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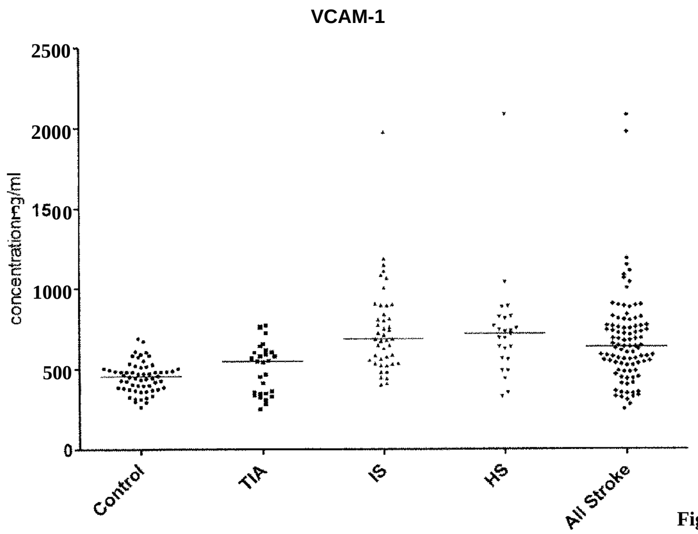

Figure 1

(57) Abstract: The present invention provides biomarker-based methods for diagnosing stoke in a patient suspected of having suffered a stroke, and also for discriminating between ischemic strokeand transient ischemic attack. Substrates comprising probes for specific combinations of biomarkers useful in the methods of the invention are also described.

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BIOMARKER-BASED METHODS AND BIOCHIPS FOR AIDING THE DIAGNOSIS OF STROKE

Background to the Invention

Stroke is the third leading cause of death worldwide and can be defined as the rapidly developing loss of brain function(s) due to interruption in the blood supply to the brain. According to the World Health Organisation, 15 million people per year suffer stroke worldwide, with 5 million dying and a further 5 million being permanently disabled. High blood pressure is estimated to be a contributing factor in 12.7 million of these 15 million stroke cases. In the UK, approximately 150,000 people have a stroke each year and stroke accounts for around 53,000 deaths per year. Stroke costs the economy an estimated £8 billion per year in England alone and stroke patients occupy approximately 20 per cent of all acute hospital beds and 25 per cent of long term beds. Stroke can be classified into three subtypes:

i) ischaemic stroke (IS) occurs when blood supply to the brain is decreased, resulting in brain damage. An ischemic stroke occurs when a blood vessel becomes blocked, usually via a blood clot. This clot may form locally at an atherosclerotic plaque (thrombotic stroke) or alternatively may occur due to a travelling particle or debris that has originated from elsewhere in the bloodstream (embolic stroke);

ii) transient ischaemic attack (TIA) is a 'mini stroke' that occurs when blood supply to the brain is temporarily decreased. A TIA is diagnosed if symptoms are quickly resolved (within 24 hours with the individual returning to normal health); and

iii) haemorrhagic stroke (HS) occurs when blood accumulates within the skull vault, usually when a weakened blood vessel ruptures. Haemorrhagic stroke can be classified into two major subtypes, namely intracerebral (within the brain tissue) and subarachnoid (around the surface of the brain and under its protective layer).

IS and TIA account for approximately 85% of all stroke cases and HS accounts for 15%. In order to minimise neurological damage following stroke it is crucial that stroke patients are rapidly and accurately diagnosed, so that appropriate treatment can be administered. For example, in order to break down clots thrombolytic therapy such as tissue plasminogen activator (TPA) can be administered. However, such therapy is only warranted in IS and is detrimental in HS. The nature of TIA does not require such therapy and blood thinners such as warfarin and aspirin are prescribed in such cases.

At present, if stroke is suspected, physical symptoms are evaluated and a computerised tomography (CT) scan is usually performed. A CT scan has good sensitivity for identifying HS patients (approximately 90% sensitivity) but poor sensitivity for identifying IS and TIA patients (approximately 20% sensitivity). In practice minimal or no tissue damage occurs for TIA due to its transient nature, therefore CT scanning is ineffective as a diagnostic technique. Magnetic Resonance Imaging (MRI) has improved sensitivity for IS diagnosis (up to approximately 80%) but increased time requirements, machine accessibility, and high cost have limited its use for stroke diagnosis. The poor sensitivity of CT scanning for the detection of IS and TIA means that a biological fluid-based diagnostic biomarker tests for detecting IS and TIA would be an invaluable tool to aid clinicians in the diagnosis of stroke sub-type. Biological fluid-based biomarkers have the potential to expedite and increase the accuracy of stroke diagnosis.

Various candidate biomarkers have been proposed for the diagnosis of stroke and stroke sub-type delineation and there are several descriptions of IS/TIA versus HS discrimination in the prior art, for example EP1 238284, WO 201 0/086697, WO 201 0/01 2834, and WO 2002/01 2892.

EP1419388 discloses data that distinguishes IS from HS and all stroke types from non-stroke controls. However, none have thus far found use in clinical practice and there is a real clinical need for biomarkers of all three stroke sub-types that have high sensitivity and specificity to enable accurate diagnosis.

Furthermore, there are currently no biomarkers for delineating IS from TIA. The delineation of IS from TIA using a blood test would facilitate a more informed clinical decision, potentially render unnecessary expensive and less expeditious neuroimaging diagnostics, and would improve the identification of patients who may be in need of thrombolytic therapeutic intervention.

Summary of the Invention

According to a first aspect, the present invention provides a method for diagnosing stroke in a patient suspected of having a stroke, comprising determining the concentration of at least two biomarkers in an *in vitro* sample obtained from the patient and establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the at least two biomarkers are selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI , D-dimer and CRP, and wherein at least one of the two biomarkers is selected from ICAM-1 , L-selectin, P-selectin and VCAM-1 .

According to a second aspect, the present invention provides a substrate comprising probes for at least two biomarkers selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI, D-dimer and CRP for use in a method according to the first aspect of the invention, wherein the substrate comprises a probe for at least one of ICAM-1 , L-selectin, P-selectin and VCAM-1 .

According to a third aspect, the invention is directed to the use of a substrate according to the second aspect in a method for diagnosing stroke according to the first aspect.

According to a fourth aspect, the present prevention provides a method of aiding the diagnosis of ischaemic stroke in a patient suspected of having a stroke, comprising

i) determining the concentration of VCAM-1 and one or more biomarkers selected from h-FABP, IL-6 and CRP in an *in* vitro sample obtained from the patient; and

ii) establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the corresponding control value is the concentration value for the corresponding biomarker determined from an *in vitro* sample obtained from a transient ischaemic attack patient or patients. This method can be used to differentially diagnose between ischemic stroke and a transient ischaemic attack.

According to a fifth aspect, the present invention provides a substrate comprising probes for VCAM-1 and at least one other biomarker selected from h-FABP, IL-6 and CRP for use in a method according to the fourth aspect of the invention.

According to a sixth aspect, the invention is directed to the use of a substrate according to the fifth aspect in a method for diagnosing stroke according to the fourth aspect.

Description of the Drawings

Figure 1 is a graph showing the concentration of VCAM-1 for all stroke patients, each stroke sub-type and the control subjects;

Figure 2 is a graph showing the concentration of ICAM-1 for all stroke patients, each stroke sub-type and the control subjects;

Figure 3 is a graph showing the concentration of E-selectin for all stroke patients, each stroke sub-type and the control subjects;

Figure 4 is a graph showing the concentration of P-selectin for all stroke patients, each stroke sub-type and the control subjects;

Figure 5 is a graph showing the concentration of L-selectin for all stroke patients, each stroke sub-type and the control subjects;

Figure 6 is a graph showing the concentration of IL-6 for all stroke patients, each stroke sub-type and the control subjects;

Figure 7 is a graph showing the concentration of sTNFRI for all stroke patients, each stroke sub-type and the control subjects;

Figure 8 is a graph showing the concentration of NGAL for all stroke patients, each stroke sub-type and the control subjects;

Figure 9 is a graph showing the concentration of D-dimer for all stroke patients, each stroke sub-type and the control subjects;

Figure 10 is a graph showing the concentration of TM for all stroke patients, each stroke sub-type and the control subjects;

Figure 11 is a graph showing the concentration of CRP for all stroke patients, each stroke sub-type and the control subjects;

Figure 12 is a graph showing the concentration of h-FABP for all stroke patients, each stroke sub-type and the control subjects;

Figure 13 is a graph showing the concentration of diluted CRP for all stroke patients, each stroke sub-type and the control subjects;

Figure 14 is a ROC curve for VCAM-1 (all stroke v control);

Figure 15 is a ROC curve for ICAM-1 (all stroke v control);

Figure 16 is a ROC curve for P-selectin (all stroke v control);

Figure 17 is a ROC curve for L-selectin (all stroke v control);

Figure 18 is a ROC curve for IL-6 (all stroke v control);

Figure 19 is a ROC curve for sTNFRI (all stroke v control);

Figure 20 is a ROC curve for CRP (all stroke v control);

Figure 21 is a ROC curve for NGAL (all stroke v control); and

Figure 22 is a ROC curve for D-dimer (all stroke v control).

Detailed Description of the Invention

The present invention relates to biomarker-based methods and biochips that can be used for rapid diagnosis of stroke, and furthermore to aid discrimination between the three stroke sub-types: haemorrhagic stroke (HS), ischemic stroke (IS) and transient ischemic attack (TIA).

Unless stated otherwise, all references herein to the term 'stroke' encompasses all three forms of stroke.

References herein to 'a patient suspected of having a stroke' or 'having had a stroke' include a patient who is suspected of currently suffering from a stroke or who is suspected of having previously had a stroke. The stroke may have been a recent event, such an event having initiated the process of the individual seeking clinical help.

The terms "subject" and "patient" may be used interchangeably herein and refer to a mammal including a non-primate (e.g. a cow, pig, horse, dog, cat, rat and mouse) and a primate (e.g. a monkey and human). Preferably the subject or patient is a human.

As used herein, the term 'biomarker' refers to a molecule present in a biological sample obtained from a patient, the concentration of which in said sample may be indicative of a pathological state. Various biomarkers that have been found to be useful in diagnosing stroke and stroke sub-types, either alone or in combination with other diagnostic methods, or as complementary biomarkers in combination with other biomarkers, are described herein. A used herein, the term 'complementary biomarker' refers to a biomarker that can be used in conjunction with other stroke biomarkers to support diagnosis.

It is well understood in the art that biomarker normal or 'background' concentrations may exhibit slight variation due to, for example, age, gender or ethnic/geographical genotypes. As a result, the cut-off value used in the methods of the invention may also slightly vary due to optimization depending upon the target patient/population.

The biological sample obtained from a patient is preferably a blood, serum or plasma sample. As used herein, the term *'in vitro'* has its usual meaning in the art and refers to a sample that has been removed from a patient's body.

When a blood sample is taken from the patient for analysis, whole blood, serum or plasma is analysed. Analysis of the blood sample can be by way of several analytical methodologies such as mass spectrometry linked to a pre-separation step such as chromatography. The preferred methodology is based on immuno-detection. Immuno-detection technology is also readily incorporated into transportable or hand-held devices for use outside of the clinical environment. A quantitative immunoassay such as a Western blot or ELISA can be used to detect the amount of protein. A preferred method of analysis comprises using a multi-analyte biochip which enables several proteins to be detected and quantified simultaneously. 2D Gel Electrophoresis is also a technique that can be used for multi-analyte analysis.

A first aspect of the invention provides a method for diagnosing stroke in a patient suspected of having a stroke, comprising determining the concentration of at least two biomarkers in an *in vitro* sample obtained from the patient and establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the at least two biomarkers are selected from ICAM-1, L-selectin, P-selectin, VCAM-1, IL-6, sTNFRI, D-dimer and CRP, and wherein at least one of the two biomarkers is selected from ICAM-1, L-selectin, P-selectin and VCAM-1.

Preferably the at least two biomarkers are selected from (i) ICAM-1 or VCAM-1 and (ii) L-selectin or P-selectin, and more preferably they are ICAM-1 and L-selectin. Combinations of three or more biomarkers are also preferred as they show the highest sensitivity and specificity.

In preferred embodiments, the method further comprises determining the sample concentration of one or more biomarkers selected from IL-6, sTNFRI, D-dimer and CRP. The method may also further comprise determining the sample concentration of h-FABP.

For the avoidance of doubt, in the context of this aspect of the invention, 'stroke' refers to 'all stroke' (i.e. all three stroke sub-types).

Preferred biomarker combinations are those listed in Table 1 or Table 2. These tables provide sensitivity, specificity and AUC data for different biomarker combinations for stoke v control.

Table 1

Biomarker(s)	% Sensitivity	% Specificity	AUC
1. VCAM-1 ICAM-1	80.6	75.0	0.831
2. VCAM-1 Psel	87.8	71.7	0.913
3. VCAM-1 Lsel	89.8	86.7	0.943
4. VCAM-1 IL-6	80.6	78.3	0.879
5. VCAM-1 CRP	78.6	75.0	0.826

6. VCAM-1 D-dimer	87.8	76.7	0.886
7. VCAM-1 NGAL	81.6	73.3	0.867
8. VCAM-1 sTNFRI	82.7	75.0	0.832
9. IL-6 sTNFRI	78.6	75.0	0.870
10. ICAM-1 Psel	92.9	76.7	0.932
11. ICAM-1 Lsel	90.8	90.0	0.954
12. ICAM-1 IL-6	83.7	83.3	0.897
13. ICAM-1 CRP	79.6	80.0	0.822
14. ICAM-1 D-dimer	86.7	76.7	0.905
15. ICAM-1 NGAL	81.6	73.3	0.836
16. ICAM-1 sTNFRI	77.6	73.3	0.832
17. IL-6 NGAL	87.8	81.7	0.909
18. Psel Lsel	88.8	65.0	0.867
19. Psel IL-6	90.8	78.3	0.937
20. Psel CRP	87.8	68.3	0.888
21. Psel D-dimer	90.8	85.0	0.931
22. Psel NGAL	86.7	58.3	0.838
23. Psel sTNFRI	86.7	65.0	0.885
24. IL-6 D-dimer	84.7	81.7	0.910
25. Lsel IL-6	84.7	85.0	0.907
26. Lsel CRP	86.7	71.7	0.863
27. Lsel D-dimer	88.8	80.0	0.894
28. Lsel NGAL	90.8	51.7	0.833
29. Lsel sTNFRI	84.7	61.7	0.862
30. IL-6 CRP	76.5	81.7	0.870
31. IL-6 NGAL sTNFRI	89.8	81.7	0.942
32. IL-6 D-dimer sTFNRI	85.7	80.0	0.908
33. IL-6 D-dimer NGAL	92.9	83.3	0.943
34. IL-6 CRP sTNFRI	75.5	78.3	0.872
35. VCAM-1 ICAM-1 Psel	91.8	80.0	0.946
36. VCAM-1 ICAM-1 Lsel	93.9	93.3	0.975
37. VCAM-1 ICAM-1 IL-6	85.7	81.7	0.906
38. VCAM-1 ICAM-1 CRP	80.6	78.3	0.853
39. VCAM-1 ICAM-1 D-dimer	88.8	80.0	0.907
40. VCAM-1 ICAM-1 NGAL	85.7	80.0	0.895
41. VCAM-1 ICAM-1 sTNFRI	82.7	75.0	0.856
42. IL-6 CRP NGAL	85.7	80.0	0.915
43. VCAM-1 Psel Lsel	92.9	88.3	0.957
44. VCAM-1 Psel IL-6	90.8	76.7	0.962
45. VCAM-1 Psel CRP	87.8	78.3	0.930
46. VCAM-1 Psel D-dimer	89.8	83.3	0.955
47. VCAM-1 Psel NGAL	89.8	76.7	0.932
48. VCAM-1 Psel sTNFRI	88.8	76.7	0.923
49. IL-6 CRP D-dimer	81.6	80.0	0.911
50. VCAM-1 Lsel IL-6	89.8	90.0	0.957
51. VCAM-1 Lsel CRP	91.8	91.7	0.951
52. VCAM-1 Lsel D-dimer	89.8	85.0	0.946
53. VCAM-1 Lsel NGAL	92.9	83.3	0.962
54. VCAM-1 Lsel sTNRI	83.3	87.8	0.947
55. Lsel NGAL sTNFRI	89.8	80.0	0.931
56. VCAM-1 IL-6 CRP	79.6	81.7	0.881
57. VCAM-1 IL-6 D-dimer	86.7	88.3	0.916
58. VCAM-1 IL-6 NGAL	91.8	86.7	0.941
59. VCAM-1 IL-6 sTNFRI	81.6	80.0	0.882
60. Lsel D-dimer sTNFRI	83.7	76.7	0.905
61. VCAM-1 CRP D-dimer	85.7	81.7	0.895

62. VCAM-1 CRP NGAL	87.8	81.7	0.911
63. VCAM-1 CRP sTNFRI	80.6	78.3	0.837
64. Lsel D-dimer NGAL	91.8	85.0	0.921
65. VCAM-1 D-dimer NGAL	90.8	96.7	0.938
66. VCAM-1 D-dimer sTNFRI	87.8	80.0	0.891
67. Lsel CRP sTNFRI	84.7	73.3	0.875
68. VCAM-1 NGAL sTNFRI	89.8	80.0	0.930
69. Lsel CRP D-dimer	86.7	76.7	0.908
70. Lsel CRP NGAL	86.7	73.3	0.882
71. ICAM-1 Psel Lsel	95.9	91.7	0.977
72. ICAM-1 Psel IL-6	93.9	91.7	0.979
73. ICAM-1 Psel CRP	92.9	83.3	0.949
74. ICAM-1 Psel D-dimer	93.9	88.3	0.969
75. ICAM-1 Psel NGAL	88.8	78.3	0.938
76. ICAM-1 Psel sTNFRI	91.8	81.7	0.946
77. Lsel IL-6 sTNFRI	84.7	81.7	0.911
78. ICAM-1 Lsel IL-6	92.9	90.0	0.975
79. ICAM-1 Lsel CRP	89.8	90.0	0.958
80. ICAM-1 Lsel D-dimer	90.8	88.3	0.964
81. ICAM-1 Lsel NGAL	91.8	86.7	0.963
82. ICAM-1 Lsel sTNFRI	91.8	88.3	0.965
83. Lsel IL-6 NGAL	90.8	83.3	0.920
84. ICAM-1 IL-6 CRP	83.7	83.3	0.896
85. ICAM-1 IL-6 D-dimer	87.8	85.0	0.931
86. ICAM-1 IL-6 NGAL	89.8	86.7	0.934
87. ICAM-1 IL-6 sTNFRI	84.7	80.0	0.903
88. Lsel IL-6 D-dimer	86.7	81.7	0.920
89. ICAM-1 CRP D-dimer	88.0	85.0	0.911
90. ICAM-1 CRP NGAL	85.7	76.7	0.882
91. ICAM-1 CRP sTNFRI	77.6	73.3	0.844
92. Lsel IL-6 CRP	87.8	81.7	0.914
93. ICAM-1 D-dimer NGAL	90.8	83.3	0.932
94. ICAM-1 D-dimer sTNFRI	87.8	80.0	0.909
95. Psel NGAL sTNFRI	89.8	76.7	0.930
97. ICAM-1 NGAL sTNFRI	87.8	83.3	0.920
98. Psel D-dimer sTNFRI	89.8	81.7	0.930
99. Psel D-dimer NGAL	91.8	86.7	0.947
100. Psel Lsel IL-6	89.8	78.3	0.943
101. Psel Lsel CRP	89.8	75.0	0.903
102. Psel Lsel D-dimer	90.8	83.3	0.936
103. Psel Lsel NGAL	88.8	70.0	0.873
104. Psel Lsel sTNFRI	90.8	71.7	0.914
105. Psel CRP sTNFRI	87.8	70.0	0.897
106. Psel IL-6 CRP	88.8	76.7	0.945
107. Psel IL-6 D-dimer	90.8	88.3	0.957
108. Psel IL-6 NGAL	92.9	88.3	0.953
109. Psel IL-6 sTNDRI	89.8	78.3	0.944
110. Psel CRP NGAL	86.7	75.0	0.907
111. Psel CRP D-dimer	91.8	85.0	0.946
112. VCAM-1 IL-6, NGAL sTNFRI	91.8	90.0	0.961
113. VCAM-1 D-dimer, NGAL sTNFRI	89.8	88.3	0.959
114. ICAM-1 , Lsel IL-6 D-dimer	92.9	90.0	0.980
115. ICAM-1 Lsel IL-6 NGAL	94.9	91.7	0.983
116. ICAM-1 Lsel IL-6 sTNFRI	92.9	91.7	0.978
117. ICAM-1 Lsel D-dimer NGAL	94.9	91.7	0.975
118. ICAM-1 Lsel D-dimer sTNFRI	93.9	90.0	0.975

119. ICAM-1 Lsel NGAL sTNFRI	96.9	95.0	0.978
120. ICAM-1 IL-6 D-dimer NGAL	91.8	88.3	0.966
121. ICAM-1 IL-6 D-dimer sTNFRI	86.7	86.7	0.932
122. ICAM-1 IL-6 NGAL sTNFRI	92.9	85.0	0.967
123. ICAM-1 D-dimer NGAL sTNFRI	91.8	85.0	0.959
124. Lsel IL-6 D-dimer NGAL	92.9	88.3	0.948
125. Psel Lsel IL-6 ICAM-1	95.9	95.0	0.995
126. Lsel IL-6 NGAL sTNFRI	93.9	85.0	0.958
127. Lsel D-dimer NGAL sTNFRI	90.8	86.7	0.946
128. VCAM-1 ICAM-1 Lsel IL-6	96.9	95.0	0.985
129. VCAM-1 ICAM-1 Lsel D-dimer	94.9	93.3	0.978
130. VCAM-1 ICAM-1 Lsel NGAL	96.9	93.3	0.984
131. VCAM-1 ICAM-1 Lsel sTNFRI	94.9	95.0	0.977
132. VCAM-1 ICAM-1 IL-6 D-dimer	86.7	86.7	0.933
133. VCAM-1 ICAM-1 IL-6 NGAL	91.8	83.3	0.954
134. Psel Lsel IL-6 VCAM-1	93.9	86.7	0.972
135. VCAM-1 ICAM-1 D-dimer NGAL	89.8	80.0	0.948
136. Psel Lsel IL-6 D-dimer	89.8	88.3	0.959
137. VCAM-1 ICAM-1 NGAL sTNRI	85.7	81.7	0.944
138. VCAM-1 Lsel IL-6 D-dimer	90.8	91.7	0.956
139. VCAM-1 Lsel IL-6 NGAL	92.9	91.7	0.972
140. VCAM-1 Lsel IL-6 sTNFRI	88.8	90.0	0.959
141. VCAM-1 Lsel D-dimer NGAL	93.9	90.0	0.968
142. VCAM-1 Lsel D-dimer sTNFRI	92.9	88.3	0.949
143. VCAM-1 Lsel NGAL sTNFRI	91.8	90.0	0.970
144. VCAM-1 IL-6 D-dimer NGAL	92.9	88.3	0.971
145. IL-6 D-dimer NGAL sTNFRI	89.8	88.3	0.971
146. Psel Lsel IL-6 NGAL	93.9	85.0	0.953
147. CRP D-dimer ICAM-1 IL-6	87.8	85.0	0.932
148. CRP D-dimer ICAM-1 Lsel	91.8	91.7	0.966
149. CRP D-dimer ICAM-1 NGAL	87.8	83.3	0.939
150. Psel Lsel ICAM-1 D-dimer	98.0	93.3	0.989
151. Psel Lsel ICAM-1 CRP	95.9	90.0	0.980
152. Psel IL-6 ICAM-1 D-dimer	95.9	93.3	0.988
153. CRP D-dimer IL-6 NGAL	91.8	85.0	0.948
154. CRP Lsel sTNFRI VCAM-1	87.8	90.0	0.952
155. Psel IL-6 ICAM-1 NGAL	94.9	90.0	0.983
156. CRP D-dimer Lsel NGAL	93.9	80.0	0.935
157. CRP Lsel NGAL sTNFRI	91.8	81.7	0.933
158. CRP D-dimer Lsel VCAM-1	88.3	91.8	0.950
159. Lsel Psel VCAM-1 ICAM-1	94.9	95.0	0.986
160. CRP D-dimer NGAL VCAM-1	90.8	85.0	0.950
161. CRP IL-6 NGAL VCAM-1	90.8	88.3	0.947
162. CRP ICAM-1 IL-6 Lsel	92.9	90.0	0.975
163. CRP ICAM-1 IL-6 NGAL	88.8	83.3	0.938
164. CRP IL-6 NGAL sTNFRI	89.8	80.0	0.947
165. CRP IL-6 Lsel VCAM-1	90.8	91.7	0.957
166. CRP ICAM-1 Lsel NGAL	94.9	88.3	0.970
167. CRP ICAM-1 Lsel sTNFRI	91.8	88.3	0.968
168. CRP ICAM-1 Lsel VCAM-1	93.9	95.0	0.976
169. CRP IL-6 Lsel NGAL	88.8	83.3	0.931
170. CRP NGAL sTNFRI VCAM-1	87.8	85.0	0.934

Table 2

Biomarkers	% Sensitivity	% Specificity	AUC
1. VCAM1 + FABP	89.8	95.0	0.960
2. ICAM1 + FABP	92.9	93.3	0.964
3. PSel + FABP	95.9	91.7	0.981
4. LSel + FABP	91.8	95.0	0.970
5. VCAM1 + ICAM1 + FABP	92.9	93.3	0.965
6. VCAM1 + PSel + FABP	95.9	91.7	0.983
7. VCAM1 + Lsel + FABP	92.9	96.7	0.971
8. VCAM1 + IL6 + FABP	90.8	95.0	0.961
9. VCAM1 + CRP + FABP	89.8	95.0	0.960
10. VCAM1 + DDimer + FABP	90.8	95.0	0.963
11. VCAM1 + NGAL + FABP	98.0	93.3	0.986
12. VCAM1 + sTNFRI + FABP	89.8	91.7	0.962
13. ICAM1 + PSel + FABP	96.9	93.3	0.990
14. ICAM1 + LSel + FABP	96.9	93.3	0.993
15. ICAM1 + IL6 + FABP	91.8	91.7	0.966
16. ICAM1 + CRP + FABP	92.9	93.3	0.964
17. ICAM1 + DDimer + FABP	92.9	95.0	0.968
18. ICAM1 + NGAL + FABP	96.9	95.0	0.984
19. ICAM1 + sTNFRI + FABP	91.8	93.3	0.966
20. PSel + LSel + FABP	95.9	93.3	0.985
21. PSel + IL6 + FABP	93.9	93.3	0.985
22. PSel + CRP + FABP	92.9	91.7	0.983
23. PSel + DDimer + FABP	93.9	93.3	0.984
24. PSel + NGAL + FABP	96.9	96.7	0.993
25. PSel + sTNFRI + FABP	93.9	91.7	0.983
26. Lsel + IL6 + FABP	90.8	93.3	0.975
27. LSel + CRP + FABP	91.8	93.3	0.970
28. IL6 + CRP + FABP	91.8	96.7	0.962
29. IL6 + DDimer + FABP	89.8	93.3	0.963
30. IL6 + NGAL + FABP	91.8	93.3	0.990
31. IL6 + sTNFRI + FABP	89.8	91.7	0.963
32. LSel + DDimer + FABP	90.8	93.3	0.973
33. LSel + NGAL + FABP	95.9	93.3	0.989
34. LSel + sTNFRI + FABP	92.9	93.3	0.972
35. FABP + CRP + DDimer	90.8	93.3	0.962
36. FABP + CRP + NGAL	95.9	93.3	0.985
37. FABP + CRP + sTNFRI	90.8	93.3	0.959
38. FABP + DDimer + NGAL	95.9	93.3	0.985
39. FABP + DDimer + sTNFRI	91.8	93.3	0.962
40. CRP + IL6 + FABP	89.8	93.3	0.962

41. DDimer + IL6 + FABP	91.8	93.3	0.963
42. NGAL + IL6 + FABP	95.9	93.3	0.990
43. sTNFRI + IL6 + FABP	89.8	91.7	0.963
44. IL6 + NGAL + FABP + DDimer	96.9	93.3	0.990
45. LSel + NGAL + FABP + DDimer	95.9	93.3	0.992
46. LSel + NGAL + FABP + IL6	94.9	93.3	0.994
47. PSel + sTNFRI + FABP + DDimer	93.9	93.3	0.985
48. PSel + sTNFRI + FABP + NGAL	96.9	96.7	0.994
49. PSel + IL6 + FABP + DDimer	93.9	91.7	0.986
50. PSel + IL6 + FABP + NGAL	96.9	95.0	0.996
51. PSel + LSel + FABP + DDimer	95.9	93.3	0.987
52. PSel + LSel + FABP + IL6	93.9	91.7	0.987
53. PSel + LSel + FABP + NGAL	96.9	96.7	0.994
54. PSel + LSel + FABP + CRP	94.9	93.3	0.985
55. ICAM1 + NGAL + FABP + IL6	95.9	93.3	0.991
56. ICAM1 + NGAL + FABP + DDimer	96.9	95.0	0.986
57. ICAM1 + NGAL + FABP + CRP	96.9	95.0	0.986
58. ICAM1 + LSel + FABP + IL6	95.9	95.0	0.994
59. ICAM1 + LSel + FABP + NGAL	99.0	96.7	0.996
60. ICAM1 + LSel + FABP + DDimer	96.9	95.0	0.993
61. ICAM1 + LSel + FABP + CRP	96.9	93.3	0.993
62. ICAM1 + LSel + FABP + sTNFRI	96.9	93.3	0.993
63. ICAM1 + PSel + FABP + IL6	98.0	95.0	0.994
64. ICAM1 + PSel + FABP + NGAL	96.9	96.7	0.996
65. ICAM1 + PSel + FABP + DDimer	96.9	93.3	0.991
66. ICAM1 + PSel + FABP + CRP	98.0	91.7	0.990
67. ICAM1 + PSel + FABP + sTNFRI	96.9	93.3	0.990
68. ICAM1 + PSel + LSel + FABP	100.0	95.0	0.997
69. VCAM1 + NGAL + FABP + DDimer	96.9	93.3	0.988
70. VCAM1 + ICAM1 + LSel + FABP	99.0	95.0	0.993
71. VCAM1 + LSel + FABP + DDimer	92.9	95.0	0.971
72. VCAM1 + LSel + FABP + NGAL	96.9	93.3	0.991
73. FABP + NGAL + sTNFRI	95.9	93.3	0.986

Biomarker concentrations can be determined by contacting the sample with a substrate having probes specific for each of the biomarkers included in the combination of biomarkers. Interactions between a biomarker and its respective probe can be monitored and quantified using various techniques that are well-known in the art. Biomarker concentrations are preferably measured in ng/ml.

Preferably, a solid state device is used in the methods of the present invention, preferably the Biochip Array Technology system (BAT) (available from Randox Laboratories Limited). More preferably, the Evidence Evolution and Evidence Investigator apparatus (available from Randox Laboratories) may be used to determine the levels of biomarkers in the sample.

Control values are derived from the concentration of corresponding biomarkers in a biological sample obtained from an individual or individuals who have not undergone a stroke. Such individual(s) who have not undergone stroke may be, for example, healthy individuals, individuals suffering from diseases other than stroke. Alternatively, the control values may correspond to the concentration of each of the biomarker in a sample obtained from the patient prior to the stroke event.

For the avoidance of doubt, the term 'corresponding biomarkers' means that concentrations of the same combination of biomarkers that are determined in respect of the patient's sample are also used to determine the control values. For example, if the concentration of ICAM-1 and L-selectin in the patient's sample is determined, then the concentration of ICAM-1 and L-selectin in the control sample will also be determined.

In a preferred embodiment, each of the patient and control biomarker concentration values is inputted into one or more statistical algorithms to produce an output value that indicates whether a stroke has occurred.

The cut-off concentrations or values are derived using a statistical technique; various different methods are available for developing statistical algorithms and are well-known to those skilled in the art. A standard method of biomarker statistical analysis is to use univariate methods to compare biomarker levels in various groups and highlight those biomarkers whose concentrations significantly differ across and between particular groups.

The accuracy of statistical methods used in accordance with the present invention can be best described by their receiver operating characteristics (ROC). The ROC curve addresses both the sensitivity, the number of true positives, and the specificity, the number of true negatives, of the test. Therefore, sensitivity and specificity values for a given combination of biomarkers are an indication of the accuracy of the assay. For example, if a biomarker combination has sensitivity and specificity values of 80%, out of 100 patients which have stroke, 80 will be correctly identified from the determination of the presence of the particular combination of biomarkers as positive for stroke, while out of 100 patients who have not suffered a stroke 80 will accurately test negative for the disease.

If two or more biomarkers are to be used in the diagnostic method a suitable mathematical model, such as logistic regression equation, can be derived. The logistic regression equation might include other variables such as age and gender of patient. The ROC curve can be used to assess the accuracy of the logistic regression model. The logistic regression equation can be used independently or in an algorithm to aid clinical decision making. Although a logistic regression equation is a common mathematical/statistical procedure used in such cases and is preferred in the context of the present invention, other mathematical/statistical procedures can also be used.

By way of example, a logistic regression equation applicable to the present invention (at a classification cut-off value of 0.5) for the biomarker combination ICAM-1 , L-selectin, D-dimer and sTNFRI for indication of stroke versus non-stroke (control) in a patient suspected of having had or currently experiencing a stroke is calculated as follows:

$$\text{Probability of Stroke} = \frac{1}{1 + e^{-(2.105+0.27[\text{ICAM-1}]-0.018[\text{L-selectin}]+0.071[\text{D-dimer}]+8.945[\text{sTNFRI}])}}$$

where [ICAM-1], [L-selectin], [D-dimer] and [sTNFRI] are the concentrations of ICAM-1 , L-selectin, D-dimer and sTNFRI measured in a blood sample taken from the patient (see number 118 of Table 1 for AUC value).

If the outcome of carrying out the method of the invention is a positive diagnosis of stoke, then the patient should be treated accordingly. However, since the most appropriate and efficacious treatment varies according to the stoke sub-type, it is useful to be able to further differentiate between the three different sub-types following a positive diagnosis of stroke. For example, if the patient has suffered an IS, thrombolytic therapy such as tissue plasminogen activator (TPA) can be administered to break-down clots. Alternatively, if the patient has suffered a TIA, blood thinners such as warfarin and aspirin may be prescribed.

Therefore, according to a further embodiment, the method according to the first aspect of the invention may optionally include carrying out additional steps for differentially diagnosing between IS and TIA as defined in the fourth aspect of this invention.

A second related aspect of the invention provides a substrate comprising probes for at least two biomarkers selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI, D-dimer and CRP for use in a method for diagnosing stroke in a patient according to the first aspect of the invention, wherein the substrate comprises a probe for at least one of ICAM-1 , L-selectin, P-selectin and VCAM-1 . Optionally, the substrate may further comprise a probe for h-FABP.

Preferably the substrate has at least two probes immobilised thereon, more preferably three, four or more probes, wherein each probe is specific to an individual biomarker. As used herein, the term 'specific' means that the probe binds only to one of the biomarkers of the invention, with negligible binding to other biomarkers of the invention or to other analytes in the biological sample being analysed. This ensures that the integrity of the diagnostic assay and its result using the biomarkers of the invention is not compromised by additional binding events.

The substrate can be any substance able to support one or more probes, but is preferably a biochip. A biochip is a planar substrate that may be, for example, mineral or polymer based, but is preferably ceramic. When identifying the various biomarkers/proteins of the invention it will be apparent to the skilled person that as well as identifying the full length protein, the identification of a fragment or several fragments of a protein is possible, provided this allows accurate identification of the protein. Similarly, although a preferred probe of the invention is a polyclonal or monoclonal antibody, other probes such as aptamers, molecular imprinted polymers, phages, short chain antibody fragments and other antibody-based probes may be used.

In a related third aspect of the invention, a substrate according to the second aspect is used in the method according to the first aspect of the invention.

The present invention also provides kits comprising probes for at least two biomarkers selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI , D-dimer and CRP, additional reagents, substrate/reaction surfaces and/or instructions for use. Such kits can be used to diagnose stroke in a patient a according to the first aspect of the invention.

A fourth aspect of the present invention provides a method of aiding the diagnosis of ischaemic stroke in a patient suspected of having a stroke, comprising

i) determining the concentration of VCAM-1 and one or more biomarkers selected from h-FABP, IL-6 and CRP in an *in* vitro sample obtained from the patient; and

ii) establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the corresponding control value is the concentration value for the corresponding biomarker determined from an *in vitro* sample obtained from a transient ischaemic attack patient or patients.

Advantageously, this method can be used to differentially diagnose between ischemic stroke and a transient ischaemic attack.

Each of the biomarkers or biomarker combinations can be used alone or as complementary biomarkers. Preferred biomarker combinations can be identified from the data in Table 4.

The control values can be established by measuring the concentration of the biomarkers VCAM-1 and one or more h-FABP, IL-6 and CRP in one or more patients clinically diagnosed as having, or having had, a TIA. The diagnosis may be derived using techniques such as clinician examination and neuroimaging analysis (which would rule out the possibility of HS).

Biomarker concentrations can be determined by contacting the sample with a substrate having probes specific for each of the biomarkers included in the combination of biomarkers. Interactions between biomarker and its respective probe can be monitored and quantified using various techniques that are well-known in the art.

In a preferred embodiment, each of the patient and control biomarker concentration values is inputted into one or more statistical algorithms to produce an output value that indicates whether ischemic stroke has occurred.

By way of example, the following concentrations ('cut-off concentration) support the diagnosis of IS in the patient: h-FABP ≥ about 10ng/ml; VCAM-1 ≥ about 570ng/ml; CRP ≥ about 30μg/ml; and IL-6 ≥ about 12pg/ml. However, biomarker normal or 'background' concentrations may exhibit slight variation due to, for example, age, gender or ethnic/geographical genotypes. As a result, the cut-off value used may also slightly vary due to optimisation depending upon the target patient/population.

The cut-off concentrations or values are usually derived using statistical techniques. A standard method of biomarker statistical analysis is to use univariate methods to compare biomarker levels in various groups and highlight those biomarkers whose concentrations significantly differ between particular groups. This is followed by Receiver Operator Characteristic (ROC) analysis.

As described above in relation to the first aspect of the invention, a ROC curve is a preferred method of assessing the accuracy of a diagnostic test. It also provides a measure of the predictive power of the test in the form of the area under the curve (AUC), which can have values of 0.5 to 1.0. As a general rule, a test with a sensitivity of about 80% or more and a specificity of about 80% or more is regarded in the art as a test of potential use, although these values vary according to the clinical application.

For discriminating between IS and TIA according to the method of the invention, a high specificity is crucial. For a given test, the closer the value of its AUC is to 1.0, the greater its predictive power. A logistic regression equation can be derived for any test involving two or more biomarkers. The logistic regression equation may include other variables, such as the age and gender of the patient. The ROC curve can be used to assess the accuracy of the logistic regression model. The logistic regression equation can be used independently or in an algorithm to aid clinical decision making. Although a logistic regression equation is a common mathematical/statistical tool, other mathematical/statistical procedures are well known in the art and can be used in accordance with the present invention.

The outcome of carrying out the method according to this aspect of the invention will be a diagnosis of either IS or TIA and the patient should then be treated accordingly. If as a result of carrying out the method of the invention it is determined that the patient has suffered an IS, appropriate treatment such as thrombolytic therapy (e.g. tissue plasminogen activator (TPA)) can be administered to break-down clots. This may be administered in conjunction with other appropriate therapies, as determined by a physician. If as a result of carrying out the method of the invention it is determined that the patient has suffered a TIA, blood thinners such as warfarin and aspirin may be prescribed and administered.

A related fifth aspect of the invention provides a substrate comprising probes for VCAM-1 and at least one other biomarker selected from h-FABP, IL-6 and CRP for use in a method for aiding the diagnosis of ischaemic stroke in a patient according to the present invention.

The substrate comprises at least two, preferably three or four probes, each probe specific to an individual biomarker. As used herein, the term 'specific' means that the probe binds only to one of the biomarkers of the invention, with negligible binding to other biomarkers of the invention or to other analytes in the biological sample being analysed. This ensures that the integrity of the diagnostic assay and its result using the biomarkers of the invention is not compromised by additional binding events.

The substrate can be any substance able to support one or more probes, but is preferably a biochip. A biochip is a planar substrate that may be, for example, mineral or polymer based, but is preferably ceramic. When identifying the various biomarkers/proteins of the invention it will be apparent to the skilled person that as well as identifying the full length protein, the identification of a fragment or several fragments of a protein is possible, provided this allows accurate identification of the protein. Similarly, although a preferred probe of the invention is a polyclonal or monoclonal antibody, other probes such as aptamers, molecular imprinted polymers, phages, short chain antibody fragments and other antibody-based probes may be used.

Preferably, a solid state device is used in the methods of the present invention, preferably the Biochip Array Technology system (BAT) (available from Randox Laboratories Limited). More preferably, the Evidence Evolution and Evidence Investigator apparatus (available from Randox Laboratories) may be used to determine the levels of biomarkers in the sample.

In a related sixth aspect of the invention, a substrate according to the fifth aspect is used in the method according to the fourth aspect of the invention.

The invention also provides kits comprising probes for VCAM-1 and at least one other biomarker selected from h-FABP, IL-6 and CRP, additional reagents, substrate/reaction surfaces and/or instructions for use. Such kits can be used to diagnose IS in a patient according to the third aspect of the invention.

A further aspect of the invention is directed to the use of one or more of h-FABP, STNFR1 , IL-6, D-dimer, L-selectin, P-selectin, ICAM-1, VCAM-1 and CRP as complementary biomarkers of stroke or stroke sub-type. As complementary biomarkers they may be used for stroke/stroke sub-type diagnosis in conjunction with proteins such as DJ-1 , BNP, S100 β, MMP-9, MCP-1 , ApoCI, ApoC3, von Willebrand factor, NMDA receptors, ADMA and Lp-PLA2.

The invention will now be described further by reference to the following non-limiting example.

Example:

Patient Group

The study consisted of 98 patients displaying stroke symptoms admitted to the Emergency Department of KAT General Hospital, Athens, Greece. Blood samples were taken at the time of admission and at days 1, 2, 3 and 7. The mean time from the onset of stroke symptoms and hospital admission was 3.2 hours. The mean age of the patients was 75.2 years (standard deviation 9.4). Clinician evaluation (using criteria highlighted in the Background section) and neuroimaging techniques identified 44 ischaemic stroke (IS), 25 haemorrhagic stroke (HS), 29 transient ischaemic attack (TIA); 60 healthy subjects served as controls (C).

Sample Analysis

The following proteins were tested against EDTA plasma samples of blood obtained from the patients of the study group: ICAM-1, VCAM-1, E-selectin, L-selectin, P-selectin, IL-6, h-FABP, CRP, D-dimer, sTNFRI, TM and NGAL. The proteins were detected and quantified using multiplexed biochips incorporating biomarker-specific antibodies and the Evidence Investigator (Randox Laboratories Ltd, Crumlin, UK). The simultaneous immunoassays were performed according to manufacturer's instructions. A nine-point calibration curve and three reference controls were assayed for each biomarker to allow validation of results. For CRP IS vs TIA analysis, samples were diluted tenfold.

Statistical Analysis

The Kruskal-Wallis test (significance limit 0.05) was used to identify analytes that were differentially expressed across the four groups (IS, HS, TIA and C). Post-hoc comparisons between the different groups were carried out using the Holm's sequential Bonferroni adjustment. Mann-Whitney test was used to compare 'All Stroke' and 'Control'. The results are shown in Figures 1-13.

Single biomarkers were subject to ROC curve analysis to assess sensitivity and specificity. Logistic regression was used to model the dependency of stroke and stroke subtype upon the concentration of various combinations of biomarkers followed by ROC curve analysis to assess the model's classification accuracy. The results are shown in Figures 1-22.

Results

Tables 1 and 2 detail the sensitivity, specificity and statistical power (AUC) of exemplary combinations of biomarkers for diagnosing stroke (all stroke v control). By combining two or more biomarkers selected from ICAM-1 , VCAM-1 , L-selectin, P-selectin, IL-6, CRP, D-dimer and sTNFRI for testing the occurrence of stroke, a test with high diagnostic performance is achieved. Also, it has been found for the first time that the blood concentration of the proteins VCAM-1 , IL-6, h-FABP and CRP are able to discriminate between IS and TIA. Critical to the usefulness of the invention is the high discriminatory power of the biomarker(s). A test which aims to discriminate IS from TIA, must have a high specificity as possible so as to rule out TIA.

If TIA cannot be ruled out by the biomarker(s), then the diagnosis will be of either an IS or TIA i.e. it will not be able to discriminate between these two stroke subtypes. Therefore, the specificity of the test should be as close to 100% as possible. The sensitivity of the test should be of sufficient magnitude to be of value to the patient and be economically viable. Table 3 shows the statistical analysis of analyte concentrations in patients who suffered TIA, IS and HS using Mann-Whitney and Kruskal-Wallis tests. Table 4 shows the ROC curve analysis (sensitivity and specificity values) of individual and grouped biomarkers for IS vs TIA. As can be seen, each of the biomarkers has 100% specificity and equal or greater sensitivity than the commonly used CAT scan. This facilitates clinical diagnosis and informs subsequent treatment decisions of suspected stroke patients in an economical and expeditious manner.

Table 3

Analyte	IS v TIA	TOA v C	IS vC	HS v C	HS v IS	All v C
VCAM-1	P<0.0001	ns	P<0.001	P<0.001	ns	P<0.0001
ICAM-1	ns	P<0.01	P<0.001	P<0.001	ns	P<0.0001
E-selectin	ns	ns	ns	ns	ns	ns
L-selectin	ns	P<0.001	P<0.001	P<0.001	ns	P<0.0001
P-selectin	ns	P<0.001	P<0.001	P<0.001	ns	P<0.01
IL-6	P<0.01	P<0.001	P<0.001	P<0.001	ns	P<0.001
h-FABP	P<0.01	P<0.001	P<0.001	P<0.001	ns	P<0.001
CRP	P<0.05	P<0.001	P<0.001	P<0.001	P<0.05	P<0.001
D-dimer	P<0.05	P<0.001	P<0.001	P<0.01	ns	P<0.001
NGAL	ns	ns	ns	ns	ns	ns
STNFR1	P<0.05	P<0.001	P<0.001	P<0.001	ns	P<0.001
TM	ns	ns	ns	ns	ns	ns

[All stroke = TIA + IS + HS; C = control; ns = not significantly different at the 5% level (P>0.05)]

Table 4

Biomarker(s)	AUC	Ischemic Stroke (IS)	
		% Sensitivity	% Specificity
VCAM-1	0.755	24.88	100
IL-6	0.727	23.26	100
h-FABP	0.700	20.45	100
VCAM-1 + IL-6	0.801	30.23	100
VCAM-1 + IL-6 + CRP	0.818	34.88	100
VCAM-1 + CRP	0.793	34.09	100
VCAM-1 + h-FABP	0.811	31.82	100
VCAM-1 + h-FABP + IL-6	0.812	31.82	100
VCAM-1 + h-FABP + CRP	0.816	34.09	100
VCAM-1 + h-FABP + IL-6 + CRP	0.820	34.09	100

Clinical Use of the Invention

Use of the invention can be envisaged in the following scenarios relating to an individual who suffers a stroke-like event:

i) in transit to the hospital a biological fluid sample is taken from the individual and tested for all stroke types using biomarkers of the invention - a positive stroke result is confirmed and further stratified into HS or IS/TIA following examination of theindividual by a clinician and analysis using a CAT scan. If HS is ruled out, a further biomarker test is implemented to delineate IS/TIA.

ii) at the hospital examination by a clinician is preceded by stroke biomarker analysis of a biological fluid sample taken from the individual in association with a CAT scan examination - if HS is ruled out, a further biomarker test is implemented to delineate IS/TIA.

Abbreviations

IL-6 - interleukin-6
ICAM-1 - intracellular adhesion molecule-1
VCAM-1 - vascular cell adhesion molecule -1
CRP - C-reactive protein
h-FABP - human fatty acid binding protein
sTNFR - soluble TNFa receptor
TM - thrombomodulin
NGAL - neutrophil-associated gelatinase lipocalin
MMP-9 - matrix metalloproteinase-9
BNP - brain natriuretic peptide
ADMA - asymmetric dimethylarginine
Lp-PLA2 - lipoprotein-associated phospholipase A2

CLAIMS

1. A method for diagnosing stroke in a patient suspected of having a stroke, comprising determining the concentration of at least two biomarkers in an *in vitro* sample obtained from the patient and establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the at least two biomarkers are selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI, D-dimer and CRP, and wherein at least one of the two biomarkers is selected from ICAM-1 , L-selectin, P-selectin and VCAM-1 .

2. A method according to claim 1, wherein the at least two biomarkers are selected from (i) ICAM-1 or VCAM-1 and (ii) L-selectin or P-selectin.

3. A method according to claim 1 or claim 2, wherein the at least two biomarkers are ICAM-1 and L-selectin.

4. A method according to claim 2 or claim 3, further comprising determining the sample concentration of one or more biomarkers selected from IL-6, sTNFRI, D-dimer and CRP.

5. A method according to any preceding claim, further comprising determining the sample concentration of h-FABP.

6. A method according to any preceding claim, wherein each of the patient and control biomarker concentration values is inputted into a statistical algorithm or algorithms to produce an output value that indicates whether a stroke has occurred.

7. A method according to claim 6, wherein the statistical algorithm includes a logistic regression equation.

8. A method according to any preceding claim, wherein the combination of two or more biomarkers corresponds to any of the biomarker combinations listed in Tables 1 and 2.

9. A method according to any preceding claim, wherein if stoke is diagnosed, the method according to any of claims 16 to 19 is carried out in order to differentially diagnose the stroke type.

10. A substrate comprising probes for at least two biomarkers selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI , D-dimer and CRP for use in a method for diagnosing stroke in a patient according to any of claims 1 to 9, wherein the substrate comprises a probe for at least one of ICAM-1 , L-selectin, P-selectin and VCAM-1 .

11. A substrate according to claim 10, further comprising a probe for h-FABP.

12. A substrate according to claim 10 or claim 11, wherein the substrate has at least two probes immobilised thereon.

13. A substrate according to any of claims 10 to 12, wherein the substrate is a biochip.

14. Use of a substrate comprising probes for at least two biomarkers selected from ICAM-1 , L-selectin, P-selectin, VCAM-1 , IL-6, sTNFRI, D-dimer and CRP in a method for diagnosing stroke in a patient according to any of claims 1 to 9, wherein the substrate comprises a probe for at least one of ICAM-1 , L-selectin, P-selectin and VCAM-1 .

15. Use according to claim 14, wherein the substrate further comprises a probe for h-FABP.

16. A method of aiding the diagnosis of ischaemic stroke in a patient suspected of having a stroke, comprising

i) determining the concentration of VCAM-1 and one or more biomarkers selected from h-FABP, IL-6 and CRP in an *in* vitro sample obtained from the patient; and

ii) establishing the significance of the concentration of the biomarkers by comparing the concentration value for each biomarker with a corresponding control value, wherein the corresponding control value is the concentration value for the corresponding biomarker determined from an *in vitro* sample obtained from a transient ischaemic attack patient or patients.

17. A method according to claim 16, wherein the method is used to differentially diagnose between ischemic stroke and a transient ischaemic attack.

18. A method according to claim 16 or claim 17, wherein each of the patient and control biomarker concentration values is inputted into a statistical algorithm or algorithms to produce an output value that indicates whether ischaemic stroke has occurred.

19. A method according to claim 18, wherein the statistical algorithm includes a logistic regression equation.

20. A substrate comprising probes for VCAM-1 and at least one other biomarker selected from h-FABP, IL-6 and CRP for use in a method for aiding the diagnosis of ischaemic stroke in a patient according to any of claims 16 to 19.

21. A substrate according to claim 20, wherein the substrate has the probes immobilised thereon.

22. A substrate according to claims 20 or 21, wherein the substrate is a biochip.

23. Use of a substrate comprising probes for VCAM-1 and at least one other biomarker selected from h-FABP, IL-6 and CRP in a method for aiding the diagnosis of ischaemic stroke in a patient according to any of claims 16 to 19.

24. Use of VCAM-1 and one or more of h-FABP, IL-6 and CRP, as biomarkers of ischemic stroke and/or as differentiators between ischemic stroke and a transient ischaemic attack.

25. A method according to any of claims 1-9 and 16-19, wherein the sample is a blood, serum or plasma sample.

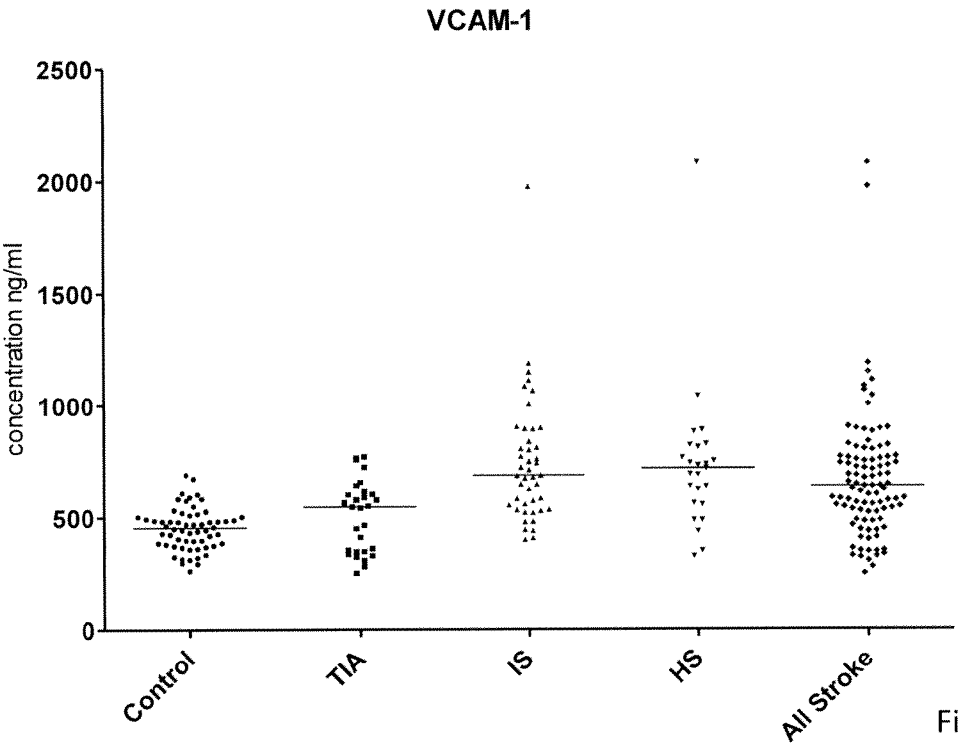


Figure 1

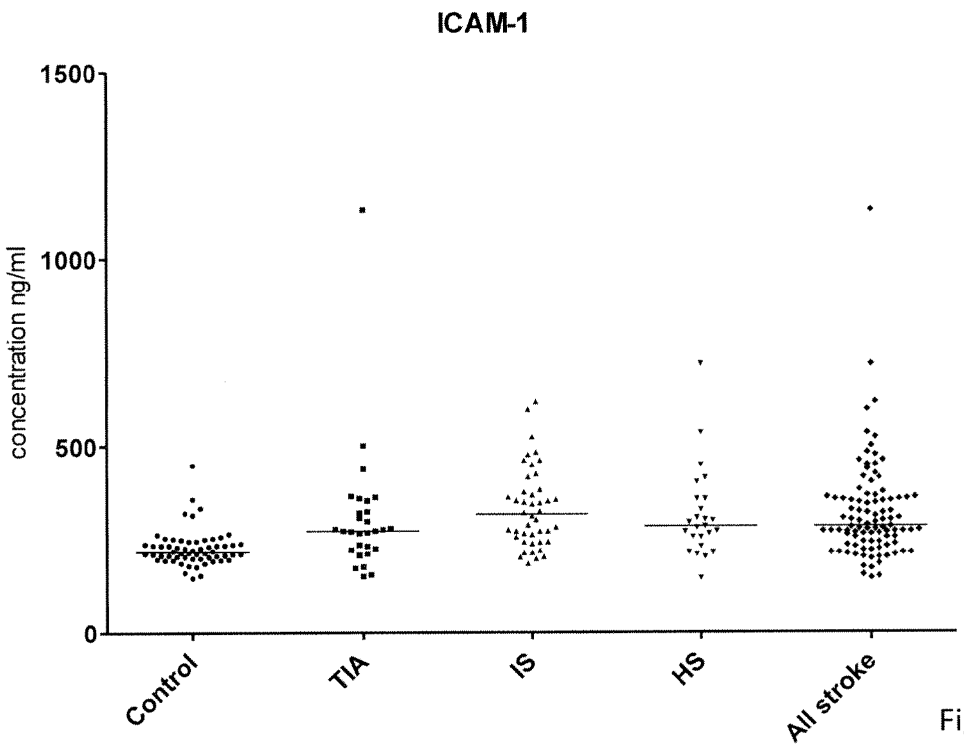
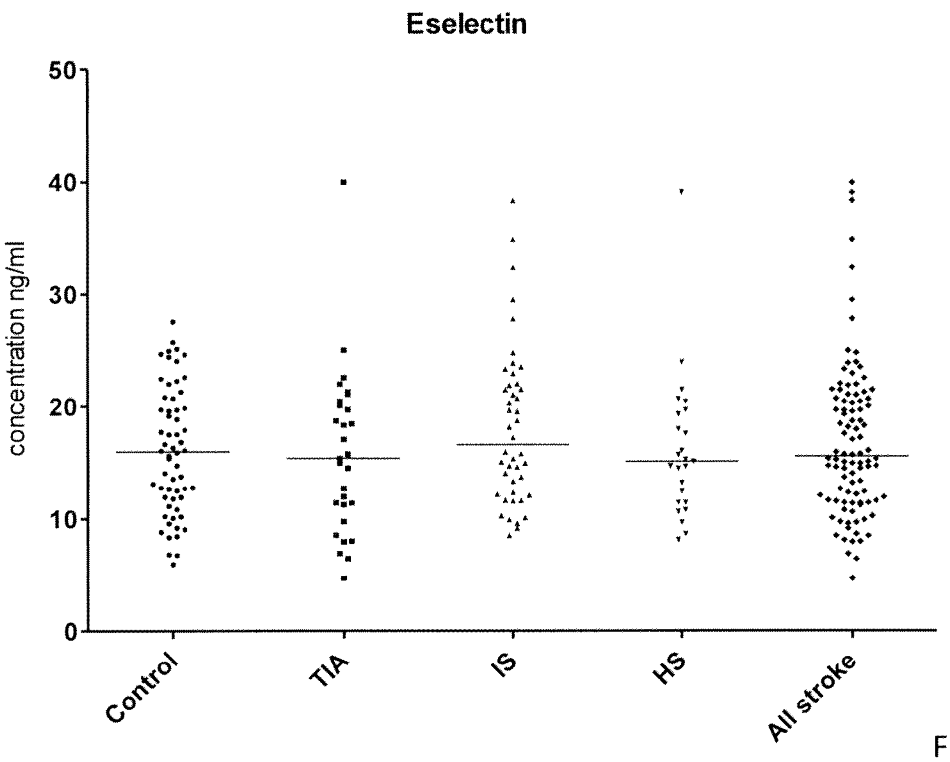


Figure 2



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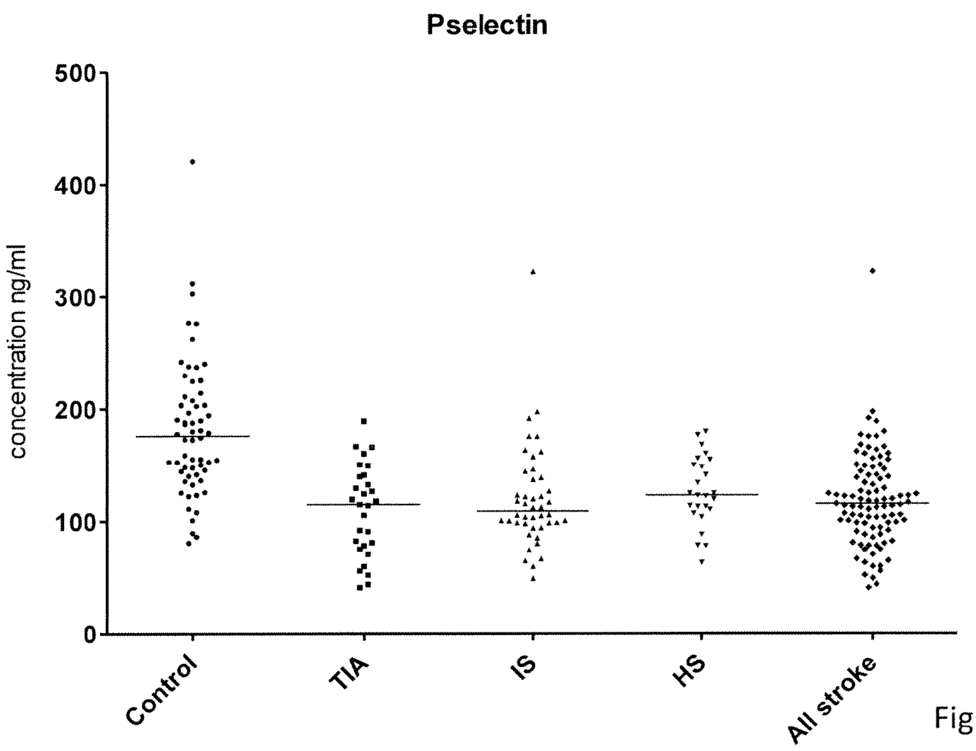

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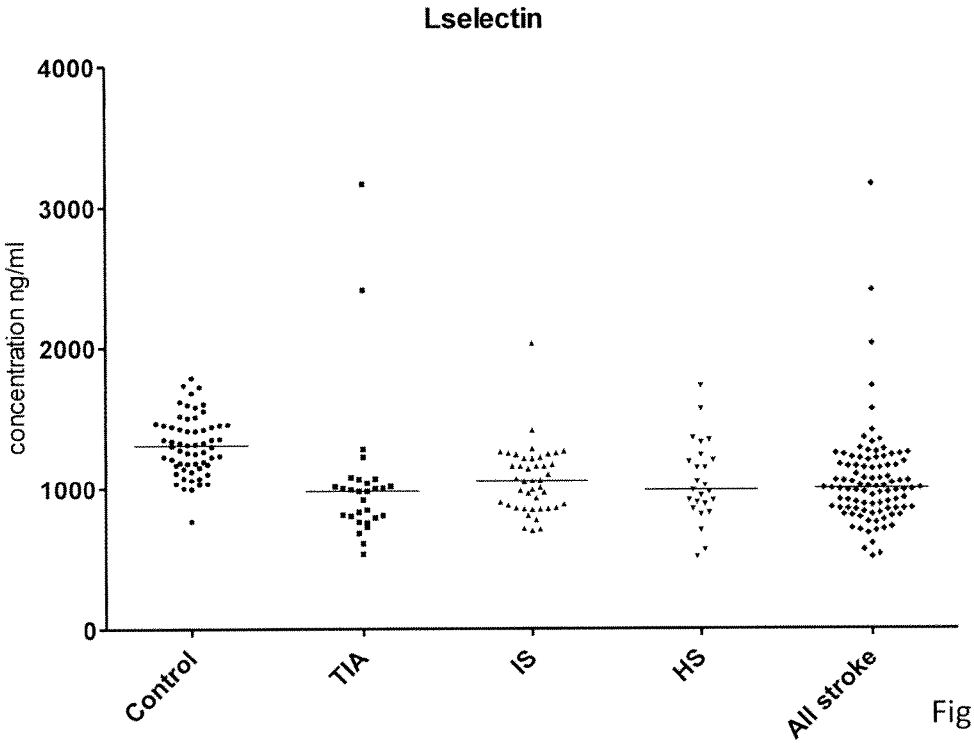

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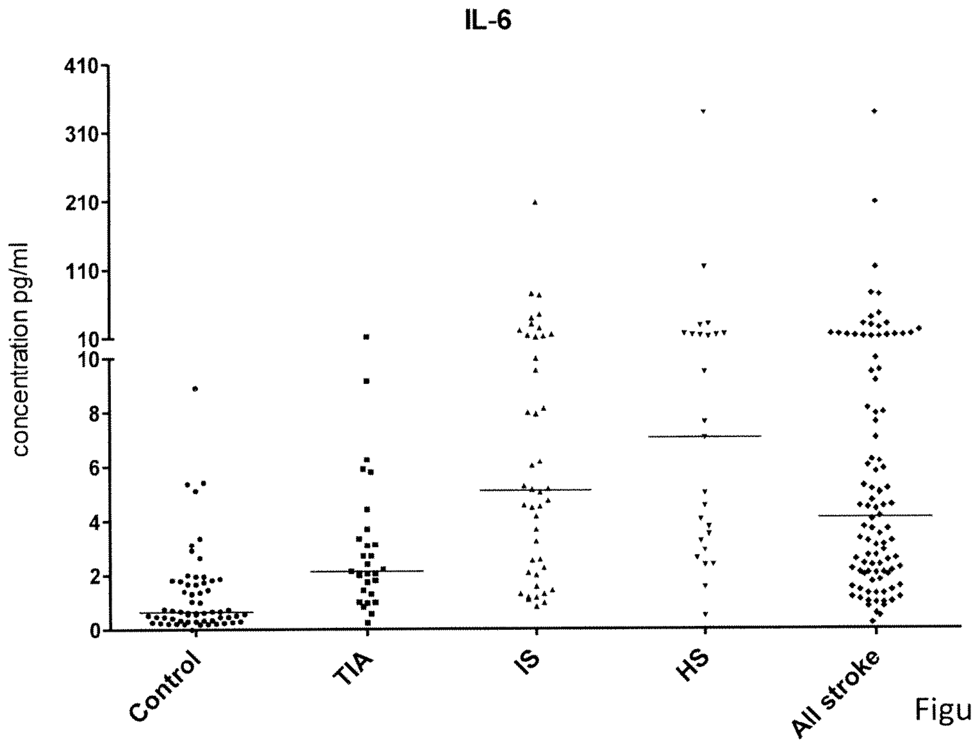

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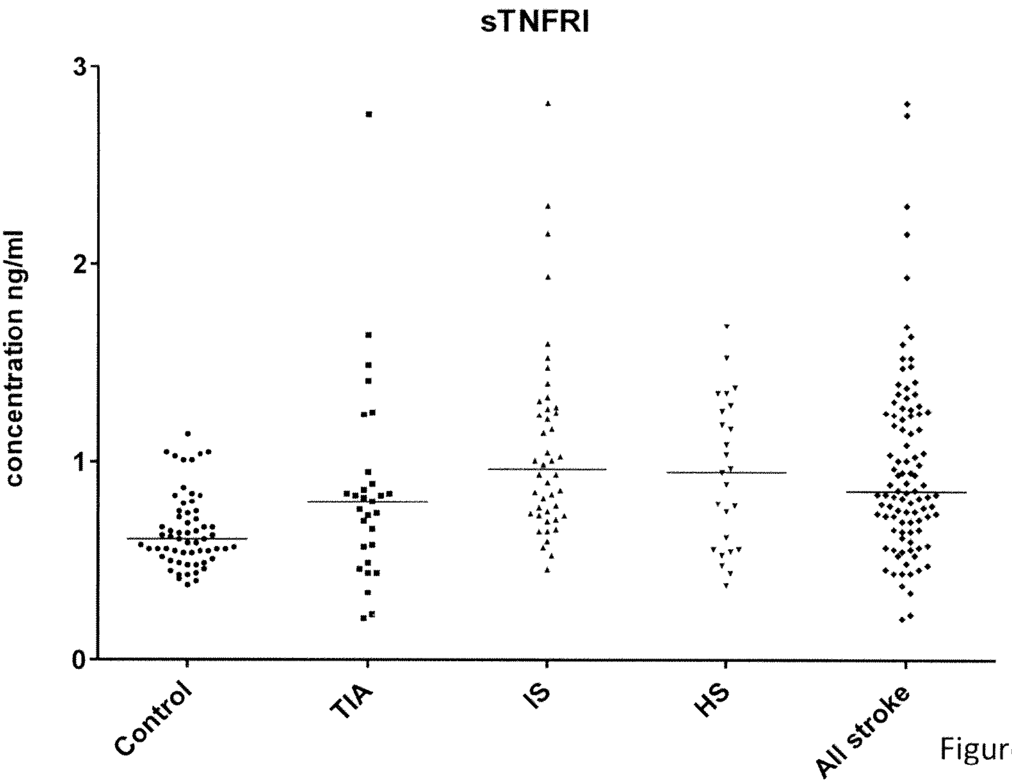
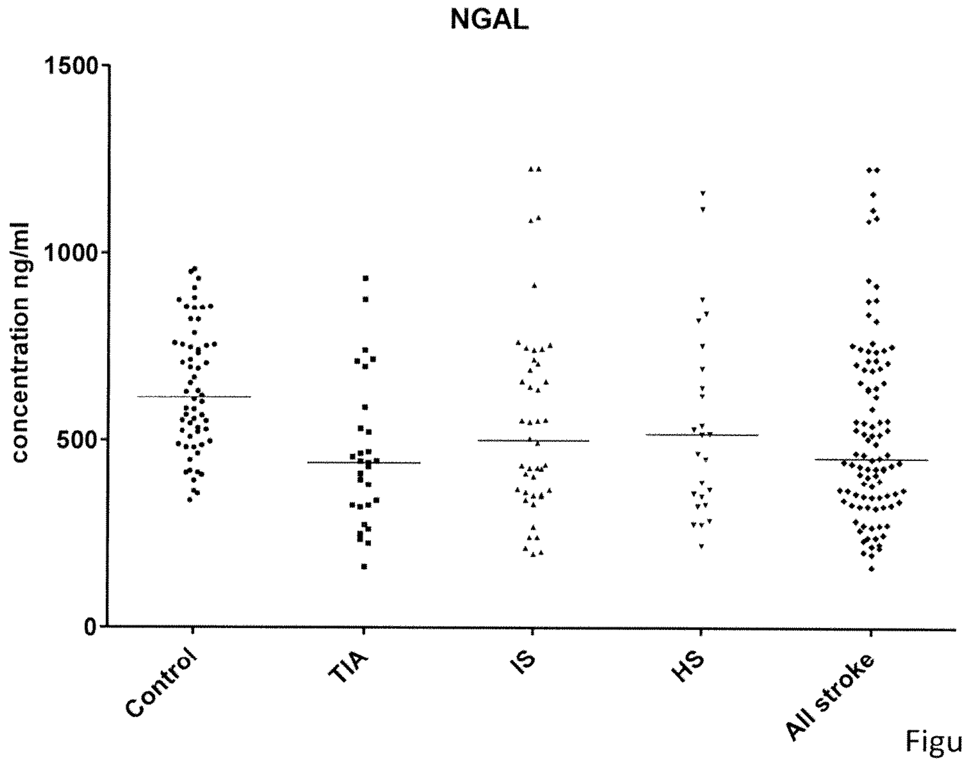

sTNFRI
concentration ng/ml
3
2
1
0
Control
TIA
IS
HS
All stroke

Figure 7



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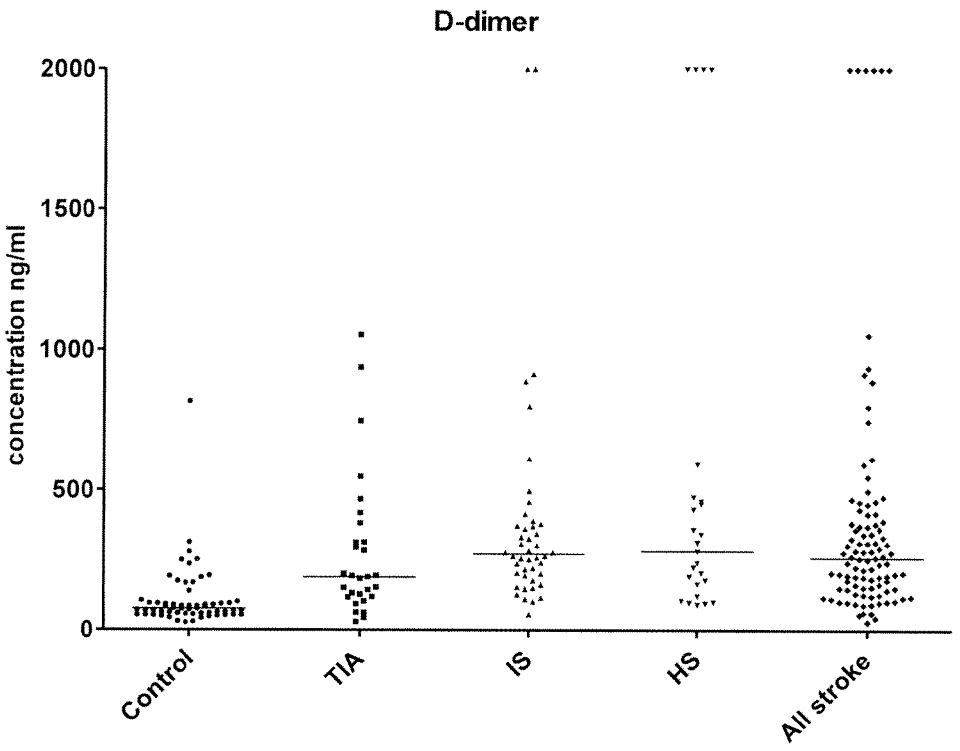

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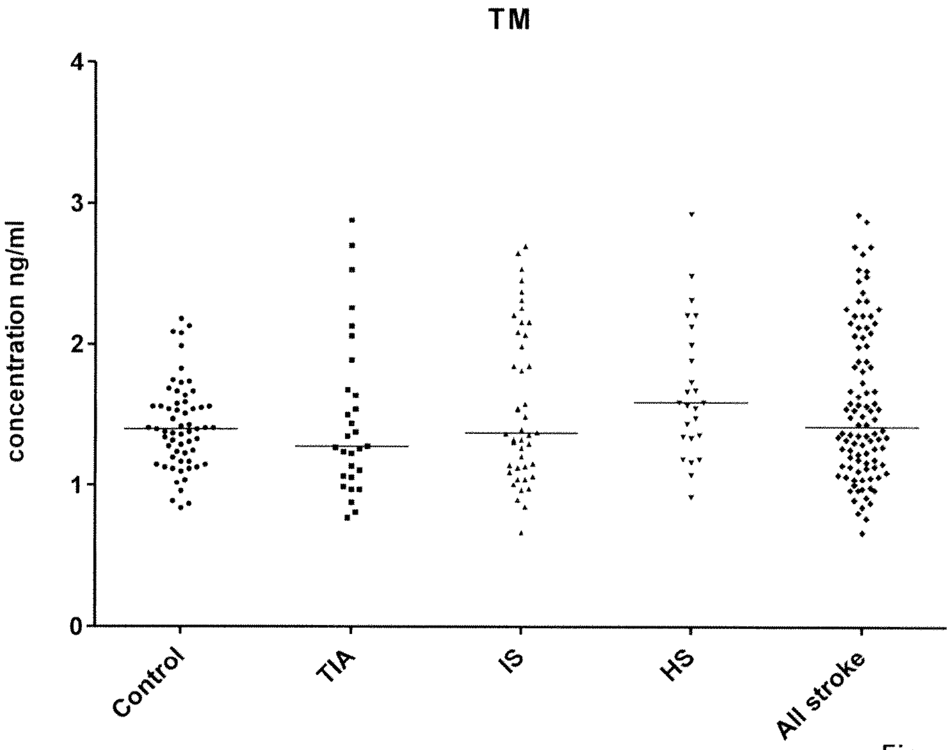

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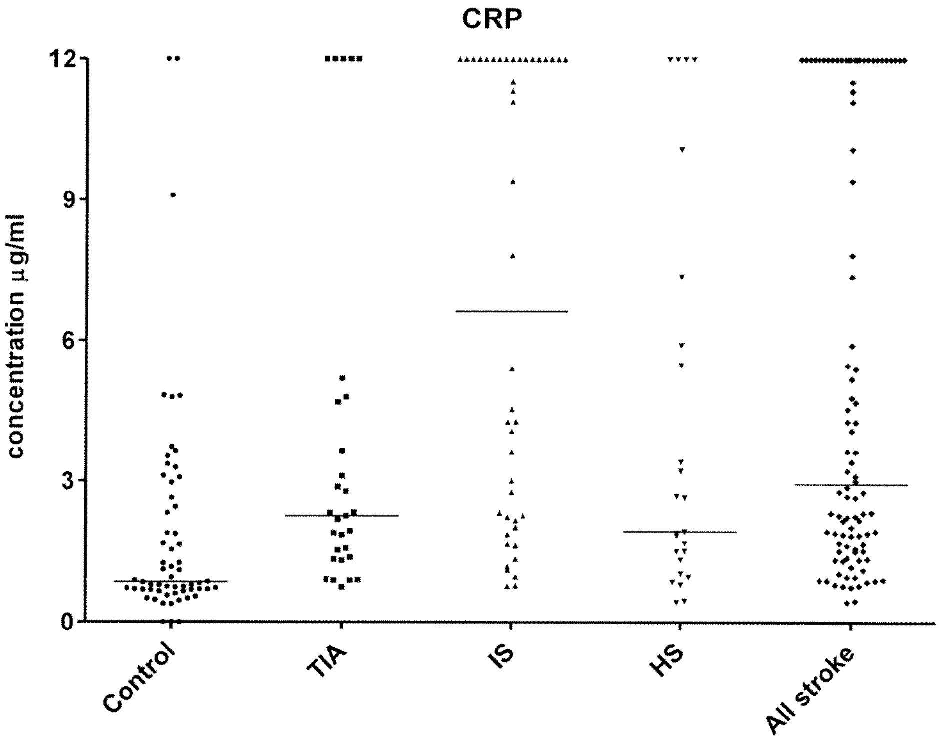

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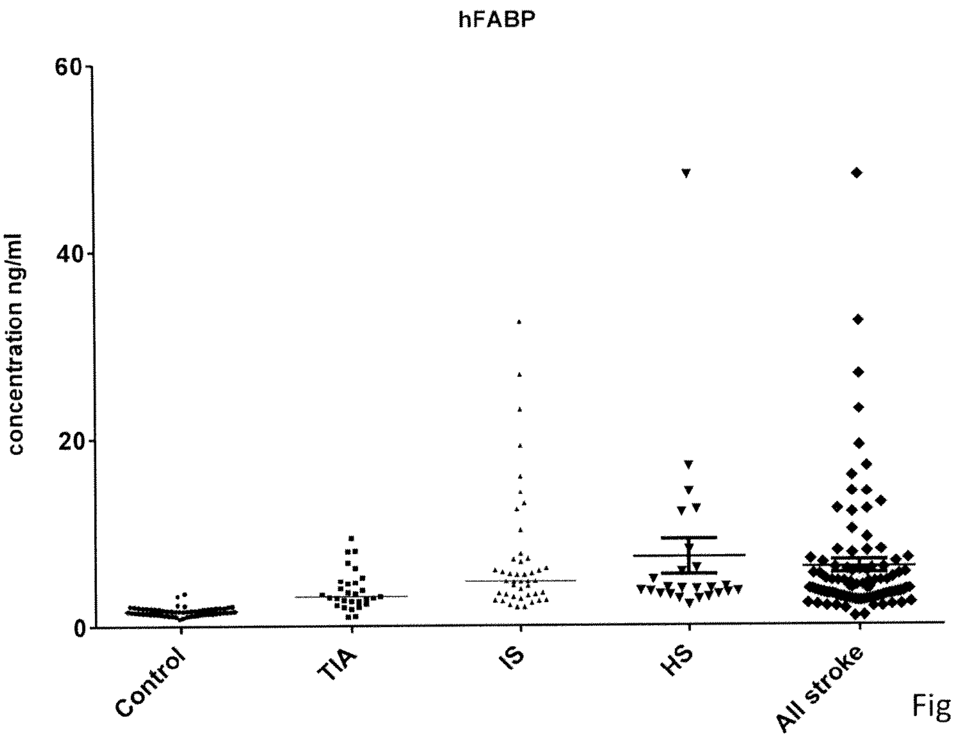

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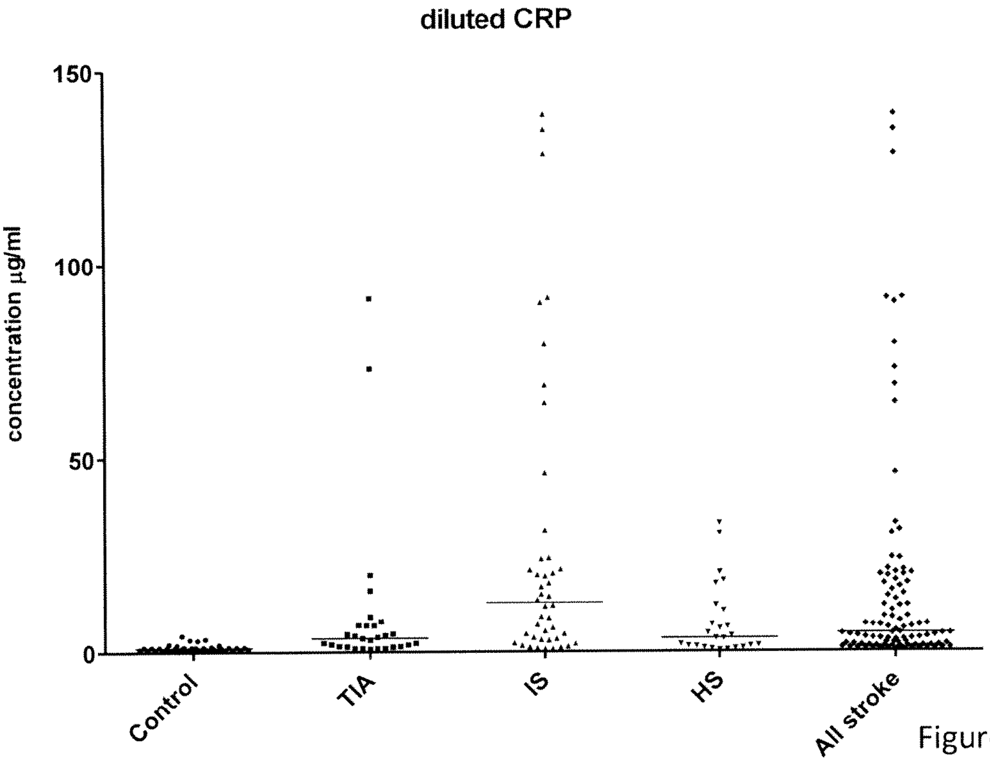

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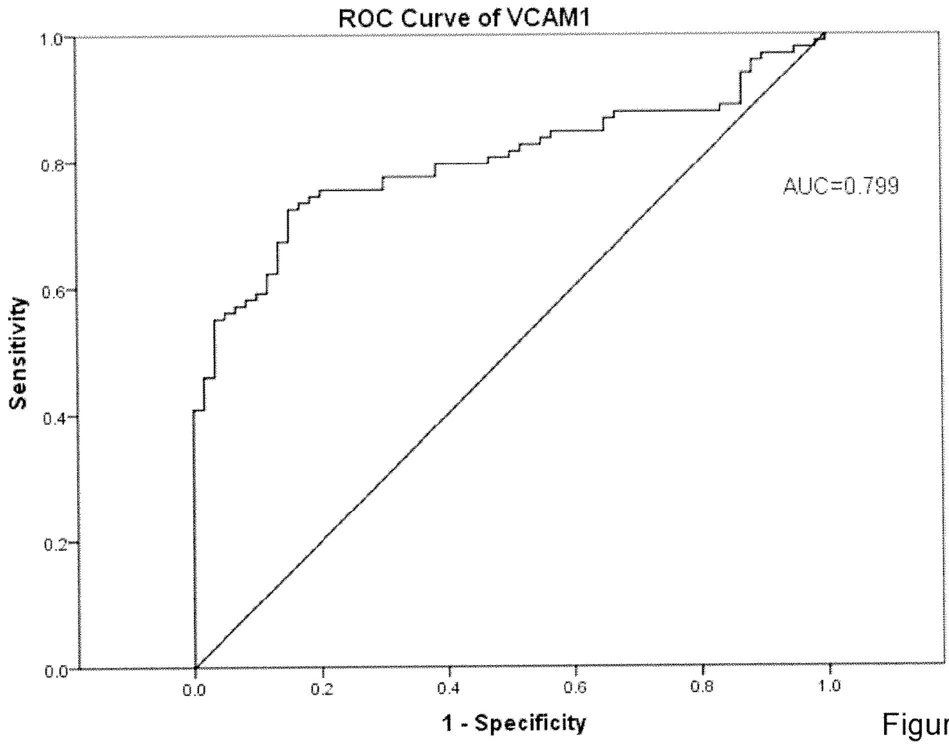

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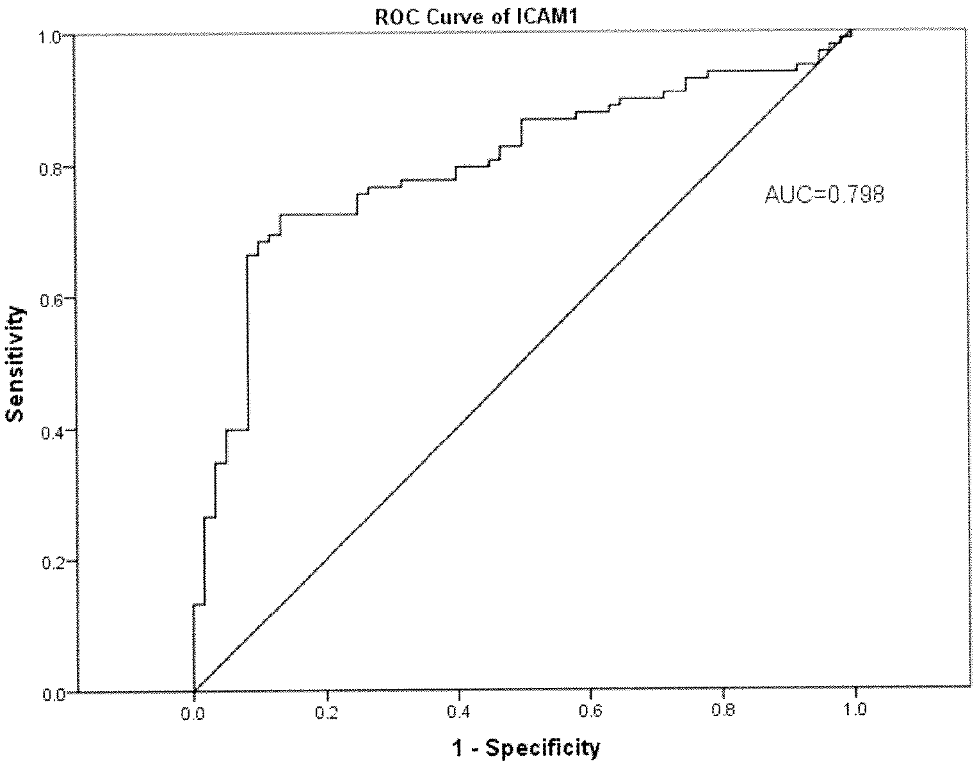

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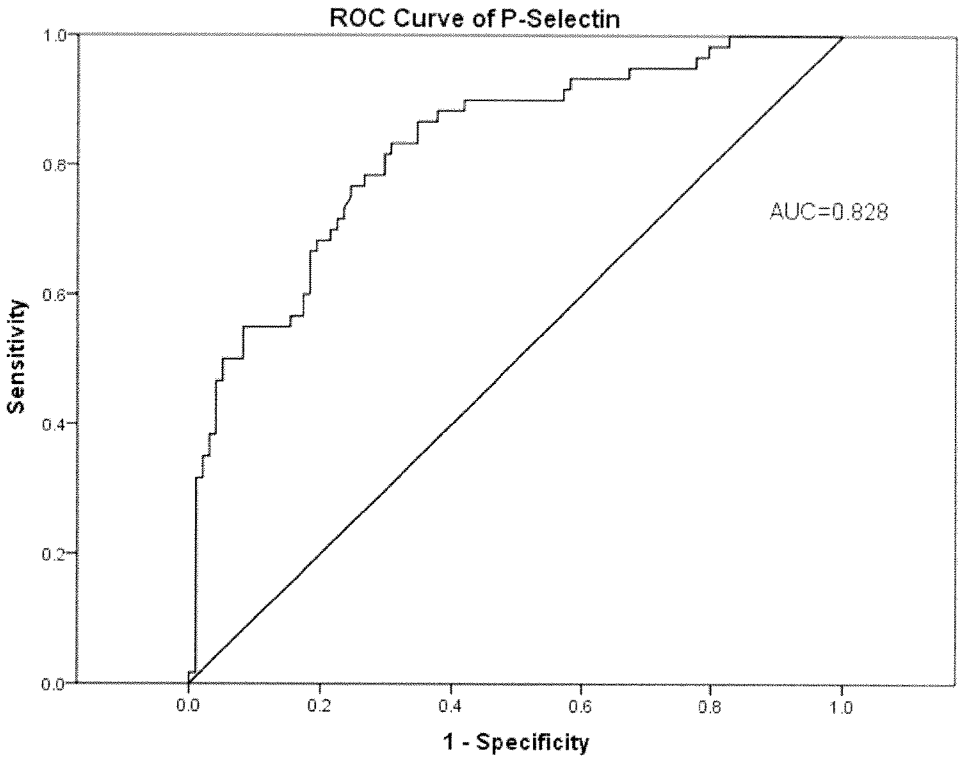
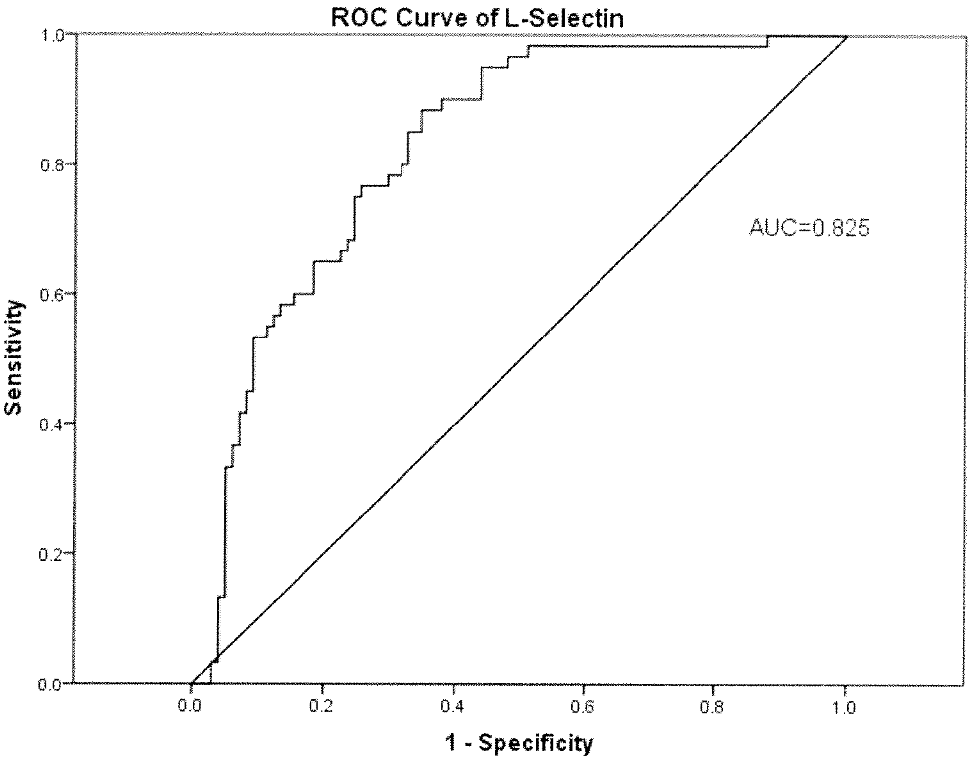


Figure 16



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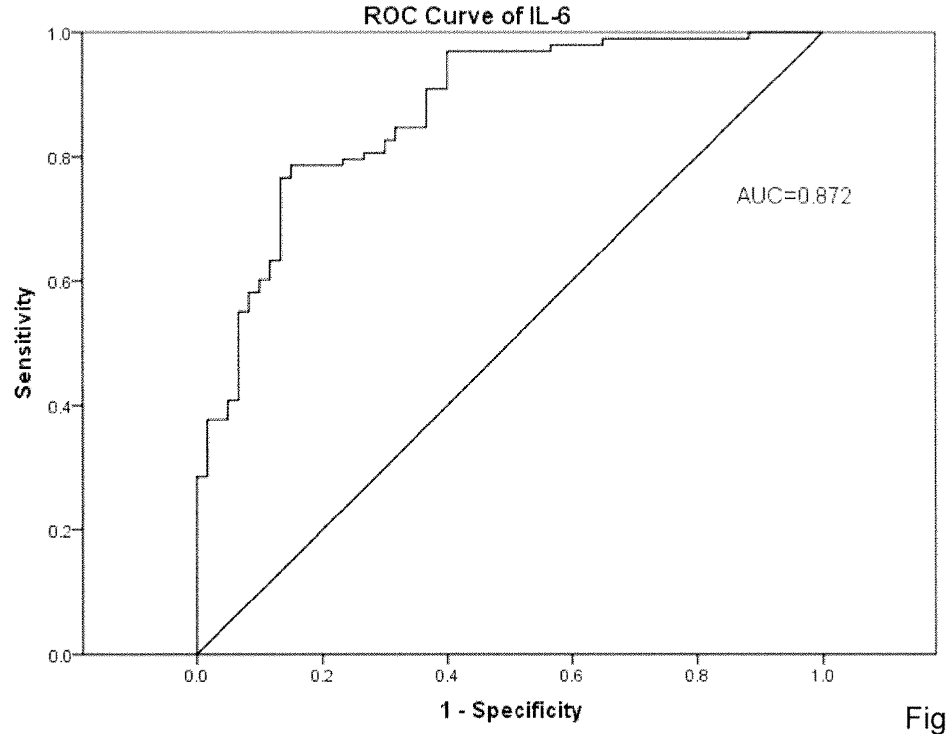

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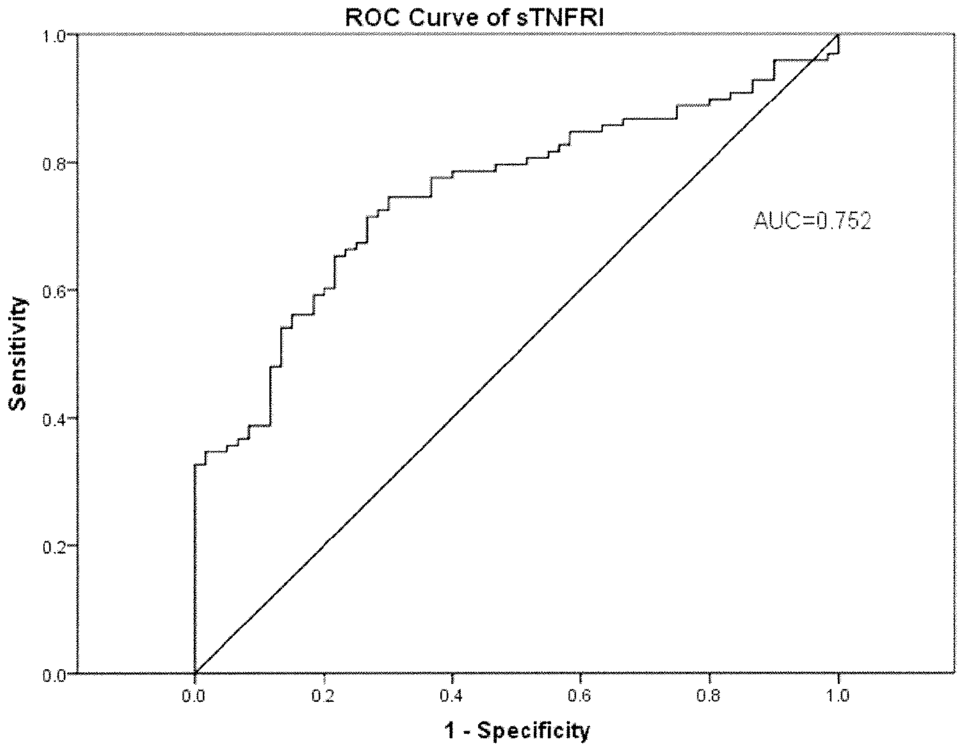

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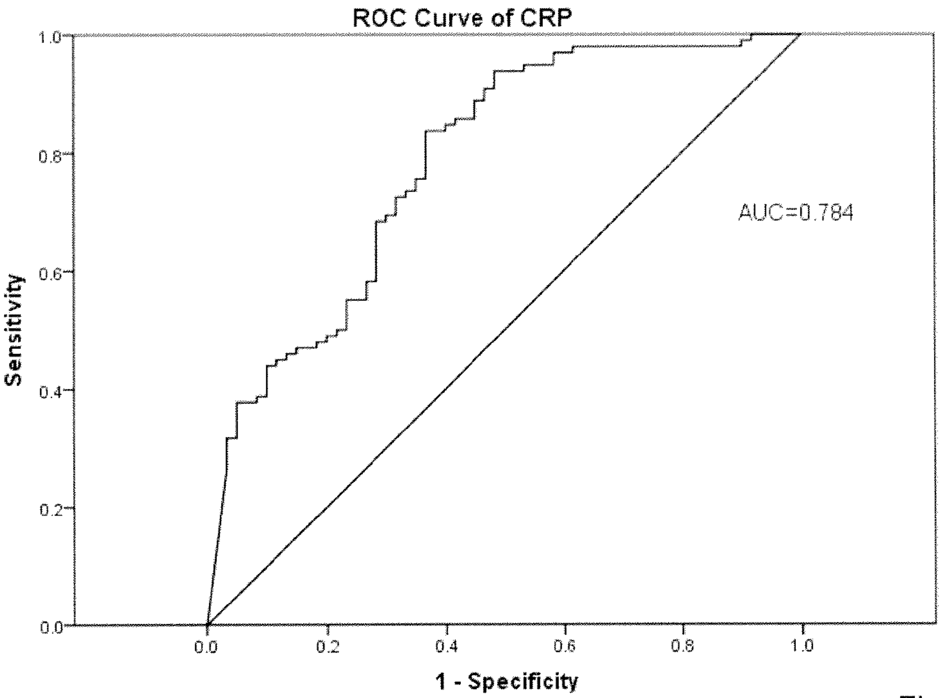

Figure 20

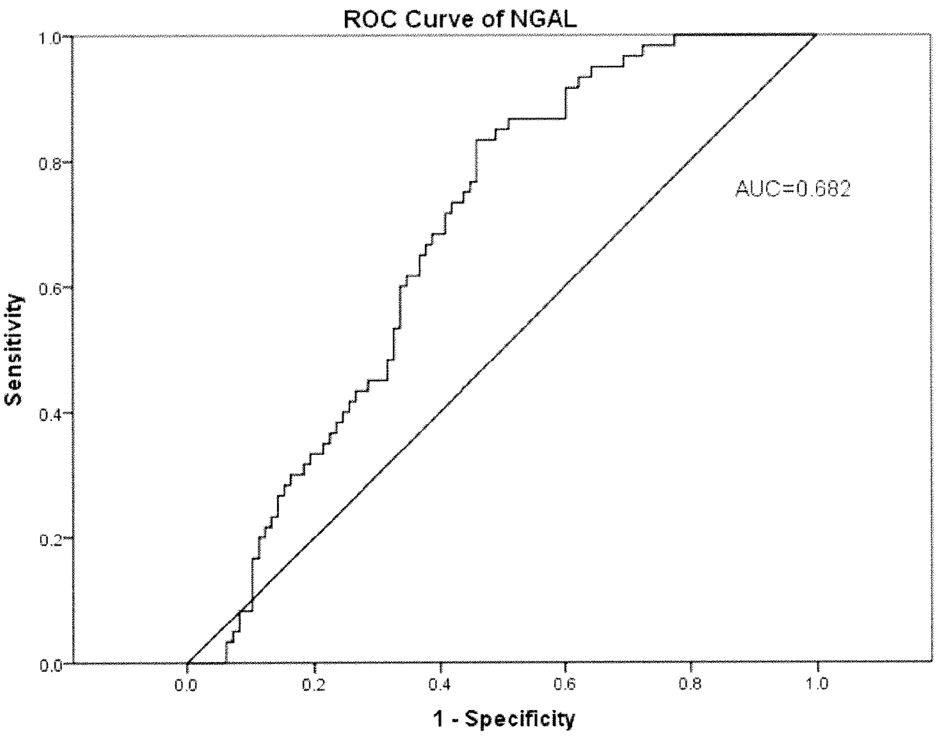

Figure 21

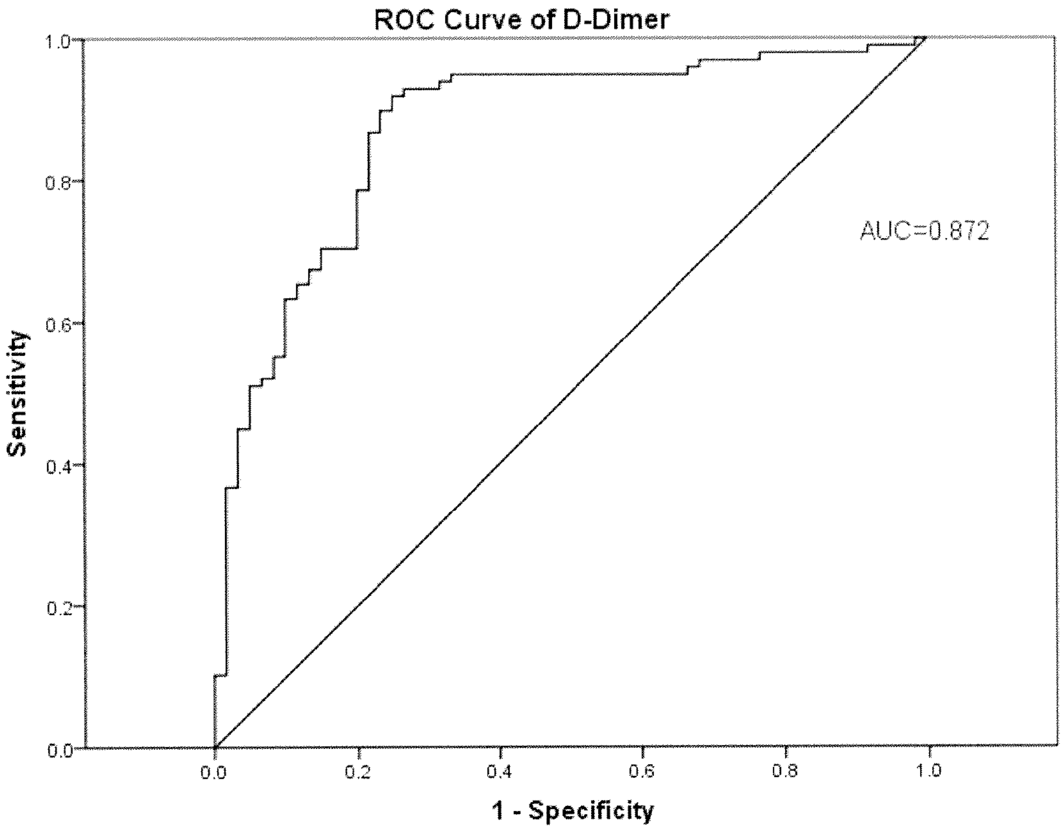
ROC Curve of D-Dimer
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
AUC=0.872

Figure 22