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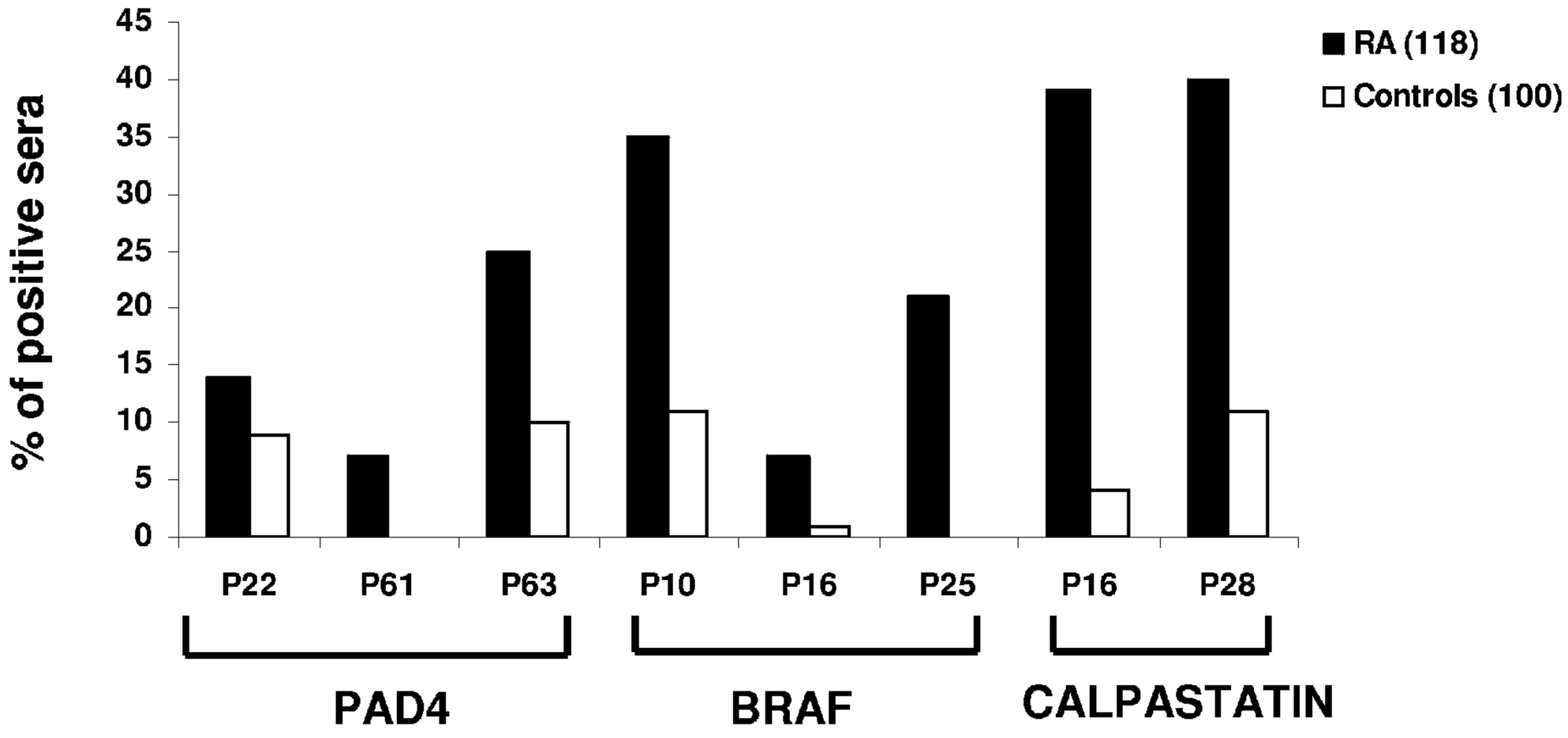

Figure 9

(57) Abrégé/Abstract:
The present invention relates to peptides biomarkers that are specifically recognized by autoantibodies present in the sera of patients with Rheumatoid Arthritis (RA). More specifically, the invention provides epitopes of PAD4, of BRAF, and of calpastatin as well as methods and kits for using these sequences for the diagnosis of RA, in particular for the diagnosis of RA in CCP-negative subjects.

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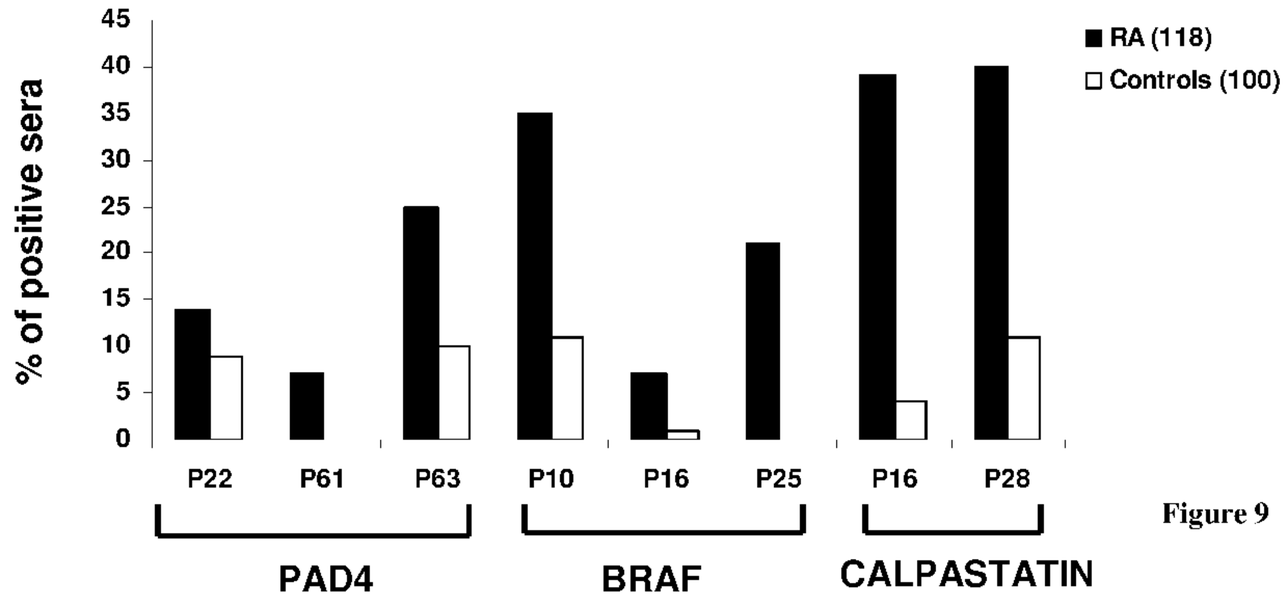

Figure 9

(57) Abstract: The present invention relates to peptides biomarkers that are specifically recognized by autoantibodies present in the sera of patients with Rheumatoid Arthritis (RA). More specifically, the invention provides epitopes of PAD4, of BRAF, and of calpastatin as well as methods and kits for using these sequences for the diagnosis of RA, in particular for the diagnosis of RA in CCP-negative subjects.

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Biomarkers, Methods and Kits for the Diagnosis of Rheumatoid Arthritis

Related Application

The present application claims priority to European Patent Application No. EP 09 305 266.1 filed on March 30, 2009 and European Patent Application No. EP 09 306 063.0 filed on November 6, 2009. The European patent applications are incorporated herein by reference in its entirety.

Background of the Invention

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the world's population. It is characterized by inflammation and cellular proliferation in the synovial lining of joints that can ultimately result in cartilage and bone destruction, joint deformity and loss of mobility. RA usually causes problems in several joints at the same time, often in a symmetric manner. Early RA tends to affect the smaller joints first, such as the joints in the wrists, hands, ankles and feet. As the disease progresses, joints of the shoulders, elbows, knees, hips, jaw and neck can also become involved. Unlike other arthritic conditions that only affect areas in or around joints, RA is a systemic disease which can cause inflammation in extra-articular tissues throughout the body including the skin, blood vessels, heart, lungs and muscles.

RA is associated with pain, deformity, decreased quality of life, and disability, which in turn affect patients' ability to lead a normal and productive life. Recent studies have shown that 5 years after the onset of the disease, approximately one third of patients with RA are no longer able to work, and within 10 years, half of the patients have substantial functional disability (A. Young *et al.*, Rheumatology, 2007, 46: 350-357). Consequently, RA imposes an important economic burden on society. Considerable data also suggest that RA is associated with lowered life expectancy.

Although RA has been extensively studied, the etiology and pathogenesis of the disease remain incompletely understood. Factors that may increase the risk for RA include: sex of the individual (women are 2 to 3 times more likely than men to develop the disease); age (RA occurs more commonly between the ages of 40 and 60, although it can also strike children, teenagers and older adults); genetics (RA was found to be strongly associated with the inherited tissue type Major Histocompatibility

Complex (MHC) antigen HLA-DR4 – more specifically DRB1*0401 and DRB1*0404); and smoking (RA is about 4 times more common in smokers than non-smokers).

There is currently no reliable cure for RA. Treatment is essentially directed towards relieving pain, reducing inflammation, and stopping or slowing joint damage and bone destruction. The current therapeutic approach is to prescribe disease-modifying antirheumatic drugs (DMARDs) early in the condition, as RA patients treated early with such drugs have better outcomes, with greater preservation of function, less work disability, and smaller risk of premature death. Recent advances in the understanding of the pathophysiology of RA have led to the development of new DMARDs, called biological response modifiers. Biological DMARDs are designed to target and block the action of certain key cells or molecules, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), T-cells, and B-cells, involved in the abnormal immune reaction associated with RA. In comparison with traditional DMARDs, the biological agents have a much more rapid onset of action and can offer better clinical response with effective long-term prevention of joint damage (J.K.D. de Vries-Bouwstra *et al.*, Rheum. Dis. Clin. North Am., 2005, 31: 745-762).

Since irreversible joint destruction can be prevented by intervention at the early stages of the disease, early diagnosis of RA is important. However, definitive diagnosis of RA can be difficult. Immunologic tests that can be performed for the diagnosis of RA include, in particular, measurement of the levels of rheumatoid factor (RF), and anti-cyclic citrullinated peptide (anti-CCP) antibodies. Serological testing for RF is complicated by moderate sensitivity and specificity, and high rates of positivity in other chronic inflammatory and infectious diseases (T. Dorner *et al.*, Curr. Opin. Rheumatol, 2004, 16: 246-253). Anti-CCP antibody testing is particularly useful in the diagnosis of RA, with high specificity, positivity early in the disease process, and ability to identify patients who are likely to have severe disease and irreversible damage. However, a negative result in anti-CCP antibody testing does not exclude RA.

Therefore, there is a great need for new biological markers of RA. In particular, biomarkers that would allow reliable diagnosis and monitoring of the early stages of

the disease and permit early intervention to potentially prevent pain, joint destruction and long-term disability, are highly desirable.

Summary of the Invention

The present invention generally relates to improved systems and strategies for the diagnosis of rheumatoid arthritis (RA). In particular, the invention relates to peptide biomarkers that specifically react with autoantibodies present in the sera of RA patients. More specifically, the present invention concerns amino acid sequences of human PAD4, BRAF and calpastatin, and methods for using such sequences in the diagnosis of RA, in particular in the diagnosis of RA in CCP-negative patients.

Thus, in one aspect, the present invention provides an *in vitro* method for diagnosing RA in a subject, said method comprising steps of: contacting a biological sample obtained from the subject with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form; and detecting any biomarker-antibody complex formed.

In this method of diagnosis, the at least one biomarker is selected from the group consisting of:

PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof,

calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and

any combination thereof; and

In certain embodiments, the at least one biomarker is selected from the group consisting of:

PAD4-peptides having an amino acid sequence consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof,

calpastatin-peptides having an amino acid sequence consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and

any combination thereof.

In certain embodiments, a plurality of peptide biomarkers (*i.e.*, two or more than two peptide biomarkers) is used in the method of diagnosis. In other words, the method of the invention may comprise steps of: contacting the biological sample with a plurality of biomarkers for a time and under conditions allowing biomarker-antibody complexes to form between one or more biomarkers and autoantibodies present in the biological sample; and detecting any biomarker-antibody complex formed.

In certain embodiments, the method of diagnosis is performed using eight peptide biomarkers including three PAD4-peptides, three BRAF-peptides and two calpastatin-peptides as described herein. In certain preferred embodiments, the three PAD4-peptides consist of SEQ ID NOs: 2-4, the three BRAF-peptides consist of SEQ ID NOs: 7-9, and the two calpastatin-peptides consist of SEQ ID NOs: 11-12.

In another aspect, the invention provides a method for detecting the presence of anti-PAD4 autoantibodies in a biological sample. The method comprises steps of: contacting the biological sample with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form, wherein the at least one biomarker is a PAD4 peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, fragments thereof, and any combination thereof; and detecting any biomarker-antibody complex formed. The detection of a biomarker-antibody complex is indicative of the presence of anti-PAD4 autoantibodies in the biological sample, and therefore of RA in the subject from whom the biological sample has been obtained.

The invention also provides a method for detecting the presence of anti-BRAF autoantibodies in a biological sample. The method comprises steps of: contacting the biological sample with at least one biomarker for a time and under conditions

allowing a biomarker-antibody complex to form, wherein the at least one biomarker is a BRAF-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, fragments thereof, and any combination thereof; and detecting any biomarker-antibody complex formed. The detection of a biomarker-antibody complex is indicative of the presence of anti-BRAF autoantibodies in the biological sample, and therefore of RA in the subject from whom the biological sample has been obtained.

A biological sample, obtained from the subject to be tested and suitable for use in a method of the present invention, may be selected from the group consisting of whole blood, serum, plasma, urine, and synovial fluid. In certain preferred embodiments, the biological sample is a serologic sample, *e.g.*, a blood sample. A blood sample, obtained from the subject and suitable for use in a method of diagnosis of the present invention, may be selected from the group consisting of whole blood, plasma, and serum.

In certain embodiments, the subject to be tested is CCP-negative or the biological sample to be tested is obtained from a CCP-negative subject.

In the methods of diagnosis provided herein, the step of detecting any biomarker-antibody complex formed between a peptide biomarker and an autoantibody present in the biological sample may be performed by any suitable method. In certain embodiments, the detection is by immunoassay.

In certain embodiments, the peptide biomarker or biomarkers used in the diagnosis method is/are immobilized on a solid carrier or support.

In certain embodiments, the methods of diagnosis may further comprise measuring, in a biological sample obtained from the subject, the concentration of at least one marker selected from the group consisting of C-reactive protein, serum amyloid A, interleukin 6, S100 proteins, osteopontin, rheumatoid factor, matrix metalloprotease 1, matrix metalloprotease 3, hyaluronic acid, sCD14, angiogenesis markers, and products of bone, cartilage and synovium metabolism.

In another aspect, the present invention provides kits for the *in vitro* diagnosis of RA in a subject. These kits comprise: at least one peptide biomarker of the invention

and at least one reagent for detecting a biomarker-antibody complex formed between the peptide biomarker and an autoantibody present in the biological sample to be tested. In the kits, the at least one peptide biomarker may be immobilized on a solid carrier or support, or alternatively, reagents may be included in the kit that can be used to immobilize the peptide biomarker on a solid carrier or support. The kits may further comprise instructions for carrying out a method of diagnosis according to the present invention. In certain embodiments, the kit comprises at least eight biomarkers including three PAD4-peptides, three BRAF-peptides and two calpastatin-peptides as described herein. In certain preferred embodiments, the three PAD4-peptides consist of SEQ ID NOs: 2-4, the three BRAF-peptides consist of SEQ ID NOs: 7-9, and the two calpastatin-peptides consist of SEQ ID NOs: 11-12.

In yet another aspect, the present invention provides arrays for the diagnosis of RA in a subject. An array according to the invention comprises, attached to its surface, at least one peptide biomarker of the invention. In particular, the invention provides an array for the diagnosis of RA in CCP-negative subjects, the array comprising, attached to its surface, at least eight peptide biomarkers including three PAD4-peptides, three BRAF-peptides and two calpastatin-peptides described herein. In certain preferred embodiments, the three PAD4-pepides consist of SEQ ID NOs: 2-4, the three BRAF-peptides consist of SEQ ID NOs: 7-9, and the two calpastatin-peptides consist of SEQ ID NOs: 11-12. In certain embodiments, an inventive array further comprises, attached to its surface, at least one additional RA biomarker for detecting the presence of RA-specific autoantibodies, such as antinuclear antibodies and anti-CCP antibodies.

In still another aspect, the present invention provides peptide biomarkers of rheumatoid arthritis, preferably comprising less than 50 amino acid residues. In particular, the invention provides peptide biomarkers of less than 50 amino acid residues that are recognized by anti-PAD4 autoantibodies and which have an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, and fragments thereof. The invention further provides peptide biomarkers of less than 50 amino acid residues that are recognized by anti-BRAF autoantibodies and which have an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ

ID NO: 9, analogues thereof, and fragments thereof. The invention also provides peptide biomarkers of less than 50 amino acid residues that are recognized by anti-calpastatin autoantibodies and which have an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and wherein the biomarker is recognized by an anti-calpastatin autoantibody.

These and other objects, advantages and features of the present invention will become apparent to those of ordinary skill in the art having read the following detailed description of the preferred embodiments.

Brief Description of the Drawing

Figure 1 is a graph showing that autoantibodies to PAD4 present in the serum of RA patients inhibit the citrullination of fibrinogen. The test was performed as described in the Examples section. A ratio of OD (test)/OD (control) > 1 indicates an activation of the citrullination of fibrinogen by PAD4, while a ratio < 1 indicates an inhibition of the citrullination of fibrinogen by PAD4.

Figure 2 is a graph showing, for each group of sera from RA patients, AS patients and healthy controls, the percentage of sera that recognize peptide **22**, peptide **28**, peptide **61** and peptide **63**. Positive sera were defined as sera for which an OD value of more than twice the background was measured (see Examples section for experimental details).

Figure 3 is a graph showing, for each group of anti-PAD4 autoantibodies that inhibit citrullination of fibrinogen (patients RA1 to RA20), that activate citrullination of fibrinogen (patients RA24 to RA29) and that have no effect on citrullination of fibrinogen (patients RA21 to RA23), the percentage of autoantibodies that recognize N terminal peptides only, C terminal peptides only, or both N terminal peptides and C terminal peptides.

Figure 4 is a graph showing, for each group of autoantibodies to PAD4 that recognize peptide **22**, peptide **28**, peptide **61** and peptide **63**, the percentage of autoantibodies that inhibit, activate or have no effect on citrullination of fibrinogen.

Figure 5 is a scheme presenting a model that explains how autoantibodies to PAD4 could inhibit PAD4. (**A**) Calcium binding could open the substrate binding domain of PAD4. (**B**) The substrate could then bind to PAD4 and be citrullinated. (**C**) Autoantibodies to PAD4 may interfere with conformation change or substrate binding.

Figure 6 is a graph showing the binding of BRAF-peptides to autoantibodies to BRAF. BRAF-peptides, **P10**, **P16**, **P25** and **P33** were tested by ELISA in the sera of 21 RA patients, 33 SPA patients, and 60 healthy volunteers. After washing, peroxydase conjugated anti human IgG was added, and optical density was read at 405 nm. Background OD was obtained by adding each serum to a well without peptide. Positive sera were defined by OD value higher than twice background OD.

Figure 7 is a table showing that BRAF-peptides (P10, P16 and P25) identify RA in 50% of CCP-negative patients. Grey indicates a positive peptide (*i.e.*, a BRAF peptide that undergoes binding to autoantibodies to BRAF present in the sera of CCP-negative patients). The binding was determined by ELISA as described in Example II.

Figure 8 is a graph showing that autoantibodies to BRAF present in the serum of RA patients activate the phosphorylation of MEK1. Purified anti-BRAF autoantibodies from patients were incubated with BRAF and MEK1. For each patient, one positive control (BRAF and MEK1) and one negative control (BRAF and MEK1 and C1 control antibody) were included. Activation or inhibition of MEK1 phosphorylation was detected by measuring the ratio test OD/positive control OD. Ratio > 1 indicated activation, ratio <1 indicated inhibition.

Figure 9 is a graph showing the binding of PAD4-, BRAF- and Calpastatin-peptides to autoantibodies present in the serum of RA patients. PAD4-peptides (peptides **22**, **61** and **63**), BRAF-peptides (**P10**, **P16** and **P25**) and Calpastatin-peptides (**P'16** and **P'28**) were contacted with the sera of 118 RA patients and of 100 control sera. Binding was determined and assessed as described in Example II.

Figure 10 is a graph showing that PAD4-peptides (peptides **22**, **61** and **63**), BRAF-peptides (**P10**, **P16** and **P25**) and Calpastatin-peptides (**P'16** and **P'28**) are recognized by CCP-negative patients. These peptides were contacted with the sera of

83 CCP-negative RA patients and 29 CCP-negative RA patients. Binding was determined and assessed as described in Example II.

Definitions

Throughout the specification, several terms are employed that are defined in the following paragraphs.

As used herein, the term "subject" refers to a human or another mammal (*e.g.*, primate, dog, cat, goat, horse, pig, mouse, rat, rabbit, and the like), that can be afflicted with RA, but may or may not have the disease. In many embodiments of the present invention, the subject is a human being. In such embodiments, the subject is often referred to as an "individual". The term "individual" does not denote a particular age, and thus encompasses children, teenagers, and adults.

The term "subject suspected of having RA" refers to a subject that presents one or more symptoms indicative of RA (*e.g.*, pain, stiffness or swelling of joints), or that is screened for RA (*e.g.*, during a physical examination). Alternatively or additionally, a subject suspected of having RA may have one or more risk factors (*e.g.*, age, sex, family history, smoking, *etc*). The term encompasses subjects that have not been tested for RA as well as subjects that have received an initial diagnosis.

The term "biological sample" is used herein in its broadest sense. A biological sample is generally obtained from a subject. A sample may be of any biological tissue or fluid with which biomarkers of the present invention may be assayed. Frequently, a sample will be a "clinical sample", *i.e.*, a sample derived from a patient. Such samples include, but are not limited to, bodily fluids which may or may not contain cells, *e.g.*, blood (*e.g.*, whole blood, serum or plasma), urine, synovial fluid, saliva, tissue or fine needle biopsy samples, and archival samples with known diagnosis, treatment and/or outcome history. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. The term "biological sample" also encompasses any material derived by processing a biological sample. Derived materials include, but are not limited to, cells (or their progeny) isolated from the sample, or proteins extracted from the sample. Processing of a biological sample may involve one or more of: filtration, distillation, extraction, concentration, inactivation of interfering components, addition of reagents, and the like. In certain

preferred embodiments of the invention, the biological sample is a serologic sample and is (or is derived from) whole blood, serum or plasma obtained from a subject.

The terms "normal" and "healthy" are used herein interchangeably. They refer to a subject that has not shown any RA symptoms, and that has not been diagnosed with RA or with cartilage or bone injury. Preferably, a normal subject is not on medication affecting RA and has not been diagnosed with any other disease (in particular an autoimmune inflammatory disease). In certain embodiments, normal subjects may have similar sex, age, and/or body mass index as compared with the subject from which the biological sample to be tested was obtained. The term "normal" is also used herein to qualify a sample obtained from a healthy subject.

In the context of the present invention, the term "control", when used to characterize a subject, refers to a subject that is healthy or to a patient who has been diagnosed with a specific disease other than RA. The term "control sample" refers to one, or more than one, sample that has been obtained from a healthy subject or from a patient diagnosed with a disease other than RA.

The term "autoantibody", as used herein, has its art understood meaning, and refers to an antibody that is produced by the immune system of a subject and that is directed against one or more of the subject's own proteins. Autoantibodies may attack the body's own cells, tissues, and/or organs, causing inflammation and damage.

As used herein, the term "autoantigen" refers to an endogenous antigen, or an active fragment thereof, that stimulates the production of autoantibodies in a subject's body, as in autoimmune reactions. The term also encompasses any substances that can form an antigen-antibody complex with autoantibodies present in a subject or in a biological sample obtained from a subject.

The terms "biomarker" and "marker" are used herein interchangeably. They refer to a substance that is a distinctive indicator of a biological process, biological event, and/or pathologic condition. In the context of the present invention, the term "biomarker of RA" encompasses BRAF-peptides, PAD4-peptides and calpastatin-peptides provided herein which are specifically recognized by anti-PAD4 autoantibodies present in a biological sample (*e.g.*, blood sample) of a RA patient. In certain preferred embodiments, the biomarkers of the invention are peptides of less than 50 amino acids.

As used herein, the term "indicative of RA", when applied to a process or event, refers to a process or event which is diagnostic of RA, such that the process or event is found significantly more often in subjects with RA than in healthy subjects and/or in subjects suffering from a disease other than RA.

The terms "protein", "polypeptide", and "peptide" are used herein interchangeably, and refer to amino acid sequences of a variety of lengths, either in their neutral (uncharged) forms or as salts, and either unmodified or modified by glycosylation, side chain oxidation, or phosphorylation. In certain embodiments, the amino acid sequence is a full-length native protein. In other embodiments, the amino acid sequence is a smaller fragment of the full-length protein. In still other embodiments, the amino acid sequence is modified by additional substituents attached to the amino acid side chains, such as glycosyl units, lipids, or inorganic ions such as phosphates, as well as modifications relating to chemical conversion of the chains such as oxidation of sulfhydryl groups. Thus, the term "protein" (or its equivalent terms) is intended to include the amino acid sequence of the full-length native protein, or a fragment thereof, subject to those modifications that do not significantly change its specific properties. In particular, the term "protein" encompasses protein isoforms, *i.e.*, variants that are encoded by the same gene, but that differ in their pI or MW, or both. Such isoforms can differ in their amino acid sequence (*e.g.*, as a result of alternative splicing or limited proteolysis), or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation).

The term "analog", when used herein in reference to a protein or polypeptide, refers to a peptide that possesses a similar or identical function as the protein or polypeptide but need not necessarily comprise an amino acid sequence that is similar or identical to the amino acid sequence of the protein or polypeptide or a structure that is similar or identical to that of the protein or polypeptide. Preferably, in the context of the present invention, an analog has an amino acid sequence that is at least 30%, more preferably, at least about: 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%, identical to the amino acid sequence of the protein or polypeptide. In certain preferred embodiments, an analog of a peptide biomarker of the invention has an amino acid sequence that is at least 80% identical or at least 85% identical to the amino acid sequence of the peptide biomarker.

The term "fragment", when used herein in reference to a protein or polypeptide, refers to a peptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, of at least about: 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, or 250 amino acid residues) of the amino acid sequence of a protein or polypeptide. The fragment of a protein or polypeptide may or may not possess a functional activity of the protein or polypeptide. In certain preferred embodiments of the present invention, a fragment of a peptide biomarker of the invention comprises an amino acid sequence of at least 10 consecutive amino acid residues of the amino acid sequence of the peptide biomarker.

The term "homologous" (or "homology"), as used herein, is synonymous with the term "identity" and refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecule. When a position in both compared sequences is occupied by the same base or same amino acid residue, then the respective molecules are homologous at that position. The percentage of homology between two sequences corresponds to the number of matching or homologous positions shared by the two sequences divided by the number of positions compared and multiplied by 100. Generally, a comparison is made when two sequences are aligned to give maximum homology. Homologous amino acid sequences share identical or similar amino acid sequences. Similar residues are conservative substitutions for, or "allowed point mutations" of, corresponding amino acid residues in a reference sequence. "Conservative substitutions" of a residue in a reference sequence are substitutions that are physically or functionally similar to the corresponding reference residue, *e.g.*, that have a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the like. Particularly preferred conservative substitutions are those fulfilling the criteria defined for an "accepted point mutation" by Dayhoff *et al.* ("Atlas of Protein Sequence and Structure", 1978, Nat. Biomed. Res. Foundation, Washington, DC, Suppl. 3, 22: 354-352).

The terms "labeled", "labeled with a detectable agent" and "labeled with a detectable moiety" are used herein interchangeably. These terms are used to specify that an entity (*e.g.*, a PAD4-peptide, a BRAF-peptide or a calpastatin-peptide) can be visualized, for example, following binding to another entity (*e.g.*, an anti-PAD4 autoantibody, an anti-BRAF autoantibody or an anti-calpastatin-autoantibody).

Preferably, a detectable agent or moiety is selected such that it generates a signal which can be measured and whose intensity is related to the amount of bound entity. In array-based methods, a detectable agent or moiety is also preferably selected such that it generates a localized signal, thereby allowing spatial resolution of the signal from each spot on the array. Methods for labeling proteins and polypeptides are well-known in the art. Labeled polypeptides can be prepared by incorporation of or conjugation to a label, that is directly or indirectly detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means, or any other suitable means Suitable detectable agents include, but are not limited to, various ligands, radionuclides, fluorescent dyes, chemiluminescent agents, microparticles, enzymes, colorimetric labels, magnetic labels, and haptens.

The terms "protein array" and "protein chip" are used herein interchangeably. They refer to a substrate surface on which different proteins or polypeptides have been immobilized, in an ordered manner, at discrete spots on the substrate. Protein arrays may be used to identify protein/protein interactions (*e.g.*, antigen/antibody interactions), to identify the substrates of enzymes, or to identify the targets of biologically active small molecules. The term "microarray" more specifically refers to an array that is miniaturized so as to require microscopic examination for visual evaluation.

The term "treatment " is used herein to characterize a method that is aimed at (1) delaying or preventing the onset of a disease or condition (*e.g.*, RA); (2) slowing down or stopping the progression, aggravating, or deteriorations of the symptoms of the condition; (3) bringing about ameliorations or the symptoms of the condition; and/or (4) curing the condition. A treatment may be administered prior to the onset of the disease, for a prophylactic or preventive action. It may also be administered after initiation of the disease, for a therapeutic action.

Detailed Description of Certain Preferred Embodiments

As mentioned above, the present invention provides biomarkers that can be used for detecting the presence of RA-specific autoantibodies in biological samples obtained from patients. These biomarkers are PAD4-peptides corresponding to epitopes on human PAD4 and which specifically react with anti-PAD4 autoantibodies present in the serum of RA patients. Other biomarkers are BRAF-peptides that are

epitopes on the autoantigen BRAF. Still other biomarkers are calpastatin-peptides. Preferably, the biomarkers are peptides of less than 50 amino acids. Also provided are methods for using these biomarkers for the diagnosis of RA.

I - Peptide Biomarkers

PAD4-Peptides

Critical antibodies in RA are directed at citrullin residues on different proteins such as Fibrin, Filaggrin, and Vimentin. Citrullin is generated by post-translational conversion of arginine residues. This process is catalyzed by a group of calcium-dependent peptidyl arginine deiminases, PADs. One of the PAD enzymes, PAD4 is widely believed to play a causative role in RA disease onset and progression because RA-associated mutations in the PAD4 gene have been identified in a variety of populations (A. Suzuki *et al.*, Nat. Genet., 2003, 34(4): 395-402; T. Iwamoto *et al.*, Rheumatology, 2006, 45: 804-807; S.M. Harney *et al.*, Rheumatology, 2005, 44: 869-872; T; Cantaert *et al.*, Ann. Rheum. Dis., 2005, 64: 1316-1320).

PAD4 is not only involved in the generation of citrullinated epitopes, it is in itself a target for RA-specific antibodies. Autoantibodies against PAD4 have already been described in RA patients (Y. Takizawa *et al.*, Scand. J. Rheumatol., 2005, 3: 212-215; E.B. Roth *et al.*, Clin. Exp. Rheumatol., 2006, 1: 12-18; E.H. Halvorsen *et al.*, Ann. Rheumatol. Dis., 2008, 67: 414-417; J. Zhao *et al.*, J. Rheumatol., 2008, 35: 969-974). In 2008, the present Applicants have identified autoantibodies against PAD4 in RA patients using protein array and ELISA analysis (I. Auger *et al.*, Rheum. Dis., 2009, 68: 591-594; EP 08 305 167, each of which is incorporated by reference in its entirety). They observed that 29% of 116 patients were positive to PAD4 compared to none of 33 patients with spondylarthropathy (AS) and 3% of 60 healthy controls (p=0 by chi square test, 116 RA patients versus 93 controls).

As described in Example I below, the present Applicants have now identified epitopes on human PAD4 that are specifically recognized by the sera of RA patients. These epitopes were identified using 65 overlapping 20-mer peptides (see Table 1 of EP 09 305 266.1) encompassing the entire sequence of human PAD4 (GenBank Accession Number: NP_036519.1; locus NM_012387; SEQ ID NO: 1). These peptides were screened with the sera of 29 RA patients and 2 controls known to contain autoantibodies to PAD4. Among the 65 peptides, 18 were recognized by the

sera tested. Four peptides (peptides **22**, **28**, **61** and **63**) were preferentially recognized by the sera of RA patients and controls containing anti-PAD4 autoantibodies (see Table 2). To further confirm these results, the Applicants have tested the sera of 33 patients with spondylarthropathy (AS) and 33 healthy individuals by ELISA using peptides **22**, **28**, **61** and **63** as immunosorbents. Autoantibodies recognizing peptides **22**, **61** and **63** were found in significantly higher percentage in the sera of RA patients than in controls (AS patients and healthy individuals). In contrast, autoantibodies recognizing peptide **28** were found in high percentage in the sera of RA patients as well as in the sera of controls (see Figure 2).

Peptide **22** identified as described herein is located in the N terminal domain of PAD4 and has the following amino acid sequence:

SEQ ID NO: 2 VRVFQATRGKLSSKCSVVLG,

which corresponds to amino acid residues 211 to 230 of human PAD4.

Peptide **61** identified as described herein is located in the C terminal domain of PAD4 and has the following amino acid sequence:

SEQ ID NO: 3 PFGPVINGRCCLEEKVCSLL,

which corresponds to amino acid residues 601 to 620 of human PAD4.

Peptide **63** identified as described herein is located in the C terminal domain of PAD4 and has the following amino acid sequence:

SEQ ID NO: 4 EPLGLQCTFINDFFTYHIRH,

which corresponds to amino acid residues 621 to 640 of human PAD4.

Accordingly, in one aspect, the present invention provides PAD4-peptides that are specifically recognized by anti-PAD4 autoantibodies present in the sera of RA patients. PAD4-peptides of the invention have an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, and homologues thereof. SEQ ID NOs: 2-4 are as described above. SEQ ID NO: 5 has the following amino acid sequence:

SEQ ID NO: 5 PFGPVINGRCCLEEKVCSLLEPLGLQCTFINDFFTYHIRH,

which corresponds to amino acid residues 601 to 640 of human PAD4.

BRAF-Peptides

BRAF (also called B-raf) is a serine-threonine kinase protein of the RAF protein family. In humans, the BRAF gene encodes a BRAF protein of 766 amino acid residues (GenBank Accession Number: NP_004324.1), whose sequence is defined in SEQ ID NO. 6.

The present Applicants have previously shown that BRAF catalytic domain (residues 456 to 712 in SEQ ID NO: 6) is a target of autoantibodies of patients affected with RA and is a specific marker of RA (I. Auger *et al.*, Rheum. Dis., 2009, 68: 591-594; EP 08 305 167). The Applicants have now identified (see Example II) three linear peptides on BRAF catalytic domain that are recognized almost uniquely by RA patients. These BRAF-peptides, **P10**, **P16** and **P25**, have been shown to be recognized by the sera of CCP-negative patients. The combined used of **P10**, **P16** and **P25** identifies 50% of CCP-negative patients.

Peptide **P10** identified as described herein has the following amino acid sequence:

SEQ ID NO: 7 RKTRHVNILLFMGYSTKPQL,

which corresponds to amino acid residues 506 to 525 of human BRAF.

Peptide **P16** identified as described herein has the following amino acid sequence:

SEQ ID NO: 8 YLHAKSIIHRDLKSNNIFLH,

which corresponds to amino acid residues 566 to 585 of human BRAF.

Peptide **P25** identified as described herein has the following amino acid sequence:

SEQ ID NO: 9 YSNINNRDQIIFMVGRGYLS,

which corresponds to amino acid residues 656 to 675 of human BRAF.

Accordingly, in one aspect, the present invention provides BARF-peptides that are specifically recognized by anti-BRAF autoantibodies present in the sera of RA patients. BRAF-peptides of the invention have an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and homologues thereof. SEQ ID NOs: 7-9 are as described above.

Calpastatin-Peptides

The present Applicants have observed that sera of patients afflicted with RA and homozygous for HLA-DRB1*0404 recognized a 100 kD synovial protein identified as calpastatin (Auger *et al.* Ann. Rheum. Dis., 2007, 66: 1588-1593). Calpastatin is an endogenous calpain (calcium dependent cysteine protease) inhibitor, present in most mammalian tissues. Calpastatin consists of an amino-terminal domain L and four repetitive calpain inhibitor domains – domains 1 to 4 (Menard *et al.*, Immun. Today, 1996, 17: 545-547). The amino acid sequence of human calpastatin isoform A is provided in SEQ ID NO: 10 (GenPept accession number: NP_001035908).

The present Applicants have previously identified (see Example III) two linear peptides on calpastatin that are recognized by the sera of CCP-negative patients: **P'16** and **P'28**, which are 15-mer peptides from locus NP_001035908.

Peptide **P'16** identified as described herein has the following amino acid sequence:

SEQ ID NO: 11 IGPDDAIDALSSDFT,

which corresponds to amino acid residues 226 to 240 of human calpastatin isoform A and is located in repetitive calpain inhibitor domain 1 .

Peptide **P'28** identified as described herein has the following amino acid sequence:

SEQ ID NO: 12 AVCRTSMCSIQSAPP,

which corresponds to amino acid residues 406 to 420 of human calpastatin isoform A and is located in repetitive calpain inhibitor domain 2.

Accordingly, in one aspect, the present invention provides calpastatin-peptides that are specifically recognized by anti-calpastatin autoantibodies present in the sera of RA patients. Calpastatin-peptides of the invention have an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and homologues thereof. SEQ ID NOs: 11-12 are as described above.

Preparation of the Peptide Biomarkers

The peptide biomarkers of the present invention may be prepared by any suitable method, including chemical synthesis and recombinant methods.

The peptides of the invention are generally sufficiently short that chemical synthesis, using standard methods is feasible. Solid-phase peptide synthesis, which was initially described by R.B. Merrifield (J. Am. Chem. Soc. 1963, 85: 2149-2154) is a quick and easy approach to synthesizing peptides and small peptidic molecules of known sequences. A compilation of such solid-phase techniques may be found, for example, in "*Solid Phase Peptide Synthesis*" (Methods in Enzymology, G.B. Fields (Ed.), 1997, Academic Press: San Diego, CA, which is incorporated herein by reference in its entirety). Most of these synthetic procedures involve the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain. For example, the carboxy group of the first amino acid is attached to a solid support *via* a labile bond, and reacted with the second amino acid, whose amino group has been, beforehand, chemically protected to avoid self-condensation. After coupling, the amino group is deprotected, and the process is repeated with the following amino acid. Once the desired peptide is assembled, it is cleaved off from the solid support, precipitated, and the resulting free peptide may be analyzed and/or purified as desired. Solution methods, as described, for example, in "*The Proteins*" (Vol. II, 3rd Ed., H. Neurath *et al.* (Eds.), 1976, Academic Press: New York, NY, pp. 105-237) may also be used to synthesize the biomarkers of the invention.

In certain embodiments, a peptide biomarker of the invention is provided immobilized onto a solid carrier or support (*e.g.*, a bead or array). Methods for immobilizing polypeptide molecules onto a solid surface are known in the art. A peptide may be immobilized by being either covalently or passively bound to the surface of a solid carrier or support. Examples of suitable carrier or support materials include, but are not limited to, agarose, cellulose, nitrocellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose, polyacrylamides, polystyrene, polyvinyl chloride, polypropylene, filter paper, magnetite, ion-exchange resin, glass, polyamine-methyl-vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, and the like. Immobilization of a peptide biomarker on the surface of a solid carrier or support may involve crosslinking, covalent binding or physical adsorption, using methods well known in the art. The solid carrier or support may be in the form of a bead, a particle, a microplate well, an array, a cuvette, a tube,

a membrane, or any other shape suitable for conducting a diagnostic method according to the invention (*e.g.*, using an immunoassay).

In particular, the invention provides an array or protein array for the diagnosis of RA, comprising, immobilized to its surface, at least one peptide biomarker of the invention. Preferably, the array comprises more than one peptide biomarker of the invention. The array may further comprise at least one additional biomarker of RA. Suitable biomarkers of RA include biomarkers allowing detection of the presence of antinuclear antibodies and/or CCP antibodies.

The present invention also provides a protein bead suspension array for the diagnosis of RA. This bead suspension array comprises a suspension of one or more identifiable distinct particles or beads, wherein each bead contains coding features relating to its size, color or fluorescence signature and wherein each bead is coated with a peptide biomarker of the present invention. Examples of bead suspension arrays include thexMAP® bead suspension array (Luminex Corporation).

II - Diagnosis Methods

As mentioned above, the peptide biomarkers disclosed herein is specifically recognized by the sera of RA patients, and in particular by the sera of CCP-negative RA patients.

Accordingly, the present invention provides methods for diagnosing RA in a subject. Such methods comprise contacting a biological sample obtained from the subject to be tested with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form; and detecting any biomarker-antibody formed.

In certain embodiments, the at least one biomarker is a PAD4-peptide having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof and fragments thereof. In these methods, the detection of a biomarker-antibody complex is indicative of the presence of anti-PAD4 autoantibodies in the biological sample, and therefore is indicative of RA in the subject. More than one PAD4-peptide may be used in these methods.

In other embodiments, the at least one biomarker is a BRAF-peptide having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof and fragments thereof. In these methods, the detection of a biomarker-antibody complex is indicative of the presence of anti-BRAF autoantibodies in the biological sample, and therefore is indicative of RA in the subject. More than one BRAF-peptide may be used in these methods.

The present invention also provides methods for diagnosing RA in a subject. Such methods comprise contacting a biological sample obtained from the subject to be tested with at least two biomarkers for a time and under conditions allowing a biomarker-antibody complex to form; and detecting any biomarker-antibody formed. The at least two biomarkers may be selected from:

PAD4-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof and fragments thereof;

BRAF-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof and fragments thereof,

Calpastatin-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof and fragments thereof; and

any combination thereof

In this method, the detection of a biomarker-antibody complex is indicative of RA in the subject.

In other embodiments, more than two peptide biomarkers are used, for example, 3, 4, 5, 6, 7 or 8 peptide biomarkers. In certain preferred embodiments, 8 peptide biomarkers are used, i.e., three PAD4-peptides, three BRAF-peptides and two calpastatin-peptides. In particularly preferred embodiments, the three PAD4-peptides consist of SEQ ID NOs: 2-4, the three BRAF-peptides consist of SEQ ID NOs: 7-9 and the two calpastatin-peptides consist of SEQ ID NOs: 11-12.

Biological Samples

The methods of diagnosis of the present invention may be applied to the study of any type of biological samples allowing one or more inventive biomarkers to be assayed. Examples of suitable biological samples include, but are not limited to, urine, whole blood, serum, plasma, saliva, and synovial fluid. Biological samples used in the practice of the invention may be fresh or frozen samples collected from a subject, or archival samples with known diagnosis, treatment and/or outcome history. Biological samples may be collected by any non-invasive means, such as, for example, by drawing blood from a subject, or using fine needle aspiration or needle biopsy. In certain embodiments, the biological sample is a serologic sample and is selected from the group consisting of whole blood, serum, plasma.

In preferred embodiments, the inventive methods are performed on the biological sample itself without, or with limited, processing of the sample.

However, alternatively, the inventive methods may be performed on a protein extract prepared from the biological sample. In this case, the protein extract preferably contains the total protein content. Methods of protein extraction are well known in the art (see, for example "*Protein Methods*", D.M. Bollag *et al.*, 2nd Ed., 1996, Wiley-Liss; "*Protein Purification Methods: A Practical Approach*", E.L. Harris and S. Angal (Eds.), 1989; "*Protein Purification Techniques: A Practical Approach*", S. Roe, 2nd Ed., 2001, Oxford University Press; "*Principles and Reactions of Protein Extraction, Purification, and Characterization*", H. Ahmed, 2005, CRC Press: Boca Raton, FL). Various kits can be used to extract proteins from bodily fluids and tissues. Such kits are commercially available from, for example, BioRad Laboratories (Hercules, CA), BD Biosciences Clontech (Mountain View, CA), Chemicon International, Inc. (Temecula, CA), Calbiochem (San Diego, CA), Pierce Biotechnology (Rockford, IL), and Invitrogen Corp. (Carlsbad, CA). User Guides that describe in great detail the protocol to be followed are usually included in all these kits. Sensitivity, processing time and costs may be different from one kit to another. One of ordinary skill in the art can easily select the kit(s) most appropriate for a particular situation.

Detection of Biomarker-Antibody Complexes

The diagnostic methods of the present invention generally involve detection of a biomarker-antigen complex formed between the peptide biomarker and an autoantibody present in the biological sample tested. In the practice of the invention, detection of such a complex may be performed by any suitable method (see, for example, E. Harlow and A. Lane, "*Antibodies: A Laboratories Manual*", 1988, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY).

For example, detection of a biomarker-antibody complex may be performed using an immunoassay. A wide range of immunoassay techniques is available, including radioimmunoassay, enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunofluorescence immunoprecipitation. Immunoassays are well known in the art. Methods for carrying out such assays as well as practical applications and procedures are summarized in textbooks. Examples of such textbooks include P. Tijssen, In: Practice and theory of enzyme immunoassays, eds. R.H. Burdon and v. P.H. Knippenberg, Elsevier, Amsterdam (1990), pp. 221-278 and various volumes of Methods in Enzymology, Eds. S.P. Colowick *et al.*, Academic Press, dealing with immunological detection methods, especially volumes 70, 73, 74, 84, 92 and 121. Immunoassays may be competitive or non-competitive.

For example, any of a number of variations of the sandwich assay technique may be used to perform an immunoassay. Briefly, in a typical sandwich assay applied to the detection of, for example, anti-PAD4 autoantibodies according to the present invention, an unlabeled PAD4-peptide biomarker is immobilized on a solid surface (as described above) and the biological sample to be tested is brought into contact with the bound biomarker for a time and under conditions allowing formation of a biomarker-antibody complex. Following incubation, an antibody that is labeled with a detectable moiety and that specifically recognizes antibodies from the species tested (*e.g.*, an anti-human IgG for human subjects) is added and incubated under conditions allowing the formation of a ternary complex between any biomarker-bound autoantibody and the labeled antibody. Any unbound material is washed away, and the presence of any anti-PAD4 autoantibody in the sample is determined by observation/detection of the signal directly or indirectly produced by the detectable moiety. Variations on this assay include an assay, in which both the biological sample

and the labeled antibody are added simultaneously to the immobilized PAD4-peptide biomarker.

The second antibody (*i.e.*, the antibody added in a sandwich assay as described above) may be labeled with any suitable detectable moiety, *i.e.*, any entity which, by its chemical nature, provides an analytically identifiable signal allowing detection of the ternary complex, and consequently detection of the biomarker-antibody complex.

Detection may be either qualitative or quantitative. Methods for labeling biological molecules such as antibodies are well-known in the art (see, for example, "*Affinity Techniques. Enzyme Purification: Part B*", Methods in Enzymol., 1974, Vol. 34, W.B. Jakoby and M. Wilneck (Eds.), Academic Press: New York, NY; and M. Wilchek and E.A. Bayer, Anal. Biochem., 1988, 171: 1-32).

The most commonly used detectable moieties in immunoassays are enzymes and fluorophores. In the case of an enzyme immunoassay (EIA or ELISA), an enzyme such as horseradish perodixase, glucose oxidase, beta-galactosidase, alkaline phosphatase, and the like, is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. The substrates to be used with the specific enzymes are generally chosen for the production of a detectable color change, upon hydrolysis of the corresponding enzyme. In the case of immunofluorescence, the second antibody is chemically coupled to a fluorescent moiety without alteration of its binding capacity. After binding of the fluorescently labeled antibody to the biomarker-antibody complex and removal of any unbound material, the fluorescent signal generated by the fluorescent moiety is detected, and optionally quantified. Alternatively, the second antibody may be labeled with a radioisotope, a chemiluminescent moiety, or a bioluminescent moiety.

RA Diagnosis

In the methods of the present invention, detection of a biomarker-antibody complex is indicative of the presence of anti-PAD4 autoantibodies, anti-BRAF autoantibodies or anti-Calpastatin-autoantibodies in the biological sample tested and is therefore indicative of RA in the subject from which the biological sample was obtained. Thus, the methods of the present invention may be used for the diagnosis of

RA in patients. In particular, methods of the invention may be used for testing subjects suspected of having RA.

It will be appreciated by one skilled in the art that diagnosis of RA may be made solely on the results obtained by a method provided herein. Alternatively, a physician may also consider other clinical or pathological parameters used in existing methods to diagnose RA. Thus, results obtained using methods of the present invention may be compared to and/or combined with results from other tests, assays or procedures performed for the diagnosis of RA. Such comparison and/or combination may help provide a more refine diagnosis.

For example, RA diagnosis methods of the present invention may be used in combination with ARA criteria (*i.e.*, the American College of Rheumatology 1987 revised criteria for the classification of RA described in F.C. Arnett *et al.*, Arthritis Rheum., 1988, 31: 315-324). According to the ARA criteria, a patient is said to have RA if the patient exhibits at least 4 of the 7 following criteria: 1) morning stiffness for at least 1 hour; 2) arthritis of 3 or more joint areas; 3) arthritis of hand joints; 4) symmetrical arthritis; 5) rheumatoid nodules; 6) serum rheumatoid factor (RF); and 7) radiographic changes, wherein criteria 1-4 must be present for at least 6 months.

Alternatively or additionally, results from RA diagnosis methods of the present invention may be used in combination with results from one or more assays that employ other RA biomarkers. Thus, in certain embodiments, diagnosis of RA may be based on results from a method of the invention and on results from one or more additional assays that use a different RA biomarker. For example, a panel of RA biomarkers may be tested either individually or simultaneously, *e.g.*, using a chip or a bead-based array technology.

Examples of suitable RA biomarkers include, but are not limited to, CCP, C-reactive protein, serum amyloid A, interleukin 6 (IL6), S100 proteins, ostopontin, rheumatoid factor, matrix metalloprotease 1 (MMP-1), matrix metalloprotease 3 (MMP-3), hyaluronic acid, sCD14, angiogenesis markers (such as the vascular endothelial growth factor or VEGF), and products of bone, cartilage or synovium metabolism (such as pyridinoline or its glycosylated form; deoxy-pyridinoline; cross-linked telopeptides; collagen neoepitopes; CS846; cartilage oligomeric matrix protein;

cartilage intermediate layer protein; matrilins, chondromodulatins, osteocalcin, and the like).

In certain embodiments, the methods of the invention are used for the diagnosis of RA in CCP-negative patients. Indeed, as reported herein in the Examples section, the applicants have shown, in particular, that a method of the invention using the combination of eight peptide biomarkers (3 PAD4-peptides, 3 BRAF-peptides, and 2 calpastatin-peptides) allows the diagnosis of RA in more than 70% of CCP-negative subjects. The terms "CCP-negative patient" and "CCP-negative subject" are used herein interchangeably. They refer to a subject whose serum contains no antibodies (or at least no detectable antibodies) directed against citrullinated proteins.

III - Kits

In another aspect, the present invention provides kits comprising materials useful for carrying out diagnostic methods according to the present invention. The diagnosis procedures provided herein may be performed by diagnostics laboratories, experimental laboratories, or practitioners. The invention provides kits that can be used in these different settings.

Materials and reagents for detecting anti-PAD4 autoantibodies, anti-BRAF-autoantibodies and/or anti-calpastatin-autoantibodies in a biological sample and/or for diagnosing RA in a subject according to the present invention may be assembled together in a kit. Each kit of the invention comprises at least one inventive peptide biomarker preferably in an amount that is suitable for detection of autoantibodies in a biological sample.

Thus, in certain embodiments, an inventive kit comprises at least one PAD4-peptide having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof and fragments thereof.

In other embodiments, an inventive kit comprises at least one BRAF-peptide having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof and fragments thereof.

In yet other embodiments, an inventive kit comprises at least two biomarkers selected from the group consisting of:

PAD4-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof and fragments thereof;

BRAF-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof and fragments thereof,

Calpastatin-peptides having an amino acid sequence, preferably of less than 50 amino acids, comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof and fragments thereof, and

any combination thereof.

In certain preferred embodiments, the kit comprises at least eight biomarkers: three PAD4-peptides, 3 BRAF-peptides and 2 calpastatin-peptides, as described above. Preferably, the three PAD4-peptides consist of SEQ ID NOs: 2-4, the three BRAF-peptides consist of SEQ ID NOs: 7-9, and the two calpastatin-peptides consist of SEQ ID NOs: 11-12.

The peptide biomarker(s) included in a kit may or may not be immobilized on as substrate surface (*e.g.*, beads, array, and the like). Thus, in certain embodiments, an inventive kit may include an array for diagnosing RA as provided herein. Alternatively, a substrate surface may be included in an inventive kit for immobilization of the peptide biomarkers.

An inventive kit generally also comprises at least one reagent for the detection of a biomarker-antibody complex formed between the peptide biomarker included in the kit and an autoantibody present in a biological sample. Such a reagent may be, for example, a labeled antibody that specifically recognizes antibodies from the species tested (*e.g.*, an anti-human IgG for human subjects), as described above.

Depending on the procedure, the kit may further comprise one or more of: extraction buffer and/or reagents, blocking buffer and/or reagents, immunodetection

buffer and/or reagents, labeling buffer and/or reagents, and detection means. Protocols for using these buffers and reagents for performing different steps of the procedure may be included in the kit.

The different reagents included in an inventive kit may be supplied in a solid (*e.g.*, lyophilized) or liquid form. The kits of the present invention may optionally comprise different containers (*e.g.*, vial, ampoule, test tube, flask or bottle) for each individual buffer and/or reagent. Each component will generally be suitable as aliquoted in its respective container or provided in a concentrated form. Other containers suitable for conducting certain steps of the disclosed methods may also be provided. The individual containers of the kit are preferably maintained in close confinement for commercial sale.

In certain embodiments, a kit comprises instructions for using its components for the diagnosis of RA in a subject according to a method of the invention. Instructions for using the kit according to methods of the invention may comprise instructions for processing the biological sample obtained from the subject and/or for performing the test, and/or instructions for interpreting the results. A kit may also contain a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products.

IV - Development of New Therapeutics for RA

The PAD4 and/or BRAF epitopes identified by the Applicants may constitute attractive targets for the identification of compounds or substances potentially useful for treating RA or preventing RA progression.

As already mentioned above, PAD4 is not only the target for RA-specific antibodies, it is also involved in the generation of citrullinated epitopes. The present Applicants have shown that anti-PAD4 autoantibodies inhibit citrullination of fibrinogen by PAD4 (see Examples section). The Applicants have also shown that anti-PAD4 autoantibodies specifically recognize 3 epitopes on PAD4, 2 of which (peptides **61** and **63**) are localized within the domain of substrate binding of PAD4. Preventing the binding of anti-PAD4 autoantibodies to these PAD4 epitopes could reverse the inhibiting action of autoantibodies and restore PAD4 enzymatic activity.

BRAF is an interesting target for autoantibodies. BRAF is a serine-threonine kinase involved in the transduction of mitogenic signals from the cell membrane to the nucleus. BRAF regulates the mitogen-activated protein kinases (MAPKs) signalling cascade. MAPKs are involved in signalling via the B cell antigen receptor, T cell receptor, Toll-like receptor and IL-1, IL-17 and TNFα receptors. MAPKs also play a role in the production of pro-inflammatory cytokines (TNFα, IL-1, IL-6). To test whether autoantibodies to BRAF may influence BRAF activity as a kinase, the Applicants have developed a phosphorylation assay using BRAF, MEK1 (its major substrate) and autoantibodies to BRAF purified from the sera of RA patients; and found that 80% of anti-BRAF autoantibodies activate phosphorylation of MEK1 by BRAF *in vitro*. This result suggests that anti-BRAF autoantibodies could activate the MAP kinase pathway through BRAF, leading to proinflammatory cytokine production and joint inflammation. The Applicants have also shown that anti-BRAF autoantibodies specifically recognize 3 epitopes on BRAF (peptides **P10**, **P16** and **P25**). Therefore, anti-BRAF antibodies may constitute a target for the control of inflammation in RA.

Thus, screens may be developed to identify compounds or substances that prevent the binding of anti-PAD4 autoantibodies to PAD4 epitopes, in particular peptide **61** and/or peptide **63**. Similarly, screens may be developed to identify compounds or substances that prevent the binding of anti-BRAF autoantibodies to BRAF epitopes, in particular to peptides **P10**, **P16** and/or **P25**.

Such screens may be carried out in any suitable biological system such as a biological fluid, a biological fluid, or isolated cells. Generally, screens are performed using cells that can be grown in standard tissue culture ware. Suitable cells include all appropriate normal and transformed cells derived from any recognized sources. Preferably, cells are of mammalian (human or animal, such as rodent or simian) origin. More preferably, cells are of human origin. Mammalian cells may be of any organ or tissue origin (*e.g.*, bone, cartilage, or synovial fluid) and of any cell types as long as the cells express PAD4 and/or PAD4. Cells to be used in the practice of the methods of the present invention may be primary cells, secondary cells, or immortalized cells (*e.g.*, established cell lines). They may be prepared by techniques well known in the art (for example, cells may be isolated from bone, cartilage or

synovial fluid) or purchased from immunological and microbiological commercial resources (for example, from the American Type Culture Collection, Manassas, VA). Alternatively or additionally, cells may be genetically engineered to contain, for example, a gene of interest. An assay developed for primary drug screening (*i.e.*, first round(s) of screening) is preferably performed using established cell lines, which are commercially available and usually relatively easy to grow, while an assay to be used later in the drug development process is preferably performed using primary and secondary cells, which are generally more difficult to obtain, maintain and/or grow than immortalized cells but which represent better experimental models for *in vivo* situation. Screening methods may be performed using cells contained in a plurality of wells of a multi-well assay plate.

As will be appreciated by one of ordinary skill in the art, any kind of compounds or agents can be screened. A candidate compound may be a synthetic or natural compound; it may be a single molecule or a mixture or complex of different molecules. Candidate compounds may be screened individually. Alternatively, compounds comprised in collections or libraries may be screened simultaneously.

Collections of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (Durham, NC). Synthetic compound libraries are commercially available from Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), Microsource (New Milford, CT), and Aldrich (Milwaukee, WI). Libraries of candidate compounds have also been developed by and are commercially available from large chemical companies, including, for example, Merck, Glaxo Welcome, Bristol-Meyers-Squibb, Novartis, Monsanto/Searle, and Pharmacia UpJohn. Additionally, natural collections, synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. Chemical libraries are relatively easy to prepare by traditional automated synthesis, PCR, cloning or proprietary synthetic methods (see, for example, S.H. DeWitt *et al.*, Proc. Natl. Acad. Sci. U.S.A. 1993, 90:6909-6913; R.N. Zuckermann *et al.*, J. Med. Chem. 1994, 37: 2678-2685; Carell *et al.*, Angew. Chem. Int. Ed. Engl. 1994, 33: 2059-2060; P.L. Myers, Curr. Opin. Biotechnol. 1997, 8: 701-707).

Using peptide **61** and/or peptide **63** of PAD4 or peptide **P10**, peptide **P16** and/or peptide **P25** of BRAF as a target(s), useful agents for the treatment of RA may be found in any of a large variety of classes of chemicals.

Examples

The following examples describe some of the preferred modes of making and practicing the present invention. However, it should be understood that the examples are for illustrative purposes only and are not meant to limit the scope of the invention. Furthermore, unless the description in an Example is presented in the past tense, the text, like the rest of the specification, is not intended to suggest that experiments were actually performed or data were actually obtained.

Some of the results presented below have been reported by the present Applicants in a scientific article (Auger *et al.*, Ann. Rheum. Dis., 2009, 68: 591–594), which is incorporated herein by reference in its entirety.

Example I: PAD4-Peptides for RA diagnosis

Patients and Methods

RA Patients and Controls

RA patients were selected from the Rheumatology Ward at the Hospital La Conception in Marseille, France. These patients fulfilled the 1987 American College of Rheumatology criteria for RA (F.C. Arnett *et al.*, Arthritis Rheum., 1988, 31: 315-324). Patients with spondylarthropathy (AS) were from the Rheumatology Ward at the Hospital La Conception in Marseille. Volunteers from the laboratory staff and the Marseille Blood Transfusion Center staff served as normal controls. All participants gave informed consent.

Purification of Autoantibodies to PAD4

ELISA plates were coated with 1 µg of PAD4 per well overnight at 4°C, using recombinant PAD4 protein (Abnova Corporation, H0023569P01). Sera from 29 RA patients and from 2 controls containing autoantibodies to PAD4 were selected (I. Auger *et al.*, Ann. Rheum. Dis., 2008 Oct 28). The sera were diluted to 1:2 in PBS and incubated for 3 hours. After washing, autoantibodies to PAD4 were purified in

PBS pH 2, neutralized in 1M Tris buffer, and quantified. The presence of anti-PAD4 autoantibodies was confirmed by dot blot.

In-house Citrullination Assay

Human Fibrinogen (Sigma) at a final concentration of 10 mg/mL was incubated with 1 µg of PAD4 (Abonova Corporation) in working buffer (100 mM Tris HCl, 5 mM $CaCl_2$, 5 mM DTT, pH 7.5) in presence of 1 µg of purified autoantibodies to PAD4, for 4 hours at 55°C. For each patient, one positive control was included (incubation of fibrinogen with PAD4 in working buffer in the presence of 1 µg of control autoantibodies anti C1 or C2). Each reaction mixture was incubated twice with protein A sepharose beads to eliminate antibodies.

Each reaction mixture was then coated in duplicate to ELISA plates and blocked with PBS containing 5% milk. To detect citrullination of fibrinogen, a serum was used that contained autoantibodies to citrullinated fibrinogen but no autoantibodies to PAD4. After washing with 0.1% Tween 20, peroxydase conjugated anti-human IgG (Sigma, France) was added. Optical density (OD) was read at 405 nm. Activation or inhibition of citrullination by PAD4 was determined by measuring the ratio of the OD measured for the test sample and OD measured for the positive control. A ratio > 1 indicated activation, a ratio < 1 indicated inhibition.

Synthetic Peptides

Peptides (Neosystem, Strasbourg, France) were synthesized using a solid phase system and purified. A total of 65 20-mer peptides overlapping on 10 amino acids derived from PAD4 (locus NM_012387), residues 1 to 663.

Mapping of Peptides in PAD4

Plates were coated overnight with 10 µg/well of PAD4 peptides diluted in phosphate buffer saline (PBS), pH 7.4. Plates were blocked with PBS containing 5% milk. Sera diluted to 1:100 in PBS were incubated for 2 hours. After washing with 0.1% Tween 20, peroxydase-conjugated anti-human IgG (Sigma, France) was added. Optical density was read at 405 nm. Background OD was obtained by adding each serum to a well without peptides. Positive sera were defined as an OD value more than twice the background OD (I Auger *et al.*, Ann. Rheum. Dis., 2007, 66: 1588-1593).

Statistical Analysis

P-values were calculated using the Chi square test.

Results

Inhibition of the Citrullination of Fibrinogen by Autoantibodies to PAD4

To test whether autoantibodies directed to PAD4 interfere with the activity of PAD4, fibrinogen citrullination was analyzed in presence of anti-PAD4 autoantibodies purified from 29 patients and 2 healthy controls. Detection of citrullination was then quantified by ELISA.

Inhibition of fibrinogen citrullination was observed for 22 out of 31 purified autoantibodies to PAD4; activation of fibrinogen citrullination was observed for 6 out of 31 purified autoantibodies to PAD4; and 3 out of 31 purified autoantibodies to PAD4 were found to have no effect on fibrinogen citrullination (Figure 1).

Four Linear Epitopes in PAD4 are recognized by Autoantibodies to PAD4

To identify B cell epitopes in PAD4, 65 overlapping 20-mer peptides encompassing the entire sequence of human PAD4 were synthesized. These peptides were screened with the sera of 29 RA patients and 2 controls known to contain anti-PAD4 autoantibodies.

Among the 65 peptides, 18 peptides were recognized by the sera tested. Ten (10) of these peptides are located in the N terminal domain and the other 8 peptides are located in the C terminal domain. Four (4) peptides (**22**, **28**, **61** and **63**) were preferentially recognized by the sera of RA patients and controls (Table 1). Indeed, 15 out of 31 sera from RA patients were positive for peptide **22**, 25 out of 31 were positive for peptide **28**, 8 out of 31 were positive for peptide **61**, and 16 out of 31 were positive for peptide **63**. Peptides **22** and **28** are located in the N terminal domain of PAD4, while peptides **61** and **63** are located in the C terminal domain of PAD4.

Three Linear Epitopes in PAD4 are associated with RA Patients

To confirm the reactivity observed, the sera of 33 patients with spondylarthropathy (AS) and 33 healthy individuals were tested by ELISA using peptides **22**, **28**, **61** and **63** as immunosorbents.

Table 1. Binding of PAD4 peptides to anti-PAD4 autoantibodies (in grey: positive peptide).

Subjects		PAD4 peptides																	
		N terminal										C terminal							
		5	9	15	16	18	**22**	25	26	27	**28**	33	49	51	59	**61**	62	**63**	64
I	RA1																		
	RA2																		
	RA3																		
	RA4																		
	RA5																		
	RA6																		
	RA7																		
	RA8																		
	RA9																		
	RA10																		
	RA11																		
	RA12																		
	RA13																		
	RA14																		
	RA15																		
	RA16																		
	RA17																		
	RA18																		
	RA19																		
	RA20																		
0	RA21																		
	RA22																		
	RA23																		
A	RA24																		
	RA25																		
	RA26																		
	RA27																		
	RA28																		
	RA29																		
I	CTL1																		
	CTL2																		

I = Inhibition of fibrinogen citrullination by PAD4; 0 = no effect on fibrinogen citrullination by PAD4; A = activation of fibrinogen citrullination by PAD4

Autoantibodies against peptide **22**, peptide **61** and peptide **63** were found in more RA patients than controls (Figure 2). Indeed, 15 of 29 RA patients were positive for peptide **22** versus 7 out of 66 controls (p=0 by Chi square test). Similarly, 8 out of 29 RA patients were positive for peptide **61** versus none out of 66 controls (p=0 by chi square test). Finally, 15 out of 29 patients were positive for peptide **63** versus 6 out of 66 controls (p=0 by Chi square test).

Autoantibodies against peptide **28** were found in high percentage in controls. Indeed, 24 of 29 patients were positive versus 20 out of 33 AS patients and 10 out of 33 healthy individuals (p=0.002 by Chi square test, 29 patients versus 66 controls).

Peptide Recognition by Autoantibodies to PAD4 and Inhibition of Citrullination by PAD4

The peptide pattern of autoantibodies to PAD4 that inhibit (patients RA1 to RA20), that activate (patients RA24 to RA29), or that have no effect (patients RA21 to RA23) on citrullination by PAD4 was analyzed.

No difference was observed in the number of peptides recognized by autoantibodies known to inhibit or activate PAD4 (Table 2). Irrespective of their action (activation or inhibition), most autoantibodies to PAD4 recognized peptides located in the N terminal domain and the C terminal domain. Among autoantibodies to PAD4 that inhibit PAD4, 5 out of 20 recognized exclusively peptides located in the N terminal domain, 1 out of 20 recognized exclusively peptides located in the C terminal domain and 14 out of 20 recognized peptides located in the N terminal domain and the C terminal domain (Figure 3).

Among autoantibodies to PAD4 that have no effect on citrullination by PAD4, 3 out of 3 recognized peptides located in the N terminal domain and the C terminal domain.

Among autoantibodies to PAD4 that activate PAD4, 1 out of 6 recognized exclusively peptides located in the N terminal domain, none of out of 6 recognized peptides located in the C terminal domain and 3 out of 6 recognized peptides located in the N terminal domain and the C terminal domain.

Peptides **22** and **61** were preferentially recognized by autoantibodies to PAD4 that inhibit PAD4 (Figure 4). Indeed, 12 out of 20 autoantibodies to PAD4 that inhibit

PAD4 were positive to peptide **22** compared to 2 out of 6 autoantibodies to PAD4 that activated PAD4. Moreover, peptide **61** was recognized by 6 out of 20 autoantibodies to PAD4 that inhibit PAD4 versus none of 6 autoantibodies to PAD4 that activate PAD4. However, among autoantibodies to PAD4 found to have no effect on citrullination of PAD4, 2 out of 3 recognized peptide **61**.

Discussion

Using protein arrays and ELISA assays, the present applicants have previously found that PAD4 is a specific autoantigen in RA patients. Autoantibodies against PAD4 have already been described in RA. PAD4 is involved in the generation of citrullinated epitopes. Autoantibodies to citrullinated epitopes are highly specific of RA. The presence of these autoantibodies before the onset of RA suggests a potential role in the pathophysiology of the disease.

The study presented herein was undertaken to test whether autoantibodies to PAD4 interfere with the activity of PAD4. The applicants have developed an in house citrullination assay with PAD4, fibrinogen and autoantibodies to PAD4 purified from the sera of RA patients. As a general rule, it was observed that autoantibodies to PAD4 inhibit the citrullination of fibrinogen by PAD4. Indeed, 22 out of 31 purified autoantibodies to PAD4 were found to inhibit fibrinogen citrullination by PAD4.

In order to identify the epitopes recognized by autoantibodies to PAD4, 65 20-mer peptides encompassing the entire sequence of PAD4 were used in a direct ELISA assay. Autoantibodies to PAD4 were found to recognize four major epitopes: peptide **22** (corresponding to amino acid residues 211-230 of human PAD4); peptide **28** (amino acid residues 271-290); peptide **61** (amino acid residues 601-620) and peptide **63** (amino acid residues 621-640). Peptides **22** and **28** are located in the N terminal domain of PAD4 (which encompasses amino acid residues 1 to 300), while peptides **61** and **63** are located in the C terminal domain of PAD4 (which encompasses amino acid residues 301 to 663). Autoantibodies that recognize peptides **22**, **61** and **63** were found in high percentage in RA patients compared to controls.

Five Ca^{2+}-binding motifs have been identified in PAD4, three are in the N terminal domain and two are in the C terminal domain (K Arita *et al.*, Proc. Natl. Acad. Sci. USA, 2006, 10: 5291-5296; K Arita *et al.*, Natl. Struct. Mol. Biol., 2004,

11: 777-783). The N terminal domain seems to be involved in conformational changes mediated by Ca^{2+}. After binding to Ca^{2+}, conformational changes generated an active cleft and substrate binds PAD4. The N terminal domain may also influence the enzymatic activity of PAD4. Indeed, the PADI4 gene presents two major haplotypes. In some populations, one is RA-susceptible and the other is not RA-susceptible. These two haplotypes contain four single nucleotide polymorphisms (SNPs) in exons of the N terminal domain (position 55, 82, 112 and 117). The C terminal domain contains the substrate binding domain and the catalytic domain.

In 2008, Harris *et al.* mapped epitopes in PAD4 by immunoprecipitation of *in vitro*-translated PAD4 truncations with autoantibodies to PAD4 (M.L. Harris *et al.*, Arthritis Rheum., 2008, 58(7): 1958-1967). Two types of sera were identified. Type I sera were found to recognize exclusively full length PAD4 (1-663) and type II sera were found to recognize both full-length (1-663) and truncated PAD4 (1-523). Type II autoantibodies to PAD4 require exclusively contributions from the N terminal domain of PAD4 (1-119). However, type I autoantibodies to PAD4 require contributions from the N terminal domain (1-119) and the C terminal domain (523-663) of PAD4.

In the protein arrays and ELISA assays performed by the present Applicants, every autoantibody to PAD4 recognized the full-length PAD4 (1-663) (I Auger *et al.*, Ann. Rheum. Dis., 2008 Oct. 28). Among autoantibodies to PAD4 from RA patients, only 3 out of 29 recognized the truncated PAD4 (residues 1 to 111) (data not shown). In the extensive peptide assay, 7 out of 29 autoantibodies to PAD4 recognized exclusively the peptides located in the N terminal domain (211-290) of PAD4. One out of 29 autoantibodies to PAD4 recognized exclusively the peptide located in the C terminal domain (611-630). Finally, 19 out of 29 autoantibodies to PAD4 were found to recognize the peptides located in the N terminal domain (211-290) and the C terminal domain (601-650).

The majority of autoantibodies to PAD4 were found to inhibit the citrullination of fibrinogen by PAD4. Most autoantibodies recognize peptides located in the N terminal domain (mostly peptide **22** or peptide **28**) and the C terminal domain (mostly peptide **61** or peptide **63**). Without wishing to be bound by theory, the applicants propose the following model to explain how autoantibodies to PAD4 inhibit

citrullination (Figure 5): interaction of autoantibodies with peptide **22** or peptide **28** may interfere with conformational changes mediated by Ca^{2+} binding. Calcium binding and substrate binding are important to generate the active site in PAD4 (K Arita *et al.*, Nat. Struct. Mol. Biol., 2004, 11: 777-783). It is also possible that because autoantibodies to PAD4 bind to 40 amino acids within the C terminal domain of PAD4, the interaction of PAD4 with its substrate is blocked. This may explain why autoantibodies to PAD4 inhibit citrullination mediated by PAD4.

It still remains to elucidate how this inhibition may influence the development of anti-citrullin immunization in RA patients. The applicants are in the process of studying this question.

Example II: BRAF-Peptides for RA Diagnosis in CCP-Negative

Patients and Methods

RA Patients and Controls

118 RA patients were selected from the Rheumatology Ward at Hospital La Conception, Marseille, France. These patients fulfilled the 1987 American College of Rheumatology criteria for RA. For all patients, HLA-DR genotyping and anti CCP titration were obtained. 89 of the 118 RA patients were CCP positive and 29 RA patients were CCP negative. Patients (33) with spondylarthropathy (AS) from the same hospital in Marseille and healthy volunteers (60) from the staff of INSERM UMR639 and of Marseille Blood Transfusion Center) were used as controls.

Synthetic Peptides

Forty 20-mer peptides encompassing residues 416 to 766 from BRAF (locus NP_004324.1) were synthesized using a solid phase system and purified (Neosystem, Strasbourg, France). BRAF sequence used for peptide synthesis displayed polymorphism at position 599 (V599E, valine replaced with glutamate), a mutation observed in human cancers and associated with increased kinase activity.

Detection of Autoantibodies by ELISA

Plates were coated overnight with 10 µg/well of peptide diluted in phosphate buffer saline (PBS), pH7.4. Plates were blocked with PBS containing 5% milk. Sera diluted to 1:100 in PBS were incubated for 2 hours. After washing with 0.1% Tween

20, peroxydase conjugated anti human IgG, (Sigma, France) was added. Optical density (OD) was read at 405 nm. Background OD was obtained by adding each serum to a well in the absence of protein. A positive serum was defined as one presenting an OD value more than twice the background OD.

Statistical Analysis

The p-values were calculated using the Chi squareTest.

Results

Autoantibodies to BRAF Recognize 4 Linear Epitopes in BRAF

To identify B cell epitopes on BRAF, 40 20-mer peptides encompassing the catalytic domain of BRAF were synthesized. These 40 peptides were screened with the sera of 21 RA patients known to contain autoantibodies to BRAF. Among the 40 peptides, 14 were recognized by the tested sera. Four peptides, **P10** (SEQ ID NO: 7, positions 506-525 of human BRAF,), **P16** (SEQ ID NO: 8, positions 566-585 of human BRAF,), **P25** (SEQ ID NO: 9, positions 656-675 of human BRAF), and **P33** (SEQ ID NO: 13) were preferentially recognized by the sera of RA patients. Indeed, 18 of 21 sera recognized **P10**, 8 recognized **P16**, 15 recognized **P25**, and 6 recognized **P33** (see Table 2).

Three Linear Epitopes on BRAF are Recognized by RA Patients

To confirm the reactivity observed, we tested by ELISA the sera of 33 patients with spondylarthropathy (AS) and of 60 healthy individuals were tested by ELISA in the presence of **P10**, **P16**, **P25** and **P33**. Autoantibodies to **P10**, **P16**, and **P25** were found in RA patients more often than in controls (see Figure 6). Indeed, 18 of 21 RA patients sera recognized **P10** versus 10 of 93 controls ($p<10^{-7}$ by Chi square test). Similarly, 8 of 21 RA patients sera recognized **P16** versus 1/93 controls ($p<10^{-7}$ by Chi square test). Finally, 15 of 21 RA patients sera recognized **P25** versus 0/93 controls ($p<10^{-7}$ by Chi square test). Antibodies to **P33** were less specific for RA. 6 of 21 RA patients, but also 6/33 AS patients and 13/60 healthy individuals had anti **P33** antibodies ($p= 0.523$ by Chi square test, 21 RA patients versus 93 controls).

Table 2. Binding of BRAF peptides to anti-BRAF autoantibodies (in grey: positive peptide).

	RA patients																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
P1																					
P2																					
P3																					
P4																					
P5																					
P6																					
P7																					
P8																					
P9																					
P10																					
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P40																					

P10, P16 and P25 Peptides Identify RA in 50% of CCP-Negative Patients

The frequency of positive sera in CCP positive and CCP negative patients was calculated by ELISA using **P10, P16** and **P25** peptides as immunosorbent. Among CCP positive patients, 29/89 recognized **P10**, 7/89 recognized **P16**, and 17/89 recognized **P25**. Among CCP negative patients, 13/29 recognized **P10**, 1/29 recognized **P16**, and 8/29 recognized **P25**. In combination, **P10, P16** and **P25** identify 50% of CCP-negative patients (Figure 7).

Conclusions

Three linear peptides on BRAF recognized almost uniquely by RA patients have been identified. **P10** (SEQ ID NO: 7), **P16** (SEQ ID NO: 8) and **P25** (SEQ ID NO: 9) peptides are located in the catalytic domain of BRAF. These peptides were also recognized by the sera of CCP-negative patients. The combination of **P10**, **P16** and **P25** of BRAF identifies 50% of CCP-negative patients.

Example III: Biomarkers for RA Diagnosis in CCP-Negative Patients

Patients and Methods

RA Patients and Controls

More than a hundred (118) RA patients are selected from the Rheumatology Ward at Hospital La Conception, Marseille, France, as described above. One hundred controls (Patients with spondylarthropathy (AS) from the Rheumatology Ward at Hospital La Conception, Marseille and volunteers from the staffs of INSERM UMR639 and Marseille Blood Transfusion Center) were also tested.

Synthetic Peptides

Sixty five 20-mer peptides encompassing residues 1 to 663 from wild type PAD4 (NM_012387), 40 20-mer peptides encompassing residues 416 to 766 from BRAF (NP_004324.1) were synthesized using the solid phase system and purified (Neosystem, Strasbourg, France). Ninety-four 15-amino-acid peptides derived from calpastatin isoform A (locus NP_001035908), residues 1–708 were used.

In particular, PAD4 peptides: **22** (SEQ ID NO: 2), **61** (SEQ ID NO: 3) and **63** (SEQ ID NO: 4) are 20-mer peptides from locus NM_012387. BRAF peptides **P10** (SEQ ID NO: 7), **P16** (SEQ ID NO: 8) and **P25** (SEQ ID NO: 9) are 20-mer peptides from locus NP_004324.1. Calpastatin peptides **P'16** (SEQ ID NO: 11) and **P'28** (SEQ ID NO: 12) are 15-mer peptides from locus NP_001741.

Detection of Autoantibodies by ELISA

The procedure used is identical to that descried in Example II.

Statistical Analysis

The p-values were calculated using the Chi squareTest.

Results

Eight Peptides (3 PAD4-peptides, 3 BRAF-peptides and 2 Calpastatin-peptides) are Recognized by RA patients.

The Applicants have identified three linear peptides on PAD4 that are preferentially recognized by sera from patients with RA: peptides **22**, **61** and **63**; three linear peptides on BRAF that are recognized almost solely by RA patients: **P10**, **P16**, P25; and two linear peptides on calpastatin that are preferentially associated with RA patients: **P'16** and **P'28** (see Figure 9).

The Eight Peptides are Recognized by CCP-Negative Patients

These 8 peptides were recognized by the sera of CCP-negative patients (see Figure 10). The most efficient binders are **P10** of BRAF and **P'28** of calpastatin. **P10** of BRAF is recognized by 44% of CCP-negative patients and **P'28** of calpastatin by 34% of CCP-negative patients.

Combination of PAD4-, BRAF- and Calpastatin-peptides Recognizes RA in 72% of CCP-Negative Patients

For one autoantigen, the best combination to identify CCP-negative patients is BRAF peptides. The combination of **P10**, **P16** and **P25** of BRAF identifies 50% of CCP-negative patients. For two autoantigens, the combination of BRAF peptides and calpastatin peptides identifies 69% of CCP-negative patients. Finally, the combination of PAD4-, BRAF- and calpastatin-peptides identifies 72% of CCP-negative patients.

Conclusions

The present Applicants have found 8 peptides (3 peptides of PAD4, 3 peptides of BRAF and 2 peptides of calpastatin) allowing the identification of 72% of CCP-negative patients.

Other Embodiments

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope of the invention being indicated by the following claims.

Claims

1. An *in vitro* method for diagnosing rheumatoid arthritis in a subject, said method comprising steps of:

contacting a biological sample obtained from the subject with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form, wherein the at least one biomarker is selected from the group consisting of:

PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof, and

any combination thereof; and

detecting any biomarker-antibody complex formed,

wherein detection of a biomarker-antibody complex is indicative of rheumatoid arthritis in the subject.

2. The *in vitro* method of claim 1, said method comprising steps of:

contacting a biological sample obtained from the subject with at least two biomarkers for a time and under conditions allowing a biomarker-antibody complex to form, wherein the at least two biomarkers are selected from the group consisting of:

PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof,

calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and

any combination thereof; and

detecting any biomarker-antibody complex formed,

wherein detection of a biomarker-antibody complex is indicative of rheumatoid arthritis in the subject.

3. The *in vitro* method of claim 2, wherein the at least two biomarkers comprise three PAD4-peptides, three BRAF-peptides and two calpastatin-peptides and wherein the three PAD4-peptides comprise or consist of SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4, the three BRAF-peptides comprise or consist of SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9, and the two calpastatin-peptides comprise or consist of SEQ ID NO: 11 and SEQ ID NO: 12.

4. An *in vitro* method for detecting the presence of anti-PAD4 autoantibodies in a biological sample, said method comprising steps of:

contacting the biological sample with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form, wherein the at least one biomarker is a PAD4 peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, fragments thereof, and any combination thereof; and

detecting any biomarker-antibody complex formed,

wherein detection of a biomarker-antibody complex is indicative of the presence of anti-PAD4 autoantibodies in the biological sample.

5. An *in vitro* method for detecting the presence of anti-BRAF autoantibodies in a biological sample, said method comprising steps of:

contacting the biological sample with at least one biomarker for a time and under conditions allowing a biomarker-antibody complex to form, wherein the at least one biomarker is a BRAF-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, fragments thereof, and any combination thereof; and

detecting any biomarker-antibody complex formed,

wherein detection of a biomarker-antibody complex is indicative of the presence of anti-BRAF autoantibodies in the biological sample.

6. The *in vitro* method according to any one of claims 1-5, wherein the at least one biomarker is a peptide of less than 50 amino acids.

7. The *in vitro* method of any one of claims 1-6, wherein the subject is CCP-negative or the biological sample is obtained from a CCP-negative subject.

8. The *in vitro* method of any one of claims 1-7, further comprising measuring, in a biological sample obtained from said subject, the concentration of at least one marker selected from the group consisting of C-reactive protein, serum amyloid A, interleukin 6, S100 proteins, osteopontin, rheumatoid factor, matrix metalloprotease 1, matrix metalloprotease 3, hyaluronic acid, sCD14, angiogenesis markers, and products of bone, cartilage, or synovium metabolism.

9. A kit for the *in vitro* diagnosis of rheumatoid arthritis in a subject, said kit comprising:

at least two biomarkers selected from the group consisting of:

PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof,

calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and

any combination thereof; and

at least one reagent for detecting a biomarker-antibody complex formed between the biomarker and an autoantibody present in a biological sample obtained from the subject, wherein detection of a biomarker-antibody complex is indicative of rheumatoid arthritis in the subject.

10. A kit for the *in vitro* diagnosis of rheumatoid arthritis in a CCP-negative subject, said kit comprising:

at least eight biomarkers comprising:

three PAD4-peptides selected from the group consisting of PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

three BRAF-peptides selected from the group consisting of BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof, and

two calpastatin-peptides selected from the group consisting of calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof; and

at least one reagent for detecting a biomarker-antibody complex formed between the biomarker and an autoantibody present in a biological sample obtained from the subject, wherein detection of a biomarker-antibody complex is indicative of rheumatoid arthritis in the subject.

11. A kit for detecting the presence of anti-PAD4 autoantibodies in a biological sample, said kit comprising:

at least one biomarker, wherein the at least one biomarker is a PAD4-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, fragments thereof, and any combination thereof; and

at least one reagent for detecting a biomarker-antibody complex formed between the biomarker and an autoantibody present in the biological sample, wherein the autoantibody is an anti-PAD4 autoantibody that is indicative of rheumatoid arthritis.

12. A kit for detecting the presence of anti-BRAF autoantibodies in a biological sample, said kit comprising:

at least one biomarker, wherein the at least one biomarker is a BRAF-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, fragments thereof, and any combination thereof; and

at least one reagent for detecting a biomarker-antibody complex formed between the biomarker and an autoantibody present in the biological sample, wherein the autoantibody is an anti-BRAF autoantibody that is indicative of rheumatoid arthritis.

13. The kit of any one of claims 9-12, wherein the biomarker(s) is/are (a) peptide(s) of less than 50 amino acids.

14. An array for diagnosing rheumatoid arthritis in a subject, said array comprising, attached to its surface at least two biomarkers, wherein the at least two biomarkers are selected from the group consisting of:

PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof,

calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and

any combination thereof.

15. An array for diagnosing rheumatoid arthritis in a CCP-negative subject, said array comprising, attached to its surface at least eight biomarkers, wherein the at least eight biomarkers comprise:

three PAD4-peptides selected from the group consisting of PAD4-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, analogues thereof, and fragments thereof,

three BRAF-peptides selected from the group consisting of BRAF-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof, and

two calpastatin-peptides selected from the group consisting of calpastatin-peptides having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof.

16. The array of claim 14 or claim 15, wherein the biomarkers are peptides of less than 50 amino acids.

17. The array of any one of claims 14-16 further comprising, attached to its surface, at least one additional biomarker for detecting the presence of RA-specific autoantibodies in a biological sample, preferably antinuclear antibodies and anti-CCP antibodies.

18. A biomarker of rheumatoid arthritis, wherein the biomarker is a PAD4-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, analogues thereof, and fragments thereof, and wherein the biomarker is recognized by an anti-PAD4 autoantibody.

19. A biomarker of rheumatoid arthritis, wherein the biomarker is a BRAF-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, analogues thereof, and fragments thereof, and wherein the biomarker is recognized by an anti-BRAF autoantibody.

20. A biomarker of rheumatoid arthritis, wherein the biomarker is a calpastatin-peptide having an amino acid sequence comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, analogues thereof, and fragments thereof, and wherein the biomarker is recognized by an anti-calpastatin autoantibody.

21. A biomarker according to any one of claims 18-20, wherein the biomarker is a peptide of less than 50 amino acids.

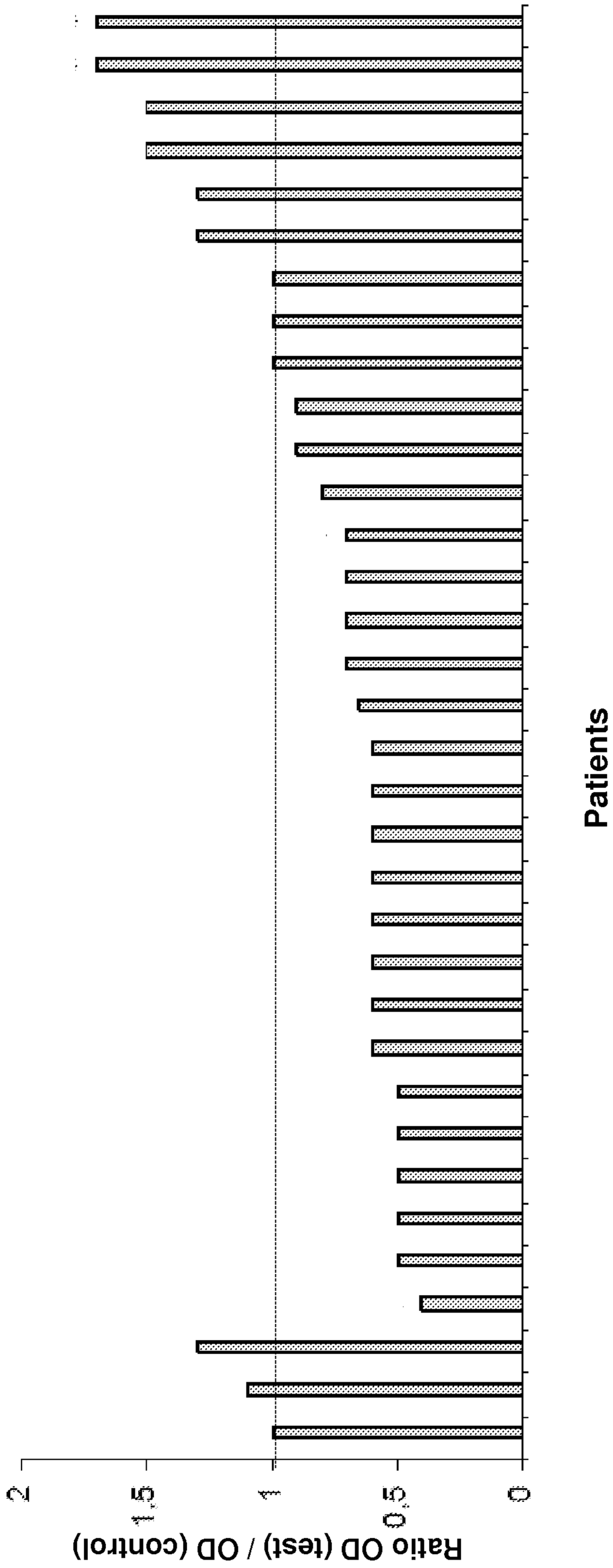


Figure 1

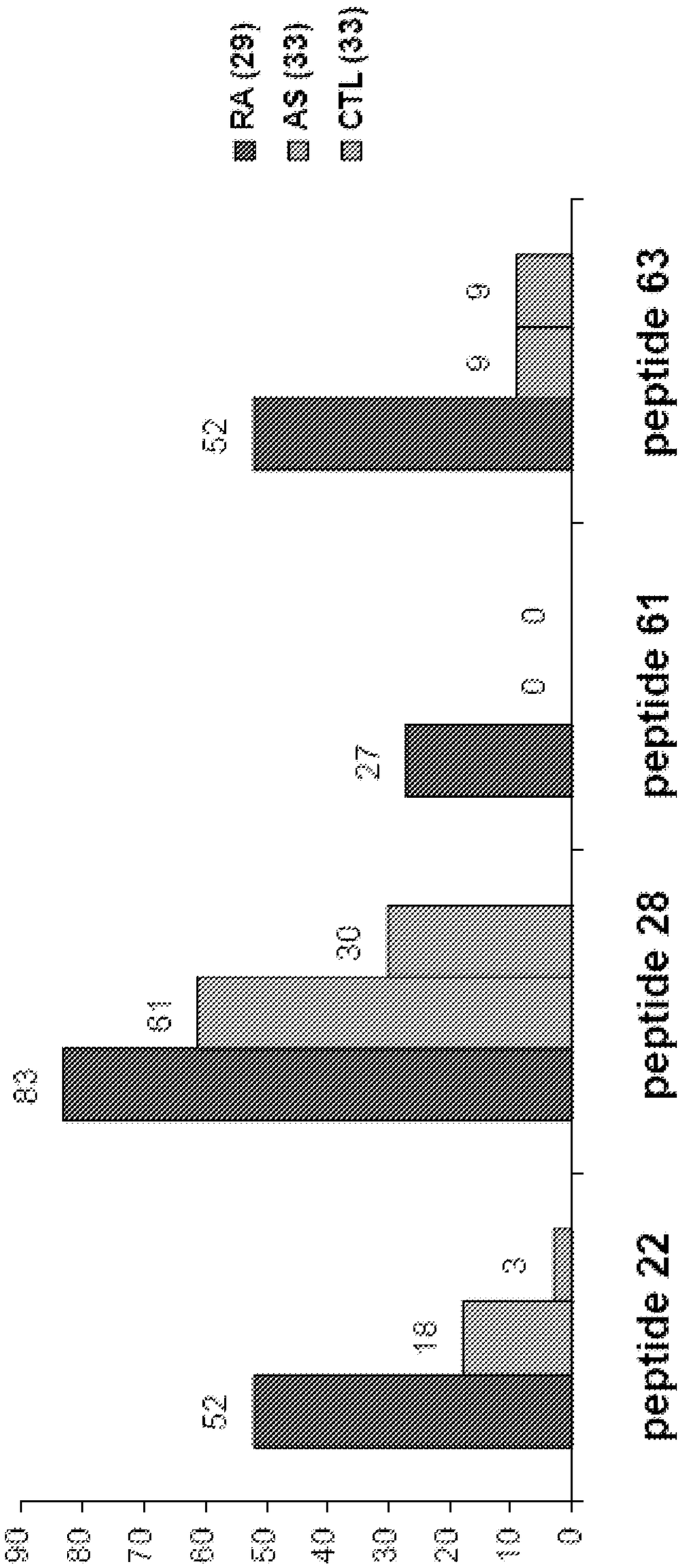

R4 (29)
AS (33)
CTL (33)
peptide 61
peptide 63
peptide 22
peptide 28
% of positive autoantibodies

Figure 2

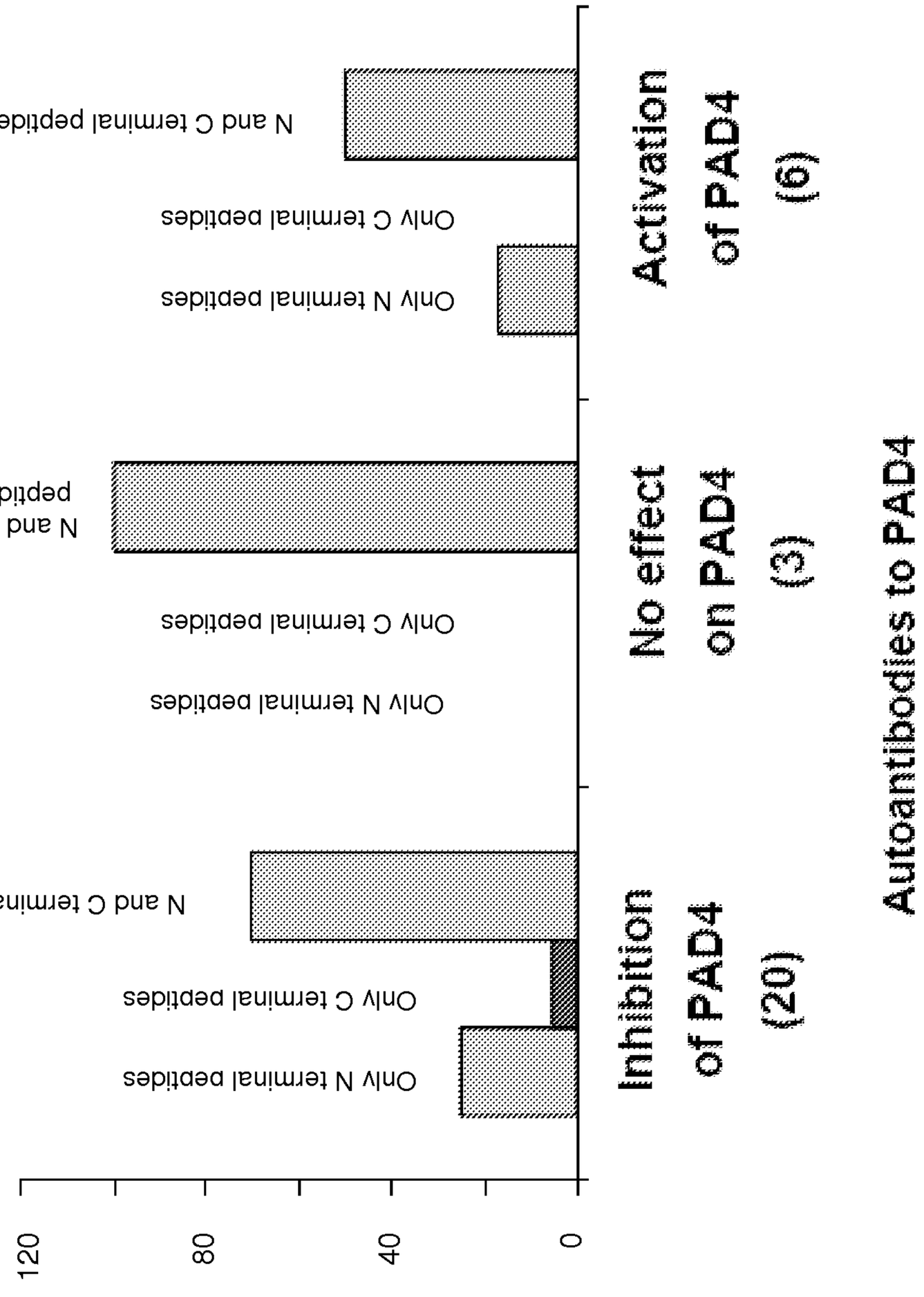

% of positive autoantibodies
0
40
80
120
Only N terminal peptides
Only C terminal peptides
N and C terminal peptides
Inhibition of PAD4 (20)
Only N terminal peptides
Only C terminal peptides
N and C terminal peptides
No effect on PAD4 (3)
Only C terminal peptides
N and C terminal peptides
Activation of PAD4 (6)
Only N terminal peptides
Only C terminal peptides
N and C terminal peptides
Autoantibodies to PAD4

Figure 3

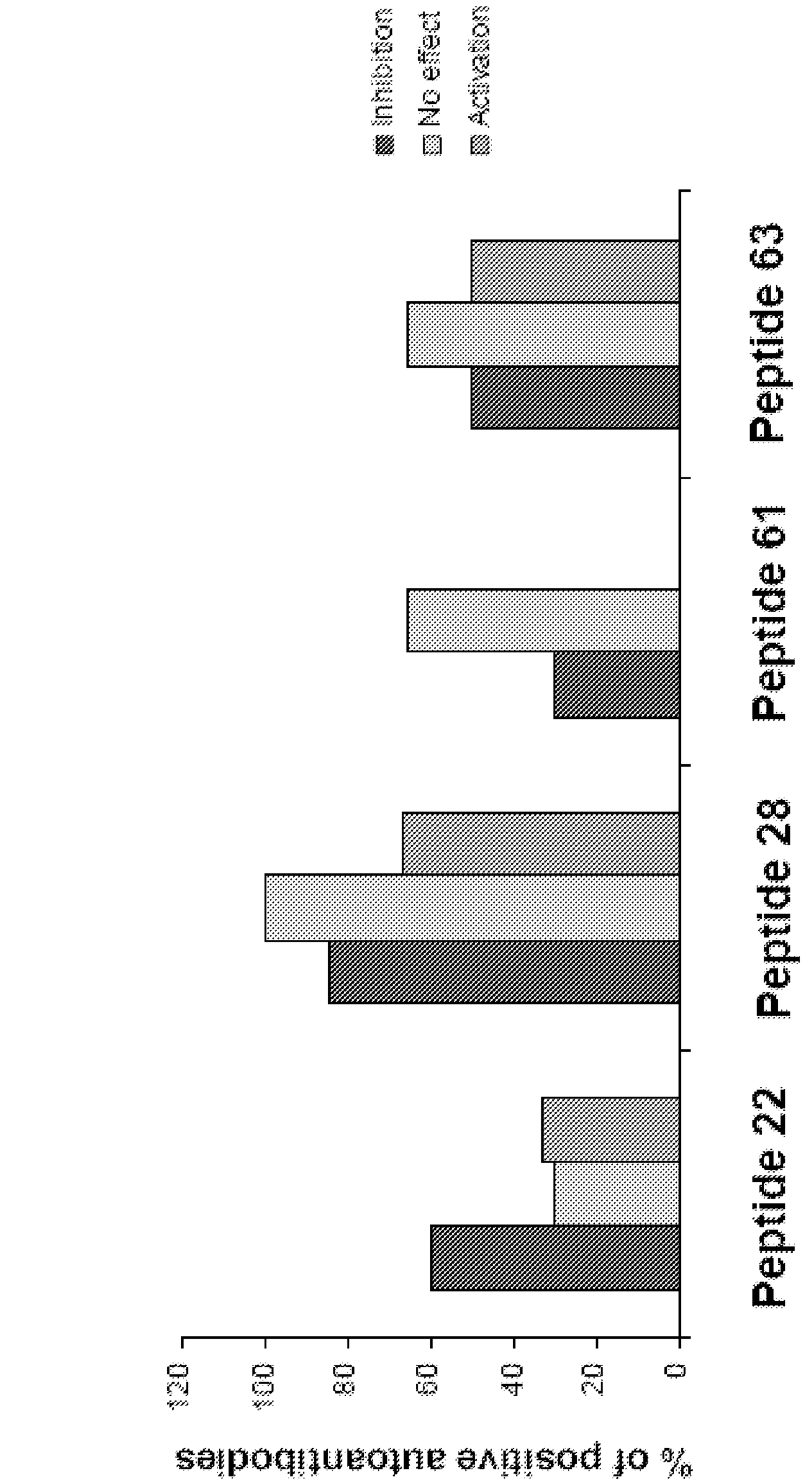
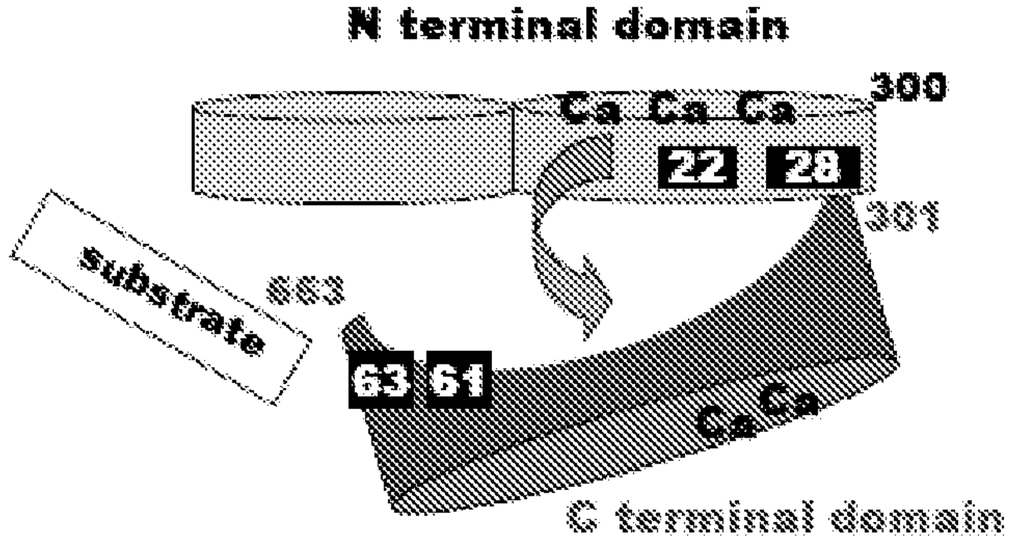
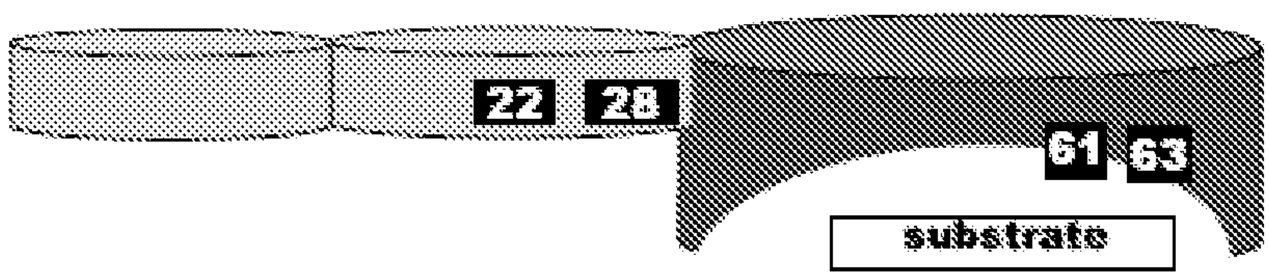

Inhibition
No effect
Activation
Peptide 63
Peptide 61
Peptide 28
Peptide 22
0
20
40
60
80
100
% of positive autoantibodies

Figure 4

A


N terminal domain
Ca Ca Ca
300
22
28
301
substrate
663
63
61
Ca Ca
C terminal domain

B


22
28
61
63
substrate

C

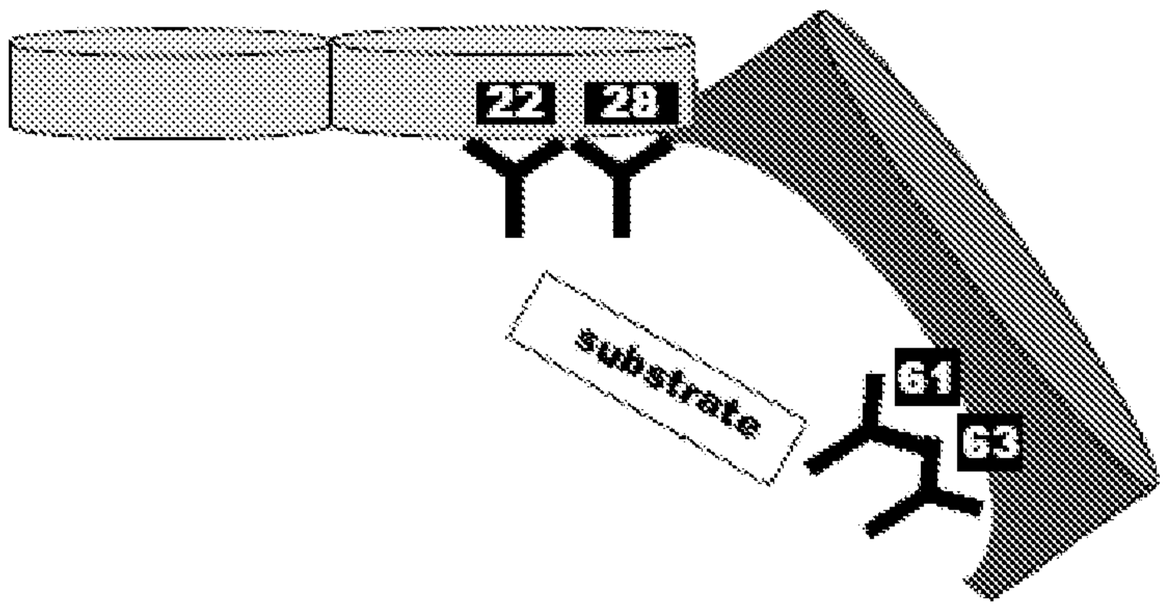

22
28
substrate
61
63

Figure 5

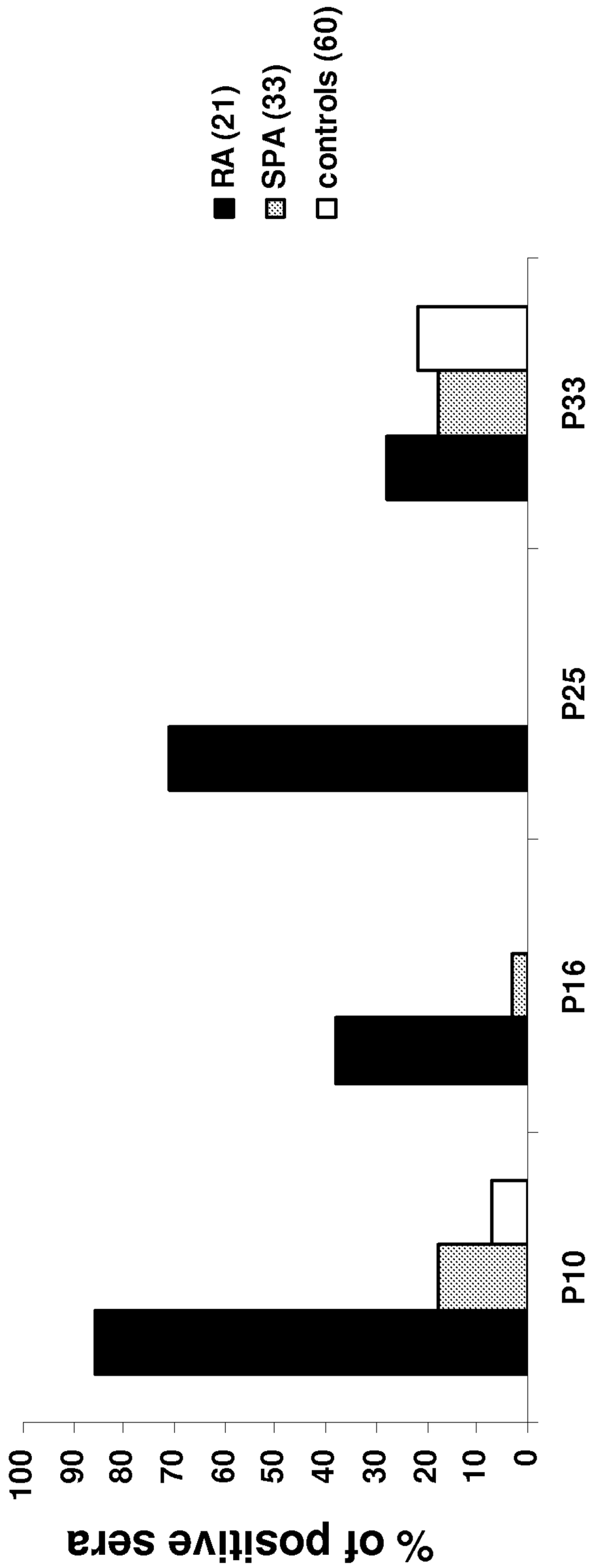

RA (21)
SPA (33)
controls (60)
P33
P25
P16
P10
100
90
80
70
60
50
40
30
20
10
0
% of positive sera

Figure 6

RA	CCP	BRAF peptides P10	P16	P25
RA1	neg			
RA2	neg			
RA3	neg			
RA4	neg			
RA5	neg			
RA6	neg			
RA7	neg			
RA8	neg			
RA9	neg			
RA10	neg			
RA11	neg			
RA12	neg			
RA13	neg			
RA14	neg			
RA15	neg			
RA16	neg			
RA17	neg			
RA18	neg			
RA19	neg			
RA20	neg			
RA21	neg			
RA22	neg			
RA23	neg			
RA24	neg			
RA25	neg			
RA26	neg			
RA27	neg			
RA28	neg			
RA29	neg			

Figure 7

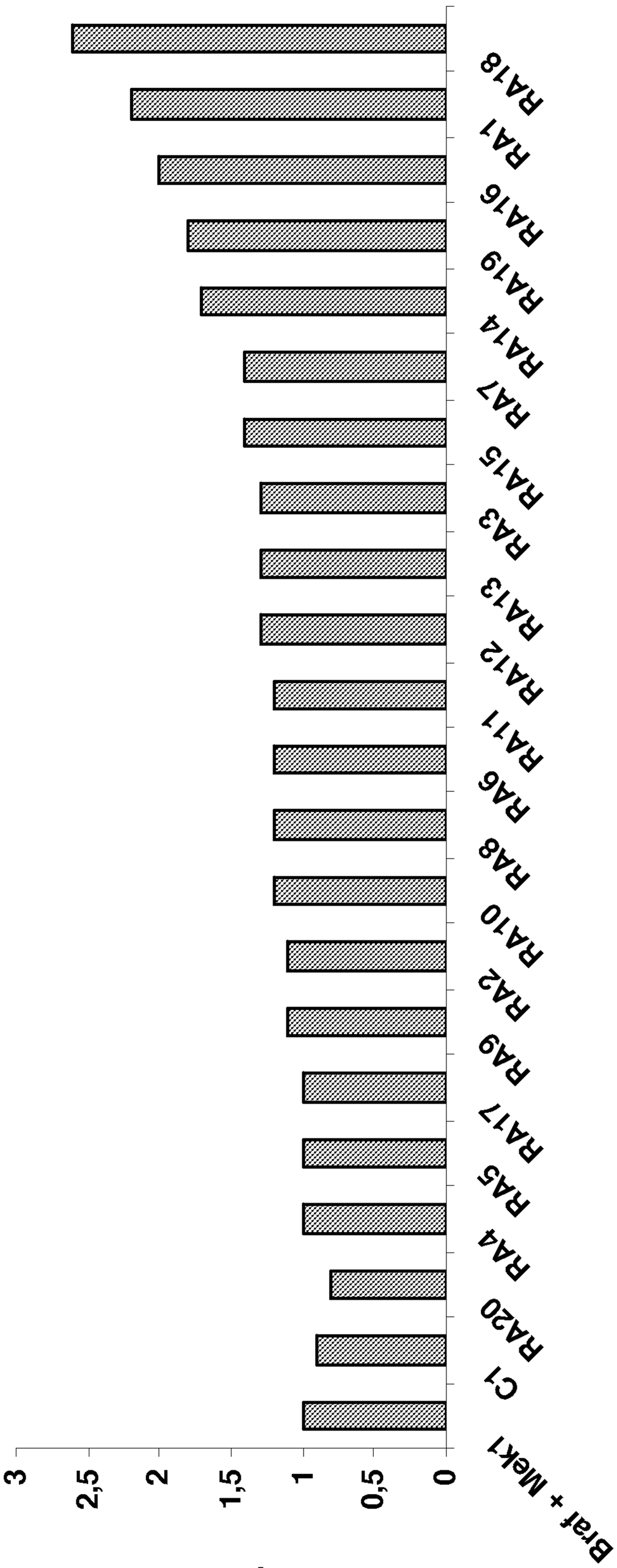

ratio test OD/ positive control OD
0
0,5
1
1,5
2
2,5
3
Braf + MeK1
C1
RA20
RA4
RA5
RA17
RA9
RA2
RA10
RA8
RA6
RA11
RA12
RA13
RA3
RA15
RA7
RA14
RA19
RA16
RA18

Figure 8

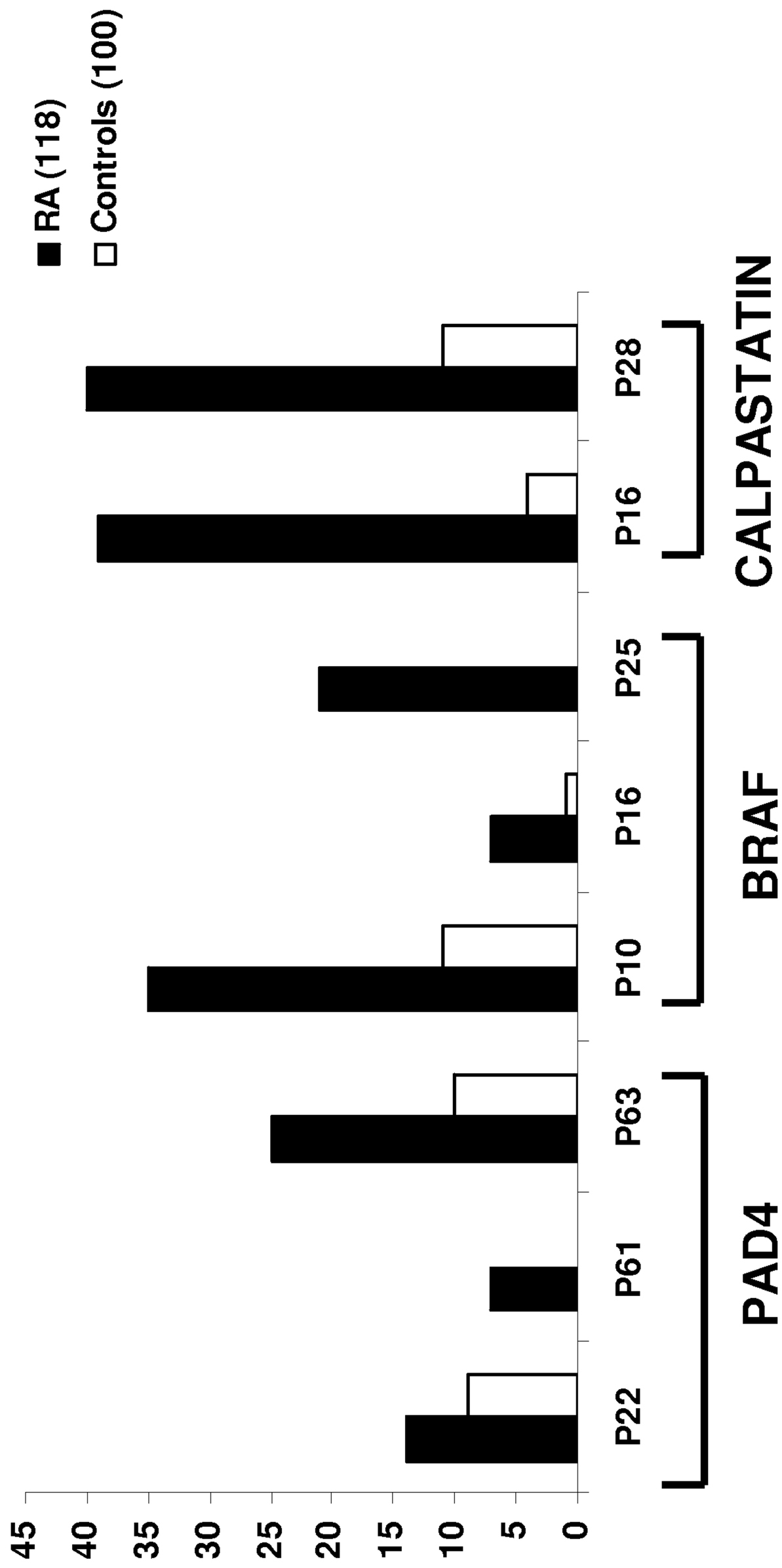

ANCA (118)
Controls (100)
CALPASTATIN
BRAF
PAD4
P28
P16
P25
P16
P10
P63
P61
P22
45
40
35
30
25
20
15
10
5
0
% of positive sera

Figure 9

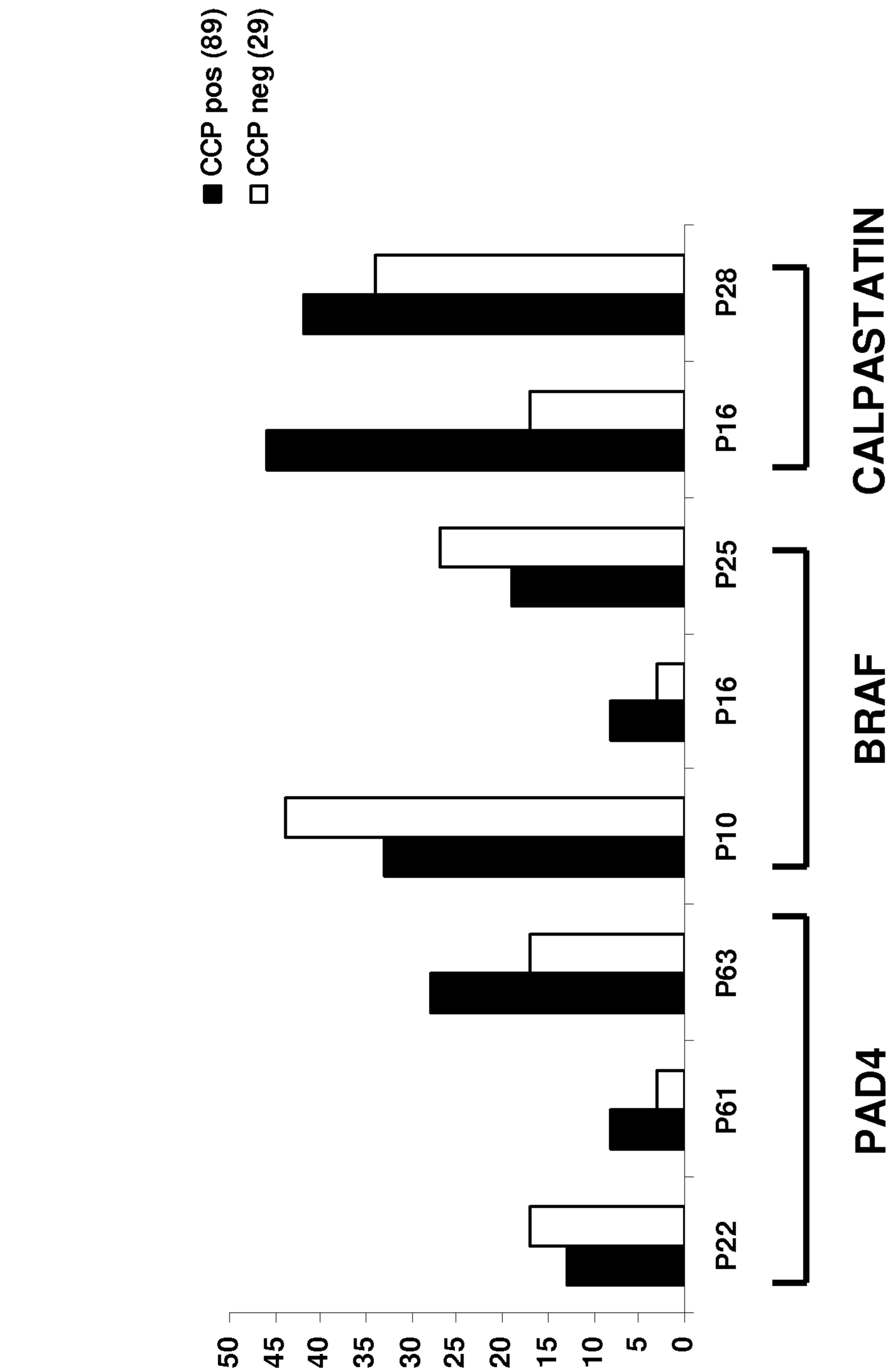

■ CCP pos (89)
□ CCP neg (29)
P28
P16
P25
P16
P10
P63
P61
P22
CALPASTATIN
BRAF
PAD4
50
45
40
35
30
25
20
15
10
5
0
% of positive sera

Figure 10

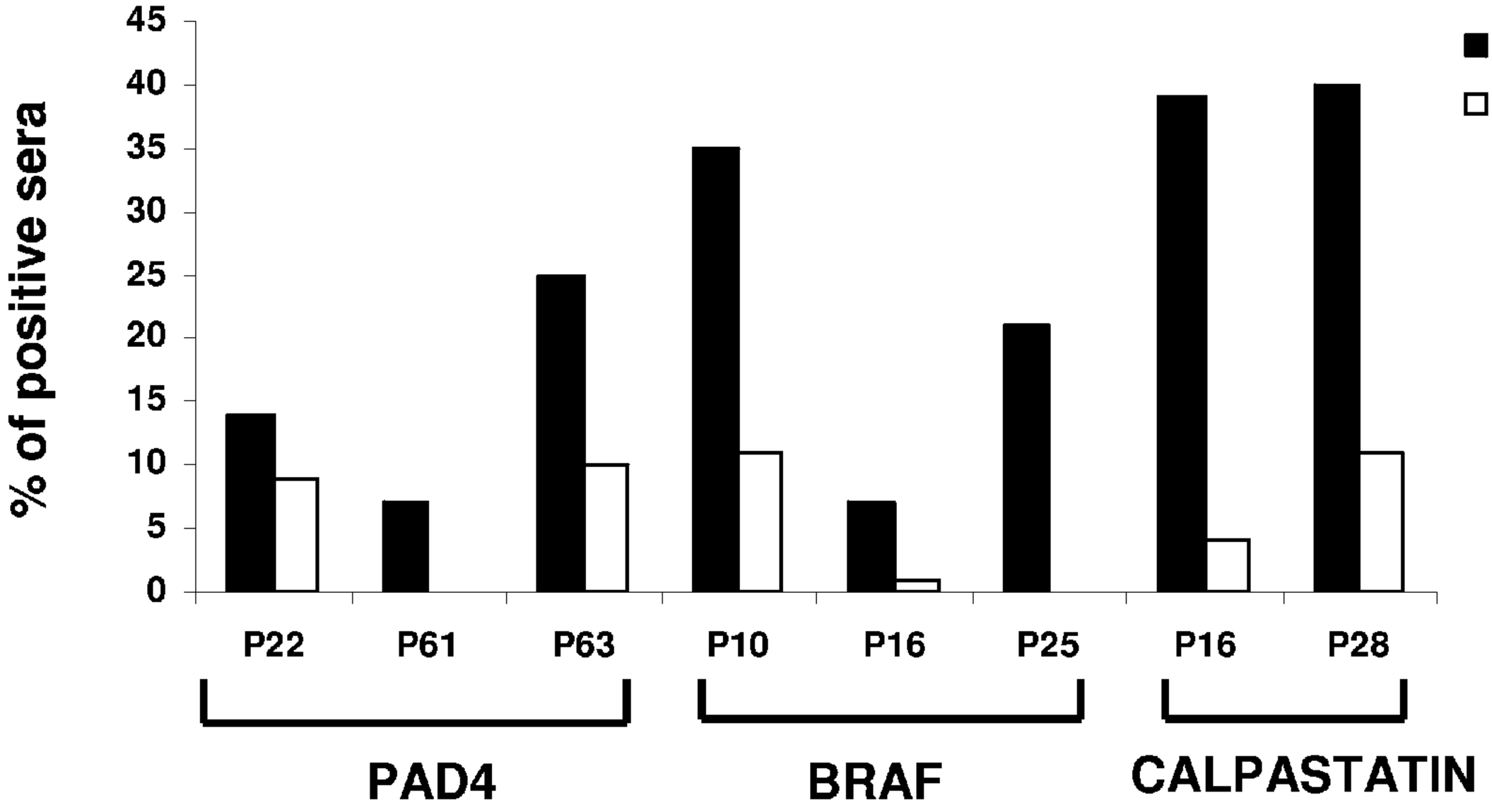

% of positive sera
45
40
35
30
25
20
15
10
5
0
RA (118)
Controls (100)
P22
P61
P63
P10
P16
P25
P16
P28
PAD4
BRAF
CALPASTATIN

Figure 9