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J Stroke and Cardiovascular Diseases, Vol 11, 2002, H Christensen et al, "Plasma Cytokines in Acute Stroke",
J Neurol, Vol 252, 2005, MT Wunderlich et al, "Release of brain-type and heart-type fatty acid-binding proteins in serum after acute ischaemic stroke", 718-724

(58) Field of Search:
INT CL **C12M, G01N**
Other: **ONLINE: EPODOC, WPI, BIOSIS, MEDLINE**

(54) Title of the Invention: **Biomarkers of stroke and stroke subtypes**
Abstract Title: **Biomarkers for stroke and stroke subtype diagnosis.**

(57) Claimed are methods for diagnosing stroke in a patient which comprise measuring in a sample from the patient the concentration of at least two or more of the possible biomarkers sTNF1, IL-6, D-dimer, L-selectin, ICAM-1, CRP, VCAM-1 and P-selectin. Alternatively claimed is a method in which stroke is diagnosed by assaying for one of more biomarkers selected from h-FABP, Il-6, CRP and VCAM-1. The methods may distinguish between different types of stroke.

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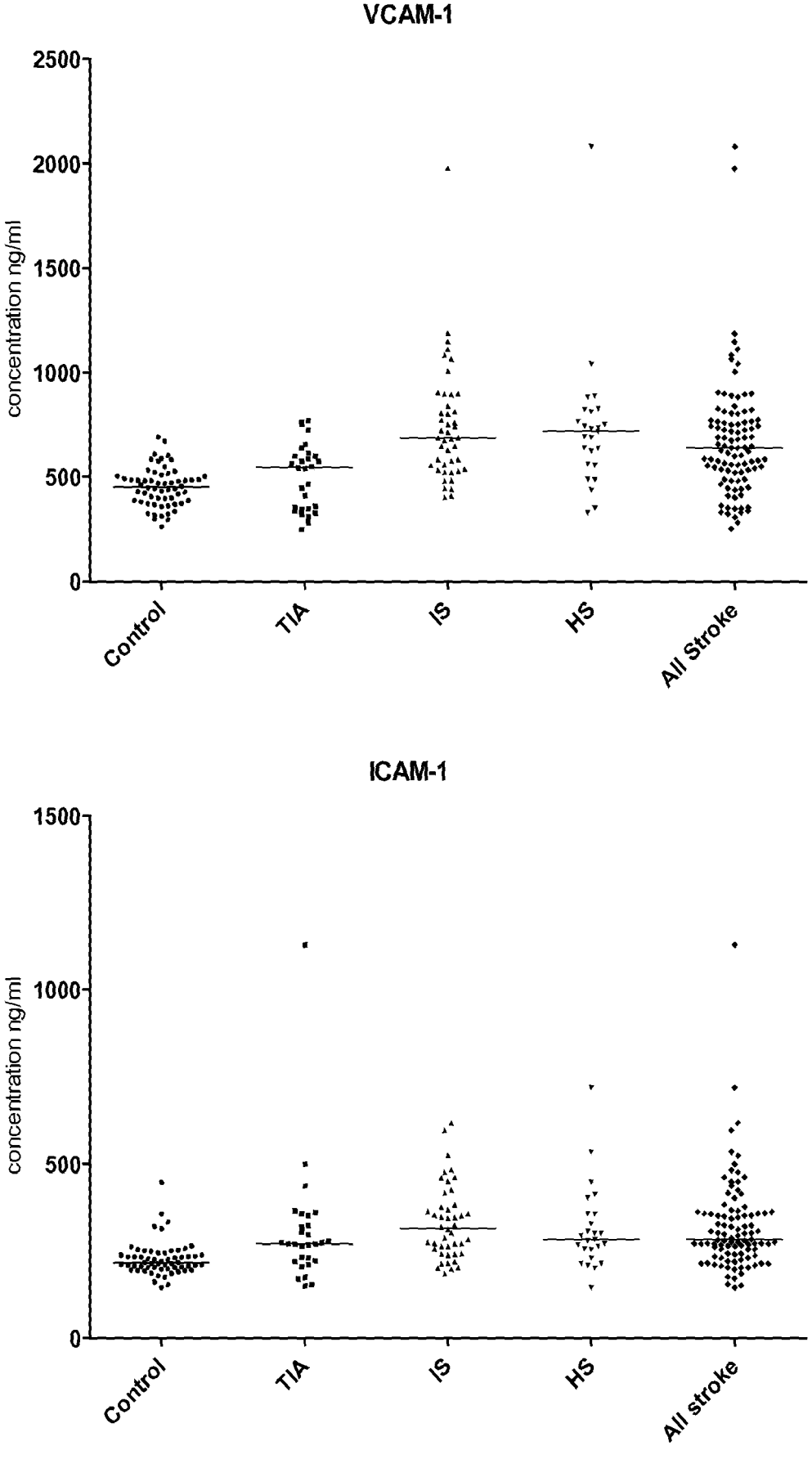

VCAM-1
2500
2000
1500
1000
500
0
concentration ng/ml
Control
TIA
IS
HS
All Stroke
ICAM-1
1500
1000
500
0
concentration ng/ml
Control
TIA
IS
HS
All stroke

Figure 1

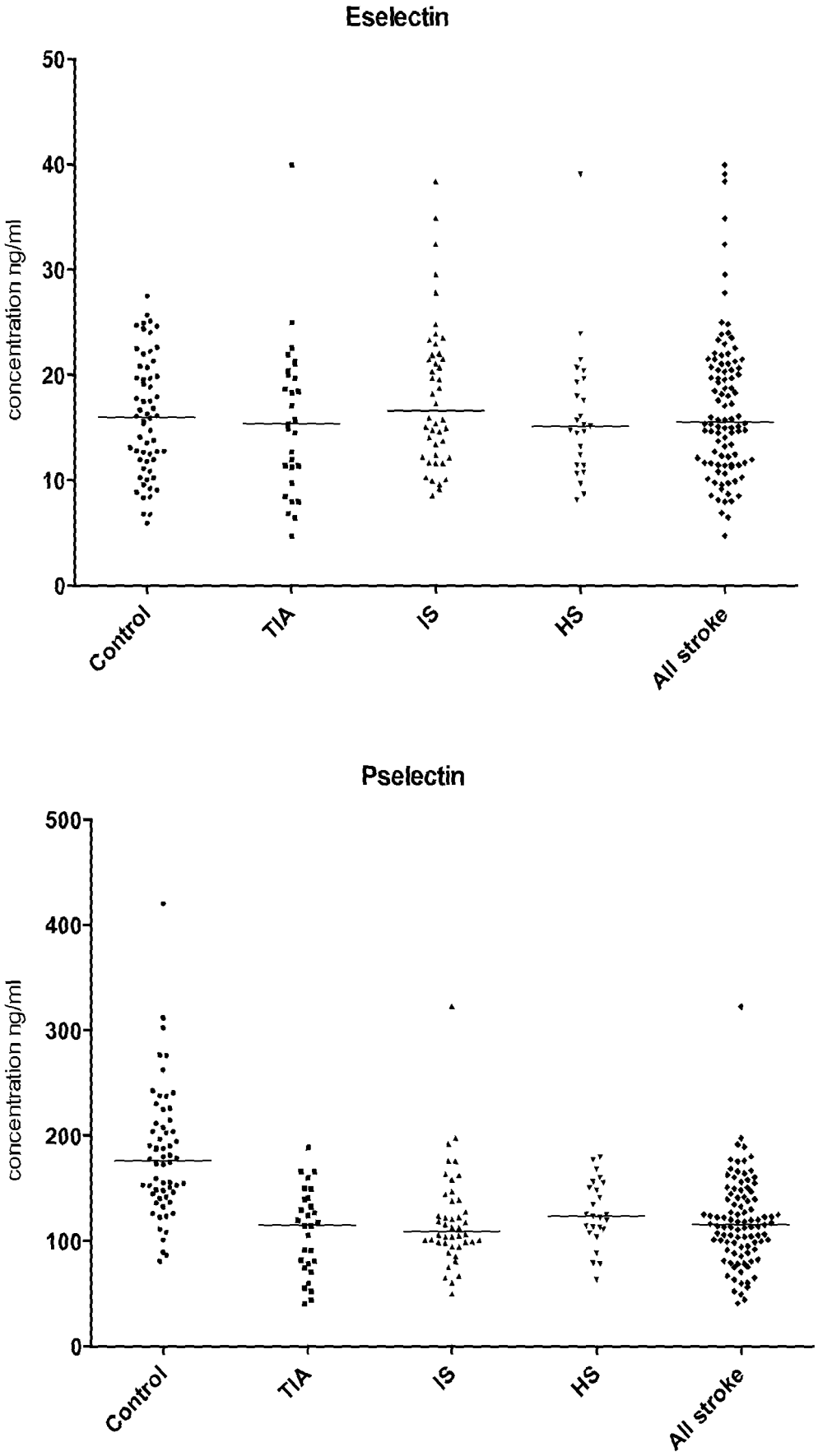

Eselectin
concentration ng/ml
50
40
30
20
10
0
Control
TIA
IS
HS
All stroke
Pselectin
concentration ng/ml
500
400
300
200
100
0
Control
TIA
IS
HS
All stroke

Figure 2

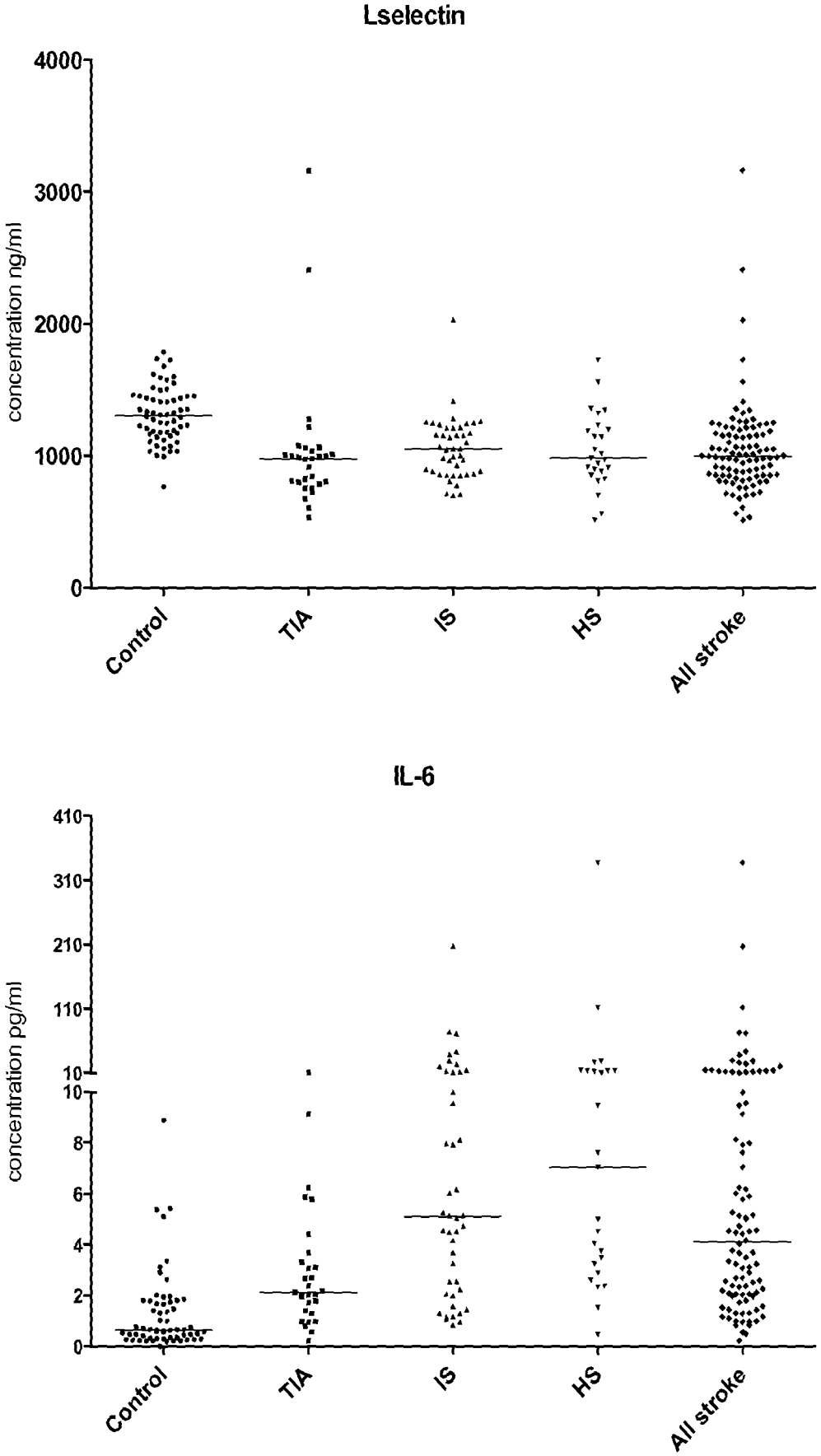

Lselectin
concentration ng/ml
4000
3000
2000
1000
0
Control
TIA
IS
HS
All stroke
IL-6
concentration pg/ml
410
310
210
110
10
10
8
6
4
2
0
Control
TIA
IS
HS
All stroke

Figure 3

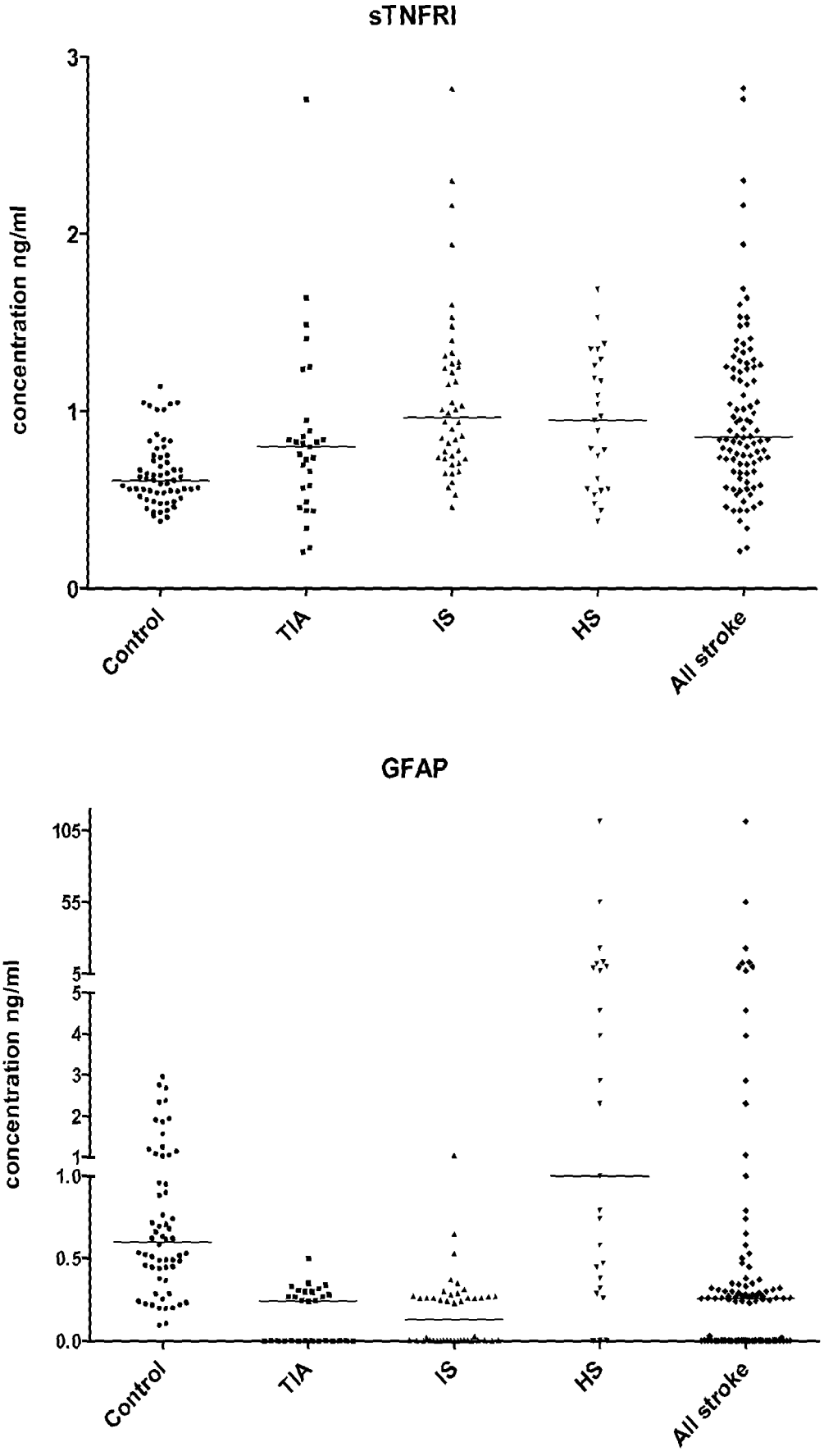

sTNFRI
concentration ng/ml
3
2
1
0
Control
TIA
IS
HS
All stroke
GFAP
concentration ng/ml
105
55
5
5
4
3
2
1
1.0
0.5
0.0
Control
TIA
IS
HS
All stroke

Figure 4

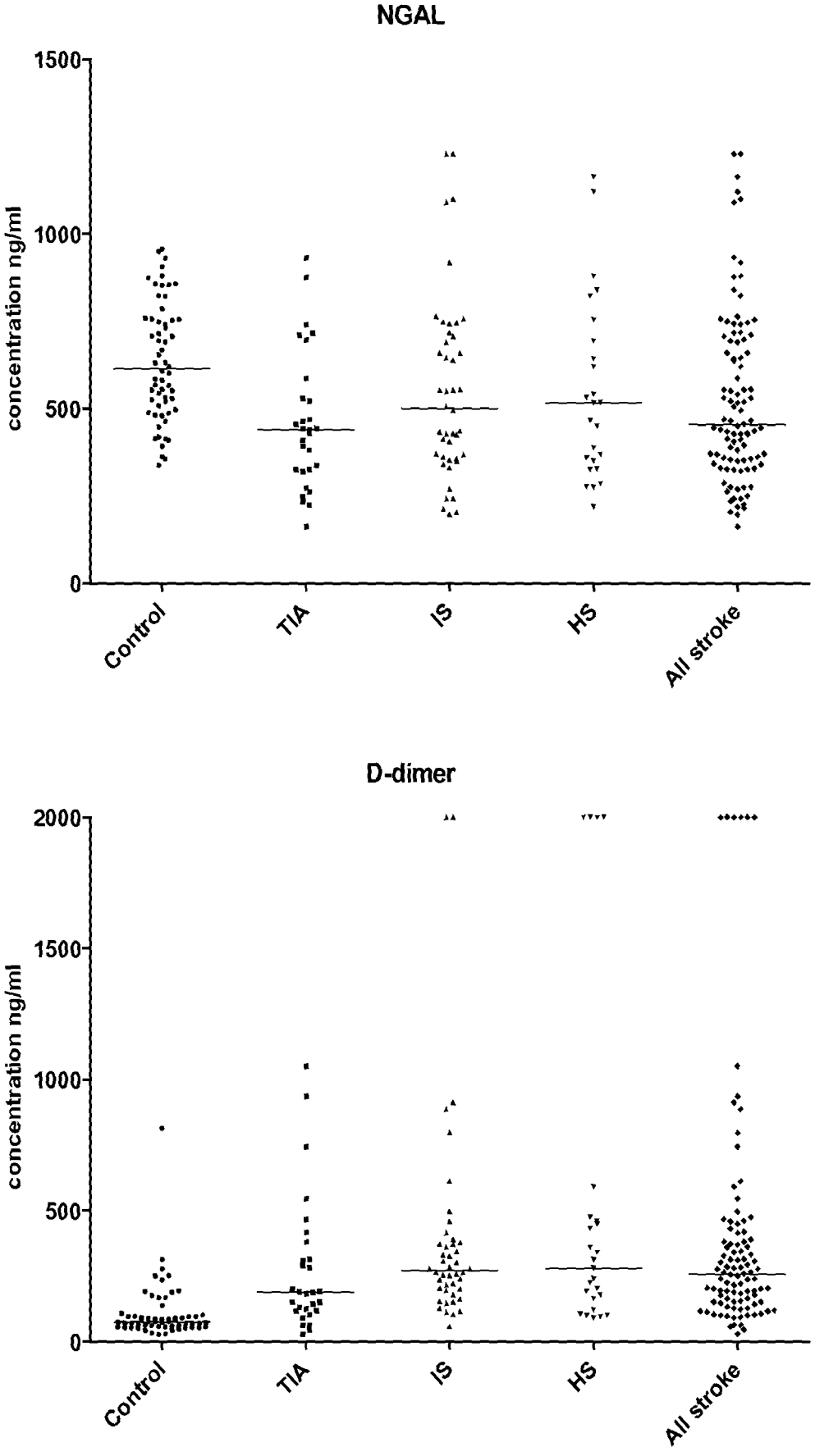

NGAL
concentration ng/ml
1500
1000
500
0
Control
TIA
IS
HS
All stroke
D-dimer
concentration ng/ml
2000
1500
1000
500
0
Control
TIA
IS
HS
All stroke

Figure 5

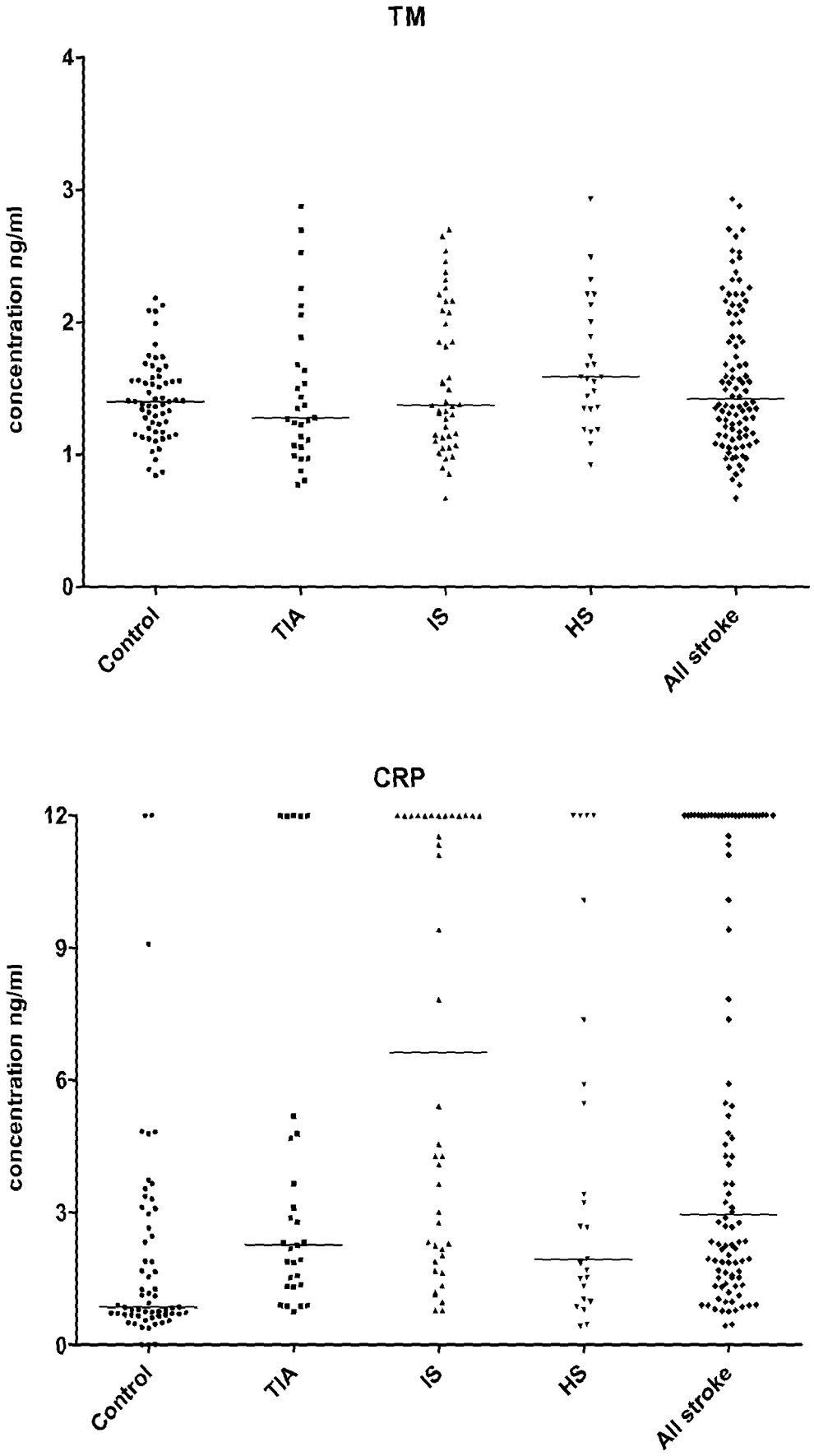

TM
concentration ng/ml
4
3
2
1
0
Control
TIA
IS
HS
All stroke
CRP
concentration ng/ml
12
9
6
3
0
Control
TIA
IS
HS
All stroke

Figure 6

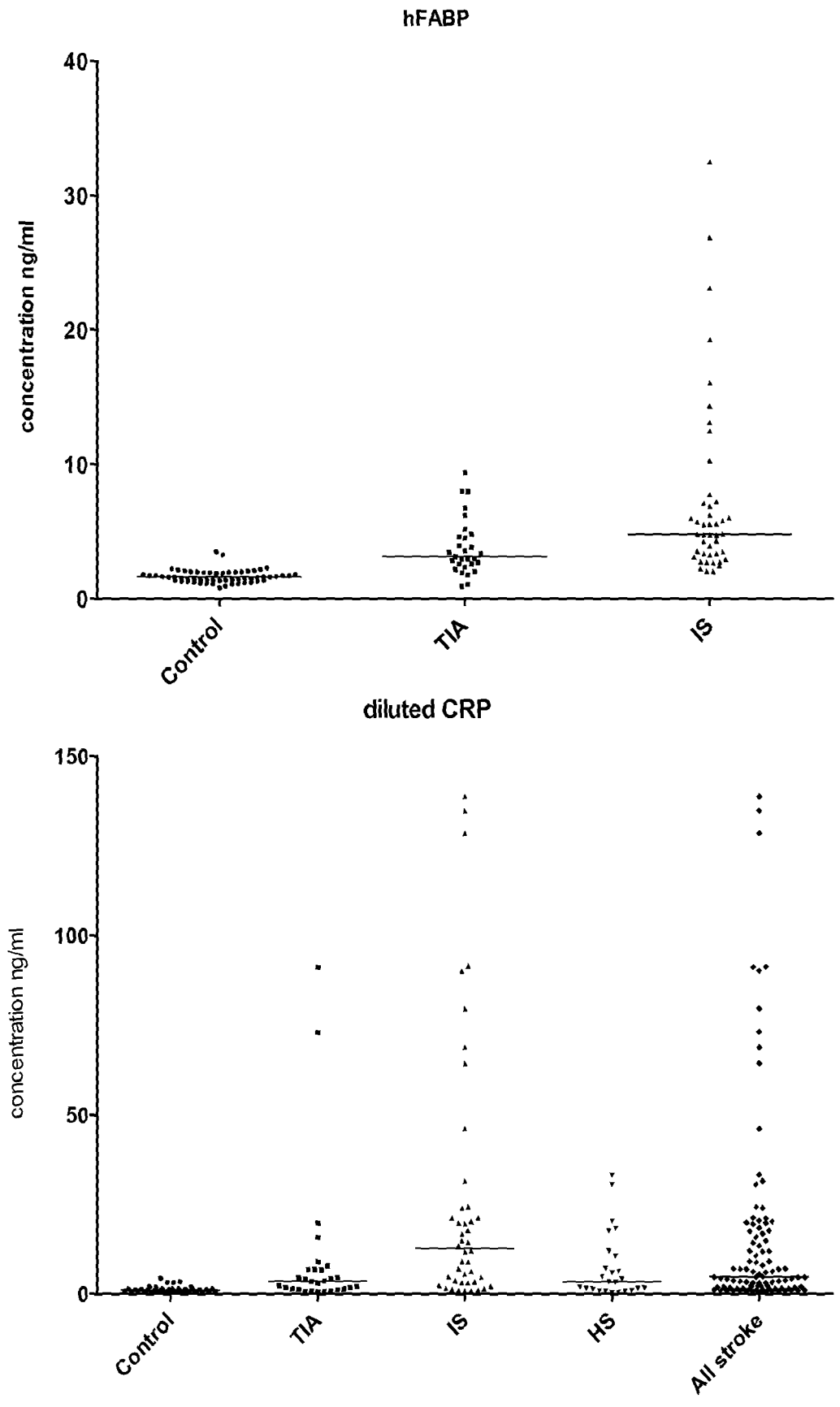
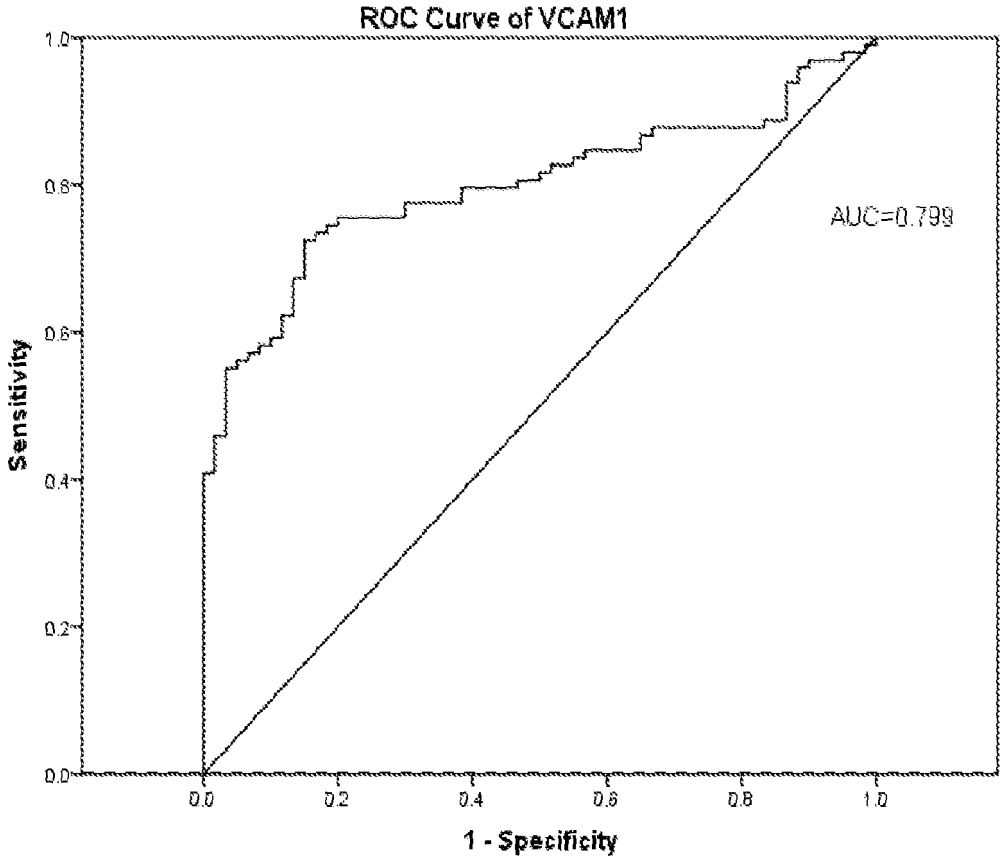

hFABP
concentration ng/ml
40
30
20
10
0
Control
TIA
IS
diluted CRP
concentration ng/ml
150
100
50
0
Control
TIA
IS
HS
All stroke

Figure 7


ROC Curve of VCAM1
Sensitivity
1 - Specificity
AUC=0.799
0.0
0.2
0.4
0.6
0.8
1.0

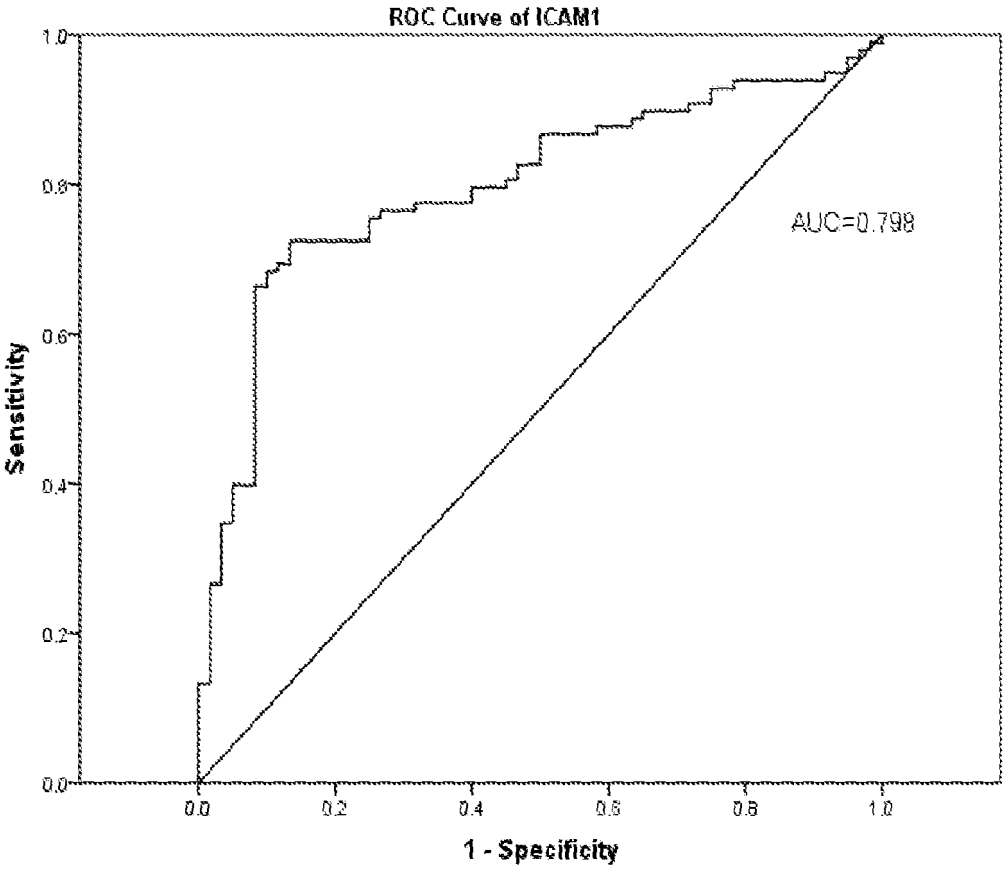
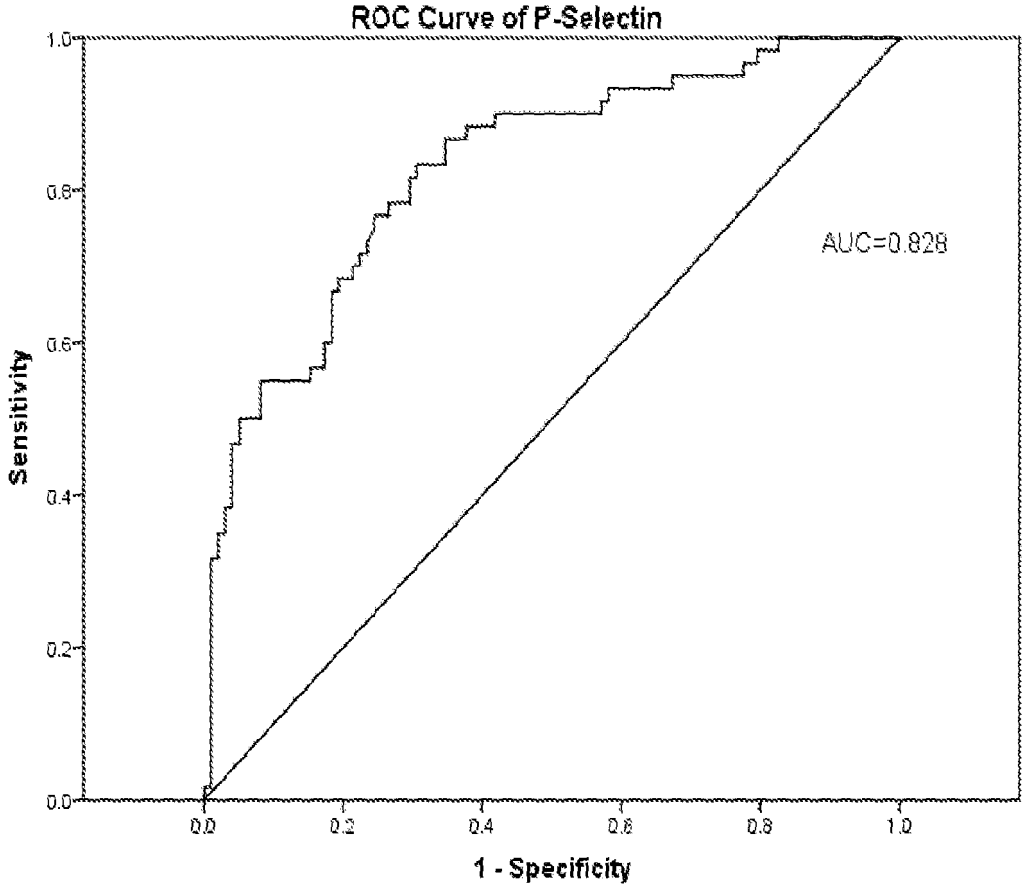

ROC Curve of ICAM1
Sensitivity
1 - Specificity
AUC=0.798
0.0
0.2
0.4
0.6
0.8
1.0

Figure 8


ROC Curve of P-Selectin
Sensitivity
1 - Specificity
AUC=0.828
0.0
0.2
0.4
0.6
0.8
1.0

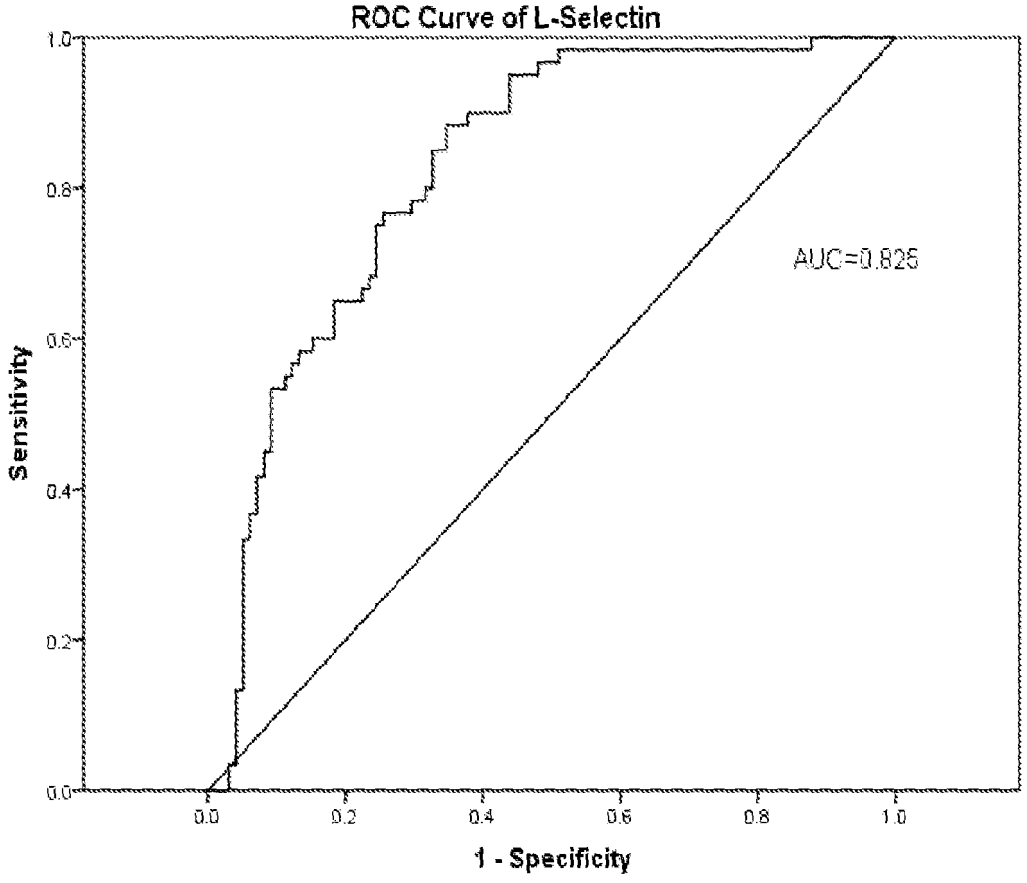
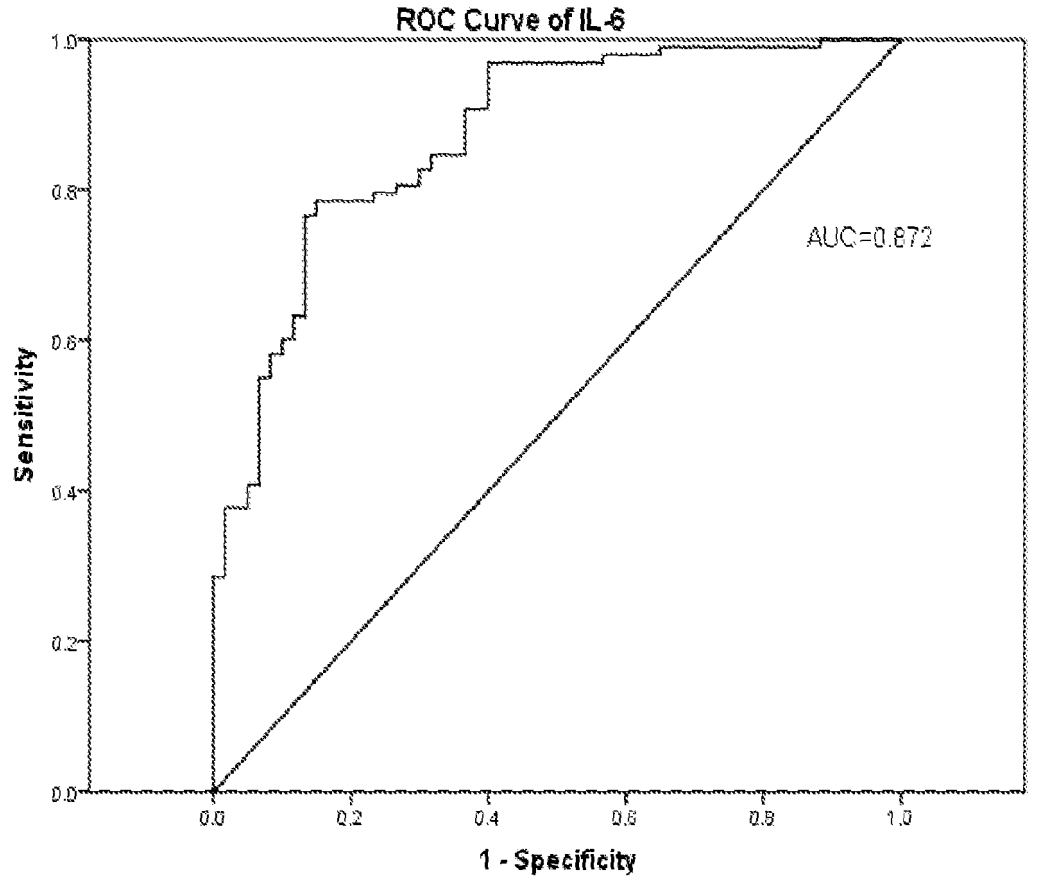

ROC Curve of L-Selectin
Sensitivity
1 - Specificity
AUC=0.825
0.0
0.2
0.4
0.6
0.8
1.0

Figure 9


ROC Curve of IL-6
Sensitivity
1 - Specificity
AUC=0.872
0.0
0.2
0.4
0.6
0.8
1.0

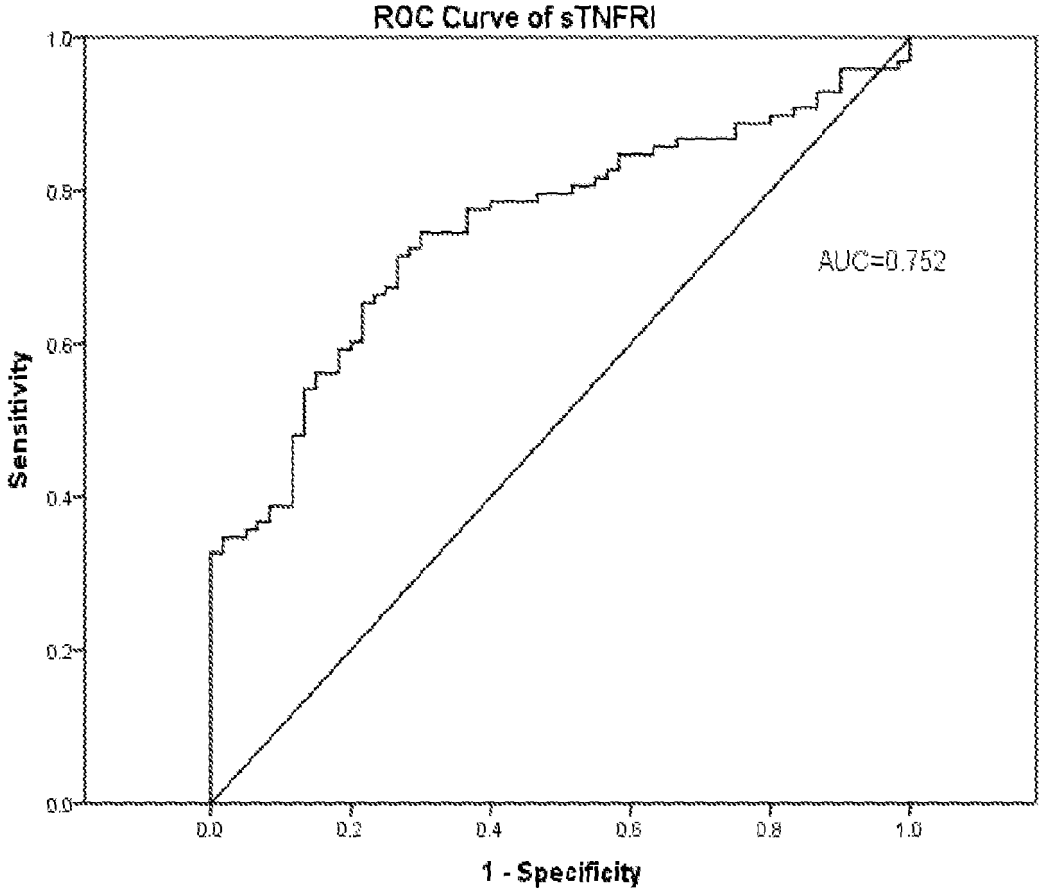
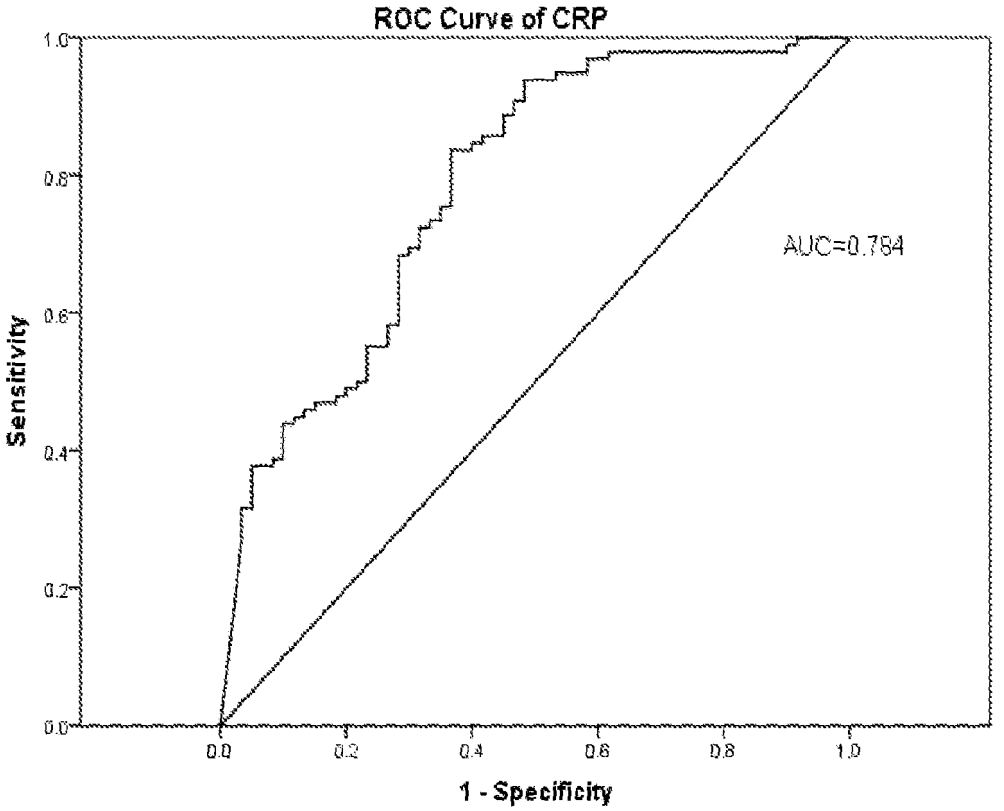

ROC Curve of sTNFRI
Sensitivity
1 - Specificity
AUC=0.752
0.0
0.2
0.4
0.6
0.8
1.0

Figure 10


ROC Curve of CRP
Sensitivity
1 - Specificity
AUC=0.784
0.0
0.2
0.4
0.6
0.8
1.0

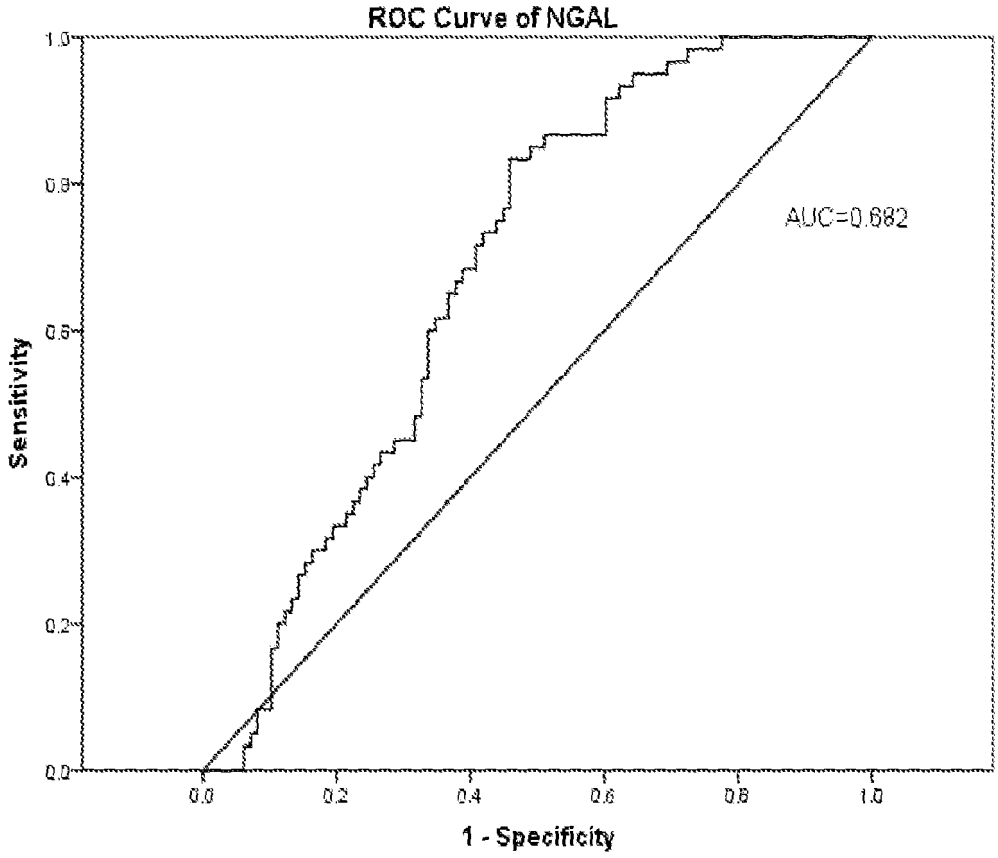

ROC Curve of NGAL
Sensitivity
1 - Specificity
AUC=0.682
0.0
0.2
0.4
0.6
0.8
1.0

Figure 11

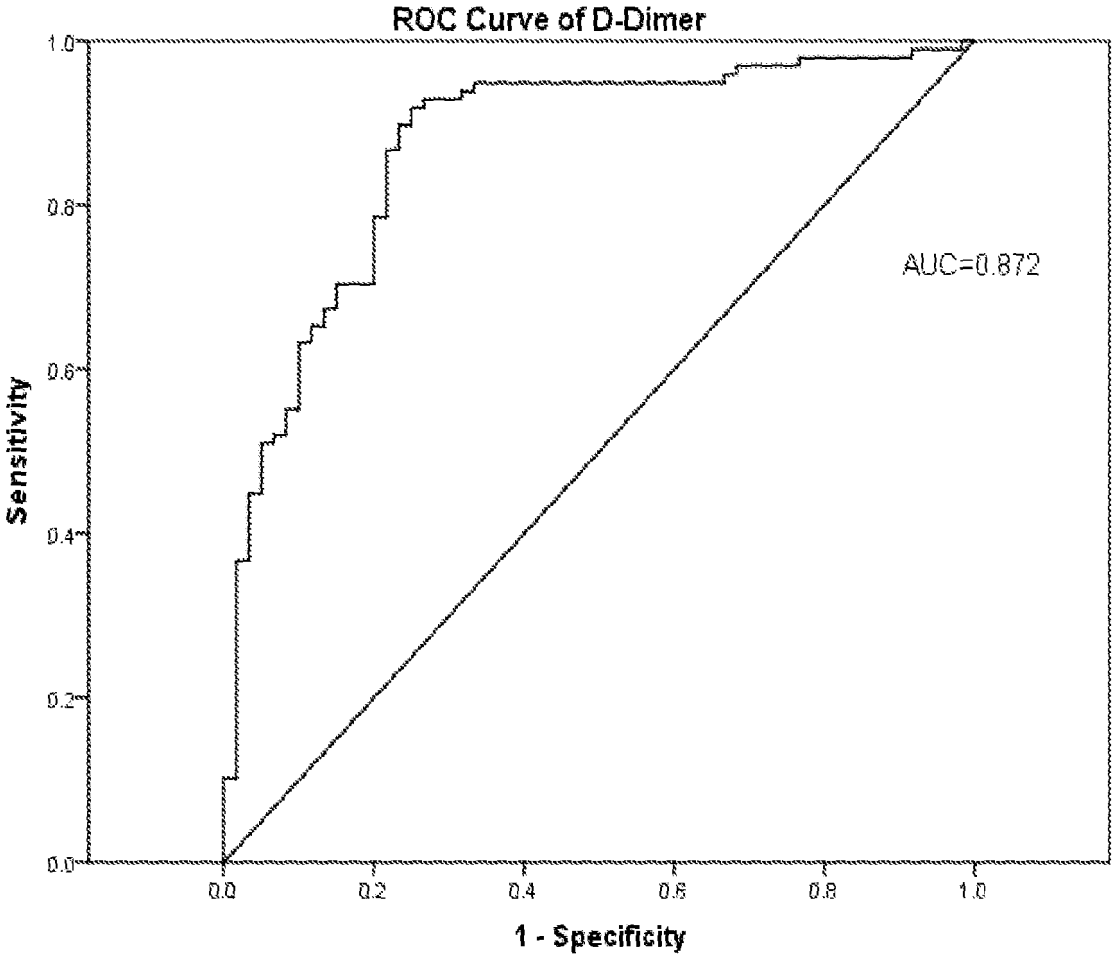

ROC Curve of D-Dimer
Sensitivity
1 - Specificity
AUC=0.872
0.0
0.2
0.4
0.6
0.8
1.0

Figure 12

Figure 13

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-1Lsel	89.8	86.7	0.943
4. VCAM-1IL-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-1Lsel 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

BIOMARKERS OF STROKE AND STROKE SUBTYPES

BACKGROUND OF 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 annum suffer stroke world-wide 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 eight billion pounds 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) – 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) – 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 iii) haemorrhagic stroke (HS) – accumulation of blood within the skull vault. A haemorrhagic stroke occurs when a weakened blood vessel ruptures. Haemorrhagic stroke can be classified into two major subtypes namely intracerebral (within the brain's tissue) and subarachnoid (around the brain's surface and under its protective layer). Ischaemic stroke and TIA accounts for approximately 85 per cent of all stroke cases and haemorrhagic stroke 15 per cent.

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, to breakdown 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 haemorrhagic stroke patients (approximately 90% sensitivity) but poor sensitivity for identifying IS and TIA patients (approximately 20% sensitivity); in practice for TIA, due to its transient nature, minimal to no tissue damage occurs, and CT scan is therefore 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 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 test for detecting IS and TIA would be an

invaluable tool to aid clinicians in the diagnosis of stroke subtype. 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 subtype delineation; there are several descriptions of IS/TIA versus HS discrimination (EP1238284; WO 2010/086697; WO 2010/012834; WO 2002/012892), while EP1419388 discloses data that distinguishes IS from HS and all stroke types from non-stroke controls. EP 1668370 proposes glial fibrillary acidic protein (GFAP) as a biomarker for HS diagnosis. However, none have thus far found use in clinical practice and there is always a need for stroke biomarkers (within this document reference to 'stroke' infers all three stroke subtypes HS, IS and TIA unless otherwise stated) of high sensitivity and specificity. Furthermore, there are 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

The invention facilitates the diagnosis of stroke and stroke subtypes through biomarkers. Specifically, biomarkers are described that delineate HS, IS and TIA individually and in combination from controls. Furthermore, biomarkers capable of discriminating IS from TIA are also described.

FIGURES

Figure 1 Graph of VCAM-1 and ICAM-1 in all stroke, stroke subtypes and control
Figure 2 Graph of E-selectin and P-selectin in all stroke, stroke subtypes and control
Figure 3 Graph of L-selectin and IL-6 in all stroke, stroke subtypes and control
Figure 4 Graph of sTNFRI and GFAP in all stroke, stroke subtypes and control
Figure 5 Graph of NGAL and D-dimer in all stroke, stroke subtypes and control
Figure 6 Graph of TM and CRP in all stroke, stroke subtypes and control
Figure 7 Graph of h-FABP in IS, TIA & Control and diluted CRP in all stroke, stroke subtypes and control
Figure 8 ROC curves of VCAM-1 and ICAM-1
Figure 9 ROC curves of P-selectin and L-selectin
Figure 10 ROC curves of IL-6 and sTNFRI
Figure 11 ROC curves of CRP and NGAL
Figure 12 ROC curve of D-dimer
Figure 13 All stroke vs controls: table of AUC, sensitivity and specificity for combinations of biomarkers. Lsel= L-selectin, Psel= P-selectin.

DETAILED DESCRIPTION OF THE INVENTION

The invention describes a biomarker-based method that can be used to support the diagnosis of stroke. Specifically, the measurement of the concentration of certain

biomarkers in a biological sample taken from a patient suspected of having undergone or undergoing a stroke, enables a biomarker-based diagnosis of HS, IS and TIA. The term 'biomarker', in the context of the current invention, unless otherwise stated, refers to a molecule present in a biological fluid of a patient, the concentration of which in said biological fluid may be indicative of a pathological state; the term 'stroke', unless otherwise stated, refers to all three stroke subtypes, namely HS, IS and TIA.

The invention describes various biomarkers for use in diagnosing stroke and stroke subtypes either alone or in combination with other diagnostic methods or as complementary biomarkers. A complementary biomarker in the current context implies a biomarker that can be used in conjunction with other stroke biomarkers to support patient diagnosis.

A first aspect of the invention describes a method for diagnosing stroke in a patient suspected of having had or of having a stroke which comprises taking an *in vitro* sample from the patient, determining the sample concentration of two or more biomarkers chosen from sTNFRI, IL-6, D-dimer, P-selectin, L-selectin, ICAM-1, CRP and VCAM-1, and establishing the significance of the concentration(s) of the one or more biomarkers. The significance of the concentration(s) of the one or more biomarkers is gauged by comparing said concentration(s) to a control value. The control value is derived from the concentrations of the one or more biomarkers in a biological sample in an individual or individuals who have not undergone a stroke. The individual(s) who have not undergone stroke can be, for example, healthy individuals, individuals suffering from diseases other than stroke or the individuals prior to the stroke event. For the biomarkers of the invention, the concentration in the biological sample is greater for sTNFRI, IL-6, D-dimer, ICAM-1, CRP and VCAM-1 and less for L-selectin and P-selectin in a stroke individual compared to the concentration in a non-stroke individual i.e. the control value. The statement 'having had' implies the stroke is a recent event, such an event having initiated the process of the individual seeking clinical help. In a preferred embodiment, the biomarkers for use in the method of the invention are chosen from any of the biomarkers or biomarker combinations 1-170 of Figure 13. Another preferred embodiment utilizes in the methods of the invention at least one biomarker chosen from ICAM-1, VCAM-1, L-selectin and P-selectin and at least one biomarker chosen from sTNFRI, IL-6, CRP and D-dimer. Other preferred biomarkers for use in the invention include two or more biomarkers chosen from P-selectin, L-selectin, ICAM-1 and VCAM-1. Combinations of three or more biomarkers are most preferred as they show the highest sensitivity and specificity. Combinations of biomarkers comprising two or more of ICAM-1, VCAM-1, L-selectin, P-selectin, IL-6 and D-dimer are especially preferred; a preferred subset of these is ICAM-1 and L-selectin in combination with one or more of VCAM-1, P-selectin, IL-6 and D-dimer,

A further aspect of the invention is a method of aiding the diagnosis of IS in a patient suspected of having had or who is having a stroke which comprises taking an *in vitro* sample from the patient and determining the sample concentration of one or more biomarkers chosen from h-FABP, VCAM-1, CRP and IL-6 and establishing the significance of the concentration(s) of the one or more biomarkers. In a preferred

embodiment the significance of the concentration of the biomarker(s) is established by comparing the determined concentration(s) to a reference value or values. The reference value or values is/are preferably the concentration of one or more of h-FABP, VCAM-1, CRP and IL-6 measured in a TIA patient or patients. This reference value or values is/are generally established by measuring the levels of the biomarkers of the invention in multiple patients clinically diagnosed as having or having had a TIA, the diagnosis derived using techniques such as clinician examination and neuroimaging analysis for HS rule out. Preferred samples for use in the invention are blood, plasma and serum.

Another aspect of the invention is a method of aiding the diagnosis of IS in a patient suspected of having had or who is having a stroke which comprises taking an *in vitro* sample from the patient and determining the sample concentration of one or more biomarkers chosen from h-FABP, VCAM-1, CRP and IL-6 and comparing the determined concentration(s) to the concentration of one or more biomarkers chosen from h-FABP,
VCAM-1, CRP and IL-6 in a TIA patient or patients (reference value) in which an increase in the concentration of the biomarker(s) in the patient compared to the reference value(s) signifies the patient is subject to IS. Preferred combinations of biomarkers for IS diagnosis are VCAM-1 + IL-6 and VCAM-1 + IL-6 + CRP.

If a single biomarker is used in any of the described methods, a cut-off reference value can be derived and used in the method; if two or more biomarkers are used in the method then a suitable model such as a logistic regression equation can be derived and used in the method.

A further aspect of the invention is a method of aiding the diagnosis of IS in a patient suspected of having had or who is having a stroke which comprises taking an *in vitro* sample from the patient and determining the sample concentration of one of h-FABP, VCAM-1, CRP and IL-6 in which the following concentrations ('cut-off' concentration) taken individually support the diagnosis of IS in the patient: h-FABP $\geq$ about 10 ng/ml, VCAM-1 $\geq$ about 570 ng/ml, CRP $\geq$ about 30 μg/ml, IL-6 $\geq$ about 12 pg/ml.

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 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. The ROC curve is a preferred method of assessing a diagnostic test's accuracy; it addresses both the sensitivity, the number of true positives, and the specificity, the number of true negatives, of the 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 example, as detailed for IS vs TIA discrimination, a high specificity is critical for the intended application. For a specific test the closer the value of its AUC to 1.0 the greater its predictive power. 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, other mathematical/statistical procedures, well known in the art, can be used.

A further aspect of the invention is the use of any of the previously described stroke or stroke subtype diagnostic methods using as stroke or stroke subtype biomarkers one or more of h-FABP, sTNFRI, IL-6, D-dimer, L-selectin, P-selectin, ICAM-1, VCAM-1 and CRP as complementary stroke or stroke subtype biomarkers. As complementary biomarkers they may be used for stroke diagnosis in conjunction with proteins such as GFAP, DJ-1, brain natriuretic peptide (BNP), S100 β, MMP-9, MCP-1, ApoC1, ApoC3, von Willebrand factor, NMDA receptors, asymmetric dimethylarginine (ADMA) and lipoprotein-associated phospholipase A2 (Lp-PLA2).

A further aspect of the invention is a substrate comprising one or more probes specific for one or more of h-FABP, sTNFRI, IL-6, D-dimer, L-selectin, P-selectin, ICAM-1, VCAM-1 and CRP for use in the methods of the invention. For delineation of IS from TIA a preferred substrate comprise a probe for IL-6 and probe for VCAM-1; preferably the substrate comprises one probe specific for IL-6, one probe specific for VCAM-1 and one probe specific for CRP. For diagnosing stroke, a preferred substrate comprises two or more probes, each probe specific to a single biomarker, at least one probe specific to one of sTNRI, CRP, D-dimer and IL-6 and at least one probe specific to one of L-selectin, P-selectin ICAM-1 or VCAM-1. For stroke diagnosis the substrate can also comprise two, preferably three or four probes each probe specific to an individual biomarker selected from a subset or all of L-selectin, P-selectin, ICAM-1 and VCAM-1. Specific implies that the probe binds only to one of the named biomarkers of the invention with negligible binding ('negligible binding' implying that the integrity of the diagnostic assay and its result using the biomarkers of the invention is not compromised) to other named biomarkers of the invention or to other analytes in the biological sample being analysed. 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 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 (the biomarker capture agent) of the invention is either 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.

A further aspect of the invention is the use of two or more of sTNFRI, IL-6, D-dimer, P-selectin, L-selectin, ICAM-1, CRP and VCAM-1 as biomarkers of stroke; a preferred embodiment makes use at least one or more of ICAM-1, VCAM-1, P-selectin and L-selectin and at least one or more of sTNFRI, IL-6, D-dimer and CRP. For example, combinations comprising ICAM-1, L-selectin and one or more of VCAM-1, IL-6 and D-dimer have particularly high sensitivities and specificties. A further embodiment makes use of a combination of two or more, preferably three or more of ICAM-1, VCAM-1, P-selectin and L-selectin as biomarkers of stroke. Another aspect of the invention is the use of one or more of h-FABP, IL-6, CRP and VCAM-1 as a biomarker or biomarkers of IS; preferred combinations are IL-6 + VCAM-1 and IL-6 + VCAM-1 + CRP. Each of these biomarkers or biomarker combinations can be used alone or as complementary biomarkers.

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, but the preferred methodology is based on immunodetection. Immunodetection 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.

Methods

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 (standard deviation) was 75.2 (9.4) years. 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.

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, GFAP, CRP, D-dimer, sTNFRI, Thrombomodulin (TM) and Neutrophil Gelatinase Associated Lipocalin (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 9 point calibration curve and

3 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 4 groups. 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. 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.

Results

Figure 13 highlights the sensitivity, specificity and statistical power (AUC) of exemplary combinations of biomarkers for diagnosing stroke. By combining two or more biomarkers selected from ICAM-1, VCAM-1, L-selectin, P-selectin, IL-6, CRP, D-dime 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, either individually or in various combinations, 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 2 shows the sensitivity and specificity values of individual and grouped biomarkers. 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 fashion. It has also been found that CRP can be used as a complementary biomarker to GFAP in HS diagnosis, by ruling out IS and TIA.

Table 1 Statistical analysis of analyte concentrations in patients who suffered transient ischaemic attack (TIA), ischaemic stroke (IS), haemorrhagic stroke (HS), TIA + IS + HS (All Stroke) and in control (C) using Mann-Whitney and Kruskal-Wallis tests. ns = not significantly different at the 5% level (P>0.05).

Analyte	IS v TIA	TIA v C	IS v C	HS v C	HS v IS	All Stroke 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
GFAP	ns	ns	ns	P<0.001	P<0.001	ns
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
sTNFRI	P<0.05	P<0.001	P<0.001	P<0.001	ns	P<0.001
TM	ns	ns	ns	ns	ns	ns

Table 2 ROC curve analysis of biomarkers for IS vs TIA

Biomarker(s)	Ischaemic Stroke (IS)		
	AUC	% 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

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 the individual 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.

CLAIMS

1. A method for diagnosing stroke in a patient suspected of having a stroke which comprises taking an *in vitro* sample from the patient, determining the sample concentration of two or more of the biomarkers sTNFRI, IL-6, D-dimer, L-selectin, ICAM-1, CRP, VCAM-1 and P-selectin, and establishing the significance of the concentration(s) of the two or more biomarkers.

2. The method of Claim 1 which establishes the significance of the concentration(s) of the two or more biomarkers by comparing the value of said concentration(s) to a control value.

3. The method of Claim 2 in which the concentrations of TNFRI, IL-6, D-dimer, ICAM-1, CRP and VCAM-1 are greater than the control value and the concentrations of L-selectin, and P-selectin are lower than the control value.

4. The method of any of the preceding claims in which the two or more biomarkers is/are the biomarker or biomarker combinations 1- 137 of Figure 13.

5. The method of any of claims 1 to 3 in which the two or more biomarkers comprise at least one biomarker selected from ICAM-1, VCAM-1, P-selectin and L-selectin and at least one biomarker selected from sTNFRI, IL-6, D-dimer and CRP.

6. The method of any of claims 1 to 3 in which the two or more biomarkers comprise L-selectin and ICAM-1 and one or more of IL-6, VCAM-1, D-dimer and P-selectin.

7. A method of aiding the diagnosis of ischaemic stroke in a patient suspected of having a stroke which comprises taking an *in vitro* sample from the patient, determining the sample concentration of one or more biomarkers selected from

a) h-FABP
b) IL-6
c) VCAM-1
d) CRP

and establishing the significance of the concentration(s) of the one or more biomarkers.

8. The method of Claim 7 which establishes the significance of the concentration(s) of the one or more biomarkers h-FABP, IL-6, VCAM-1 and CRP by comparing the value of said concentration(s) to a reference value.

9. The method of Claim 8 in which the reference value is the concentration of one or more of h-FABP, IL-6, CRP and VCAM-1 in an *in vitro* sample or samples taken from a TIA patient or patients.

10. The method of Claim 9 in which the concentrations of one or more of h-FABP, IL-6, CRP and VCAM-1 is greater in a patient subject to ischaemic stroke than in a patient subject to TIA.

11. The sample of any of the preceding claims which is a blood, serum or plasma sample.

12. Use of two or more of sTNFRI, IL-6, D-dimer, L-selectin, P-selectin, ICAM-1, CRP, and VCAM-1 as biomarkers of stroke or as complementary biomarkers of stroke.

13. Use of one or more of ICAM-1, VCAM-1, P-selectin and L-selectin and one or more of sTNFRI, IL-6, D-dimer and CRP as biomarkers of stroke or as complementary biomarkers of stroke.

14. Use of two or more of ICAM-1, VCAM-1, P-selectin and L-selectin as biomarkers of stroke or as complementary biomarkers of stroke.

15. Use of one or more of h-FABP, IL-6, CRP and VCAM-1 as a biomarker/biomarkers of ischaemic stroke or as a complementary biomarker/biomarkers of ischaemic stroke.

INTELLECTUAL PROPERTY OFFICE

Application No: GB1120781.8 **Examiner:** Dr Patrick Purcell

Claims searched: 1- **Date of search:** 13 September 2012

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
Y	1, 12 at least	US2004/0121343 A (BUECHLER ET AL) see whole document, esp. paras 0111-0112, 0204
Y	1, 12 at least	US2004/253637 A (BUECHLER ET AL) see whole document, esp. paras 0131-0132, 0227
Y	1, 12 at least	US2003/199000 A (VALKIRS ET AL) see whole document, esp. para 0135
Y	1, 12, at least	J Stroke and Cardiovascular Diseases, Vol 11, 2002, H Christensen et al, "Plasma Cytokines in Acute Stroke", see whole document
A		WO2008/149148 A (DOMANTIS LTD.)

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

C12M; G01N

The following online and other databases have been used in the preparation of this search report

ONLINE: EPODOC, WPI, BIOSIS, MEDLINE

INTELLECTUAL PROPERTY OFFICE

International Classification:

Subclass	Subgroup	Valid From
G01N	0033/68	01/01/2006
C12Q	0001/68	01/01/2006

Application No:	GB1120781.8	**Examiner:**	Dr Patrick Purcell
Claims searched:	7-10, 15	**Date of search:**	8 October 2012

Patents Act 1977
Further Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	7-10, 15	J Neurol, Vol 252, 2005, MT Wunderlich et al, "Release of brain-type and heart-type fatty acid-binding proteins in serum after acute ischaemic stroke", 718-724 see whole document, esp. http://www.springerlink.com/content/pw744x81g9262nh4/fulltext.pdf
X	7-10, 15	WO2005/103720 A (MEDSTAR RESEARCH INSTITUTE) see whole document, esp. page 17, lines 25-29, page 19, lines 15-32
X	7-10, 15	US2005/0181386 A (DIAMOND ET AL) see whole document, esp. page 1, paras 0013-0021, page 6, para 0057, page 11, para 0079, page 13, para 0092, page 29, para 0343
X	7-10, 15	EP2206726 A (UNIVERSITE JOSEPH) see whole document, esp. page 5, paras 0041-44, 0048-0052
X	7-10, 15	US2004/0121343 A (BUECHLER ET AL) see whole document, esp. paras 0111-0112, 0204
X	7-10, 15	US2004/253637 A (BUECHLER ET AL) see whole document, esp. paras 0131-0132, 0227
X	7-10, 15	US2003/199000 A (VALKIRS ET AL) see whole document, esp. para 0135

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC
The following online and other databases have been used in the preparation of this search report
ONLINE: EPODOC, WPI, BIOSIS, MEDLINE

International Classification:

Subclass	Subgroup	Valid From
G01N	0033/68	01/01/2006
C12Q	0001/68	01/01/2006