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(54) Titre : ANTICORPS DIRIGES CONTRE UNE PROTEINE CARBAMYLEE ET LE RISQUE DE PRESENTER UNE ARTHRITE

(54) Title: ANTI-CARBAMYLATED PROTEIN ANTIBODIES AND THE RISK FOR ARTHRITIS

(57) Abrégé/Abstract:
Antibodies against citrullinated protein antigens (ACPA) have shown their relevance for the diagnosis and possibly pathogenesis in arthritis. The present invention provides means and methods for determining antibodies against homocitrulline containing proteins or carbamylated proteins/peptides (anti-Car P) for the classification of individuals suffering from or at risk of suffering from arthritis.

Abstract

Antibodies against citrullinated protein antigens (ACPA) have shown their relevance for the diagnosis and possibly pathogenesis in arthritis. The present invention provides means and methods for determining antibodies against homocitrulline containing proteins or carbamylated proteins/peptides (anti-Car P) for the classification of individuals suffering from or at risk of suffering from arthritis.

Title: Anti-carbamylated protein antibodies and the risk for Arthritis

The invention relates to the fields of post-translational modification and arthritis. The invention in particular relates to methods classifying samples of individuals based on the detection of post-translationally modified proteins or peptides or antibodies specific for post-translationally modified proteins or peptides in a sample containing a body fluid of the individual.

There are over 100 different forms of arthritis. The most common form is osteoarthritis (degenerative joint disease). Osteoarthritis is most commonly the result of a trauma or of an infection of the joint, albeit that there are also not readily identifiable causes. The latter are often collectively referred to age related osteoarthritis. Other arthritis forms are for example rheumatoid arthritis, psoriatic arthritis, and related autoimmune diseases.

The major complaint by individuals who have arthritis is joint pain. Pain is often a constant and may be localized to the joint affected. The pain from arthritis is often the result of the damage that is induced to the joint or the result of the inflammation that occurs around the joint. Other complaints are pain as a result of muscle strains caused by forceful movements against stiff, painful joints and fatigue.

The diagnosis of patients with joint associated pain is not easy as the complaints are typically vague and can be attributed to a variety of different causes some of which are not arthritis. Indeed many patients that first see a doctor with joint pain associated complaints go into remission and don't develop a chronic form of arthritis, whereas a significant minority progress to develop Rheumatoid Arthritis (RA). It is clear that individuals that go into remission spontaneously do not need to receive treatment, whereas

individuals that progress would benefit considerably from early treatment. To discriminate between the respective groups the field has developed a series of tests with which the diagnosis of arthritis can be made with more certainty. Such tests presently involve the screening of tissue samples and/or body fluid samples for the presence of arthritis indicators therein. Such indicators are among others determination of "chronic" inflammation indicators such as for instance certain chemokines, cytokines and other immune cell signalling factors; the determination of "accumulation of" active immune cells in joints, and/or the presence of the certain factors in the blood, the most notable of which is rheumatoid factor. Recently, tests directed toward the detection of citrullinated protein or peptides or antibodies specific for such citrullinated protein or peptide have been developed as a useful tool for such tests. The availability of such tests has greatly improved the diagnosis of individuals suspected of having a form of arthritis. These tests also aid the clinician in giving a more accurate prognosis for the future development of the disease to individuals suffering from arthritis. However, in spite of these developments the diagnosis of arthritis or individuals at risk thereof still leaves much to be desired.

For example, in the Netherlands the recommendation to diagnose RA is based on a probability score generated by the ACR/EULAR 2010 criteria. This criteria combines clinical features such as involvement of type and number of joints, presence or absence of the serological factors rheumatoid factor and anti-CCP antibodies, presence or absence of acute phase proteins such as CRP and duration of complaints. Patients that are diagnosed with RA according to this protocol need to have more than 6 points. The fact that only the clinical involvement and duration of complaints are sufficient for a positive diagnosis indicates that the arthritis population is very heterogeneous and in fact often a wrong diagnosis is made.

In the Netherlands and in other countries, tests for the typing of arthritis or diagnoses of an individual at risk of developing arthritis presently include tests for the detection of antibodies specific for citrullinated protein or peptide in samples of body fluids of such patients. These tests have led to the insight that arthritis patients can be classified on the basis of positivity for antibodies directed towards citrullinated proteins (ACPA). The identification of ACPA has had an important impact on the understanding of RA [1]. Major differences have been observed when comparing ACPA positive vs. ACPA negative RA patients regarding genetic- and environmental risk factors [2], progression [3] remission [4] and response to treatment [5]. Over the recent years much more insight has been gained into the occurrence and etiophathology of ACPA positive RA. However, much less information is available on ACPA negative RA. In part this is because it is relatively difficult to identify or even subgroup these individuals as no good assays are currently available. Interestingly, rituximab treatment has been reported to also be beneficial in patients negative for RF and ACPA [6,7].

The post-translational modification of Arginine residues into Citrulline residues by the PAD enzymes is the essential step to generate antigens for ACPA [1]. Under physiological circumstances this citrullination is important in tissues like hair and skin to generate layers of tissue that are not-well connected [8]. Also in the nucleus citrullination plays a role in epigenetic regulation [9] and condensation of chromatin, which is important both in translation [8] and in host defense against pathogens [10]. Under pathological conditions where cell death may overwhelm the phagocytic capacity, necrotic cells death may release PAD into the extracellular space, where higher Calcium concentrations now also allows other host molecules to become citrullinated [8]. Since many of these molecules will be presented to the immune system as non-self, it can induce an antibody response in some individuals. Citrulline highly resembles (Fig. 1) another post-translationally modified amino acid called homocitrulline [11]. Homocitrulline is only one

carbon longer, but similar in structure [11]. Homocitrulline is generated from a Lysine residue following an attack from cyanate, which exists in the body in equilibrium with urea. Under physiological conditions the urea concentration may be too low to allow extensive carbamylation (the process of changing Lysine to Homocitrulline). In conditions of renal failure, the urea concentration increases and carbamylation can be readily detected. However, most carbamylation is taking place during inflammation when myeloperoxidase (MPO) is released from neutrophils [12]. This enzyme strongly shifts the equilibrium of urea towards cyanate, now allowing more carbamylation to occur [13]. It has been shown recently that homocitrulline containing proteins are present in the RA joint and that this may affect T cell triggering and autoantibody formation in animal models [11,14]. Although highly similar, carbamylation differs from citrullination as, next to their structural difference, Lysine and not Arginine is modified. Therefore, homocitrulline will, by definition, be located at other positions in proteins then citrulline. In the present invention autoantibodies against carbamylated proteins were found to be present in arthritis and we determined that measurement of such antibodies is useful in the diagnosis, prognosis and the management of early arthritis and RA.

The invention now provides a method for classifying an individual that is suffering from or at risk of suffering from a form of arthritis said method comprising determining whether a sample comprising a body fluid of said individual comprises an Anti-Carbamylated Protein (anti-CarP) antibody.

An individual that is suffering from arthritis can be classified on the basis of anti-CarP antibodies as low or high risk of developing a more severe form arthritis. Individuals with arthritis that are positive for anti-CarP tend to develop a more severe form of arthritis than anti-CarP negative individuals with arthritis, at least within any given time period. The anti-CarP positive individuals also tend to progress into more severe forms more quickly when

compared with anti-CarP negative individuals. The method is thus preferably used to allocate an individual that is suffering from a form of arthritis to a group with a lower or a higher than average risk for progressing to a more severe, or chronic form of arthritis. In a preferred embodiment the individual is suffering from undifferentiated arthritis at presentation. In a preferred aspect of this embodiment said more severe or chronic form is RA. In another preferred aspect of this embodiment said more severe or chronic form is juvenile arthritis, preferably juvenile idiopathic arthritis. In a preferred embodiment said group with a lower than average risk is a group that has a higher than average incidence of spontaneous remission of the arthritis complaints.

The at risk population may be healthy individuals, patients suffering from undifferentiated arthritis or arthralgia, autoantibody-positive individuals with joint complaints, autoantibody positive individuals, family members from patients with arthritis. Juvenile idiopathic arthritis is the term used for a subset of arthritis seen in childhood, which may be transient and self-limiting or chronic. Children with juvenile idiopathic arthritis are considered an at risk population, as some of these patients may develop a chronic form of arthritis.

The present invention shows that detection of anti-CarP antibodies is useful in individuals that present with undifferentiated arthritis, arthralgia, and other joint complaints, and/or with juvenile arthritis. The presence or absence of anti-CarP antibodies is predictive of the development of RA or persistent arthritis later in life. This predictive power is observed both in ACPA negative and ACPA positive subjects.

The method is thus preferably used to predict whether the individual is at risk of developing RA or persistent arthritis later in life.

For instance human carbamylated Fibrinogen can be used as a target for anti-CarP antibodies. Carbamylated fibrinogen is recognized by both IgG and IgA anti-CarP antibodies. Intact fibrinogen, or any of peptides derived

from carbamylated fibrinogen can be used as targets in assays to detect anti-CarP antibodies. The intact protein or any of the peptides derived from fibrinogen can be used directly in the assay or can be immobilized via an attached biotin group that will bind to streptavidin coated surfaces or can be used in any other way to detect anti-CarP antibodies. In a preferred embodiment, a method according to the invention is provided, wherein the anti-CarP antibody is capable of specifically binding carbamylated fibrinogen. Throughout the description, the abbreviation anti-Ca-Fib antibody is used to indicate such antibody capable of specifically binding carbamylated fibrinogen.

Progression of disease in arthritis, for instance progressive pain complaints or progressive joint damage, is not a constant. The progression may be faster or slower for some period of time. When in this invention mention is made of a lower or higher risk of progression, this is typically compared to the average risk of progression within the group of individuals that is studied.

Progression of diseases is at a group level established every year, so that follow up data can be analyzed using repeated measurement analysis. Yet at the individual levels positivity for anti-CarP, preferably anti-Ca-Fib, is predictive for future progression. Currently, several methods including MRI, Ultra-sound and other techniques are available to measure progression of disease in the short term.

To assess whether a sample comprises anti-CarP, preferably anti-Ca-Fib, antibodies a test for the presence of the antibodies is performed. Such tests can involve but are not limited to ELISA and/or Westernblot, using one/or several carbamylated proteins and / or peptides. Commonly, though not necessarily, the result obtained for the sample is compared with a reference. The reference is typically the result of a similar, and preferably the same test, performed on one, or a number of healthy individuals (i.e. not known to suffer from arthritis and not known to be at immediate risk of developing arthritis). The result of the sample can be directly compared with the result of the

reference, or the reference can be used to determine a threshold, below which any sample is to be judged a negative for anti-CarP, preferably anti-Ca-Fib, antibodies when below the threshold or positive when above the threshold.

The invention further provides a method for providing a prognosis for the development of arthritis to an individual suffering from said arthritis, said method comprising determining whether a sample comprising a body fluid of said individual comprises an Anti-Carbamylated Protein (anti-CarP) antibody, and estimating the future severity of said arthritis based on the detection of said anti-CarP antibody in said sample. In a preferred embodiment, said anti-CarP antibody is capable of specifically binding carbamylated fibrinogen (anti-Ca-Fib antibody). This estimation is typically accompanied by a time interval within which the more severe form or progression becomes apparent or not (see herein above).

One advantage of the classifications as indicated herein above is that the groups of individuals have more homogeneous genetic profile within each group than with other methods. Another advantage is that the groups of individuals are more homogeneous in their response to (prophylactic) treatment. Since it has been shown that early aggressive treatment is beneficial [18,19], the invention provides methods for arthritis treatment of individual suffering from or at risk of suffering from arthritis said method comprising an arthritis diagnosis of said individual wherein said diagnosis comprises a method for determining an anti-CarP antibody, preferably an anti-Ca-Fib antibody, in a sample comprising a body fluid of said individual. Preferably said sample was determined to contain an anti-CarP antibody, preferably an anti-Ca-Fib antibody. A more stringent treatment of said anti-CarP or anti-Ca-Fib positive individual is beneficial to the patient. The treatment is typically a therapeutic treatment given to a patient that was diagnosed with arthritis prior to receiving said treatment. The invention further provides a method of treating an individual suffering from arthritis

with an arthritis medication and/or treatment, said method characterized in that said individual was diagnosed with a method of the invention prior to said treatment. Preferably, said individual was diagnosed as suffering from said arthritis with a method for classifying individuals suffering from arthritis of the invention, prior to receiving said arthritis medication and/or treatment. The invention further provides a method for the prophylactic treatment of an individual at risk of developing arthritis with an arthritis medication and/or treatment said method characterized in that said individual was diagnosed being at risk of developing said arthritis with a method for classifying individuals of the invention, prior to receiving said arthritis medication and/or treatment. Treatment, in this case, prophylactic, can also be given to individuals that are classified to be at risk of developing the disease in the near future, typically within one year of classification. Such prophylactic treatments are capable of at least postponing the onset of the disease and/or reducing the severity of the disease.

The methods to classify arthritis patients or individuals at risk of developing arthritis typically involve a number of different tests. Such tests may also be combined with a method of the invention to arrive at a more accurate assessment of the classification. To this end the invention further provides means and methods to further classify individuals that suffer from arthritis or that are suspected/at risk of having arthritis. In a preferred embodiment a method of the invention is combined with a test for Anti-citrullinated Protein Antibodies (ACPA).

The ACPA test has been used for quite some time (reviewed among others in: Venrooij et al. (2002): Anticitrullinated protein/peptide antibody and its role in the diagnosis and prognosis of early rheumatoid arthritis: The Netherlands Journal of Medicine Vol 60: pp 383-388.; and Klareskog L, Ronnelid J, Lundberg K, Padyukov L, Alfredsson L. Immunity to

citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol* 2008; 26:651-75.

Many different citrullinated proteins or peptides can be used in tests to detect anticitrullinated protein antibodies (ACPA). Citrullinated filaggrin has been used to detect the so-called antifilaggrin antibodies (AFA). In first attempts to find suitable substrates for RA auto-antibodies a number of linear peptides containing one citrulline residue were developed. These citrullinated peptides were specifically recognised by the RA auto-antibodies and, more important, their arginine-containing counterparts were not. However, most peptides reacted with only 30 to 45% of the RA sera, although more than 75% of RA sera reacted with at least one of a total of nine peptides tested (Schellekens et al. (1998). J. Clin. Invest. Vol 101: pp 271-281). Although linear peptides may be used, it has been found that cyclic peptides render the tests more sensitive. Tests that include cyclic citrullinated protein/peptide are typically referred to CCP tests. The CCP1 test was already sensitive, but it has been found that a novel selection of cyclic citrullinated protein/peptides with improved immune recognition properties has increased the sensitivity of the CCP test to at least 80%. This latter test is typically referred to as the CCP2 test. Thus in one embodiment of this aspect of the invention the method for detecting ACPA comprises detecting anti-cyclic citrullinated protein/peptide in said sample. Preferably, said method for detecting ACPA is a CCP2 test as described in: The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis.Pruijn GJ, Wiik A, van Venrooij WJ.Arthritis Res Ther. 2010;12(1):203. Epub 2010 Feb 15. Review and "A comparison of the diagnostic accuracy and prognostic value of the first and second anti-cyclic citrullinated peptides (CCP1 and CCP2) autoantibody tests for rheumatoid arthritis." van Gaalen FA, Visser H, Huizinga TW. Ann Rheum Dis. 2005 Oct;64(10):1510-2.

As is the case for ACPA, also for an anti-CarP antibody in principle, many different carbamylated proteins or peptides can be used in tests to detect anti-carbamylated protein antibodies. In a preferred embodiment said carbamylated protein or peptide is a cyclic peptide. A good source of carbamylated proteins is carbamylated fetal calfs serum. Serum proteins from other species are, however, also suitable. Following optimization also human proteins might be used. For reasons of inevitable background human serum can not be used in carbamylated form without extensive depletion of Ig. In a preferred embodiment, said carbamylated protein is fibrinogen, preferably human fibrinogen.

A peptide for use in a method of the invention, be it for the detection of ACPA in general or of anti-CarP antibodies or of anti-Ca-Fib antibodies, is typically a peptide of between 6-50 amino acids. Preferably said peptide is a peptide of between 12 and 30 amino acids, more preferably of between 18 and 22 amino acids, most preferably of about 21 amino acids. The mentioned ranges include the number mentioned i.e. a range of between 12 and 30 amino acids includes peptides of 12 and 30 amino acids, respectively. The peptide may or may not be a cyclic peptide depending on the sensitivity and/or specificity than the comparable linear peptide. Circular peptides can be generated in any molecular composition as to generate the cyclic nature. Any method may be used to couple peptides and or proteins in carbamylated, citrullinated or native form to plates and or beads being either direct coating or using biotin-streptavidin or any other available coating method.

In a preferred embodiment, such peptide for use in a method of the invention is a peptide derived from human fibrinogen. Said peptide preferably comprises a contiguous amino acid of between between 12 and 30 amino acids, more preferably of between 18 and 22 amino acids, most preferably of about 21 amino acids present in the amino acid sequence of any one of fibrinogen alpha, fibrinogen beta or fibrinogen beta. In a

more preferred embodiment, the peptide is any one of the peptides depicted in Table I, Table II, or Table III.

The invention further provides, as indicated herein above, a method for classifying an individual that is suffering from or at risk of suffering from a form of arthritis said method comprising determining whether a sample comprising a body fluid of said individual comprises an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and determining whether said sample comprises Anti-citrullinated Protein Antibodies (ACPA) and classifying said individual on the basis of the detection of said anti-CarP antibody and/or said ACPA.

The anti-CarP antibody, the anti-Ca-Fib antibody, and ACPA can be of any immunoglobulin isotype. The art typically focuses on one or more of the IgG subclasses. In the present invention it has been found that the level of an anti-CarP antibody, anti-Ca-Fib antibody and/or ACPA of both the Ig-subtype A (IgA) and the Ig-subtype G (IgG) in a sample comprising body fluid of said individual is predictive for clinical outcome measures such as joint destruction. It has also been found that whereas a sample can be negative for anti-CarP or anti-Ca-Fib IgG it can be positive for anti-CarP or anti-Ca-Fib IgA and vice versa. Thus in a preferred embodiment of a method of the invention said method comprises determining whether a sample comprising a body fluid of said individual comprises an anti-CarP and/or anti-Ca-Fib antibody of Ig-subtype IgA or an anti-CarP and/or anti-Ca-Fib antibody of Ig-subtype IgG, or both, and wherein detection of said IgA and/or IgG anti-CarP and/or anti-Ca-Fib antibody indicates that said individual is suffering from or at risk of suffering from arthritis. In a preferred embodiment said method further comprises determining whether a sample comprising a body fluid of said individual comprises ACPA. As mentioned herein above, a method of the invention is particularly useful in subdividing the heterogeneous group of ACPA negative individuals. Using a method of the invention this ACPA negative group can be divided in a group that is an anti-CarP and/or anti-Ca-

Fib antibody positive and that can be classified as suffering from or at risk of suffering from arthritis, and a group of an anti-CarP and/or anti-Ca-Fib antibody negative group that is classified as not suffering from arthritis or not at risk of suffering from arthritis. This determination can also be done on an individual basis. Thus a method of the invention preferably further comprises determining whether a sample comprising a body fluid of said individual comprises Anti- Citrullinated Protein Antibodies (ACPA) and wherein the level of said ACPA in said sample is below the detection limit and or cut-off of positivity.

A sample of an individual tested for the presence of an anti-CarP antibody, preferably of isotype IgA or of isotype IgG, or both and ACPA can be classified on the basis of the result as an

a) an anti-CarP and/or anti-Ca-Fib antibody+, ACPA- sample an;

b) an anti-CarP and/or anti-Ca-Fib antibody+, ACPA+ sample an;

c) an anti-CarP and/or anti-Ca-Fib antibody-, ACPA+ and a

d) an anti-CarP and/or anti-Ca-Fib antibody-, ACPA- sample.

Results a), b) and c) classify the sample as a sample of an individual that is at high risk to be currently suffering from or at risk to develop arthritis. Result d) classifies the sample as a sample of an individual that have a low risk to be currently suffering from or to develop arthritis. In the event that said individual presented with undifferentiated arthritis than classifying said sample in group d) indicates that said individual has a high chance of spontaneous remission. Such an individual is not expected to benefit on the long term from an anti-arthritis treatment. The invention further provides a method for typing a sample comprising a body fluid of an individual suffering from undifferentiated arthritis, said method comprising determining whether said sample comprises an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and typing said sample as derived from an individual that has a higher than average chance of spontaneous remission of said arthritis when

said sample was determined to be negative for said anti-CarP antibody, preferably said anti-Ca-Fib antibody, and typing said sample as derived from an individual that has a higher than average chance of having or progressing to RA when said sample was determined to be positive for said anti-CarP antibody, preferably said anti-Ca-Fib antibody. Said higher than average chance is typically arrived at by comparison of said chance with the average chance arrived at for a number of unselected individuals presenting with undifferentiated arthritis.

In another aspect the invention provides a method for estimating the severity of arthritis for an individual that is suffering from a form of arthritis has an increased risk of developing a more severe form of said arthritis, said method comprising determining whether a sample comprising a body fluid of said selected individual comprises Anti-Homocitrulline Containing Protein Antibodies (an anti-CarP antibody), preferably Anti-Homocitrulline Containing Fibrinogen Antibodies (an anti-Ca-Fib antibody), and estimating the severity of said arthritis based on the detection of said anti-CarP antibodies, preferably said anti-Ca-Fib antibody in said sample.

In yet another aspect the invention provides a method for providing a prognosis for the development of arthritis to an individual suffering from said arthritis, said method comprising determining whether a sample comprising a body fluid of said individual comprises an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and estimating the future severity of said arthritis based on the detection of said anti-CarP antibody, preferably an anti-Ca-Fib antibody, in said sample. Said method is preferably combined with a method for determining ACPA is said sample and the combined result of said anti-CarP and/or anti-Ca-Fib antibody test and said ACPA test is used to estimate the future severity of said arthritis for said individual.

The sample to be tested for the presence of an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and/or ACPA can in principle be any type of sample as long as it contains body fluid of the individual. Typically, however, the sample is a sample of body fluid. In a preferred embodiment said sample comprising body fluid is a serum sample or a synovial fluid sample.

Arthritis is, as mentioned herein above a complex disease and many different forms of arthritis have presently been identified. The arthritis that the individual is suffering from or at risk of suffering from is preferably an arthritis selected from Rheumatoid arthritis, Juvenile arthritis, Psoriatic arthritis, Osteoarthritis, Polymyalgia rheumatica, Ankylosing spondylitis, Reactive arthritis, Gout, Pseudogout, Autoimmune arthritis, Systemic lupus erythematosus, Polymyositis, Fibromyalgia, Lyme disease, Undifferentiated arthritis, non-rheumatoid arthritis or Spondyloarthropathy. More preferably the arthritis that the individual is suffering from or at risk of suffering from is an arthritis selected from Rheumatoid arthritis, Psoriatic arthritis, Osteoarthritis, Polymyalgia rheumatica, Ankylosing spondylitis, Reactive arthritis, Gout, Pseudogout, Autoimmune arthritis, Systemic lupus erythematosus, Polymyositis, Fibromyalgia, Lyme disease, Undifferentiated arthritis, non-rheumatoid arthritis or Spondyloarthropathy. Preferably, said arthritis is selected from rheumatoid arthritis, juvenile arthritis, more preferably juvenile idiopathic arthritis, or undifferentiated arthritis. More preferably, said arthritis is selected from rheumatoid arthritis or undifferentiated arthritis.

The invention further provides a method for typing the arthritis of an individual suffering from a form of arthritis, said method comprising determining whether a sample comprising a body fluid of said individual comprises an Anti-Carbamylated Protein (anti-CarP) antibody, preferably an Anti-Carbamylated Fibrinogen (anti-Ca-Fib) antibody. The arthritis can be

typed on the basis of the detected presence or absence of said anti-CarP and/or anti-Ca-Fib antibody. A sample in which said anti-CarP antibody, preferably said anti-Ca-Fib antibody, is detected is more likely to be derived from an individual with Rheumatoid Arthritis. Thus this method can be used alone, or in combination with another test for RA to assess the likelihood that said individual is suffering from RA. In a preferred embodiment said further test comprises a test for the presence of ACPA, preferably a CCP2 test and or a test for Rheumatoid Factor.

The present invention shows that anti-CarP antibodies can be detected in asymptomatic, healthy individuals before RA-development. Thus, also in the healthy population, detection of anti-CarP antibodies are useful for the early identification of patients at risk to develop RA or persistent arthritis later in life. In addition, the detection of anti-CarP antibodies is useful to identify persons that would benefit from early treatment, preferably before they fulfil the current classification for RA.

In a further aspect the invention provides a method for determining whether an individual is at risk of developing or suffering from a form arthritis, and wherein said individual was not known to suffer, or preferably not suffering from arthritis at the time that the sample comprising body fluid was collected, said method comprising determining whether a sample comprising a body fluid of said individual comprises an Anti-Carbamylated Protein (anti-CarP) antibody. In a preferred embodiment, a method according to the invention is provided wherein said anti-CarP antibody is an Anti-Carbamylated Fibrinogen (anti-Ca-Fib) antibody. A sample in which said anti-CarP antibody, preferably said anti-Ca-Fib antibody, is detected is likely to be derived from an individual that is at risk of suffering from or developing arthritis, in particular RA in the near future, particularly within 5 years from the date of sample collection, particularly within three years and more

particularly within 1,5 years from the date of sample collection. This method can be used alone, or in combination with another test for RA to assess said risk. In a preferred embodiment said further test comprises a test for the presence of ACPA, preferably a CCP2 test and or a test for Rheumatoid Factor.

In another aspect the invention provides a kit for the detection of anti-CarP antibodies, preferably anti-Ca-Fib antibodies, in a body fluid of an individual said kit comprising a carbamylated protein or peptide. Preferably, said carbamylated protein or peptide is carbamylated fibrinogen or a fibrinogen derived peptide. In a preferred embodiment said protein or peptide is a carbamylated protein or peptide as indicated herein above. In particular, said kit comprises at least one peptide comprising a contiguous amino acid of between between 12 and 30 amino acids, more preferably of between 18 and 22 amino acids, most preferably of about 21 amino acids present in the amino acid sequence of any one of fibrinogen alpha, fibrinogen beta or fibrinogen beta. In a more preferred embodiment, said kit comprises at least one of the peptides depicted in Table I, Table II, or Table III.

As also mentioned herein above. In a preferred embodiment said kit further comprises an anti-human IgG antibody and/or an anti-human IgA antibody. Preferably said anti-human Ig antibody comprises a label that can be detected. Non-limiting examples of such labels are a direct labeling with HRP or AP. Alternatively, said kit preferably further comprises another antibody, which antibody is specific for the anti-human Ig antibody used. In this embodiment said another antibody comprises a label that can be detected such as biotin or DIG. This nesting can of course continued with label being present on the last and/or one or more (earlier) antibodies. In a preferred embodiment said anti-human Ig antibody comprises an anti human IgA antibody. In a more preferred embodiment, said kit comprises an anti-human IgA antibody and an anti-human IgG antibody. The anti-human Ig antibody as indicated herein

above is typically a full antibody, however, a fragment that contains the antigen binding site is also within the use of the term antibody. Similarly, there are presently a great variety of different binding proteins or peptides available that can be tailored to specifically bind to human Ig. Such human Ig specific binding proteins or peptides are equivalents of an anti-human Ig antibody as defined herein. Similarly, the one or more further antibodies in a nesting setting can be replaced with binding proteins and/or peptides that specifically bind the earlier antibody and/or binding protein/peptide in the nesting tree.

In a particularly preferred embodiment said kit further comprises a citrullinated protein or peptide. In a preferred embodiment said protein or peptide is a citrullinated protein or peptide as indicated herein above. As also mentioned herein above, said citrullinated protein or peptide is preferably a protein or cyclic peptide. Preferably said kit comprises a cyclic citrullinated peptide of a CCP1 or CCP2 test. Preferably said kit comprises all cyclic peptides of a CCP test, preferably a CCP2 test.

A method of the invention is, as mentioned herein above, preferably combined with another arthritis classifier test. Thus in a preferred embodiment of a method of the invention said method further comprises determining a further factor as an arthritis classifier for said individual. Preferably said further factor comprises determining ACPA, rheumatoid factor, C-reactive protein, and/or erythrocyte sedimentation rate.

Carbamylation is defined herein as the process of providing a protein or peptide with a modification that generates a homocitrulline residue. Where in this document reference is made to a carbamylated protein or peptide or collection thereof, reference is made to said protein or peptide having a homocitrulline modification, or a collection of proteins or peptide having homocitrulline modifications.

The invention further provides an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and ACPA for use in determining whether an individual is suffering from or at risk of suffering from a form of arthritis. The invention further provides an anti-CarP antibody, preferably an anti-Ca-Fib antibody, and ACPA for use in the classification of arthritis. Said determining and/or classification being a determining or classification as indicated herein above.

The invention further provides a method for determining whether an individual that is suffering from a form of arthritis has an increased risk of developing a more severe form of said arthritis, said method comprising selecting an individual that is suffering from arthritis but that does not have the most severe form of arthritis and determining whether a sample comprising a body fluid of said selected individual comprises an anti-Carbamylated Protein Antibody (an anti-CarP antibody), wherein detection of said anti-CarP indicates that said individual has increased risk of developing a more severe form of said arthritis. In a preferred embodiment, a method according to the invention is provided, wherein said anti-CarP antibody is an anti-Carbamylated Fibrinogen Antibody (an anti-Ca-Fib antibody).

Where in this document reference is made to detection, determination or otherwise assessment of an anti-CarP antibody, an anti-Ca-Fib antibody or an ACPA, said reference includes that more than one anti-Carp antibody, anti-Ca-Fib antibody and/or more than one ACPA is detected, determined or otherwise assessed. Anti-CarP or anti-Ca-Fib antibody can recognize a homocitrulline modification per se or, more typically, recognize said modification in the context of one or more of the amino acids of the protein or peptide in the immediate vicinity of the homocitrulline modification. A method or kit of the invention is more accurate when anti-CarP antibodies and/or anti-Ca-Fib antibodies of more than one specificity is detected, determined or otherwise

assessed. A method of the invention thus preferably comprises determining two or more, and preferably three or more, more preferably 5 or more, more preferably at least 7 anti-CarP and/or anti-Ca-Fib antibodies in said sample. Similarly, a kit of the invention preferably comprises two or more, and preferably three or more, more preferably 5 or more, more preferably at least 7 carbamylated proteins and/or peptides, preferably fibrinogen and/or fibrinogen derived peptides.

Brief description of the drawings

Figure 1

Development of a novel and specific assay for the detection of anti-CarP antibodies.

(A) Dose response curves of the anti-CarP antibody positive standard on carbamylated FCS and native FCS in ELISA. (B) Inhibition studies where anti-CarP antibody binding to ELISA plates coated with Ca-FCS was inhibited using pre-incubations with fluid-phase inhibitors as indicated. Only Ca-FCS inhibited the binding of anti-CarP antibodies. (C) Coomassie staining showing equal loading of Ca-FCS and FCS, and Westernblot showing a positive staining of Ca-FCS loaded lanes and not FCS loaded lanes by a serum sample of a anti-CarP positive sample and not by a negative sample.

Figure 2

Anti-CarP IgG and IgA antibodies are present in RA sera.

ELISA was performed for the detection of anti-CarP IgG and IgA in sets of sera of healthy controls and RA patients. A cut-off was established using the mean plus 2 times the standard deviation of the healthy controls. Shown is the titer expressed as arbitrary units per ml following calculation based on the standard curve. Below the graph both the number of samples tested and the percentage positivity is indicated.

Figure 3

Anti-CarP antibodies and ACPA are two separate autoantibody systems

Piecharts showing the percentages of RA patients pos and negative for ACPA and or anti-CarP antibodies.

Figure 4

Anti-CarP IgA antibodies are associated with the conversion of undifferentiated arthritis (UA) to RA.

Sera of patients that presented with UA at baseline were measured for anti-CarP IgG and IgA antibodies and analyzed for their conversion towards RA at 1 year follow up. Data shown are split up also on the basis of ACPA positivity. IgA anti-CarP antibodies associate strongly with development of RA from a UA population.

Figure 5

Anti-CarP IgA antibodies are associated with more severe radiological progression in RA.

The extent and rate of joint destruction were analyzed in the RA patients split up on the basis of positivity for ACPA and anti-CarP antibodies. Positivity for anti-CarP IgA antibodies is associated with a more severe radiological damage in both ACPA negative and ACPA positive RA patients.

Figure 6

Illustration of citrullination and carbamylation.

Citrullination and carbamylation occur on different amino acid via different mechanisms, but yield similar end-products.

Figure 7

Antibodies against carbamylated proteins are present in sera of RA patients.

The reactivity of IgG (A,B) and IgA (D,E) from sera of healthy controls (NHS) or RA patients (RA) to wells coated with non-modified FCS (FCS) or carbamylated FCS (Ca-FCS) is depicted. Data expressed as absorbance at 415 nm. (C,F) Absorbance units of FCS were subtracted from the absorbance units of Ca-FCS, representing the specific anti-carbamylated protein response.

Figure 8

Anti-CarP antibodies and ACPA are two separate autoantibody systems

(A) IgG reactivity of 76 sera from RA patients, towards several forms of a Fib peptide is depicted. (B,C) Antibody binding to Ca-FCS or Ci-FCS was inhibited using pre-incubations with fluid-phase inhibitors is depicted. (D) FCS, Ca-FCS and Ci-FCS were separated by SDS-page gels and blotted. The presence of antibodies reactive to proteins on the blots was analyzed by incubating these blots with either Anti-CarP positive ACPA-negative and Anti-CarP negative ACPA-positive sera.

Figure 9

Anti-CarP antibodies bind to Ca-Fib via variable domains.

(A) IgG reactivity against Fib, Ci-Fib and Ca-Fib of 54 healthy controls and 214 RA patients was analyzed by ELISA. (B) Specificity of anti-Ca-Fib reactivity was confirmed using inhibition studies. One sample is shown, where data are expressed relative to inhibition with PBS. (C) The molecular nature of purified IgG and F(ab')2 was confimed by Coomassie Stained SDS page gel. (D) F(ab')2 fragments were generated from purified IgG of 2 anti-CarP positive patients and 2 negative controls. Only F(ab')2 from patients reacted with Ci-Fib and Ca-Fib. (E) Inhibition experiments confirm that also F(ab')2 are not necessarily cross-reactive between Ci-Fib and Ca-Fib.

Figure 10

Anti-CarP IgG and IgA antibodies are present in RA sera.

(A,B) Dose response curves of the anti-CarP antibody positive standard (IgG and IgA) on Ca-FCS and FCS in ELISA. (C,D) ELISA was performed for the detection of anti-CarP IgG and IgA in sera of healthy controls (NHS) and RA patients. A cut-off was established using the mean plus 2 times the standard deviation of the healthy controls as described in the methods. Reactivity is depicted as arbitrary units per mL. The number of samples tested and the %

positivity is indicated below the graph. (E,F) Pie charts showing the % of RA patients positive and negative for anti-CCP2 and/or anti-CarP antibodies. (G,H) Pie charts showing the % of anti-CarP IgG or IgA positive patients negative for anti-CCP2.

Figure 11

Anti-CarP IgG antibodies are associated with a more severe radiological progression in ACPA negative RA.

The extent and rate of joint destruction were analyzed in all RA patients included, or analyzed separately for ACPA-negative or ACPA-positive subgroups (Fig. 13). The severity of joint destruction is depicted as median Sharp / van der Heijde score (SHS) on the Y-axis and the follow-up years on the X-axis. Below the X-axis the patient number is listed for each time point. Radiological progression for the anti-CCP2-negative RA patients is shown. The P-value is derived from the analysis model as described in the methods section.

Figure 12

Anti-CarP IgG antibodies are associated with the conversion of pre-disease to RA.

(A) Sera of patients that presented with Undifferentiated Arthritis (UA) at baseline were measured for anti-CarP IgG antibodies and analyzed for their conversion towards RA at 1 year follow up. Data shown are split up also on the basis of ACPA positivity. Anti-CarP antibodies associate strongly with development of RA from a UA population. (B) Also in sera of patients that presented with arthralgia at baseline, anti-CarP antibodies are predictive for development of RA. (C) Sera of healthy persons that do not develop RA or that do develop RA later in life are compared for anti-CarP positivity. The presence of anti-Carp is associated with future development of RA.

Figure 13

Anti-CarP IgA antibodies are associated with more severe radiological progression in RA.

The extent and rate of joint destruction were analyzed in the RA patients split up on the basis of positivity for ACPA and anti-CarP antibodies. Positivity for anti-CarP IgG antibodies is associated with a more severe radiological damage in ACPA negative RA patients.

Figure 14

Anti-CarP antibodies are present in sera of patients suffering from juvenile arthritis.

ELISA was performed for the detection of anti-CarP IgG in sera of healthy children (Ctr) and in sera of patients suffering from Juvenile Arthritis. A cut-off was established using the mean plus 2 times the standard deviation of the healthy controls as described in the methods. Reactivity is depicted as arbitrary units per mL. The number of samples tested and the % positivity is indicated below the graph.

EXAMPLES

EXAMPLE 1

Materials and Methods

Generation of antigens

As a source of antigens we have used fetal calf serum (FCS) (Bodinco, batch No, 212-192909). This was carbamylated, citrullinated or employed as an unmodified source.

Carbamylated FCS (Ca-FCS) was generated by diluting FCS in bidest to 4mg/ml. Potassium cyanate (sigma, cat No, 215074) was added at 80mg/ml. Following incubation at 37 °C for 12 hours the sample was extensively dialyzed against bidest.

As a control we have also generated citrullinated FCS (Ci-FCS), for this purpose we incubated 50ul FCS (24mg/ml) with 24ul 0,5M Tris-HCl pH7,6 + 15ul 0,125M CaCl2 + 31ul PAD4 (Sigma P1584) for 24 hours at 37 °C.

Detection of anti-CarP antibodies by ELISA

Non-modified FCS and Ca-FCS were coated at 10ug/ml (diluted in pH 9.6 0,1M carbonnate-bicarbonate buffer) 50ul on Nunc immunoplates (Thermo scientfic, cat No, 430341), overnight at 4 °C. Following washing for 4 times in phosphate buffered saline (PBS) containing 0.05% tween (Sigma, cat No, 27, 434-8) (PT), the plates were blocked by incubating 100ul PBS/1% bovine serum albumin (BSA) (sigma, cat No, A2153) for 1 hour at 37 °C. Following additional washing the sera were incubated in 50ul at a 1/50 (in PBS/0.05% tween/1% BSA buffer (PTB)) to both FCS and Ca-FCS coated wells and incubated at 37 °C for 1 hour. Serial dilutions of a standard serum (diluted in PTB) were incubated on Ca-FCS coated wells. Following washing bound human IgG or IgA was detected by incubating the wells with 50ul 1/5000 diluted (in PTB) rabbit anti human IgG antibody (Dako, cat No, A0423) or 1/1000 diluted (in PTB) rabbit anti human

IgA antibody (Dako, cat No, A0262) incubating at 37 °C for 1 hour. Following washing, wells were incubated at 37 °C for 1 hour with 50ul 1/2000 diluted (in PTB) goat anti rabbit IgG HRP labeled antibody (Dako, cat No, P0448). Following the last washings HRP enzyme activity was visualized by incubating 50 ul 2,2'Azino-bis-(3-ethylbenzo-thiazole-6-sulfonic acid) diammonium salt (ABTS) and H_2O_2, measuring absorbance at 415nm on a standard ELISA reader.

Detection of anti-CarP antibodies by Western blot

Both FCS and Ca-FCS were loaded onto regular 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham, Diegem, Belgium). Blots were then incubated in blocking buffer (3% ELK Milk/PBS/0.05% Tween) 1hr at RT, following washing with PBS/0.05% Tween. The blots were subsequently incubated with 5 ml serum 1:500 diluted in blocking buffer for 1 hr at RT. After three washes with PBS/0.05% Tween, blots were incubated with 3 ml horseradish peroxidase conjugated rabbit anti-human IgG (DAKO, Heverlee, Belgium) 1:50 000 diluted in blocking buffer for 1hr at RT. Next, blots were washed and bound antibodies were visualized using enhanced chemiluminescence (ECL; Amersham). Equal protein loading was verified using Coomassie Brilliant Blue (Bio-Rad, Veenendaal, The Netherlands)

Sera and Synovial Fluids

The sera analyzed were from patients participating in the Leiden Early Arthritis clinic (EAC) cohort. The Leiden EAC is an inception cohort of patients with recent-onset arthritis (symptoms duration <2 years) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 [15]. All RA patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the classification of RA [16] within 1 year of follow up (EAC cohort).

A total of 1007 patients were analyzed of which 582 were diagnosed as RA and 425 as UA of which 151 developed RA on follow up. These patient samples were compared to 280 healthy control samples also derived from the Leiden area. An additional set of paired serum / synovial fluid of RA patients was analyzed. The protocols were approved by the relevant local ethics committee and all participants provided informed consent.

ELISA for the detection of ACPA

Total IgG anti-CCP2, as a measure of ACPA, was measured in sera collected at baseline by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions. Individuals with antibodies against CCP2 were considered ACPA-positive.

ELISA for the detection of anti-CaFib antibodies.

Non-modified Fib and Ca-Fib were coated at 20 µg/ml in 50 µl (diluted in pH 9.0 PBS) on Nunc Maxisorp plates ON. Following washing in PBSTween, the plates were blocked by incubating 200µl pH 9.0 PBS/2% BSA for 2 hours at 4 °C. Following additional washing the wells were incubated with 50µl serum at a 1/50 dilution in RIA buffer (10 mM Tris pH 7.6; 350 mM NaCl; 1% TritonX; 0.5% Na-deoxycholate; 0.1% SDS) (Sigma) on ice for 3h. All subsequent incubations are performed in RIA buffer. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using HRP-labeled rabbit anti human IgG antibody (DAKO) incubated on ice for 2 h. Following the last washings HRP enzyme activity was visualized using ABTS. We transformed the absorbance on Fib and Ca-Fib to aU/mL. We established the cut-off for a positive response as the mean plus 2 x the standard deviation of the specific anti-CarP reactivity of the healthy controls.

We analyzed 67 sera of healthy children and 110 sera of patients suffering from Juvenile Arthritis.

Statistics

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) 17.0 using logistic regression. P-values below 0.05 were considered to be statistically significant.

EXAMPLE 2

Materials and Methods

Patient and control sera.

The sera analyzed were from patients participating in the Leiden Early Arthritis clinic (EAC) cohort. The Leiden EAC is an inception cohort of patients with recent-onset arthritis (symptoms duration <2 years) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 (42). All RA patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the classification of RA (43) within 1 year of follow up. A total of 571 RA patients were involved in the analyses. Patient samples were compared to 305 healthy control samples also living in the Leiden area. The protocols were approved by the local ethics committee and informed consent was obtained.

Detection of anti-CarP antibodies by ELISA.

In brief, Non-modified FCS and modified-FCS were coated on Nunc Maxisorp plates (Thermo scientific), over night. Following washings and blocking, the wells were incubated with serum. Bound human IgG or IgA was detected using rabbit anti human IgG or IgA antibodies (DAKO). Followed by HRP-labeled goat anti-rabbit IgG antibody (Dako). Following the last washings HRP

enzyme activity was visualized using ABTS (44). A more detailed description of the protein modifications and ELISA assays based on FCS and Fib, including F(ab)2, is available online (SI-materials and Methods). We established the cut-off for a positive response as the mean plus 2 x the standard deviation of the specific anti-CarP reactivity of the healthy controls. The methods for the detection of ACPA and Westernblotting are available online (SI-materials and Methods).

ELISA for Fib peptides.

Streptavidin (Invitrogen) was coated at 2 µg/ml in 100µl on Nunc plates at 4°C ON. After washing, Fib peptides containing either an arginine, citrulline, homocitruline or a lysine (Fig. 8A) (45) were incubated at 10 µg/ml in 100µl PTB for 1h at RT. Next the reactivity of antibodies reactive to these antigens was detected as described above.

Inhibition studies.

To determine whether anti-CarP antibodies and ACPA are cross-reactive antibodies, we performed inhibition studies in which autoantibody positive serum samples, positive for both ACPA and anti-CarP antibodies, were pre-incubated with increasing concentrations of either non-modified FCS, Ca-FCS, Ci-FCS or the citrulline- or arginine containing form of the CCP1 peptide (46). Following pre-incubation at room temperature (RT), the samples were tested for reactivity against Ca-FCS and Ci-FCS as described above. Serum and F(ab')2 samples positive for both Ci-Fib and Ca-Fib were pre-incubated with Fib, Ci-Fib and Ca-Fib at 4°C ON and subsequently analyzed on the Fib ELISA (SI-materials and Methods).

Radiological progression.

In the EAC cohort, radiographs of the hands and feet, which had been obtained in a longitudinal fashion, were scored according to the Sharp / van der Heijde

method (47). Scoring and analysis have been described in detail before (24). Data are analyzed directly, or using repeated measurement analysis as to optimally make use of the longitudinal data obtained for each patient (24). More detailed information is available online (SI-materials and Methods).

Generation of antigens.

As we did not know whether antibodies against carbamylated proteins would be present in sera of RA patients, or which proteins they would recognize, we set out to study a diverse set of carbamylated proteins, to maximize the chances to detect as many of the anti-CarP reactivities. For this purpose we have used fetal calf serum (FCS) (Bodinco) that was carbamylated, citrullinated or left untreated. For generating carbamylated FCS (Ca-FCS), FCS was diluted in H_2O to 4mg/ml and potassium cyanate (Sigma) was added to a concentration of 1M. Following incubation at 37 °C for 12 h the sample was extensively dialyzed against H_2O. Carbamylated fibrinogen (Ca-Fib) was generated by incubating 5mg/ml fibrinogen (Fib) with 0,5M potassium cyanate at 4 °C for 3 days followed by extensively dialyzed against PBS. Citrullinated FCS (Ci-FCS) and citrullinated fibrinogen (Ci-Fib) was generated by incubation of 10 mg FCS or Fib in a volume of 1 mL containing 0.1 M Tris-HCl pH7,6, 0,015 M $CaCl_2$ and 40U PAD4 (Sigma) for 24 h at 37 °C. We have confirmed the presence of citrulline and homocitrulline residues using mass-spectrometry analysis. For Fib we observed, in the protein segments that we analyzed, extensive citrullination and complete carbamylation.

Detection of anti-CarP antibodies by ELISA.

Non-modified FCS and modified-FCS were coated at 10 µg/ml in 50 µl (diluted in pH 9.6 0,1M carbonate-bicarbonate buffer) (CB) on Nunc Maxisorp plates (Thermo scientific), over night (ON). Following washing in PBS containing 0.05% tween (Sigma) (PT), the plates were blocked by incubating 100µl PBS/1% bovine serum albumin (BSA) (Sigma) for 6 hours at 4 °C. Following

additional washing the wells were incubated with 50µl serum at a 1/50 dilution in PBS/0.05% tween/1% BSA buffer (PTB) on ice overnight. All subsequent incubations are performed in PTB. As a standard, serial dilutions of a pool of positive sera were used. Human IgG or IgA was detected using rabbit anti human IgG antibody (DAKO) or rabbit anti human IgA antibody (Dako) incubated on ice for 3.5 h. Following washing, wells were incubated on ice for 3.5 h with HRP-labeled goat anti-rabbit IgG antibody (Dako). Following the last washings HRP enzyme activity was visualized using ABTS as described before (25). Sera of healthy subjects (n=305) were used as controls. We transformed the absorbance on both Ca-FCS and FCS to aU/mL and subtracted the background signal (aU/mL) of FCS from the signal (aU/mL) of Ca-FCS as to analyze the specific anti-CarP reactivity (Fig. 7). We established the cut-off for a positive response as the mean plus 2 x the standard deviation of the specific anti-CarP reactivity of the healthy controls.

ELISA for Fibrinogen.

Non-modified Fib Ci-Fib and Ca-Fib were coated at 20 µg/ml in 50 µl (diluted in pH 9.0 PBS) on Nunc Maxisorp plates ON. Following washing in PT, the plates were blocked by incubating 200µl pH 9.0 PBS/2% BSA for 2 hours at 4 °C. Following additional washing the wells were incubated with 50µl serum at a 1/50 dilution in RIA buffer (10 mM Tris pH 7.6; 350 mM NaCl; 1% TritonX; 0.5% Na-deoxycholate; 0.1% SDS) (Sigma) on ice for 3h. All subsequent incubations are performed in RIA buffer. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using HRP-labeled rabbit anti human IgG antibody (DAKO) incubated on ice for 2 h. Following the last washings HRP enzyme activity was visualized using ABTS. We analyzed sera of 214 RA patients and 54 healthy subjects as controls. We transformed the absorbance on Fib Ci-Fib and Ca-Fib to aU/mL. We established the cut-off for a positive response as the mean plus 2 x the

standard deviation of the specific anti-CarP reactivity of the healthy controls. These assays were repeated three times showing the same data.

F(ab')2 preparation.

Total IgG from 2 Anti-CarP positive and 2 control sera were isolated via a HiTrap ™ protein A HP column (GE healthcare) following the protocol for the column provided by the manufacturer. F(ab')2 fragments were generated from purified IgG samples using a F(ab')2 Preparation Kit (Thermo Scientific) following the protocol provided by the manufacturer. We have verified the molecular nature of the intact IgG and the F(ab')2 using Coomassie stained SDS-page gels. These F(ab')2 were used in ELISA as described above, now using either HRP-labeled rabbit anti human IgG, IgA, IgM kappa, lamda antibody (anti-light chain) (Dako) or HRP-labeled rabbit anti human IgG (Dako).

Detection of ACPA by ELISA.

ACPA were measured by the CCP2 ELISA (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions. A small percentage of ACPA-positive RA patients may be outside the anti-CCP2 reactivity, and therefore both terms will be used to explicitly indicate what has been used in our analyses.

ACPA reactivity towards Ci-FCS was detected using ELISA plates that were coated with Ci-FCS (50µl / well 10 µg/ml) diluted with CB in the Nunc Maxisorp plates ON at 4 °C. The plates were washed in PT followed by blocking with 100µl PBS/1%BSA solution at 37 °C for 1h. Following washing, sera were incubated at a 1/50 dilution in 50µl PTB and incubated at 37 °C for 1h. After washing, human IgA and IgG were detected as described above.

Detection of anti-CarP antibodies by Western blot.

FCS, Ca-FCS and Ci-FCS were loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham). Blots were incubated in blocking buffer (3% ELK Milk/PBS/0.05% Tween) for 1h at RT, following washing with PT. The blots were subsequently incubated with 2.5 ml 1:500 diluted serum in blocking buffer for 1.5 h at RT. The sera were either ACPA-positive anti-CarP negative or ACPA-negative anti-CarP positive as determined by ELISA. After three washes with PT, blots were incubated with 5 mL HRP-labeled rabbit anti-human IgG diluted in blocking buffer for 1 h at RT. Next, blots were washed and bound antibodies were visualized using enhanced chemiluminescence (Amersham).

Statistics of Radiological Progression.

Association between Anti-CarP antibodies positivity and radiographic progression was analyzed using the Statistical Package for the Social Sciences (SPSS) 17.0 as described before. P-values below 0.05 were considered statistically significant. A multivariate normal regression analysis for longitudinal data was used with radiological score as response variable. This method analyses repeated measurements at once and takes advantage of the correlation between these measurements, which results in a more precise standard error. Radiological scores were log-transformed to obtain a normal distribution. The rate of joint destruction over time was tested by an interaction of time with anti-CarP. The effect of time was assumed to be linear in the interaction term. The effect of time was entered as factor in the model as well, allowing a mean response profile over time. Age, gender and inclusion period as proxy for treatment were included as correction variables in all analyses. In a separate analysis the effect of anti-CarP antibodies was corrected for the effect of anti-CCP and RF.

EXAMPLE 1

Results

Detection of anti-CarP antibodies.

We have generated a novel ELISA to detect anti-CarP antibodies from serum and synovial fluid using plates coated with, in vitro generated, carbamylated FCS. We generated a standard from a pool of positive sera showing a dose dependent binding of both IgG and IgA to Carbamylated FCS (Ca-FCS) and no binding to the native FCS (Figure 1A). Since homocitrulline and citrulline are very similar residues but differ from each other by one atom, we wished to exclude the possibility that the anti-CarP antibodies were actually ACPA. As a first test to exclude this we performed an inhibition studies that show that anti-CarP antibody binding to Ca-FCS can only be inhibited by Ca-FCS itself and not by citrullinated FCS (Ci-FCS), native FCS or by peptides used to detect ACPA (Figure 1B) indicating that anti-CarP is truly a different reactivity. Also binding of ACPA to CCP2 plates was not inhibited by addition of Ca-FCS (data not shown).

Since these methods rely on ELISA we also wished to confirm our findings using Western Blotting. We ran FCS and Ca-FCS on non-reducing gels, and following westernblotting stained the blots using sera of patients that were positive or negative for anti-CarP antibodies as detected by ELISA. Serum of anti-CarP antibody positive patients tested positive on lanes loaded with Ca-FCS while no reactivity was seen in lanes loaded with FCS (Figure 1C). Sera from anti-CarP antibody negative patients did not show such staining. Using coomassie staining on loaded gels we confirm that the FCS loaded lanes did contain similar protein amounts. Collectively these data indicate that we now have generated a novel assay that can specifically detect anti-CarP antibodies, both IgG and IgA.

Anti-CarP antibodies are present in RA

From the Leiden Early Arthritis Clinic (EAC) we have used patients suffering from UA or RA, according to the 1987-inclusion criteria. In addition we have used healthy controls also from the Leiden region. We measured the presence of anti-CarP antibodies in patients and controls simultaneously. OD values were calculated to arbitrary units per mL using a standard. We used the healthy persons to calculate the cut-off for positivity as defined by the mean plus 2 x the standard deviation of the healthy controls. We considered samples to be positive when they had a titer higher than the cut-off and an absorbance which was at least 0.1 units higher on Ca-FCS as compared to FCS [17]. Using this approach we established that 42% of the RA patients were positive for IgG anti-CarP antibodies whereas 54% of sera from RA patients tested were positive for IgA anti-CarP-antibodies (Figure 2). Analysis of paired serum / synovial fluid samples revealed that anti-CarP IgG and IgA can also be found in synovial fluid of patients that are positive for these autoantibodies in serum (data not shown).

Anti-CarP antibodies are present in serum and synovial fluid in a substantial proportion of RA patients.

Anti-CarP antibodies are independent from ACPA

Next we wished to analyze whether the anti-CarP antibodies occur independently of ACPA. To this end we analyzed the relationship between ACPA and anti-CarP antibodies in a set of 373 RA patients. Our data show that 14% and 23% of the RA patients did not display ACPA but did harbor anti-CarP IgG and IgA respectively. Likewise, 22% and 19% of the RA patients were positive for ACPA but negative for anti-CarP IgG and IgA respectively (Figure 3). Zooming in on the ACPA negative individuals we observe that around 50% of all ACPA negative RA patients tested positively for anti-CarP antibodies. Thus anti-CarP antibodies occur independently from ACPA.

Anti-CarP antibodies are predictive for development of RA.

Patients presenting themselves at baseline with a diagnosis of undifferentiated arthritis can go into remission, develop another form of arthritis or can develop RA. Clinically it would be relevant to be able to discriminate between the patients in need for treatment and the patients that will remit spontaneously. Therefore we analyzed 425 patients that had UA at baseline for the presence of anti-CarP antibodies and the development of RA (n=151). We observed that positivity for IgG anti-CarP antibodies did not associate with RA-development according to the 1987 criteria in a statistical significant manner (p=0.11). In contrast, IgA anti-CarP antibodies were strongly associated with future development of RA in the UA group as a whole (p=0.002) (Figure 4). This effect was especially prominent in the ACPA negative individuals (Figure 4).

Measuring anti-CarP antibodies in patients suffering from undifferentiated arthritis is useful to indentify persons at risk to develop RA.

Anti-CarP antibodies are associated with more sever radiological damage.

Finally we analyzed whether RA patients that at baseline are positive for anti-CarP antibodies would have a different clinical course of their disease and

therefore we compared the extent of joint damage over time. Also in this analysis, positivity for IgG anti-CarP antibodies did not associate with radiological damage in a statistical significant manner (p=0.43). However, IgA anti-CarP antibodies are strongly associated with more severe damage to the joints (p=0,002) (figure 5), an effect that was independent from Rheumatoid Factor (RF) or ACPA. Together these data indicate that anti-CarP antibodies are associated with a more sever disease course, independent of the presence of RA and or ACPA.

Discussion

Here we describe a novel family of autoantibodies that recognize carbamylated proteins (anti-CarP). These anti-CarP antibodies can be detected in both the IgG and IgA isotypes. Both inhibition studies and cohort studies show that anti-CarP antibodies are different from ACPA. Interestingly positivity for anti-CarP, especially IgA, has clinical implications as individuals positive for anti-CarP IgA have an increased risk to progress from UA to RA and anti-CarP IgA positive RA patients have a worse outcome compared to anti-CarP IgA negative RA patients.

We decided to use a complex protein mix as an initial source of carbamylated protein antigens and therefore we generated Ca-FCS. We observed that antibodies exist that are specifically directed against the carbamylated form of FCS which do not bind to native or citrullinated FCS in both ELISA and Western blot systems. These antibodies are of both the IgG and IgA isotypes indicating that they are derived from class-switched B cells, a process that would require T cell help. Indeed, data indicate that homocitrulline directed T cells can be induced by immunization with carbamylated model antigens [11].

We show here that detection of these antibodies in early arthritis can predict the future development of RA and predict a more sever disease course. Since it has been shown that early aggressive treatment is beneficial [18,19], the invention provides methods for arthritis treatment of individual suffering

from or at risk of suffering from arthritis said method comprising an arthritis diagnosis of said individual wherein said diagnosis comprises a method for determining an anti-CarP antibody in a sample comprising a body fluid of said individual. Preferably sample was determined to contain an anti-Carp antibody. A more stringent treatment of said anti-CarP positive individual is beneficial to the patient.

In conclusion next to the autoantibody system that recognizes citrullinated proteins (ACPA) also an autoantibody system is present against carbamylated proteins (anti-CarP). Detection of such antibodies is useful since its presence is, independently of ACPA, associated with development of UA to RA and is associated with a more severe disease course.

EXAMPLE 2

Results

Anti-CarP antibodies and ACPA are different antibody families.

To detect antibodies against carbamylated proteins (anti-CarP antibodies) we developed an ELISA using carbamylated FCS (Ca-FCS) and non-modified FCS as antigens. Analysing sera of 40 RA patients and 40 controls, we observed that sera of RA-patients reacted with Ca-FCS as compared to sera obtained from healthy subjects with both IgG (Fig. 7A,B) and IgA (Fig. 7C,D) reactivity. The enhanced reactivity of RA-sera to Ca-FCS is further emphasized after subtraction of the reactivity against unmodified FCS (Fig. 7C,E). Since citrulline and homocitrulline are two rather similar amino acids (Fig. 6), we next wished to determine whether ACPA also recognizes homocitrulline when located at the same position as citrulline in a peptide. For this purpose we performed ELISAs using a citrullinated Fib peptide known to be recognized by ACPA (23). Within this peptide backbone a citrulline, an arginine, a homocitrulline or a lysine residue was introduced for further analysis. Analysing a set of 76 RA sera we observed that ACPA only recognized the citrullinated peptide, but not the arginine-containing, or the homocitrulline-

containing peptide (Fig. 8A). These data indicate that ACPA, can discriminate between citrulline and homocitrulline present within the same peptide backbone. Next, we wished to analyze whether there is cross-reactivity between anti-CarP antibodies and ACPA for binding to post-translationally modified proteins. Therefore we performed inhibition studies using sera that were reactive to both citrullinated- and carbamylated antigens. We analyzed the binding of anti-CarP antibodies to Ca-FCS coated plates following pre-incubation with Ca-FCS, citrullinated FCS (Ci-FCS), native FCS or by citrullinated peptides used to detect ACPA (CCP1). Following pre-incubation, we observed that anti-CarP antibody binding to Ca-FCS can only be inhibited by Ca-FCS but not by citrullinated FCS (Ci-FCS), native FCS or by peptides used to detect ACPA (Fig. 8B). We also performed the reverse inhibition experiment where we analyzed the binding of ACPA to plates coated with Ci-FCS following the same pre-incubation procedure. We observed that ACPA binding to Ci-FCS could only be inhibited by Ci-FCS and the citrullinated peptide but not by Ca-FCS, non-modified FCS, or the arginine form of the peptide (Fig. 8C). Together, these data indicate that anti-CarP antibodies and ACPA are not- or only limited cross-reactive and specifically directed against homocitrulline, respectively citrulline containing antigens. Since all observations described above were made using ELISA, we also wished to confirm our findings using a different technique. For this reason we performed a Western blot-analysis using FCS, Ca-FCS and Ci-FCS on reduced gels, followed by Western Blotting. The different blots were incubated with sera of individuals that were either anti-CarP positive and ACPA-negative or anti-CarP negative and ACPA-positive. We observed a positive staining of the anti-CarP positive sample only on Ca-FCS but not on Ci-FCS or FCS (Fig. 8D) In contrast, the anti-CarP negative, ACPA-positive sample reacted to Ci-FCS, but not to Ca-FCS and FCS (Fig. 8D). To confirm the presence of anti-CarP antibodies we repeated these experiments using a more defined protein, human Fib, as target antigen. Fib was citrullinated by PAD (Ci-Fib) or

carbamylated by cyanate (Ca-Fib). The non-modified form (Fib), Ci-Fib and Ca-Fib were used as antigens in ELISA. Similar to the observations for FCS, we observed significant binding of antibodies to the Ci-Fib and the Ca-Fib but not to the Fib coated wells (Fig. 9A). This was largely restricted to the RA sera and not the controls ($p =< 0.0001$). To analyze cross-reactivity we also performed inhibition studies as described above. ELISA analyses confirmed that ACPA and anti-CarP antibodies are largely non-cross-reactive. To ensure that reactivity towards carbamylated proteins is mediated by the antigen-binding-part of the antibodies, we generated F(ab')2. As expected, F(ab')2, generated from anti-CarP IgG positive samples but not from negative samples display anti-CarP reactivity (Fig. 9C,D). As observed using intact antibodies, also F(ab')2-reactivity towards Ca-Fib could be inhibited specifically by Ca-Fib, whereas F(ab')2-reactivity towards Ci-Fib could only be inhibited specifically by Ci-Fib (Fig. 9E).

Collectively these data indicate that anti-CarP antibodies and ACPA recognize different antigens, one recognizing citrullinated proteins (ACPA) and the other carbamylated proteins (anti-CarP). Likewise, these data indicate that antigen-recognition is most likely mediated via the variable domains present in the F(ab')2 fragments.

Anti-CarP antibodies are present in RA.

Following the identification of anti-CarP antibodies as an autoantibody family separate from ACPA we wished to quantify the presence of these anti-CarP antibodies in a large population of RA patients and controls. For this reason, we first generated a standard, comprising of a pool of anti-CarP antibody positive sera. This standard displayed a specific, dose dependent, binding of both IgG and IgA to carbamylated FCS (Ca-FCS) but no binding to unmodified FCS (Fig. 10A,B). For this analysis we again used the FCS based assay in an attempt to capture as many anti-CarP reactivities as possible. We established a cut-off for positivity using sera of 305 healthy individuals as described in the

methods section. Using this approach we observed that 45% of the sera of RA patients analyzed are positive for IgG anti-CarP antibodies (Fig. 10C). Likewise, 43% of sera from RA patients tested are positive for IgA anti-CarP-antibodies (Fig. 10D).

Anti-CarP antibodies are also present in sera of anti-CCP2 negative RA patients.

The group of RA patients analyzed in this study consisted of both ACPA-positive and ACPA-negative individuals, as measured by the CCP2 assay. Therefore, we analyzed next the association between anti-CarP antibodies and anti-CCP2 antibodies. The presence of anti-CarP antibodies and anti-CCP2 antibodies showed a limited degree of correlation when analyzing the entire RA population (r^2=0.27, $p<0.001$ for anti-CarP IgG or r^2=0.15, $p<0.001$ for IgA). However, we also indentified substantial numbers of RA patients that are only positive for anti-CCP2 antibodies as well as a group of patients that is only positive for anti-CarP antibodies (Fig. 10E,F). We observed that approximately 16% of the anti-CCP2-negative RA patients displayed anti-CarP IgG antibodies, whereas 30% of the anti-CCP2-negative RA patients tested positive for anti-CarP IgA (Fig. 10G,H). These data indicate that the presence of anti-CarP antibodies overlaps with the occurrence of anti-CCP2 antibodies, but that this overlap is not absolute as over 30% of the anti-CCP2 negative patients harbor anti-CarP antibodies. In total more than 35% of all anti-CCP2 negative patients have either anti-CarP IgG or IgA antibodies.

Anti-CarP antibodies are associated with more severe radiological damage.

The presence of ACPA is associated with a more severe clinical disease course as measured by radiological damage. To analyze whether the presence of anti-CarP antibodies are also predictive for a more severe disease course, we compared the extent of joint damage over time between anti-CarP positive and

negative patients participating in the Leiden EAC cohort. This cohort is an inception cohort of patients with recent-onset arthritis where X-rays of hands and feet are taken of all RA-patients at yearly intervals to assess radiological damage using the Sharp -van der Heijde method (24). We observed that the presence of anti-CarP IgG strongly associates with a more severe disease progression. Patients positive for anti-CarP IgG had more joint destruction over 7-years than IgG negative patients without (β=2.01, 95%CI 1.68-2.40, p=8.68x10^{-14}) or with correction of ACPA and RF (β=1.41, 95%CI 1.13-1.76, p=0.002) (Fig 13). Anti-CarP IgA was associated with more joint destruction over 7-years than anti-CarP IgA negative patients without correction of ACPA and RF (β=1.21, 95%CI 1.01-1.45, p=0.041) but not after correction (p=0.855) (Fig 13). As the analysis described above does not show whether anti-CarP antibodies predict radiological progression in the anti-CCP2-negative, anti-CCP2-positive or both RA-subgroups, we next performed a stratified analysis. Importantly this analysis revealed that the presence of anti-CarP IgG is associated with a more severe joint damage in the anti-CCP2-negative subgroup (β=1.86, 95%CI 1.41-2.66, p=1.8·10^{-5}) (Fig. 11). Likewise, a similar trend towards more joint damage over time was observed for anti-CCP2-negative patients tested positive for IgA anti-CarP antibodies (β=1.25, 95%CI 0.98-1.58, p=0.071) (Fig 13). In contrast, in the anti-CCP2-positive subgroup, which is already characterized by severe joint destruction, no additional increase was observed in individuals that also harbored anti-CarP antibodies (Fig 13). Together, these data indicate that the detection of anti-CarP antibodies at baseline is predictive for a more destructive disease course in anti-CCP2-negative RA as measured by the sharp / van der Heijde method.

Discussion

A family of autoantibodies that recognize carbamylated proteins, anti-CarP antibodies, can be detected in sera of RA patients. Both inhibition studies and cohort studies show that anti-CarP antibodies and ACPA represent two

different and independent autoantibody families, one recognizing carbamylated proteins and the other citrullinated proteins. Our data show that anti-CarP antibodies and ACPA are, by and large, non-cross-reactive although we do not exclude that some cross-reactivity exists at the population level as is also indicated in recent data obtained in rabbits after vaccination with carbamylated proteins (14). Interestingly, positivity for anti-CarP antibodies, is related to clinical outcome as individuals positive for anti-CarP IgG, but negative for anti-CCP2 antibodies, have a more destructive disease course as compared to anti-CarP IgG negative RA patients.

It is currently unknown which proteins undergo posttranslational modifications like carbamylation. Carbamylation is mediated by cyanate which is in equilibrium with urea. Increased urea concentrations, smoking and inflammation have been reported to shift this equilibrium towards cyanate and hence enhanced carbamylation (13). Since currently no in-vivo relevant targets for anti-CarP antibodies are known, we used a complex protein mixture as an initial source of carbamylated protein antigens for the detection of anti-CarP antibodies. Western blot analyses indicate the recognition by anti-CarP antibodies of at least one dominant protein present in FCS after carbamylation employing Cyanate (representing high Urea concentrations) (Fig. 8D).

However, these data are likely not to represent the in vivo situation where carbamylation is a more gradual, but constantly occurring process (25). In this respect, it is likely that especially long-lived proteins acquire homocitrulline residues over time as carbamylation is nearly irreversible and thus will lead to the accumulation of homocitrulline-residues on proteins with a long half-life. Intriguingly, the joint is known for the presence of long-lived proteins such as collagens and other cartilage-expressed proteins. Therefore, it is conceivable that such matrix-proteins will accumulate homocitrulline residues during life, especially under conditions of inflammation. Indeed, it has been shown that homocitrulline is present in the joint (11), possibly representing the long-lived nature of many joint-derived proteins. It will be interesting to know the

identity of these proteins and whether these can serve as a target for anti-CarP antibodies.

The molecular nature of the antigens recognized by ACPA has been identified more than 15 years ago by describing that citrulline is an essential constituent of antigens recognized by these RA-specific antibodies (26, 27). This finding has made considerable impact as it has opened up the way to relevant and novel insights into RA-diagnosis and etiopathology (1). For example, ACPA are now part of the new ACR/EULAR-criteria for RA (28), and have been implicated in RA-pathogenesis, both in animal models (29, 30, 31) and in ex-vivo human studies (32, 33, 34, 35). Importantly, the description of ACPA has led to the realization that RA constitutes at least two clinical syndromes that share many clinical features, but differ with respect to genetic background, predisposing environmental factors and clinical progression / remission (36, 3, 4, 37, 38). Although it is clearly too early to allow any firm conclusions, it is tempting to speculate that anti-CarP antibodies also contribute to disease pathogenesis and / or display diagnostic value, given the similar nature of the antigens recognized and their presence in ACPA negative disease.

The presence of anti-CarP-antibodies in anti-CCP2-negative disease is highly intriguing as it could potentially represent a novel biomarker that positively identifies at least part of this manifestation of RA. To gain further insight into this possibility it is important to establish whether the presence of anti-CarP-antibodies is specific for RA or also found in other rheumatic diseases as well as whether their presence predict the development of (ACPA-negative) RA in patients suffering from early unclassified RA and/or joint complaints such as arthralgia.

To establish a cut-off to define a positive sample we have analyzed the presence of IgG and IgA directed against Ca-FCS and FCS in sera of healthy controls. All samples were tested for reactivity towards Ca-FCS and FCS, and absorbance values were converted into aU/mL using an anti-CarP antibody positive standard present on the same plate. Since sera from several individual

subjects also displayed reactivity towards non-modified FCS, we subtracted the 'FCS-reactivity' from the reactivity towards Ca-FCS using aU/mL as defined by the standard curve. We calculated subsequently, the cut-off as the mean plus 2 times standard deviation and applied the cut-off to the data of the RA patients following a similar strategy. The disadvantage of this method is that a standard is used on Ca-FCS for the determination of aU/mL towards FCS, another antigenic entity. However, this method did allow the calculation of a specific response to the post-translational modification.

Every method of establishing a cut-off has advantages and limitations. Therefore we subsequently confirmed our observations using another strategy as well, by calculating the cut-off as the mean plus 2 times standard deviation of the anti-Ca-FCS response in controls. This cut-off was applied to the data of the RA patients as was also employed before (39). The association with radiological progression of IgG in ACPA negative RA remains significant, albeit with a lower level of significance ($p=0.001$).

From a clinical perspective, the detection of anti-CarP antibodies in early arthritis could be highly rewarding since they predict a more severe disease course. Since early aggressive treatment in RA has been shown to prevent future damage (40, 41), the detection of anti-CarP antibodies might be beneficial to identify anti-CCP2-negative patients at risk to develop severe disease. The identification of such patients might be important to guide treatment decisions early after onset of symptoms, especially in early arthritis patients that are difficult to classify.

In conclusion, in addition to the autoantibody system that recognizes citrullinated proteins (ACPA) an autoantibody system against carbamylated proteins (anti-CarP) is present in sera of RA patients. Detection of anti-CarP antibodies could offer new possibilities to identify patients at risk for a severe disease course.

Aminoacid sequence of Fibrinogen alpha

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        10          20          30          40          50          60
MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS DWPFCSDEDW
        70          80          90         100         110         120
NYKCPSGCRM KGLIDEVNQD FTNRINKLKN SLFEYQKNNK DSHSLTTNIM EILRGDFSSA
       130         140         150         160         170         180
NNRDNTYNRV SEDLRSRIEV LKRKVIEKVQ HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC
       190         200         210         220         230         240
RGSCSRALAR EVDLKDYEDQ QKQLEQVIAK DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ
       250         260         270         280         290         300
LQKVPPEWKA LTDMPQMRME LERPGGNEIT RGGSTSYGTG SETESPRNPS SAGSWNSGSS
       310         320         330         340         350         360
GPGSTGNRNP GSSGTGGTAT WKPGSSGPGS TGSWNSGSSG TGSTGNQNPG SPRPGSTGTW
       370         380         390         400         410         420
NPGSSERGSA GHWTSESSVS GSTGQWHSES GSFRPDSPGS GNARPNNPDW GTFEEVSGNV
       430         440         450         460         470         480
SPGTRREYHT EKLVTSKGDK ELRTGKEKVT SGSTTTTRRS CSKTVTKTVI GPDGHKEVTK
       490         500         510         520         530         540
EVVTSEDGSD CPEAMDLGTL SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF
       550         560         570         580         590         600
VSETESRGSE SGIFTNTKES SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS
       610         620         630         640         650         660
YKMADEAGSE ADHEGTHSTK RGHAKSRPVR DCDDVLQTHP SGTQSGIFNI KLPGSSKIFS
       670         680         690         700         710         720
VYCDQETSLG GWLLIQQRMD GSLNFNRTWQ DYKRGFGSLN DEGEGEFWLG NDYLHLLTQR
       730         740         750         760         770         780
GSVLRVELED WAGNEAYAEY HFRVGSEAEG YALQVSSYEG TAGDALIEGS VEEGAEYTSH
       790         800         810         820         830         840
NNMQFSTFDR DADQWEENCA EVYGGGWWYN NCQAANLNGI YYPGGSYDPR NNSPYEIENG
       850         860
VVWVSFRGAD YSLRAVRMKI RPLVTQ
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Aminoacid sequence of Fibrinogen beta

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        10          20          30          40          50          60
MKRMVSWSFH KLKTMKHLLL LLLCVFLVKS QGVNDNEEGF FSARGHRPLD KKREEAPSLR
        70          80          90         100         110         120
PAPPPISGGG YRARPAKAAA TQKKVERKAP DAGGCLHADP DLGVLCPTGC QLQEALLQQE
       130         140         150         160         170         180
RPIRNSVDEL NNNVEAVSQT SSSSFQYMYL LKDLWQKRQK QVKDNENVVN EYSSELEKHQ
       190         200         210         220         230         240
LYIDETVNSN IPTNLRVLRS ILENLRSKIQ KLESDVSAQM EYCRTPCTVS CNIPVVS
       250         260         270         280         290         300
CEEIIRKGGE TSEMYLIQPD SSVKPYRVYC DMNTENGGWT VIQNRQDGSV DFGRKWDPYK
       310         320         330         340         350         360
QGFGNVATNT DGKNYCGLPG EYWLGNDKIS QLTRMGPTEL LIEMEDWKGD KVKAHYGGFT
       370         380         390         400         410         420
VQNEANKYQI SVNKYRGTAG NALMDGASQL MGENRTMTIH NGMFFSTYDR DNDGWLTSDP
       430         440         450         460         470         480
RKQCSKEDGG GWWYNRCHAA NPNGRYYWGG QYTWDMAKHG TDDGVVWMNW KGSWYSMRKM
       490
SMKIRPFFPQ Q
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Aminoacid sequence of Fibrinogen gamma

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        10          20          30          40          50          60
MSWSLHPRNL ILYFYALLFL SSTCVAYVAT RDNCCILDER FGSYCPTTCG IADFLSTYQT
        70          80          90         100         110         120
KVDKDLQSLE DILHQVENKT SEVKQLIKAI QLTYNPDESS KPNMIDAATL KSRKMLEEIM
       130         140         150         160         170         180
KYEASILTHD SSIRYLQEIY NSNNQKIVNL KEKVAQLEAQ CQEPCKDTVQ IHDITGKDCQ
       190         200         210         220         230         240
DIANKGAKQS GLYFIKPLKA NQQFLVYCEI DGSGNGWTVF QKRLDGSVDF KKNWIQYKEG
       250         260         270         280         290         300
FGHLSPTGTT EFWLGNEKIH LISTQSAIPY ALRVELEDWN GRTSTADYAM FKVGPEADKY
       310         320         330         340         350         360
RLTYAYFAGG DAGDAFDGFD FGDDPSDKFF TSHNGMQFST WDNDNDKFEG NCAEQDGSGW
       370         380         390         400         410         420
WMNKCHAGHL NGVYYQGGTY SKASTPNGYD NGIIWATWKT RWYSMKKTTM KIIPFNRLTI
       430         440         450
GEGQQHHLGG AKQVRPEHPA ETEYDSLYPE DDL
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Table I List of Lysine containing peptides of Fibrinogen alpha and their homocitrulline containing counterparts

	Lysine containing peptides of Fibrinogen alpha		Homocitrulline containing peptides of Fibrinogen alpha
1	RVVERHQSACKDSDWPFCSDE	1	RVVERHQSAChomocitDSDWPFCSDE
2	PFCSDEDWNYKCPSGCRMKGL	2	PFCSDEDWNYhomocitCPSGCRMhomocitGL
3	NYKCPSGCRMKGLIDEVNQDF	3	NYhomocitCPSGCRMhomocitGLIDEVNQDF
4	VNQDFTNRINKLKNSLFEYQK	4	VNQDFTNRINhomocitLhomocitNSLFEYQhomocit
5	QDFTNRINKLKNSLFEYQKNN	5	QDFTNRINhomocitLhomocitNSLFEYQhomocitNN
6	KLKNSLFEYQKNNKDSHSLTT	6	homocitLhomocitNSLFEYQhomocitNNhomocitDSHSLTT
7	NSLFEYQKNNKDSHSLTTNIM	7	NSLFEYQhomocitNNhomocitDSHSLTTNIM
8	EDLRSRIEVLKRKVIEKVQHI	8	EDLRSRIEVLhomocitRhomocitVIEhomocitVQHI
9	LRSRIEVLKRKVIEKVQHIQL	9	LRSRIEVLhomocitRhomocitVIEhomocitVQHIQL
10	IEVLKRKVIEKVQHIQLLQKN	10	IEVLhomocitRhomocitVIEhomocitVQHIQLLQhomocitN
11	EKVQHIQLLQKNVRAQLVDMK	11	EhomocitVQHIQLLQhomocitNVRAQLVDMhomocit
12	KNVRAQLVDMKRLEVDIDIKI	12	homocitNVRAQLVDMhomocitRLEVDIDIhomocitI
13	MKRLEVDIDIKIRSCRGSCSR	13	MhomocitRLEVDIDIhomocitIRSCRGSCSR
14	SRALAREVDLKDYEDQQKQLE	14	SRALAREVDLhomocitDYEDQQhomocitQLE
15	VDLKDYEDQQKQLEQVIAKDL	15	VDLhomocitDYEDQQhomocitQLEQVIAhomocitDL
16	QQKQLEQVIAKDLLPSRDRQH	16	QQhomocitQLEQVIAhomocitDLLPSRDRQH
17	SRDRQHLPLIKMKPVPDLVPG	17	SRDRQHLPLIhomocitMhomocitPVPDLVPG
18	DRQHLPLIKMKPVPDLVPGNF	18	DRQHLPLIhomocitMhomocitPVPDLVPGNF
19	PVPDLVPGNFKSQLQKVPPEW	19	PVPDLVPGNFhomocitSQLQhomocitVPPEW
20	VPGNFKSQLQKVPPEWKALTD	20	VPGNFhomocitSQLQhomocitVPPEWhomocitALTD
21	SQLQKVPPEWKALTDMPQMRM	21	SQLQhomocitVPPEWhomocitALTDMPQMRM
22	SSGTGGTATWKPGSSGPGSTG	22	SSGTGGTATWhomocitPGSSGPGSTG
23	PGTRREYHTEKLVTSKGDKEL	23	PGTRREYHTEhomocitLVTShomocitGDhomocitEL
24	EYHTEKLVTSKGDKELRTGKE	24	EYHTEhomocitLVTShomocitGDhomocitELRTGhomocitE
25	TEKLVTSKGDKELRTGKEKVT	25	TEhomocitLVTShomocitGDhomocitELRTGhomocitEhomocitVT
26	SKGDKELRTGKEKVTSGSTTT	26	ShomocitGDhomocitELRTGhomocitEhomocitVTSGSTTT
27	GDKELRTGKEKVTSGSTTTTR	27	GDhomocitELRTGhomocitEhomocitVTSGSTTTTR
28	STTTTRRSCSKTVTKTVIGPD	28	STTTTRRSCShomocitTVThomocitTVIGPD
29	TRRSCSKTVTKTVIGPDGHKE	29	TRRSCShomocitTVThomocitTVIGPDGHhomocitE
30	TKTVIGPDGHKEVTKEVVTSE	30	ThomocitTVIGPDGHhomocitEVThomocitEVVTSE
31	IGPDGHKEVTKEVVTSEDGSD	31	IGPDGHhomocitEVThomocitEVVTSEDGSD
32	AAFFDTASTGKTFPGFFSPML	32	AAFFDTASTGhomocitTFPGFFSPML
33	GSESGIFTNTKESSSHHPGIA	33	GSESGIFTNThomocitESSSHHPGIA
34	PGIAEFPSRGKSSSYSKQFTS	34	PGIAEFPSRGhomocitSSSYShomocitQFTS
35	PSRGKSSSYSKQFTSSTSYNR	35	PSRGhomocitSSSYShomocitQFTSSTSYNR
36	YNRGDSTFESKSYKMADEAGS	36	YNRGDSTFEShomocitSYhomocitMADEAGS
37	GDSTFESKSYKMADEAGSEAD	37	GDSTFEShomocitSYhomocitMADEAGSEAD
38	EADHEGTHSTKRGHAKSRPVR	38	EADHEGTHSThomocitRGHAhomocitSRPVR
39	GTHSTKRGHAKSRPVRDCDDV	39	GTHSThomocitRGHAhomocitSRPVRDCDDV
40	SGTQSGIFNIKLPGSSKIFSV	40	SGTQSGIFNIhomocitLPGSShomocitIFSV
41	IFNIKLPGSSKIFSVYCDQET	41	IFNIhomocitLPGSShomocitIFSVYCDQET
42	LNFNRTWQDYKRGFGSLNDEG	42	LNFNRTWQDYhomocitRGFGSLNDEG
43	VRGIHTSPLGKPSLSP	43	VRGIHTSPLGhomocitPSLSP

Table II List of Lysine containing peptides of Fibrinogen beta and their homocitrulline containing counterparts

	Lysine containing peptides of Fibrinogen beta		Homocitrulline containing peptides of Fibrinogen beta
1	MKRMVSWSFHKL	1	MhomocitRMVSWSFHhomocitL
2	MKRMVSWSFHKLKTMKHLLLL	2	MhomocitRMVSWSFHhomocitLhomocitTMhomocitHLLLL
3	RMVSWSFHKLKTMKHLLLLLL	3	RMVSWSFHhomocitLhomocitTMhomocitHLLLLLL
4	SWSFHKLKTMKHLLLLLLCVF	4	SWSFHhomocitLhomocitTMhomocitHLLLLLLCVF
5	LLLLLCVFLVKSQGVNDNEEG	5	LLLLLCVFLVhomocitSQGVNDNEEG
6	FSARGHRPLDKKREEAPSLRP	6	FSARGHRPLDhomocithomocitREEAPSLRP
7	SARGHRPLDKKREEAPSLRPA	7	SARGHRPLDhomocithomocitREEAPSLRPA
8	SGGGYRARPAKAAATQKKVER	8	SGGGYRARPAhomocitAAATQhomocithomocitVER
9	ARPAKAAATQKKVERKAPDAG	9	ARPAhomocitAAATQhomocithomocitVERhomocitAPDAG
10	RPAKAAATQKKVERKAPDAGG	10	RPAhomocitAAATQhomocithomocitVERhomocitAPDAGG
11	AAATQKKVERKAPDAGGCLHA	11	AAATQhomocithomocitVERhomocitAPDAGGCLHA
12	SSSFQYMYLLKDLWQKRQKQV	12	SSSFQYMYLLhomocitDLWQhomocitRQhomocitQV
13	YMYLLKDLWQKRQKQVKDNEN	13	YMYLLhomocitDLWQhomocitRQhomocitQVhomocitDNEN
14	LLKDLWQKRQKQVKDNENVVN	14	LLhomocitDLWQhomocitRQhomocitQVhomocitDNENVVN
15	DLWQKRQKQVKDNENVVNEYS	15	DLWQhomocitRQhomocitQVhomocitDNENVVNEYS
20	PVVSCEEIIRKGGETSEMYLI	20	PVVSCEEIIRhomocitGGETSEMYLI
21	MYLIQPDSSVKPYRVYCDMNT	21	MYLIQPDSSVhomocitPYRVYCDMNT
22	VDFGRKWDPYKQGFGNVATNT	22	VDFGRhomocitWDPYhomocitQGFGNVATNT
23	FGNVATNTDGKNYCGLPGEYW	23	FGNVATNTDGhomocitNYCGLPGEYW
24	LPGEYWLGNDKISQLTRMGPT	24	LPGEYWLGNDhomocitISQLTRMGPT
25	TELLIEMEDWKGDKVKAHYGG	25	TELLIEMEDWhomocitGDhomocitVhomocitAHYGG
26	LIEMEDWKGDKVKAHYGGFTV	26	LIEMEDWhomocitGDhomocitVhomocitAHYGGFTV
27	EMEDWKGDKVKAHYGGFTVQN	27	EMEDWhomocitGDhomocitVhomocitAHYGGFTVQN
28	GGFTVQNEANKYQISVNKYRG	28	GGFTVQNEANhomocitYQISVNhomocitYRG
29	EANKYQISVNKYRGTAGNALM	29	EANhomocitYQISVNhomocitYRGTAGNALM
30	NDGWLTSDPRKQCSKEDGGGW	30	NDGWLTSDPRhomocitQCShomocitEDGGGW
31	LTSDPRKQCSKEDGGGWWYNR	31	LTSDPRhomocitQCShomocitEDGGGWWYNR
32	WGGQYTWDMAKHGTDDGVVWM	32	WGGQYTWDMAhomocitHGTDDGVVWM
33	TDDGVVWMNWKGSWYSMRKMS	33	TDDGVVWMNWhomocitGSWYSMRhomocitMS
34	NWKGSWYSMRKMSMKIRPFFQ	34	NWhomocitGSWYSMRhomocitMSMhomocitIRPFFQ
35	SWYSMRKMSMKIRPFFPQQ	35	SWYSMRhomocitMSMhomocitIRPFFPQQ

Table III List of Lysine containing peptides of Fibrinogen gamma and their homocitrulline containing counterparts

	Lysine containing peptides of Fibrinogen gamma		Homocitrulline containing peptides of Fibrinogen gamma
1	IADFLSTYQTKVDKDLQSLED	1	IADFLSTYQThomocitVDhomocitDLQSLED
2	FLSTYQTKVDKDLQSLEDILH	2	FLSTYQThomocitVDhomocitDLQSLEDILH
3	LEDILHQVENKTSEVKQLIKA	3	LEDILHQVENhomocitTSEVhomocitQLIhomocitA
4	HQVENKTSEVKQLIKAIQLTY	4	HQVENhomocitTSEVhomocitQLIhomocitAIQLTY
5	NKTSEVKQLIKAIQLTYNPDE	5	NhomocitTSEVhomocitQLIhomocitAIQLTYNPDE
6	QLTYNPDESSKPNMIDAATLK	6	QLTYNPDESShomocitPNMIDAATLhomocit
7	KPNMIDAATLKSRKMLEEIMK	7	homocitPNMIDAATLhomocitSRhomocitMLEEIMhomocit
8	MIDAATLKSRKMLEEIMKYEA	8	MIDAATLhomocitSRhomocitMLEEIMhomocitYEA
9	KSRKMLEEIMKYEASILTHDS	9	homocitSRhomocitMLEEIMhomocitYEASILTHDS
10	LQEIYNSNNQKIVNLKEKVAQ	10	LQEIYNSNNQhomocitIVNLhomocitEhomocitVAQ
11	NSNNQKIVNLKEKVAQLEAQC	11	NSNNQhomocitIVNLhomocitEhomocitVAQLEAQC
12	NNQKIVNLKEKVAQLEAQCQE	12	NNQhomocitIVNLhomocitEhomocitVAQLEAQCQE
13	QLEAQCQEPCKDTVQIHDITG	13	QLEAQCQEPChomocitDTVQIHDITG
14	DTVQIHDITGKDCQDIANKGA	14	DTVQIHDITGhomocitDCQDIANhomocitGA
15	TGKDCQDIANKGAKQSGLYFI	15	TGhomocitDCQDIANhomocitGAhomocitQSGLYFI
16	DCQDIANKGAKQSGLYFIKPL	16	DCQDIANhomocitGAhomocitQSGLYFIhomocitPL
17	GAKQSGLYFIKPLKANQQFLV	17	GAhomocitQSGLYFIhomocitPLhomocitANQQFLV
18	GSGNGWTVFQKRLDGSVDFKK	18	GSGNGWTVFQhomocitRLDGSVDFhomocithomocit
19	QKRLDGSVDFKKNWIQYKEGF	19	QhomocitRLDGSVDFhomocithomocitNWIQYhomocitEGF
20	KRLDGSVDFKKNWIQYKEGFG	20	homocitRLDGSVDFhomocithomocitNWIQYhomocitEGFG
21	VDFKKNWIQYKEGFGHLSPTG	21	VDFhomocithomocitNWIQYhomocitEGFGHLSPTG
22	GTTEFWLGNEKIHLISTQSAI	22	GTTEFWLGNEhomocitIHLISTQSAI
23	RTSTADYAMFKVGPEADKYRL	23	RTSTADYAMFhomocitVGPEADhomocitYRL
24	AMFKVGPEADKYRLTYAYFAG	24	AMFhomocitVGPEADhomocitYRLTYAYFAG
25	GFDFGDDPSDKFFTSHNGMQF	25	GFDFGDDPSDhomocitFFTSHNGMQF
26	QFSTWDNDNDKFEGNCAEQDG	26	QFSTWDNDNDhomocitFEGNCAEQDG
27	EQDGSGWWMNKCHAGHLNGVY	27	EQDGSGWWMNhomocitCHAGHLNGVY
28	GVYYQGGTYSKASTPNGYDNG	28	GVYYQGGTYShomocitASTPNGYDNG
29	YDNGIIWATWKTRWYSMKKTT	29	YDNGIIWATWhomocitTRWYSMhomocithomocitTT
30	ATWKTRWYSMKKTTMKIIPFN	30	ATWhomocitTRWYSMhomocithomocitTTMhomocitIIPFN
31	TWKTRWYSMKKTTMKIIPFNR	31	TWhomocitTRWYSMhomocithomocitTTMhomocitIIPFNR
32	RWYSMKKTTMKIIPFNRLTIG	32	RWYSMhomocithomocitTTMhomocitIIPFNRLTIG

Claims

1. A method for classifying an individual that is suffering from, or at risk of developing, a form of arthritis, said method comprising determining whether a sample comprising a body fluid obtained from blood or a joint of said individual comprises an Anti-Carbamylated Fibrinogen (anti-Ca-Fib) antibody, wherein the presence of the anti-Ca-Fib antibody in the sample classifies the individual to be suffering from or at risk of developing arthritis.

2. A method for providing a prognosis for the development of arthritis to an individual suffering from said arthritis, said method comprising determining whether a sample comprising a body fluid obtained from blood or a joint of said individual comprises an Anti-Carbamylated Protein (anti-CarP) antibody, wherein the presence or absence of the anti-CarP antibody in the sample predicts the future severity of said arthritis.

3. The method according to claim 2, wherein said Anti-Carbamylated Protein (anti-CarP) antibody is an Anti-Carbamylated Fibrinogen (anti-Ca-Fib) antibody.

4. The method according to any one of claims 1-3, wherein said body fluid is a serum sample or a synovial fluid sample.

5. The method according to any one of claims 1-4, wherein said anti-CarP and/or anti-Ca-Fib antibody is of Ig-subtype IgA or of the Ig-subtype IgG.

6. The method according to any one of claims 2-5, wherein said sample is negative for Anti-citrullinated Protein Antibody (ACPA).

7. The method according to claim 1, wherein for determining whether said individual is at risk of developing arthritis, said individual was not suffering from arthritis at the time the fluid sample was obtained.

Date Reçue/Date Received 2021-11-17

8. The method according to any one of claims 1-7, wherein said arthritis comprises Rheumatoid arthritis, Juvenile arthritis, Psoriatic arthritis, Osteoarthritis, Polymyalgia rheumatica, Ankylosing spondylitis, Reactive arthritis, Gout, Pseudogout, Autoimmune arthritis, Systemic lupus erythematosus, Polymyositis, Fibromyalgia, Lyme disease, Undifferentiated arthritis, non-rheumatoid arthritis or Spondyloarthropathy.

9. The method according to claim 8, wherein said arthritis is rheumatoid arthritis, juvenile arthritis or undifferentiated arthritis.

10. The method according to any one of claims 1-9, wherein said anti-Carp and/or anti-Ca-Fib antibody is specific for a carbamylated protein or peptides derived from fetal calfs serum (FCS) and or its equivalents.

11. The method according to any one of claims 1-10, further comprising determining a further factor as an arthritis classifier for said individual.

12. The method according to claim 11, wherein said further factor comprises determining ACPA, rheumatoid factor, C-reactive protein, and/or erythrocyte sedimentation rate.

13. Use of a kit for the detection of anti-CarP antibodies in a body fluid of an individual in a method according to any one of claims 1-12, said kit comprising a carbamylated protein or peptide.

14. Use according to claim 13, wherein said kit comprises carbamylated fibrinogen or a peptide derived therefrom.

15. Use according to claim 13 or 14, wherein said anti-CarP antibodies are anti-Ca-Fib antibodies.

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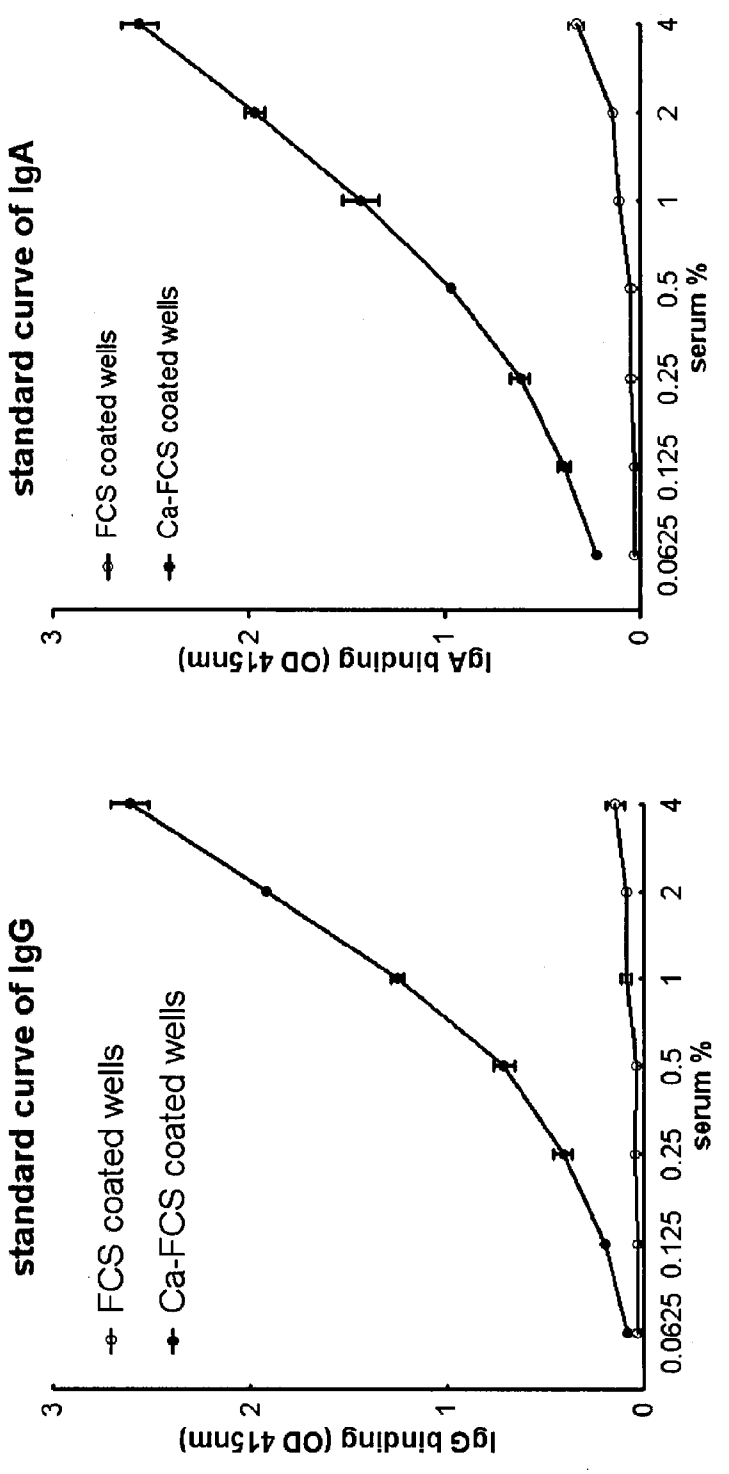
standard curve of IgA
FCS coated wells
Ca-FCS coated wells
IgA binding (OD 415nm)
0
1
2
3
0.0625 0.125 0.25 0.5 1 2 4
serum %
standard curve of IgG
FCS coated wells
Ca-FCS coated wells
IgG binding (OD 415nm)
0
1
2
3
0.0625 0.125 0.25 0.5 1 2 4
serum %

Fig. 1A

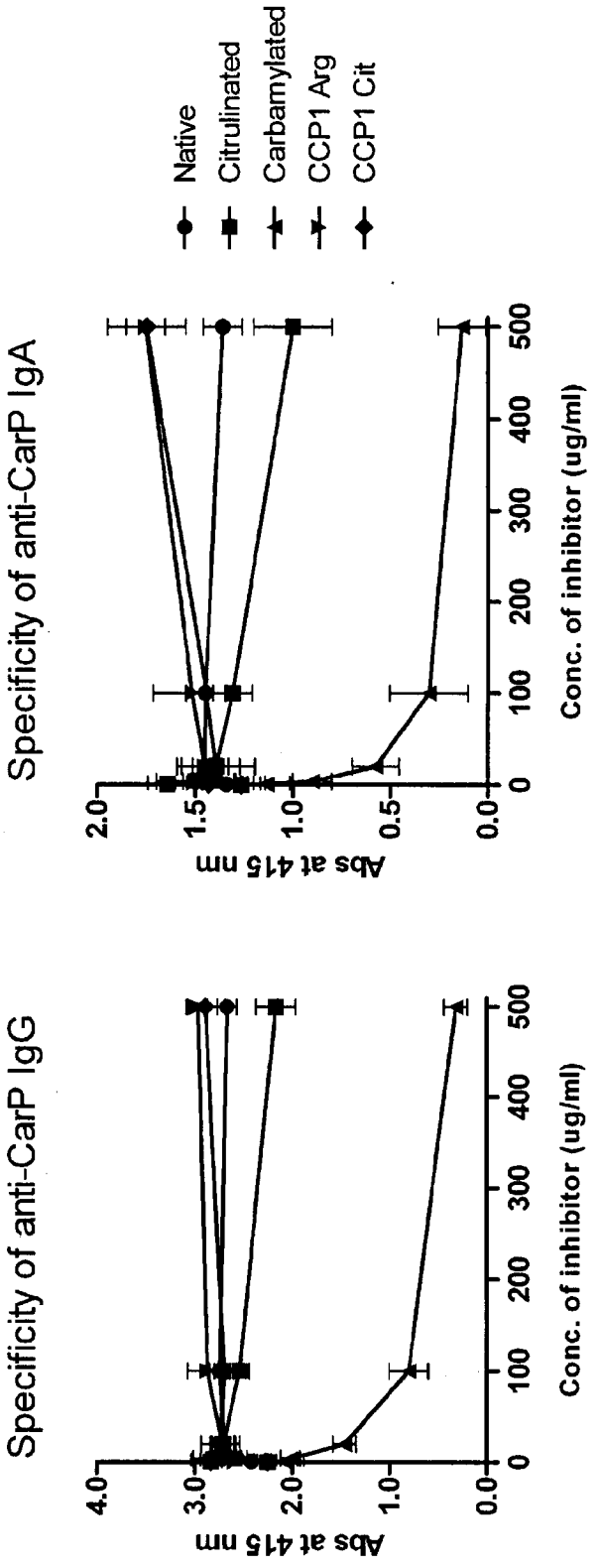
Specificity of anti-CarP IgA
Native
Citrulinated
Carbamylated
CCP1 Arg
CCP1 Cit
Abs at 415 nm
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
Conc. of inhibitor (ug/ml)
Specificity of anti-CarP IgG
Abs at 415 nm
4.0
3.0
2.0
1.0
0.0
0
100
200
300
400
500
Conc. of inhibitor (ug/ml)

Fig. 1B

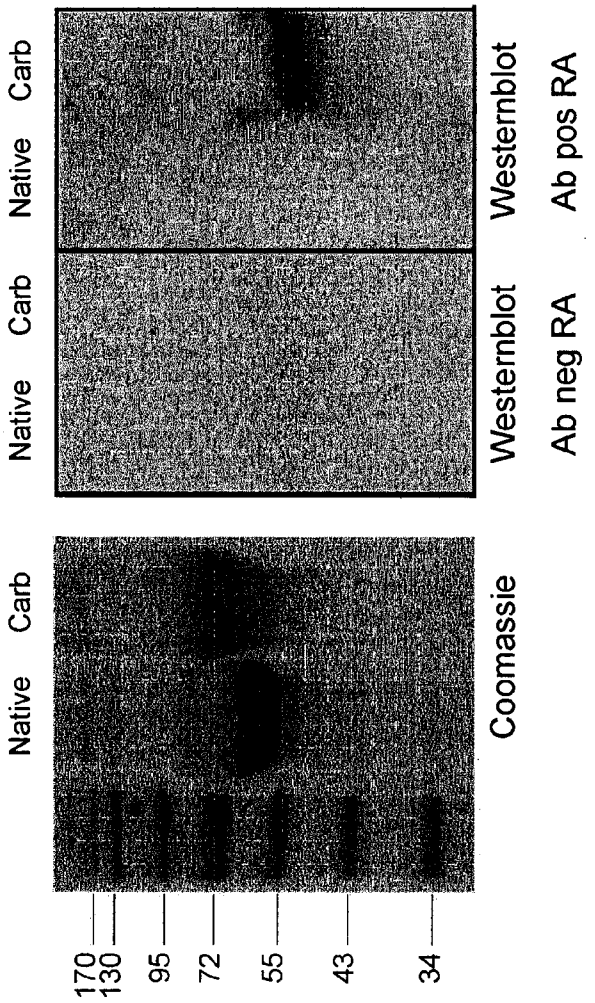
Native
Carb
Native
Carb
Native
Carb
170
130
95
72
55
43
34
Coomassie
Westernblot
Ab neg RA
Westernblot
Ab pos RA

Fig. 1C

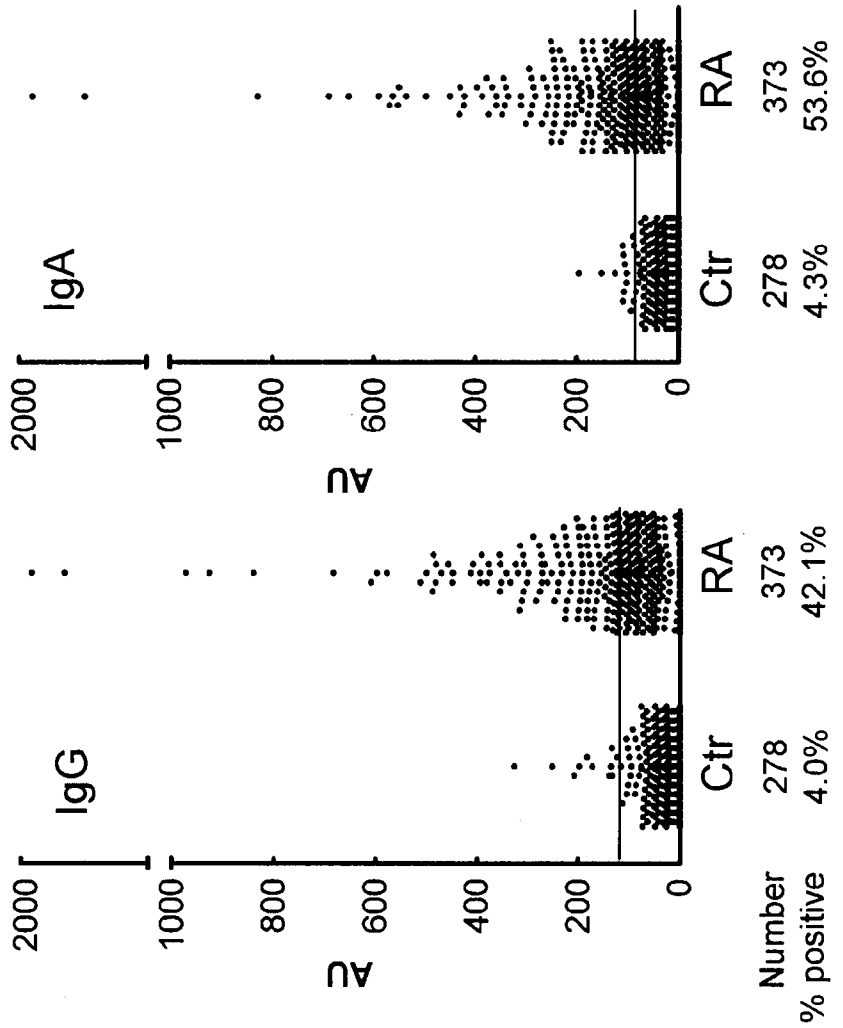
IgG
IgA
AU
2000
1000
800
600
400
200
0
Ctr
RA
Number
% positive
278
4.0%
373
42.1%
278
4.3%
373
53.6%

Fig. 2

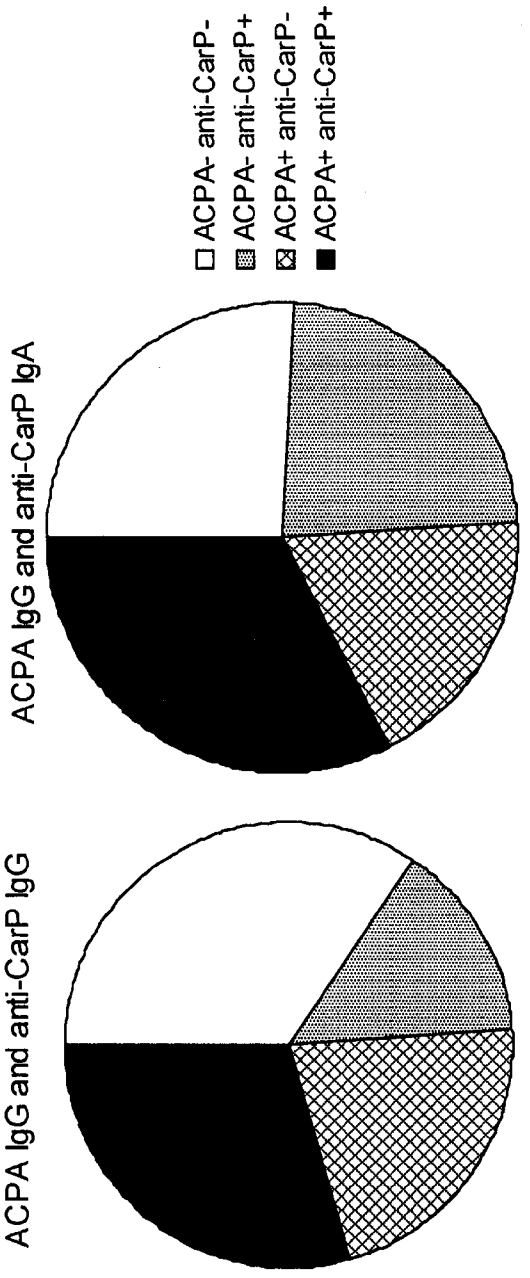
ACPA- anti-CarP-
ACPA- anti-CarP+
ACPA+ anti-CarP-
ACPA+ anti-CarP+
ACPA IgG and anti-CarP IgA
ACPA IgG and anti-CarP IgG

Fig. 3

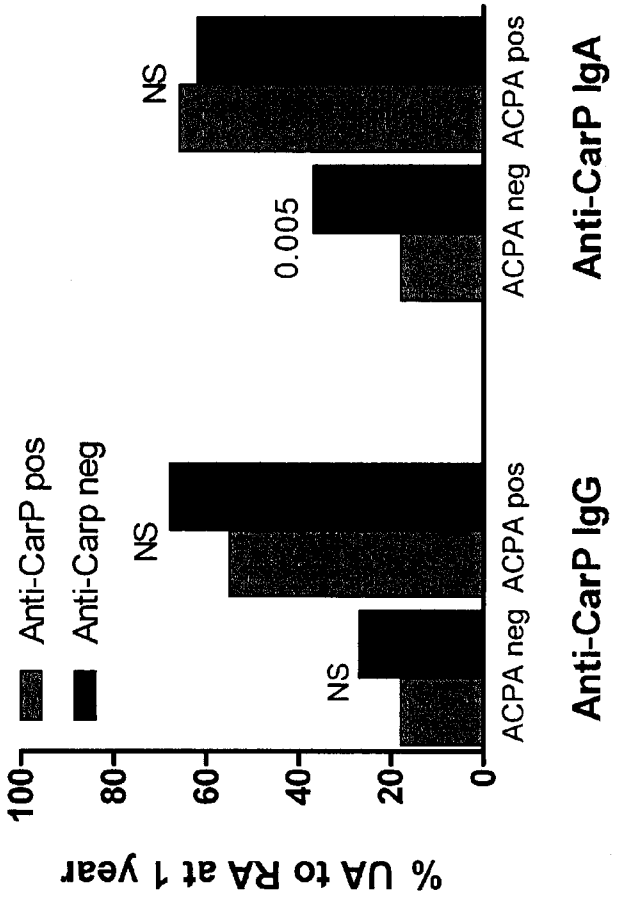
% UA to RA at 1 year
100
80
60
40
20
0
Anti-CarP pos
Anti-Carp neg
NS
NS
0.005
NS
ACPA neg
ACPA pos
ACPA neg
ACPA pos
Anti-CarP IgG
Anti-CarP IgA

Fig. 4

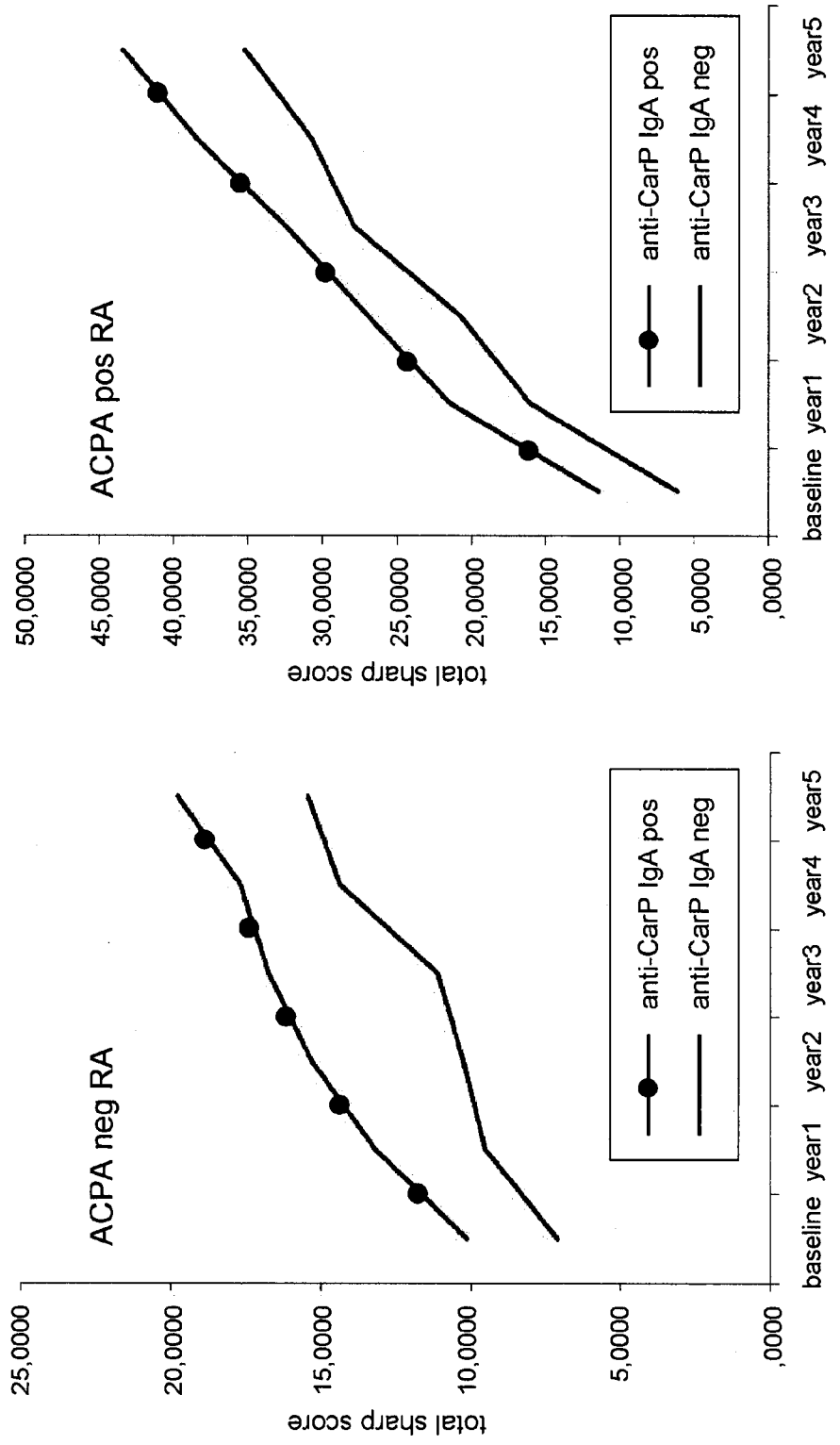
ACPA neg RA
ACPA pos RA
total sharp score
25,0000
20,0000
15,0000
10,0000
5,0000
,0000
50,0000
45,0000
40,0000
35,0000
30,0000
25,0000
20,0000
15,0000
10,0000
5,0000
,0000
baseline
year1
year2
year3
year4
year5
anti-CarP IgA pos
anti-CarP IgA neg

Fig. 5

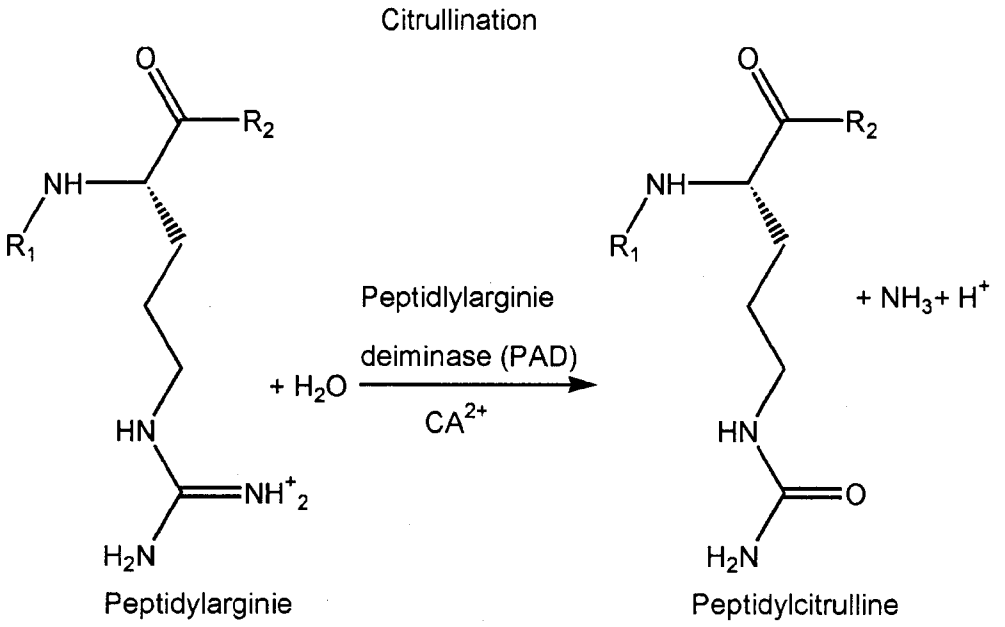
Citrullination
Peptidylarginie
deiminase (PAD)
+ H2O
CA2+
+ NH3+ H+
Peptidylarginie
Peptidylcitrulline

Fig. 6A

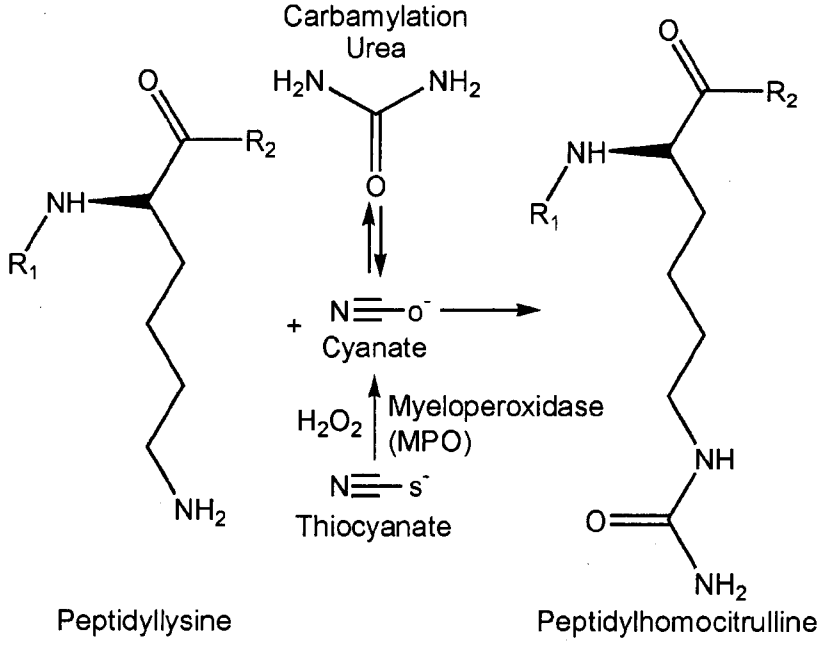
Carbamylation
Urea
Cyanate
Myeloperoxidase
(MPO)
H2O2
Thiocyanate
Peptidyllysine
Peptidylhomocitrulline

Fig. 6B

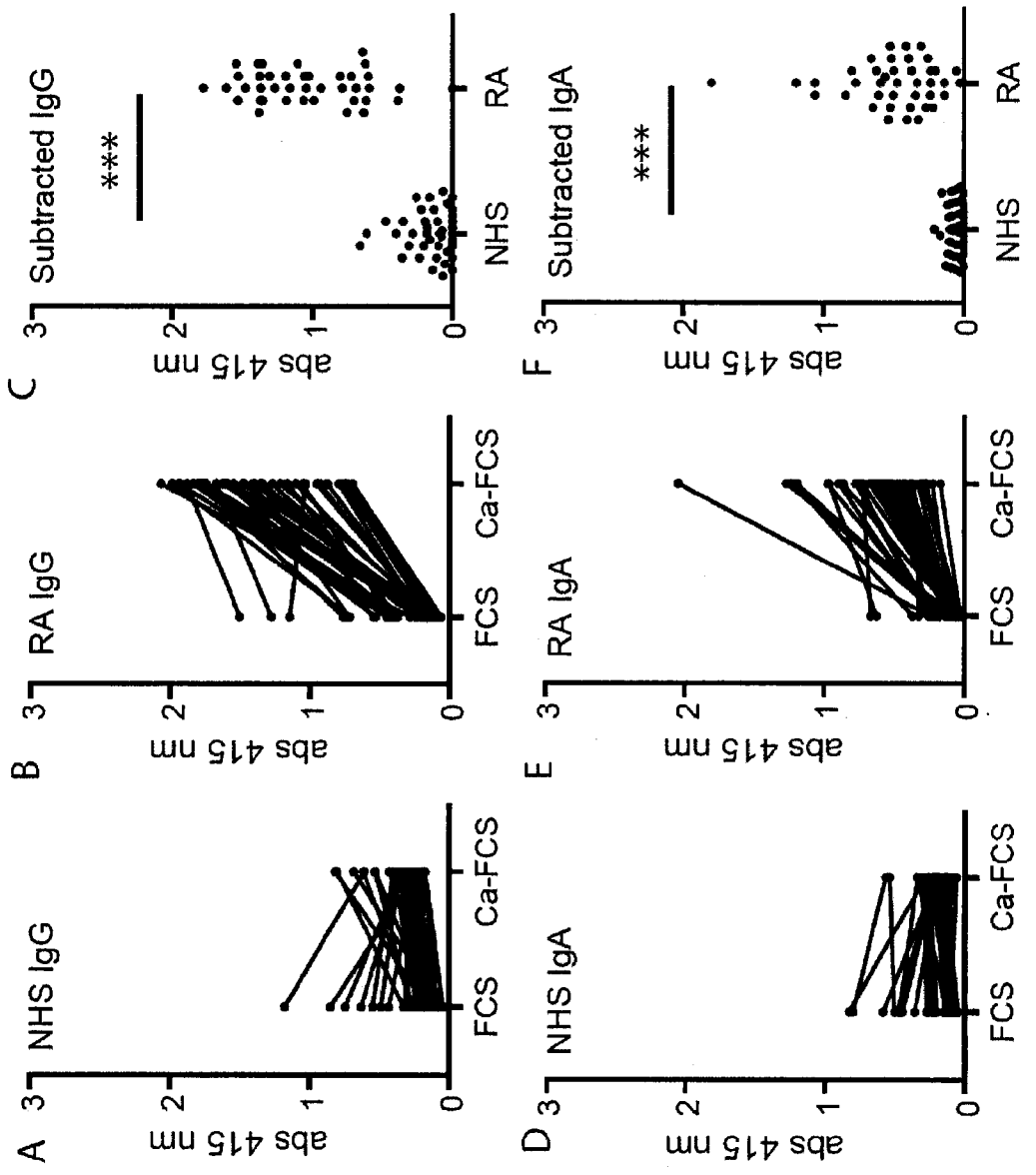
A
NHS IgG
abs 415 nm
FCS
Ca-FCS
B
RA IgG
C
Subtracted IgG
***
NHS
RA
D
NHS IgA
E
RA IgA
F
Subtracted IgA

Fig. 7

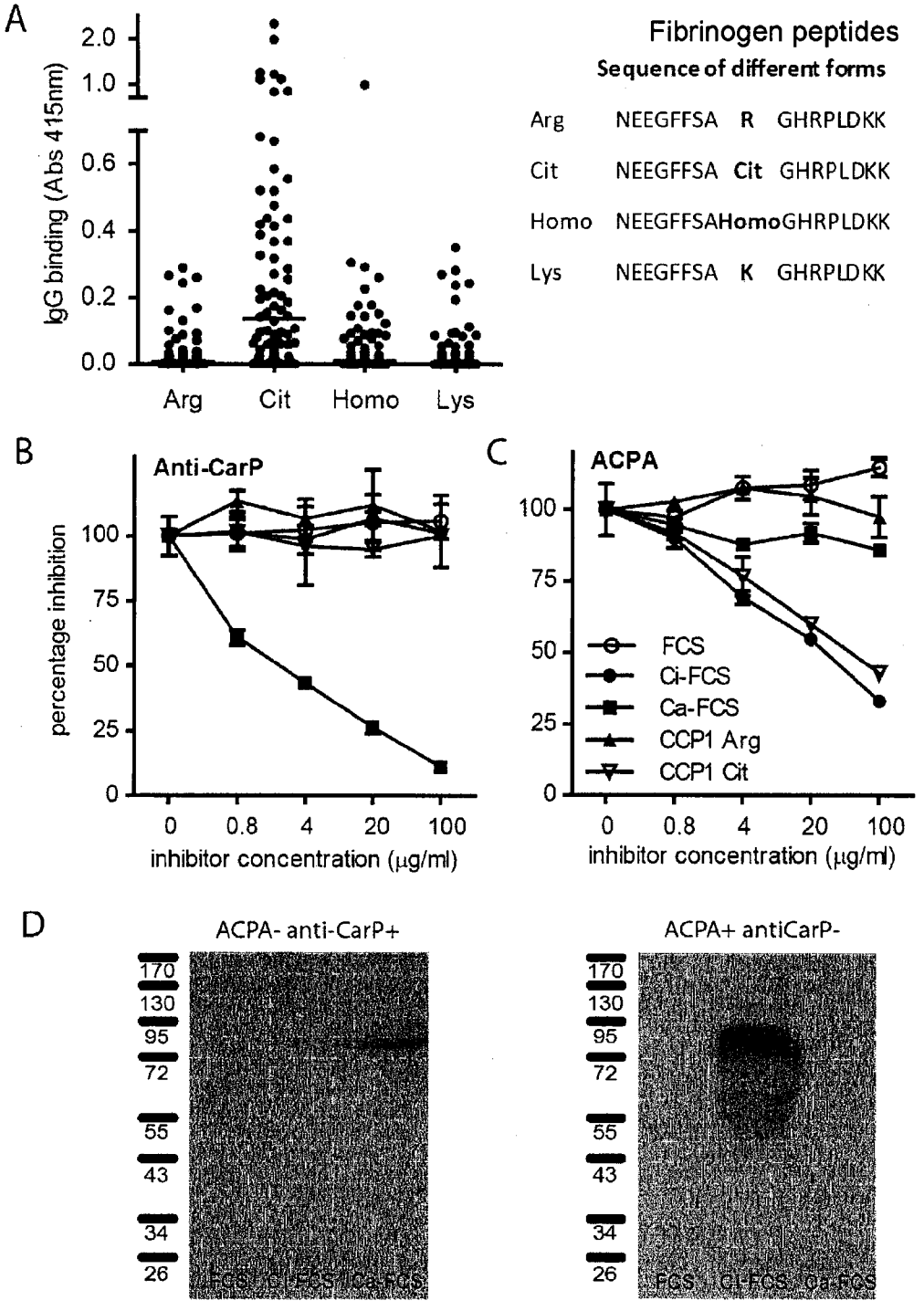
A
IgG binding (Abs 415nm)
2.0
1.0
0.6
0.4
0.2
0.0
Arg
Cit
Homo
Lys
Fibrinogen peptides
Sequence of different forms
Arg NEEGFFSA R GHRPLDKK
Cit NEEGFFSA Cit GHRPLDKK
Homo NEEGFFSAHomoGHRPLDKK
Lys NEEGFFSA K GHRPLDKK
B
Anti-CarP
percentage inhibition
100
75
50
25
0
0
0.8
4
20
100
inhibitor concentration (µg/ml)
C
ACPA
100
75
50
25
0
FCS
Ci-FCS
Ca-FCS
CCP1 Arg
CCP1 Cit
0
0.8
4
20
100
inhibitor concentration (µg/ml)
D
ACPA- anti-CarP+
170
130
95
72
55
43
34
26
FCS
Ci-FCS
Ca-FCS
ACPA+ antiCarP-
170
130
95
72
55
43
34
26
FCS
Ci-FCS
Ca-FCS

Fig. 8

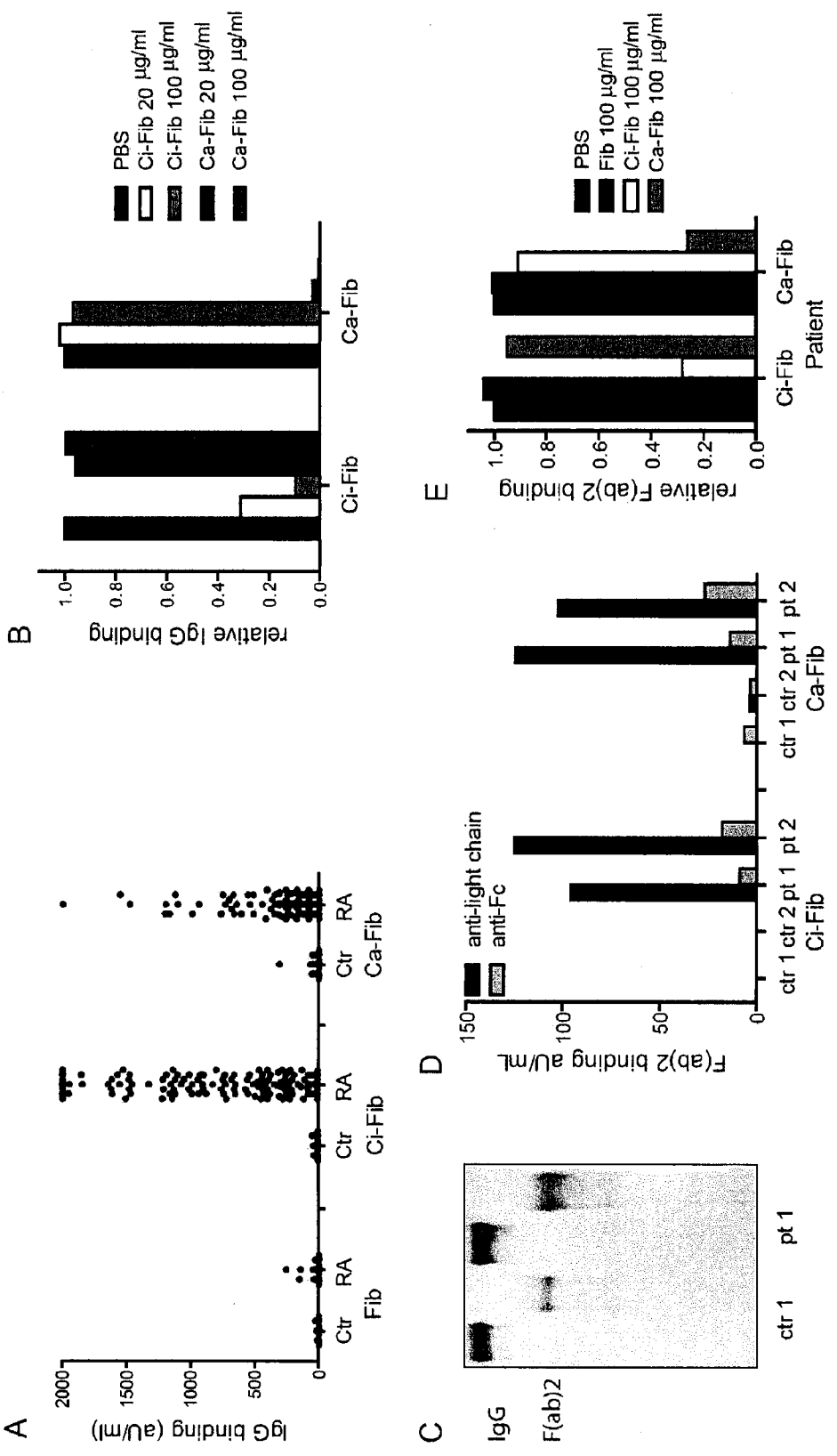
A
IgG binding (aU/ml)
2000
1500
1000
500
0
Ctr
RA
Ctr
RA
Fib
Ci-Fib
Ctr
RA
Ca-Fib
B
relative IgG binding
1.0
0.8
0.6
0.4
0.2
0.0
PBS
Ci-Fib 20 µg/ml
Ci-Fib 100 µg/ml
Ca-Fib 20 µg/ml
Ca-Fib 100 µg/ml
Ci-Fib
Ca-Fib
C
IgG
F(ab)2
ctr 1
pt 1
D
F(ab)2 binding aU/mL
150
100
50
0
anti-light chain
anti-Fc
ctr 1 ctr 2 pt 1 pt 2
Ci-Fib
ctr 1 ctr 2 pt 1 pt 2
Ca-Fib
E
relative F(ab)2 binding
1.0
0.8
0.6
0.4
0.2
0.0
PBS
Fib 100 µg/ml
Ci-Fib 100 µg/ml
Ca-Fib 100 µg/ml
Ci-Fib
Ca-Fib
Patient

Fig. 9

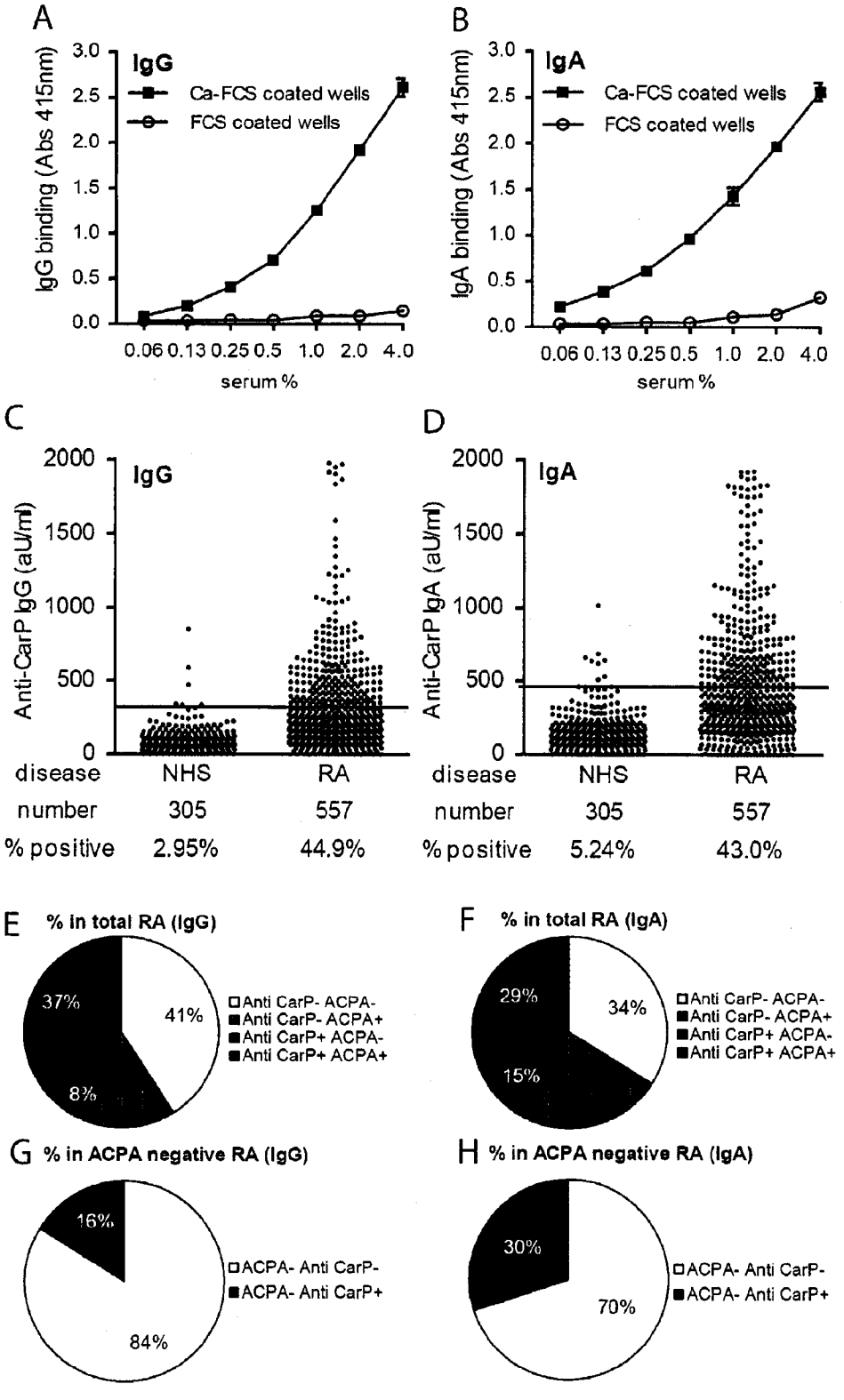
A
IgG
Ca-FCS coated wells
FCS coated wells
IgG binding (Abs 415nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.06 0.13 0.25 0.5 1.0 2.0 4.0
serum %
B
IgA
Ca-FCS coated wells
FCS coated wells
IgA binding (Abs 415nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.06 0.13 0.25 0.5 1.0 2.0 4.0
serum %
C
IgG
Anti-CarP IgG (aU/ml)
2000
1500
1000
500
0
disease NHS RA
number 305 557
% positive 2.95% 44.9%
D
IgA
Anti-CarP IgA (aU/ml)
2000
1500
1000
500
0
disease NHS RA
number 305 557
% positive 5.24% 43.0%
E % in total RA (IgG)
37%
41%
8%
Anti CarP- ACPA-
Anti CarP- ACPA+
Anti CarP+ ACPA-
Anti CarP+ ACPA+
F % in total RA (IgA)
29%
34%
15%
Anti CarP- ACPA-
Anti CarP- ACPA+
Anti CarP+ ACPA-
Anti CarP+ ACPA+
G % in ACPA negative RA (IgG)
16%
84%
ACPA- Anti CarP-
ACPA- Anti CarP+
H % in ACPA negative RA (IgA)
30%
70%
ACPA- Anti CarP-
ACPA- Anti CarP+

Fig. 10

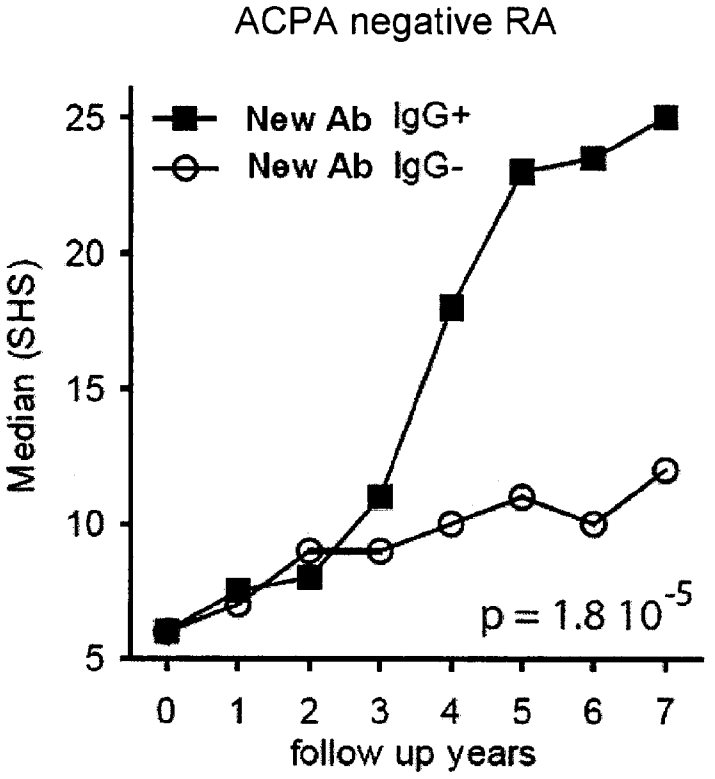
ACPA negative RA
New Ab IgG+
New Ab IgG-
Median (SHS)
25
20
15
10
5
p = 1.8 10-5
0 1 2 3 4 5 6 7
follow up years

Fig. 11

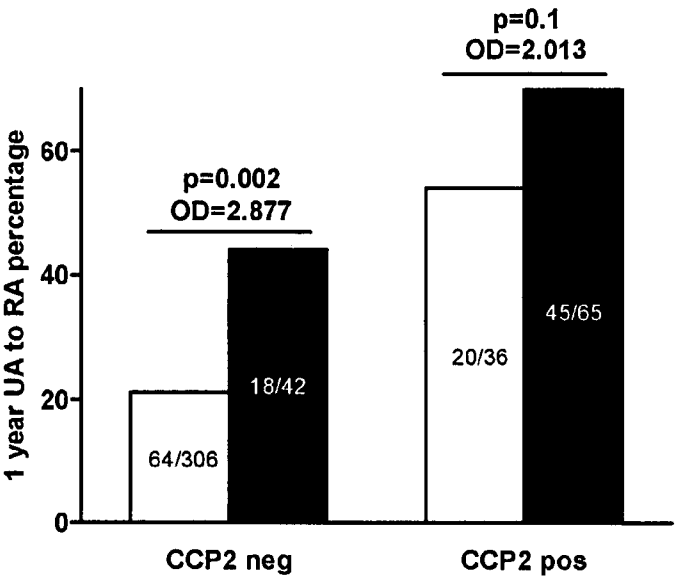

Fig. 12A

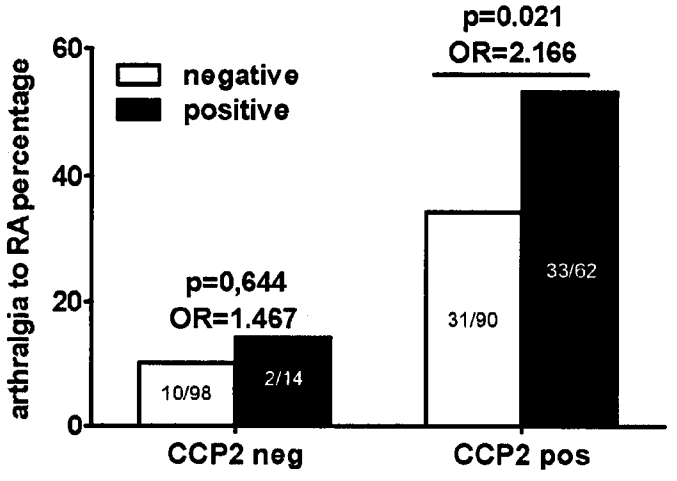

Fig. 12B

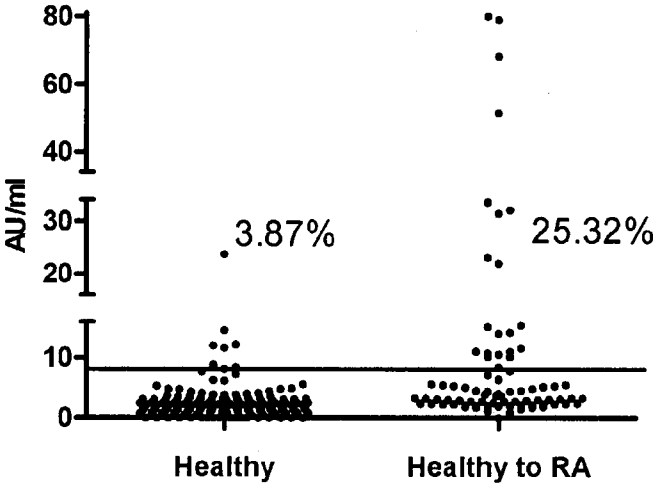

Fig. 12C

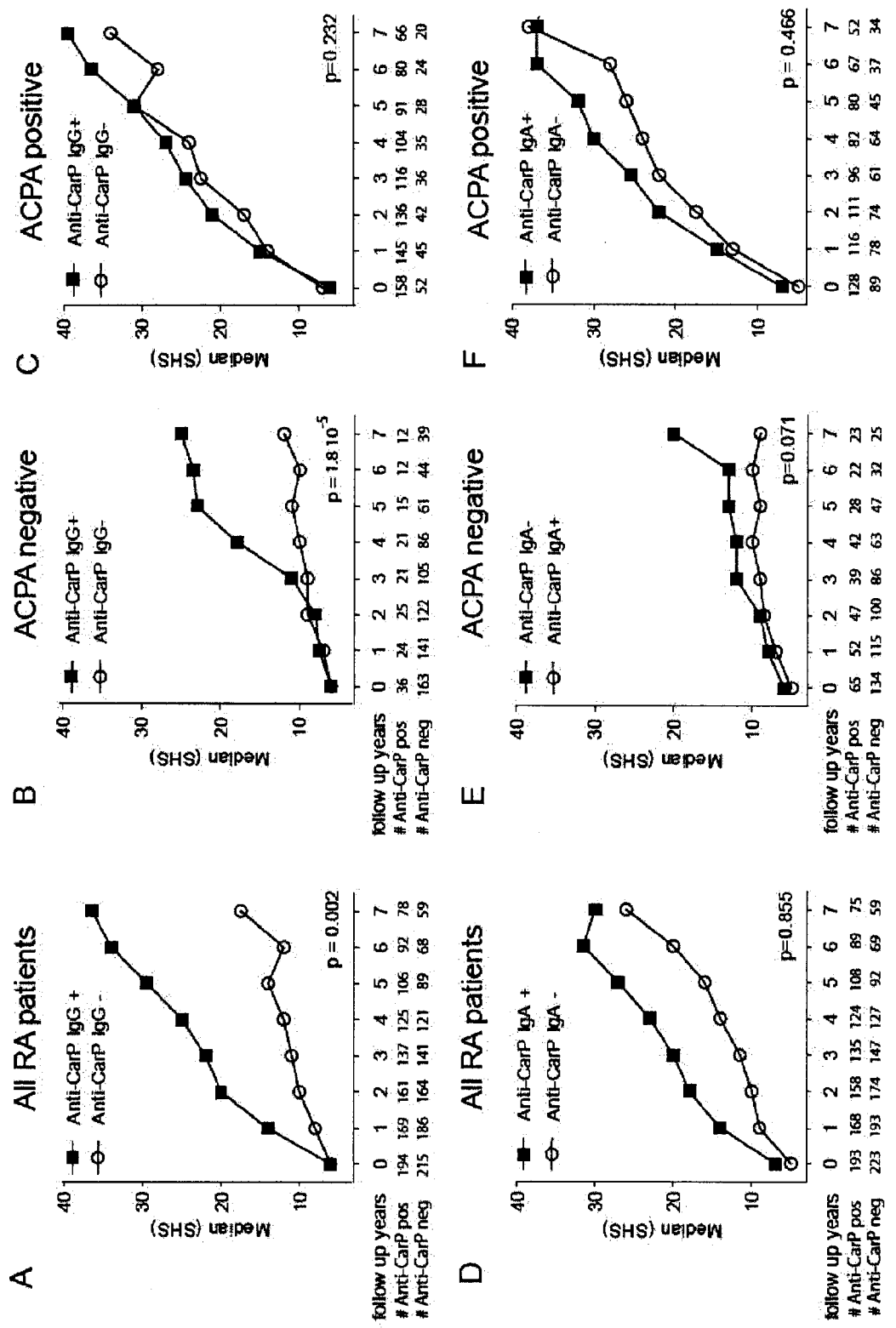
A
All RA patients
Anti-CarP IgG +
Anti-CarP IgG -
p = 0.002
Median (SHS)
follow up years 0 1 2 3 4 5 6 7
Anti-CarP pos 194 169 161 137 125 106 92 78
Anti-CarP neg 215 186 164 141 121 89 68 59
B
ACPA negative
Anti-CarP IgG+
Anti-CarP IgG-
p = 1.8 10-5
Median (SHS)
follow up years 0 1 2 3 4 5 6 7
Anti-CarP pos 36 24 25 21 21 15 12 12
Anti-CarP neg 163 141 122 105 86 61 44 39
C
ACPA positive
Anti-CarP IgG+
Anti-CarP IgG-
p=0.232
Median (SHS)
0 1 2 3 4 5 6 7
158 145 136 116 104 91 80 66
52 45 42 36 35 28 24 20
D
All RA patients
Anti-CarP IgA +
Anti-CarP IgA -
p=0.855
Median (SHS)
follow up years 0 1 2 3 4 5 6 7
Anti-CarP pos 193 168 158 135 124 108 89 75
Anti-CarP neg 223 193 174 147 127 92 69 59
E
ACPA negative
Anti-CarP IgA-
Anti-CarP IgA+
p=0.071
Median (SHS)
follow up years 0 1 2 3 4 5 6 7
Anti-CarP pos 65 52 47 39 42 28 22 23
Anti-CarP neg 134 115 100 86 63 47 32 25
F
ACPA positive
Anti-CarP IgA+
Anti-CarP IgA-
p = 0.466
Median (SHS)
0 1 2 3 4 5 6 7
128 116 111 96 82 80 67 52
89 78 74 61 64 45 37 34

Fig. 13

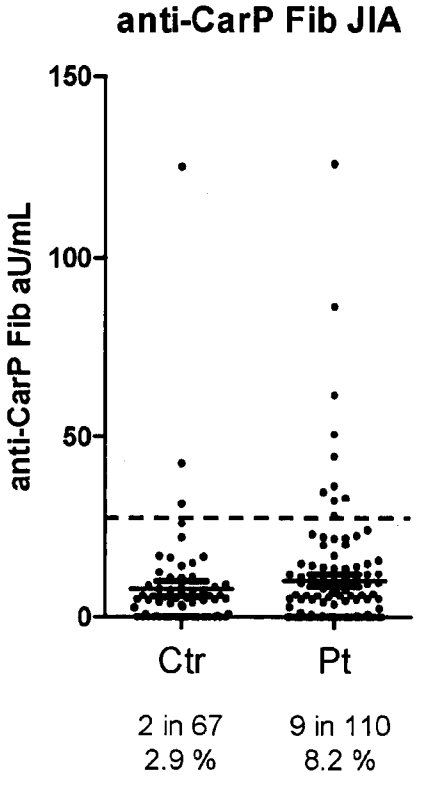
anti-CarP Fib JIA
anti-CarP Fib aU/mL
150
100
50
0
Ctr
Pt
2 in 67
2.9 %
9 in 110
8.2 %

Fig. 14