Antitijela na citrulinirane proteine/peptide u reumatoidnom artritisu: što smo dosad naučili?

Antibodies to citrullinated proteins/peptides in rheumatoid arthritis: what have we learned so far?

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Sažetak

Reumatoidni artritis (RA) najčešća je upalna sistemska autoimuna bolest s progresivnim narušavanjem strukture zglobova, ali i višestrukim organskim oštećenjem koje rezultira smanjenjem kvalitete života i povećanjem smrtnosti. Iako je etiologija RA još uvijek nerazjašnjena, značajan napredak postignut je u razumijevanju patofiziologije, što je s jedne strane dovelo do identifikacije novih terapijskih ciljeva, a s druge do otkrića antigena koje prepoznaju antitijela specifična za RA. Glavna odrednica ciljnih antigena je citrulin pa se čini da je posttranslacijska enzimska citrulinacija proteina u sinovijalnom tkivu ključna karika autoimunog procesa u RA. Ubikvitarnost citruliniranih proteina u fiziološkim i patološkim stanjima govori u prilog tome da autoimuni odgovor specifičan za RA pokreće interakcija procesa citruliniranja i još nedefiniranih genetskih čimbenika te čimbenika okoliša.

Ovaj rad daje pregled dosadašnjih saznanja o mehanizmu autoimune reakcije usmjerene na citrulinirane proteine, kao i o kliničkom značenju određivanja ovih antitijela u RA.

Ključne riječi: reumatoidni artritis; citrulinacija; peptidilarginin deiminaza; anti-CCP antitijela; anti-MCV antitijela

Abstract

Rheumatoid arthritis (RA) is the most common inflammatory systemic autoimmune disease with progressive joint destruction but also with multiple organs lesions that result in decreased quality of life and higher mortality rate. Although the etiology of RA remains unclear, a significant progress has been done in the understanding of its pathophysiology. It has led to identification of new therapeutic targets on the one hand, and to discovering of antigens that can recognize RA-specific antibodies on the other hand. The main determinant of target antigens is citrulline, so enzymatic post-translational citrullination of protein in synovial tissue appears to be the key link of the autoimmune process in RA. The ubiquity of citrullinated proteins in physiological and pathological conditions confirms that RA-specific autoimmune response is run by the interaction between citrullination and some undefined genetic and environmental factors.

This paper reviews current knowledge of the mechanism of autoimmune reaction to citrullinated proteins and clinical significance of determination of these antibodies in RA.

Key words: rheumatoid arthritis; citrullination; peptidylarginine deiminase; anti-CCP antibodies; anti-MCV antibodies

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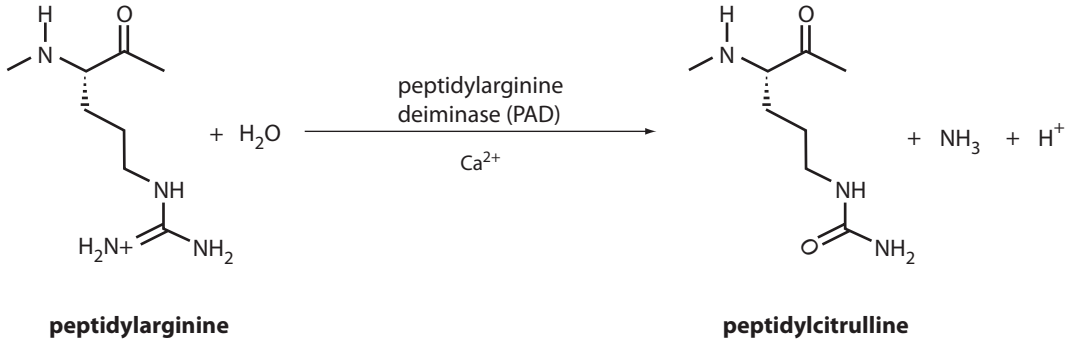
Uvod

Reumatoidni artritis (RA) s učestalošću od 0,5% do 1% u općoj populaciji najčešća je sistemska autoimuna bolest. Obilježena kroničnom upalom s posljedičnim ireverzibilnim oštećenjem zglobova, ali i drugih organskih sustava, ova bolest značajno utječe na kvalitetu života i time predstavlja nezanemarivi socioekonomski problem. S obzirom

Introduction

Rheumatoid arthritis (RA) with a prevalence of 0.5% to 1% in the general population is the most common inflammatory systemic autoimmune disease. Characterized by chronic inflammation and consequent irreversible joint damage, but also damage to other organ systems, the disease considerably affects the patient's quality of life,



Slika 1. Deiminacija peptidilarginina u peptidilcitrulin djelovanjem peptidilarginin deiminaze (PAD), ovisno o Ca^{++} ionima.

Figure 1. Peptidylarginine deimination to peptidylcitrulline by Ca^{++} ion-dependent peptidylarginine deiminase (PAD).

na nedavnu spoznaju da ireverzibilna oštećenja zglobova nastaju tijekom prve dvije godine od pojave simptoma, moderni terapijski pristup RA pretpostavlja primjenu agresivne antireumatske terapije već u prvim mjesecima bolesti. Zbog potencijalno toksičnih lijekova koji mogu izazvati vrlo opasne nuspojave neophodan je visokospecifičan biljeg koji bi identificirao bolesnike s RA prije pojave zglobnih oštećenja, a u kojih bi korist od primjene takve terapije nadvladala potencijalne rizike. Kriteriji Američke udruge za reumatologiju (engl. *American College of Rheumatology,* ACR) za dijagnozu RA (1) koji se, uz iznimku reumatoidnog faktora (RF), prvenstveno zasnivaju na kliničkim pokazateljima, nedovoljno su osjetljivi upravo u toj kritičnoj, ranoj fazi bolesti. Godine nakon otkrića biljega RF, koji je pokazao prihvatljivu osjetljivost, ali nedovoljnu specifičnost za RA, obilježila su nastojanja za identifikacijom drugih, specifičnijih antitijela. Većina opisanih antitijela nisu pokazala dijagnostičku superiornost u odnosu na RF, sve do nedavnog otkrića obitelji antitijela usmjerenih na citrulinirane epitope na peptidima/proteinima (ACPA) koja su se pokazala specifičnim dijagnostičkim, ali i prognostičkim biljegom RA.

Citrulinacija – ključni proces u autoimunom odgovoru u RA

Danas je poznato da su antitijela iz obitelji ACPA odavno identificirana u serumu bolesnika s RA, ali nedovoljno poznavanje strukture antigenskih determinanti rezultiralo je testovima nezadovoljavajućih dijagnostičkih značajki. Tako povijest ACPA datira još od 1964. godine kada su Nienhuis i Mandema (2) metodom indirektne imunofluorescencije (IIF) otkrili antitijela u serumu bolesnika s RA koja se vežu na komponente keratohijalinih granula koje

thus posing a major socioeconomic problem. Considering a recent concept according to which irreversible joint lesions occur in the first two years of the symptom onset, current therapeutic approach to RA implies aggressive anti-rheumatic therapy already in the first months of the disease. Due to the potentially toxic drugs that can cause severe side effects, a highly specific marker is necessary to identify RA patients before the onset of joint lesions, where the benefit of this therapy would overcome the potential risks. The American College of Rheumatology (ARA) criteria for RA diagnosis (1) that are primarily based on clinical indicators, except for rheumatoid factor (RF), are insufficiently sensitive, especially in this critical early stage of the disease. After discovery of the RF marker, which showed acceptable sensitivity but insufficient RA specificity, years have been spent on efforts to identify another, more specific antibodies. The majority of described antibodies have not shown diagnostic superiority regarding RF, until recent discovery of a family of antibodies directed to citrullinated epitopes on proteins/peptides (ACPA), which have been found to be a specific diagnostic as well as prognostic marker of RA.

Citrullination – a key process in the autoimmune response in RA

Today it is known that antibodies from ACPA family had long been identified in serum of RA patients, but insufficient knowledge of the structure of antigenic determinants resulted in tests of unsatisfactory diagnostic characteristics. The history of ACPA dates back to 1964 when Nienhuis and Mandema (4) using the method of indirect immunofluorescence (IIF), discovered antibodies in serum of RA patients that bound to the components

okružuju jezgru humanih epitelnih stanica sluznice usne šupljine, dajući znakovitu perinuklearnu fluorescenciju. S obzirom na tip fluorescencije, ova antitijela dobila su naziv antiperinuklearni faktor (APF). Prema rezultatima ispitivanja koja su uslijedila nakon ovoga otkrića, antitijela APF prisutna su u 49–91% bolesnika s RA sa specifičnošću od 73–99% (3). Međutim, unatoč visokoj specifičnosti, određivanje ovih antitijela nije zaživjelo u rutini iz dva razloga. Prvi je ograničena dostupnost odgovarajućeg supstrata, jer se u svega oko 5% davatelja mogu naći epitelne stanice dovoljno diferencirane da sadrže perinuklearni faktor. Drugi razlog su negativne osobine metode IIF, kao što su nestandardiziranost i iskustvo neophodno za pravilnu interpretaciju.

Godine 1979. Young i sur (4) su metodom IIF identificirali antitijela čiji se ciljni antigen nalazi u filamentima rožnatog sloja epitela štakorskog jednjaka. Pretpostavljajući da je ciljni antigen keratin, ova antitijela su nazvali antikeratinska antitijela (AKA). Osjetljivost AKA za RA kreće se od 36% do 59%, a specifičnost od 88% do 99% (3). Autoimuno podrijetlo ovih antitijela dokazano je njihovim vezanjem i za tada još nepoznatu sastavnicu u rožnatom sloju humanog epidermisa. Nekoliko argumenata podupiralo je hipotezu da su antitijela AKA i APF iz iste obitelji: 1) ciljni antigen za oba antitijela eksprimiran je u epitelu istog histološkog tipa (epitel štakorskog jednjaka, humani epidermis, kao i epitel sluznice usne šupljine spadaju u skvamozni epitel sa sličnim putovima završne diferencijacije), 2) njihova prisutnost i titar koreliraju međusobno, s RF te s težinom i aktivnosti bolesti, 3) i jedno i drugo antitijelo su uglavnom izotipa IgG, 4) oba su visoko specifična za RA, 5) njihova učestalost neovisna je o spolu i dobi, 6) oba antitijela su prisutna u ranoj fazi bolesti i mogu prethoditi pojavi simptoma. Godine 1993. Simon i sur. (5) identificirali su protein veličine 40 kDa ekstrahiran iz humanog epidermisa i specifično obilježen serumom bolesnika s RA na Western blotu, kao kiselu/neutralnu izoformu filagrina. Dvije godine kasnije, Sebbag i sur. (6) su utvrdili da je frakcija IgG, pročišćena iz seruma bolesnika s RA imunoadsorpcijom upotrebom spomenutog 40 kDa proteina, reaktivna i u testu AKA i u testu APF te time potvrdili da ova antitijela prepoznaju isti antigen. Identificirani ciljni antigen bio je, dakle, filagrin, protein koji sudjeluje u agregaciji citokeratinskih filamenata tijekom orožnjavanja epidermisa. Ovim otkrićem uveden je novi naziv za obitelj antitijela koja uključuje AKA i APF, antifilagrinska antitijela (AFA). Filagrin se sintetizira u *stratumu granulosumu* kao fosforilirani prekursor, profilagrin (200–400 kDa), koji se nakuplja u citoplazmatskim organelama specifičnim za keratinocite, keratohijalinim granulama. Tijekom završne diferencijacije epidermnih keratinocita molekule profilagrina se defosforiliraju i proteolitički cijepaju u funkcionalne filagrinske jedinice. Kako filagrin sadrži 10–12% bazične aminokiseline histidina, postavilo se pitanje pod-

of keratohyaline granules surrounding the nucleus of human buccal mucosal epithelial cells, giving characteristic perinuclear fluorescence. Regarding the fluorescence type, these antibodies were named antiperinuclear factor (APF). According to the results of studies that followed this discovery, APF antibodies are present in 49%–91% of RA patients, with 73%–99% specificity (3). However, despite high specificity, determination of these antibodies has not been accepted in daily routine for two reasons. The first is limited availability of appropriate substrate because epithelial cells differentiated enough to contain perinuclear factor can only be found in about 5% of donors. The second reason refers to unfavorable characteristics of the IIF method such as the lack of standardization and of experience required for correct interpretation.

In 1979, using the IIF method Young *et al*. (4) identified antibodies with target antigen located in filaments of the keratinized epithelial layer of rat esophagus. Assuming keratin as the target antigen, they named these antibodies antikeratin antibodies (AKA). AKA sensitivity for RA is 36%–59% and specificity 88%–99% (3). The autoimmune origin of these antibodies was proven by their binding to, at the time unknown, component in the keratinized layer of human epidermis. Several arguments have supported the hypothesis on the AKA and APF to belong to the same family: 1) target antigen for both antibodies is expressed in the epithelium of the same histologic type (rat esophageal epithelium, human epidermis and buccal mucosa belong to squamous epithelium with similar pathways of final differentiation); 2) their presence and titer correlate with each other, with RF and with the disease severity and activity; 3) both antibodies are mostly of IgG isotype; 4) both are highly specific for RA; 5) their prevalence is neither sex nor age dependent; and 6) both antibodies are present in the early stage of the disease and may precede the onset of symptoms. In 1993, Simon *et al*. (5) identified a 40kDa protein extracted from human epidermis and specifically marked by serum from RA patient on Western blot as the acidic/neutral isoform of filaggrin. Two years later, Sebbag *et al*. (6) found the IgG fraction purified from RA patient serum by immunoadsorption using the mentioned 40kDa protein to be reactive on both AKA and APF tests, demonstrating that these antibodies recognized the same antigen. Thus, the target antigen identified was filaggrin, a protein involved in the cytokeratin filament aggregation during epidermis keratinization. With this discovery, a new name for the antibodies has been introduced, including AKA and APF, i.e. anti-filaggrin antibodies (AFA). Filaggrin is synthesized in *stratum granulosum* as a phosphorylated precursor, profilaggrin (200–400 kDa), that accumulates in cytoplasmic organelles specific for keratinocytes, keratohyaline granules. During final differentiation of epidermal keratinocytes, profilaggrin molecules are dephosphorylated

rijetla kisele, odnosno neutralne izoforme filagrina koju prepoznaju AFA. Bazične molekule filagrina, oslobođene proteolizom prekursora profilagrina, posttranslacijski se modificiraju enzimom peptidilarginin deiminazom (PAD), pri čemu se 20% argininskih ostataka deiminira (ovisno o Ca^{++} ionima) u aminokiselinu citrulin (Slika 1).
Postupkom citruliniranja od bazične nastaje neutralna, odnosno kisela izoforma filagrina koja ima manji afinitet za citokeratinske filamente. Godine 1998. Schellekens i sur. (7) uspješno su identificirali citrulin kao glavnu antigensku odrednicu upotrebom citruliniranih peptida sintetiziranih prema karboksi-terminalnom kraju molekule profilagrina. Samo godinu dana kasnije ovo otkriće potvrdili su Girbal-Neuhauser i sur. (8) pokazavši da antitijela specifična za RA prepoznaju rekombinantne filagrinske fragmente nakon enzimatske deiminacije *in vitro*.

Citrulinirani proteini u reumatoidnom sinovijalnom tkivu

Kako je ekspresija (pro)filagrina ograničena isključivo na skvamozni epitel i ne nalazi se u sinovijalnom tkivu, pretpostavlja se da deiminacija različitih proteina u upaljenom sinovijalnom tkivu aktivira imunu reakciju, a citrulinirani (pro)filagrin je samo križno-reaktivni supstrat (9). U prilog ovoj hipotezi govori nalaz Masson-Bessierea i sur. (10) koji su otkrili 7,5 puta veći udio AFA u ukupnoj koncentraciji IgG u ekstraktu reumatoidnog sinovijalnog tkiva u odnosu na pripadajući serum. Ovaj nalaz upućuje na zaključak da su u sinovijalnom tkivu bolesnika s RA prisutne AFA-secernirajuće plazma stanice, a lokalno izlučena antitijela potom difundiraju u cirkulaciju. Isti autori sugeriraju deiminirane oblike α- i β-lanaca fibrina koji se odlažu u reumatoidnoj sinovijalnoj membrani kao glavne ciljne antigene za AFA (11). Međutim, nedavno istraživanje pokazalo je da prisutnost citruliniranog fibrina nije specifična za reumatoidno sinovijalno tkivo, nego se nalazi i u upaljenom sinovijalnom tkivu spondiloartropatija (psorijatični artritis, ankilozni spondilitis, reaktivni artritis) i osteoartritisa. Deiminacija fibrina, dakle, predstavlja nespecifičan fenomen vezan za sinovitis, koji nužno ne inducira autoimuni odgovor sa stvaranjem antitijela AFA (12). Među ostalim citruliniranim sinovijalnim proteinima identificirani su humani kolagen tipa I. (13), vimentin (14) i fibronektin (15). Nadalje, Kinloch i sur. (16) su dokazali reaktivnost 46% seruma RA s *in vitro* citruliniranom α-enolazom iz lizata promijelocitne stanične linije koja se diferencira u stanice monocitnog i granulocitnog fenotipa kakve prevladavaju u upaljenom sinovijalnom tkivu. Iako je prisutnost α-enolaze dokazana u sinovijalnom tkivu, nije potvrđena njena *in vivo* citrulinacija. Imajući na umu da je za vezanje AFA kritičan citrulin vezan za neutralne aminokiseline kao što su glicin, serin ili treonin, Pratesi i sur. (17) ispitali su hipotezu može li deiminacija nuklearand proteolytically cleaved into functional filaggrin units. As filaggrin contains 10%–12% of the basic amino acid histidine, the question was raised of the origin of the acidic and neutral filaggrin isoform recognized by AFA. The basic filaggrin molecules released by the profilaggrin precursor proteolysis are post-translationally modified by the peptidylarginine deiminase (PAD) enzyme, whereby 20% of arginine residues are being deiminated (depending on Ca^{++} ions) to the citrulline amino acid (Figure 1).
By the procedure of citrullination, the basic isoform transforms to the neutral or acidic filaggrin isoform, which has lower affinity for cytokeratin filaments. In 1998, Schellekens *et al*. (7) successfully identified citrulline as the main antigen determinant using citrullinated peptides synthesized towards the COOH-terminal end of the profilaggrin molecule. Only a year later, this discovery was confirmed by Girbal-Neuhauser *et al*. (8), showing the RA-specific antibodies to recognize recombinant filaggrin fragments after enzymatic deimination *in vitro*.

Citrullinated proteins in rheumatoid synovial tissue

Due to the fact that (pro)filaggrin expression is limited exclusively to squamous epithelium and is not present in synovial tissue, it is assumed that deimination of different proteins in inflamed synovial tissue activates immune reaction, and citrullinated (pro)filaggrin is merely a cross-reactive substrate (9). In favor of this hypothesis speaks the result of Masson-Bessiere *et al*. (10) who discovered a 7.5-fold higher AFA ratio in total IgG concentration in the extract of rheumatoid synovial tissue than in the respective serum. This result points to a conclusion that AFA-secreting plasma cells are present in synovial tissue of RA patients and the locally secreted antibodies are then being diffused into the circulation. The same authors suggest deiminated isoforms of α- and β-fibrin chains, deposited in rheumatoid synovial membrane, as the main target antigens for AFA (11). However, a recent study has shown that the presence of citrullinated fibrin is not specific for rheumatoid synovial tissue, but is also found in inflamed synovial tissue in spondyloarthropathies (psoriatic arthritis, ankylosing spondylitis and reactive arthritis) and osteoarthritis. Therefore, fibrin deimination is a nonspecific phenomenon associated with synovitis which need not necessarily induce autoimmune response with the generation of AFA antibodies (12). Human type I collagen (13), vimentin (14) and fibronectin (15) have been identified among other citrullinated synovial proteins. Furthermore, Kinloch *et al*. (16) demonstrated reactivity of 46% of RA sera with *in vitro* citrullinated α-enolase from promyelocytic cell line lysate that is differentiated into cells of monocyte and granulocyte phenotype, which prevail in inflamed synovial tissue. Although the presence

nog antigena 1 virusa Epstein-Barr (EBNA-1) koji sadrži 6 ponavljajućih arginin-glicin sekvenca, a često se povezuje s RA, stvoriti epitope koje prepoznaju AFA. Antitijela specifična za ovu sekvencu EBNA-1 otkrivena su u 50% seruma RA i u < 5% seruma kontrolnih ispitanika, ali nije još dokazana prisutnost deiminiranog EBNA-1 u reumatoidnom sinovijalnom tkivu. Konačno, iako prisutnost citruliniranih histona u upaljenom sinovijalnom tkivu nije dokazana, ovi proteini čine se nezanemarivim potencijalnim antigenima s obzirom na to da je njihova citrulinacija opisana *ex vivo* tijekom apoptoze granulocita (18). U upaljenom sinovijalnom tkivu prisutan je velik broj granulocita čiji je životni vijek oko 3 dana pa će i velik broj odjednom biti podvrgnut apoptozi. Pri tome dolazi do citruliniranja histona koji mogu potaknuti lokalni imuni odgovor.
Ako se deiminacija proteina događa lokalno, u sinovijalnom tkivu, mora biti prisutan i enzim PAD. Ovaj enzim je ubikvitaran u tkivima sisavaca i do danas je identificirano 5 izoforma s tkivno-specifičnom ekspresijom. PAD2 i PAD4 su, između ostalih tkiva, eksprimirani i u hematopoetskim stanicama (19) i stoga su od posebnog interesa, jer ove stanice infiltriraju upaljeno reumatoidno sinovijalno tkivo. Chapuy-Regaud i sur. (20) dokazali su ekspresiju ove dvije izoforme u sinovijalnoj membrani bolesnika s RA.

Mehanizam RA-specifičnog imunog odgovora na citrulinirane antigene

Izoforme enzima PAD prisutne su u raznim tkivima pa je tako proces citrulinacije dio normalnih fizioloških procesa, kao što je već opisana završna diferencijacija epidermnih keratinocita ili diferencijacija mijelinske ovojnice tijekom razvoja središnjeg živčanog sustava (21). Osim u reumatoidnom sinovijalnom tkivu, ekspresija citruliniranih proteina otkrivena je i u sinovijalnom tkivu bolesnika s osteoartritisom, reaktivnim artritisom i drugim artropatijama (22). Nadalje, citrulinirani proteini otkriveni su u neurodegenerativnim bolestima kao što su Alzheimerova bolest (23) i multipla skleroza (24), ali i u mišićima bolesnika s polimiozitisom, crijevu oboljelih od ulceroznog kolitisa i Crohnove bolesti te kronično upaljenim tonzilama (25). Čini se da je poveznica procesa citrulinacije i navedenih bolesti upala. Međutim, u navedenim slučajevima prisutnost citruliniranih antigena nije povezana s istodobnom prisutnošću autoantitijela, što znači da citrulinirani proteini, čak ni u upalnom okolišu, nisu dostatni za aktivaciju specifične imune reakcije. Kako onda objasniti prisutnost ovih visoko specifičnih autoantitijela u RA? Tri su predložena mehanizma koji bi mogli biti uključeni u RA-specifičan imuni odgovor na ubikvitarne antigene kao što su citrulinirani proteini: 1) poznato je da imuni odgovor ne određuje samo prisutnost, nego i količina prisutnog antigena, što bi značilo da u reumatoidnom sinovijumu dolazi do pretjerane ekspresije citruliniranih antigena, 2)

of α–enolase has been demonstrated in synovial tissue, its *in vivo* citrullination has not yet been confirmed. Bearing in mind that citrulline bound to neutral amino acids like glycine, serine or threonine is critical for AFA binding, Pratesi *et al*. (17) investigated the hypothesis whether deimination of Epstein-Barr nuclear antigen 1 (EBNA-1) that contains 6 repeating arginine-glycine sequences and is often related to RA, can generate epitopes recognizable by AFA. Antibodies specific for this EBNA-1 sequence have been detected in 50% of RA sera and in <5% of sera from normal controls, but the presence of deiminated EBNA-1 in rheumatoid synovial tissue has not been proven. Finally, although the presence of citrullinated histones in inflamed synovial tissue has not yet been proven, these proteins do not seem as negligible potential antigens, since their citrullination has been described *ex vivo* during granulocyte apoptosis (18). A great number of granulocytes with a life span of some 3 days are present in inflamed synovial tissue, and many of them will undergo apoptosis at one time. It is accompanied by citrullination of histones, which may trigger local immune response.
If protein deimination occurs locally, in synovial tissue, the PAD enzyme must be present. This enzyme is ubiquitous in mammalian tissues and 5 isoforms with tissue-specific expression have been identified to date. PAD2 and PAD4 are, among other tissues, also expressed in hematopoietic cells (22), and are therefore of special interest because they infiltrate inflamed rheumatoid synovial tissue. Chapuy-Regaud *et al*. (23) demonstrated expression of these two isoforms in synovial membrane of RA patients.

The mechanism of RA-specific immune response to citrullinated antigens

PAD enzyme isoforms are present in different tissues, so the process of citrullination is part of the normal physiologic processes such as already described final differentiation of epidermal keratinocytes or of myelin sheath during central nervous system development (21). Besides rheumatoid synovial tissue, citrullinated protein expression was also observed in synovial tissue of patients suffering from osteoarthritis, reactive arthritis and other arthropathies (22). Furthermore, citrullinated proteins were detected in neurodegenerative diseases like Alzheimer's disease (23), multiple sclerosis (24), but also in muscles of patients suffering from polymyositis, in the intestine of patients with ulcerative colitis and Crohn's disease and chronic tonsillitis (25). It appears that the link between the process of citrullination and these diseases lies in inflammation. However, in these cases the presence of citrullinated antigen is not connected with simultaneous presence of autoantibodies, which means that citrullinated proteins, even in inflammatory environment, are not sufficient to activate specific immune reaction. How then

nenormalan humoralni imuni odgovor na uobičajeno prisutne citrulinirane proteine u upaljenom tkivu, 3) prisutnost citruliniranih epitopa specifičnih za RA (26). Čini se da bi određeni genetski čimbenici mogli biti odgovorni za prva dva mehanizma. Suzuki i sur. (27) otkrili su povezanost polimorfizama gena *PADI4* koji kodira enzim PAD4 i RA u japanskoj populaciji. Pokazalo se da ovi polimorfizmi dovode do mRNA trostruko veće stabilnosti, što pak upućuje na povećanu sintezu enzima PAD4 u osoba s ovim haplotipom. Ovaj enzim prisutan je u jezgri granulocita i monocita koji u velikom broju infiltriraju upaljeno reumatoidno sinovijalno tkivo pa će povećana razina enzima PAD4 u ovim stanicama imati kumulacijski učinak na konačnu količinu citruliniranih proteina. Dakle, velika količina citruliniranih antigena prezentira se imunom sustavu, što može dovesti do gubitka tolerancije na vlastite antigene. Ipak, povezanost polimorfizama *PADI4* s RA treba uzeti s rezervom, jer nije potvrđena i u drugim populacijama (28). Drugi mehanizam koji bi mogao biti odgovoran za povećanu ekspresiju citruliniranih antigena je neučinkovito uklanjanje mrtvih stanica. Unutarstanična koncentracija Ca^{++} iona preniska je za aktivaciju enzima PAD i on se nalazi u neaktivnom stanju. Tek kad se naruši homeostaza Ca^{++} iona, što se događa npr. pri završnoj diferencijaciji epitelnih stanica, dolazi do njegove aktivacije. Tijekom stanične smrti dolazi do narušavanja integriteta stanične membrane, što može imati dvojak učinak: influks Ca^{++} iona iz izvanstaničnog prostora i aktivaciju unutarstaničnog enzima PAD ili pak istjecanje enzima PAD u izvanstanični prostor s posljedičnim opsežnim citruliniranjem proteina. U stanjima u kojima prevladava neučinkovito uklanjanje mrtvih stanica (zbog masivne stanične smrti ili neučinkovitog mehanizma uklanjanja), kao što je to slučaj u RA, velika količina citruliniranih proteina izložit će se imunom sustavu koji će ih prepoznati kao strane antigene (26). Slijedeći genetski čimbenik koji može utjecati na abnormalan humoralni odgovor na uobičajeno prisutne citrulinirane proteine su RA-rizični HLA-DR aleli, prvenstveno HLA-DRB1*0401 i HLA-DRB1*0404. Naime, nedavno istraživanje je pokazalo da se citrulinirani peptidi derivirani iz vimentina, ali ne i oni koji na istoj poziciji imaju arginin, s visokim afinitetom vežu za molekulu HLA-DRB1*0401 (29). Ova interakcija mogla bi biti osnova citrulin specifičnog imunog odgovora, jer su u pokusu s HLA-DRB1*0401 transgeničnim mišem isti autori dokazali kako stimulacija citruliniranim peptidima, ali ne i argininskom varijantom peptida, potiče proliferaciju i aktivaciju T limfocita. Međutim, odsutnost ACPA antitijela u HLA-DRB1*0401 pozitivnih bolesnika sa spondiloartritisom i psorijatičnim artritisom, u čijoj sinovijalnoj membrani je otkrivena prisutnost deiminiranog fibrina, upućuje na zaključak da samo RA-rizičan haplotip nije dostatan za ACPA specifičan imuni odgovor (12). Nadalje, potencijalni genetski čimbenik koji bi mogao sudjelovati u RA-specifičnom imunom odgovoru

to explain the presence of these highly specific autoantibodies in RA? There are three suggested mechanisms that might be involved in RA-specific immune response to ubiquitous antigens like citrullinated proteins: 1) it is known that immune response is defined not only by the mere presence but also by the quantity of the antigen present, which would mean that there is an over-expression of citrullinated antigens in rheumatoid synovium; 2) abnormal humoral immune response to commonly present citrullinated proteins in inflamed tissue; and 3) presence of RA-specific citrullinated epitopes (26). It seems that certain genetic factors could be responsible for the first two mechanisms. Suzuki *et al*. (27) found an association between polymorphisms of the *PADI4* gene encoding PAD4 enzyme and RA in the Japanese population. These polymorphisms were found to result in a mRNA of threefold stability, pointing to enhanced PAD4 enzyme synthesis in persons with this haplotype. This enzyme is present in the nuclei of granulocytes and monocytes that grossly infiltrate inflamed rheumatoid synovial tissue; so, the elevated level of PAD4 enzyme in these cells will have a cumulative effect on the final quantity of citrullinated proteins. Thus, a high quantity of citrullinated antigenes is presented to the immune system, which can lead to the loss of tolerance to own antigens. However, the association of *PADI4* polymorphisms and RA should be considered with caution, since it has not been confirmed in other populations (28). Another mechanism that could be responsible for excess citrullinated antigen expression is inefficient clearing of dying cells. The intracellular Ca^{++} ion concentration is too low for PAD enzyme activation, so it remains inactive. It is only activated when Ca^{++} ion homeostasis has been disturbed, e.g., upon final epithelial cell differentiation. During cell death, the integrity of cellular membrane is impaired, which can have a dual effect, i.e. Ca^{++} ion influx from extracellular space and activation of intracellular PAD enzyme or PAD enzyme efflux into extracellular space with consequential massive protein citrullination. In conditions predominated by inefficient dead cell removal (due to massive cell death or inefficient mechanism of their elimination) such as RA, a large quantity of citrullinated proteins will be exposed to the immune system, which will recognize them as foreign antigens (26). The next genetic factor that can affect abnormal humoral response to commonly present citrullinated proteins are HLA-DR alleles associated with an increased risk of RA, primarily HLA-DRB1*0401 and HLA-DRB1*0404. Namely, a recent study has shown that vimentin derived citrullinated proteins but not those having arginine on the same locus, bind to HLA- DRB1*0401 molecule with high affinity (29). This interaction may underlay the citrulline-specific immune response, because in an experiment with HLA-DRB1*0401 transgenic mice the same authors proved that stimulation with citrullinated peptides, but not with the arginine variant of peptide, stimulated T

na citrulinirane proteine je polimorfizam gena za citokine koji imaju važnu ulogu u upalnom procesu znakovitom za RA. Jedan od takvih polimorfizama je –2849 A/G u promotorskoj regiji gena za IL-10 koji je odgovoran za povećanu proizvodnju IL-10. Ovaj interleukin, uz brojne protuupalne učinke, djeluje i proupalno u smislu da stimulira proliferaciju i diferencijaciju B limfocita s posljedičnom proizvodnjom antitijela. Pokazalo se da ACPA pozitivni bolesnici s RA s haplotipom koji rezultira visokom koncentracijom IL-10 imaju viši titar antitijela i teže erozivne promjene u odnosu na ACPA pozitivne bolesnike s RA bez ovog haplotipa (30). Viši titar antitijela stvoren lokalno dovodi i do stvaranja više imunokompleksa koji se posredstvom Fcγ receptora vežu za makrofage koji se potom aktiviraju i izlučuju veću količinu proupalnih citokina. Različiti polimorfizmi citokina i njihovih receptora povezuju se s RA (31). Ovi genetski čimbenici uzrokuju otpuštanje veće količine citokina nakon stimulacije ili uzrokuju veću osjetljivost stanica na citokine. Kako su citokini ključne molekule u upalnom odgovoru koji uključuje influks velikog broja upalnih stanica iz cirkulacije i njihovu aktivaciju, time se nastavlja ciklus koji vodi u kroničnu upalu znakovitu za RA. Uz nabrojene genetske čimbenike koji mogu doprinjeti RA-specifičnom imunom odgovoru na ubikvitarne citrulinirane proteine, danas se sve više zastupa i hipoteza o postojanju RA-specifičnih unutarstaničnih citruliniranih epitopa. Imunohistokemijskom metodom, upotrebom mišjeg monoklonskog antitijela, u reumatoidnom sinovijumu su otkriveni unutarstanični citrulinirani proteini koji su, za razliku od izvanstaničnih, specifični za RA. Ovi proteini pokazuju kolokalizaciju s enzimom PAD2 koji je značajno jače eksprimiran u sinovijalnom tkivu bolesnika s RA u odnosu na sinovijalno tkivo kontrolnih ispitanika. Nadalje, utvrđena je korelacija ekspresije unutarstaničnih citruliniranih proteina s lokalnom i sistemskom razinom ACPA antitijela (32). Biokemijski identitet ovih deiminiranih unutarstaničnih proteina još je u fazi ispitivanja.

Metode određivanja ACPA i njihovo kliničko značenje

U testovima ELISA za određivanje ovih antitijela rabe se sintetski citrulinirani peptidi ili citrulinirani proteini prisutnost kojih je dokazana u ekstraktu sinovijalnog tkiva. U izboru proteinskog supstrata prednost imaju oni bogati argininom kao što su fibrinogen i vimentin. Nakon *in vitro* citruliniranja ovi će proteini imati više različitih epitopa koje čine citrulin, ali i susjedne aminokiseline čime se, usporedbom sa sintetskim peptidima, teoretski povećava osjetljivost na poliklonski imuni odgovor. Međutim, s druge strane, upotreba proteina kao supstrata ima svoje nedostatke kao što je problem standardizacije *in vitro* enzimskog citruliniranja te moguća reaktivnost seruma s drugim ne-citruliniranim epitopima.

lymphocyte proliferation and activation. However, the absence of ACPA antibodies in HLA-DRB1*0401 positive patients with spondyloarthritis and psoriatic arthritis, with the presence of deiminated fibrin detected in their synovial membrane, suggests that the haplotype associated with an increased risk of RA alone is not sufficient for ACPA-specific immune response (12). Furthermore, polymorphism of the gene for cytokines that play an important role in inflammatory process characteristic of RA is a potential genetic factor that may be involved in RA-specific immune response to citrullinated proteins. One of these polymorphisms is -2849 A/G in the promotor region of the IL-10 gene responsible for enhanced production of IL-10. Besides numerous anti-inflammatory effects, this interleukin also exerts proinflammatory action by stimulating B lymphocyte proliferation and differentiation with consequential antibody production. It has been shown that ACPA positive RA patients with a haplotype that results in high IL-10 concentration have higher antibody titer and more severe erosive lesions in comparison with ACPA positive RA patients without this haplotype (30). A higher locally formed antibody titer also leads to higher production of immunocomplexes, which bind *via* Fcγ receptors to macrophages that are then activated and secret an increased amount of proinflammatory cytokines. Different polymorphisms of cytokines and their receptors have been associated with RA (31). These genetic factors cause the release of a higher amount of cytokines after stimulation or cause higher cell sensitivity to cytokines. As cytokines are the key molecules in inflammatory response that includes influx of a great number of inflammatory cells from the circulation and their activation, this continues the cycle that leads to chronic inflammation characteristic of RA. Besides the mentioned genetic factors that can contribute to RA-specific immune response to ubiquitous citrullinated proteins, the hypothesis on the existence of RA-specific intracellular citrullinated epitopes has currently been increasingly adopted. Intracellular citrullinated proteins which, unlike extracellular ones, are RA-specific, have been detected in rheumatoid synovium by immunohistochemical method using a mouse monoclonal antibody. These proteins show colocalization with PAD2 enzyme, which is significantly more expressed in RA synovial tissue than in synovial tissue of normal controls. Furthermore, correlation of intracellular citrullinated protein expression with local and systemic level of ACPA antibodies has been established (32). Biochemical identity of these deiminated intracellular proteins is still in the phase of research.

Methods of ACPA determination and their clinical significance

Synthetic citrullinated cyclic peptides or citrullinated proteins the presence of which has been demonstrated

Antitijela na cikličke citrulinirane peptide (anti-CCP)

Identifikacija filagrina kao ciljne molekule koju prepoznaju RA–specifična antitijela AKA i APF omogućila je novi metodološki pristup otkrivanju ovih antitijela specifičnih za RA. Prve metode koje su se upotrebljavale bile su metode imunoblot, nešto kasnije i metode ELISA, u kojima je kao antigeni supstrat upotrebljen filagrin ekstrahiran iz humanog epidermisa (33,34). Međutim, pokazalo se da je gotovo nemoguće prirediti standardizirani supstrat s filagrinom kao antigenom. Razlog tome je činjenica da je gotovo 30–40% aminokiselinskih ostataka na molekuli filagrina promjenjivo i da je citrulinacija argininskih ostataka samo djelomična, pa ove izoforme obilježava velika heterogenost u naboju. Ovaj problem odnosi se i na *in vitro* enzimatski deiminirane bakterijski eksprimirane rekombinantne filagrinske fragmente. Suprotno heterogenom prirodnom supstratu, sintetski peptidi imaju više značajki idealnog antigenog supstrata. Peptidi prethodno definirane sekvence mogu se sintetizirati u velikim količinama s visokim stupnjem čistoće, a njihova mala veličina smanjuje mogućnost nespecifičnih interakcija koje se događaju s drugim sastavnicama u serumu. Ipak, upotreba linearnih sintetskih peptida (sintetiziranih prema molekuli profilagrina) kao supstrata u testu ELISA bila je razočaravajuća, jer se zadovoljavajuća osjetljivost postigla tek upotrebom više različitih sintetskih peptida, što je bilo tehnički prezahtjevno (35). Jedan od problema s upotrebom linearnih peptida kao supstrata je u tome što se standardnim postupkom pasivne adsorpcije ne postiže adsorpcija svih peptida na jažice pločica ELISA. Djelomično rješenje ovoga problema je kovalentno vezanje peptida na kruti nosač. Drugi problem upotrebe relativno kratkih (linearnih) peptida je u tome što oni u otopini uglavnom ne zauzimaju stabilnu konformaciju, pa stoga samo frakcija peptida zauzima konformaciju usporedivu onoj originalnog veznog mjesta na proteinskom antigenu. Ovaj učinak, nazvan "konformacijska dilucija", ima neposredan negativni utjecaj na afinitet antitijela za peptid unatoč činjenici da peptidna sekvenca obuhvaća potpunu antigensku odrednicu. Način da se riješi ovaj problem je omogućiti da peptidi vezani na jažice zauzmu konformaciju koja pogoduje vezanju antitijela, odnosno koja će što više oponašati konformaciju originalnog veznog mjesta na proteinu. Imajući na umu da peptidi često zauzimaju β-konformaciju u kompleksu Ag-At, kao i činjenicu da cistinom premošteni ciklički peptidi oponašaju β-konformaciju originalne antigenske determinante i povećavaju afinitet vezanja za antitijelo, Schellekens i sur. (35) su upotrijebili cikličke peptide formirane supstitucijom serinskih ostataka s cisteinom koji je potom oksidiran u cistin. Tako je stvorena prva generacija testova ELISA s cikličkim citruliniranim peptidima (CCP1) kao antigenim supstratom.

in synovial tissue extract are used in enzyme-linked immunosorbent assays (ELISA) for determination of these antibodies. On choosing protein substrate, those rich in arginine, such as fibrinogen and vimentin, are preferred. After *in vitro* citrullination, these proteins will have more different epitopes that constitute citrulline, but also neighboring amino acids, thus theoretically increasing the sensitivity to polyclonal immune response as compared with synthetic cyclic peptides. On the other hand, however, the use of protein as substrate has some disadvantages such as the problem of standardization of *in vitro* enzyme citrullination and the possible serum reactivity with other non-citrullinated epitopes.

Antibodies to cyclic citrullinated peptides (anti-CCP)

Identification of filaggrin as a target molecule recognized by RA-specific AKA and APF antibodies has enabled a new methodological approach to detection of these RA-specific antibodies. Immunoblot methods were the first methods employed, then also ELISAs were used with filaggrin extracted from human epidermis as antigen substrate (33,34). However, it turned out almost impossible to prepare a standardized substrate with filaggrin used as antigen. The reason is the fact that almost 30%–40% of amino acid residues on the filaggrin molecule are variable and citrullination of arginine residues is only partial, so these isoforms are characterized by great charge heterogeneity. This problem also applies to *in vitro* enzymatic deimination of bacterially expressed recombinant filaggrin fragments. Contrary to heterogeneous natural substrate, synthetic peptides carry more characteristics of an ideal antigenic substrate. Peptides of predefined sequence can be synthesized in large amounts with high purity level and their small size reduces the possibility of nonspecific interactions that occur with other serum components. Still, the use of linear synthetic peptides (synthesized according to profilaggrin molecule) as a substrate in ELISAs proved disappointing because satisfactory sensitivity could only be achieved with the use of several different synthetic peptides, which was technically too demanding (35). One of the problems with the use of linear peptides as substrate is that adsorption of all peptides on the wells of ELISA microtiter plates cannot be achieved by the standard procedure of passive adsorption. Partial solution of this problem is covalent peptide binding to a solid carrier. Another problem encountered on the application of relatively short (linear) peptides is that they generally do not assume stable conformation in a solution and therefore only a fraction of the peptides assumes conformation comparable to that of the original binding site on the protein antigen. This effect named "conformational dilution" has a direct unfavorable effect on the an-

Medijan osjetljivosti dobiven u istraživanjima u kojima se rabio test CCP1 bio je 54% (raspon od 41% do 68%), a specifičnosti 97% (raspon od 90% do 99%) (36). Nekoliko godina poslije osjetljivost testa poboljšana je na način da je biblioteka citruliniranih peptida pretraživana pomoću „poola" seruma bolesnika s RA, što je rezultiralo identifikacijom brojnih visoko reaktivnih peptida koji se trenutno rabe u testovima ELISA druge generacije (CCP2) (37). Godine 2005. na tržištu se pojavila i treća generacija testa anti-CCP (CCP3) u kojem su uključeni dodatni citrulinirani epitopi te verzija testa s istodobnim otkrivanjem IgG i IgA anti-CCP antitijela (CCP 3.1), što bi trebalo poboljšati osjetljivost. Iako su neki autori (38,39) utvrdili poboljšanje osjetljivosti za oko 5% u odnosu na CCP2, rezultati drugih istraživanja pokazali su podjednaku dijagnostičku učinkovitost testova CCP2 i CCP3 (40,41).

Dijagnostička točnost testa anti-CCP2 u RA

Od 2003. godine objavljena su brojna istraživanja koja su se bavila ispitivanjem dijagnostičke točnosti testa anti-CCP2 za RA. Dobiveni rezultati pokazuju značajnu varijabilnost, što se pripisuje razlikama u značajkama ispitivanih skupina bolesnika s RA s jedne strane, odnosno heterogenosti skupina kontrolnih ispitanika s druge strane. Tako se osjetljivost anti-CCP2 u ispitanika s uznapredovalim RA kreće od 64% do čak 96%, dok se u onih s ranim RA ili još nediferenciranim artritisom kreće u rasponu od 14,4% do 83,5% (42). Na specifičnost testa odražava se pak izbor kontrolne skupine. Vrlo često su se u kontrolne skupine uključivali zdravi ispitanici, što precjenjuje specifičnost testa. Isti učinak se postiže s kontrolnom skupinom koju čine ispitanici s istom bolešću. Stvarna dijagnostička svojstva nekog testa proizaći će samo iz istraživanja u kojem su uključeni bolesnici koji predstavljaju stvarnu populaciju na kojoj će se test primjenjivati u kliničkoj praksi. Stoga, uzimajući u obzir samo istraživanja u kojima kontrolna skupina najviše odgovara ovoj pretpostavci, specifičnost anti-CCP2 za RA kreće se od 90,4% do 97,3% (42). Uz one s RA, anti-CCP antitijela nađena su i u oko 9% bolesnika sa sistemskim eritematoznim lupusom (SLE), 5% bolesnika sa Sjögrenovim sindromom, 8% onih s psorijatičnim artritisom te u 2–5% bolesnika s juvenilnim idiopatskim artritisom (36).

Prediktivna vrijednost anti-CCP antitijela za razvoj RA

Rantapaa-Dahlqvist i sur. utvrdili su prisutnost anti-CCP antitijela, kao što je to slučaj i s RF, mnogo godina prije pojave prvih simptoma RA. Retrospektivnom analizom krvi dobrovoljnih davalaca identificirali su 83 bolesnika u kojih se naknadno razvio RA. Osjetljivost anti-CCP antitijela pokazala je tendenciju porasta od 4% za period do

tibody affinity for peptide despite the fact that peptide sequence spans the complete antigen determinant. The way to solve this problem is to enable the peptides bound to microplate wells to assume conformation that favors antibody binding, i.e. which will imitate conformation of the original binding site on the protein as close as possible. Having in mind that peptides often assume β-conformation in Ag-At complex and the fact that the cystine-bridged cyclic peptides imitate β-conformation of the original antigen determinant and enhance the antibody binding affinity, Schellekens *et al*. (35) used cyclic peptides formed by substitution of serine residues with cysteine which is then oxidized into cystine. Thus the first generation of ELISAs with cyclic citrullinated peptides (CCP1) as antigenic substrate were created. Median sensitivity calculated from studies using CCP1 test was 54% (ranging from 41% to up to 68%) and median specificity 97% (ranging from 90% to up to 99%) (36). Several years later, test sensitivity was improved in a way that a library of citrullinated peptides was searched using pool serum of RA patients, which resulted in identification of numerous high-reactive peptides that are currently used in second generation ELISAs (CCP2) (37). In 2005, third generation of the anti-CCP test (CCP3) appeared on the market, including additional citrullinated epitopes and a test version with simultaneous detection of IgG and IgA anti-CCP antibodies (CCP 3.1), which should improve test sensitivity. Although some authors (38,39) found improvement of sensitivity by about 5% in relation to CCP2, results of other studies showed an equal diagnostic efficiency of CCP2 and CCP3 tests (40,41).

Diagnostic accuracy of anti-CCP2 test in RA

Since 2003, a number of studies assessing diagnostic accuracy of anti-CCP2 test in RA have been published. The results show significant variability, which is attributed to differences in the characteristics of RA patient groups on the one hand and heterogeneity of control groups on the other hand. The sensitivity of anti-CCP2 in patients with advanced RA stage spans from 64% to even 96%, while in patients with early RA stage or those with undifferentiated arthritis it ranges from 14.4% to 83.5% (42). The choice of control groups influences test specificity. Healthy subjects have quite frequently been included in control groups, thus overestimating test specificity. Inclusion of patients with the same disease in control group has the same effect. The real diagnostic characteristics of a test can only be obtained in a study including patients that represent the real population in which the test will be used in clinical routine. Therefore, taking into consideration only studies where control groups corresponded best to this presumption, the specificity of anti-CCP2 for RA ranges from 90.4% to 97.3% (42). In addition to RA, an-

9 godina pa do 52% za period do 1,5 godine prije prve posjete liječniku (43). Za podskupinu iz iste kohorte, 2 godine prije pojave simptoma su anti-CCP antitijela pokazala veću prediktivnu vrijednost u odnosu na RF i antigene HLA-DRB1 lokusa za razvoj RA, s omjerom vjerojatnosti (engl. *odds ratio*, OR) od 15,9 (44). Prediktivna vrijednost anti-CCP antitijela za RA potvrđena je i u longitudinalnim istraživanjima na kohortama ispitanika s nespecifičnim ranim upalnim artritisom (nediferencirani artritis). U istraživanju van Gaalena i sur., nakon prve godine je kriterije ACR za dijagnozu RA zadovoljilo 75% bolesnika koji su bili anti-CCP pozitivni u početku, a nakon 3 godine čak 93% (OR 38,6 u odnosu na 9,8 za RF). Među bolesnicima koji su bili anti-CCP negativni u početku, 25% ih je dijagnosticirano kao RA nakon 3 godine (45). U drugom istraživanju, bolesnici sa sinovitisom u trajanju ≤ 3 mjeseca praćeni su 18 mjeseci i pritom se pokazalo da kombinacija anti-CCP i RF ima pozitivnu prediktivnu vrijednost (PPV) od 58% i negativnu prediktivnu vrijednost (NPV) od 88% za dijagnozu RA (46). Iz navedenih rezultata može se zaključiti da je pojava anti-CCP, kao i RF, dio vrlo ranog procesa u razvoju RA. Kliničko značenje prediktivne vrijednosti ovih antitijela za razvoj RA prvenstveno je u skraćenju vremena od pojave prvih simptoma sinovitisa do terapijske intervencije.

Prognostička vrijednost anti-CCP antitijela za razvoj RA

Već se upotrebom testova anti-CCP prve generacije ukazalo na potencijal ovih antitijela u predviđanju erozivne progresije RA. Sve je veći broj istraživanja koja to potvrđuju i upotrebom testova druge generacije. U okviru švedskog istraživanja (TIRA projekt) je 242 bolesnika s ranim RA (trajanje bolesti ≤ 1 god.) praćeno tijekom 3 godine redovitim mjerenjem laboratorijskih [sedimentacija eritrocita (SE) i C-reaktivni protein (CRP)] i kliničkih pokazatelja aktivnosti bolesti (DAS28, engl. *28 joint disease activity score*; broj otečenih zglobova; liječnikova globalna procjena aktivnosti bolesti; procjena funkcionalne sposobnosti pomoću HAQ, engl. *Health Assessment Questionnaire*) te RF (izotipa IgM i IgA) i anti-CCP. Iako je dijagnostička osjetljivost anti-CCP i RF bila podjednaka, pozitivna anti-CCP antitijela pri dijagnozi pokazala su se boljim predskazateljem aktivnosti bolesti u slijedeće 3 godine. Anti-CCP pozitivan status ostao je gotovo nepromijenjen tijekom 3 godine, neovisno o primijenjenoj antireumatskoj terapiji (47). U drugom istraživanju, Forslind i sur. su ispitivali prediktivnu vrijednost više čimbenika, uključujući i anti-CCP antitijela, za radiološki mjerenu progresiju zglobnog oštećenja metodom po Larsenu u 379 bolesnika s ranim RA tijekom 2 godine. Bolesnici s pozitivnim anti-CCP antitijelima imali su značajno veće oštećenje zglobova i na početku i na kraju praćenja u odnosu na one s negativ-

ti-CCP antibodies have also been found in 9% of patients with systemic lupus erythematosus, 5% of patients with Sjögren's syndrome, 8% of those with psoriatic arthritis and 2%–5% of patients suffering from juvenile idiopathic arthritis (36).

Predictive value of anti-CCP antibodies for RA development

Rantapaa-Dahlqvist *et al*. established the presence of anti-CCP antibodies, as in case of RF, many years before the initial RA symptoms. Retrospective analysis of donor blood had identified 83 patients that subsequently developed RA. The sensitivity of anti-CCP antibodies has shown an increasing tendency from 4% in period of 9 years up to 52% in period of 1.5 years before the first visit to physician (43). In a subgroup of the same cohort, anti-CCP antibodies showed a higher predictive value than RF and HLA-DRB1 antigens of the locus for RA development, with odds ratio (OR) of 15.9 two years before the onset of symptoms (44). Predictive value of anti-CCP antibodies for RA was also confirmed in longitudinal studies in cohorts of subjects presenting with nonspecific early inflammatory arthritis (undifferentiated arthritis). In the study by van Gaalen *et al*., at one year ARA criteria for RA diagnosis were satisfied by 75% of patients that initially were anti-CCP positive, and at 3 years the percentage increased to even 93% (OR 38.6 *vs*. 9.8 for RF). Among patients that initially were anti-CCP negative, 25% were diagnosed with RA after 3 years (45). In another study, patients suffering from synovitis for a period of ≤3 months were monitored for 18 months. A combination of anti-CCP and RF was found to have a positive predictive value (PPV) of 58% and negative predictive value (NPV) of 88% for RA diagnosis (46). These results suggest that the occurrence of anti-CCP, like RF, is part of a very early process in the development of RA. Clinical significance of the predictive value of anti-CCP antibodies for RA development lies primarily in reducing the time elapsed from the first synovitis symptom onset to therapeutic intervention.

Prognostic value of anti-CCP antibodies in RA

The use of first generation anti-CCP assays pointed to the potential of these antibodies in predicting erosive progression of RA. There are an increasing number of studies confirming it with the use of second generation assays. In the scope of a Swedish study (TIRA project), 242 patients with early stage RA (disease duration ≤1 year) were followed-up for 3 years by regular measurement of laboratory [erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)] and clinical indicators of disease activity (28 joint disease activity score (DAS28); swollen joint count; physician's global assessment of disease ac-

nim anti-CCP antitijelima. Isti trend u odnosu na anti-CCP status pokazali su i laboratorijski i klinički pokazatelji aktivnosti bolesti (48). Logističkom regresijskom analizom najvažnijim varijablama u predviđanju progresije zglobnog oštećenja pokazali su se stupanj oštećenja zglobova po Larsenu, anti-CCP i SE izmjereni u početku. Ovaj model omogućuje ispravnu klasifikaciju u smislu progresije oštećenja u 75% slučajeva. Rönnelind i sur. su praćenjem 279 bolesnika s ranim RA potvrdili da prisutnost anti-CCP antitijela u početku predskazuje nepovoljan tijek bolesti kroz 5 godina i progresivnije oštećenje zglobova tijekom 2 godine (49). Treba naglasiti da je rezultat ovoga istraživanja pokazao kako promjene u koncentraciji anti-CCP antitijela ne odražavaju aktivnost bolesti. U nedavno objavljenom istraživanju su se anti-CCP, IgM-RF, SE i ženski spol pokazali neovisnim prediktorima progresije zglobnog oštećenja tijekom 10 godina, pri čemu su se pozitivna anti-CCP antitijela pokazala najjačim prediktorom (50).

Prediktivna vrijednost anti-CCP antitijela komplementirana drugim varijablama kao što su SE, radiološki procijenjeno oštećenje zglobova, RA-rizični HLA-aleli ili RF, omogućuje vrlo ranu identifikaciju bolesnika s visokim rizikom za razvoj progresivnog erozivnog artritisa u kojih je indicirana agresivna antireumatska terapija.

Antitijela na citrulinirani vimentin (anti-CV) i mutirani citrulinirani vimentin (anti-MCV)

Prije nešto više od desetljeća, Despres i sur. (51) opisali su antitijela u serumu bolesnika s RA koja su, prema njegovu prezimenu (Savoie), nazvali Sa-antitijela. Sa-antitijela prepoznaju proteinsku vrpcu od 50 kDa na imunoblotu ekstrakta humane slezene, odnosno 2 vrpce (50 i 55 kDa) na imunoblotu ekstrakta humane placente. Ova antitijela nalaze se u 21–43% bolesnika s RA uz vrlo visoku specifičnost od 92–100% (52). Godine 2004. Vossenaar i sur. (14) su sekvencioniranjem Sa antigena pročišćenog iz ekstrakta placente otkrili nekoliko peptidnih sekvenca koje odgovaraju sekvencama vimentina. Western blot analizom isti autori potvrdili su vezanje anti-Sa antitijela na citrulinirani rekombinantni vimentin, ali ne i za nemodificirani vimentin, što upućuje na to da je prisutnost citrulina bitna za autoantigeničnost Sa. Ovim otkrićem su Sa-antitijela, identificirana kao antitijela na citrulinirani vimentin (anti-CV), ubrojena u veliku obitelj antitijela na citrulinirane proteine. Polimerizirane molekule vimentina čine strukturu intermedijarnih filamenata, glavne sastavnice citoskeleta u mezenhimskim stanicama (hondrociti, sinovijalni fibroblasti) i makrofazima pa je stoga očekivana njegova prisutnost u sinovijalnom tkivu. Intermedijarni filamenti, zajedno s aktinskim mikrofilamentima i mikrotubulima, tvore mrežu odgovornu za mehanički integritet stanice i uključeni su u kritične procese kao što je dioba stanice i pokretljivost. Polimerizacija slobodnih podjedinica

tivity; assessment of functional ability by the Health Assessment Questionnaire (HAQ); RF values (of IgM and IgA isotypes) and anti-CCP values. Although the diagnostic sensitivity of anti-CCP and RF was almost equal, positive anti-CCP antibodies at diagnosis proved to be better predictor of disease activity in the next 3 years. Anti-CCP positive status remained almost unchanged during 3 years irrespective of the antirheumatic therapy applied (47). In another study, Forslind *et al*. examined predictive value of several factors including anti-CCP antibodies for radiologically measured progression of articular damage using the method of Larsen in 379 patients with early RA during 2 years. Patients with positive anti-CCP antibodies had a significantly greater articular damage both at the beginning and at the end of monitoring than those with negative anti-CCP antibodies. The same tendency in relation to anti-CCP status was observed in laboratory and clinical indicators of disease activity (48). Logistic regression analysis pointed to the grade of articular damage according to Larsen, anti-CCP and the ESR measured initially as the most important variables in predicting the progression of articular damage. This model enables correct classification in terms of damage progression in 75% of cases. By monitoring 279 patients with early stage of RA, Rönnelind *et al*. confirmed the presence of anti-CCP antibodies initially to predict unfavorable course of the disease in the next 5 years and more progressive joint damage in 2 years (49). It should be noted that the results of this study have revealed that changes in anti-CCP antibody concentration do not reflect disease activity. In a recently published study, anti-CCP, IgM-RF, ESR and female sex were found to be independent predictors of articular damage progression over 10 years, with positive anti-CCP antibodies as the strongest predictor (50).

The predictive value of anti-CCP antibodies complemented with other variables such as ESR, radiologically assessed joint damage, RA-risk HLA-alleles or RF allows for very early identification of patients at a high risk of developing progressive erosive arthritis, where aggressive antirheumatic therapy is indicated.

Antibodies to citrullinated vimentin (anti-CV) and modified citrullinated vimentin (anti-MCV)

More than a decade ago, Despres *et al*. (51) described new antibodies in serum of RA patient, which they named Sa antibodies after family name of the patient where it was first identified (Savoie). Sa antibodies recognize protein band of 50 kDa on the human spleen extract immunoblot and 2 bands (50 and 55 kDa) on the human placenta extract immunoblot. These antibodies are present in 21%–43% of RA patients with a very high specificity of 92%–100% (52). In 2004, Vossenaar *et al*. (14) discovered

nica vimentina u filamente je reverzibilni proces u kojem dolazi do defosforilacije (53). *In vivo*, vimentin obično nije u citruliniranom obliku, ali je dokazano da se deiminacija argininskih ostataka na molekuli vimentina događa u makrofazima tijekom apoptoze (54). Kako je poznato da *in vitro* deiminacija amino-terminalne domene molekule vimentina uzrokuje nestabilnost mreže intermedijarnih filamenata, pretpostavlja se da bi citrulinacija vimentina *in vivo* mogla biti uključena u kolabiranje citoskeleta tijekom apoptoze kad se filamenti vimentina odlažu u perinuklearne agregate. U stanjima praćenim nedostatnim uklanjanjem apoptotičnog materijala, kao što je to slučaj u RA, citrulinirani vimentin se izlaže imunokompetentnim stanicama koje reagiraju stvaranjem autoantitijela. Međutim, kako citrulinacija *per se* nije dovoljna za pokretanje ovakve imune reakcije, pretpostavilo se da upalni mikrookoliš u kojem prevladavaju reaktivni radikali kisika i dušika, u sinovijalnim fibroblastima i makrofazima može izazvati mutacije gena za vimentin i tako stvoriti nove antigene. Iz fibroblasta i sinovijalne tekućine bolesnika s RA pročišćene su izoforme vimentina karakterizirane LC/MS spektroskopijom i identificirane mutacije kao i citrulinacija. Rekombinantne mutirane izoforme vimentina, eksprimirane su u *E. coli*, a pročišćeni protein citruliniran *in vitro* i iskorišten kao antigen u ELISA testu za određivanje IgG antitijela na mutirani citrulinirani vimentin (anti-MCV).

Dijagnostička točnost i prognostička vrijednost anti-MCV antitijela u RA

Unatoč očekivanju da bi primjena citruliniranog proteina kao ciljnog antigena umjesto sintetskih peptida mogla povećati osjetljivost testa za otkrivanje ACPA antitijela, rezultati dosadašnjih istraživanja ukazuju na podjednaku dijagnostička točnost testova anti-MCV i anti-CCP2. Dejaco i sur. su usporedbom 164 bolesnika s RA i kontrolne skupine bolesnika s drugim upalnim i neupalnim reumatskim bolestima utvrdili osjetljivost testa anti-MCV od 69,5% i specifičnost 90,8%, u odnosu na test anti-CCP2 čija je osjetljivost bila 70,1% i specifičnost 98,7% (55). U drugom istraživanju su anti-MCV antitijela pokazala 9% veću osjetljivost u odnosu na anti-CCP2 antitijela i 4% veću u odnosu na RF, međutim, ukupna dijagnostička točnost prema ROC analizi bila je nešto niža od anti-CCP2, ali ne značajno. Iako su ova antitijela značajno međusobno korelirala, što upućuje na brojne zajedničke epitope, prisutnost samo jednog antitijela u oko 12% bolesnika navodi na zaključak da citrulinirani antigeni u oba testa sadrže i jedinstvene epitope koje prepoznaje samo jedno ili drugo antitijelo. Stoga autori predlažu kombinirano određivanje anti-CCP2 i anti-MCV u svrhu poboljšanja dijagnostičke učinkovitosti (56). Mathsson i sur. određivali su anti-CCP i anti-MCV antitijela u 273 bolesnika s ranim RA u trenutku pojave simptoma, te nakon 3 mjeseca i 1 godine, a

several peptide sequences matching vimentin sequences by sequencing Sa gene purified from placenta extract. Using Western blot analysis, the same authors confirmed anti-Sa antibody binding to citrullinated recombinant vimentin but not to non-modified vimentin, suggesting the presence of citrulline to be essential for Sa autoantigenicity. With this discovery, Sa antibodies identified as antibodies to citrullinated vimentin (anti-CV) were included in the large family of antibodies to citrullinated proteins. Polymerized vimentin molecules constitute a structure of intermediary filaments, the main component of cytoskeleton in mesenchymal cells (chondrocytes, synovial fibroblasts) and macrophages, thus its presence in synovial tissue is expected. Intermediary filaments together with actin microfilaments and microtubules constitute a network responsible for mechanical cell integrity and are involved in critical processes like mitosis and motility. Polymerization of free vimentin subunits into filaments is a reversible process where dephosphorylation occurs (53). Vimentin is usually not found in citrullinated form *in vivo*, but deimination of arginine residues on vimentin molecule has been demonstrated to occur in macrophages during apoptosis (54). As *in vitro* deimination of the amino-terminal domain of the vimentin molecule is known to cause instability of the intermediary filament network, it has been postulated that vimentin citrullination *in vivo* might be involved in cytoskeletal collapse during apoptosis, when vimentin filaments are being deposited into perinuclear aggregates. In conditions accompanied by inadequate apoptotic material clearance, as in RA, citrullinated vimentin is exposed to immunocompetent cells that react by the production of autoantibodies. However, as citrullination *per se* is insufficient to trigger such an immune reaction, it has been postulated that inflammatory microenvironment predominated by reactive oxygen and nitrogen radicals can induce gene mutations for vimentin and thus create new antigens in synovial fibroblasts and macrophages. Vimentin isoforms were purified from fibroblasts and synovial fluid from RA patients, characterized by LC/MS spectroscopy, and mutations and citrullination were identified. Recombinant mutated vimentin isoforms were expressed in *E. coli*, whereas purified protein was citrullinated *in vitro* and used as antigen in ELISA for determination of IgG antibodies to mutated citrullinated vimentin (anti-MCV).

Diagnostic accuracy and prognostic value of anti-MCV antibodies in RA

Despite expectations that the use of citrullinated protein as target antigen instead of synthetic peptides could increase test sensitivity for ACPA antibody detection, results of the studies reported to date point to comparable diagnostic accuracy of anti-MCV and anti-CCP2 tests. Dejaco

za 72 bolesnika određivanje anti-CCP i anti-MCV antitijela nastavljeno je tijekom 2, 3 i 5 godina (57). Osjetljivost anti-MCV antitijela u ovom istraživanju bila je značajno viša (70,7%) u odnosu na anti-CCP2 (57,9%) uz podjednaku specifičnost (95% *vs*. 96%). Međutim, treba napomenuti da se dobivena dijagnostička točnost odnosi na usporedbu bolesnika s ranim RA i zdravih ispitanika. Pozitivna anti-MCV antitijela pri pojavi prvih simptoma pokazala su se podjednako dobrim predskazateljem progresivnog erozivnog artritisa kao i anti-CCP antitijela. Čini se pak da su anti-MCV antitijela osjetljiviji pokazatelj aktivnosti bolesti. Naime, promjene u koncentraciji anti-MCV antitijela tijekom 1 godine pokazale su snažniju korelaciju s promjenama laboratorijskih (SE i CRP) i kliničkih pokazatelja aktivnosti bolesti (DAS28, HAQ, liječnikova procjena aktivnosti bolesti, broj otečenih zglobova). U nedavno objavljenom radu Innala i sur. potvrdili su bolju povezanost anti-MCV antitijela s težim tijekom bolesti u odnosu na anti-CCP antitijela određena testovima druge i treće generacije, uz podjednaku prediktivnu vrijednost za progresiju radiološki procijenjenog zglobnog oštećenja (58).

Na osnovi rezultata dosadašnjih istraživanja čini se da anti-MCV antitijela nisu donijela značajan doprinos u dijagnostičkom smislu, međutim, klinička važnost njihovog određivanja u ranom RA leži u prognostičkoj vrijednosti za teži tijek bolesti.

Zaključak

Moderni terapijski pristup RA koji podrazumijeva ranu primjenu agresivnih antireumatika u svrhu sprječavanja ireverzibilnih zglobnih oštećenja nameće potrebu za ranom potvrdom dijagnoze, kao i prognoze tijeka bolesti. Dosadašnji dijagnostički pristup koji se zasniva na kliničkim parametrima i RF kao jedinom serološkom biljegu pokazao se nedovoljno osjetljivim i specifičnim u kritičnoj, ranoj fazi bolesti. Otkriće citrulina kao glavne odrednice ciljnih antigena visoko specifičnih antitijela u RA označilo je početak nove ere u dijagnozi RA. Upotreba sintetskih cikličkih citruliniranih peptida (CCP) kao antigena u testovima ELISA povećala je osjetljivost ovih antitijela za RA. Nadalje, ova visoko specifična antitijela pokazala su se i vrijednim prognostičkim biljegom. Ubikvitarnost citruliniranih proteina u fiziološkim i patološkim stanjima, kao i prisutnost antitijela na citrulinirane proteine mnogo godina prije pojave simptoma RA govori u prilog tome da citruliniranje *per se* nije dostatno za razvoj RA. Stoga je razumijevanje mehanizma RA specifičnog imunog odgovora na citrulinirane proteine u žarištu istraživanja posljednjih nekoliko godina. Jedan od najvažnijih ciljeva u tim istraživanjima je identifikacija citruliniranih proteina kao potencijalnih antigena u upaljenom sinovijalnom tkivu. Do danas su identificirani brojni proteini kao potencijalni nativni antigeni, kao što su citrulinirani vimentin,

et al. compared 164 RA patients and control group of patients suffering from other inflammatory and non-inflammatory rheumatic diseases, and found 69.5% sensitivity and 90.8% specificity for anti-MCV test *vs*. 70.1% sensitivity and 98.7% specificity for anti-CCP2 test (55). In another study, anti-MCV antibodies showed sensitivity higher by 9% and 4% in comparison with anti-CCP2 antibodies and RF, respectively. However, diagnostic accuracy according to ROC analysis was somewhat but not significantly lower than for anti-CCP2. Although these antibodies were in significant correlation, pointing to many common epitopes, the presence of only one antibody in about 12% of patients suggests that citrullinated antigens in either test contain unique epitopes that are only recognized by one or the other antibody. Therefore, the authors suggest combined determination of anti-CCP2 and anti-MCV in order to improve diagnostic efficiency (56). Mathsson *et al*. determined anti-CCP and anti-MCV antibodies in 273 patients with early RA at the time of symptom onset, at three months and at one year; in 72 patients anti-CCP and anti-MCV antibody determination was continued at 2, 3 and 5 years (57). In this study, the sensitivity of anti-MCV antibodies significantly exceeded (70.7%) that of anti-CCP2 (57.9%), with comparable specificity (95% *vs*. 96%). However, it should be noted that diagnostic accuracy refers to comparison of patients with early RA and healthy subjects. Positive anti-MCV antibodies at first symptom onset turned out to be as good predictor of progressive erosive arthritis as anti-CCP antibodies. It seems that anti-MCV antibodies are a more sensitive indicator of disease activity. Namely, changes in the concentration of anti-MCV antibodies during one year yielded higher correlation with changes in laboratory (ESR and CRP) and clinical indicators of disease activity (DAS28, HAQ, doctor's assessment of disease activity, swollen joint count). In a recent study, Innala *et al*. confirmed higher association of anti-MCV antibodies with more severe course of the disease in comparison with anti-CCP antibodies determined by second and third generation tests, with almost equal predictive value for progression of radiologically assessed articular damage (58).

Based on the results of the studies reported to date, it seems that anti-MCV antibodies failed to make any significant diagnostic contribution, however, their determination in early RA is clinically relevant for their prognostic value for a more severe course of the disease.

Conclusion

Modern therapeutic approach to RA that assumes early introduction of aggressive antirheumatics to prevent irreversible articular damage poses the need of early verification of the diagnosis and prognosis of the course of disease. Current diagnostic approach based on clinical

fibrin i histoni. Prema najnovijim saznanjima, čini se da su za indukciju imunog odgovora prije odgovorni točno određeni citrulinirani epitopi negoli sama prisutnost citruliniranih proteina, pa je definiranje ovih epitopa glavni izazov u daljnjim istraživanjima.

parameters and RF as the only serologic marker has proved inadequately sensitive and specific in the critical, early stage of the disease. The discovery of citrulline as the main determinant of target antigens of highly specific antibodies in RA has marked the beginning of a new era in the diagnosis of RA. The use of synthetic cyclic citrullinated peptides (CCP) as antigens in ELISAs has increased the sensitivity of these antibodies for RA. Furthermore, these highly specific antibodies have turned out to be a valuable prognostic marker. The ubiquity of citrullinated proteins in physiological and pathological conditions as well as the presence of antibodies to citrullinated proteins many years before the first RA symptom onset suggest that citrullination *per se* is not sufficient for RA development. Therefore, understanding of the mechanisms of RA-specific immune response to citrullinated proteins has been in the focus of research in the last years. One of the most important goals in these researches is identification of citrullinated proteins as potential antigens in inflamed synovial tissue. Up to now, numerous proteins have been identified as potential native antigens, such as citrullinated vimentin, fibrin and histones. According to latest findings, it seems that distinct citrullinated epitopes rather than mere presence of citrullinated proteins are responsible for the induction of immune response. Therefore, defining of these epitopes remains as the major challenge in forthcoming researches.

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