

**PEPTIDES FOR USE IN DIAGNOSING THE PRESENCE
OF RUPTURED ATHEROSCLEROTIC LESIONS IN AN INDIVIDUAL**

5 The present invention relates to peptides that interact with ruptured atherosclerotic plaque-associated antibodies in an individual and to their use in diagnostic reagents and diagnostic assays for determining the presence in an individual of ruptured atherosclerotic lesions.

10 Atherosclerosis is a chronic inflammatory disease of the arteries and is characterized by the accumulation of lipids, cells, cellular waste products, extracellular matrix and calcification in the inner lining (the intima) of an artery. This buildup forms the so-called atherosclerotic plaque.

15 Cardiovascular disease, currently the leading cause of death and illness in Western Society, will soon become the pre-eminent health problem worldwide. The vast majority of acute clinical manifestations including Myocardial Infarction (MI) and stroke are not due to progressive luminal narrowing due to the presence of a slowly growing, stable,
20 atherosclerotic plaque, but are caused by the formation of an occluding thrombus on the surface of an unstable plaque.

25 Morphological criteria to discriminate between stable and unstable plaques are well-described and include the presence of a large lipid core, a thin fibrous cap, erosion of the endothelium and the presence of an inflammatory process inside the fibrous cap or the shoulder region of unstable plaques.

30 The major limitation of these morphological criteria is that they can only be applied after an interventional procedure. As a result, markers to identify unstable or ruptured plaques non-invasively and a fast, sensitive and specific *in vitro* test for the identification of patients having ruptured or rupture prone lesions is not available.

Despite the increasing knowledge on the pathogenesis of atherosclerosis and the suggestion that several circulating markers of inflammation, such as C-reactive protein, fibrogen and interleukins, are associated with cardiovascular disease, biomarkers for non-invasive diagnosis of ruptured atherosclerotic lesions and a high prognostic value for the identification of individual patients at high risk of future cardiovascular events are not readily available.

It is therefore the object of the present disclosure to provide such biomarkers for non-invasive diagnosis of ruptured atherosclerotic lesions.

In the research that led to the present disclosure, peptides were identified that bind specifically to antibodies that are present in sera of individuals suffering from ruptured plaques.

For the identification of these peptides, a cDNA expression library was used. To create an expression library of cDNAs predominantly expressed in ruptured atherosclerotic lesions, a SSH library of >3000 cDNAs preferentially expressed in ruptured plaques (as described by Faber et al. (Circ Res. 89:547-554 (2001)) was re-cloned and the cDNAs were expressed as fusion proteins with the minor coat protein pVI of filamentous phage M13. To enrich for peptides that bind specifically to antibodies in pooled sera derived from patients known to suffer from ruptured plaques (identified herein as "ruptured sera"), four successive rounds of Serologic Antigen Selection (SAS) were performed. Subsequently, the individual phage clones were tested in ELISA for interaction with sera from individuals suffering from ruptured atherosclerotic plaques.

While the majority of the phage-displayed peptides showed a positive reaction in both pooled sera from patients with

ruptured atherosclerotic lesions and pooled sera from patients with stable plaques and healthy controls, the positive reaction of four clones could distinguish pooled sera from patients with ruptured lesions from the sera from patients with stable plaques and control sera (See **Figure 10**).

Sequence analysis revealed that these four clones represented two different antigenic peptides. The inventors then further focussed on the two antigens, E1 (**Figure 1**) and E12 (**Figure 2**), that were only reactive with ruptured sera. Clones E1 and E12 were tested on a large panel of individual sera (n= 38 for ruptured sera, n=23 for stable sera and n=10 for normal sera).

Clone E1 showed significant reactivity (ratio mean OD₄₅₀ sample/(mean OD₄₅₀ empty phage + 3*SD) > 1) to 22 out of 38 ruptured sera (58%) whereas no reactivity towards the stable and age and sex matched control sera tested was observed.

In addition, clone E12 showed enhanced reactivity in 15 out of 38 patient sera (40%), while none of the stable and control sera tested positive (see **Figures 11A and B**).

Combination of the serum reactivity against clones E1 and E12 even further increased the sensitivity to 74%, while the specificity remained at 100% (**Figure 11C**).

To determine whether the observed positive reaction of ruptured sera was indeed due to the ruptured plaque derived sequences, five ruptured sera were pre-incubated with the synthetic peptides Ac-E1-Lys-NH₂ (Ac-VRGFTMLTRLVLNLK-NH₂) and Ac-E12-Lys-NH₂ (Ac-VHIIRSSLIYALFTRISISNYK-NH₂), representing the cDNA inserts of clones E1 and E12, and with a non-specific peptide.

As shown in **Figure 12A**, pre-incubation with peptides Ac-E1-Lys-NH₂ and Ac-E12-Lys-NH₂, clearly inhibited the formation of specific IgG antibody/phage E1 and IgG antibody/phage E12

complexes. In contrast, the serum reactivity against clones E1 and E12 was not inhibited by addition of the random peptide.

Since testing the sera against the combination of clones E1 and E12 raised the sensitivity of the assay to 74%, it was tested whether inclusion of additional phage-displayed peptides would further increase the sensitivity. To this end, a panel of 192 randomly chosen recombinant phages from selection round 4 was tested with six ruptured, six stable and six control sera. Of the 192 clones tested, 118 phage-displayed peptides showed a positive reaction with at least one ruptured serum, while 106 clones interacted solely with one or more ruptured sera.

The panel of 106 clones comprised seven different recombinant phages that led to 100% sensitivity and 100% specificity for the identification of ruptured sera. **Table 2** lists the additional peptides that specifically react with sera from patients suffering from ruptured plaques. The sequences are also shown in **Figures 3-7**.

Table 2

clone	amino acid sequence
mp 1 (SEQ ID NO:3)	GSGGGSGGGQVGQIF
mp23 (SEQ ID NO:4)	RYKDFVNVIQGPQHPCVCRVPSKLSRSSRCQPVNFPPYPRI CYSPNHFPVSEQ
mp36 (SEQ ID NO:5)	REFITRYV
mp64 (SEQ ID NO:6)	EFRAGTTALVLDYWNNIQPRTEKLNRLTIVCSLD GLTASHLPEPFQNEQRSPPHHYVFFFDKFRREEMC VPRGGEPDHTGSRVPPKKKN
mp179 (SEQ ID NO:7)	NQKLNTIGNW

In one aspect, there is provided peptides that interact with ruptured atherosclerotic plaque-associated antibodies in an

individual, the peptides comprising an immunoreactive part of one of the following amino acid sequences:

SEQ ID NO:1: VRGFTMLTRLVLNL
 SEQ ID NO:2: VHIIRSSLIYALFTRISISNY
 5 SEQ ID NO:3: GSGGGSGGGQVGQIF
 SEQ ID NO:4: RYKDFVNVIQGPQHPCVCRVPSKLSRSSRCQPVN
 FPPYPRICYSPNHFPVSFQ
 SEQ ID NO:5: REFITRYV
 SEQ ID NO:6: EFRAGTTALVLDYWNNIQPRTEKLNRLTIVCSLD
 10 GLTASHLPEPFQNEQSPHHYVFFFDKFRREEMC
 VPRGGEPDHTGSRVPPKKKN
 SEQ ID NO:7: NQKLNTIGNW

In a further embodiment, the present disclosure relates to antigens, comprising an immunoreactive part of one of the following amino acid sequences:

15 SEQ ID NO:8: GQVRGFTMLTRLVLNL
 SEQ ID NO:9: GQVHIIRSSLIYALFTRISISNY
 SEQ ID NO:10: SSREFITRYV
 SEQ ID NO:11: SSREFRAGTTALVLDYWNNIQPRTEKLNRLTIVCSL
 20 DGLTASHLPEPFQNEQSPHHYVFFFDKFRREEMCVPRG
 GEPDHTGSRVPPKKKN

In another embodiment, the present disclosure relates to antigens, comprising an immunoreactive part of one of the following amino acid sequences:

25 SEQ ID NO:12: GSGGGSGGGPSRPDLLENSGQVRGFTMLTRLVLNL
 SEQ ID NO:13: GSGGGSGGGPSRPDLLENSGQVHIIRSSLIYALFTR
 SISNY
 SEQ ID NO:14: GSGGGSGGGPSRPDLLENSGSGGGSGGGQVGQIF
 SEQ ID NO:15: GSGGGSGGGPSRPDLLENSRYKDFVNVIQGPQHPCV
 30 CRVPSKLSRSSRCQPVNFPPYPRICYSPNHFPVSFQ
 SEQ ID NO:16: GSGGGSGGWAKSARSSREFITRYV
 SEQ ID NO:17: GSGGGSGGWAKSARSSREFRAGTTALVLDYWNNIQPR

RTEKLNRLTIVCSLDGLTASHLPEPFQNEQRSPHHYVFF
FDKFRREEMCVPRGGEPDHTGSRVPPKKKN

SEQ ID NO:18: **GSGGGSGGGPSRPDLLENS**NQKLNTIGNW

In a still further embodiment, the present disclosure
relates to antigens, comprising an immunoreactive part of one
of the following amino acid sequences:

SEQ ID NO:19: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^GKIA
IAISLFLALIIGLNSILVGYLSDISAQLPSDFVQGVQLI
LPSNALPCFYVILSVKAAIFIFDVKQKIVSYLDWDK**GSG**
GSGGGPSRPDLLENSGQVRGFTMLTRLVLNL

SEQ ID NO:20: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^GKIA
IAISLFLALIIGLNSILVGYLSDISAQLPSDFVQGVQLI
LPSNALPCFYVILSVKAAIFIFDVKQKIVSYLDWDK**GSG**
GSGGGPSRPDLLENSGQVHIIRSSLIYALFTRISINY

SEQ ID NO:21: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^G
KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
KIVSYLDWDK**GSGGGSGGGPSRPDLLENS**GSGGG
SGGGQVGQIF

SEQ ID NO:22: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^G
KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
KIVSYLDWDK**GSGGGSGGGPSRPDLLENS**RYKDF
VNVIQGPQHPCVCRVPSKLSCSSRCQPVNFPPYP
RICYSPNHFPVSFQ

SEQ ID NO:23: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^G
KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
KIVSYLDWDK**GSGGGSGGWAKSARS**SREFITRYV

SEQ ID NO:24: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGF^G
KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ

KIVSYLDWDK**GSGGGSGGWAKSARSSREFRAGTT**
 ALVLDYWNNIQPRTEKLNRLTIVCSLDGLTASHL
 PEPFQNEQQRSPHHYVFFFDKFRREEMCVPRGGEP
 DHTGSRVPPKKKN
 SEQ ID NO:25: MPVLLGIPLLLRLFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
 KIVSYLDWDK**GSGGGSGGGPSRPDLLENSNQKLN**
 TIGNW

In another aspect, there is provided peptides as defined above for use in diagnosis.

In still another aspect, there is provided the use of one or more of the peptides as defined above for the preparation of a diagnostic reagent.

In yet another aspect, there is provided a diagnostic reagent for the detection of ruptured atherosclerotic lesions in an individual, characterized in that said reagent comprises at least one peptide, comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 1-25.

In a further aspect, there is provided an *in vitro* method for detecting antibodies that are indicative of the presence of ruptured atherosclerotic plaques in an individual, the method comprising detecting, in a sample taken from said individual, antibodies that form an immune complex with at least one amino acid sequence selected from the group consisting of SEQ ID NOS:1-25.

In still a further aspect, there is provided a test kit for the diagnosis of a sample from an individual for the presence therein of antibodies that are indicative of the presence of ruptured atherosclerotic plaques in the individual, characterized in that said test kit comprises a diagnostic reagent as defined above and optionally means for visualizing the formation of immune complexes.

The part MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFGKIAIAISLFLALIIGLN
 SILVGYLSDISAQLPSDFVQGVQQLILPSNALPCFYVILSVKAAIFIFDVKQKIVSYLDWDKG
SGGGSGGGPSRPDLLENS in SEQ ID NOS: 19, 20, 22, 23 and 25 is
 derived from the pVI part and linker of pSP6B. The linker
 sequence is shown in bold. Amino acids shown in italics are
 derived from an artificial cloning sequence. The part
 MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFGKIAIAISLFLALIIGLNSILVG
 YLSDISAQLPSDFVQGVQQLILPSNALPCFYVILSVKAAIFIFDVKQKIVSYLDWDKG**SGGGGS**
GGWAKSAR in SEQ ID NOS 23-24 is derived from the pVI part and
 linker of vector pSP6C. The linker sequence is shown in bold.
 Amino acids shown in italics are derived from an artificial
 cloning sequence.

"Immunoreactive part" is defined herein as a part of the
 amino acid sequence according to SEQ ID NOS: 1-25 that shows
 enhanced reactivity to ruptured serum as compared to its
 reactivity with stable and normal serum. Such immunoreactive
 parts can be identified without undue burden with well-known
 techniques such as the PepScan™ method.

"Enhanced reactivity" as used herein is in general any
 statistically significant difference between the reaction of a
 peptide according to the invention with ruptured sera and the
 reaction of the same peptide with stable and/or normal sera.

More in particular "enhanced reactivity" means that the ratio (mean OD₄₅₀ sample / (mean OD₄₅₀ empty phage + X*SD) is > 1. X is in particular more than one, more in particular at least 2, more in particular 3 or more.

5 "Ruptured serum" is serum from an individual or pooled serum of multiple individuals that have ruptured plaques.

"Stable serum" is serum from an individual or pooled serum of multiple individuals that have stable plaques but no evidence of the presence of ruptured plaques. "Normal serum" is serum
10 from an individual or pooled serum of multiple individuals that do not have clinical relevant cardiovascular disease.

Peptides of the invention comprise an amino acid sequence that is structurally identical to or functionally similar to an immunoreactive part of the amino acid sequences according
15 to SEQ ID NOS: 1-25 or conservative variants thereof. The peptides may comprise further amino acids in addition to the amino acids that constitute the immunoreactive part and that may or may not contribute to the immunoreactivity of the complete peptide or may be present to facilitate binding of
20 the peptides to a solid support, enable labelling or for other purposes. The amino acids that constitute the immunoreactive part are not necessarily consecutive amino acids.

Conservative variations of the peptides are also part of the present invention. The term "conservative variation" as
25 used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine or methionine, for another, or the substitution of one polar
30 residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like.

The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid and/or of a non-natural amino acid in place of a natural amino acid provided that antibodies to the peptide comprising the substituted and/or natural amino acid(s) also immunoreact with the peptide comprising the unsubstituted and/or non-natural amino acid(s). By using a routine screening method, such as by testing a conservative variant antigen with sera from a patient that is known to suffer from ruptured plaques, one of skill in the art can readily determine if the variant peptide has the requisite biological activity of the peptide of the invention without having to resort to undue experimentation.

According to a further aspect thereof the invention relates to a diagnostic reagent for the detection of ruptured atherosclerotic lesions in an individual, wherein said reagent comprises at least one peptide, said peptide comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS:1-25.

In preferred embodiments of the invention, the diagnostic reagent comprises combinations of peptides to increase the sensitivity as compared to the use of only one peptide. Suitable combinations are peptides comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS:1, 8, 12 and 19 with peptides comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS:2, 9, 13 and 20. In a further embodiment one or more peptides are added to this mixture that comprise an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS:3-7, 10-11, 14-18 and 21-25.

Alternatively, a peptide comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 1, 8, 12 and 19 is combined with one or more peptides that comprise an immunoreactive part of the amino acid
5 sequence as shown in any one of the SEQ ID NOS: 3-7, 10-11, 14-18 and 21-25.

In another alternative embodiment, a peptide comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 2, 9, 13 and 20 is combined with,
10 one or more peptides that comprise an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 3-7, 10-11, 14-18 and 21-25.

Each combination of peptides can furthermore comprise more than one peptide that comprises an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS:
15 1, 8, 12 and 19, and/or more than one peptide that comprises an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 2, 9, 13 and 20 and/or more than one peptide that comprises an immunoreactive part of the amino
20 acid sequence as shown in any one of the SEQ ID NOS: 3-7, 10-11, 14-18 and 21-25.

The above combinations of peptides can be considered a panel of peptides that can be used for determining a fingerprint of antibodies present in an individual suffering
25 from ruptured atherosclerotic lesions.

According to the invention, a sample is positive when there is a reaction between antibodies in the sample to be tested with at least one peptide of the invention.

The peptides of the invention can be isolated peptides
30 that comprise at least the immunoreactive part of any one of the SEQ ID NOS: 1-25. However, these peptides or immunoreactive parts thereof may still be present in the

phage. Thus the antigen used for diagnosing the presence in an individual of ruptured atherosclerotic lesions may be isolated peptide but also phage-displayed peptide or phage lysate containing peptide of the invention. The phage may be any
5 filamentous phage and is in particular M13 or a variant thereof.

According to a further aspect thereof the invention relates to a method for diagnosing a sample from an individual for the presence therein of antibodies that are indicative of
10 the presence of ruptured atherosclerotic plaques in the individual, wherein the sample is contacted with the peptide or the diagnostic reagent and immune complexes formed between said peptide or reagent and the antibodies are detected.

In a further embodiment the invention relates to a test
15 kit for the diagnosis of a sample from an individual for the presence therein of antibodies that are indicative of the presence of ruptured atherosclerotic plaques in the individual, wherein the test kit comprises one or more antigens or the diagnostic reagent of the invention and
20 optionally means for visualizing formation of immune complexes.

The sample from the individual to be tested can be a body fluid, such as blood, serum, plasma etc. or a tissue sample, in particular atherosclerotic plaque tissue. Antibody present in the sample will bind to the antigen. After removal of the
25 unbound antigen the antigen-antibody complex can be determined and quantitated by means of various known techniques.

In a first embodiment, a predetermined quantity of antigen (which is the peptide or diagnostic reagent) is adsorbed on a solid phase protein-binding surface. The test
30 sample to be assayed for antibodies is then contacted with the surface having the antigen bound thereto. Antibodies in the test sample bind to the immobilized antigen thus forming an

antigen-antibody complex. The complex is subsequently detected by any detecting agent such as an antibody-enzyme conjugate that binds to the immobilized antibody in the antigen-antibody complex. A substrate that changes colour when acted on by the enzyme is used to quantify the amount of bound antibody-enzyme conjugate, which is indicative of the antibody concentration in the test sample. Alternatively, the detecting agent that binds to the antibody in the antigen-antibody complex is a radioactively labelled agent, in particular an antibody. The amount of bound detecting agent is then quantitated by means of the amount of bound radioactivity.

In another embodiment immunocomplexes between the antigen and the antibodies to be detected are formed and then separated from non-bound antigen and antibodies, for example by capturing the complexes on a solid support, and quantitated.

A solid support can be any solid support that is used in diagnostic assays of the type described above and can be for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH).

In a particular embodiment, a diagnostic reagent of the invention may thus comprise one or more peptides in any form, i.e. as isolated peptides, as phage-displayed peptides, as peptides in phage lysates etc., and a suitable support. A test kit of the invention comprises in a particular embodiment such diagnostic reagent and a labelling substance for visualizing the immunocomplexes between the peptides of the invention and

the ruptured-plaque associated antibodies in the sera to be tested.

Labelling substances which can be used are, inter alia, a radioactive isotope, a fluorescent compound, a chemiluminescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle, etc. Examples of enzymatic labelling systems comprise the peroxidase/anti-peroxidase (PAP) system, the alkaline phosphatase/anti-alkaline phosphatase (APAAP) system, the avidin-biotin and biotin-streptavidin system.

Depending on the nature and further characteristics of the reagents, the immunochemical reaction that takes place is a so-called sandwich reaction, an agglutination reaction, a competition reaction, an inhibition reaction or other assay formats. These assay formats are well-known in the art.

The techniques for detecting the formation of immunocomplexes in a sample are well known and all fall within the scope of this invention which is based on the use of the newly identified peptides that bind specifically to ruptured plaque-associated antibodies. "Ruptured plaque-associated antibodies" as used herein are antibodies that are present in serum from an individual that suffers from ruptured atherosclerotic plaques and can be detected therein by binding to the peptides of the invention. Normal serum or serum from an individual having stable plaques does not have a significant detectable amount of antibodies that specifically bind to these peptides.

The present invention will be further illustrated in the examples that follow and that are not intended to limit the invention in any way. In the description and examples reference is made to the following figures.

Figure 1A shows the nucleotide sequence of clone E1.

Figure 1B shows the amino acid sequence of clone E1.

Figure 1C shows the full length fusion protein of clone E1.

Figure 2A shows the nucleotide sequence of clone E12.

5 **Figure 2B** shows the amino acid sequence of clone E12.

Figure 2C shows the full length fusion protein of clone E12.

Figure 3 shows the full length fusion protein of clone mp1.

10 **Figure 4** shows the full length fusion protein of clone mp23.

Figure 5 shows the full length fusion protein of clone mp36.

15 **Figure 6** shows the full length fusion protein of clone mp64.

Figure 7 shows the full length fusion protein of clone mp179.

20 **Figure 8** shows the nucleotide sequence and amino acid sequence of the empty phage display vector pSP6B with the pVI sequence, the linker and the multiple cloning sequence.

Figure 9 shows the nucleotide sequence and amino acid sequence of the empty phage display vector pSP6C with the pVI sequence, the linker and the multiple cloning sequence.

25 **Figure 10** shows the reactivity of different antigens to pooled ruptured serum. Reactivity is represented as the ratio of mean OD₄₅₀ sample/(mean OD₄₅₀ empty phage + 3*SD). # = clones with identical inserts.

30 **Figure 11** shows the serum reactivity to clone E1 (A) and clone E12 (B). **Figure 11C** shows the sensitivity and specificity of clones E1 and E12.

Figure 12 shows the ability of synthetic peptides Ac-E1-Lys-NH₂ and Ac-E12-Lys-NH₂ to deplete human ruptured sera from

antibodies directed against phage E1(A) and E12 (B), respectively. Reactivity is represented as the ratio of mean OD₄₅₀ sample/(mean OD₄₅₀ empty phage + 3*SD).

EXAMPLES

EXAMPLE 1

ELISA of antigens of the invention on pooled plasma

Two clones of a phage displayed ruptured plaque enriched cDNA expression library were used in an ELISA to test the reactivity of these clones with pooled patient sera. To select the clones an expression library of cDNAs predominantly expressed in ruptured atherosclerotic lesions, a SSH library of over 3000 ruptured plaque enriched cDNAs (described in Faber et al. Circ Res. 89:547-554 (2001)) was used. This library was re-cloned into the phage display vectors pSP6A, B and C (Hufton et al. J Immunol Methods. 231: 39-51 (1999)). Using these vectors, the SSH cDNA fragments are expressed as a fusion protein with the minor coat protein pVI of filamentous phage M13 in all reading frames.

After various rounds of selection with pooled sera from patients known to suffer from ruptured atherosclerotic plaques (= ruptured sera) the two clones E1 and E12 as shown in **Figures 1 and 2** were selected because they showed enhanced reactivity (ratio mean OD₄₅₀ sample/mean OD₄₅₀ empty phage + 3xSD) to pooled ruptured serum (**Figure 10**; background = empty phage).

Selection was performed in a 96-well flat-bottom micro-titer plate (Falcon, Franklin Lakes, NJ) coated o/n at 4°C with 200 µl rabbit anti-human IgG (10 µg/ ml in coating buffer)/well. Subsequently, the plate was washed twice with PBST (0.1 % (v/v) Tween 20 in PBS) and twice with PBS, blocked with 2% MPBS (2 % Marvel (=skimmed milk) in PBS) for 2 hrs at

RT, and washed again 3 times with PBST and 3 times with PBS. In a 96 wells round bottom plate (Costar, Corning, NY), 50 μ l pre-adsorbed pooled serum (by repeated passage through a column of Sepharose 6 MB (Pharmacia, Uppsala, Sweden) coupled to *E. coli* TG1 and bacteriophage infected *E. coli* TG1 lysates to remove antibodies reactive to antigens related to the phage-host infection; diluted 1:100 in 2% MPBS) was added to 100 μ l crude phage supernatant diluted 1:1 in 4% MPBS.

The mixture was incubated at 37°C for 1 hr followed by a 30 min incubation at RT. Next, the pre-incubated serum-phage mixture was transferred to the blocked and washed rabbit anti-human IgG coated micro-titer plate and incubated at 37°C for 1 hr followed by 30 min incubation at RT.

After washing, 150 μ l (1:5000 in 2% MPBS) horseradish peroxidase (HRP) conjugated anti-phage (anti-M13) monoclonal antibody (Amersham, Uppsala, Sweden) was added and incubated 1 hr at RT. After washing, 100 μ l 3,3',5,5'-tetramethylbenzidine dihydrochloride chromogen (TMB) solution (10 mg/ml) was added to each well. The reaction was stopped by the addition of 50 μ l 2N H₂SO₄/well.

Plates were read at 450nm in a Novapath micro-titer plate reader (Biorad, Hercules, CA).

ELISA using individual patient sera was essentially as described above. However, adsorption of sera against bacterial and phage-related proteins was not performed, as no difference in background signals between adsorbed and non-adsorbed sera was observed. Each determination was done in triplicate. Both the intra- and interassay variability were < 5%.

Four clones (A1, A7, E1 and E12) showed enhanced reactivity (ratio mean OD₄₅₀ sample/ mean OD₄₅₀ empty phage + 3*SD) to pooled ruptured serum as compared to their reactivity with stable and normal serum (see **Figure 10**). These antigens

thus showed a ruptured plaque specific antibody signature. Furthermore, both dilution of sera and decreasing the amount of recombinant phages resulted in diminished ELISA reactivity, clearly indicating the specificity of the observed interaction (data not shown).

EXAMPLE 2

Detailed serological analysis of antigens E1 and E12

Clones E1 and E12 were tested on a large panel of individual sera (n= 38 for ruptured sera, n=23 for stable sera and n=10 for normal sera). **Table 1** shows detailed patient characteristics.

Serum samples were obtained from patients undergoing peripheral vascular surgery (Department of General Surgery, Academic Hospital Maastricht) and stored at -20EC. Control sera (n=10) were derived from age and sex matched blood donors (n=10) from the Dutch blood bank (Sanquin).

Table 1

	Ruptured (n=38)	Stable (n=23)
Age (mean +/- SEM)	64.5 ± 1.7	64.6 ± 2.0
Male (%)	76 %	61%
Hypertensive (%)	41 %	50%
Smoker (%)	52 %	37%
Diabetic (%)	47 %	52%
Statín use (%)	50 %	48%
CRP (mean +/- SEM)	3.2 ± 2.0	3.2 ± 2.0
Cholesterol (mean +/- SEM)	5.7 ± 0.2	6.1 ± 0.3
LDL (mean +/- SEM)	4.0 ± 0.2	4.0 ± 0.2
HDL (mean +/- SEM)	1.2 ± 0.1	1.2 ± 0.1
Triglycerides (mean +/- SEM)	2.1 ± 0.2	2.0 ± 0.3

Clone E1 showed significant reactivity to 22 out of 38 ruptured sera (58%) whereas no reactivity towards the stable and age and sex matched control sera tested was observed. In addition, clone E12 showed enhanced reactivity in 15 out of 38 patient sera (40%), while again none of the stable and control sera tested positive (see **Figures 11A and B**).

Combination of the serum reactivity against clones E1 and E12 even further increased the sensitivity to 74 %, while the specificity remains at 100% (**Figure 11C**).

EXAMPLE 3

Serological analysis of a ruptured plaque specific Ag panel

The combination of reactivity against clones E1 and E12 raised the sensitivity of the assay to 74%. Therefore, it was tested whether extension of the amount of clones included would further increase the sensitivity. To this end, 6 ruptured sera, 6 stable sera and 6 normal sera were tested for there reactivity to a panel of 192 randomly chosen recombinant phages from selection round 4. With this panel of 192 clones a 100% sensitivity and 100% specificity for the identification of ruptured sera was obtained.

55 Of the clones that reacted solely with one or more ruptured sera were identical to clone E1, while 36 clones were identical to clone E12. Another 15 clones interacted also solely with ruptured sera. These 15 clones had five different amino acid sequences as given in **Table 2**.

EXAMPLE 4

Inhibition of reactivity of ruptured sera with synthetic peptides

In order to determine whether the observed reactivity of ruptured sera was due to the clone specific, ruptured plaque

derived, cDNA insert sequences, 5 ruptured sera were pre-incubated with the synthetic peptides E1 (Ac-VRGFTMLTRLVLNLK-NH₂) and E12 (Ac-VHIIRSSLIYALFTRSISNYK-NH₂) representing the clone specific part of the protein VI-ruptured plaque specific antigen fusion proteins expressed by phage clones E1 and E12 and with a non-specific peptide.

Synthetic peptides Ac-E1-Lys-NH₂ and Ac-E12-Lys-NH₂ were prepared by standard manual solid-phase peptide synthesis and subsequent purification by reversed-phase HPLC.

Fifteen µl serum of 5 individual patients were pre-incubated for 20 min at 37°C, 20 min at RT and 20 min at 4°C in the presence of 50 ng of random peptide, synthetic peptide Ac-E1-Lys-NH₂ (Ac-VRGFTMLTRLVLNLK-NH₂) or Ac-E12-Lys-NH₂ (Ac-VHIIRSSLIYALFTRSISNYK-NH₂). Subsequently, peptide/IgG complexes were precipitated by spinning at 20,000 g and discarded. After two such depletion rounds, the reactivity to phage E1 and E12 was determined in ELISA as described above.

As is shown in **Figure 12**, pre-incubation with the Ac-E1-Lys-NH₂ ("peptide E1") and Ac-E12-Lys-NH₂ ("peptide E12") peptides, clearly inhibited the formation of specific IgG antibody/phage E1 and IgG antibody/phage E12 complexes, while the serum reactivity against clones E1 and E12 was not inhibited by addition of the random peptide.

CLAIMS

1. Peptides that are able to interact with ruptured atherosclerotic plaque-associated antibodies, which peptides
5 comprise an immunoreactive part of one of the following amino acid sequences:

SEQ ID NO:1: VRGFTMLTRLVLNL
SEQ ID NO:2: VHIIRSSLIYALFTRISISNY
SEQ ID NO:3: GSGGGSGGGQVGQIF
10 SEQ ID NO:4: RYKDFVNVIQGPQHPCVCRVPSKLSRSSRCQPVN
 FPPYPRICYSPNHFPVSFQ
SEQ ID NO:5: REFITRYV
SEQ ID NO:6: EFRAGTTALVLDYWNNIQPRTEKLNRLTIVCSLD
 GLTASHLPEPFQNEQSPHHYVFFFDKFRREEMC
15 VPRGGEPDHTGSRVPPKKKN
SEQ ID NO:7: NQKLNTIGNW

2. Peptides as claimed in claim 1, comprising an immunoreactive part of one of the following amino acid
20 sequences:

SEQ ID NO:8: GQVRGFTMLTRLVLNL
SEQ ID NO:9: GQVHIIRSSLIYALFTRISISNY
SEQ ID NO:10: SSREFITRYV
SEQ ID NO:11: SSREFRAGTTALVLDYWNNIQPRTEKLNRLTIVC
25 SLDGLTASHLPEPFQNEQSPHHYVFFFDKFRRE
 EMCVPRGGEPDHTGSRVPPKKKN

3. Peptides as claimed in claim 1 or 2, comprising an immunoreactive part of one of the following amino acid
30 sequences:

SEQ ID NO:12: GSGGGSGGGPSRPDLLENSGQVRGFTMLTRLVLNL

SEQ ID NO:13: **GSGGGSGGGPSRPDLLENS**GQVHIIRSSLIYALF
 TRSISNY

SEQ ID NO:14: **GSGGGSGGGPSRPDLLENS**GSGGGSGGGQVGQIF

5 SEQ ID NO:15: **GSGGGSGGGPSRPDLLENS**RYKDFVNVIQGPQHP
 CVCRVPSKLSRSSRCQPVNFPPYPRICYSPNHFP
 VSFQ

SEQ ID NO:16: **GSGGGSGGWAKSARSS**REFITRYV

SEQ ID NO:17: **GSGGGSGGWAKSARSS**REFRAGTTALVLDYWNNI
 QPRTEKLNRLTIVCSLDGLTASHLPEPFQNEQRS
 10 PHHYVFFFDKFRREEMCVPRGGEPTHTGSRVPPKKKN

SEQ ID NO:18: **GSGGGSGGGPSRPDLLENS**NQKLNTIGNW

4. Peptides as claimed in any one of the claims 1-3,
 comprising an immunoreactive part of one of the following
 15 amino acid sequences:

SEQ ID NO:19: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVQKIVSY
 LDWDK**GSGGGSGGGPSRPDLLENS**GQVRGFTMLTRLVLN
 20 L

SEQ ID NO:20: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVQK
 KIVSYLDWDK**GSGGGSGGGPSRPDLLENS**GQVHI
 25 IRSSLIYALFTRSISNY

SEQ ID NO:21: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVQK
 KIVSYLDWDK**GSGGGSGGGPSRPDLLENS**GSGGG
 30 SGGGQVGQIF

SEQ ID NO:22: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD

FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
 KIVSYLDWDK**GSGGGS****GSGGPSR****PDLL****ENS**RYKDF
 VNVIQGPQHPCVCRVPSKLSRSSRCQPVNFPPYP
 RICYSPNHFPVSFQ

5 SEQ ID NO:23: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
 KIVSYLDWDK**GSGGGS****GSGGWAKSAR**SSREFITRYV

10 SEQ ID NO:24: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
 KIVSYLDWDK**GSGGGS****GSGGWAKSAR**SSREFRAGTT
 ALVLDYWNNIQPRTEKLNRLTIVCSLDGLTASHL
 PEPFQNEQRSPHHYVFFFDKFRREEMCVPRGGEP
 15 DHTGSRVPPKKKN

 SEQ ID NO:25: MPVLLGIPLLLRFLGFLLVTLFGYLLTFLKKGFG
 KIAIAISLFLALIIGLNSILVGYLSDISAQLPSD
 FVQGVQLILPSNALPCFYVILSVKAAIFIFDVKQ
 KIVSYLDWDK**GSGGGS****GSGGPSR****PDLL****ENS**NQKLN
 20 TIGNW

5. Peptides as claimed in any one of the claims 1-4 for use in diagnosis.

25 6. Peptides as claimed in claim 5, wherein the use is for the diagnosis of the presence of ruptured atherosclerotic plaques in an individual.

30 7. Peptides as claimed in claim 6, wherein the diagnosis comprises detection in a sample of body fluid or tissue of an individual of antibodies that are indicative for the presence

of ruptured atherosclerotic plaques in the said individual by binding to one or more of the peptides.

8. Peptides as claimed in claim 7, wherein the sample of
5 body fluid is blood, serum, plasma.

9. Peptides as claimed in claim 7, wherein the sample of tissue is atherosclerotic plaque tissue.

10 10. Use of one or more of the peptides as claimed in any one of the claims 1-4 for the preparation of a diagnostic reagent.

11. Use as claimed in claim 10, wherein the diagnostic
15 reagent is for use in the diagnosis of the presence of ruptured atherosclerotic plaques in an individual.

12. Use as claimed in claim 11, wherein the diagnosis
20 comprises detection in a sample of body fluid or tissue of an individual of antibodies that are indicative for the presence of ruptured atherosclerotic plaques in the said individual by binding to the diagnostic reagent.

13. Diagnostic reagent for the detection of ruptured
25 atherosclerotic lesions in an individual, characterized in that said reagent comprises at least one peptide, comprising an immunoreactive part of the amino acid sequence as shown in any one of the SEQ ID NOS: 1-25.

30 14. Diagnostic reagent as claimed in claim 13, wherein the one or more peptides are bound to a solid phase.

15. An *in vitro* method for detecting antibodies that are indicative of the presence of ruptured atherosclerotic plaques in an individual, the method comprising detecting, in a sample taken from said individual, antibodies that form an immune complex with at least one amino acid sequence selected from the group consisting of SEQ ID NOS:1-25.

16. Test kit for the diagnosis of a sample from an individual for the presence therein of antibodies that are indicative of the presence of ruptured atherosclerotic plaques in the individual, characterized in that said test kit comprises a diagnostic reagent according to claim 13 or 14 and optionally means for visualizing the formation of immune complexes.