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RASSEGNA

Citrullination: the loss of tolerance and development of autoimmunity in rheumatoid arthritis

Citrullinazione: perdita della tolleranza e comparsa di autoimmunità nell'artrite reumatoide

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RIASSUNTO

L'artrite reumatoide (AR) è una malattia caratterizzata da infiammazione cronica, da progressiva formazione di panno sinoviale e distruzione ossea. Le proteine, nel genoma sono codificate da un limitato numero di geni. Modifiche post-traduzionali, come la citrullinazione nei residui di arginina, possono creare diversità strutturali e funzionali. Molte proteine autologhe sono considerate possibili autoantigeni capaci di indurre il processo autoimmune. È stato dimostrato che la presenza di anticorpi anticitrullina sia antecedente la comparsa di sintomi di artrite. È ipotizzabile che la flogosi sinoviale e la reazione di sintesi del nitrossido, in particolare nel contesto di una genetica predisponente, porti alla risposta autoimmune, nei confronti di antigeni post-traduzionalmente strutturalmente modificati.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and pannus formation, which can lead to severe destruction of cartilage and bone. Several self proteins have been suggested to be disease-driving autoantigens. Moreover the presence of autoantibodies to citrullinated proteins in sera of patients with RA enhances the strength of this hypothesis.

Proteins are encoded by a limited number of genes in our genome. Post-translational modifications such as phosphorylation, glycosylation and citrullination can increase the morphological and the functional diversity of the proteome. Post-translational modifications (PTM) are very common processes that modify specific parts of a protein after its synthesis. To date all known proteins undergo some form of post-translational modification, and every amino acid can be altered by these processes. The modified proteins obtain rare amino acids with critical influence on the structure and function of the original molecule. One of these peptide-bound residues is arginine that can undergo this modification and replaced by citrulline that remains part of the protein as peptidyl citrulline (1). Citrulline is not a natural amino acid in proteins structure and may induce immune response.

THE CITRULLINATION REACTION

After the conversion of arginine (Arg) to citrulline (Cit), there are changes of the charge of the amino acid. Arginine is a strongly basic residue due to the presence of a guanidino group. The resulting citrulline loses the strong basic character because of its neutral nature (2).

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The conversion process is an enzymatic reaction catalyzed by a family of calcium-binding enzymes called peptidylarginine deiminases (PAD).
During the reaction there is a 1 Dalton mass reduction for each Arginine modified and the basic charge (s) lost influences the overall charge, charge distribution and the isoelectric point of the resulting protein. Even the interactions of the protein with others might be altered (3).
As said before, the deimination process is catalysed by PAD whose five isoenzymes have been identified (4).
These isoenzymes are distributed in many mammalian tissues: PAD1 is mainly expressed in the epidermis and in the female reproductive system; PAD3 in the hair follicles and PAD4, the human homologue of the mouse one, previously named PAD5, in neutrophils and eosinophils. PAD6 has been found in ovaries, in embryos at the early stage of development and in eggs and PAD2 has an ubiquitous distribution (for example skeletal muscles, spleen, brain, secretory glands) (5).
The mechanism of deimination involves the active site of the enzyme with Cys residue that links the guanidino group of the arginine and establishes a tetrahedral transition state while ammonia residue is released. The intermediate is then cleaved after the attack of a water molecule. This last step of the process regenerates the Cys residue and leads to the formation of a ketogroup and a new primary amine (6).
The catalytic process could change the primary, secondary and tertiary structures of proteins. Several in vitro analysis showed that a high degree of citrullination could denature proteins (3) and experiments with filaggrin revealed that change of 5% of the arginine residues modifies the tertiary structure, and modification of more than 10% of the arginines leads to a complete denaturation of the protein. So, In vivo, under physiological conditions, it seems possible to assume that the modification alters the protein structure and results in a less organised and more open configuration (3). Consequently this process may influence the interaction of the new molecule with other proteins.
Any arginine residue could be deiminated and represent a physiological substrates of PAD enzymes. Several studies have shown various proteins citrullinated in vitro although with different kinetic parameters (7, 8). Data reveals some preferences of peptidylarginine deiminases on the primary and secondary structure of the substrate. For example the beta-turn secondary configuration represents the most susceptible region for deimination (3).
Only few proteins in healthy conditions contain citrulline. Some of these are nuclear histones, which have other different post-traslational modifications such as methylation of arginine residues in the aminoacid chain. It seems that citrullination and methylation of arginine residues are competing processes. Pruijn and colleagues, evaluating PAD enzymes from several mouse tissues, showed that they are able only to convert non-methylated arginine into citrulline (9).
Calcium is the key regulator of PAD enzymes. In 2004, Sato and colleagues analysed the structural basis for Calcium-induced activation of human PAD4. They presented the crystal structure of Ca-free wild type PAD4, which have five Calcium binding sites in its 3D conformation. The ion is able to induce conformational changes that generate the opening of the active site in the C-terminal domain. The new conformation is essential for the catalysis (10).
In vitro citrullination is only detectable in the presence of high calcium concentration (11) but in healthy conditions, its concentration range is 10^{-8}-10^{-6} M, so PADs are inactive (12).This suggests that the citrullination process happens only under 'extreme' conditions, such as during apoptosis or terminal differentiation of the epidermis. PADs are enzymes primarily involved in apoptotic and differentiation events in physiological conditions and recently they have also been involved in other cellular processes, such as gene regulation (13, 14).

AUTOANTIBODIES AGAINST CITRULLINATED PROTEINS

The history of anti-citrullinated protein antibodies starts in 1964, when Nienhuis and Mandema, performing IIF with RA sera on human buccal mucosa cells, noticed reactivity with an unknown protein component that they called the perinuclear factor (15, 16).
Many years later, Young and colleagues (17), through IIF on rat, described the specific staining in the stratum corneum of the esophageal epithelium. Thinking that the target antigen was keratin, these antibodies were called AKA (anti-keratin antibodies). Later it was found that both the APF and the AKA reacted with filaggrin, a citrulline containing protein associated with keratin

filaments in the citoskeletal structure. These antibodies were named anti-filaggrin antibodies (AFA) (18).
The anti-CCP antibodies are produced locally in the inflamed synovium of RA patients (19, 20), suggesting that citrullinated proteins are located in the inflamed synovium. The absence of filaggrin expression in the (inflamed) synovium suggested that other citrullinated proteins should exist in the joint. To investigate this, a library of citrullinated peptides was screened with a pool of RA sera and this resulted in the identification of a number of highly reactive peptides that are currently used in the second generation CCP test (CCP2). This CCP2 test has a sensitivity of about 82% for RA with 98–99% of specificity (21). In our cohort of 177 patients with long-standing RA (mean DAS44 value = 2.88 ± 1.30) we found a prevalence of 67.7% for anti-citrullinated proteins antibodies, compared to a prevalence of 46.3% for IgM rheumatoid factor and 37.8% for IgA rheumatoid factor (Tab.I).

ANTI-CCP ANTIBODIES ARE PRESENT EARLY IN RA

Two different studies, using stored samples of sera of patients with RA who were blood donors, showed the presence of anti-CCP antibodies prior to the appearance of the first clinical symptoms of arthritis (22, 23).
These studies demonstrated that the positivity of anti-citrullinated peptides autoantibodies and rheumatoid factor autoantibodies is an early stage of the disease development. Data from longitudinal studies underline the predictive power of anti-CCP2 tests for RA development (24-26).
Van Gaalen and colleagues, studying patients attending an early arthritis clinic who were at first classified as undifferentiated arthritis (UA), tried to predict which of these patients would progress to RA in the next years. One year later, 75% of UA patients who were anti CCP positive developed RA. From the UA group, anti CCP negative at baseline, only 25% of patients was classified as RA after three years (24). Even Vittecq and colleagues reached similar data. Of 314 UA patients, 90% of the anti CCP ones were classified as RA at one-year follow up (25). This early aspect may be useful for the clinician in the early diagnosis and in the selection of patients to treat with aggressive therapeutic protocols very early on. In our cohort of 110 early rheumatoid arthritis (ERA) patients the prevalence of anti-CCP positivity is 70.0% compared to the prevalence of 60.9% for IgM Rheumatoid Factor and of 43.6% for IgA rheumatoid factor. In long standing RA (LSRA) we observed a slightly lower prevalence of IgM-RF likely due to the underlying therapy with aggressive combination protocols (Tab. I).

INTRA-ARTICULAR CITRULLINATION

The first evaluation to address this hypothesis was the clear demonstration of citrullinated proteins in the RA synovium. In synovial tissue specimens the first evidence of the presence of citrullinated peptides was provided by performing immunoblotting analysis (27, 28).
Then Serre and colleagues showed the abundant presence of citrullinated alpha and beta fibrin chains at the immunostaining procedure, even if its presence is not specific of RA, since it could be detected even in spondylarthropaties and inflammed osteoarthritis synovium (27). During the years, multiple possible deiminated synoval proteins were found such as: fibronectin, associated with citrullinated fibrin because of their parallel staining (29, 30); α-enolase, even if Western blotting did not confirm its citrullination in vivo (31);

Table I - Autoimmune profile in patients with early and long standing rheumatoid arthritis. Personal cohort with a long term follow-up.

	Long standing RA patients n = 177	*Early RA patients n = 110*
Age, years (mean ± SD)	57.1±13.6	54.9±13.8
Disease duration, years (mean ± SD)	13.7±10	0.51±0.73
Anti CCP positive, n (%)	120 (67.7)	77 (70.0)
RF IgM positive, n (%)	82 (46.3)	67 (60.9)
RF IgA positive, n (%)	67 (37.8)	48 (43.6)
DAS44 value, (mean ± SD)	2.9±1.3	4.2±1.1

Sa antigen,(at first found in human placenta, spleen and RA synovium) was later seen to correspond to vimentin (32, 33), Epstein-Barr nuclear antigen 1 (EBNA-1) and nuclear proteins such as histones (34, 35).
Different studies showed that PAD4 is expressed in the synovial tissue. Chang and colleagues found that PAD4 is widely expressed in T cells, B cells, macrophages, neutrophils, fibroblast-like cells and endothelial cells in the lining and in the sublining areas of the RA synovium. They underlined that this expression has intra and extracellular distribution, generally associated with fibrin deposits.
This co-localization suggests that PAD4 is responsible for fibrin citrullination that may be a potential antigen of RA autoimmunity (36).
The anti-citrulline autoantibodies are thought to be produced by local plasma cells, since significant amounts of anti-citrullinated peptides antibodies have been measured in synovial cells cultures supernatants of tissue from anti-filaggrin antibody-positive RA patients (37).
Moreover anti CCP producing B cells were found in the synovial fluid of RA patients. Isolating B cells from synovial fluid or bone marrow from anti CCP-positive patients, it was found that they are able to produce IgM anti-CCP antibodies without stimulation, whereas B cells from peripheral blood required stimulation (38). So the clear presence of such active B cells directly in the synovial cavity underlines the antigen-driven nature of the inflammatory process. Alternatively, it is likely that the response to citrullinated proteins, rather than their presence, is specific for RA (39).
Recently, citrullinated proteins were found even in synovial exosomes (40). Exosomes are saucer shaped vesicles with a diameter of 30-10 nm, that are released from cells after the fusion of multiple endosomes to the cell surface. These particles with their modified content could stimulate T cell-proliferation and play an important role in B cell activation even directly stimulating the BCR through the presentation of autoantigenic peptides on their surface (41).
Distler and colleagues showed that during specific cells conditions, apoptosis or activation, there is an increased release of microparticles from T cells and monocytes. These phenomena are very frequent and abundant in inflammatory disease such as Rheumatoid Arthritis and may represent a novel stimulatory way for the release of cytokines, chemokines and inflammatory mediators (42).

EXTRA-ARTICULAR SITES OF CITRULLINATION

This enzymatic process normally occurs in specific backgrounds as epidermal differentiation, growth of hair follicles and maturation and differentiation of myelin during the development of the central nervous system (43). Makrygiannakis and colleagues in 2006, evaluated the presence of citrullinated proteins in synovial biopsies specimens from patients with RA, healthy controls, myositis-affected muscles, inflamed tonsil tissues and intestinal tissue from patients with inflammatory bowel disease. The presence of citrullinated proteins in all the specimens characterized by chronic inflammation suggested to the authors that this process is inflammation rather than disease dependent (44).
The citrullination phenomenon is not only present in synovial tissue of patients with RA but can also occur in extra-articular targets involved by the disease. Moreover it is not clear if it could contribute to the local disease process by an anti citrullinated peptides autoantibodies mediated way. Bongartz and colleagues detected the presence of citrulline in lung specimens of patients with RA associated interstitial pneumonia (IP) and patients with idiopathic IP compared with healthy controls (45).
They found that, although anti citrulline antibodies has high specificity for RA, citrullination was not restricted to patients with RA associated IP but can also be found in idiopathic IP (45). The citrulline staining was found to be principally with an intracellular location, inside mononuclear cells. this aspect seems to be of great relevance in the light of recent insight in the role of positive and negative selection of autoreactive B-cells depending on the cellular antigen distribution: intracellular antigens may lead to positive B-ell selection and enhances autoantibodies production (46).The next step will be the biochemical characterization of deiminated peptides from fresh RA lung tissue and the comparison with the citrullinated ones in the synovium (45).

GENETIC LINKS WITH CITRULLINATION IN RA

1. PAD polymorphisms

Suzuki an colleagues described the existence of several single nucleotide polymorphisms (SNPs)

in the PAD gene (located on chromosome 1p36). 8 of the 17 SNPs in PAD4 gene were clearly associated with RA. These different SNPs are characterized by a linkage disequilibrium association and this is the reason of their segregation together in distinct haplotypes. Four of the 17 SNPs of PAD4 involves the exons of the same gene. Three of them leads to an aminoacid substitution but only one of the three aminoacid substitution is associated with a change in the electrostatic charge of the residue (39).
Different SNPs in the gene encoding for PAD4, that have been assigned as a susceptibility haplotype to the development of RA, are thought to make PAD4 mRNA more resistant to cellular degradation even if few is known about the relationship between the aminoacid replacement and the final protein function. Suzuki and colleagues evaluated the mRNA stability in vitro and found that stability of susceptible transcripts is higher (approximately threefold) than that of nonsusceptible transcripts (39).
They did not evaluate the possible differences in PAD4 mRNA and protein levels comparing patients with susceptible haplotype versus those with nonsusceptible one. However they suggested that the over-resistance and stability of PAD4 mRNA could lead to a higher citrullination level and the appearance of autoantigens. This hypotesis is supported by the finding that patients carrying the susceptible haplotype in homozygosis have significantly higher anti citrulline antibodies titer (87% versus 67%, $p<0,05$) compared with heterozygosis or nonsusceptible haplotype homozygosis (47).

2. HLA genes: the shared epitope

Since thirty years ago, several studies about the genes-linkage have shown a strong correlation between RA and some HLA-DR alleles, in particular HLADRB1*0401 and HLA-DRB1*0404 (48, 49).
The so called "shared epitope" has been shown to be contained on the third hypervariable (HV3) region of DRB1. This HV3 motif in RA-associated alleles consists of ^{70}QKRAA^{74} or ^{70}QRRAA^{74} in DRB1*0401 and DRB1*0404, respectively (50). These five-AA sequence is crucial to determine the p4 peptide-binding pocket of the DRB1 molecule. Thanks to the positive charge at position 71 this pocket has a very high affinity for negatively charged or uncharged polar molecules (51). Therefore, the SE hypothesis became one of the most well-known hypothesis in the field of HLA and disease associations.
During the citrullination process there is the conversion of the positively charged iminogroup of arginine to the uncharged carbonyl group of citrulline. This may increase the peptide affinity for SE-positive DRB1 molecules (52).
Huizinga and colleagues demonstrated that the SE epitope was associated with anti citrullinated peptides autoantibodies(ACPA)-positive RA patients and not with ACPA-negative RA (53). Analysing if the RF and ACPA associations with SE were independent they found that the association between SE and RF was secondary to the association between SE and ACPA. Analysing the influence of the SE and ACPA on the evolution of recent onset undifferentiated arthritis (UA) to RA, no contribution of the SE was observed on the progression to RA (53).
In order to evaluate if SE-positive HLA DRB1 alleles was associated with the onset of ACPA positive RA or only with ACPA positivity, De Vries and colleagues analysed SE and ACPA influence in the progression of recent undifferentiated arthritis (UA) into RA in a cohort of patients. No contribution on the progression to RA from UA was observed for SE and its presence was associated only with a higher titer of these autoantibodies. According to the authors, the SE alleles do not contribute to the progression of UA into RA but clearly in the development of anti-citrullinated peptides autoantibodies (54).

CITRULLINATION: UNIQUE ORIGIN?

As said before, citrulline formation depends on the conversion of arginine catalysed by PAD enzymes. However citrulline can be formed even as a side product when nitric oxide (NO) is generated from arginine by NO syntetase (NOS).
NOS enzymes can be divided in two separated domains: a C-terminal reductase domain and an N-terminal oxygenase domain (55). There are three isoforms of NOS: endothelial NOS (eNOS) and neuronal NOS (nNOS) that are constitutively expressed, Calcium dependent enzymes, even if eNOS can also be activated in a Calcium independent way; inducible NOS (iNOS) whose activation depends on the presence of cytokines ad inflammatory molecules with a calcium-independent activity (55).
One of the feature of a good-working immune

system is the production of NO: dendritic cells, NK cells, mast cells and phagocytic cells including monocytes, macrophages, Kupffer cells, neutrophils and other cells involved in immune reactions generate NO (56).
Several factors are involved in the regulation of the expression and the action of NOS. IL-12 and IL-18 are thought to be important in the induction of iNOS expression in macrophages in an IFN-gamma dependent manner (57). Many cytokines, as TNF, IL-1, IL-4, TGF-beta are able to induce/suppress guanosine triphosphate cyclohydrolase I, the key enzyme of BH4 synthesis, an essential metabolite for NOS catalysis (56, 58).
At first, NO produced in the site of inflammation, by macrophages expressing iNOS was considered a damaging molecule (59, 60). Analyses on experimental autoimmune diseases such as arthritis, uveitis or encephalomyelitis showed the NOS ability to function as a negative feedback molecule of the TH1 cell response (61, 62). In fact, treating an experimental autoimmune arthritis (EAA) with an arginine analogue able to inhibit all NOS isoforms, there is a improvement of the disease, while using a selective iNOS inhibitor there is no protective effect but even a worsening of arthritis (63, 64).
In a recent study, Ling and colleagues investigated the possible link between NO and the shared epitope effect. They found that cells with LA-DRB1 alleles presented an increased constitutive NO production. Even murine cells expressing human SE DR molecules after transfection, showed the same increasing, underlining that there is no linkage disequilibrium with another gene (65).

ENVIRONMENTAL FACTORS: *SMOKING HABIT*

Cigarette smoking has been shown to have a high association with RA (66-73). At first smoking was

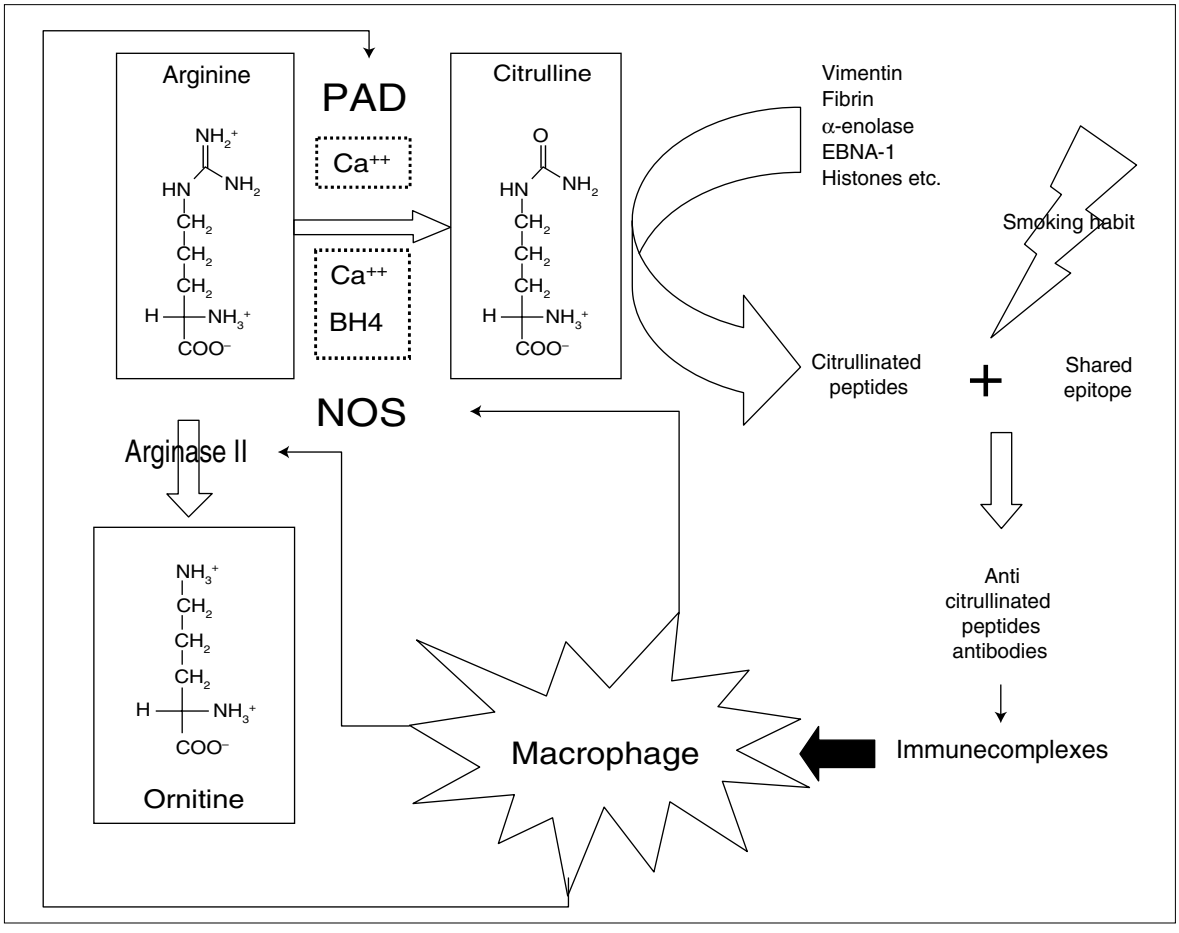

Figure 1 - Loss of tolerance to citrullinated proteins in RA: possible biological mechanisms.

associated with Rheumatoid factor-positive RA and considered to interact with the HLA-DRB1 alleles (74, 75). More recently, Klareskog and colleagues demonstrated that smoking habit and HLA-DRB1 SE were risk factors only for anti-citrulline positive, but not anti-citrulline negative RA and that HLA-DRB1 SE was linked to presence of anti-citrulline immunity rather than to presence of rheumatoid factors (76). The same authors demonstrated even an increased level of citrullination in bronchoalveolar lavage fluid cells from smokers even if another group did not find a significant difference in citrulline staining lung specimens from patients with RA associated IP comparing with idiopathic IP (45). In order to confirm the interaction between cigarette smoking and shared epitope, Lee and colleagues recently performed an analysis of three North American RA cohorts. They found that the shared epitope correlated with the anti citrullinated peptides autoantibodies in all three cohorts but only in two a significant correlation between smoking and ACPA was found. After a multiple logistic regression analysis, carrying the shared epitope is still the most significant risk factor for ACPA development but a possible interaction between shared epitope and smoking for ACPA positivity was not confirmed (77). Recent data suggest that environmental factors contribute to anti-CCP autoantibodies in patients carrying the shared epitope, but not in those without the genetic setting (78).

BIOLOGICAL INTERPRETATIONS OF GENE, ENVIRONMENT AND IMMUNITY INTERACTION IN RA

All these biological and clinical data suggest that citrullination in a definite genetic setting allows the emergence of the loss of tolerance to several proteins (Fig. 1). Why the loss of tolerance occurs, because of the citrullination, is not yet fully defined, but the increased affinity for the DRB1 task of the shared epitope might provide the clue to understand the autoreactivity. How to control the loss of tolerance and how to possibly rescue the control of autoreactivity in the earliest phases of the disease remains a matter of further investigations.

SUMMARY

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and pannus formation leading to destruction of cartilage and bone. Several self proteins have been suggested to be disease-driving autoantigens. Proteins are encoded by a limited number of genes in our genome. Post-translational modifications such as citrullination of the arginine residues, can increase the morphological and the functional diversity of the proteome. The positivity of anti-citrullinated peptides autoantibodies occurs then at an early stage of the disease development. Several factors, among which the synovial tissue inflammatory and the nitric oxide reaction, are involved in the regulation of the citrullination reaction. All of them have to be analysed and considered to understand the loss of tolerance and the development of autoimmunity leading to the disease.

Parole chiave - Artrite reumatoide, citrullina, anticorpi anti-peptidi citrullinati, perdita della tolleranza, autoimmunità.
Key words *- Rheumatoid arthritis, citrulline, anti-citrullinated peptides antibodies, loss of tolerance, autoimmunity.*

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