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Autoantibodies to protein-arginine deiminase (PAD) 4 in rheumatoid arthritis: immunological and clinical significance, and potential for precision medicine

Anti-PAD4 antibodies in RA

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ABSTRACT

Introduction: The protein-arginine deiminase (PAD) 4 enzyme plays an important role in the pathogenesis of rheumatoid arthritis (RA) and also represents an antigenic target. Anti-PAD4 antibodies can be present in RA and are associated with specific clinical features.

Areas covered: This review aims to analyze the current knowledge and recent findings on anti-PAD4 antibodies in RA and their clinical and immunological significance.

Expert opinion: Anti-PAD4 antibodies are not currently used in clinical practice for the management of RA. Nevertheless, there is growing evidence of their relevance in RA, and of their potential utility to improve diagnosis, patient stratification, and prognosis.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic, inflammatory disease that affects 0.5–1% of the population [1]. It is a complex heterogeneous condition with variable clinical manifestations, characterized by chronic synovial inflammation, and articular cartilage and bone damage. Several risk factors have been identified [2], including genetic and environmental factors, as well as lifestyle. Nevertheless, the underlying pathogenic mechanisms are still not fully understood.

The presence of autoreactive T- and B-cells and the production of autoantibodies are key features of RA, with Rheumatoid Factor (RF) and anti-citrullinated protein antibodies (ACPA) being the main two biomarkers. Yet, up to 60% of the early RA patients do not present these antibodies, often referred to as the serological gap [3]. Although the combination of ACPA and RF can increase the diagnostic efficiency [3], seronegative patients persist. Consequently, numerous studies aimed to identify novel biomarkers to further improve the diagnosis of RA [4]. In addition, biomarkers that aid in establishing prognosis, in patient stratification, in disease activity monitoring and in predicting response to treatment are desired. In this context, several novel autoantibodies have been described in RA patients over the past few years [4], including antibodies targeting carbamylated proteins (CarP) [5–8] and the protein-arginine deiminase (PAD) enzymes [9–12]. The PAD proteins are enzymes that catalyze the conversion of peptidyl-arginine into peptidyl-citrulline, a post-translational modification (PTM) known as citrullination or deimination, which is an important part in the pathogenesis of RA. In addition to their role in citrullination, three members of the PAD family of enzymes have been recently identified as autoantigens in RA, including PAD2, PAD3, and PAD4 [11–13], and among them, antibodies to PAD4 are the most characterized. In the last few years, PAD enzymes captured growing attention due to the cumulative understanding of their role in the pathogenesis of RA and the potential to block PAD activity as a novel treatment strategy [14–16]. The objective of this review is to provide an overview of the current understanding on anti-PAD4 antibodies in RA, including their role in pathogenesis, their clinical significance and their potential for precision medicine (PM) approaches.

2. The PAD enzymes and citrullination

Citrullination is an important PTM in normal physiological conditions of key cellular processes such as apoptosis, organization of structural proteins [14,17] and gene regulation, especially during early embryonic development [18,19]. The activity of the PAD enzymes is stringently regulated in normal conditions; however, dysregulation of the citrullination pathway can occur in association with several diseases, including several types of cancer [20], neurodegenerative diseases [21], and...
autoimmune diseases [22]. Particularly relevant is the case of RA, characterized by hypercitrullination and accumulation of citrullinated products and antibodies to the modified antigens present in the joints. Thus, citrullination can promote generation of neo-(auto) antigens and help trigger the autoimmune response [23].

Citrullination and the PAD enzymes also play an important role during apoptosis, autophagy and the formation of neutrophil extracellular traps (NETs) [24], processes well known for their involvement in autoimmunity. During infection or inflammation, PAD4 becomes activated in neutrophils resulting in the citrullinating of multiple autoantigens [25–27] and the ejection of chromatin from the cell, generating the NETs, important tools in the protection against infection [28]. Citrullination of histones represents an important step in this process [29]. L1ttle is known about the role of PAD2 in NETs formation; limited data seems to indicate that PAD2 is required for tumor necrosis factor alpha (TNFα)-induced citrullination and arthritis, but that it is not required for the generation of NETs [30]. The release of NETs and PADs by neutrophils is likely followed by citrullination of extracellular antigens [31] that together with the infectious agents [2], the complement activation [32] and the formation of immune complexes [33] could challenge the immune system and potentially compromise the immune tolerance. Once tolerance is broken, the presence of an additional ‘hit’ to the immune system and/or additional co-factors that increase PAD activity (such as autoantibodies [11]) could be helping maintain efficient citrullination and contributing to autoimmunity. Moreover, it was recently demonstrated that PAD4 directly citrullinates nuclear factor κB (NF-κB) which has a critical role in the expression of pro-inflammatory cytokines IL-1β and TNFα [34]. Therefore, citrullination can help propagate inflammation in RA.

Citrullination is an irreversible process resulting in conformational structure changes of the modified protein, that can lead to unfolding [17], and alterations of the intra- and intermolecular interactions. The PAD enzymes require calcium for their catalytic activity; however, the calcium concentration identified for maximum PAD4 activity in-vitro is higher than found in the synovial fluid [27,35,36], suggesting the existence of additional factors that modulate this process during normal physiology and in the pathogenesis of RA.

The PAD enzymes were first described in 1977 by Rogers and colleagues [37]. Today, a total of five isotypes are known (reviewed in [27], summarized in Table 2) of which PAD2, PAD3, and PAD4 have been identified as antigenic targets in RA. As expected by the similar cellular function, there is a significant protein sequence homology between these three enzymes (Table 1). The members of the PAD family differ in their substrate specificities and tissue-specific expression and studies suggest that the PAD enzymes have the capacity to select unique protein targets and that this capability may play a role in autoantigen selection in RA [26].

PAD2 is the most broadly expressed isoform. Many proteins have been identified as substrates of PAD2, with the main being myelin basic protein (MBP) in the central nervous system, and vimentin in skeletal muscle and macrophages [26,27,35,36]. Studies suggest a tissue-specific hormonal regulation of PAD2 expression [64,65]. Although PAD2 is mainly localized in the cytoplasm, data suggest that a fraction of PAD2 can also be found in the nucleus despite lacking a nuclear translocation signal, and that this nuclear PAD2 may citrullinate histones H3 and H4 and play a role in gene regulation [66].

On the other hand, PAD3 is expressed in hair follicles and has a cytoplasmic intra-cellular localization [27]. Its natural substrate, trichohyalin, is a major structural protein of inner root sheath cells of hair follicles [67].

PAD4 is mainly found in the nucleus and expressed in white blood cells (granulocytes, monocytes) and can be detected in several tissues [27]. In addition to upregulated enzymatic activity, PAD2 and PAD4 are overexpressed by neutrophils and monocytes in the synovium of RA patients in co-expression with numerous citrullinated proteins [68]. PAD4 has been confirmed as a susceptibility gene for RA [69–71] and an association between single nucleotide polymorphisms (SNPs) and RA has been reported in numerous ethnic groups [72–74], although these findings have not been replicated in certain populations [75,76]. Besides, it has been reported that PAD4 can autocitrullinate itself influencing the enzyme structure and immune response and that this process contributes to the regulation of citrullinated proteins generation during cell activation [77]. Nevertheless, the clinical implications of these observations need to be further explored.

A relatively broad range of targets has been described for PAD4, with certain overlap with PAD2 [78–83]. The enzymes’ specificity for cellular substrates and synthetic peptides seems to be different for PAD2 and PAD4, with the latest being more restricted by the amino acid composition surrounding the acceptor arginine residue [36]. It is unclear whether one of the isoforms dominates in the generation of citrullinated self-proteins that are targeted by ACPAs. A recent study showed that very high-titers ACPA preferentially bind fibrinogen citrullinated by PAD4 vs. PAD2 [84]. However, in a more recent

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**Table 1.** Protein sequence homology between the protein-arginine deiminase (PAD) 2, 3, and 4 enzymes. Data were generated by BLASTp analysis [38] using the FASTA sequences from UniProt (The UniProt Consortium). The UniProt IDs of each isoform can be found in Table 2.

<table>
<thead>
<tr>
<th>Proteins compared</th>
<th>PAD2 vs. PAD3</th>
<th>PAD2 vs. PAD4</th>
<th>PAD3 vs. PAD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identities</td>
<td>345/667 (52%)</td>
<td>337/668 (50%)</td>
<td>374/669 (56%)</td>
</tr>
<tr>
<td>Positives</td>
<td>452/667 (67%)</td>
<td>440/668 (65%)</td>
<td>461/669 (68%)</td>
</tr>
<tr>
<td>Gaps</td>
<td>5/667 (0%)</td>
<td>8/668 (1%)</td>
<td>11/669 (1%)</td>
</tr>
<tr>
<td>E value</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

PAD, protein-arginine deiminase.

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**Article highlights**

- Five members of the PAD family have been described in humans, and three of them, PAD2, 3 and 4, have been identified as autoantigens in RA.
- Anti-PAD4 antibodies are found in 30–40% of the RA patients with specificity of >95%, and in 2–18% of the RF and ACPA seronegative individuals.
- Anti-PAD4 antibodies are associated with joint erosions and a more severe disease phenotype.
- Testing for anti-PAD4 antibodies might provide diagnostic and prognostic value.
Overview of human protein-arginine deiminases (PAD) isoforms and their characteristics.

Table 2. Overview of human protein-arginine deiminases (PAD) isoforms and their characteristics.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Mass (kDa)</th>
<th>Length (aa)</th>
<th>Substrate</th>
<th>Subcellular location</th>
<th>Tissue and cellular expression under normal conditions</th>
<th>Physiological roles</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD1 (Q9ULC6)</td>
<td>74.7</td>
<td>663</td>
<td>Keratin K1, filaggrin</td>
<td>Cytoplasm</td>
<td>Epidermis, prostate, testis, placenta, uterus, spleen and thymus</td>
<td>Skin differentiation, terminal differentiation of keratinocytes</td>
<td>[27,39–41]</td>
</tr>
<tr>
<td>PAD2 (Q9Y2J8)</td>
<td>75.6</td>
<td>665</td>
<td>Vimentin, MBP, GFAP, β and γ-actins, histone H3, fibrinogen, collagen II, alphanelase</td>
<td>Cytoplasm, may become nuclear in human mammary epithelial cells</td>
<td>Skeletal muscle, brain, pancreas, glial cells, macrophages, bone marrow, muscle, breast, colon, embryo, eye, kidney, epidermal, uterus, thymus</td>
<td>May play a role in brain development, innate immune defense, female reproduction, gene expression</td>
<td>[26,27,35,42–48,64,66,81,83]</td>
</tr>
<tr>
<td>PAD3 (Q9ULW8)</td>
<td>74.7</td>
<td>664</td>
<td>Trichohyalin, filaggrin</td>
<td>Cytoplasm</td>
<td>Hair follicles and keratinocytes</td>
<td>Skin differentiation, hair follicle formation, terminal differentiation of keratinocytes</td>
<td>[27,37,41,49,50]</td>
</tr>
<tr>
<td>PAD4 (Q9UM07)</td>
<td>74.1</td>
<td>663</td>
<td>Histones H2A, H3, H4, vimentin, p300, nucleophosmin/ B23, ING4, fibrinogen, collagen II, alphanelase</td>
<td>Nucleus and cytoplasmic granules (eosinophils, neutrophils)</td>
<td>Eosinophils, neutrophils, granulocytes, macrophages; several cancerous tissues</td>
<td>Chromatin decondensation, transcription regulation, tumorigenesis, cellular differentiation, transcriptional co-repressor for the estrogen receptor and p53, NETs formation</td>
<td>[27,45,51–59,81,83]</td>
</tr>
<tr>
<td>PAD6 (Q6TGC4)</td>
<td>77.7</td>
<td>694</td>
<td>Keratin</td>
<td>Cytoplasm</td>
<td>Egg, ovary, early embryo, thymus, oocyte, peripheral blood leukocytes</td>
<td>Embryonic development, oocyte cytoplasmic lattice formation, fertility</td>
<td>[27,60–63]</td>
</tr>
</tbody>
</table>

PAD, protein-arginine deiminase; kDa, kilo Dalton; aa, amino acids; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; NETs, Neutrophil Extracellular Traps.

publication, both enzymes seemed to be equally efficient in generating citrullinated targets for ACPAs on fibrinogen and enolase, but autoantibodies to histone H3 in these patients seemed to have a preference for the antigen that had been citrullinated by PAD4 [81].

3. Anti-citrullinated protein autoimmunity in the pathogenesis of RA

3.1. Animal models

Experimental models of inflammatory arthritis have proven essential to understanding the underlying mechanisms and developing therapeutics for the treatment of RA. Findings in multiple mouse models of RA support a key role for PADs and citrullinated antigens in the pathogenesis of RA.

Animal models that recapitulate certain clinical and mechanistic characteristics of RA have been developed over the years. Collagen-induced arthritis (CIA) is the classical animal model of RA, first established in rats [85] and later adapted to mice [86]. CIA develops following immunization with type II collagen that was derived from either bovine or chicken. In addition to recapitulating certain histologic features of RA, including inflammation, pannus formation, and cartilage and bone damage, pathogenic antibodies including ACPA have been described to develop in mice with CIA [87–90]. These findings support the possibility that ACPA and anti-citrullinated protein autoimmunity could contribute to the pathogenesis of this model.

Two common citrullinated autoantigens targeted in RA include citrullinated fibrinogen and citrullinated histone 2B (H2B). A significant proportion of ACPA+ patients express antibodies targeting these two antigens [89,91–93]. Immunization of mice with citrullinated fibrinogen or citrullinated H2B both resulted in inflammatory arthritis [89,92,94]. Further, fibrin-induced arthritis (FIA) in mice was associated with the development of RF as well as antibodies against other citrullinated proteins.

There are also mouse models in which antibody transfer results in inflammatory arthritis. Examples of such antibody transfer models of RA include anti-collagen antibody-induced arthritis (CAIA), the K/BxN serum transfer model in which anti-glucose-6-phosphate isomerase (GPI) antibodies induce inflammatory arthritis, and other models in which transfer of serum antibodies from arthritic mice results in arthritis in the recipients. These antibody transfer models enable characterization of the role of antibodies against particular targets in the induction or exacerbation of inflammatory arthritis. For example, in the first study to demonstrate that an antibody against a citrullinated target could contribute to inflammatory arthritis in mice, a recombinant antibody specific for citrullinated fibrinogen exacerbated suboptimal CAIA [87]. Subsequently, it was shown that transfer of polyclonal antibodies derived from mice immunized with either citrullinated fibrinogen or citrullinated H2B induced arthritis in naive recipients [89,92]. Together, these studies demonstrate that citrullinated fibrinogen and citrullinated H2B can be pathogenic autoantigens that mediate autoimmune arthritis in mice.

3.2. PAD inhibition

Genetic and pharmacologic inhibition of PADs in mouse models of RA has been used to further define the roles and mechanisms of PAD2 and PAD4, citrullination and NETosis in inflammatory arthritis. Mice deficient for PAD4 have been studied for development of both CIA [95] and K/BxN serum transfer arthritis [96], as well as in other models of arthritis...
Global PAD as well as PAD4-selective inhibitors have also been tested in mouse models of RA, and the results provide further insights into the mechanisms by which PADs contribute to inflammatory arthritis.

PAD4-deficient DBA1J mice exhibited modestly reduced arthritis severity following immunization with type II collagen (CII) to induce CIA [95]. Although an initial study reported that genetic PAD4 deficiency did not protect mice against K/BxN serum transfer arthritis despite reducing levels of citrullination in arthritic joints [96], and it is possible that PAD4 does not play a role in the effector phase of the anti-GPI passive transfer model. In contrast, a subsequent study demonstrated that genetic PAD4-deficiency protected mice against GPI-immunization-mediated inflammatory arthritis [97]. In addition to inhibiting clinical arthritis following GPI-immunization, PAD4-deficiency also was associated with reduced serum IL-6 and reduced synovial myeloid and Th17 cells [97]. Together, these data suggest that PAD4 plays an important pathogenic role in immunization-induced autoimmune arthritis.

In a series of studies using TNF-transgenic mice that spontaneously develop inflammatory arthritis, PAD2-deficient mice exhibited reduced inflammatory arthritis [30]. The authors further demonstrated that TNFα-induced arthritis resulted in increased citrullination in inflamed ankle joints, and that genetic deficiency in PAD2 reduced this citrullination while the levels of citrullination in inflamed ankle joints were not significantly altered by PAD4 deficiency. The authors also demonstrated that PAD2-deficient neutrophils appeared to undergo normal NETosis, while PAD4 was critical for neutrophil NETosis [30].

The importance of PADs in RA mouse models has been corroborated by studies utilizing small molecule PAD inhibitors. Cl-amidine, a pan-PAD inhibitor, reduced the severity of inflammatory arthritis in the CIA model [98]. Further, the CII-immunized Cl-amidine-treated mice exhibited reduced synovial and serum citrullination, and decreased anti-CII antibody titers in these mice [98]. While Cl-amidine also significantly reduced arthritis severity following K/BxN serum transfer, it had no significant impact on ACPA titers [99].

While Cl-amidine globally inhibits PADs (including both PAD2 and PAD4), BB-Cl-amidine exhibits increased potency against PAD2 [100,101]. Treatment of mice with BB-Cl-amidine-reduced joint inflammation and destruction to a greater degree as compared to Cl-amidine in mice with CIA [101]. While a shift from pro-inflammatory Th1 and Th17-type responses to pro-resolution Th2-type responses were associated with BB-Cl-amidine treatment, minimal change in antibodies to collagen or citrullinated peptides was observed [101].

More recently, treatment of mice with GSK199, a PAD4-selective inhibitor significantly reduced arthritis severity in CIA. In this study, a subset of ACPA was reduced by GSK199 treatment, but global citrulline levels and circulating anti-CII antibody levels were not affected [16].

In addition to development of small molecule PAD inhibitors, several groups are developing monoclonal antibodies specific for PAD2 or PAD4. Although such reagents will inhibit specific targeting of PAD2 or PAD4, due to their relatively large size as monoclonal antibodies they will not fully penetrate joint tissues and/or cells and thereby likely be most effective at examining inhibition of extracellular PAD2 and/or 4 released by neutrophils, macrophages and/or other cells in animal models of RA.

4. Anti-PAD antibodies in RA

4.1. Anti-PAD4 antibodies

Anti-PAD4 antibodies were first described in patients with RA by Nissinen and colleagues in 2003 [13] (Figure 1). Since this initial description, numerous studies over the past years have...
Table 3. Summary of studies on anti-PAD4 antibodies, including number and origin of patients, detection method used and diagnostic findings.

<table>
<thead>
<tr>
<th>Study, year of publication</th>
<th>Number of RA patients</th>
<th>RA patients origin</th>
<th>RA stage</th>
<th>Number of controls</th>
<th>Controls composition</th>
<th>Detection method</th>
<th>Sensitivity/Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nissinen et al., 2003 [13]</td>
<td>57/51</td>
<td>NP</td>
<td>57 early (baseline and follow up 3 years later); 51 established</td>
<td>172</td>
<td>43 SLE, 19 pSjS, 20 MS, 90 HI (BD)</td>
<td>ELISA</td>
<td>Early: 88% at baseline, 70% at follow up; Established: 22% 50% 95%</td>
</tr>
<tr>
<td>Takizawa et al., 2005 [103]</td>
<td>42</td>
<td>NP</td>
<td>Established</td>
<td>82</td>
<td>19 SLE, 23 other rheumatic diseases, 40 HI</td>
<td>ELISA and WB</td>
<td>31% all patients (25% MTX, 35% non-MTX) 97%</td>
</tr>
<tr>
<td>Roth et al., 2006 [104]</td>
<td>184</td>
<td>Rheumatological Unit of Malmo University Hospital</td>
<td>Early</td>
<td>59</td>
<td>67 SLE, 48 pSjS, 41 SSc, 34 OA, 23 DM/PM, 19 AS, 106 HI (DB)</td>
<td>ELISA</td>
<td>50% 97%</td>
</tr>
<tr>
<td>Zhao et al., 2008 [105]</td>
<td>109</td>
<td>Department of Rheumatology and Immunology, People's Hospital, Peking University, Beijing</td>
<td>Established</td>
<td>338</td>
<td>67 SLE, 48 pSjS, 41 SSc, 34 OA, 23 DM/PM, 19 AS, 106 HI (DB)</td>
<td>ELISA</td>
<td>42%</td>
</tr>
<tr>
<td>Harris et al., 2008 [106]</td>
<td>38/129</td>
<td>John Hopkins Arthritis Center/ESCAPE RA TRIAL</td>
<td>Established</td>
<td>158</td>
<td>32 HI, 31 myositis, 31 SSc, 32 SjS, 32 SLE</td>
<td>IP</td>
<td>42%/36%</td>
</tr>
<tr>
<td>Halvorsen et al., 2008 [102]</td>
<td>237/177</td>
<td>EURIDISS RA cohort/Oslo RA Register</td>
<td>Established</td>
<td>232</td>
<td>84 SLE, 148 HI</td>
<td>ELISA</td>
<td>22%/25% 91.4%</td>
</tr>
<tr>
<td>Halvorsen et al., 2009 [107]</td>
<td>40</td>
<td>NP</td>
<td>Established [at baseline (n = 40) and follow up after one year (n = 33)]</td>
<td>NP</td>
<td>NP</td>
<td>ELISA</td>
<td>42.5% at baseline, 45.5% at follow up</td>
</tr>
<tr>
<td>Auger et al., 2009 [108]</td>
<td>116</td>
<td>Rheumatology Unit La Conception Hospital, Marseille, France</td>
<td>Established</td>
<td>93</td>
<td>33 A5, 60 HI</td>
<td>ELISA</td>
<td>29% 98%</td>
</tr>
<tr>
<td>Koffenbach et al., 2010 [109]</td>
<td>83</td>
<td>Military Cohort – Walter Reed Army Medical Center Rheumatology Clinic</td>
<td>Preclinical</td>
<td>83</td>
<td>83 HI</td>
<td>IP</td>
<td>18% 99%</td>
</tr>
<tr>
<td>Wang et al., 2011 [110]</td>
<td>102</td>
<td>NP</td>
<td>Established [with active disease (n = 50), without active disease (n = 52)]</td>
<td>239</td>
<td>84 SLE, 35 pSjS, 20 SSc, 100 HI</td>
<td>ELISA</td>
<td>32.4% 96%</td>
</tr>
<tr>
<td>Ishigami et al., 2013 [111]</td>
<td>32</td>
<td>Department of Rheumatology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology</td>
<td>Established</td>
<td>30</td>
<td>10 OA, 20 HI</td>
<td>ELISA</td>
<td>37.5% 100%</td>
</tr>
<tr>
<td>Ferucci et al., 2013 [112]</td>
<td>82</td>
<td>Two indigenous North American populations in Canada and the United States; First Nations or Alaska Native people</td>
<td>Established</td>
<td>191</td>
<td>147 FDR, 44 HI</td>
<td>IP</td>
<td>29.3% 98.90%</td>
</tr>
<tr>
<td>Darrah et al., 2013 [11]</td>
<td>194</td>
<td>ESCAPE RA Cohort</td>
<td>Established</td>
<td>66</td>
<td>36 HI, 30 PsA</td>
<td>IP</td>
<td>37.1% NP</td>
</tr>
<tr>
<td>Reyes-Castro et al., 2015 [113]</td>
<td>170</td>
<td>Rheumatology service of two hospitals in Jalisco, Mexico</td>
<td>Early and established</td>
<td>103</td>
<td>103 HI</td>
<td>ELISA</td>
<td>24% (18% early, 29% established) 95%</td>
</tr>
<tr>
<td>Umeda et al., 2015 [114]</td>
<td>148</td>
<td>University of Tsukuba Hospital</td>
<td>Established</td>
<td>113</td>
<td>36 SLE, 37 SjS, 40 HI</td>
<td>ELISA</td>
<td>20% 89%</td>
</tr>
<tr>
<td>Navarro-Millan et al., 2016 [115]</td>
<td>192</td>
<td>CLEAR Registry</td>
<td>Early and established</td>
<td>NP</td>
<td>NP</td>
<td>IP</td>
<td>24% NP</td>
</tr>
<tr>
<td>Martinez-Prat et al., 2018 [116]</td>
<td>640</td>
<td>MTX PATH, IMPACT and CAPITAL studies</td>
<td>Early and established</td>
<td>833</td>
<td>369 SLE, 64 SjS, 33 SSc, 29 IIM, 14 OAARD, 85 FMS, 42 OAID, 197 HI</td>
<td>PMAT</td>
<td>35% (16% Early) (99.1% early)</td>
</tr>
</tbody>
</table>

(Continued)
confirmed the presence those antibodies in RA patients (Table 3) [9–11,102–119]. Anti-PAD4 antibodies are usually found in a subgroup of RA patients with a prevalence of 20–45% and are associated with the presence of ACPA [11,102,117,120]. Meta-analysis [121] showed a pooled sensitivity of 38.0% with 96.0% specificity of anti-PAD4 antibodies in RA, and good discrimination between RA patients and controls has consistently been observed. Several studies have looked into the presence of anti-PAD4 antibodies in independent cohorts, summarized in Table 3. Interestingly, anti-PAD4 antibodies have been found in ACPA negative patients, suggesting a utility to help close the serological gap in RA [116].

The effect of the autoantibodies on the enzyme functionality and activity is not completely understood (Figure 2). Some data indicate that anti-PAD4 antibodies seem to inhibit the enzymatic activity of the protein [9]. In the same study, peptides located in both the N-terminal domain (211–290) and the C-terminal domain (601–650) of protein were recognized and information provided. The exact location, sequence, and structure of the epitopes recognized by anti-PAD4 antibodies still need to be fully understood.

Interestingly, anti-PAD4 can precede the onset of disease and are found in the pre-clinical phase in a subset of RA patients [109]. The exact timing of appearance of anti-PAD4 antibodies during the evolution of RA still requires systematic studies. In a study using a military cohort with samples collected over decades, it was demonstrated that anti-PAD4 antibodies might occur after ACPA and RF. Since immunoprecipitation was used for the measurement of anti-PAD4 antibodies which sometimes lacks sensitivity, those findings should be verified with newer methods such as ELISA or the recently developed PMAT system [116]. Only then it will be possible to conclude whether anti-PAD4 antibodies arise after ACPA and RF and whether they hold value in the prediction and potential prevention of RA [122]. The finding that anti-PAD4 antibodies might appear later is also somewhat controversial to the findings that immunization with PAD enzymes can stimulate the production of ACPA in mice [123]. However, this might be explained by the fact that animal models not always translate to human disease, especially when generated via immunization (vs. spontaneous models). Additional insights will also come from the sequencing of the B-cell repertoire of RA patients and the subsequent analysis of potential mutations [124,125]. Pollman et al. [120] studied the levels of anti-PAD4 in RA patients over a period of 10 years and demonstrated that anti-PAD4 positive patients remained positive over time, and some patients that initially did not present these antibodies became positive later in the disease course.

The potential effect of treatment on the levels of anti-PAD4 is also an area of interest. Limited data have indicated that anti-TNF-alpha therapy does not impact the levels of anti-PAD4 antibodies over a 12-month period time and the researchers suggested that this could be indicative of a specific phenotype characterized by inadequate response
to this treatment type [120]. Somewhat contrary to these findings, Darrah et al. recently reported that anti-PAD4 positive patients are characterized by a worse radiographic joint damage at baseline, but a more favorable response to treatment escalation therapy, which was more effective in slowing the progression of the disease and decreasing disease activity [118]. Significant differences in study design between these two studies could explain the discrepant outcomes. Additional studies in larger cohorts treated with drugs with different mechanism of action are needed to further investigate a potential effect of therapy on these antibodies and their associations with treatment response.

The discovery and characterization of a PAD enzyme expressed by the primary periodontal pathogen Porphyromonas gingivalis, known as the as PPAD, together with the fact that periodontitis is a known RA risk factor RA, formed the basis for the hypothesis that PPAD may represent a mechanistic link between periodontitis and RA [126]. Several studies have suggested that PPAD can perform protein citrullination at the inflamed periodontal sites and trigger a cascade of events that can lead to NET formation, generation of citrullinated neoantigens, and ACPA production [127]. The link between these events and the clinical onset of RA remains an area of interest. Recently, antibodies to PPAD were described in RA patients [128]; however, the association between these antibodies, ACPA and their potential role in the development of RA needs to be further investigated.

4.2. Antibodies to other PAD enzymes

Besides PAD4, PAD2 and PAD3 have also been described as targets of autoantibodies in patients with RA. Compared to anti-PAD4 antibodies, the body of literature on the autoantibodies to other PAD enzymes is scarce. In addition, although no studies have described antibodies targeting PAD1 or PAD6, it is likely that those proteins serve as autoantigens in RA. In the section below, the current knowledge on anti-PAD2 and anti-PAD3 antibodies is summarized.

4.2.1. Anti-PAD2 antibodies

Several factors point to PAD2 as an important driver in RA, including the association of PAD2 polymorphism with the development of the disease, the expression of this enzyme in the tissue and synovial fluid from inflamed joints and for its capacity to generate citrullinated autoantigens [81,129–131]. Yet, PAD2 had not been identified as a target of the immune response in RA until very recently, when Darrah et al. described for the first time antibodies targeting this protein in the sera of RA patients [12]. In this pilot study, in contrast to anti-PAD4 or the anti-PAD3/4XR antibodies, anti-PAD2 antibodies seemed to be characteristic of a genetically and clinically distinct subtype of RA patients with less severe baseline joint inflammation, slower joint disease progression, and less lung disease. Together with the lack of association between anti-PAD2 and ACPA and the low overlap with anti-PAD3/4XR antibodies observed, these data suggest that these antibodies define a different subpopulation of RA patients, potentially with milder progress and better outcome. Further studies are needed to verify the interesting but preliminary findings.

4.2.2. Anti-PAD3 antibodies

In 2013 PAD3 was also identified as an antigenic target in RA [11]. Anti-PAD3 antibodies are present in 10-20% of the RA patients. Similar to anti-PAD4, anti-PAD3 antibodies are also associated with ACPA and the HLA-DRB1 SE [11]. Experiment competition demonstrated that this subset of anti-PAD3 antibodies cross-react with PAD4, leading to the identification of a cross-reactive epitope in these two enzymes. Although the characteristics of these antibodies remained to be further understood, data suggests that affinity maturation has a role in defining the function of these antibodies [132]. It was demonstrated that these anti-PAD3/4 cross-reactive (anti-PAD3/4XR) antibodies mimic the calcium-ion binding to the enzyme and increase the catalytic efficiency of PAD4 by decreasing the calcium concentration needed for its activation (Figure 2). Thus, they support protein citrullination at physiologically relevant calcium concentrations and therefore, represent important drivers of dysregulated protein citrullination and RA pathogenesis.

5. Clinical associations of anti-PAD4 antibodies

The high number of ‘seronegative’ RA patients, with negative RF and/or ACPA, have driven the demand for novel diagnostic biomarkers. In the last few years, anti-PAD4 antibodies have been identified in between 2% and 17.7% of seronegative RA patients in numerous cohorts [105,116,133]. However, the utility of biomarkers goes beyond diagnosis, and several clinical studies have recently focused on the clinical associations and the prognostic value of anti-PAD4 antibodies (Table 4). Nevertheless, the majority of these studies were cross-sectional in design, limiting their power to determine a temporal association between the clinical feature and the presence of anti-PAD4 antibodies.

5.1. Erosions, radiographic progression, and clinical activity

Out of the 13 identified studies, 10 described the presence of erosive disease [11,102,105–107,109,115,117,118,120,134] and/or longitudinal radiographic progression [102,107,115,118,120] in patients with anti-PAD4 antibodies. Additionally, seven studies found a higher modified Sharp score and overall more erosive disease at the time of recruitment in patients with anti-PAD4 antibodies when compared to PAD4 negative patients [102,105,106,117,118,120,134]. In the three studies focused on the cross-reactive anti-PAD3/4XR antibodies, the presence of the cross-reactive antibodies was strongly associated with baseline erosive disease and more severe radiographic progression [11,115,117].

Two studies evaluated response to treatment (DMARDs, biologics, and glucocorticoids) in terms of radiographic progression of anti-PAD4 and anti-PAD3 positive versus negative patients [11,118]. In 2013 Darrah and colleagues suggested that patients with both anti-PAD4 and anti-PAD3 antibodies represent a subset individuals with more erosive disease and severe radiographic damage despite therapy [11]. A few years later, the very same group conducted a prospective study and demonstrated that anti-PAD4 antibodies may represent
a biomarker for better response to therapy despite more aggressive erosive disease at baseline [118].

Clinical activity in RA was generally assessed by erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and/or disease activity score (DAS) 28 [102,105,113,115,118,119,134]. Overall, only Zhao and colleagues found higher levels of ESR and DAS-28 in patients with anti-PAD4 antibodies, whereas the other studies found no differences between the two groups, except for higher CRP in patients positive for both anti-PAD4 and anti-CCP antibodies [102]. Surprisingly, Cappelli et al. found lower CDAI score in patients with anti-PAD3/4XR antibodies [117].
**Table 4.** Summary of studies on anti-PAD4 antibodies and clinical associations.

<table>
<thead>
<tr>
<th>Study, year of publication</th>
<th>Erosive disease</th>
<th>Radiographic progression</th>
<th>CRP</th>
<th>ESR</th>
<th>DAS28</th>
<th>Rheumatoid nodules</th>
<th>Physical disability</th>
<th>Lung involvement</th>
<th>Response to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-PAD4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harris et al., 2008</td>
<td>More</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Halvorsen et al., 2008</td>
<td>More</td>
<td>Yes</td>
<td>Higher, but related to ACPA presence</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>More severe</td>
<td>NA</td>
</tr>
<tr>
<td>Zhao et al., 2008</td>
<td>More</td>
<td>NA</td>
<td>Higher</td>
<td>Higher</td>
<td>Higher</td>
<td>More</td>
<td>NA</td>
<td>No difference</td>
<td>NA</td>
</tr>
<tr>
<td>Halvorsen et al., 2009</td>
<td>More</td>
<td>More</td>
<td>No difference</td>
<td>No difference</td>
<td>Higher</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Kolenbach et al., 2010</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pollmann et al., 2012</td>
<td>More</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Darrah et al., 2013</td>
<td>More, but only for anti-PAD3/4XR</td>
<td>Yes, but only for anti-PAD3/4XR</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>NA</td>
<td>No difference</td>
<td>More, but only of anti-PAD3/4XR; no difference for anti-PAD4</td>
</tr>
<tr>
<td>Giles et al., 2014*</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Reyes-Castillo et al., 2015</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Navarro-Millán et al., 2016*</td>
<td>More, but only for anti-PAD3/4XR</td>
<td>Yes, but only for anti-PAD3/4XR</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cappelli et al., 2018</td>
<td>More, but only for anti-PAD3/4XR</td>
<td>More at baseline</td>
<td>Better response to therapy</td>
<td>NA</td>
<td>No difference</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>Better</td>
</tr>
<tr>
<td>Darrah et al., 2018</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Guderud et al., 2018</td>
<td>NA</td>
<td>NA</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Anti-PAD2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seanan et al., 2016</td>
<td>More, but only for high serum levels</td>
<td>No difference</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Anti-PAD3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darrah et al., 2018</td>
<td>No difference, but few swollen joints</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>No difference</td>
<td>Less, but without statistical significance</td>
<td>No difference</td>
<td>No difference</td>
</tr>
</tbody>
</table>

The items ‘more’, ‘higher’ and ‘no difference’ are referred to the comparison with anti-PAD negative patients. When multivariate analysis is present, only this item is taken into consideration, while crude analysis was excluded.

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score 28; NA, not analyzed; ACPA, anti-citrullinated protein antibodies.
The presence of rheumatoid nodules may be associated with a more aggressive disease [135]. Zhao et al. evaluated this extra-articular manifestation of RA in their cohort, finding a positive association of rheumatoid nodules with anti-PAD4 antibody positivity [105].

More than 25% of the RA patients are at risk of a certain grade of physical impairment and identification of high-risk patients needing early and aggression therapy is needed [136]. Three studies assessed the correlation between anti-PAD4 levels and the presence of physical disability, with conflicting results [102,119,134]. However, longitudinal studies that look into this aspect are still lacking.

It is important to mention that certain differences between the studies could represent a limitation in these comparisons. These include the use of different methods for the measurement of anti-PAD antibodies, different scales for measurement of erosions and radiographic progression and even different scores for the evaluation of joint erosion. Joint space narrowing score and modified Sharp score were used in the majority, whereas some used ad hoc erosion scores.

5.2. Lung involvement

Lung involvement is common in RA and might affect the airways, pleura, parenchyma, and vasculature. Interstitial lung disease (ILD) in patients with RA is the second most common cause of death, after cardiovascular disease [137]. To date, two studies have investigated the relationship between anti-PAD4 antibodies and lung involvement, and one focused on ILD. Zhao and colleagues found no differences among patients positive or negative for anti-PAD4 for pulmonary fibrosis. A cross-sectional study by Giles and colleagues found that anti-PAD3/4XR antibodies were associated with ILD; however, no correlation with abnormalities of pulmonary function tests or the reported respiratory symptoms was observed [134]. No correlation was found for patients with anti-PAD4 monospecific antibodies. It is important to note, however, that anti-PAD3/4XR positive individuals presented a longer standing disease, limiting any ability to establish temporality in this association.

Little is known about whether there is an etiological association of cigarette smoking and the development of these antibodies. Although differences in terms of smoking profile between the anti-PAD4 positive and negative patients have been reported [11,134], very recent data have indicated that there is not a direct link between smoking and the development of anti-PAD4 antibodies [117]. The data around this topic are quite controversial and systematic studies are needed to better understand the link between smoking and the development of anti-PAD antibodies.

5.3. Clinical associations of antibodies to other PADs

5.3.1. Anti-PAD3

Only one cross-sectional study investigated the presence of anti-PAD3 alone in RA patients, showing that only higher levels of these antibodies correlated to the presence of erosion [138]. Nevertheless, disease duration at the time of sampling was not assessed in this study and very limited clinical information was available on these patients.

5.3.2. Anti-PAD2

Very recently, Darrah and colleagues reported that higher levels of anti-PAD2 antibodies are associated with fewer swollen joints, but no difference was found regarding the presence of erosions. Only five patients positive for anti-PAD2 antibodies presented ILD at CT-scan and their ILD-score assessed by an expert radiologist resulted significantly lower than the control group [12].

6. Anti-PAD4 antibodies and precision medicine

The knowledge and understanding of RA have evolved significantly during the last decades which help to improve patient care transforming RA from a destructive and disabling disease with limited therapeutic options, to a disease for which remission is an achievable goal through early intervention, control of inflammation and prevention of joint destruction [139,140]. Opportunities for PM approaches in RA are emerging but its complete implementation will require a paradigm shift in several aspects, including the use and application of autoantibodies and other biomarkers.

In this context, anti-PAD4 antibodies represent very interesting serological tools. Although more studies are needed, similarly to RF IgM and ACPA, they seem to precede onset of RA, so they could be useful in disease prediction and earlier diagnosis [109]. Furthermore, anti-PAD4 antibodies can be found in ACPA negative patients and can help to close the serological gap [116,133]. Consequently, it could be anticipated that multi-parametric approaches that integrate ACPA and RF, and that this novel biomarker will be helpful in improving diagnosis. In addition, anti-PAD4 antibodies can help stratify patients based on risk for erosion and ILD potentially guiding therapeutic decisions [11,118,134]. However, whether they can help predict or monitor response to treatment still needs to be investigated.

Several treatment options targeting different pathways are currently available; however, many patients still do not respond or achieve remission with current therapies [139]. Therefore, there is a need for new treat-to-target strategy and new therapies. In this respect, PAD inhibitors are emerging as a new class of drugs to treat RA [15,16,98,141,142], and anti-PAD antibodies could represent useful biomarkers to stratify patients for prediction of response to this new therapeutic approach.

Hence, anti-PAD4 antibodies can be helpful in addressing several needs in RA and represent a useful biomarker to facilitate the implementation of PM approaches in RA.

7. Conclusion

In conclusion, there is a growing body of evidence that anti-PAD4 antibodies represent a promising biomarker in the diagnosis and stratification of RA. In particular, the presence in ACPA/RF negative individuals holds promise to help close the serological gap. In addition, the validated association with joint erosions indicates that anti-PAD4 antibodies might have utility beyond the diagnosis and allow for stratification of patients according to the disease severity. Less is known about autoantibodies to the other PAD enzymes. While some studies have looked at antibodies targeting PAD2 and PAD3,
no study has reported antibodies to PAD1 or PAD6 until today. Further research is needed to better understand the role of anti-PAD antibodies in RA and their clinical significance.

8. Expert opinion

Although known for almost 20 years now, anti-PAD4 antibodies are not used in clinical practice for the diagnosis and management of RA patients. This is mostly related to the lack of standardized and validated immunoassays for the detection of anti-PAD antibodies. However, based on the growing body of evidence on the relevance of anti-PAD antibodies in RA and their potential utility in the diagnosis of RA, it is likely that commercial assays will become available. From the recent data, the main benefit of anti-PAD4 antibodies lies in the detection of early RA patients that are negative for the classification criteria markers ACPA and RF as well as in the identification of patients with more aggressive disease the manifest in joint erosion and damage. These findings were recently summarized in meta-analysis performed by different groups and using different approaches [121,143]. It was concluded that anti-PAD4 antibodies can be found in about 35% of the RA patients accompanied with a specificity of more than 95%. In the patients negative for ACPA and RF, the sensitivity decreases, but still provides high OR for the differentiation between RA and controls. In light of the trend toward precision medicine, it is important to mention that testing for anti-PAD4 antibodies also has the potential to define a subset of RA patients that benefit from treatment escalation. Based on the different associations of antibodies to the other PAD isoforms (e.g. PAD2 and PAD3), multi-analyte testing for anti-PAD antibodies might provide a useful tool for patient stratification and PM. According to current knowledge, patients with anti-PAD4 and especially anti-PAD3/4XR might benefit from more aggressive treatment, whereas patients positive for anti-PAD2 antibodies might present a more benign form which would require less attention. The recent study by Darrah et al. [118] indicated that treatment escalation through additional DMARDs including TNFα inhibitor provided benefits to anti-PAD4 positive patients. Whether this also applies for other DMARDs available on the market today or under development remains unclear. Of special interest could be the combination of anti-PAD4 testing with potential anti-PAD inhibitors as a companion or complementary diagnostic approach. However, further studies are required and assays need to be developed and taken through regulatory scrutiny (including but not limited to CE mark and FDA clearance).

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Declaration of interest

L Martinez-Prat and M Mahler are employees of Inova Diagnostics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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Papers of special note have been highlighted as of interest (-) to readers.


16. Willis VC, Banda NK, Cordova KN, et al. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced


- **Meta-analysis that summarizes the diagnostic performance of anti-PAD4 antibodies in RA.**


- In this study, anti-PAD antibodies and ACPA were detected in mice after immunization with PAD2 or PAD4. These results are of relevance in the context of understanding the role of the PAD enzymes and the anti-PAD antibodies in RA.


