The Role of Citrullination in Inflammatory Bowel Disease: A Neglected Player in Triggering Inflammation and Fibrosis?

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Citrullination is a posttranslational modification of proteins mediated by a specific family of enzymes called peptidylarginine deiminases (PAD). Dysregulation of these enzymes is involved in the etiology of various diseases, from cancer to autoimmune disorders.

In inflammatory bowel disease (IBD), data for a role of citrullination in the disease process are starting to accumulate at different experimental levels including gene expression analyses, RNA, and protein quantifications. Most data have been generated in ulcerative colitis, but data in Crohn disease are lacking so far.

In addition, the citrullination of histones is the fundamental process promoting inflammation through the formation of neutrophil extracellular traps (NETs). Interestingly, NETs have also been shown to activate fibroblasts into myofibroblasts in fibrotic interstitial lung disease. Therefore, citrullination merits more thorough study in the bowel to determine its role in driving disease complications such as fibrosis.

In this review we describe the process of citrullination and the different players in this pathway, the role of citrullination in autoimmunity with a special focus on IBD, the emerging role for citrullination and NETs in triggering fibrosis, and, finally, how this process could be therapeutically targeted.

Key Words: citrullination, inflammatory bowel disease, neutrophil extracellular traps

INTRODUCTION

Posttranslational modifications (PTMs) are enzymatic reactions regulating the function and the folding of proteins by converting the substrate in new active groups. These processes result in the formation of new epitopes, potentially acting as neoantigens that are able to alter the immune system

homeostasis.¹ Among others, the role of citrullination in activating inflammation and immune response is only partially understood.

Citrullination is a PTM that consists of the deimination, or demethylination, of positive arginine residues to produce neutral citrulline via specific calcium-binding enzymes called peptidylarginine deiminases (PAD, Fig. 1).² To date, it is believed that citrullination is an irreversible modification of proteins, because no decitrullinating enzymes have been discovered in human tissues.³

Five members belong to the PAD family (PAD1, PAD2, PAD3, PAD4, and PAD6), with PAD2 and PAD4 being the most widely expressed and studied.⁴ The unbalanced expression of these enzymes has been shown to be involved in the etiopathogenesis of various diseases, from cancer to many autoimmune and degenerative disorders (Table 1).

INFLAMMATION AND CITRULLINATION

At first, citrullination was considered a specific trigger for rheumatoid arthritis (RA) because intracellular citrullinated proteins were found in RA synovia but not in samples derived from other synovitides.⁵ The role of citrullination in RA was further confirmed by a significant expression of PAD2 and PAD4 in the monocytes and macrophages of the synovial fluid⁶ and the synovial membrane.⁷ In addition, this hypothesis was corroborated by in vivo models of induced arthritis where PAD4-knockout mice were showing reduced inflammatory cytokine production (such as anti-tumor necrosis factor- α and interleukin-6 [IL-6]) and less severe joint damage.^{9, 10} The impact of citrullination as

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FIGURE 1. Enzymatic reaction of citrullination. Citrullination of positive arginine residues to produce citrulline determines a change in protein folding. This process can result in the modification of protein interactions, with the formation of new epitopes.

TABLE 1. Tissue Expression of the Different PAD enzymes And their Dysregulation in Diseases.

	Enzyme Main distribution	Proven Biological Function	Disease Involvement
PAD1	Epidermis; hair follicles; uterus; cancer	Keratinization of epidermis ¹¹² ; epithelial-to-mesenchymal transition	Psoriasis ¹¹³ ; bullous congenital ichthyosiform erythroderma ¹¹⁴ ; breast cancer ¹¹⁵
PAD2	Brain; pituitary gland; immune cells; spleen; skeletal muscle; uterus; hepatic stellate cells and portal fibroblasts; kidney; inner ear; pancreas; cancer	Innate immune defense; CNS plasticity and homeostasis; transcription regulation; hormone signaling; female reproduction ¹¹⁶	Rheumatoid arthritis ^{6, 7} ; multiple sclerosis ^{17–19} ; Alzheimer disease ²⁰ ; prion disease ²⁴ ; UC ^{48, 117} ; RA-ILD and IPF ²⁵ ; breast cancer ¹¹⁸ ; prostate cancer ¹¹⁹
PAD3	Hair follicles; epidermis	Control of epidermal keratinization and hair growth	Loss of barrier function and epidermal homeostasis ¹²⁰ ; uncombable hair syndrome ¹²¹
PAD4	Cancer cells; neutrophils; NK cells; monocytes/macrophages; mammary gland epithelium; brain; bone marrow	NET formation; innate immune response; transcription regulation via chromatin decondensation and demethylation ^{122, 123} ; tumor aggressive behavior ^{89, 90, 124}	Tumors ^{125–128} ; IBD ⁴⁸ ; RA ⁶ ; multiple sclerosis ¹²⁹ ; Alzheimer disease ²² ; thrombosis and atherosclerosis ¹³⁰ ; RA-ILD and IPF ^{25, 31}
PAD6	Early embryo; ovaries and oocytes; testicles	Female reproduction and oocytes formation ^{131, 132} ; embryo development (mouse)	Infertility ^{133, 134}

CNS indicates central nervous system; IPF, idiopathic pulmonary fibrosis; NK, natural killer; RA-ILD, rheumatoid arthritis–associated interstitial lung disease.

a PTM in the pathogenesis of RA is so important that anti-citrullinated protein antibodies (ACPAs) are the most specific serological test for the disease today¹¹ and are part of the criteria set for diagnosis.¹² Interestingly, recent evidence has linked (oral) microbial dysbiosis and hypercitrullination in RA: The predominant pathogen of periodontal disease, *Porphyromonas gingivalis*, expresses a high level of PAD enzymes that lead to the generation of citrullinated peptides as ACPAs and thus the activation of the immune system in individuals genetically predisposed for RA.¹³

Some studies have shown an increased presence of citrullinated peptides in the synovium of other arthritides,^{14, 15} and immunohistochemical analysis of various tissues has shown a higher level of citrullination in several other inflammatory contexts (polymyositis, chronic tonsillitis, and inflammatory bowel diseases [IBD]).¹⁶ In our experience with Crohn disease (CD), we did not observe a clear-cut difference of citrullination levels between normal and inflamed tissue from the same patients, but rather variations in the density and nature of the positive staining inflammatory cells (Fig. 2).

FIGURE 2. Citrulline expression in CD. Comparison of citrullinated peptide expression via immunohistochemistry (F95 anti-peptidyl-citrulline, Merck, Germany) between normal (A, B) and inflamed (C, D) ileum of a patient with CD. A, B, Detail of the deeper mucosa layer of a proximal healthy margin after resection for stricturing CD; some intraepithelial and lamina propria lymphocytes show a clear positive nuclear staining (magnification 400x in A, 630x in B). C, D, Positive staining in the nucleus of many marginated neutrophils overlying an ulcer base (magnification 400x in C, 630x in D). Personal unpublished data.

Excessive citrullination of myelin basic protein by PAD2-expressing oligodendrocytes results in the formation of new epitopes along the axon sheaths, leading to autoimmune demyelination typical of multiple sclerosis.¹⁷⁻¹⁹ Alzheimer disease is another neurodegenerative disorder that has been linked to excessive citrullination and consequent production of auto-antibodies, in this case through increased activity of PAD2 and PAD4 in the hippocampal cells.²⁰⁻²² Accordingly, increased citrullinated peptides have also been shown in the substantia nigra neurons of Parkinson disease patients²³ and in many damaged cerebral areas of patients with prion disease.²⁴

Citrullination is also upregulated in lung autoimmune diseases, such as RA-associated interstitial lung disease and idiopathic pulmonary fibrosis, where increased levels of citrullinated peptides and both PAD2 and PAD4 were found in patient granulocytes and macrophages when compared to those of control groups.^{25, 26}

Taken together, citrullination has to be considered an important step of the inflammatory process across different tissues rather than a disease-specific event. The precise pathogenic mechanism of each inflammatory disorder related to citrullination is currently under investigation, but a common line is probably present in every condition. It is believed that

either external insults trigger the upregulation of PAD enzymes in different cell types (mainly innate immune cells) or that bacteria derived from microbial dysbiosis directly overexpress citrullinating enzymes, resulting in the citrullination of various proteins. These new citrullinated peptides could then act as neoantigens, stimulating the formation of antibodies and the activation of adaptive immunity toward the host in genetically predisposed individuals.^{27, 28}

CITRULLINATION IN FIBROSIS

In addition to a role in inflammation, data on the potential role of PAD enzymes in fibrosis are starting to accumulate. In RA, citrullination has been associated with a more inflammatory and destructive behavior in synovial fibroblasts,²⁹ but recent data on cirrhosis³⁰ and idiopathic pulmonary fibrosis³¹ report that citrullination may also activate a profibrogenic response.

The expression of PAD enzymes in liver diseases has been associated with the stage of fibrosis in a series of biopsies from patients with cirrhosis.³² In addition, increased serum levels of citrullinated vimentin have been found in a carbon tetrachloride rat model of liver fibrosis.³³ To selectively study a potential role during fibrosis, Kim et al³⁰ studied an experimental mouse

model of bile duct ligation-induced liver fibrosis and found increased expression of PAD2 together with citrullinated proteins in hepatic stellate cells and α -smooth muscle actin-positive portal fibroblasts.

The PAD4-dependent citrullination of histones H1 and H3 in the nucleus of neutrophils is the crucial process allowing chromatin decondensation and the subsequent expulsion of neutrophil extracellular traps (NETs).³⁴ This process constitutes a unique form of cell death called NETosis. NETs are made of neutrophil granules bound to DNA filaments and are extruded during the first steps of innate immune response in various situations such as cancer, thromboembolic episodes, sepsis, and autoimmunity in general.^{35, 36} In addition, they can directly trigger the immune response toward the self, because some of their granules are potentially modified by citrullination or other PTM reactions³⁷ and DNA is able to activate dendritic cells to produce type-1 interferon.^{38, 39}

Among other functions, NETs have been shown to mediate the activation of fibroblasts into myofibroblasts in vitro in fibrotic interstitial lung disease.³¹ In particular, after stimulation with NETs, fibroblasts increased their expression of α -smooth muscle actin and connective tissue growth factor together with enhanced collagen production and proliferation/migration rates. In the same study, the authors showed a co-localization of myofibroblasts and NETs in lung fibrosis and in skin scar tissue with immunofluorescence, suggesting that this process could be reproduced in different tissue environments.³¹ Moreover, these data have been indirectly corroborated by the findings that age-related myocardial and pulmonary fibrosis are reduced in PAD4-knockout mice because of the reduced amount of NET formation.⁴⁰

Therefore, citrullination-associated enzymes and their products could enhance fibroblast activity via intrinsic and extrinsic pathways, but additional and more robust data are needed in this field.

IBD AND CITRULLINATION

Improved insights into the pathways driving inflammation in IBD have resulted in the development of monoclonal antibodies or small molecules targeting specific cytokines or their receptors, such as infliximab,⁴¹ adalimumab,⁴² and golimumab⁴³ for tumor necrosis factor- α ; vedolizumab^{44, 45} for integrin $\alpha 4\beta 7$ -dependent lymphocyte diapedesis; ustekinumab⁴⁶ for IL-12/IL-23 Th1 lymphocyte proliferation and differentiation; and tofacitinib⁴⁷ for JAK/STAT nuclear signaling.

However, despite these therapeutic advances, clinical and endoscopic remission rates have reached a ceiling at 30% to 40%. Explanations may be found in the outcomes used, in the patient populations studied, or in the fact that not all cell types or processes driving inflammation are targeted. In this respect, citrullination may be considered a good candidate because it intervenes in neutrophil and macrophage activation during the

initial phases of the innate immune response. The result is the creation of new citrullinated peptides that stimulate the formation of autoantibodies, which are then responsible for the perpetuation of the inflammation (Fig. 3).

The first evidence for the role of citrullination in the context of IBD comes from Makrygiannakis et al,¹⁶ who found a numerically—albeit not significant—higher expression of citrullinated peptides in colonic biopsies of patients with IBD ($n = 10$) than in control patients ($n = 10$). Immunohistochemical analysis in both acute dextran sulfate sodium (DSS)-induced colitis and human ulcerative colitis (UC) biopsies has shown a strong staining for PAD2 and PAD4 in inflammatory cells and in the lamina propria cells of affected tissue, confirming the correlation between citrullination and inflammation.⁴⁸ In the same study, a significant reduction in inflammation was seen when mice were treated with Cl-amidine, a potent pan-PAD inhibitor.

Further evidence comes from genetic studies. In a Japanese cohort of 114 patients with UC, an association between PAD4 haplotype 2 and RA and an inverse association between PAD4 haplotype 1 and UC was reported.⁴⁹ In addition, a selected analysis of PAD enzymes extracted from a genome-wide expression profiling of 236 biopsies of newly diagnosed pediatric patients with IBD showed a positive correlation with PAD4 and a negative correlation with PAD2 expression in CD and UC, in both colon and ileum biopsies when compared with those of control patients⁵⁰ (data available at <https://www.ebi.ac.uk/gxa/home>).

Data about the role of these enzymes in CD, in inflammation and in the context of intestinal fibrosis, are lacking. Interestingly, no study has yet investigated in IBD the potential role of the gut microbiome in enhancing the citrullination cascade, as reported in RA, independently from eventual external insults.

NETS IN IBD

In consideration of the recent discovery of NETs,⁵¹ only few studies have been published in IBD on this topic, with some authors claiming their presence in both UC and CD^{52–54} and some others confirming this evidence only in UC.^{55, 56}

The first publication analyzed colonic biopsies from 10 patients with macroscopically inactive UC and 10 control patients at the proteomic and histological level, with the conclusion that NET-related proteins were more abundant in the UC group (on average 42.2 folds; $P < 0.0005$).⁵⁷ An additional pathological evaluation of active CD and UC, with biopsies taken from the terminal ileum and colon, respectively, showed an abundant presence of NETs in both diseases in comparison to no NET detection in the healthy control group.⁵² These findings were recently confirmed in actively inflamed colonic biopsies of patients with UC and CD when compared with sites of inactive IBD and control patients.⁵⁴ Moreover, to show a link between

FIGURE 3. Proposed pathogenesis of citrullination in IBD. External insults to the intestinal mucosa trigger innate immune activation in genetically predisposed individuals. Neutrophils and monocytes/macrophages increase their expression of PAD2 and PAD4 enzymes, resulting in modification of the physiological citrullinome and the formation of new autoantibodies. In addition, PAD4 is responsible for NET formation in the inflammatory infiltration.

NETs and the activation of a hypercoagulable state, He and colleagues⁵³ described the presence of these extracellular filaments in the peripheral blood of patients with active UC and active CD, but not in patients with inactive disease.

Another analysis of colonic biopsies and peripheral blood neutrophils of 23 patients with UC and 11 patients with CD showed an abundance of NETs in UC but not in CD.⁵⁵ Dinallo et al⁵⁶ confirmed the UC specificity by Western blot analysis and immunohistochemistry.

The reasons for these conflicting results in IBD inflammation should probably be found in the relatively small cohorts investigated and the heterogeneous sampling of ileal and colonic biopsies. More data on larger numbers are needed to clarify whether the presence of NETs is exclusive to UC or at least more relevant in its pathogenesis compared with CD.

Interestingly, in the lung NETs can trigger the fibrogenic process when put in co-culture with fibroblasts; they enhance the activation of fibroblasts into myofibroblasts and the production of collagens.³¹ A similar mechanism is thought to be involved in age-related cardiac fibrosis as shown in a PAD4-knockout model of mouse fibrosis.⁴⁰ Therefore, citrullination and NETs also merit study in IBD to determine their role in driving disease complications (Fig. 3).

From a therapeutic point of view, a profibrogenic role of NETs in the human intestine could open avenues for the development of powerful drugs with the ability to control both

inflammation and fibroblast activation. This dual action is what could break the above-mentioned therapeutic efficacy ceiling, because intestinal fibrosis is autopropagative when triggered by inflammation and hence should also be targeted separately.⁵⁸

Specifically, combined regimens tackling the innate and adaptive inflammatory responses together with a treatment to contrast fibroblast activation are presumably the primary strategies that will be tested and adopted in the future of IBD management, until preventive measures are available.

THE CITRULLINOME

There is now robust evidence for increased citrullination and hyperexpression of PAD enzymes in many autoimmune disorders. The total amount of citrullinated proteins constitutes the so-called citrullinome. The challenge for research in this field is to identify the specific proteins that are citrullinated in the different immune-mediated diseases.

To date, only few substrates of PADs have been found because of technical issues related to the exiguous mass change of a protein after citrullination (only +0.98 Da).⁵⁹ Two probes (rhodamine-phenylglyoxal and biotin-phenylglyoxal)^{60, 61} have thus recently been developed with the ability to chemoselectively bind and isolate citrullinated substrates. These probes can then be subsequently highlighted with specific fluorescent staining. This novel technique enables researchers to “split” the citrullinome into different groups of proteins based on their

weight. As an example, Bicker et al⁶⁰ reported in their previous model of colitis that CI-amidine reduced citrullination in proteins placed at 25, 50, 70, and 100 kDa but not in those at 10 kDa.⁶⁰ Yet the limitation of this novel method is still the inability to identify the specific proteins within a given cluster. Proteomic analysis is therefore needed to provide more insights about citrullination biology.⁶¹

Initial experiments in RA tried to identify citrullinated proteins in the synovium, synovial fluid, and serum of patients. In their comparison between 16 patients with RA and 16 healthy control patients, Tilwala et al⁵⁹ discovered approximately 150 new substrates of citrullination such as serpins, serine proteases, and glycolytic enzymes. Additional proteomics analysis on the synovial fluid of patients with RA using a mass-spectrometry approach identified 9 new sites of the citrullination of fibrinogen chains, which may help in acquiring more information on the disease's evolution.⁶²

In general, the step of protein identification is fundamental for further selective downstream analyses toward the understanding of citrullination mechanisms. In fact, proteomic studies may not tell whether a protein is physiologically citrullinated or whether it is involved in disease pathogenesis.

A thorough examination of the citrullinome pool in IBD is crucial to provide more insights on the effect of citrullination in normal, inflamed, and fibrotic bowel and to offer information about the class of inhibitors to be preferred in these contexts.

BIOMARKERS OF CITRULLINATION

In the field of citrullination, the first and only markers that are currently used in clinical practice are ACPAs, which

are significantly more sensitive and specific than the rheumatoid factor for the diagnosis of RA.⁶³ In addition, ACPAs are predictive of RA onset because increased levels are found very early in the preclinical context of the disease.⁶⁴ For other autoimmune diseases in which citrullination plays a role, there is no evidence that ACPAs can be used for these purposes, including IBD.⁶⁵⁻⁶⁸ Therefore, the study of the citrullinome could also help identify new specific antibodies to be used for diagnostic and prognostic purposes in IBD (Fig. 4).

In the last few years, many groups have started to address the potential value of NET serum markers, in both inflammation and thromboembolism. Despite the lack of standardized methods to assess their formation,⁶⁹ a few components have been tested in different scenarios.

The citrullinated histone H3 (H3cit) is produced during the PAD4-dependent histone citrullination as a fundamental step in chromatin decondensation and the NETosis process. Increased blood levels of H3cit have been found in 2 rodent models of lipopolysaccharide-induced septic shock in the first hours after disease onset^{70,71}; these results have been confirmed in a human model of endotoxemia, suggesting that H3cit could predict the lethality of sepsis in the early phases of disease.⁷² Serum H3cit levels have been linked to an enhanced inflammatory state related to advanced cancer and have been associated with poor prognosis.^{73,74}

Another component of NETs, the myeloperoxidase (MPO)-DNA complex, has been studied in inflammatory conditions. High serum levels of MPO-DNA have been associated with worse outcomes (clinical instability, prolonged stay in the hospital, and overall mortality at 30 days) in patients with

WHO	WHEN	HOW
Citrullinome-associated antibodies	IBD diagnosis	Serum/Plasma
H3cit	Inflammatory burden (ASUC prognosis?)	Feces
MPO/DNA complex	Response to treatments	Urines metabolites
	Crohn's disease POR prediction	Endoscopic biopsies
	Thrombotic risk assessment (severe flare? Tofacitinib treatment?)	

FIGURE 4. Citrullination biomarkers and IBD. Potential clinical applications where biomarkers of citrullination in IBD could play a role. ASUC indicates acute severe ulcerative colitis; POR, postoperative recurrence.

community-acquired pneumonia.⁷⁵ Accordingly, the MPO-DNA complex is increased in the bronchoalveolar lavage fluid of patients with ventilator-associated pneumonia when compared with that of critically ill patients without inflammatory burden and infection in the lung.⁷⁶

Neutrophil granules released with chromosomal DNA during NET formation are able to trigger thrombosis by trapping platelets and various procoagulant factors.^{77, 78} As a consequence, NETs have been found in clots, in both coronary and venous systems.⁷⁹⁻⁸¹ For all these reasons, it is not surprising that H3cit levels are increased in patients with acute ischemic stroke, especially in those with more severe course and higher risk of mortality at 1 year.⁸² In addition, serum H3cit levels correlate with the risk of venous thromboembolism in patients with advanced cancer,⁸³ although data are conflicting.⁷⁴

In addition, the MPO-DNA complex has been studied and found to be increased in the context of antiphospholipid syndrome.⁸⁴ However, the MPO-DNA complex was not associated with clinical outcomes or hypercoagulability in a cohort of 1001 patients with stable coronary artery disease,⁸⁵ despite its positive correlation with severe calcifications and atherosclerotic evolution of coronaries.⁸⁶

In IBD, only 1 work has addressed this topic, showing that MPO-DNA complexes are predominantly expressed in patients with active UC and CD when compared with patients with inactive IBD and control patients.⁵⁴ The same authors reported an attenuation of platelet activation and thrombus formation with the use of DNase in a murine DSS-induced model of acute colitis, showing a link between NETs and the prothrombotic state of active IBD.⁵⁴

In consideration of the prognostic value of H3cit and MPO-DNA in some acute inflammatory events, it could be interesting to evaluate these proteins in patients with acute severe UC to see whether they can predict colectomy.

In addition, in light of the recent warnings about deep vein thrombosis and pulmonary embolism with higher doses of the JAK inhibitor tofacitinib,^{87, 88} the aforementioned markers merit investigation to see if a prognostic role can be identified.

TARGETING CITRULLINATION: PAD-INHIBITORS

The PAD enzymes (especially PAD4) are aberrantly expressed in some tumors with more aggressive behavior and are involved in cancer-related neoangiogenesis and stromal activation^{89, 90}; thus, many efforts are currently being undertaken to find specific inhibitors to be used as antineoplastic agents. The strategy in chronic inflammatory disorders is to test the same components as anti-inflammatory and antifibrotic drugs. Several potential drugs with irreversible (covalent) and reversible (noncovalent) PAD inhibition have been tested (Table 2).

Initially, the role of the first-generation PAD-inhibitors F-amidine and Cl-amidine was investigated: Both compounds have been shown to reduce the vitality *in vitro* of leukemic cells and epithelial cancer cells derived from colon and mammalian

cancer at micromolar concentration, with no toxic effect on normal cells.⁹¹ Promising results have also been reported for the anti-inflammatory effect of Cl-amidine in mouse models of disease including acute DSS-related colitis,⁴⁸ collagen-induced arthritis,⁹² murine lupus,^{93, 94} and atherosclerosis.⁹⁵

Despite the wide utilization of Cl-amidine in animal models, many limitations have impeded its clinical evaluation: low selectivity for a specific PAD enzyme, short half-life *in vivo*, low potency, limited bioavailability, and irreversible binding. For all these reasons, second-generation irreversible inhibitors with structure modification were developed: o-F-amidine and o-Cl-amidine.⁹⁶ In detail, o-F-amidine is 65 times more potent than the corresponding F-amidine and is at least 6 times more selective for PAD1; o-Cl-amidine is also more selective than first-generation Cl-amidine, more specifically blocking PAD1 and PAD4.⁹⁶ Another interesting component of this “family” is BB-Cl-amidine with a benzimidazol tail, which has resulted in higher selectivity toward PAD2 in a model of collagen-induced arthritis.⁹⁷

The unmet need of highly selective inhibitors together with the improvement of bioengineering and pharmaceutical technologies eventually resulted in the third-generation compounds Thr-Asp-F-amidine and Thr-Asp-Cl-amidine, with high selectivity and high efficacy in irreversibly blocking PAD4.⁹⁸ Streptonigrin also has high specificity for PAD4, around 37 times higher than Cl-amidine, and is considered today as one of the most powerful selective inhibitors.⁹⁹ It is not a surprise that this molecule has also started to be tested in *in vivo* experiments of IBD, as in the acute DSS model of colitis.⁵⁶

In the end, the research on selective but reversible inhibitors is still ongoing. In fact, the early discovery of a few antibiotics (streptomycin, minocycline, chlorotetracycline)¹⁰⁰ and antineoplastic agents (taxol)¹⁰¹ with a relatively low inhibitory action for PAD4 has not been followed by improvements in subsequent years. Recently, some new reversible inhibitors of PAD1,¹⁰² PAD2,¹⁰³ and PAD4¹⁰⁴⁻¹¹⁰ have been tested in biochemical assays and in cell-based models and *in vivo* with promising results, but they need further validation before consideration for clinical human studies.

OPEN QUESTIONS AND FUTURE SCENARIOS

In conclusion, citrullination is involved in many different steps of human physiology, but also in pathology. A better understanding of its mechanisms in IBD could provide a new target for anti-inflammatory treatments. In order to achieve this goal, many questions remain to be solved. First, there are no data on PAD enzyme expression levels in CD, and only UC has been studied so far. In addition, it is unknown which proteins specifically are citrullinated during the inflammatory process in IBD, and a detailed analysis of the citrullinome is mandatory to increase knowledge on this topic. Moreover, some products of neutrophil citrullination (ie, NET compounds) have shown the potential of reliable inflammatory markers. Therefore, the

TABLE 2. PAD Inhibitors Tested in Experimental Contexts.

Agent	Binding Reversibility	Relative Selectivity*	Setting of Experimental Testing
Cl-amidine	Irreversible	Pan-inhibitor	In vitro ^{91, 107, 123, 135–137} and in vivo ^{48, 92–95, 138, 139}
F-amidine	Irreversible	Pan-inhibitor	In vitro ^{91, 140}
o-Cl-amidine	Irreversible	PAD1 and PAD4	Biochemical assays and in vitro ⁹⁶
o-F-amidine	Irreversible	PAD1	Biochemical assays and in vitro ⁹⁶
BB-Cl-amidine	Irreversible	PAD2	In vitro ¹⁰⁷ and in vivo ⁹⁷
Cl4-amidine and F4-amidine	Irreversible	PAD3	Biochemical assays ¹⁴¹
Thr-Asp-F-amidine	Irreversible	PAD4	In vitro ⁹⁸
Thr-Asp-Cl-amidine	Irreversible	PAD1 and PAD4	In vitro ⁹⁸
d-Cl-amidine and d-o-F-amidine	Irreversible	PAD1	Biochemical assays and in vitro ¹⁴²
2-chloroacetamidine	Irreversible	PAD2	In vitro and in vivo ¹⁴³
Biphenyl tetrazole tert-butyl Cl-amidine	Irreversible	PAD4	Biochemical assays and in vitro ¹⁴⁴
Streptonigrin	Irreversible	PAD4	Biochemical assays ⁹⁹ and in vivo ⁵⁶
Fluoroacetamidine-containing inhibitor	Reversible	PAD1	Biochemical assays and in vitro ¹⁰²
GSK199 and GSK484	Reversible	PAD4	Biochemical assays, in vitro, ^{106, 107} and in vivo ^{108, 109}
5,5-Dimethyl-3-(1-piperazinylmethyl)-2,4-imidazolidinedione	Reversible	PAD4	In vivo ¹¹⁰
LT02193089	Reversible	PAD4	Biochemical assays and in vitro ¹⁰⁵
YW3-56	NA	PAD4	Biochemical assays and in vitro ¹⁴⁵
N-imidazole hydantoin compound	Reversible	PAD2	Biochemical assays and in vitro ¹⁰³
Benzamide analogue compound	NA	PAD3	Biochemical assays and in vitro ¹⁴⁶

*Most of the compounds can block many of the PAD enzymes; the ones with relative selectivity are the enzymes with lower concentration needed to block their activity. In vivo refers to animal models.
NA indicates not available.

accuracy of these markers in the context of active inflammation should be studied and compared to established markers, such as CRP and fecal calprotectin.¹¹¹ Whether accuracy should be checked in serum, feces, or urine is not yet known and requires studies.

Finally, the stimulating effect of NETs in pulmonary fibroblast cell lines should be also investigated in the context of the intestine, because a confirmation of these findings could result in new targets for antifibrotic therapy research.

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