

# The Central Role of Acute Phase Proteins in Rheumatoid Arthritis: Involvement in Disease Autoimmunity, Inflammatory Responses, and the Heightened Risk of Cardiovascular Disease

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## Abstract

Rheumatoid arthritis (RA) is an autoimmune disease of complex etiopathogenic origin and traditionally characterized by chronic synovitis and articular erosions. Furthermore, there is strong evidence that infectious agents, including those that become dormant within the host, play a major role in much of the etiology of RA and its hallmark of inflammation. A combination of genetic predisposition, environmental exposure, and presence of infectious agents may therefore lead to a loss of immune tolerance to citrullinated proteins, which present as self-antigens to the human immune system. This results in generation of highly RA-specific autoantibodies, known as anti-citrullinated protein antibodies (ACPAs). Protein citrullination occurs via posttranslational deamination of arginine residues by peptidylarginine deiminase enzymes, which have confirmed sources of both endogenous and infectious origins. A recognized plasma protein target of citrullination and RA autoantibody generation is fibrin and its soluble precursor fibrinogen, both key components of hemostasis and acute phase reaction. Increased titers of ACPAs that accompany rapid progression to clinical RA disease have been shown to drive a variety of proinflammatory processes, and therefore results in aberrant fibrin clot formation and increased cardiovascular risk. However, the full extent to which hemostasis is affected in RA remains controversial, owing to the differential impact that citrullinated fibrin(ogen) and concurrent systemic inflammation may have on resulting hemostatic outcome. This review highlights key events in initiation of autoimmune-driven inflammatory events, including the role of bacterial infectious agents, which subsequently result in clinical RA disease and associated secondary cardiovascular disease risk, with specific focus on plasma proteins that are heavily involved throughout the immunopathological progression process.

## Keywords

- ▶ rheumatoid arthritis
- ▶ hemostasis
- ▶ coagulation
- ▶ citrullination
- ▶ periodontitis

Rheumatoid arthritis (RA) is the most common autoimmune disease, displaying a worldwide prevalence of 0.5 to 1%.<sup>1,2</sup> RA typically has a middle-aged onset that disproportionately affects females (threefold higher prevalence than males).<sup>3</sup> Symptomatic RA is characterized by chronic systemic inflammation, resulting in bilateral and symmetrical polyarticular

arthritis, persistent erosive synovitis, and development of autoantibodies.<sup>1,4</sup> The clinical manifestations of chronic inflammation in RA are most commonly observed in the small joints of hands and feet.<sup>5</sup> Common disease symptoms include pain, swelling of the joint, stiffness, and progressive destruction of joint structure.<sup>6</sup> Irreversible damage to joint structure

and subsequent loss of mobility can occur within 3 years of diagnosis in 20 to 30% of RA patients when appropriate treatment measures are not undertaken.<sup>7</sup>

RA is considered to be a prototypic polygenic disorder, as disease development is thought to occur as a result of the interaction between predisposing genetic risk factors and environmental exposures.<sup>8</sup> The strongest genetic link associated with RA is the single nucleotide polymorphisms in the human leukocyte antigen (HLA)-DR $\beta$ 1 gene, which in some study cohorts have shown to be present in approximately 64 to 70% of RA patients and 55% of their first-degree relatives.<sup>9,10</sup> HLA molecules belong to the major histocompatibility complex (MHC) and is strongly linked to anti-citrullinated protein antibodies (ACPA)<sup>+</sup>-RA.<sup>5</sup> HLA-DRB1 genes exert major influence on the cluster of differentiation 4 (CD4)<sup>+</sup>  $\alpha\beta$  T cell repertoire, through their “shared epitope” (SE) alleles that present as self-peptides to CD4<sup>+</sup> T-cells in the thymus.<sup>5,11</sup>

The most compelling current explanation for the development and perpetuation of clinical RA disease is the infection of certain mucosal sites (oral, intestinal, and pulmonary) where microbes, under the influence of environmental factors such as smoke and drugs along with genetic predisposition, trigger mucosal inflammation.<sup>12</sup> Subsequent migration of mucosal macrophages and lymphocytes to articular spaces results in synovial inflammation and joint destruction.<sup>13</sup> Research by Rashid and Ebringer has strongly linked the bacterium *Proteus mirabilis* to the development of RA,<sup>14</sup> stemming from an increased incidence rate of urinary tract infections (UTIs) observed in affected individuals.<sup>15</sup> *P. mirabilis* is a major causative agent of UTIs and is detected to a greater extent in RA patient urine, along with increased levels of antibodies directed toward the bacterium.<sup>16</sup> The closely matched amino acid sequences of “E-S-R-R-A-L” contained in *Proteus spp.* hemolysins and “E-Q/K-R-R-A-A” in the “SE” of RA-linked HLA-DR molecules result in the binding of cross-reactive anti-*P. mirabilis* antibodies to various “SE” containing immune cells, causing a plethora of proinflammatory responses related to clinical RA disease.<sup>17</sup> The nature of the *Proteus spp.* relationship with RA possibly explains the disproportionate occurrence of disease in middle-aged women, owing to inherent difference in the genitourinary tract resulting in higher frequencies of UTIs in females.<sup>18</sup>

Lipopolysaccharides (LPS) are major endotoxin components of gram-negative bacterial cell walls, and act as strong pathogen-associated molecular patterns (PAMPs) to stimulate various innate immune responses.<sup>19</sup> High levels of LPS are present within the gut lumen as a result of numerous residing commensal bacteria, and LPS is normally unable to penetrate across healthy intestinal epithelium.<sup>20</sup> However, LPS-mediated inflammation and breach of intestinal permeability occurs when homeostasis of the normal gut microbiome is disturbed.<sup>21</sup> LPS is therefore able to enter blood circulation and contribute to various systemic inflammatory processes. The disruption of the normal gut microbiota, known as gut dysbiosis, is highly prominent in RA patients and could therefore contribute significantly to the chronic inflammatory state.<sup>22,23</sup> LPS ligates with toll-like receptor (TLR-4) leading to the production of various proinflammatory cytokines (tumor necrosis factor  $\alpha$  [TNF $\alpha$ ], interleukin-6

[IL-6], interleukin-8 [IL-8], interleukin-12 [IL-12]) through the nuclear factor kappa B (NF $\kappa$ B) signal transduction pathway.<sup>24</sup> TLR-4 is expressed on various immune cells involved in RA inflammatory signaling.

The high levels of LPS associated with gut dysbiosis, which is common in RA patients, might also influence the hypercoagulable state seen in a high proportion of affected individuals. As discussed previously, LPS stimulate the release of proinflammatory cytokines via TLR-4 dependent pathways. This process has been shown to facilitate the increased expression of tissue factor (TF) by immune cells, thereby amplifying coagulation via the TF pathway.<sup>25,26</sup> It has also been demonstrated that LPS, due to its highly lipophilic nature, is able to bind to fibrinogen and cause more acute fibrin polymerization.<sup>27</sup>

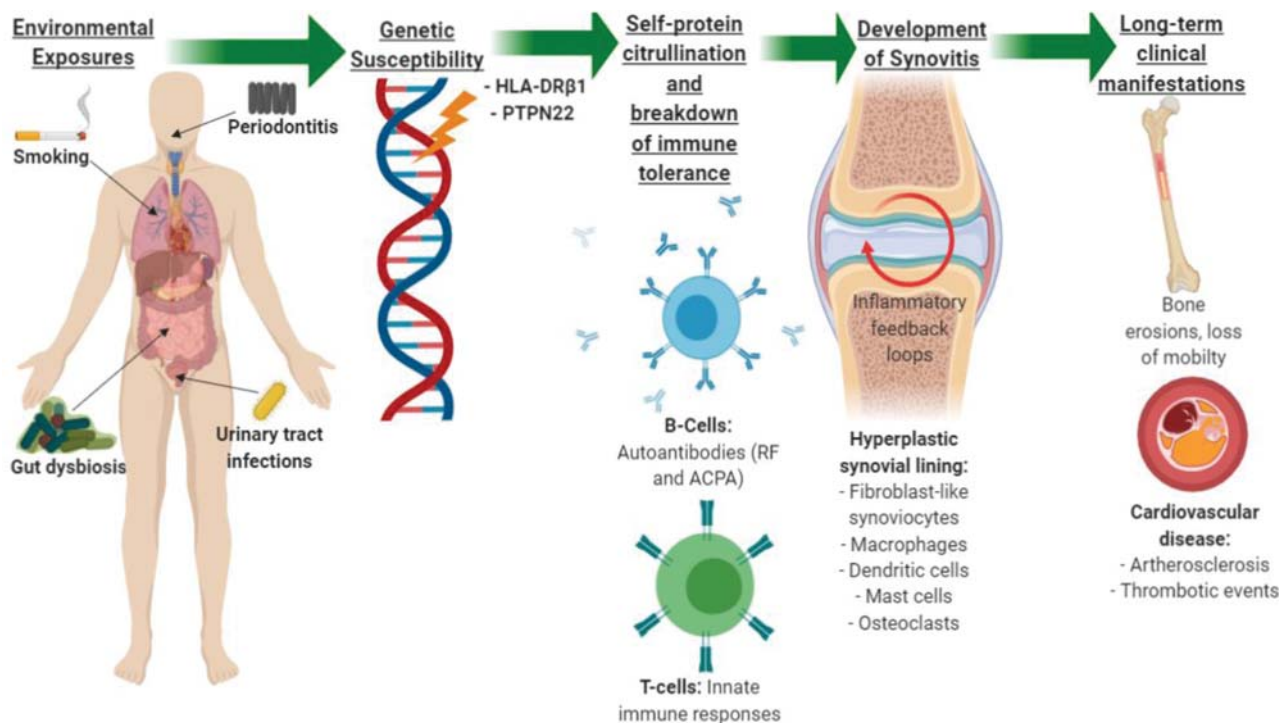
The general overview of the key immunopathogenic events in the clinical RA disease development is illustrated in **Fig. 1**. We consider RA pathogenesis within a systems biology approach, providing evidence of bacterial infectious agents generating the autoimmune inflammatory response, while emphasizing the importance of hematological biomarkers of acute systemic inflammation (hypercoagulation and  $\beta$ -amyloid formation) in disease diagnosis and treatment.<sup>28</sup>

## Cardiovascular Complications in Rheumatoid Arthritis

Together with the immunopathogenic profile in RA, there is evidence for increased cardiovascular disease (CVD) risk in these patients.<sup>29–31</sup> Conventional risk factors (age, sex, obesity, hypertension, and hypercholesterolemia) do not fully account for premature development of CVD in RA, thus indicating a prominent role for chronic systemic inflammation in alterations of hemostatic and thrombotic function.<sup>32</sup> Highly sensitive molecular markers of hemostatic function such as prothrombin factor 1 + 2 (PF<sub>1+2</sub>), thrombin-antithrombin complex (TAT), D-dimer, thrombin-activatable fibrinolysis inhibitor (TAFI), TF, and fibrinogen are significantly elevated in RA patients compared with healthy individuals.<sup>33–35</sup>

Increased prevalence of an activated platelet profile has been observed in RA patients.<sup>36</sup> Platelets have a dual role in affecting both inflammatory and hemostatic responses in RA. Activated platelets express P-selectin which binds soluble CD40 ligand (sCD40L), triggering the expression of inflammatory adhesion receptors, proinflammatory cytokines, and matrix metalloproteinases (MMPs).<sup>37</sup> Increased levels of platelet activity markers (sCD40L, P-selectin) are associated with RA disease activity.<sup>36</sup> In addition to stimulation of platelets by TNF $\alpha$ , ACPAs are also capable of directly influencing platelet activity through Fc $\gamma$ -RIIa-dependent binding.<sup>36</sup> Some proteins released from platelet granules are associated with formation of more compact fibrin clot structures (e.g., increasing concentrations of platelet factor 4 and factor XIII which have fibrin cross-linking properties).<sup>38,39</sup>

TF is a transmembrane glycoprotein that functions as a cellular receptor and an important initiator of coagulation.<sup>40</sup> TF expression is upregulated in the RA synovium and present on key proinflammatory mediators such as fibroblast-like synoviocytes, B-cells, and macrophages.<sup>41</sup> Proinflammatory



**Fig. 1** The outline of key events in the immunopathogenesis of rheumatoid arthritis (RA). (Original figure; created with Biorender.com).

cytokines (TNF $\alpha$ , IL-1) facilitate thrombus formation by stimulating the procoagulant activity of endothelial cells, leading to increased TF expression.<sup>42</sup>

Fibrin fiber generation and structure are abnormally altered in RA as a result of the underlying inflammatory processes.<sup>43</sup> Aberrant fibrin deposition in RA plasma as seen by scanning electron microscopy (SEM) is illustrated in **Fig. 2**. The increased levels of fibrin deposition contribute the structural integrity of pannus in RA synovia.<sup>44</sup> Pannus refers to the abnormal proliferation of synovial tissue lining, composed of stimulated fibroblasts, immune cells, and enriched vasculature.<sup>45</sup> The presence of pannus formation in the synovium contributes to perpetual stimulation of local inflammation and erosion of extra-articular cartilage and bone.<sup>45</sup>

Levels of hemostasis factors responsible for thrombus generation (fibrinogen, von Willebrand factor, plasminogen-activator inhibitor 1) are increased in RA patients.<sup>31,35</sup> Characteristics of fibrin clot structure in RA include thick, matted layers of fibrin fibers with increased diameter.<sup>43</sup> Unusual projections in RA patient fibrin fibers are also present owing to interactions with various proinflammatory mediators.<sup>34</sup> These ultrastructural alterations result in fibrin clots that are significantly less stable, permeable, and lysable compared with clots formed under physiological conditions.<sup>34</sup>

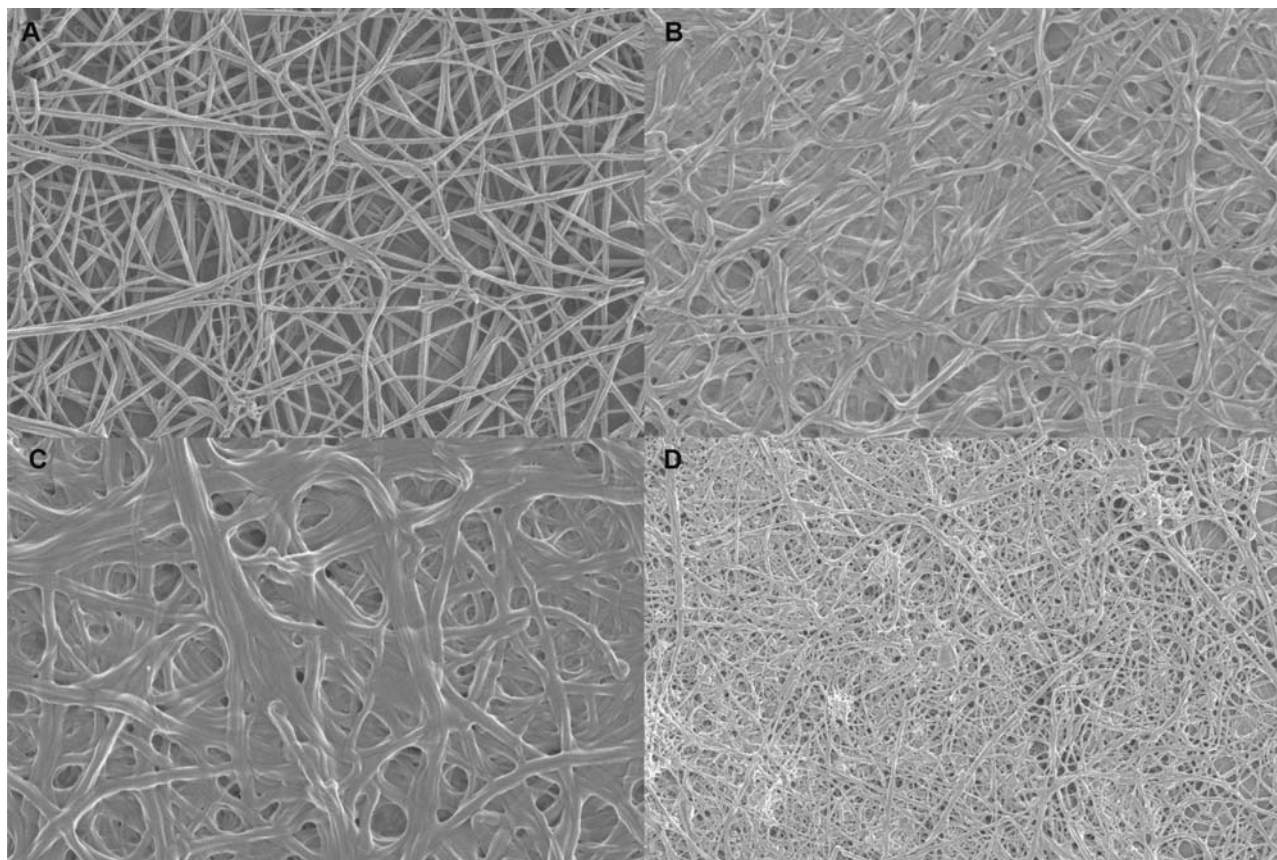
Increased levels of fibrinolysis biomarkers (i.e., D-dimer, PF<sub>1+2</sub>, TAT) have been associated with RA disease activity and inflammation.<sup>33,35</sup> However, inflammation also induced TAFI, which favors fibrin persistence and prevents the protein breakdown.<sup>35</sup> Previously described ultrastructural alterations also contribute to decreased breakdown of fibrin clots.<sup>34,43</sup> Citrullinated-fibrinogen immune complexes impair the activity of plasmin, leading to decreased levels of fibrinolysis and sustained synovial damage through proinflammatory effec-

tors.<sup>46</sup> These findings further support the notion of an impaired hemostatic balance of fibrin clot formation and breakdown in RA patients. CVD risk, inflammation, hypercoagulation, and immunopathology may be closely interlinked in RA pathology. The main immunological hallmarks and diagnostic biomarkers of RA are known to be rheumatoid factor (RF) and ACPAs. The development of autoimmunity in RA may be driven by a bacterial presence that may play important roles in the development of hypercoagulation in RA.

### Diagnostic Markers in Rheumatoid Arthritis with Specific Focus on Anti-Citrullinated Protein Antibodies

The presence of two notable autoantibodies in RA patient sera, namely RF and ACPA, is a hallmark characteristic of the disease. RFs were the first identified autoantibodies to be linked with RA and target the Fc portion of immunoglobulin G (IgG).<sup>47</sup> RF has very low specificity for RA, as it is detected in other rheumatic disorders and in healthy individuals,<sup>48,49</sup> whereas there is strong evidence for the role of ACPAs in RA immunopathogenesis.<sup>50</sup> However, the presence of both IgA-RF and ACPA in the preclinical phase is predictive of more rapid disease onset, with increased severity and decreased possibility of remission.<sup>51</sup> The concomitant effect of RF and ACPAs in RA patients stimulates *in vivo* proinflammatory cytokine production, which increases the risk of significant bone mineral density loss.<sup>52</sup> Recently, the cross-reactivity of RF with citrullinated fibrinogen, which is distinct from its primary IgG1-Fc target, has been reported.<sup>53</sup> This occurs as a result of sequence homology between the Fc and citrullinated fibrinogen epitopes, both of which contain arginine residues.<sup>53</sup> These findings could therefore mechanistically explain the





**Fig. 2** A series of scanning electron microscopy images illustrating the difference in fibrin clot structure of a healthy control (A) and RA patients (B–D). (Unpublished work by authors).

occurrence of more severe disease severity in RA patients with positive sera for both RFs and ACPAs.

Citrullination is a physiologically occurring process during apoptosis, inflammation, or epithelial keratinization.<sup>54</sup> The amino-acid arginine is posttranslationally modified by peptidylarginine deaminase (PAD) enzymes to form citrulline, a nonstandard amino acid.<sup>54</sup> Modification of proteins in this manner results in an increase of molecular mass (1 kDa compared with regular arginine residues)<sup>55</sup> and a reduction of the net positive charge, causing the loss of potential ionic bonds and interference of hydrogen bonds.<sup>56</sup> These conformational changes ultimately result in unfolding of protein structure.<sup>56</sup> Various protein targets for citrullination exist, including human fibrin(ogen),<sup>57</sup> histones,<sup>58</sup> vimentin,<sup>59</sup>  $\alpha$ -enolase,<sup>60</sup> and types I- and II collagen.<sup>61,62</sup> Citrullination enhances peptide affinity toward MHC molecules, leading to activation of CD4<sup>+</sup> T-cells.<sup>63</sup> ACPAs are then produced in response to the presence of citrullinated peptides or proteins.<sup>54,64</sup> ACPAs are highly specific to RA,<sup>65</sup> present prior to clinical disease onset,<sup>66</sup> and are indicative of more severe disease activity and rapid structural damage.<sup>67</sup>

#### Generation of Citrullinated Autoantigens

PADs are intracellular, Ca<sup>2+</sup>-dependent deaminase enzymes that are usually inactive under normal physiological conditions.<sup>68</sup> PAD enzymes become activated during large-scale apoptotic or inflammatory events as a result of plasma or

organelle membrane disintegration, causing extracellular Ca<sup>2+</sup> influx or PAD enzyme liberation from cellular compartments.<sup>69–71</sup> The abnormal humoral response to the presence of citrullinated proteins results from insufficient phagocytic clearance of apoptotic remnants under severe inflammatory conditions.<sup>70–72</sup> Five mammalian isoforms of PAD are known (PAD1, PAD2, PAD3, PAD4–5, PAD6), of which PAD2 and PAD4 are primarily responsible for the generation of autoantibodies in RA.<sup>4,73</sup> PAD2 and PAD4 are expressed by several peripheral blood leukocytes present in the RA synovium, in addition to performing regulatory functions related to gene expression, central nervous system development, and tumorigenesis.<sup>4,71,73,74</sup> PAD2 and PAD4 exhibit different subcellular localization and antigen target specificities in RA and are associated with the presence of inflammatory markers.<sup>75,76</sup> The most prominent protein targets for PAD-driven citrullination are human fibrin(ogen),<sup>57</sup> histones,<sup>58</sup> vimentin,<sup>59</sup>  $\alpha$ -enolase,<sup>60</sup> and types I- and II collagen.<sup>61,62</sup>

The primary environmental risk factors associated with RA development are infection<sup>76</sup> and smoking.<sup>77</sup> There has also been strong emerging evidence of the role of infectious agents in the initiation and progression of rheumatic diseases. The prevalence of periodontitis in RA patients (35% in all patients and 37% in ACPA<sup>+</sup>-RA patients) is significantly increased compared with individuals not diagnosed with RA.<sup>78</sup> Periodontitis has been associated with various RA disease markers, such as disease activity scores (DAS-28), C-reactive protein

(CRP), erythrocyte sedimentation rate, and anticyclic citrullinated peptide (CCP) antibodies.<sup>78–80</sup> Okada et al also demonstrated that clinical disease presentation is improved with periodontal treatment in RA patients.<sup>81</sup> Emerging evidence suggests that RA may develop at extra-articular mucosal sites (lungs and oral cavities), where exposure of HLA-DR $\beta$ 1 risk genes to environmental sources of inflammation (tobacco smoke and bacterial or viral infection) is most likely to occur.<sup>76</sup> This results in the generation of an aggressive autoimmune response and subsequent assault of self-tissues.<sup>76</sup>

### Anti-Citrullinated Autoantibodies

Synovial fluid-derived B cells are responsible for generating ACPAs in RA.<sup>82</sup> Varying degrees of ACPA cross-reactivity have been demonstrated, ranging from monoreactive antibodies to reactivity with multiple citrullinated antigens.<sup>83,84</sup> Li et al indicated that 66.7% of plasmablast-derived ACPAs from RA patients demonstrated cross-reactivity with different citrullinated epitopes, while 33% of the patients had monoreactive ACPA subsets that recognize either citrullinated fibrinogen, citrullinated enolase, or citrullinated vimentin.<sup>85</sup> The mode in which ACPAs target citrulline-containing peptide antigens is dependent on appropriate molecular motifs, specifically sequences in which citrulline residues are flanked by other small and neutral amino acids.<sup>86</sup>

In the preclinical stage of RA development, increased ACPA titers are accompanied by increased levels of epitope spreading to recognize a broader range of citrullinated antigens.<sup>87,88</sup> However, no further expansion of epitope recognition is observed after disease onset.<sup>89</sup> The presence and level of antigen recognition by ACPAs in the RA synovium determine the magnitude of the immune response.<sup>90</sup> The magnitude of the patient ACPA isotype profile is also strongly predictive of long-term radiographic progression.<sup>91</sup>

The presence of ACPAs represent a specified subgroup that constitutes 70 to 80% of all RA patients.<sup>74,75</sup> These observations indicate a strong role of ACPAs in development of clinical RA, ACPAs are therefore listed as an important criterion for RA disease classification in American College of Rheumatology/European League against Rheumatism (ACR-EULAR) guidelines.<sup>92</sup> The discovery of the first citrullinated antigens (filaggrin) and ACPAs (antikeratin factor and antiperinuclear factor) in RA<sup>93,94</sup> led to the development of enzyme-linked immunosorbent assay (ELISA)-based methods for detecting ACPAs.<sup>95</sup> Known as anti-CCP tests, these detection methods are considered the gold standard for detection of ACPA presence in RA patients. First generation anti-CCP tests displayed 68% sensitivity and 97 to 98% specificity for RA diagnosis,<sup>95</sup> with improved results in second generation anti-CCP2 tests (80% sensitivity and 98% specificity).<sup>96</sup> However, anti-CCP detection captures overlapping ACPA cross-reactivity and therefore cannot determine single ACPA-subtype specificity.<sup>83</sup>

### Fibrinogen in Rheumatoid Arthritis

Fibrinogen is a large (340 kDa) glycoprotein that is synthesized in the liver and circulates in blood at concentrations between 2 and 4 g/L.<sup>97</sup> The triglobular structure consists of paired  $\alpha$ -,  $\beta$ -, and  $\gamma$ -polypeptide chains linked by disulfide bonds via their N-

terminal regions.<sup>97–99</sup> Fibrinogen is the soluble precursor of insoluble fibrin, the terminal component of the common coagulation pathway with thrombin responsible for the proteolysis of fibrinogen.<sup>98,100</sup> Thrombin, a trypsin-like serine protease, cleaves fibrinogen between Arg-16 and Gly-17 of the A $\alpha$  chain and Arg-14 and Gly-14 of the B $\beta$  chain to expose polymerization sites, resulting in release of fibrinopeptide A and fibrinopeptide from their respective N-terminal regions.<sup>97,98</sup> Fibrin monomers polymerize spontaneously, mediated by activated factor XIII (FXIIIa) that cross-link individual fibrin molecules to form a complex, stable, and insoluble branching network of fibrin fibers.<sup>97</sup> In the event of vascular injury, fibrinogen is digested by thrombin, and generates clots consisting of dense fibrin fiber networks.<sup>43,98</sup> During systemic inflammatory states, characteristic of clinical RA, excessive fibrinogen generation and degradation occur.<sup>101</sup>

The role that fibrinogen and fibrin (in both native and citrullinated forms) play in RA disease development and progression will be extensively discussed further in this review, with specific focus on cardiovascular disease as a secondary risk in RA patients. Fibrinogen fulfils a unique role in rheumatic disease as it not only is involved in the conventional interplay between chronic local and systemic inflammation with dysregulated coagulation, but also actively contributes to the development of the autoimmune response. Insights into the role that fibrinogen/fibrin plays at each level of disease progression may provide the necessary knowledge to identify novel therapeutic targets and treatment strategies to alleviate severe disease symptoms.

As mentioned previously, there is a strong genetic link associated with RA, where the HLA-DR $\beta$ 1 gene is the most well-known implicated gene.<sup>9,10</sup> The extent to which citrullinated fibrinogen peptides may influence HLA-DR $\beta$ 1-mediated immune responses in RA remains unclear. Several studies observed no significant T-cell response or arthritogenic induction to citrullinated-fibrinogen immunization of HLA-DR $\beta$ 1 transgenic mice.<sup>100,102,103</sup> However, the presence of antibodies to citrullinated fibrinogen (ACF) is associated with human HLA-DR $\beta$ 1-0404\* allele along with early disease onset and radiographic progression.<sup>104</sup> The arthritogenic potential of citrullinated-fibrinogen in HLA-DR4 transgenic mice has also been reported by Rajmakers et al.<sup>55</sup> Possible reasons for these discrepancies include increased ACPA heterogeneity in human RA, increased number of sites available for citrullination on human fibrinogen, and differences between the T-cell repertoire of humans and murine models.<sup>103</sup> The humoral response in murine models is also dependent on the strain, immunogen, and the immunization protocol used.<sup>102</sup> Citrullinated fibrinogen has also been associated with the presence of protein tyrosine phosphatase, non-receptor type 22 (PTPN22) R620W risk alleles.<sup>100</sup> PTPN22 promotes the survival of B-cells in RA and other inflammatory conditions, such as type 2 diabetes, by evading tolerance checkpoints.<sup>105–107</sup>

### Fibrinogen and Fibrin Are Endogenous Sources of RA Autoantigens

As described previously, fibrin and fibrinogen are major targets for PAD citrullination and could therefore play a role

in the immunopathogenesis of RA.<sup>55,57,108</sup> RA is characterized by excessive generation and breakdown of fibrinogen, in addition to deposition of fibrin within the inflamed RA synovium, even in cases of well-controlled disease.<sup>109,110</sup> Increased plasma fibrinogen levels in RA have been reported to positively associate with disease activity scores and acute-phase markers such as CRP and serum amyloid A (SAA).<sup>110</sup> The presence of immune complexes containing citrullinated fibrin(ogen) and autoantibodies directed toward the citrullinated forms of fibrin(ogen) has been confirmed in human RA sera and synovial fluid in various studies.<sup>55,57,100,101,108</sup> The presence of circulating immune complexes containing citrullinated fibrinogen has been reported in half of anti-CCP<sup>+</sup> RA patients in a study by Zhao et al.<sup>111</sup> ACF antibodies have predominantly nonoverlapping reactivity, and target citrullinated fibrinogen peptides more favorably than native fibrinogen.<sup>100</sup> ACF-ELISA detection tests display similar performance to commercial anti-CCP2 assays and are highly predictive of rapid radiographic progression in terms of RA specificity.<sup>112,113</sup> The detection of both citrullinated fibrinogen antigens and autoantibodies directed toward such peptides, along with reported findings of arthritis development in certain murine models when immunized with both native and citrullinated forms of fibrinogen,<sup>102,114</sup> strongly suggests a pathogenic role for fibrinogen and fibrin in human RA.

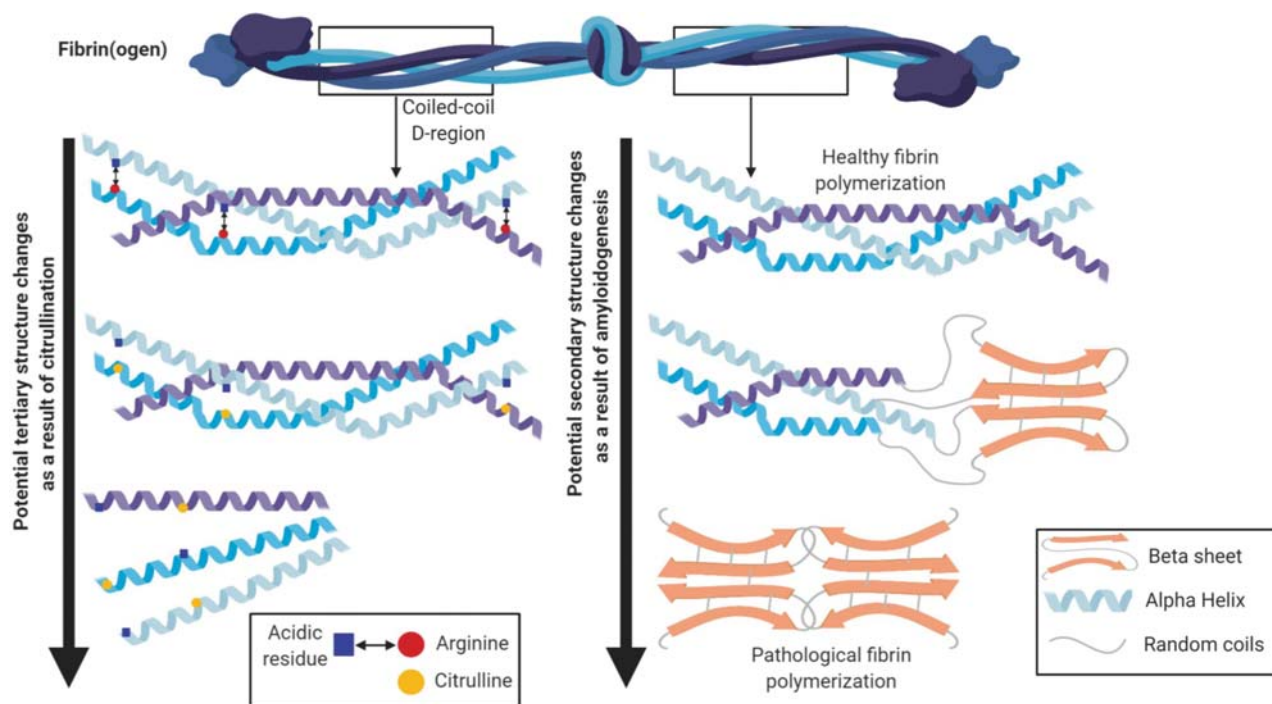
Deamination of arginine residues to citrulline leads to an increase in nominal molecular mass of 1 kDa per altered amino acid residue.<sup>55</sup> This leads to an overall molecular mass increase of 31 kDa in the  $\alpha$  fibrinogen subunit and 21 kDa in the  $\beta$  fibrinogen subunit, respectively.<sup>97</sup> Two-thirds of arginine residues in fibrinogen are susceptible to citrullination by both PAD2 and PAD4 isoforms, with the most prominent citrullination sites located on the  $\alpha$  (highest amount of target sites) and  $\beta$  fibrinogen subunits.<sup>46,97,115</sup> Fibrinogen  $\gamma$ -chains and fibrinogen-derived degradation products are not significantly targeted for citrullination by PAD enzymes.<sup>97</sup> PAD2 and PAD4 enzymes recognize different epitopes for citrullination in fibrinogen and fibrin peptide chains.<sup>116</sup> Studies have demonstrated that PAD2 is more efficient in deamination of arginine residues in fibrinogen and fibrin chains<sup>75</sup> and induces a significantly higher proinflammatory response<sup>117</sup> than PAD4. The enhanced efficiency of PAD2 for deaminating arginine residues is due to the enzyme being less restricted by the amino acid composition surrounding acceptor arginine residues in fibrinogen and fibrin compared with PAD4.<sup>75</sup> There is no significant discrimination of ACF-directed reactivity toward citrullinated peptides generated by either PAD2 or PAD4, as there is a great degree of overlap in the citrullination sites targeted by both PAD2 and PAD4.<sup>75</sup> The mapping of citrullinated epitopes in fibrinogen and fibrin has been conducted by a large number of studies. The identification of 42 citrullinated residues in fibrinogen (27 in the  $\alpha$ -chain, 11 in the  $\beta$ -chain and 4 in the  $\gamma$ -chain) currently represents the most comprehensive epitope list for citrullinated fibrinogen compiled thus far.<sup>115</sup> Fibrin contains a decreased amount of susceptible arginine residues for citrullination compared with soluble fibrinogen.<sup>4,46</sup> The major citrullination epitopes contained in fibrin have been reported as  $\alpha$ <sub>36-50</sub> and  $\beta$ <sub>60-74</sub>, respectively.<sup>46,57</sup>

Despite evidence of significantly increased levels of fibrin clot formation and fibrinolysis in various studies, limited research regarding the proteolysis of citrullinated fibrinogen peptides has been conducted. Paradoxical observations regarding the involvement of citrullinated fibrinogen in the thrombin-catalyzed fibrin polymerization reaction have been made in some literature. Studies by Nakayama-Hamada et al and Okumura et al found that citrullinated fibrinogen peptides are resistant to thrombin digestion under reverse phase high-performance liquid chromatography (HPLC) conditions.<sup>97,98</sup> The inhibition of thrombin-catalyzed fibrinogen polymerization most likely occurs due to the citrullination of arginine residues within the thrombin cleavage sites in  $\alpha$  and  $\beta$  fibrinogen chains, preventing thrombin binding.<sup>97,98,118</sup> SEM analysis by Okumura et al of fibrin clot structure comparing samples containing native fibrinogen and native fibrinogen with citrullinated fibrinogen in combination indicated no significant difference in terms of fibrin fiber density and diameter between samples.<sup>97</sup> These findings confirm that citrullinated-fibrinogen does not participate in thrombin-catalyzed fibrin clot formation. Citrullinated fibrinogen could therefore be regarded as an uncompetitive inhibitor of the thrombin reaction in the coagulation process.<sup>98</sup> This impairment may suggest an effect of citrullination on the hemostatic balance in RA patients, but this directly conflicts with multiple observations of excessive fibrinogenesis and fibrinolysis in previous studies. Possibly explaining this phenomenon is that excessive levels of thrombin in RA synovial fluid increase the likelihood that high amount of fibrinogen is polymerized within the inflamed synovium, despite the concurrent presence of deaminating enzymes.<sup>35,119</sup> The reduced fibrinolytic ability of plasmin has also been observed following fibrin citrullination.<sup>120</sup> Plasmin is a serine proteinase that cleaves peptide bonds near basic amino acid residues such as arginine, and therefore deamination will reduce the amount of potential degradation sites.<sup>120</sup>

### Possible Interaction between Protein Deamination and Amyloidogenic Clot Formation

As discussed earlier in this review, the deamination of arginine residues in plasma proteins such as fibrinogen is observable in RA patient sera and brings about a change in structural conformation. Arginine residues contribute to tertiary protein structure by forming noncovalent intramolecular bonds with acidic residues.<sup>121</sup> Citrulline lacks the polarity of arginine, and the conversion of arginine to citrulline by PADs will therefore disrupt key structural features within protein molecules (**►Fig. 3**).<sup>121</sup> It has been reported that 54 of the 81 arginine residues (66%) within human fibrinogen are susceptible to deamination by either PAD2 or PAD4,<sup>115</sup> indicating that substantial protein structural modification could take place. The normal function of fibrinogen would inevitably also be altered, as discussed previously in the form of autoantibody-driven inflammatory responses and disrupted fibrin polymerization and degradation. The major sites of the fibrinogen molecule affected by citrullination are the paired  $\alpha$ ,  $\beta$ , and  $\gamma$  peptide chains within the D-regions that flank the central nodule of the





**Fig. 3** Comparison of protein structure modification during citrullination and amyloidogenesis. Deamination of polar arginine residues interferes with intramolecular bonds, causing potential unfolding of tertiary protein structure. Under increasing mechanical force or pathological stress,  $\alpha$ -helices transition to  $\beta$ -sheets in fibrin, resulting in a pathologically altered clot. (Original figure; created using Biorender.com).

fibrinogen molecule.<sup>115</sup> Paired structures within the fibrinogen molecule are held in place by 29 disulfide bridges formed between cysteine amino acid residues.<sup>122</sup> These individual peptide chains consist of an  $\alpha$ -helical secondary protein structure, that wind around each other to form an  $\alpha$ -helical coiled-coil structure.<sup>123</sup> The  $\alpha$ -helical coiled-coil conformation comprises approximately 25% of the amino acid architecture of the entire fibrinogen molecule.<sup>124</sup> The normal elastic and plastic properties of fibrin(ogen) are highly dependent on the ability of coiled-coils to unwind, and the conversion of  $\alpha$ -helical coiled-coils to a  $\beta$ -sheet formation under mechanical stress has been demonstrated.<sup>125,126</sup> Staining of these  $\beta$ -sheet structures in mechanically altered fibrin(ogen) by Congo-red revealed that these structures closely resemble the  $\beta$ -sheet aggregation seen in amyloid proteins.<sup>127</sup>

The relevant biophysical definition of amyloid is described as “unbranched protein fibers whose repeating substructure consists of  $\beta$  strands that run perpendicular to the fiber axis, forming a cross  $\beta$ -sheet of undefined length.”<sup>128</sup> Amyloid proteins have been traditionally linked with conditions such as Alzheimer’s disease (deposition of amyloid- $\beta$  in senile plaques) and type 2 diabetes (islet amyloid polypeptide).<sup>129</sup> Amyloidosis are a category of protein-misfolding disorders in which soluble proteins undergo conformational changes and are deposited in extracellular locations as aberrant, insoluble fibrillar structures that disrupt tissue structure and function.<sup>130</sup> Fibrinogen is not generally considered amyloidogenic, except in rare cases of mutations in the *fibrinogen A $\alpha$*  genes, which seem to be the most common cause of hereditary renal amyloidosis.<sup>131</sup> However, our group has demonstrated anomalous fibrin clot formation that is amyloid in nature in several

inflammatory diseases.<sup>132–134</sup> The same aberrant clot formation has also been demonstrated in a purified fibrinogen model in the presence of certain inflammatory stimuli such as bacterial LPS<sup>27,135</sup> and SAA.<sup>136</sup>

It therefore begs the question whether any interaction may occur between the processes of citrullination and amyloid transformation of fibrin(ogen) as outlined in **Fig. 3**, and if it could play a major role in disease outcome in RA. This notion is substantiated by the occurrence of abnormal clot formation and presence of bacterial LPS from a dormant blood microbiome (seen as causal agents of amyloid fibrin formation) that seems intrinsically related to RA as well. However, limited research has been conducted with regards to the direct impact these two protein modification processes have upon each other. Osaki and Hiramatsu conducted research on the effect of citrullination on the single arginine residue conserved in the two isoforms of amyloid  $\beta$  protein ( $A\beta_{1-40}$  and  $A\beta_{1-42}$ ).<sup>137</sup>  $A\beta$  is the major component of insoluble amyloid plaques in Alzheimer’s disease (AD) neural tissue, with the 42-peptide isoform considered the most neurotoxic. The soluble, prefibrillar amyloid- $\beta$  oligomers are also implicated in causing significant neurotoxicity and cytotoxicity in AD.<sup>138</sup> Citrullination of  $A\beta_{1-40}$  resulted in an increased fraction of soluble amyloid oligomers, and citrullination of  $A\beta_{1-42}$  caused in an increased fraction of  $\beta$ -sheet rich oligomers.<sup>137</sup> These findings suggest that citrullination causes structural changes bearing implication for the aggregative nature of amyloid proteins and providing some compelling additional research questions. Could citrullination of fibrin(ogen)  $\alpha$ -helical coiled-coil regions that result in tertiary structural changes create favorable conditions for conversion to pathogenic, amyloid-like

$\beta$ -sheet structures by LPS and SAA molecules? Conversely, could fibrin clots that become amyloid in nature provide suitable epitopes for PAD enzymes, thus resulting in enhanced autoantibody driven inflammatory responses? Further investigation to assess the extent that the processes of citrullination and amyloidogenesis affect each other could further enhance current knowledge on the nature of aberrant fibrin clot formation seen in RA.

### Acute Phase Reaction

The acute phase response is highly prominent in RA as both a result and a contributor to the chronic inflammatory state in affected individuals. Acute SAA (A-SAA) is of particular significance, as it is a highly sensitive marker of acute inflammation (increases 1,000-fold above normal) and is therefore highly elevated in RA patients, even within individuals that present with improved clinical symptoms.<sup>139–142</sup> A-SAA is the circulating precursor of amyloid A protein, a fibrillar, insoluble apolipoprotein that is deposited in major organs (amyloidosis) leading to the severe risk of organ damage and possible death.<sup>143–145</sup> Despite being primarily produced by the liver, extrahepatic sources of SAA have also been identified.<sup>146</sup> A-SAA is expressed by synovial fibroblasts,<sup>146,147</sup> monocytes/macrophages,<sup>146,148</sup> and endothelial cells.<sup>146</sup> A-SAA synthesis is also stimulated by various proinflammatory cytokines such as IL-6, IL-1, TNF- $\alpha$ , and IFN- $\gamma$ .<sup>142</sup> A-SAA is also able to induce several proinflammatory processes contributing to RA immunopathogenesis. A-SAA promotes the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and the adhesion of peripheral blood mononuclear cells to synovial fibroblasts and thus accelerating pannus formation.<sup>149</sup> A-SAA also mediates the production of MMPs by synovial fibroblasts, thus contributing to joint destruction.<sup>149</sup>

Recently our group has demonstrated the ability of SAA to bind to fibrin(ogen), and that relatively low concentrations of SAA-induced amyloid fibrillation of fibrin clots.<sup>136</sup> Amyloidogenic formation of proteins occurs when  $\alpha$ -helices uncoil resulting in  $\beta$ -sheet structure<sup>125</sup> with misfolded proteins leading to an increased thrombotic tendency.<sup>150</sup> With previous reported findings of significantly elevated levels of A-SAA in RA patients, including patients with improved clinical symptoms, A-SAA may significantly contribute to coagulopathy seen in RA individuals and should thus be closely monitored in clinical settings. LPS-mediated  $\beta$ -amyloid formation of fibrin networks through the ability of LPS to bind human fibrin(ogen) has also been demonstrated.<sup>27</sup>

## Involvement of Bacterial Components in Immunopathogenesis and Hemostatic Complications of Rheumatoid Arthritis

### *Porphyromonas gingivalis* as Environmental Source of (Fibrinogen) Citrullination

As mentioned in the introduction, gingivitis and periodontitis are now increasingly implicated in development and progression of RA. *Porphyromonas gingivalis*, the major etiological cause of periodontitis, has received significant

attention as a possible risk exposure for RA development. *P. gingivalis* is a gram-negative, asaccharolytic, and highly proteolytic bacterium,<sup>151,152</sup> and is the only known prokaryote to produce deaminating enzymes similar to that of human isoforms, termed *P. gingivalis* peptidylarginine deaminase (PPAD).<sup>152</sup> Human PAD enzymes, particularly PAD2 and PAD4, are the major sources of citrullinated antigens that drive the development of RA autoimmunity. It is therefore suggested that an external source of citrullination through bacterial PPAD activity could significantly contribute to the generation of RA-specific autoantibodies. Previous studies have demonstrated the ability of PPAD to citrullinate conventional RA-specific self-peptide targets, such as human fibrin(ogen), vimentin, and  $\alpha$ -enolase.<sup>153,154</sup> The mode of citrullination by PPAD is distinct from human PAD isoforms in that it targets C-terminal arginine residues for deamination in proteins, whereas human PAD preferentially deaminates internal arginine residues.<sup>155</sup> PPAD is also not Ca<sup>2+</sup> dependent like human PAD isoforms.<sup>156</sup>

The major bacterial co-virulence factor of PPAD secreted by *P. gingivalis* is a class of proteolytic enzymes termed gingipains. Gingipains catalyze the cleavage of proteins or peptides at either lysine or arginine residues.<sup>155</sup> Arginine-specific gingipains (RgpA or RgpB) yield suitable peptide fragments with C-terminal Arginine residues for citrullination by PPAD.<sup>155–157</sup> RgpB has been shown to effectively cleave arginine residues in fibrinogen, with PPAD able to execute citrullination of the majority of resulting peptide fragments.<sup>158</sup> PPAD and Rgp are both expressed on the outer membrane of *P. gingivalis*, and operate in concert to elicit autoimmune inflammatory responses in the human host.<sup>154,156</sup>

Positive correlations between serum levels of anti-PPAD antibodies with CRP and IL-6 indicate a role of *P. gingivalis* infection in producing systemic inflammation.<sup>80</sup> Yamakawa et al demonstrated that *P. gingivalis* infection of the spontaneous keratoconus (SKC) mouse model produces rapid RA development, with significant increases in arthritis score, APCAs, proinflammatory signaling molecules, and a decrease in bone density observed.<sup>159</sup> PPAD citrullination of histone proteins aids *P. gingivalis* in evading neutrophil extracellular traps (NETs).<sup>152</sup> *P. gingivalis* in turn can induce NETosis.<sup>160</sup> The development of arthritis through *P. gingivalis*-induced inflammatory lesions has been demonstrated in an animal model.<sup>161</sup>

The activity of *P. gingivalis* and its virulence factors may also contribute to the increased cardiovascular disease risk in RA and Parkinson's disease (PD) patients.<sup>162,163</sup> It has been demonstrated that Arginine-specific gingipains (RgpA, RgpB) are able to activate the coagulation cascade at multiple levels. Gingipains proteolytically stimulate coagulation factors IX,<sup>164</sup> X,<sup>165</sup> and II (prothrombin).<sup>166</sup> However, unstable fibrin clots are formed as a result of concurrent fibrinogen degradation by lysine-gingipains (Kgp).<sup>167</sup> The proteolytic activities of the virulence factors of *P. gingivalis* have been shown to degrade apolipoproteins essential for normal lipoprotein metabolism, producing proinflammatory and proarthritogenic events that could account for increased risk of atherosclerosis.<sup>162</sup> The proteolysis of human fibrinogen through the combined effect of PPAD and RgpB inhibits



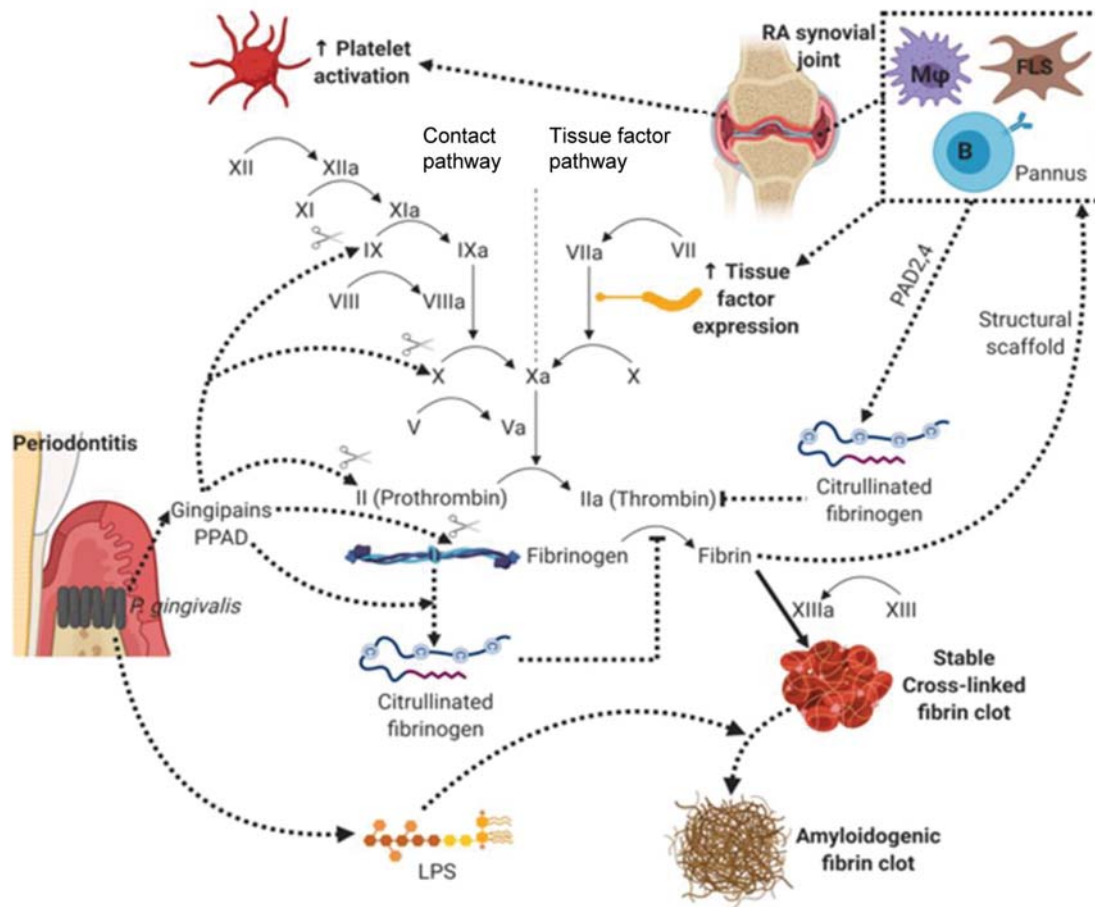
fibrin polymerization, which results in localized bleeding and a potentially dysregulated coagulation cascade.<sup>168</sup> Rgp is able to activate platelets via platelet activation receptors one (PAR-1) and four (PAR-4).<sup>169</sup> Activation of PARs by gingipains is similar to platelet activation by thrombin, as gingipains target N-terminal regions of PARs that contain cleavage sites for thrombin.<sup>151</sup>

Recently, LPS and gingipains from *P. gingivalis* were also shown to cause amyloid formation in purified fibrinogen.<sup>170</sup> The presence of gingipains in whole blood clots as detected by a fluorescent anti-RgpA antibody was confirmed in a PD patient population, and this was associated with increased levels of circulating proinflammatory cytokines, hypercoagulation, amyloid fibrin formation, and altered platelet ultrastruc-

ture.<sup>170</sup> These findings could therefore also be observable within RA patient sera, as the presence of *P. gingivalis* and its associated gingipains has a long-established relationship with disease as well. ► **Fig. 4** illustrates the human coagulation cascade, indicating the effects RA-specific inflammatory and autoimmune events may have on overall hemostatic outcome.

### RA Inflammatory Pathways: Interactions between Citrullinated Fibrinogen and Prominent Immune Cells

The chronic inflammatory state that is a hallmark of RA is characterized by an accumulation of inflammatory leukocytes into the synovium and articular spaces.<sup>54,171</sup> The



**Chart key**  
**PPAD:** *Porphyromonas gingivalis* peptidylarginine deaminase **PAD:** peptidylarginine deaminase  
**B:** B lymphocyte **Mφ:** Macrophage **FLS:** Fibroblast-like synoviocyte **LPS:** lipopolysaccharide  
 \_\_\_\_\_ Physiological hemostasis reactions  
 - - - - - RA-specific interferences with hemostasis

**Fig. 4** Chronic synovitis and invasive pannus formation, characteristic of clinical rheumatoid arthritis (RA), may result in the amplification of coagulation by increased stimulation of clotting components in both the contact pathway (heightened activated platelet phenotype) and tissue factor pathway (enhanced tissue factor expression by immune cells). Insoluble fibrin in return acts as a structural scaffold agent for pannus formation in RA synovial joints. Activation of coagulation may also be proteolytically propagated by *Porphyromonas gingivalis* virulence factors (gingipains) at various levels. However, previous studies have demonstrated that citrullinated fibrinogen is not able to undergo thrombin digestion and subsequent fibrin polymerization. It has also been shown that LPS from gram-negative bacteria (such as *P. gingivalis*) is able to inflict fibrin alteration that are amyloid in nature, resulting in structurally unstable clots. (Original figure; created with Biorender.com).

cellular composition of inflamed RA synovium consists of immune cells from both innate and humoral systems, fibroblast-like synoviocytes, chondrocytes, and bone-remodeling cells such as osteoclasts and osteoblasts.<sup>54</sup>

The RA autoimmune response is likely elicited due to an abnormal humoral response to the presence of citrullinated proteins in affected individuals.<sup>172</sup> The major genetic risk factor for RA ACPA generation, HLA-DR $\beta$ 1 “shared epitope” alleles, exerts major influence on  $\alpha\beta$  CD4<sup>+</sup> T-cell selection.<sup>11,173</sup> The altered CD4<sup>+</sup> T-cell repertoire is responsible for inflammatory signaling and generation of anticitrullinated autoantibodies by synovial B-cells through the self-presentation of citrullinated proteins by MHC molecules (►Fig. 5).<sup>11</sup> Deaminating enzymes responsible for generation of citrullinated antigens (PAD2 and PAD4) are also expressed by inflammatory leukocytes within the synovial microenvironment, which include synovial T and B cells, macrophages, neutrophils, and fibroblast-like synoviocytes.<sup>174,175</sup>

Human fibrin(ogen) is capable of directly stimulating a variety of leukocyte activities (►Fig. 6). Fibrin(ogen) engagement with macrophages via TLR-4 and other integrins induces expression of proinflammatory chemokines,<sup>176</sup> and cytokines such as TNF- $\alpha$ .<sup>177</sup> Fibrinogen stimulates the proinflammatory activity of neutrophils by binding to the  $\alpha$ -subunit of CD11b/CD18.<sup>178</sup> Fibrinogen regulates NF $\kappa$ B signaling and inflammatory chemokine expression of endothelial cells.<sup>179</sup> Human synovial fibroblasts express ICAM-1 and inflammatory chemokines under the influence of fibrinogen.<sup>180</sup> These findings indicate that fibrin(ogen), which is present in high amounts within RA articular spaces, may fulfil a pronounced role in mediating various RA-related inflammatory processes. Fibrin displays even stronger involvement in propagation of inflammatory processes in RA than its soluble precursor.<sup>117</sup> Fibrin deposits are present within the RA synovial tissue lining, and thus enable the proliferation of RA synovial fibroblasts and local cytokine release.<sup>44,181</sup> The insoluble nature of the protein increases the difficulty of its clearance from articular spaces, thus leading to increased likelihood of posttranslational modifications and generation of an autoimmune inflammatory response.<sup>117</sup>

### Macrophages/Monocytes

ACPAs directly contribute to initiating and sustaining the state of synovial inflammation by their ability to activate monocytes and macrophages, to produce inflammatory cytokines.<sup>182</sup> Citrullinated-fibrinogen containing immune complexes stimulate TNF $\alpha$  secretion by macrophages through Fc $\gamma$ R-TLR4 engagement.<sup>108,183</sup> The presence of ACPAs causes a dysregulated M1/M2 macrophage subset polarization, favoring M1 (classical) activation.<sup>184</sup> The M1 subset is characterized by its inflammatory, microbicidal, and tumoricidal properties.<sup>184</sup> There is an overexpression of TLR2 and TLR4 receptors by macrophages and synovial fibroblasts in RA.<sup>117</sup> TLRs recognize disease-associated molecular patterns (DAMPs) and PAMPs, resulting in innate immune responses.<sup>185</sup> PAMPs are microbial-derived molecules such as peptidoglycan, bacterial and viral deoxyribonucleic acid (DNA) that are structurally distinguish-

able from host molecules.<sup>185,186</sup> DAMPs are derived from clinical symptoms associated with the pathophysiological responses, with fibrinogen/fibrin and dsRNA from apoptotic cells present in the RA joint serving as DAMPs.<sup>185,186</sup>

### RA Synovial Fibroblasts

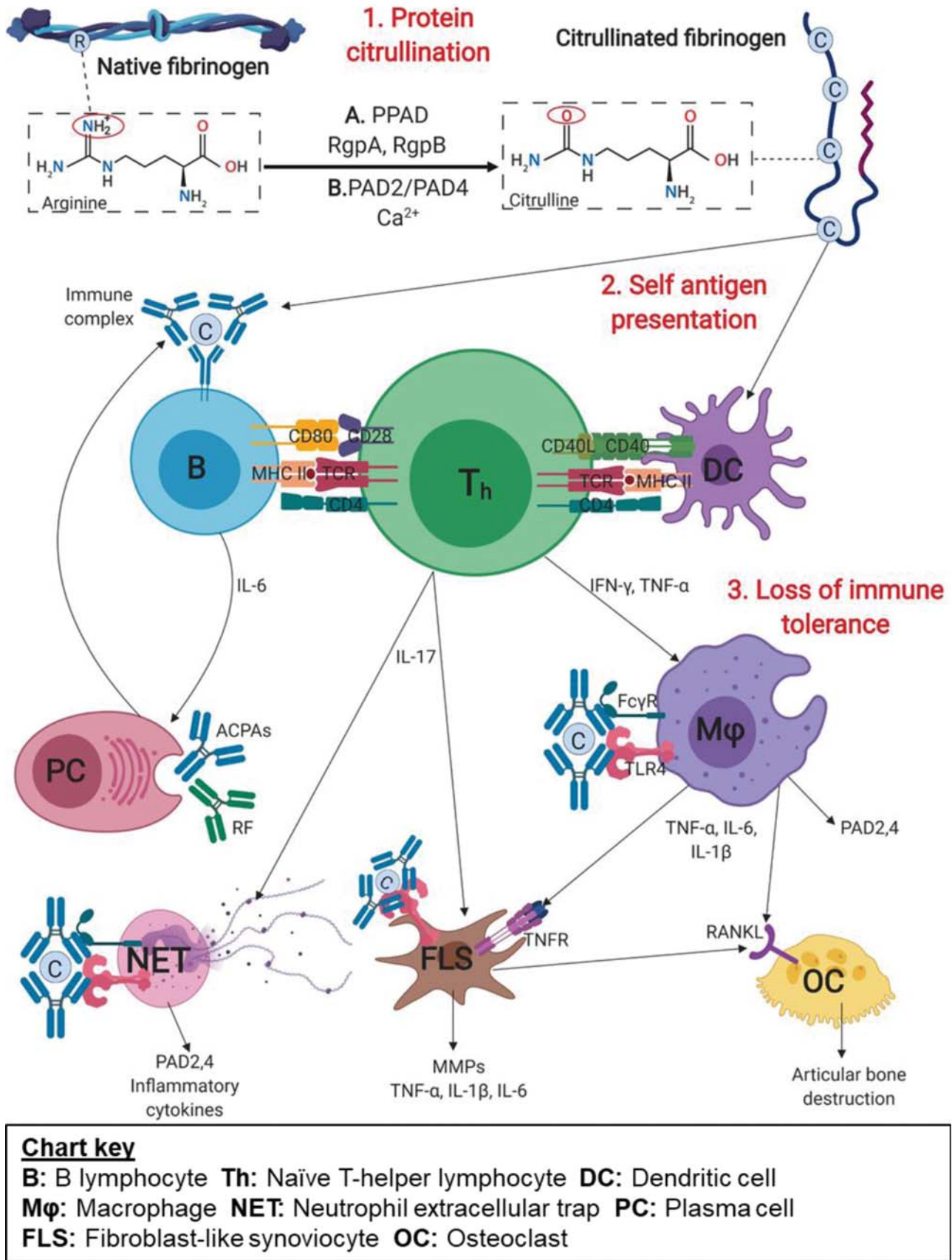
The porous structural arrangement of the synovial lining that lacks epithelial cells, basement membranes, tight junctions, or desmosomes allows accumulation of immune complexes and bacterial cell wall components within rheumatic joints.<sup>187</sup> The synovial intimal lining layer consists of two distinct cell types—macrophage-like synoviocytes (Type A) and fibroblast-like synoviocytes (Type B).<sup>187</sup> The inflammatory milieu of the RA synovium transforms the surrounding tissues to become increasingly hyperplastic, invasive, and immunogenic.<sup>187</sup> Transcription factor NF $\kappa$ B is ubiquitously expressed in monocytes and synovial fibroblasts, and regulates the expression of proinflammatory cytokines, chemokines, and adhesion molecules in RA.<sup>188</sup> There is an overexpression of TLRs in RA synovial fibroblasts (RASf), with both fibrin deposits in the RA joint and soluble fibrinogen acting as candidate TLR ligands.<sup>44,186</sup> TLR ligation potentiates the inflammatory cytokine production of RASfs, with prominent secreted mediators including IL-6, IL-8, TNF $\alpha$ , MMPs, and vascular endothelial growth factor (VEGF).<sup>186</sup> Citrullination of peptides further amplifies the proinflammatory potential of RASfs, with a significant increase in cytokine secretion.<sup>117</sup>

### Tumor Necrosis Factor: Apical RA Cytokine

TNF $\alpha$  is considered the most prominent cytokine involved in the RA inflammatory response, as it in turn stimulates further monocyte and macrophage activity and proinflammatory cytokine release.<sup>189–191</sup> TNF $\alpha$  has been demonstrated to be increased by 62% in RA patient sera compared with controls.<sup>192</sup> The suggested importance of TNF $\alpha$  in RA pathogenesis has been highlighted by the relative efficacy of TNF $\alpha$  inhibitor (infliximab) treatment in the attenuation of disease symptoms and synovial inflammation.<sup>193,194</sup> TNF $\alpha$  is produced by T cells within the synovial lining, local macrophages, and monocytes resulting in chronic synovitis and activates osteoclasts and MMPs leading to cartilage and bone destruction.<sup>195,196</sup> TNF $\alpha$  is also able to activate platelets through the arachidonic pathway, with platelets further propagating RA synovial inflammation and the associated risk of thrombotic cardiovascular events.<sup>197</sup> The chronic exposure of synovial T-cells to TNF $\alpha$  leads to decreased immune tolerance and unresponsiveness to TNF $\alpha$  inhibitor treatment (prevalent in 30–40% of RA patients).<sup>191</sup>

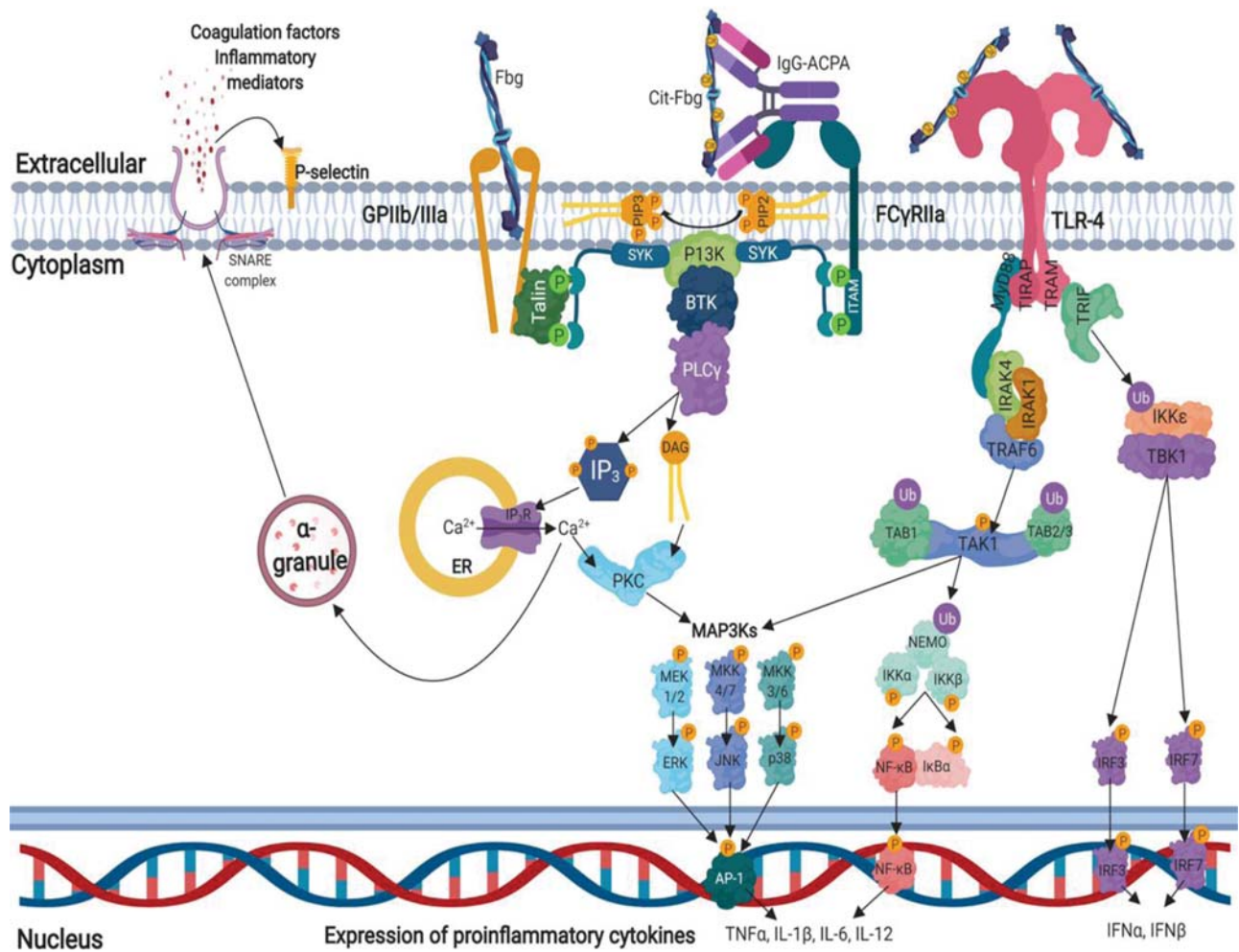
### Neutrophils and NETosis

The presence of hyperactive neutrophils in RA synovial fluid and pannus tissue, in particular NETosis (formation of neutrophil extracellular traps or NETs), has been confirmed by multiple studies.<sup>198–200</sup> NETs are chromatin-derived extracellular snares that are externalized from neutrophils in response to microbial infections or inflammation.<sup>201</sup> Neutrophils and NETosis are key components in the perpetual inflammatory response encountered in RA patients.



**Fig. 5** The rheumatoid arthritis (RA) “shared epitope” risk gene results in citrullinated proteins being presented as self-antigens via MHCII complexes to CD4+ T-helper cells, resulting in a breakdown of immune tolerance to the presence of citrullinated proteins, with T-lymphocyte differentiation resulting in the production of RA-specific autoantibodies (ACPAs and RFs), as well as direct stimulation of inflammatory effector cells via cytokine signaling. ACPAs, anti-citrullinated protein antibodies; MHC, major histocompatibility complex; RF, rheumatoid factor (Original figure; created with Biorender.com).





**Fig. 6** The prominent cell surface receptors recognized as ligating with human fibrinogen, citrullinated fibrinogen, and citrullinated-fibrinogen immune complexes and their downstream signaling pathways are summarized. (Original figure; created with Biorender.com).

Neutrophils express multiple cell surface receptors, and NETosis is induced by proinflammatory mediators such as bacterial LPS,<sup>202</sup> platelet TLR-4,<sup>203</sup> and cytokines such as TNFα, IL-1β, and IL-8.<sup>204</sup> Enhanced NETosis is associated with local and peripheral presence of ACPAs, with NETs prominent sources of citrullinated autoantigens that express high levels of PAD2 and PAD4 enzymes.<sup>205</sup> NETs along with their individual components (DNA and histones) have also been shown to possess significant prothrombotic and antifibrinolytic properties, and have been discussed at length in a recent review by Varjú and Kolev.<sup>206</sup> NETs provide a platform for the accumulation of integral hemostatic components such as platelets, erythrocytes, von Willebrand factor, and fibrinogen.<sup>207</sup> Histones promote fibrin deposition through its ability to bind to and inactivate antithrombin.<sup>208</sup> This protective mechanism against the inhibitory effects of antithrombin is also achieved by the citrullination of antithrombin by PAD4.<sup>209</sup> Integration of DNA–histone complexes into fibrin networks results in thicker fibrin fibers that consequently improve clot stability and rigidity but decreases clot permeability.<sup>208,210</sup> The presence of these NET-derived complexes within the fibrin network also impedes fibrinolysis, through the ability of both histones and DNA to bind large fibrin

degradation products (such as plasminogen) and inhibit its activity.<sup>208,210</sup>

## Conclusion

In this review, we have discussed in detail the role that specific plasma proteins play at every stage of RA immunopathogenic progression. All major themes and the clinical relevance considered in this review are summarized in ►Table 1. Endogenous and exogenous sources of protein citrullination, coupled with the genetic propensity to present citrullinated antigens to CD4<sup>+</sup> T-lymphocytes present the most convincing current paradigm to explain the development of RA autoimmunity. The insoluble clotting protein fibrin, along with its soluble precursor fibrinogen, has been demonstrated as prime targets for PAD citrullination and the generation of fibrin(ogen)-specific autoantibodies in RA. The excessive rate of fibrin generation and breakdown in the RA synovium actively contributes to RA pannus structure and inflammatory signaling. Both native and citrullinated forms of fibrin(ogen) have been shown to interact with and regulate the proinflammatory activities of various immune cells in RA, including macrophages,

**Table 1** Key points of this review

<ul style="list-style-type: none"> <li>To date, a combination of genetic risk, the presence of infectious agents, including those that become dormant within the host, along with certain environmental exposures present the most plausible etiological explanation for the development of clinical rheumatoid arthritis (RA) disease.</li> </ul>
<ul style="list-style-type: none"> <li>Ebringer's theory (with experiments) states that <i>Proteus</i> infection from the urinary tract is a major cause of RA. Also, the periodontal pathogen <i>Porphyromonas gingivalis</i> possibly presents a major exogenous contributor to RA autoimmune generation through the expression of its unique virulence factors.</li> </ul>
<ul style="list-style-type: none"> <li>In addition to destructive synovitis, a high risk of cardiovascular disease as a result of dysregulated thromboinflammatory function is well established in RA patients.</li> </ul>
<ul style="list-style-type: none"> <li>The presence of autoantibodies directed toward citrullinated proteins (ACPAs) is highly correlated with disease development and influences various proinflammatory processes.</li> </ul>
<ul style="list-style-type: none"> <li>Human fibrin(ogen), a key component of the coagulation process, is a prime candidate ligand for citrullination by peptidylarginine deaminase (PAD) enzymes.</li> </ul>
<ul style="list-style-type: none"> <li>Further research is necessary to define the involvement of citrullination with regards to the formation of abnormal fibrin clots observed in RA patients.</li> </ul>
<i>Clinical relevance</i>
<ul style="list-style-type: none"> <li>Infections of certain mucosal tissues and bacterial components are important factors for consideration in the possible prevention, diagnosis, and treatment of RA.</li> </ul>
<ul style="list-style-type: none"> <li>Acute phase proteins, such as fibrinogen and serum amyloid A, present significant clinical value due to their involvement at various levels of disease development.</li> </ul>
<ul style="list-style-type: none"> <li>By monitoring highly sensitive markers of thrombotic function in RA patients through an individualized medicine approach may represent an improved diagnostic and treatment strategy for systemic inflammation and secondary cardiovascular disease.</li> </ul>

synovial fibroblasts, and neutrophils. The increased CVD risk in RA is independent of traditional risk factors, and the interactions between dysregulated inflammatory and hemostatic processes present a major contribution to this secondary disease complication. The extent to which the protein modification processes of citrullination and amyloidogenesis of fibrinogen may interact and influence the other represents an area of novel research that could explain the presence of excessive and aberrant clot formation in RA patients.  $\beta$ -amyloid formation of fibrin fibers as a result of increased A-SAA levels and bacterial LPS constitutes additional coagulopathic complications in RA patients. Despite observations of increased thrombotic tendency coupled with structurally abnormal fibrin clot formation in RA patients, the exact role that citrullinated proteins fulfil in this regard remains controversial and requires further investigation. The contribution of bacterial LPS to the generation of autoantibodies in RA is also yet to be determined. Whether LPS could directly stimulate increased rates of protein citrullination as a result of perhaps autocrine TNF $\alpha$ -induced apoptosis of immune cells,<sup>211</sup> or through indirect mechanisms certainly warrants further investigation. Furthermore, it is yet to be determined how free LPS in circulation might interact directly with plasma proteins like fibrinogen which can lead to citrullination, amyloid formation, and anomalous blood clot formation.

To conclude, it is suggested that specific proteins involved in chronic synovitis and thrombotic tendency in RA patients, such as fibrin(ogen) and amyloid A proteins be considered more intensively in research. The extensive signaling processes these proteins are involved with regards to disease

development may present novel areas for identifying potential diagnostic biomarkers and therapeutic interventions.

#### Conflicts of Interest

None.

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