Diagnosis and Treatment of Atherosclerosis and Coronary Heart Disease

The present invention concerns the use of human HSP60 (heat shock protein 60) in methods of treatment or diagnosis of the human body, more particularly diagnostic test methods, the manufacture of diagnostic tests, and diagnostic test kits for patients with vascular diseases due to atherosclerosis, having a tendency to heat shock protein-induced complement activation, for example with myocardial disorders such as coronary heart disease.

The complement system (see McAleer, M.A. and Sim, R.B. in Activators and Inhibitors of Complement, Kluwer Academic Publishers, Dordrecht, ed R.B. Sim, 1993, p. 1-15; Reid, K.B.M. and Law, A., 1988, Complement, IRL Press, Oxford) is concerned with host defence against infection - upon activation of the system a catalytic set of reactions and interactions occur resulting in the targeting of the activating cell, organism or particle for destruction. Due to the destructive nature of the system it has the potential to cause severe damage to a host system if incorrectly triggered (Davis, A.E., 1988, Ann. Rev. Immunol., 6: 595-628; Frank, M.M., 1993, In: Complement in Health and Disease, 2nd Edition, Whaley, K. *et al.* eds., Kluwer Academic Publishers, Dordrecht, p. 229) and if its activity is diminished then it has the potential to leave the host open to attack from infecting pathogens.

It has been shown that complement activation has an important role in the development of coronary heart disease and other vascular complications of atherosclerosis (Prohászka, Z. *et al.*, September 1999, Int. Immunol, 11(9): 1363-1370; Torzewski *et al.*, 1997, Atherosclerosis, 132: 131), including for example peripheral atherosclerosis and strokes. Recent publications such as Xu, Q. *et al.* (1993, The Lancet, 341: 255), Hoppichler, F. *et al.* (1996, Atherosclerosis, 126:333), AT398495 and Birnie,

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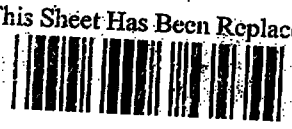
D.H. *et al.* (1998, Eur. Heart J., 19: 387) have been concerned with the association between coronary heart disease and antibody specific against the mycobacterial 65 kDa heat shock protein (HSP) in atherosclerosis. The heat shock proteins (also referred to as chaperons), for example the HSP60 family of proteins, are highly conserved and antibodies against them are found to be highly cross-reactive (Ellas, D. *et al.*, 1990, PNAS USA, 87: 1576; Daniele, M.G. *et al.*, 1992, 5: 443; de Graeff-Meeder, E.R. *et al.*, 1994, Pediatr. Res., 152: 3656; Metzler, B, *et al.*, 1997, Atheroscler. Thromb. Vasc. Biol., 17: 536; Latif, N. *et al.*, 1997, Transplant. Proc., 29: 1039-1040; Schett, G. *et al.*, 1995, J. Clin. Invest., 96(6): 2569-2577; Schett, G. *et al.*, 1997, Atherosclerosis, 128(1): 27-38). These cross-reactive antibodies against HSPs are known to be associated with atherosclerosis, and complement activation is also known to be associated with atherosclerosis although the exact nature of these associations remains unknown, and there is no known causative relationship between atherosclerosis, complement activation and cross-reactive anti-HSP antibodies. Other uses of HSPs are detailed in for example WO 97/06821

The generally held belief that conserved heat shock proteins from pathogens cause heart disease is confirmed by e.g. Bachmaler, K. *et al.*, 1999, Science, 283: 1335-1339.

The present inventors have succeeded in identifying a cause of myocardial disorders (indeed, a cause of atherosclerosis related diseases such as coronary heart disease, myocardial infarctions and strokes) which has not been suggested in the prior art. It has been found that (see "Experiments" below):

i) the formation of immune complexes of human HSP60 (hHSP60) and anti-hHSP60 antibodies causes activation of the complement system *via* the classical pathway (CP) in a dose-dependent manner, but hHSP60 alone does not cause complement activation; and

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ii) the hHSP60 epitopes bound by anti-hHSP60 antibodies in the complexes and which cause complement activation are unique to hHSP60.

There is a strong positive correlation between whether or not a patient suffers from coronary heart disease, as well as the severity of coronary heart disease, and the level of antibody in patient sera specific against hHSP60 (i.e. antibody which does not bind to other HSPs such as mycobacterial HSP65). This may therefore be used as the basis of diagnostic tests for identifying patients and samples from same prone to heat shock protein-induced complement activation.

Thus according to a first aspect of the present invention there is provided hHSP60 or an immunogenic fragment thereof for use in a method of treatment or diagnosis of the human body.

Naturally, a recombinant hHSP60 may be used and such recombinant molecules may have minor modifications to them, for example as a result in changes in their amino acid sequence, or as a result of post-translational modifications effected (or not effected) in an expression system. Such molecules may have at least 50, for example at least 60, 70, 80, 90 or 95% homology with hHSP60 as determined using the algorithms employed by the BLAST2 program (www.ncbi.nlm.nih.gov). As discussed above, the homology of hHSP60 with other HSP60 molecules is very high and therefore in order to fall within the scope of the present invention the modified molecule must display the same functional properties as hHSP60 with regard to those essential to the invention, i.e. they must display the hHSP60 specific epitope(s). For example, this may be determined by performing comparative tests with hHSP60 and the modified molecule. A sample taken from a patient having antibody which is capable of binding with hHSP60 and effecting complement activation should also be able to achieve the same result with the modified hHSP60 replacing hHSP60. Such modified molecules are referred to herein as

"essentially comprising hHSP60" and reference to hHSP60 is also reference to such molecules essentially comprising hHSP60.

Inappropriate activation of the complement system can lead to a wide range of disorders including progression or complication of atherosclerosis, particularly coronary heart disease. Thus the present invention may allow for the diagnosis of patients prone to coronary heart disease and other vascular complications of atherosclerosis. Extremely surprisingly, experiments (below) have shown that the ability to produce high levels of complement activating anti-human HSP60 antibodies is an inheritable trait diagnosable in juveniles which is associated with an increased susceptibility (i.e. a tendency) to the development of coronary heart disease in adulthood. This means that high level results in the assays of the present invention are indicative of susceptibility to this disease amongst otherwise wholly healthy juvenile patients.

Also provided according to the present invention is a diagnostic test method for a tendency to heat shock protein-induced complement activation, comprising the steps of:

i) taking a sample from a patient;

ii) contacting the sample with hHSP60;

iii) detecting any complement activation; and

iv) correlating the results of detection step (iii) with a tendency to heat shock protein-induced complement activation.

Also provided according to the present invention is a diagnostic test method for a tendency to heat shock protein-induced complement activation, comprising the steps of:

i) taking a sample from a patient;

ii) detecting any antibody in the sample of step (i) which is specific solely to hHSP60; and

iii) correlating the results of detection step (ii) with a tendency to heat shock protein-induced complement activation.

Detection step (ii) above may comprise the steps of:

i) contacting the sample with a member of the family of HSP60 proteins, being other than hHSP60 (for example HSP65);

ii) contacting the resulting sample from step (i) with hHSP60; and

iii) detecting any antibody-hHSP60 binding reaction.

Alternatively, detection step (ii) may comprise the steps of:

i) contacting the sample with antigen displaying an epitope unique to hHSP60, and not displaying any shared HSP60 epitopes; and

ii) detecting any antibody-antigen binding reaction.

Antibody-hHSP60 binding reactions may be detected by the detection of any complement activation, particularly by the binding of C4b (complement factor 4b).

The diagnostic test method may be a method of diagnosis of the human body.

Diagnostic tests such as ELISA (enzyme linked immunosorbent assay) and immunochromatographic tests such as the Rapid-Test (EP 291194) may be used in the diagnostic test methods of the present invention and other suitable test methods and kits will be readily apparent to one skilled in the art and may be used as appropriate.

As well as determining when a patient is prone to hHSP60-mediated complement activation, diagnostic tests may be used to determine how the therapy of a patient prone to heat shock protein-induced complement activation is proceeding - reduced levels of hHSP60-induced complement activation or of anti-hHSP60 antibodies

may indicate that a theraby is effective. Similarly other results may indicate, as appropriate, that a therapy is preventing progression (worsening) of a condition or may indicate that a condition is worsening.

The present invention also provides the use of hHSP60 in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation. The present invention also provides the use of hHSP60 and a member of the family of HSP60 proteins, being other than hHSP60, in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation. Also provided is the use of antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes, in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation. As described below, such antigens mat be readily produced using techniques such as epitope mapping and mimotope design or using the various other technologies available. Similarly, binding agents which competitively inhibit the binding of anti-hHSP60 antibodies to hHSP60 may be useful. Molecules which may be screened for their ability to act as such binding agents include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL), a method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacopeia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/18980. The libraries of compounds are screened for binding to hHSP60 in competition with anti-hHSP60 antibodies. Inhibition of binding of anti-hHSP60 antibodies is detected by an inhibition of complement activation in comparison with results obtained from a Control. The identification of such compounds may be particularly useful in the drug discovery process, identifying

compounds which specifically bind with hHSP60 or anti-hHSP60 antibodies and which may therefore inhibit the complement inducing activity of anti-hHSP60 antibodies.

Also provided by the present invention is a diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises hHSP60. Also provided by the present invention is a diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises hHSP60 and a member of the family of HSP60 proteins, being other than hHSP60. Also provided by the present invention is a diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes.

As well as being diagnostically useful, the present invention also provides therapeutic opportunities. The hHSP60 epitope (or epitopes) required to bind to anti-hHSP60 antibody to cause heat shock protein-induced complement activation in patients may be readily determined using sera from a patient prone to (or suffering from) heat shock protein-induced complement activation. Using epitope mapping (Geysen, H.M *et al.*, 1987, Journal of Immunological Methods, 102: 259-274; Geysen, H.M. *et al.*,1988, J. Mol. Recognit., 1(1):32-41; Jung, G. and Beck-Sickinger, A.G., 1992, Angew. Chem. Int. Ed. Eng., 31: 367-486) a series of overlapping peptides may be produced for the sequence of hHSP60. Each of the overlapping peptides can then be mixed with a sample of the patient sera. Those that cause complement activation (i.e. binding of C4b) are displaying the epitope required for antibody binding and complement activation. From them, a core epitope sequence may be determined and peptides (or other molecules) produced which display (i.e. carry) the epitope but which do not cause complement activation. If epitope mapping fails then other techniques such as mimotope design (Geysen, H.M. *et al.*, 1987, 1988 *supra*; Jung, G. and Beck-Sickinger, A.G., 1992, *supra*) may be used to synthesise molecules displaying the epitope, using e.g. patient

antibody as a reference material in assays of their efficacy. In particular this allows the determination and replication of epitopes having e.g. tertiary and quarternary structural features which might not otherwise be identified by epitope mapping. Thus the invention also extends to the use of fragments of hHSP60. Clones producing monoclonal antibodies (Harlow, E. and Lane, D., "Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998) may be readily identified by their ability to bind the hHSP60 epitope (above) required for complement activation, or by their ability to cause complement activation in the presence of hHSP60, and the present invention extends to the uses of such monoclonal antibodies and antigen binding fragments thereof. Similarly, hHSP60 may be fragmented and specific fragments carrying and displaying hHSP60-specific epitopes identified. Other techniques (as discussed above) are available for identifying antibody targets and competitive inhibitors of binding and may be readily employed.

Thus also provided according to the present invention is the use of hHSP60 in the manufacture of a medicament for the treatment of heat shock protein-induced complement activation, particularly for the treatment of vascular disorders due to atherosclerosis. A medicament may be for the maintenance and/or restoration of health, i.e. it may cure, alleviate, remove or lessen the symptoms of, or it may prevent or lessen the possibility of, heat shock protein-induced complement activation. The medicament may for example be a vaccine. The use may comprise the use of an epitope or peptide carrying an epitope displayed by hHSP60 but which is not displayed by other members of the family of HSP60 molecules. Also provided is a method of manufacture of a medicament for the treatment of heat shock protein-induced complement activation, characterised in the use of hHSP60.

Now that hHSP60 has been identified as a cause of heat shock protein-induced complement activation, hHSP60 (or fragments of it as discussed above) may be used in the drug discovery process, allowing the identification of agents which prevent

its triggering complement activation. Thus also provided according to the present invention is a method for the identification of a compound, substance or agent which inhibits hHSP60-induced complement activation, characterised in the use of hHSP60 (or a fragment thereof). Also provided is the use of hHSP60 (or a fragment thereof) in a method of identification of a compound, substance or agent which inhibits hHSP60-induced complement activation. Also provided is the use of an assay of the present invention in the drug discovery process, for example screening combinatorial chemistry libraries do detect chemicals or drugs which may inhibit hHSP60-induced complement activation or the onset of atherosclerosis or coronary heart disorders

The invention will be further apparent from the following description with reference to the accompanying figures which show, by way of example only, forms of detection of patients having a tendency to heat shock protein-induced complement activation.

Of the Figures:

Figure 1 shows the dose-dependent complement activation of hHSP60. Human HSP60 was incubated either with normal human serum (NHS; solid squares) or with heat-inactivated human serum (HIHS; solid circles). The amount of C4b fixed to the hHSP60 was determined with anti-C4b antibody. An uncoated control plate was incubated with NHS (open circles) or with HIHS (open triangles). The figure shows mean OD values and standard deviations of four parallel measurements. Results from one representative experiment out of a total of three repeats are shown. Significantly increased C4b binding was seen in the indicated (**) case as compared to the uncoated control plate (Student t-test, $p<0.01$);

Figure 2 shows the lack of classical pathway activation by hHSP60 in agammaglobulinaemic serum (AGS). Human HSP60 (0.1 µg/ml) was incubated either

with NHS or with two different AGS. The amount of C4b fixed to the plate was determined with anti-C4b antibody. Uncoated wells were used as controls. AGS + IgG indicates AGS reconstituted with antibodies (5% heat inactivated serum). Results from one representative experiment out of a total of two repeats are shown. Significantly increased C4b binding was seen in the indicated (***, $p<0.0001$; *, $p<0.05$) case as compared to the uncoated control plate (Student t-test);

Figure 3 shows (panel A) the correlation of anti-hHSP60 antibody levels to the quantity of hHSP60-fixed C4b. A highly significant ($r=0.459$, $p<0.0001$) positive correlation was observed between the level of anti-HSP60 antibodies and hHSP60-fixed C4b. Panel B shows the lack of correlation between anti-*M.bovis* HSP65 antibody levels to the quantity of HSP65-fixed C4b ($r=0.1068$, $p=0.554$). Normalisation of results obtained on different plates was achieved by using the results of 8 individual sera tested on each plate as standards;

Figure 4 shows the distinction between anti-hHSP60 and anti-*M.bovis* HSP65 antibodies by competitive ELISA. ELISA plates were coated either with hHSP60 (left panels) or with *M.bovis* HSP65 (right panels) and incubated with serial dilutions of patient sera. The binding of IgG-type antibodies to the plate was blocked by 5 µg/ml hHSP60 (open circles) or HSP65 (dashed line) added to the incubation mixture (buffer was added in control cases, solid squares). Results from one representative experiment out of a total of three repeats are shown. Values represent mean and SEM OD values of three parallel measurements; and

Figure 5 shows results from an investigation into the prediction of susceptibility of coronary heart disease in children at risk due to family history. Y-axis shows Complement Activation Index. X-axis shows two sets of data for median (interquartile ranges) for (data on the left) control-children (median 0.58, interquartile

range 0.33-1.07) and for (data on the right) at-risk children (median=1.27, interquartile range 0.53-2.33). P=0.0002 for the results using a Mann-Whitney test.

EXPERIMENTS

Experiments were performed as detailed below and results show that:

i) hHSP60 is capable of inducing the activation of the complement cascade through anti-HSP60 antibodies;

ii) there is a correlation between hHSP60-induced complement activation and the level of anti-HSP60 antibodies; and

iii) from the set of anti-HSP60 antibodies it is hHSP60-specific antibodies which do not cross-react with other HSP60 proteins which cause the activation of the complement cascade by interaction with hHSP60.

The results show a positive correlation between the level of anti-hHSP60 antibodies (i.e. which do not cross-react with other HSP60 proteins) in patient sera and coronary heart disease due to atherosclerosis.

An additional independently performed study shows that assays of the present invention provide an extremely strong correlation between complement activating anti-hHSP60 antibodies and the occurrence of coronary events, with odds ratios for second and third tertiles of 5.24 and 6.37.

A study has also been performed with samples from healthy children with parents and/or grandparents who had myocardial infarction before the age of 45. The results show that the ability to produce high levels of complement activating anti-hHSP60 antibodies is an inheritable trait associated with an increased susceptibility for the development of coronary heart disease in adulthood, and is thus an extremely useful diagnostic for juveniles.

Definitions

The term "**antibody**" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Such molecules are also referred to as "**antigen binding fragments**" of immunoglobulin molecules.

Illustrative antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')2 and F(v).

"**Therapy**" is any treatment which is designed to cure, alleviate, remove or lessen the symptoms of, or prevent or reduce the possibility of contracting any disorder or malfunction of the human or animal body

Modes of carrying out the invention

Example 1

Materials and Methods

1. Heat-shock proteins

Recombinant hHSP60 and the recombinant *Mycobacterium bovis* HSP65 were produced and purified from heat-induced (42 °C) *Escherichia coli* K12 strain M1164, which carries plasmid pRIB1300 (Thole, J.E.R. *et al.*, 1987, Infect. Immun., 55: 1466-1475). Cells were lysed by lysozyme treatment and sonication. The soluble protein was purified by anion exchange chromatography. The buffer was changed to 10 mM ammonium bicarbonate by dialysis and the protein aliquoted and lyophilized.

2. Complement-activation ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine the complement activating ability of solid-phase hHSP60 or mycobacterial HSP65 as described by Prohászka Z. (1998, Annal. N.Y. Acad. Sci, 851: 94-98), Prohászka, Z. *et al.* (1995, Immunology, 85: 184) and Prohászka, Z. *et al.* (1997, Mol. Immunol., 34: 809). ELISA plates were coated with different amounts of hHSP60 or with 0.1 µg/well *M.bovis* HSP65. After washing, wells were incubated either with 50 µl of normal human serum (NHS, pooled serum from 10 young, healthy individuals) or with heat-activated (56 °C, 30 minutes) human serum prediluted 1:1 with veronal buffered saline containing Ca^{2+} and Mg^{2+} or with Mg^{2+} EGTA-chelated serum or with a serum of a homozygous C2-deficient SLE (systemic lupus erythematosis) -patient for 30 minutes at 37°C. In another set of experiments, individual patients' sera were used prediluted 1:1 with veronal buffered saline containing Ca^{2+} and Mg^{2+}. The amounts of complement proteins fixed to the plate were determined with specific goat anti-C4b antibodies and goat anti-C3b antibodies (Atlantic Antibodies, Stillwater, MN, USA).

3. Agammaglobulinaemic sera

Two serum samples with very low levels of antibodies were used in the complement activation enzyme-immune assays. Agammaglobulinaemic serum AGS-1 was obtained from a four year old boy. The levels of IgG, IgA and IgM type antibodies were 0.375, <0.01 and 0.037 g/l, respectively. All complement parameters tested (CH_{50}, C3, C4 levels and haemolytic activity) were in the normal range. The agammaglobulinaemic serum AGS-2 was donated by a 33-year old woman. IgG, IgA and IgM levels were 0.380, <0.063 and <0.042 g/l respectively. All complement parameters tested (C3, C4 levels and haemolytic activity) were in the normal range.

4. Determination of anti-hHSP60/HSP65 antibodies

The amounts of IgG-type antibodies reacting with chaperonin 60 family (recombinant hHSP60, recombinant *M. bovis* HSP65) proteins were assessed by ELISA as described previously (Prohászka, Z. *et al.*, 1995, *supra*). Plates were coated with 0.1 µg/well

hHSP60 or *M.Bovis* HSP65. After washing and blocking (PBS, 0.5% gelatin), wells were incubated with 100 µl of serum samples diluted 1:500 in PBS containing 0.5% gelatin and 0.05% Tween 20. Binding of anti-HSP antibodies was determined using γ-chain specific anti-human IgG peroxidase labelled antibodies (Sigma, St Louis, USA) and o-phenylene-diamine (Sigma) detection system. The optical density was measured at 492 nm (reference at 620 nm) and mean results were calculated from duplicate wells. A serial dilution of a control anti-HSP60 rabbit polyclonal antiserum (StressGen SPA-804) was used as standard. Data obtained as optical density values were calculated to unit/ml value relative to this standard.

5. Competitive ELISA

One step of the above described ELISA test was modified in order to assess the antibody competing effect of recombinant HSPs. The sera to be tested were diluted in PBS containing 0.5% gelatin and 0.05% Tween 20 and additional 5 µg/ml hHSP60 or HSP65. PBS was added in control samples. The assay was performed as described above.

6. Statistical analysis

Correlation coefficients were calculated by the non-parametric Spearman method. Group comparisons were computed either with Student t-test or with the non-parametric Mann-Whitney test using the GraphPad Prism 2.0 software package (San Diego, CA USA).

7. Patients

Seventy-four patients (60 males, 14 females, median age 60 years) with coronary heart disease were enrolled in this study. The basic data of the patients are summarised in Table 1. All patients underwent coronary angiography and by-pass operation. Samples were taken 6 months after the operation to rule out the influence of acute disease and stress. Sera were aliquoted and kept at -70°C until use. The 74 patients were randomly selected from 357 patients according to their anti-HSP60 antibody levels (37 patients with "high" (higher than median) and 37 with "low" (lower than median) levels).

Experiments

1. Complement activation by hHSP60

In order to test whether solid phase hHSP60 can induce the activation of the complement cascade, the solid-phase ELISA test system of Prohászka, Z. *et al.* (1995, *supra*) and Prohászka, Z. *et al.* (1997, *supra*) was applied. Human HSP60-coated ELISA plates were incubated with NHS (containing medium-level (114.8 ± 15.7 U/ml, mean ± SD) of anti-HSP60 antibodies) and C4b and C3b binding was measured (Table 2). Markedly higher binding of both complement proteins to hHSP60 coated plates than uncoated ones indicated that hHSP60 can trigger the complement cascade. Since heat-inactivation of NHS reduced the binding of C4b and C3b to a background level, the activation of the complement system by hHSP60 appears to require the interaction of serum and hHSP60. This activation takes place through the classical pathway since neither C4b nor C3b binding was seen in Mg^{2+}-EGTA containing serum. The chelation of the Ca^{2+} ions is known to inhibit the CP. The CP activation by hHSP60 was further evidenced by showing the lack of C3b binding from a serum obtained from a homozygous C2-deficient SLE patient, despite the fact that the initiation of the CP took place, since significant C4b binding was seen in this serum (Table 2). The specificity of the CP activation by hHSP60 was proved by the demonstration of its dose-dependence (Figure 1).

2. hHSP60 triggers the CP through anti-HSP60 antibodies

The initiation of the CP of the complement cascade can proceed through two ways: by the formation of immune complexes or by direct binding of C1q (the first component of the CP) by certain molecules, viruses or bacterial products. In order to determine how hHSP60 initiates the CP, solid-phase hHSP60 was incubated with AGS samples. As shown in Figure 2, no significant CP activation was observed in either AG serum. The reconstitution of the AGS-1 serum with antibodies (provided by HIHS) resulted in a significant CP activation, indicating that hHSP60 triggers the CP only in the presence of antibodies, i.e. by the formation of immune complexes.

3. Correlation between hHSP60-induced CP and the level of anti-HSP60 antibodies

In order to assess the influence of specific anti-HSP60 antibodies (known to be present in different amounts in sera of patients with atherosclerotic lesions) on the CP activation by hHSP60, the extent of hHSP60-induced CP activation (binding of C4b to hHSP60-coated plates) was tested in individual serum samples with known amounts of anti-hHSP60 and anti-*M.bovis* HSP65 antibodies. These samples were taken 6 months after by-pass surgery of 74 coronary heart disease patients. A highly significant positive correlation ($r=0.459$. $p<0.0001$) between the extent of hHSP60-induced CP activation (measured by C4b-binding) and the amount of anti-hHSP60 antibodies was seen (Figure 3, panel A). By contrast, the extent of CP activation did not correlate with the level of anti-*M.bovis* HSP65 ($r=0.09$. $p=0.451$) antibodies despite the fact that the amount of anti-HSP65 antibodies correlated very strongly with the amount of anti-hHSP60 antibodies ($r=0.425$. $p=0.0005$). In a part of the same serum samples the classical pathway activation by *M.bovis* HSP65 was analysed (Figure 3, panel B). The CP activation in individual sera by HSP65 was much weaker as compared to hHSP60 and seemingly not influenced by the amount of anti-HSP65 antibodies (Spearman correlation coefficient $r=0.106$. $p=0.554$). An analysis was then performed for any difference in the extent of hHSP60-induced CP activation in serum specimens with high or low level of anti-hHSP60 antibodies. The CP activation by hHSP60 in sera with higher-than-median levels of anti-HSP60 antibodies was significantly higher than in sera with lower-than-median levels of anti-HSP60 antibodies ($p=0.0001$, Mann-Whitney test). The same difference was not seen in the case of mycobacterial HSP65 induced CP activation and anti-HSP65 antibodies ($p=0.787$).

4. Differentiation between anti-hHSP60 and anti-HSP65 antibodies

Competitive ELISA was used to determine whether the anti-HSP60/65 antibodies are specific for the same epitopes on the different antigens (i.e. whether the same antibodies cross-react with different antigens) or are specific for different epitopes displayed by the different antigens. ELISA plates were coated either with hHSP60 or with *M.bovis*

HSP65 proteins and incubated with serial dilution of the test sera. The incubation mixture contained 5 µg/ml hHSP60 or HSP65 as competitive antigen in each case (or buffer as control). A total of 10 sera from the study group were tested in this assay. Figure 4 shows the results obtained in the sera of three patients. The serum of patient 4101 contained significant amounts of anti-hHSP60 antibodies but not anti-HSP65 antibodies. Incubation of this serum with hHSP60 markedly decreased the binding activity of antibodies to solid phase hHSP60, whereas incubation with HSP65 did not influence antibody fixation. Serum of patient KM exhibited a strong activity against HSP65 but not against hHSP60. Incubation of this serum with HSP65 markedly decreased the binding activity of antibodies to solid phase HSP65, whereas incubation with hHSP60 did not influence antibody fixation. The serum of patient 4395 contained antibodies reacting with both hHSP60 and HSP65. In this sample, incubation with the homologous HSP preparations (i.e. the other one of the pair of hHSP60 and HSP65) almost fully abolished antibody binding while hHSP60 only slightly inhibited anti-HSP65 fixation and *vice versa*. Similar results were obtained in the other 7 serum samples not shown in Figure 4.

Conclusions

Two important conclusions can be drawn from the results of the experiments. First, hHSP60 alone does not activate the complement system but complexing of antibodies to solid phase hHSP60 leads to a marked complement activation which is dependent on the amount of specific antibodies. Second, the antibodies against hHSP60 and *M.bovis* HSP65 differ from each other in their antigen specificity and complement activating ability.

Furthermore, solid phase recombinant hHSP60 is able to activate the complement system in pooled normal human serum as well as in the sera of coronary heart disease patients. Since inhibition of the first complement component by Mg^{2+}-EGTA blocked C4 activation and binding and no C3b binding to hHSP60 occurred in the serum of a patient

with homozygous C2 deficiency, it can be concluded that hHSP60 activates complement in human sera *via* the CP.

The CP can be activated by different substances either directly or with the contribution of specific antibodies i.e. by immune complex formation. In the case of hHSP60, the initiation of the CP seems to occur by immune complex formation since no complement activation was observed in agammaglobulinaemic serum samples.

The present findings indicate that specific antibodies directed to hHSP60 trigger, and dose-dependently enhance, complement activation by hHSP60.

In addition anti-IgG antibodies (rheumatoid factors) may also influence this activation. Furthermore, mycobacterial HSP65 was also found to activate the CP, but the extent of this activation does not correlate either to anti-HSP60 or anti-HSP65 antibody levels. Since anti-HSP65 and anti-*H.pylori* antibodies correlate to each other (Birnie, D.H. *et al.*, 1998, *supra*) a test was performed to determine whether the amounts of anti-*H. pylori* antibodies correlate to the extent of HSP65-induced complement activation. A very strong positive correlation was found between the amount of C4b fixed to HSP65 and levels of anti-*H.pylori* antibody in the sera of CHD patients. Another possible explanation for the lack of correlation between HSP65-induced CP activation and anti-HSP65 antibody levels might be a different (complement non-activating) subtype of anti-HSP65 antibodies.

These findings show that the complement system may be efficiently activated when it is exposed to complexes of hHSP60 and specific antibodies. Different pathological conditions and stress situations may markedly increase hHSP60 expression in endothelial cells (Wick, G. *et.al.*, 1995, Immunol. Today, 16: 27; Xu, Q. and Wick, G., 1996, Mol. Med. Today, 2(9): 372). For instance, non-toxic ischemia-reperfusion was found to render human umbilical vein endothelial cells capable of activating the complement

system in human serum (Collard, C.D. *et al.*, 1997, Circulation, 96: 326). The mechanism of this process is not fully understood. However, the present results suggest that over-expression of hHSP60 on the surface of endothelial cells could be one of the factors that increases the complement activating ability of the cultured endothelial cells, especially when high titers of anti-HSP65 antibodies are present. Since complement activation has an important role both in the early and late phase of atherogenesis, the present findings have important pathological implications.

The assumed role of anti-HSP65 antibodies in the development of atherosclerosis is supported by several recent findings. Xu *et al.* (1993, *supra*) found an association of elevated level of anti-mycobacterial HSP65 antibody with carotid atherosclerosis. More recently, Hopplicher *et al.* (1996, *supra*) measured significantly increased anti-HSP65 titres in 114 patients with coronary heart disease as compared to 76 age and sex matched healthy controls. Similarly, Birnie *et al.* (1998, *supra*) demonstrated a positive correlation between the extent of coronary atherosclerosis and the titre of anti-HSP65 antibodies.

The demonstration of differences between anti-hHSP60 and anti-HSP65 antibodies in their antigen recognition and complement activating ability is extremely important and has not been previously suggested. Antibodies to heat shock proteins of the 60 kDa family are elevated in patients with several diseases as compared to heathy subjects. If antibodies against the different members of the chaperonin family are measured in parallel, usually (but not always (Handley, H.H. *et al.*, 1996, Clin. Exp. Immunol., 103: 429)) a strong correlation is found between their amounts. It has also been found that a significant correlation exists between the level of anti-HSP60 and anti-HSP65 antibodies in the sera of HIV patients (Prohaszka, Z. *et al.*, 1998, Ann. NY Acad. Sci., 851: 94-98) and patients with coronary heart disease.

Example 2

Study into the Prediction of Susceptibility of Coronary Heart Disease in Middle Aged Men

The following investigation was undertaken by an independent third party and was performed by Professor G J Miller, MD FRCP and Dr David Howarth of the MRC Epidemiology and Medical Care Unit, Wolfson Institute of Preventive Medicine, Charterhouse Square, London EC1M 6BQ, United Kingdom using a diagnostic test kit as detailed below supplied by CardioPath Ltd.

A total of 265 citrated plasma samples were assayed as detailed below. For each sample taken from a case, i.e. a patient who has suffered a coronary event, two samples were selected from patients with no signs of any heart problems.

- 56 samples were taken from cases prior to event with 110 controls.

- 33 samples were taken from cases after the event with 66 controls.

All samples came from middle-aged men and the case and two control samples were age matched. The samples were taken and then immediately centrifuged and frozen at -45 °C before being transported on dry ice to the MRC unit. The plasma were then stored at -80 °C and were only thawed specifically for this evaluation. The actual testing of the assay was done blind with no analysis started until all the testing was complete.

1. Principle of the test

Purified recombinant human HSP60 protein is coated onto the surface of microtitration wells. Test samples are then applied. Anti-hHSP60 antibodies complex to the antigen in the well. Unbound material is washed away and peroxidase conjugated anti-C4b is applied. If antibodies have bound and activated the complement cascade the C4b

conjugate will bind to the C4b fragment covalently bound to the antigen. Unbound material is again washed away. On addition of the substrate, stabilised 3,3', 5,5', Tetramethyl Benzidine (TMB), a colour will develop only in those wells in which enzyme is present indicating the presence of human C4b. The enzyme reaction is then stopped by the addition of Sulphuric Acid and the absorbance is measured at 450 nm.

2. Materials

Component/Chemical	Commercial Source	Product Code
Plate	Dynex	Immulon 4
Plate Lid	Griener	n/a
hHSP60	Stressgen	SPP-740
Na_2CO_3	Fisher	S/2920/53
$NaHCO_3$	BDH	10247
Sucrose	Sigma	S9378
Tris	Sigma	T1503
NaCl	Sigma	S9625
Tween 20	Sigma	P1379
Gelatin	Sigma	G0262
Rabbit Anti-hHSP60	Stressgen	SPA-804-1
C4b	Diasorin	80255
Stablzyme	Surmodics	SZ02
Goat anti Rabbit Ab	Amdex	AX01-0301X
TMB Substrate	Dako	S1600
H_2SO_4	Sigma	102761C

The HSP60 (www.stressgen.com) is a recombinant hHSP60 cloned from a human promyelocytic leukemia (HL-60) cDNA library.

Plate coating: 50µl of 1µg/ml hHSP60 in 0.05M Carbonate buffer pH 9.6 is added to each well and incubated overnight at 4 °C. The plates are then washed before blocking with 200µl of 1% Gelatin, 5% sucrose, 0.15M NaCl, 0.05M Tris, 0.1% Tween 20 and incubated for 1 hour at RT (room temperature) before the liquid is removed and the plates dried overnight at 37 °C.

Negative Control: 0.05M Tris, 0.15M NaCl, 5% Gelatin, pH 7.5.

Low Positive Control: The Low Positive Control contains rabbit anti hHSP60 at an approximate dilution of 1/500 in 0.05M Tris, 0.15M NaCl, 5% Gelatin, pH 7.5. This control is used to monitor assay to assay variation and calculate Complement Activation Indexes.

Conjugate: Heterobifunctional HRP conjugate of C4b protein at an approximate dilution of 1/500 in Stablzyme Conjugate Diluent. Also contains goat anti rabbit HSP conjugate at approximate dilution of 1/60 000 for Low Positive Control.

Wash Buffer: 0.05M Tris, 0.15M NaCl, 0.1% Tween 20, pH 7.5.

Stop Solution: 0.2% H_2SO_4.

Assay Kit:

1. Instruction Leaflet, EIA Data Recording Sheet	1+1
2. Microtitration Plate and Plate Lid: Wells coated with hHSP60 protein, sealed with a plastic seal, covered with a plate lid and contained in a resealable foil bag with a desiccant.	12 X 8 wells + 1
3. Negative Control (Blue) (REAGENT 2)	1 X 1ml
4. Low Positive Control (LP) (Green) (REAGENT 3)	1 X 1ml
5. Anti-C4b1e HRP IgG Conjugate (Purple) (REAGENT 4)	1 X 11ml
6. Wash Buffer: 20 X concentrate (Colourless) (REAGENT 5)	1 X 50ml
7. TMB Substrate (Colourless) (REAGENT 7)	1 X 11ml
8. Stop Solution (Colourless) (REAGENT 8)	1 X 11ml

Reagent Preparation:

Wash Buffer: Dilute the concentrated Wash Buffer using 1 part Wash Buffer concentrates with 19 parts distilled water. For every 8 well strip prepare 80ml of diluted Wash Buffer by adding 4ml of concentrated Wash Buffer to 76ml of distilled water. Prepare fresh diluted Wash Buffer prior to every assay run.

3. Test Procedure

1. Bring all the kit components and the test sample to room temperature (20 °C to 25 °C) prior to the start of the assay.

2. The kit Control should be run with each batch of specimens to check the test performance. Kit controls should be run in duplicate. Select sufficient microwell strips for the number of samples to be tested including the Kit Control. Locate the strips in the frame and remove the protective plastic seals. Record the positions of the Kit Control and the test sample on the EIA Data Recording Sheet provided. Unused strips should be stored in the resealable foil bag, containing the desiccant, before being replaced at 2 °C to 8 °C for storage.

3. Without diluting the controls or test, dispense 50 µl of sample or control sera (REAGENTS 2, 3) into the appropriate wells. The control should be added last to ensure accurate interpretation of the results. Gently shake for 5 seconds. Cover the plate with the plate lid provided and place it on top of moist absorbent paper at RT 20-30 °C for 20 minutes.

4. At the end of the incubation period, remove the plate leaving the moist absorbent paper and wash the plate as described below.

5. Dilute Wash Buffer (REAGENT 5) as described in the Reagent preparation Section. Wash plate with the diluted Wash Buffer either three times by hand or using an automatic plate washer. For machine washing ensure that 300 µl are dispensed per well and that an appropriate disinfectant is added to the waste collection bottle. After washing remove excess fluid by tapping the inverted plate on absorbent paper.

   For hand washing, rinse the wells by carefully emptying the contents into a vessel containing an appropriate disinfectant and then fill the wells into the waste container. Repeat this procedure a further two times. After washing remove excess fluid by tapping the inverted plate on absorbent paper. Appropriate precautions should be taken bearing in mind the potential biohazard of the test sera. These precautions should adhere to regional laboratory legislation. The washing step is critical, as insufficient washing will result in poor precision and falsely elevated absorbencies.

6. Dispense 100 µl of Conjugate (REAGENT 4) into each well. Gently shake the plate for 5 seconds before replacing the lid onto the plate and returning to the incubator ensuring that the plate is positioned on top of the moist absorbent paper. Incubate at RT 20-30 °C for 60 minutes.

7. At the end of the incubation period repeat step 4 and the washing procedure described in step 5.

8. Dispense 100 µl of stabilised TMB Substrate (REAGENT 7) into each well. Gently shake for 5 seconds before replacing the lid onto the plate and placing in the dark at room temperature (20-30 °C) for 15 minutes.

9. Stop the reaction by adding 100 µl of Stop Solution (REAGENT 8) to each well. This will produce a colour change from blue to yellow in wells containing enzyme, which indicates the presence of complement activation. Blank the plate reader on air. Measure the absorbance of each well at 450 nm immediately after stopping the reaction.

4. Calculation and Interpretation of Results

For each test and Control, the OD obtained in the wells is determined.

Assay Validation: The average OD of the Low positive Control should be greater than 0.3 for the assay results to be valid.

OD levels showing positive results i.e. significant levels of complement activating anti-hHSP60 antibodies can be expected to vary between sample populations, for example between adult and juvenile populations. For example, a result considered positive in one population may be insignificant in another. Therefore standard curves correlating ODs with actual risk and defining cut-off values at which results are considered positive are established for populations which are studied.

For comparisons between assays Complement Activation Indexes (CAI) must be calculated:

CAI = OD of sample / Average OD of Low Positive Control

This use of the average OD of Low Positive Controls allows for standardisation of results between different sets of experiments for example using different equipment.

5. Results

After the raw data had been collected the following statistical analyses were performed.

Complement Activation Index in cases and controls:

Results are shown in Table 3. 1 outlier was removed from the controls.

Conditional Logistic Regression Analysis:

Odds Ratio (OR) is for a 1 sd increase (0.94) in log CAI.

This is equivalent to a 2.6 fold increase in CAI.

Data is shown in Table 4.

Time of Event:

56 of the cases had samples taken prior to their event. In 33 cases the samples were taken after the event.

There was a significant interaction with time of event (p=0.007). CAI was significantly raised in cases when the sample was taken prior to the event but the association was not found when the sample was taken after the event.

Sample prior to the Event:

Data for samples taken prior to the event is shown in Table 5.

Samples taken after the Event:

Data for samples taken after the event is shown in Table 6.

In those with samples taken prior to the event there is no evidence that the association changes according to the proximity of the event (p=0.65).

6. Analysis by Tertiles

Analysis of CAI by tertiles indicates that the association is non-linear with risk being reduced only in the lowest tertile. Cut-off points for the CAI tertiles are 0.27 and 0.6. Again there is a significant interaction with time of event (p=0.007)

Overall results are shown in tertiles in Table 7. Results in tertiles for samples prior to event are shown in Table 8, and results in tertiles for samples after event are shown in Table 9.

Univariate associations in Complement Activation Index (CAI) nested case-control studies are shown in Table 10.

Complement Activation Index:
With samples taken before the event, the association between tertiles of Complement Activation Index and case/control status remains (p=0.003) after adjustment for smoking status, systolic BP, cholesterol, triglyceride, fibrinogen and body mass index.

Relative to tertile 1 the odds ratios for tertiles 2 and 3 are 5.24 (1.50 - 18.26) and 6.37 (1.80 - 22.58) respectively.

The full model is shown in Table 11. The analysis uses 155 observations - 53 cases and 102 matched controls. Odds Ratios are for a 1 sd increase int the variable except for smoking and antibody index. Triglyceride and fibrinogen are log-transformed.

Using tertiles of Complement Activation Index alone in the model, the area under the ROC (Receiver Operating Characteristic) curve is 0.65.

7. Conclusions

The data shows that the Complement Activation Index (CAI) as determined by the assay of the present invention is strongly related to the risk of coronary disease. This is shown in Table 8.

Table 10 shows that cholesterol, smoking, fibrinogen and systolic blood pressure are also significantly related to risk in these men.

Table 11 is a multivariate analysis for the strength of independent associations of these factors with risk. It can be seen from the p values and 95% confidence intervals in this table that only this assay was independently associated with risk. Compared to men with levels in the low third of the distribution, men in the middle third were 5.2 times at risk, and men in the top third were 6.4 times at risk.

In this evaluation the assay kit used was therefore considered to be highly predictive of coronary risk.

Example 3

Study into the Prediction of Susceptibility of Coronary Heart Disease in Children at risk due to family history

1. Introduction

It is known that children who come from a family who have suffered a coronary event are much more susceptible to the development of coronary heart disease when they grow up than their peers with a negative family history. A study was undertaken to investigate whether healthy children with parents and/or grandparents who had myocardial infarction before 45 years of age gave significantly higher results with the assay kit of the present invention than the control samples.

A total of 117 serum samples were analysed.

At risk children – 64 healthy children aged between 10 and 16 years of age who had at least one parent or grandparent who had a myocardial infarction before 45 years of age.

Controls – 53 aged matched randomly selected healthy children before elective surgery or after trauma. The controls are not fully adequate since only some of them were asked

for the family history so it cannot be excluded that this group also contained at risk children. It is therefore expected that the difference shown in the results (below) would be even higher when the at risk children are compared with proper controls i.e. those with proven negative family history for coronary heart disease.

The sera samples were stored at -70 °C prior to testing.

1. Principle of the test

- as detailed above.

2. Materials

- as detailed above.

3. Test Procedure

- as detailed above.

4. Calculation and Interpretation of Results

- as detailed above.

5. Results

After the raw data had been collected the following statistical analyses were performed.

The Odds Ratio (OR) of the At Risk Children was calculated using two separate cut off limits of the 75% and 95% percentile measured in the Control Group. This gives cut off values using Complement Activation Indexes (CAI) of 1.07 and 2.0 respectively.

Data is given in Tables 12 and 13 and Figure 5.

The results show that children in the At Risk Group give significantly elevated results when compared to the Control Group.

7. Conclusions

The data shows that in this evaluation the ability to produce high levels of complement activating anti-hHSP60 antibodies is an inheritable trait which is associated with an increased susceptibility for the development of coronary heart disease in adulthood.

This means that high level results in this assay are indicative of susceptibility to this disease amongst otherwise wholly healthy juvenile patients.

Exampe 4

Alternatively, to identify patient samples having anti-hHSP60 antibodies, prior to contacting the patient sample with hHSP60, an additional microtitre plate is provided which is coated with a non-human HSP60. Samples are contacted with the non-human HSP60 and allowed to bind it. Unbound sample is then separated from the well and contacted with the microtitre plate having hHSP60 in its wells and the process continued as above. This helps ensure that only antibody specific to epitopes found only on hHSP60 bind hHSP60.

Example 5

Instead of using hHSP60 bound to wells of a microtitre plate to detect samples from patients with a tendency to hHSP60-induced complement activation, hHSP60-specific antibodies are isolated from patient sera and are then used for mimotope design to produce mimotopes of hHSP60-specific epitopes. These mimotopes are then used to coat wells of microtitre plates and are contacted patient samples. Binding of any patient

antibodies to these mimotopes is indicative of anti-hHSP60 antibodies in a patient sample and therefore of a patient's tendency to hHSP60-induced complement activation.

Example 6

The mimotopes of Example 4 are injected into patients with a tendency to hHSP60-induced complement activation in order that they can compete with hHSP60 to bind anti-hHSP60 antibodies and thereby reduce complement induction by hHSP60. Similarly, hHSP60 specific binding agents other than anti-hHSP60 antibodies are injected into patients to competitively inhibit the binding of anti-hHSP60 antibodies with hHSP60.

Table 1 - Basic data of the patients with known level of anti-HSP60 antibodies.

	Patients with low (< median) level of anti-HSP60 antibodies (n=37)	Patients with high (> median) level of anti-HSP60 antibodies (n=37)
Age, year	60, 35-76	60, 40-69
Sex	31 male, 6 female	29 male, 8 female
BMI (kg/m_2)	27.5, 22-34	29, 24-38
Family History of MI	48.64%	45.94%
Serum cholesterol, mmol/l	6.1, 3.4-9.0	6.75, 3.5-11.0
HDL-cholesterol	1.26, 0.7-1.4	1.25, 0.73-1.92
LDL-cholesterol	4.11, 1.7-6.5	3.98, 2.0-8.1
Serum triglycerides, mmol/l	1.77, 0.3-9.6	2.2, 0.6-5.5
Never smoked	44%	31%
Ex-smoker	53%	55%
Current smoker	3%	14%
Anti-hHSP60 (unit/ml)	22.5, 0-71.8	447.9, 137.6-2410
Anti-*M.bovis* HSP65 (unit/ml)	6.6, 0-103.3	20.63, 2.0-380.5

Variables presented as median, range; BMI indicates body mass index; MI - myocardial infraction; HDL - high-density lipoprotein; LDL - low-density lipoprotein.

Table 2 - Complement activation by solid-phase hHSP60

	C4b-binding to:		C3b-binding to:	
	HSP60-coated plate	uncoated plate	HSP60-coated plate	uncoated plate
NHS	0.383±0.036*	0.083±0.02	0.507±0.028	0.28±0.037
Student t-test, p	<0.0001	-	<0.0001	-
HIHS	0.121±0.015	0.118±0.008	0.217±0.022	0.255±0.027
Student t-test, p	0.65	-	0.023	-
Mg^{2+}EGTA-NHS	0.077±0.012	0.081±0.025	0.1±0.005	0.123±0.034
Student t-test, p	0.68	-	0.13	-
C2D	0.179±0.036	0.088±0.017	0.242±0.022	0.285±0.011
Student t-test, p	0.0002	-	0.0014	-

* the numbers are mean OD values ± standard deviation of 6 (NHS, HIHS) or 3 (Mg^{2+}EGTA-NHS, C2D) parallel measurements. One representative experiment out of three similar ones is shown. NHS = normal human serum; HIHS = heat inactivated human serum; C2D = serum of a homozygous C2-deficient patient.

Table 3 - Complement Activation Index (CAI) in cases and controls

	Controls	Cases
Geom mean (approx. sd)	0.43 (0.41)	0.52 (0.47)
Median	0.36	0.47
N	N=176	N=89

Table 4 - Conditional Logistic Regression Analysis

OR (95% CI)	p value
1.23 (0.94 - 1.62)	p=0.14

Table 5 - Samples taken prior to the event

	Controls	Cases	OR (95% CI) p value
Geom mean (approx. sd)	0.39 (0.37)	0.6 (0.54)	1.74 (1.18 - 2.56)
Median	0.33	0.55	p=0.003
N	110	56	

Table 6 - Samples taken after the event

	Controls	Cases	OR (95% CI) p value
Geom mean (approx. sd)	0.50 (0.50)	0.40 (0.36)	0.80 (0.53 - 1.21)
Median	0.41	0.39	p=0.29
N	66	33	

Table 7 - Overall analysis of results by tertiles

Tertile of CAI	OR (95% CI)	p value
1	1.00	p=0.02
2	2.48 (1.24-4.97)	
3	2.16 (1.08-4.31)	

Table 8 - analysis by tertiles of samples prior to event.

Tertile of CAI	OR (95% CI)	p value
1	1.00	p=0.0002
2	5.57 (1.96-15.84)	
3	5.92 (2.04-17.21)	

Table 9 - analysis by tertiles of samples after event.

Tertile of CAI	OR (95% CI)	p value
1	1.00	p=0.67
2	0.92 (0.32-2.68)	
3	0.65 (0.24-1.78)	

Table 10 - Univariant associations in Complement Activation Index nested case-control study.

	Odds Ratio	P>|z|	(95% Conf. Interval)
Body Mass Index	1.17	0.370	0.83 - 1.63
Cholesterol	1.74	0.003	1.20 - 2.53
Triglyceride	1.22	0.249	0.87 - 1.71
Smoking	2.04	0.052	0.99 - 4.18
Fibrinogen	1.59	0.009	1.12 - 2.25
Systolic BP	1.50	0.021	1.06 - 2.12

Table 11 - Full CAI model

	Odds Ratio	P>|z|	(95% Conf. Interval)
Body Mass Index	0.98	0.950	0.60 - 1.60
Cholesterol	1.52	0.060	0.98 - 2.36
Triglyceride	1.02	0.936	0.64 - 1.62
Smoking	1.49	0.361	0.63 - 3.54
Fibrinogen	1.22	0.367	0.80 - 1.86
Systolic BP	1.13	0.600	0.72 - 1.78
CAI tertile 1	1.00		
CAI tertile 2	5.24	0.009	1.50 - 18.26
CAI tertile 3	6.37	0.004	1.80 - 22.58

Table 12 - CAI Sample Distribution by 75% and 95% Percentiles

CAI Values	N° in Control Group	N° in Risk Group
< 1.07	40	28
> 1.07	13	36
< 2.0	49	41
> 2.0	4	23

Table 13 - Odds Ratios (OR)

Cut Off (CAI)	OR (95% CI)	P value
1.07	3.95 (1.78 - 8.73)	p = 0.0007
2.0	6.87 (2.19 - 21.49)	p = 0.0003

CLAIMS

1. hHSP60 or an immunogenic fragment thereof for use in a method of treatment or diagnosis of the human body.

2. A diagnostic test method for a tendency to heat shock protein-induced complement activation, comprising the steps of:

i) contacting a patient sample with hHSP60;

ii) detecting any complement activation; and

iii) correlating the results of detection step (ii) with a tendency to heat shock protein-induced complement activation.

3. A diagnostic test method for a tendency to heat shock protein-induced complement activation, comprising the steps of:

i) taking a sample from a patient;

ii) detecting any antibody in the sample of step (i) which is specific solely to hHSP60; and

iii) correlating the results of detection step (ii) with a tendency to heat shock protein-induced complement activation.

4. A diagnostic test method according to claim 3, detection step (ii) comprising the steps of:

i) contacting the sample with a member of the family of HSP60 proteins, being other than hHSP60;

ii) contacting the resulting sample from step (i) with hHSP60; and

iii) detecting any antibody-hHSP60 binding reaction.

5. A diagnostic test method according to claim 3, detection step (ii) comprising the steps of:

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i) contacting the sample with antigen displaying an epitope unique to hHSP60, and not displaying any shared HSP60 epitopes; and

ii) detecting any antibody-antigen binding reaction.

6. A diagnostic test method according to either one of claims 4 or 5, an antibody-hHSP60 binding reaction being detected by the detection of any complement activation.

7. A diagnostic test method according to either one of claims 2 or 6, complement activation being detected by the binding of C4b.

8. The use of hHSP60 in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation.

9. The use of hHSP60 and a member of the family of HSP60 proteins, being other than hHSP60, in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation.

10. The use of antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes, in the manufacture of a diagnostic test for patients having a tendency to heat shock protein-induced complement activation.

11. A diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises hHSP60 or antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes.

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12. A diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises hHSP60 and a member of the family of HSP60 proteins, being other than hHSP60.

13. A diagnostic test kit for patients having a tendency to heat shock protein-induced complement activation, characterised in that it comprises antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes.

14. The use of hHSP60 or antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes in the manufacture of a medicament for the treatment of heat shock protein-induced complement activation.

15. The use of hHSP60 or antigen displaying an epitope unique to hHSP60 and which does not display any shared HSP60 epitopes according to claim 14, the medicament comprising a vaccine.

16. A method of manufacture of a medicament for the treatment of heat shock protein-induced complement activation, characterised in the use of hHSP60.

17. A diagnostic test method according to any one of claims 2-7, being a method of diagnosis of the human body.

18. The diagnostic test method, use or diagnostic test kit according to any one of claims 2-17, the tendency to heat shock protein-induced complement activation being a tendency to an atherosclerosis-related disease.

19. The diagnostic test method, use or diagnostic test kit according to any one of claims 2-17, the tendency to heat shock protein-induced complement activation being a tendency to a myocardial disorder.

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20. The diagnostic test method, use or diagnostic test kit according to any one of claims 2-17, the tendency to heat shock protein-induced complement activation being a tendency to coronary heart disease.

21. The use of hHSP60 in a method of identifying a compound or substance or agent which inhibits hHSP60-induced complement activation.

22. A method of identifying a compound or substance or agent which inhibits hHSP60-induced complement activation, characterised in the use of hHSP60.

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