogenase 1	[103]	placenta	[3,5]	x	x		human
	voltage-dependent anion channel 1	[103]	rectum	[3,5]	x	x		human
	peroxiredoxin 1	[103]	colon	[3,16]	x	x		human
	cofilin 1	[103]	salivary gland	[3,5]	x	x		human
	peptidylprolyl isomerase a	[103]	gall bladder	[3]	x	x		human
	heat shock protein 8	[103]	spleen	[3,5]	x	x		human
	MMP-9	[104]	epidermis	[4]		x	x	human
	actin	[105]	pancreas	[56]	x	x	x	mouse
	GRP-78	[56]	spinal cord	[7]	x		x	mouse
	CXCL8*	[106]	pituitary gland	[102]		x		mouse
	CXCL10*	[35]	inner ear	[102]		x		mouse
	TNF-α	[75]	kidney	[102]		x		mouse
			olfactory tissue	[102]		x		mouse
			thymus	[102]		x		mouse
			neutrophils	[17,18,22]	x	x	x	human
			macrophages	[17,23,24]	x	x	x	human
			monocytes	[17,23]		x	x	human
PAD3	trichohyalin	[25]	hair follicles	[4]	x	x	x	human
	S100A3	[26]	neural stem cells	[29]	x	x	x	human
	filaggrin	[4]	urinary bladder	[5]		x		human
	apoptosis-inducing factor	[29]	muscle	[5]		x		human
	vimentin	[29]	proximal digestive tract	[5]		x		human
	tubulin	[28]	prostate	[5]		x		human
	histone H2	[28]	vagina	[5]		x		human
			epidermis	[5]	x	x	x	human
			thymus	[102]		x		mouse
			mammary gland	[28]		x	x	mouse
			neutrophils	[105]	x	x		human
PAD4	ADAMTS13	[40]	blood	[5,17]	x	x	x	human
	histone H3	[40]	lung	[21]	x	x	x	human
	ING4	[38]	bone marrow	[5]	x	x		human
	p300	[3]	spleen	[3,5]	x	x		human
	nucleophosmin	[91]	brain	[64]	x	x		human
	nuclear lamin c	[91]	fat	[3]	x			human
	GSK3β	[92]	vagina	[5]		x		human
	NF-κB-p65	[74]	placenta	[5]		x		human
	collagen type I	[34]	eye	[65]	x	x	x	mouse
	collagen type II	[108]	neutrophils	[5]	x	x	x	human
			monocytes	[23]	x	x	x	human
			macrophages	[23]	x		x	human
			eosinophils	[53]	x	x	x	human
PAD6	Not determined		blood	[17]		x		human
			muscle	[5]		x		human
			bone marrow	[5]		x		human
			testis	[109]		x		human
			spleen	[109]		x		human
			small intestine	[109]		x		human
			lung	[109]		x		human
			liver	[109]		x		human
			ovary	[42]	x	x	x	mouse
			thymus	[102]		x		mouse

*** purified from human cell cultures**

Table 2. Disease clusters amongst PAD-related pathologies

Disease	Effect citrullination	ACPA level[a]	PAD expression	Refs
Alzheimer's disease	Altered protein integrity	ND	Patient sample	[66]
Atherosclerosis	Altered protein integrity	ND	Animal model	[85]
Central centrifugal cicatricial alopecia	Altered protein integrity	ND	Patient sample	[68]
Congenital anonychia	Altered protein integrity	ND	Patient sample	[69]
Deep vein thrombosis	Altered protein integrity	ND	Animal model	[39]
Parkinson's disease	Altered protein integrity	ND	Patient sample	[67]
Glaucoma	Altered protein integrity	ND	Patient sample	[107]
Uncombable Hair Syndrome	Altered protein integrity	ND	Animal model	[69]
Multiple sclerosis	Altered protein integrity Generation of auto antigens	ND	Patient sample	[8]
Juvenile idiopathic arthritis	Generation of auto antigens	Low	Patient sample	[94]
Mixed connective tissue disease	Generation of auto antigens	Low	Patient sample	[94]
Periondontitis	Generation of auto antigens	Intermediate[b]	Patient sample	[50, 94]
Primary Sjören syndrome	Generation of auto antigens	Low	Patient sample	[94]
Psoriatic arthritis	Generation of auto antigens	Low	Patient sample	[94]
Spondyloarthritis	Generation of auto antigens	Low	Patient sample	[94]
St-segment elevation myocardial infarction	Generation of auto antigens	Low	Patient sample	[110]
Systemic lupus erythematous	Generation of auto antigens	Low	Patient sample	[94, 111]
Chronic obstructive pulmonary disease	Generation of auto antigens Hyperinflammation	Low	Patient sample	[98]
Type I diabetes mellitus	Generation of auto antigens Hyperinflammation	ND	Patient sample	[56]
Rheumatoid arthritis	Generation of auto antigens Hyperinflammation	High	Patient sample	[44]
Endotoxic shock	Hyperinflammation	ND	Patient sample	[77,78]
Haemorrhagic shock	Hyperinflammation	ND	Patient sample	[79]
Covid19	Hyperinflammation	ND	Patient sample	[80]
Juvenile dermatomyositis	Hyperinflammation	ND	Patient sample	[112]
Kidney ischemia reperfusion	Hyperinflammation	ND	Animal model	[82-84]
Hypoxic ischemia injury	Hyperinflammation	ND	Animal model	[87]
Pancreatitis	Hyperinflammation	ND	Patient sample	[113]
Sendai virus-induced asthma	Hyperinflammation	ND	Animal model	[114]
Cancer	Hyperinflammation	ND	Patient sample	[33,88,92]
Creutzfeldt–Jakob disease	Undetermined	ND	Animal model	[10]
Retinal gliosis	Undetermined	ND	Animal model	[115]

[a]*Abbreviation: ND, not determined.*
[b]*Intermediate in the specific subpopulation of ACPA-positive RA patients.*

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Declaration of interests

The authors declare no competing interests.

References

1. Vossenaar, E.R. et al. (2003) PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays 25 (11), 1106-18.

2. Verheul, M.K. et al. (2018) Pitfalls in the detection of citrullination and carbamylation. Autoimmun Rev 17 (2), 136-141.

3. Lee, C.Y. et al. (2018) Mining the Human Tissue Proteome for Protein Citrullination. Mol Cell Proteomics 17 (7), 1378-1391.

4. Nachat, R. et al. (2005) Peptidylarginine deiminase isoforms 1-3 are expressed in the epidermis and involved in the deimination of K1 and filaggrin. J Invest Dermatol 124 (2), 384-93.

5. Uhlén, M. et al. (2015) Proteomics. Tissue-based map of the human proteome. Science 347 (6220), 1260419.

6. Mondal, S. et al. (2019) Halogen Bonding Increases the Potency and Isozyme Selectivity of Protein Arginine Deiminase 1 Inhibitors. Angew Chem Int Ed Engl. 58(36), 12476-12480.

7. Falcão, A.M. et al. (2019) PAD2-Mediated Citrullination Contributes to Efficient Oligodendrocyte Differentiation and Myelination. Cell Rep 27 (4), 1090-1102.e10.

8. Moscarello, M.A. et al. (2007) The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis. Neurochem Res 32 (2), 251-6.

9. Moscarello, M.A. et al. (1994) Myelin in multiple sclerosis is developmentally immature. J Clin Invest 94 (1), 146-54.

10. Jang, B. et al. (2018) Myelin Basic Protein Citrullination, a Hallmark of Central Nervous System Demyelination, Assessed by Novel Monoclonal Antibodies in Prion Diseases. Mol Neurobiol 55 (4), 3172-3184.

11. Petrozziello, T. et al. (2020) Neuroinflammation and histone H3 citrullination are increased in X-linked Dystonia Parkinsonism post-mortem prefrontal cortex. Neurobiol Dis, 144, 105032.

12. Zhang, X. et al. (2012) Peptidylarginine deiminase 2-catalyzed histone H3 arginine 26 citrullination facilitates estrogen receptor α target gene activation. Proc Natl Acad Sci U S A 109 (33), 13331-6.

13. Zheng, L. et al. (2019) Calcium Regulates the Nuclear Localization of Protein Arginine Deiminase 2. Biochemistry 58(27), 3042-3056.

14. Li, F. et al. (2019) Inhibiting PAD2 enhances the anti-tumor effect of docetaxel in tamoxifen-resistant breast cancer cells. J Exp Clin Cancer Res 414 (38).

15. Makrygiannakis, D. et al. (2006) Citrullination is an inflammation-dependent process. Ann Rheum Dis 65 (9), 1219-22.

16. Chumanevich, A.A. et al. (2011) Suppression of colitis in mice by Cl-amidine: a novel peptidylarginine deiminase inhibitor. Am J Physiol Gastrointest Liver Physiol 300 (6), G929-38.

17. Uhlen, M. et al. (2019) A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. Science 366 (6472).

18. Zhou, Y. et al. (2017) Spontaneous Secretion of the Citrullination Enzyme PAD2 and Cell Surface Exposure of PAD4 by Neutrophils. Front Immunol (8) 1200.

19. Damgaard, D. et al. (2014) Demonstration of extracellular peptidylarginine deiminase (PAD) activity in synovial fluid of patients with rheumatoid arthritis using a novel assay for citrullination of fibrinogen. Arthritis Res Ther 16 (6), 498.

20. Tian, Y. et al. (2020) Peptidylarginine deiminase 2 has potential as both a biomarker and therapeutic target of sepsis. JCI Insight 5 (20).

21. Damgaard, D. et al. (2015) Smoking is associated with increased levels of extracellular peptidylarginine deiminase 2 (PAD2) in the lungs. Clin Exp Rheumatol 33 (3), 405-8.

22. Spengler, J. et al. (2015) Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. Arthritis Rheumatol 67 (12), 3135-45.

23. Vossenaar, E.R. et al. (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Ann Rheum Dis 63 (4), 373-81.

24. Yu, H.C. et al. (2020) The Essential Role of Peptidylarginine Deiminases 2 for Cytokines Secretion, Apoptosis, and Cell Adhesion in Macrophage. Int J Mol Sci 21 (16), 5720.

25. Steinert, P.M. et al. (2003) Trichohyalin mechanically strengthens the hair follicle: multiple cross-bridging roles in the inner root sheath. J Biol Chem 278 (42), 41409-19.

26. Kizawa, K. et al. (2008) Specific citrullination causes assembly of a globular S100A3 homotetramer: a putative Ca2+ modulator matures human hair cuticle. J Biol Chem 283 (8), 5004-13.

27. Ite, K. et al. (2020) Optimal Mutant Model of Human S100A3 Protein Citrullinated at Arg51 by Peptidylarginine Deiminase Type III and Its Solution Structural Properties. ACS Omega 5 (8), 4032-4042.

28. Li, G. et al. (2020) Identification and Characterization of the Lactating Mouse Mammary Gland Citrullinome. Int J Mol Sci 21 (7), 2634.

29. U, K.P. et al. (2014) Modulation of calcium-induced cell death in human neural stem cells by the novel peptidylarginine deiminase-AIF pathway. Biochim Biophys Acta 1843 (6), 1162-71.

30. Delettre, C. et al. (2006) AIFsh, a novel apoptosis-inducing factor (AIF) pro-apoptotic isoform with potential pathological relevance in human cancer. J Biol Chem 281(10), 6413-27.

31. Sørensen, O.E. and Borregaard, N. (2016) Neutrophil extracellular traps - the dark side of neutrophils. J Clin Invest 126(5), 1612-20.

32. Konig, M.F. and Andrade F. (2016) A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. Front Immunol (7), 461.

33. Yuzhalin, A.E. et al. (2018) Colorectal cancer liver metastatic growth depends on PAD4-driven citrullination of the extracellular matrix. Nat Commun 9 (1), 4783.

34. Martinod, K. et al. (2017) Peptidylarginine deiminase 4 promotes age-related organ fibrosis. J Exp Med 214 (2), 439-458.

35. Loos, T. et al. (2008) Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. Blood 112 (7), 2648-56.

36. Wang, Y. et al. (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. Science 306 (5694), 279-83.

37. Kearney, P.L. et al. (2005) Kinetic characterization of protein arginine deiminase 4: a transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. Biochemistry 44 (31), 10570-82.

38. Guo, Q. and Fast, W. (2011) Citrullination of inhibitor of growth 4 (ING4) by peptidylarginine deminase 4 (PAD4) disrupts the interaction between ING4 and p53. J Biol Chem 286 (19), 17069-78.

39. Martinod, K. et al. (2013) Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. Proc Natl Acad Sci U S A 110 (21), 8674-9.

40. Sorvillo, N. et al. (2019) Plasma Peptidylarginine Deiminase IV Promotes VWF-Platelet String Formation and Accelerates Thrombosis After Vessel Injury. Circ Res 125 (5), 507-519.

41. Wong, S.L. et al. (2020) Recombinant human ADAMTS13 treatment and anti-NET strategies enhance skin allograft survival in mice. Am J Transplant 20 (4), 1162-1169.

42. Esposito, G. et al. (2007) Peptidylarginine deiminase (PAD) 6 is essential for oocyte cytoskeletal sheet formation and female fertility. Mol Cell Endocrinol 273 (1-2), 25-31.

43. Yurttas, P. et al. (2008) Role for PADI6 and the cytoplasmic lattices in ribosomal storage in oocytes and translational control in the early mouse embryo. Development 135 (15), 2627-2636.

44. Curran, A.M. et al. (2020) PAD enzymes in rheumatoid arthritis: pathogenic effectors and autoimmune targets. Nat Rev Rheumatol 16 (6), 301-315.

45. Wegner, N. et al. (2010) Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. Arthritis Rheum 62 (9), 2662-72.

46. Martinez-Prat, L. et al. (2020) Decephering the anti-protein-arginine deiminase (PAD) response identifies PAD1 and PAD6 as novel autoantigens in Rheumatoid Arthritis. Ann Rheum Dis (79), 78-79.

47. Sipilä, K.H. et al. (2017) Joint inflammation related citrullination of functional arginines in extracellular proteins. Sci Rep7(1), 8246.

48. Damgaard, D. et al. (2016) Reduced glutathione as a physiological co-activator in the activation of peptidylarginine deiminase. Arthritis Res Ther 18(1), 102.

49. Kinloch, A. et al. (2008) Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis. Arthritis Rheum 58(8), 2287-95.

50. Quirke, A.M. et al. (2014) Heightened immune response to autocitrullinated Porphyromonas gingivalis peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. Ann Rheum Dis 73 (1), 263-9.

51. Muñoz-Atienza, E. et al. (2020) The P. gingivalis Autocitrullinome Is Not a Target for ACPA in Early Rheumatoid Arthritis. J Dent Res. 99(4), 456-462.

52. Gómez-Bañuelos, E. et al. (2019) Rheumatoid Arthritis-Associated Mechanisms of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans. J Clin Med 8 (9), 1309.

53. Moelants, E.A. et al. (2014) Citrullination and proteolytic processing of chemokines by Porphyromonas gingivalis. Infect Immun 82 (6), 2511-9.

54. Bielecka, E. et al. (2014) Peptidyl arginine deiminase from Porphyromonas gingivalis abolishes anaphylatoxin C5a activity. J Biol Chem 289 (47), 32481-7.

55. Konig MF et al. (2016) Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Sci Transl Med 8(369):369ra176.

56. Sodré, F.M.C. et al. (2021) Peptidylarginine Deiminase Inhibition Prevents Diabetes Development in NOD Mice. Diabetes 70 (2), 516-528.

57. Yang, M.L. et al. (2021) Citrullination and PAD Enzyme Biology in Type 1 Diabetes - Regulators of Inflammation, Autoimmunity, and Pathology. Front Immunol (12) 678953.

58. Nicholas, A.P. et al. (2004) Increased citrullinated glial fibrillary acidic protein in secondary progressive multiple sclerosis. J Comp Neurol 473 (1), 128-36.

59. Jang, B. et al. (2020) Vimentin citrullination probed by a novel monoclonal antibody serves as a specific indicator for reactive astrocytes in neurodegeneration. Neuropathol Appl Neurobiol 46(7), 751-769.

60. Musse, A.A. et al. (2006) Deimination of membrane-bound myelin basic protein in multiple sclerosis exposes an immunodominant epitope. Proc Natl Acad Sci U S A 103 (12), 4422-7.

61. Deraos, G. et al. (2008) Citrullination of linear and cyclic altered peptide ligands from myelin basic protein (MBP(87-99)) epitope elicits a Th1 polarized response by T cells isolated from multiple sclerosis patients: implications in triggering disease. J Med Chem 51 (24), 7834-42.

62. Moscarello, M.A. et al. (2013) Inhibition of peptidyl-arginine deiminases reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. Dis Model Mech 6(2), 467-78.

63. Standiford, M.M. et al. (2021) Citrullinated myelin induces microglial TNFα and inhibits endogenous repair in the cuprizone model of demyelination. J Neuroinflammation 305 (18).

64. Mastronardi, F.G. et al. (2006) Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. J Neurosci 26 (44), 11387-96.

65. Wizeman, J.W. and Mohan, R. (2017) Expression of peptidylarginine deiminase 4 in an alkali injury model of retinal gliosis. Biochem Biophys Res Commun 487 (1), 134-139.

66. Mukherjee, S. et al. (2021) Citrullination of Amyloid-β Peptides in Alzheimer's Disease. ACS Chem Neurosci 12(19), 3719-3732.

67. Nicholas, A.P. (2013) Dual immunofluorescence study of citrullinated proteins in Alzheimer diseased frontal cortex. Neurosci Lett 545, 107-11.

68. Malki, L. et al. (2019) Variant PADI3 in central centrifugal cicatricial alopecia. N Engl J Med 380 (9), 833-841.

69. Hsu, C.K. et al. (2017) Congenital Anonychia and Uncombable Hair Syndrome: Coinheritance of Homozygous Mutations in RSPO4 and PADI3. J Invest Dermatol 137 (5), 1176-1179.

70. Kim, H.J. et al. (2020) Lysophosphatidylserine induces eosinophil extracellular trap formation and degranulation: Implications in severe asthma. Allergy 75 (12), 3159-3170.

71. Li, P. et al. (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med 207 (9), 1853-62.

72. Claushuis, T.A.M. et al. (2018) Role of Peptidylarginine Deiminase 4 in Neutrophil Extracellular Trap Formation and Host Defense during. J Immunol 201 (4), 1241-1252.

73. Tsourouktsoglou, T.D. et al. (2020) Histones, DNA, and Citrullination Promote Neutrophil Extracellular Trap Inflammation by Regulating the Localization and Activation of TLR4. Cell Rep 31 (5), 107602.

74. Sun, B. et al. (2017) Citrullination of NF-κB p65 promotes its nuclear localization and TLR-induced expression of IL-1β and TNFα. Sci Immunol 2 (12), eaal3062.

75. Moelants, E.A. et al. (2013) Citrullination of TNF-α by peptidylarginine deiminases reduces its capacity to stimulate the production of inflammatory chemokines. Cytokine 61 (1), 161-7.

76. Li, Y. et al. (2011) Identification of citrullinated histone H3 as a potential serum protein biomarker in a lethal model of lipopolysaccharide-induced shock. Surgery 150 (3), 442-51.

77. Tian, Y. et al. (2021) Serum citrullinated histone H3 concentrations differentiate patients with septic verses non-septic shock and correlate with disease severity. Infection 49 (1), 83-93.

78. Deng, Q. et al. (2019) Citrullinated Histone H3 as a Therapeutic Target for Endotoxic Shock in Mice. Front Immunol (10), 2957.

79. Zhou, J. et al. (2020) Peptidylarginine Deiminase 2 Knockout Improves Survival in hemorrhagic shock. Shock 54(4), 458-463.

80. Traby, L. et al. (2021)Extracellular Vesicles and Citrullinated Histone H3 in Coronavirus Disease 2019 Patients. Thromb Haemost 122(1), 113-122.

81. Savchenko, A.S. et al. (2014) VWF-mediated leukocyte recruitment with chromatin decondensation by PAD4 increases myocardial ischemia/reperfusion injury in mice. Blood 123 (1), 141-8.

82. Raup-Konsavage, W.M. et al. (2018) Neutrophil peptidyl arginine deiminase-4 has a pivotal role in ischemia/reperfusion-induced acute kidney injury. Kidney Int 93 (2), 365-374.

83. Rabadi, M.M. et al. (2019) Peptidyl arginine deiminase-4 exacerbates ischemic AKI by finding NEMO. Am J Physiol Renal Physiol 316 (6), F1180-F1190.

84. Ham, A. et al. (2014) Peptidyl arginine deiminase-4 activation exacerbates kidney ischemia-reperfusion injury. Am J Physiol Renal Physiol 307 (9), F1052-62.

85. Knight, J.S. et al. (2014) Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. Circ Res 114 (6), 947-56.

86. Westman, J. et al. (2019) Phagocytosis of Necrotic Debris at Sites of Injury and Inflammation. Front Immunol (10), 3030.

87. Lange, S. et al. (2014) Peptidylarginine deiminases: novel drug targets for prevention of neuronal damage following hypoxic ischemic insult (HI) in neonates. J Neurochem 130(4), 555-62.

88. Yuzhalin, A.E. (2019) Citrullination in Cancer. Cancer Res 79(7), 1274-1284.

89. Shi, L. et al. (2020) Endogenous PAD4 in Breast Cancer Cells Mediates Cancer Extracellular Chromatin Network Formation and Promotes Lung Metastasis. Mol Cancer Res 18 (5), 735-747.

90. Yao, H. et al. (2008) Histone Arg modifications and p53 regulate the expression of OKL38, a mediator of apoptosis. J Biol Chem 283 (29), 20060-8.

91. Tanikawa, C. et al. (2009) Regulation of protein Citrullination through p53/PADI4 network in DNA damage response. Cancer Res 69 (22), 8761-9.

92. Zhou, Q. et al. (2019) Peptidylarginine deiminase 4 overexpression resensitizes MCF-7/ADR breast cancer cells to adriamycin via GSK3β/p53 activation. Cancer Manag Res 11, 625-636.

93. Cook, K. et al (2017) Citrullinated α-enolase is an effective target for anti-cancer immunity. Oncoimmunology. 7(2), e1390642.

94. Payet, J. et al. (2014) Anticyclic citrullinated peptide antibodies in rheumatoid and nonrheumatoid rheumatic disorders: experience with 1162 patients. J Rheumatol 41 (12), 2395-402.

95. Ting, Y.T. et al. (2018) The interplay between citrullination and HLA-DRB1 polymorphism in shaping peptide binding hierarchies in rheumatoid arthritis. J Biol Chem 293(9), 3236-3251.

96. Yoshida, K. et al. (2019) Passive Smoking Throughout the Life Course and the Risk of Incident Rheumatoid Arthritis in Adulthood Among Women. Arthritis Rheumatol 73(12), 2219-2228.

97. Ikari, K. et al. (2005) Association between PADI4 and rheumatoid arthritis: a replication study. Arthritis Rheum 52 (10), 3054-7.

98. Ruiz-Esquide, V. et al. (2012) Anti-citrullinated peptide antibodies in the serum of heavy smokers without rheumatoid arthritis. A differential effect of chronic obstructive pulmonary disease? Clin Rheumatol 31 (7), 1047-50.

99. Inoue, M. et al. (2018) Plasma redox imbalance caused by albumin oxidation promotes lung-predominant NETosis and pulmonary cancer metastasis. Nat Commun 5116 (9).

100. Bozonet, S.M. et al. (2019) The Role of Physiological Vitamin C Concentrations on Key Functions of Neutrophils Isolated from Healthy Individuals. Nutrients 11(6), 1363.

101. Guerrin, M. et al. (2003) cDNA cloning, gene organization and expression analysis of human peptidylarginine deiminase type I. Biochem J 370 (Pt 1), 167-74.

102. Balandraud, N. et al. (2005) A rigorous method for multigenic families' functional annotation: the peptidyl arginine deiminase (PADs) proteins family example. BMC Genomics 6, 153.

103. Jang, B. et al. (2008) Accumulation of citrullinated proteins by up-regulated peptidylarginine deiminase 2 in brains of scrapie-infected mice: a possible role in pathogenesis. Am J Pathol 173 (4), 1129-42.

104. Boon, L. et al. (2021) Citrullination as a novel posttranslational modification of matrix metalloproteinases. Matrix Biol (95), 68-83.

105. Darrah, E. et al. (2012) Peptidylarginine deiminase 2, 3 and 4 have distinct specificities against cellular substrates: novel insights into autoantigen selection in rheumatoid arthritis. Ann Rheum Dis 71 (1), 92-8.

106. Proost, P. et al. (2008) Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. J Exp Med 205 (9), 2085-97.

107. Bhattacharya, S.K. et al. (2006) Proteomics implicates peptidyl arginine deiminase 2 and optic nerve citrullination in glaucoma pathogenesis. Invest Ophthalmol Vis Sci 47 (6), 2508-14.

108. Yoshida, M. et al. (2006) Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. Mod Rheumatol 16 (5), 276-81.

109. Chavanas, S. et al. (2004) Comparative analysis of the mouse and human peptidylarginine deiminase gene clusters reveals highly conserved non-coding segments and a new human gene, PADI6. Gene 330, 19-27.

110. Hermans, M.P.J. et al. (2017) Long-term mortality in patients with ST-segment elevation myocardial infarction is associated with anti-citrullinated protein antibodies. Int J Cardiol 240, 20-24.

111. Lande, R. et al. (2020) Native/citrullinated LL37-specific T-cells help autoantibody production in Systemic Lupus Erythematosus. Scientific Reports 10 (1), 5851.

112. Duvvuri, B. et al. (2020) Neutrophil Extracellular Traps in Tissue and Periphery in Juvenile Dermatomyositis. Arthritis Rheumatol 72 (2), 348-358.

113. Madhi, R. et al. (2019) Targeting peptidylarginine deiminase reduces neutrophil extracellular trap formation and tissue injury in severe acute pancreatitis. J Cell Physiol 234 (7), 11850-11860.

114. Akk, A. et al. (2016) Neutrophil Extracellular Traps Enhance Early Inflammatory Response in Sendai Virus-Induced Asthma Phenotype. Frontiers in Immunology 7 (325).

115. Wizeman, J.W. et al. (2016) Citrullination of glial intermediate filaments is an early response in retinal injury. Mol Vis 22, 1137-1155.

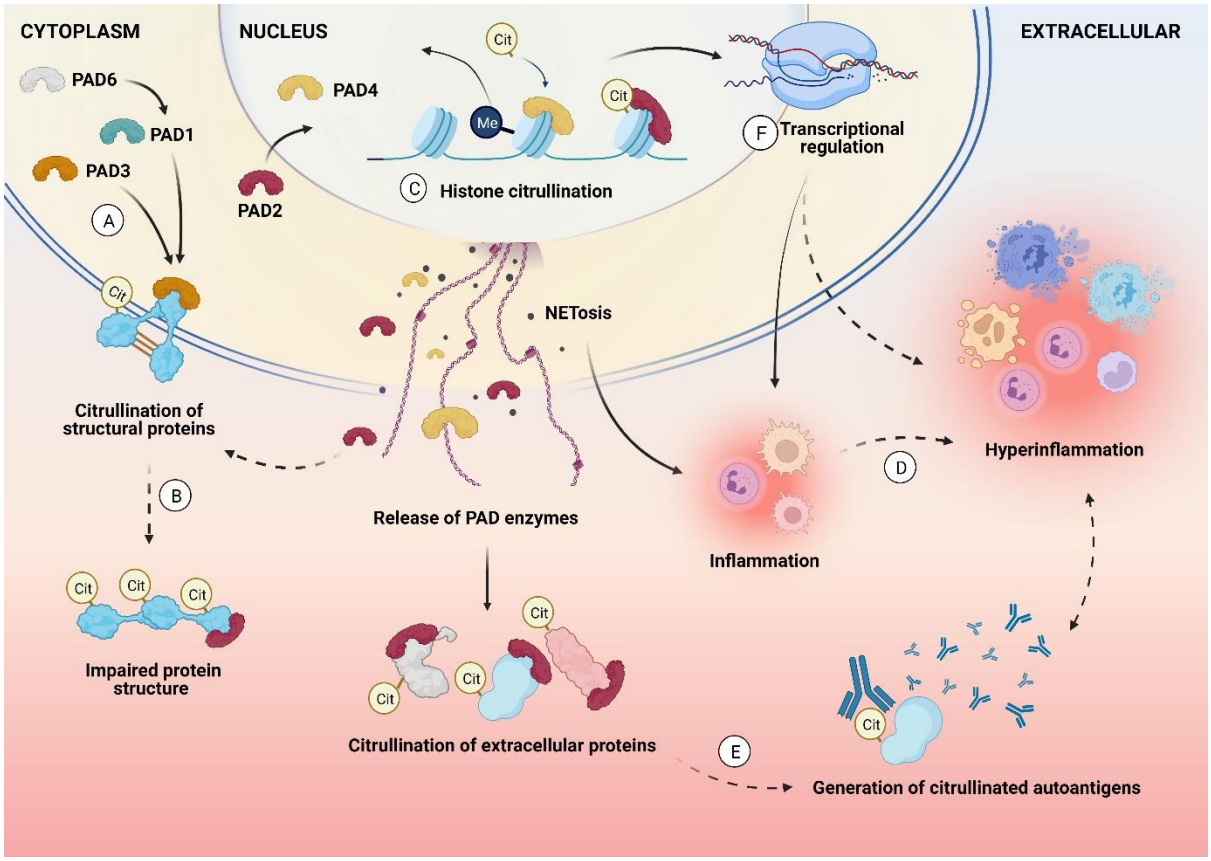

Figure 1. Citrullination pathways in health and disease.

Cytoplasmic PAD enzymes mainly focus on the citrullination of structural proteins to maintain the cell architecture. Upon citrullination and loss of positive charges structural proteins may undergo conformational changes resulting in unfolding or polymerisation, which provide flexibility and strength to the cytoskeleton, respectively **(A).** In disease however, aberrant citrullination by any of the PAD enzymes can modify structural proteins to lose their protein integrity and compromise cell architecture **(B).** Nuclear expression of PAD4 and translocation of PAD2 to the nucleus allow both enzymes to mediate histone citrullination **(C)**, which can trigger neutrophil extracellular trap(NET)-osis in leukocytes and elicit an inflammatory response as part of cellular host defense, but excessive PAD activity leads to hyperinflammation **(D)**. Due to cell lysis, more PAD enzymes are released and gain access to extracellular substrates**.** In rheumatoid arthritis, extracellular citrullination by PAD2 could contribute to the formation of neo-epitopes for autoreactive T and B cells and fuel the (auto-

)immune response **(E)**. Meanwhile, PAD4 has epigenetic effects on gene expression by histone citrullination, demethylimination or direct modification of transcription factors. NF-κB-signalling in particular is heavily influenced by PAD activity, which in turn, has a significant impact on the inflammatory response **(F)**. Uninterrupted arrows represent citrullination pathways in health; dashed arrows represent citrullination in disease.

Glossary

Aberrant citrullination: Uncontrolled citrullination when PAD enzymes are too active and modify too many arginine residues or citrullinate substrates that should remain unmodified.

Anti-cyclic citrullinated protein antibodies (ACPAs): Antibodies that recognise citrullinated proteins. ACPAs are typically generated during the autoimmune response observed in patients with Rheumatoid Arthritis. Therefore, ACPAs are currently used for the diagnosis of Rheumatoid Arthritis.

Chemokines: Chemotactic cytokines that mediate the directional recruitment of immune cells through G protein-coupled receptors.

Cl-amidine: A chemical inhibitor of PAD activity.

Deimination: The chemical reaction that converts a citrulline to an arginine amino acid

Epitope spreading: The development of immune responses against epitopes that differ from the epitope that was recognized by T cell receptors or antibodies at the onset of a specific immune reaction. These new epitopes may be present on endogenous proteins instead of the disease-causing epitope and therefore sustains autoinflammation.

G1/S checkpoint: In the cell cycle, cells grow in the G1 phase and transition to the S-phase, during which DNA is replicated. P53 controls this transition phase and interrupts cell cycles that contain damaged DNA.

Lipopolysaccharide (LPS): Gram-negative bacteria express large molecules that consist of a lipid and a sugar chain and are considered endotoxins.

Molecular mimicry: Foreign antigens that resemble self-antigens in their structure or sequence, making them more prone to break self-tolerance.

NETosis: A form of cellular defence where neutrophils expulse DNA to the extracellular environment to stimulate microbial clearance.

Nuclear factor-κ B (NF-κB): NF-κB is a complex of transcription factors that mediate the expression of proinflammatory genes such as cytokines and chemokines.

Peptidylarginine/Peptidylcitrulline: To distinguish between free amino acids and protein-bound amino acids, 'peptidyl' precedes the specific amino acid to indicate it is incorporated in a protein.

Polymerization: Single molecules or proteins that combine to form a complex with multiple entities.

Posttranslational modification: Translated proteins can be modified chemically or enzymatically in a posttranslational modification that may change their biological function.

Saltatory conduction: The propagation of action potentials along myelinated axons.

Single Nucleotide Polymorphisms (SNPs): A genetic polymorphism that only affects a single nucleotide in the DNA sequence.

Thrombosis: Blood clotting process.

Toll-like receptor (TLR): A class of receptors that are expressed by macrophages and dendritic cells that recognize microbial molecules such as LPS.

Highlights

- Although PAD enzymes have high sequence homology, each enzyme isoform has its own features, including unique functions and specific tissue expression.
- PADs are expressed throughout the body but have a preference for structural proteins and nuclear substrates such as histones. Therefore, the latest insights reveal how PAD activity affects cytoskeletal organization and gene expression.
- Evidence for extracellular citrullination is emerging in physiology and pathology
- Recent insights in periodontitis show that exogenous proteins citrullinated by *P. gingivalis* do not generate autoreactivity and dispute the role of *P. gingivalis* in loss of tolerance.
- Aberrant citrullinated proteins disturb normal physiological processes in many inflammatory disorders and cancer outside rheumatoid arthritis or multiple sclerosis.
- Citrullinated histones in plasma are markers for bacterial and viral sepsis.

Outstanding Questions Box

- Does PAD activity result in anti-inflammatory effects through chemokine citrullination?
- Which form of NETosis is highly correlated with PAD activity?
- Is protein citrullination the initial trigger towards rheumatoid arthritis?
- What is the role of extracellular PAD?
- What are substrates of PAD6?
- What is the effect of citrullination of receptor dependent proteins?
- How are PAD2 and PAD4 transported to the nucleus?
- What is the role of PAD in the gastrointestinal system?
- Are PAD enzymes actively released in the extracellular milieu?