(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 November 2011 (24.11.2011)

PCT

(10) International Publication Number
WO 2011/144914 A2

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/GB2011/000784

(22) International Filing Date:
23 May 2011 (23.05.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1008541.3 21 May 2010 (21.05.2010) GB

(71) Applicants *(for all designated States except US)*: **UNIVERSITE DE GENEVE** [CH/CH]; 24, rue du Général-Dufour, CH-1211 Geneva 4 (CH). **RECERCA INSTITUT DE RECERCA HOSPITAL UNIVERSITARI VALL D'HEBRON** [ES/ES]; Pg. Vall d'hebron, 119-129, E-08035 Barcelona (ES). **ELECTROPHORETICS LIMITED** [GB/GB]; Coveham House, Downside Bridge Road, Cobham Surrey KT11 3EP (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DAYON, Loïc, Gérard** [FR/GB]; 144 Crystal Place Road, London SE22 9ER (GB). **SANCHEZ, Jean-Charles** [CH/CH]; Department of Structural Biology and Bioinformatics, University Medical Centre (CMU), Univesity of Geneva, 1 ru Michel Servet, CH-1121 Geneva 4 (CH). **MONTANER VILLALONGA, Joan** [ES/ES]; Passeig Sant Joan 149 5° 1a, E-08037 Barcelona (ES).

(74) Agents: **RICHARDS, William** et al.; Bryer, Shelley, John Amor, Greenwood Llp, 7, Gay Street, Bath BA1 2PH (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2011/144914 A2

(54) **Title**: DIAGNOSTIC METHODS

(57) **Abstract**: The invention relates to a method of aiding the diagnosis of acute brain damage in a subject, said method comprising (i) assaying the concentration of at least one oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1 in a sample from said subject; and (ii) assaying the concentration of at least one further polypeptide selected from Panel A; (Hi) comparing the concentrations of (i) and (ii) to the concentrations of the polypeptides in a reference standard and determining quantitative ratios for said polypeptides; (iv) wherein a finding of a quantitative ratio of each of the assayed polypeptides in the sample to the polypeptides in the reference standard of greater than 1.3 indicates an increased likelihood of acute brain damage having occurred in said subject.

Diagnostic Methods

Field of the Invention

The invention relates to aiding the diagnosis of acute brain injury. In particular the invention relates to aiding the diagnosis of stroke. Novel biomarkers and panels of biomarkers are described in the methods of the invention.

Background to the invention

Stroke is a leading cause of death and disability in industrialized countries. The rapid diagnosis of an acute stroke is essential to triage suspected patients and transfer confirmed ones in specialized stroke units.

It is well known that non-cerebrovascular conditions can present with a clinical picture mimicking stroke, so that early accurate differentiation of such "mimics" from true stroke is essential to direct patients towards appropriate care. At present, the absence of a simple and widely available diagnostic test for acute cerebral ischemia remains a problem in the diagnosis (mostly based on clinical grounds and neuroimaging techniques) and management of stroke. In addition, the prognosis of stroke patients is relevant to rationalize the treatment and the follow-up.

The need for markers to diagnose a stroke and/or to predict probable course and outcome of the disease is therefore a major problem for the medical workforce (1).

Human cerebral microdialysis is an in vivo sampling technique to monitor the changes in composition of extracellular fluid (ECF) in the brain. Basically, a flexible microprobe is inserted into the patient's brain and a solution with composition very close to that of cerebrospinal fluid (CSF) is perfused (2). The probe simulates the function of a fenestrated capillary. The endogenous substances, that can pass the semi-permeable membrane situated at the probe tip, diffuse from the interstitial fluid to the microdialysis solution.

In the past few years, several studies measuring small molecules in human brain microdialysates, such as substrates (e.g., glucose), metabolites (e.g., pyruvate, lactate), and neurotransmitters (e.g., glutamate) were carried out (3-5). Conversely, few proteomic studies of these rare materials have been reported to date (6, 7). The ability to recover proteins depends on several physico-chemical factors such as their molecular weight, hydrophobicity/hydrophilicity, charge, shape, radius of gyration and

interactions with other molecules. The structure of the microdialysis catheters, the pore size of the membrane, the flow rate, the temperature, and the diffusion properties of the proteins inside the perfused fluid influence both the protein and fluid recovery (8). As an example, *in vitro* recovery of protein S100-B (S100B), a 12 kDa calcium binding protein with important intracellular and extracellular function (9), was improved with catheter MW cut-off of 100 kDa with respect to formal cut-off value of 20 kDa (10). The accumulation of biological debris within the catheter was also shown to decrease recovery over time (8). Thus microdialysis approaches remain technically extremely challenging. Furthermore, they represent an invasive procedure requiring incision and access to the inner parts of the brain, which is an extremely specialised and difficult procedure to carry out.

In this context, Maurer et al. carried out a proteomic analysis of human brain microdialysate with two-dimensional gel electrophoresis and mass spectrometry (MS), and identified 27 proteins from the non-infarcted (i.e. contralateral (CT)) hemisphere of stroke patients (11). Many of those proteins were previously detected in CSF but few appeared to be exclusively present in the brain microdialysate. None appeared to show sufficient utility as a biomarker of stroke. In more recent research, microdialysate samples of patients with subarachnoid hemorrhage (SAH), developing or not a vasospasm, were compared (12). Glyceraldehyde-3-phosphate and heat-shock cognate 71 kDa proteins were respectively increased and decreased in the group that suffered a posterior vasospasm that may produce a cerebral infarction as a side effect. The authors concluded that these proteins might be used as early markers for the development of symptomatic vasospasm after SAH.

In view of the above, the identification of markers indicative of acute brain injury and/or the diagnosis of stroke remains an unanswered problem in the field.

The present invention seeks to overcome problem(s) associated with the prior art.

Description of the Invention

Due to their permitting real-time monitoring and sampling in close proximity to the damaged tissue, human brain microdialysates are a highly valuable source material for the discovery of brain-specific biomarkers. Proteomic analysis of human brain microdialysis samples has been applied by the inventors to find innovative molecules for the diagnosis and prognosis of cerebrovascular disorders such as stroke.

These studies allowed the identification of particular biomarkers and further allowed them to be interrogated for association with stroke. The biomarkers could be further characterised in terms of their association with particular forms or elements of the injury such as the proximity to the core of the damaged region or other such property.

The insights gained from these demanding studies have permitted the identification of certain biomarkers for acute brain injury such as stroke. Thus the inventors have been able to devise methods for aiding diagnosis of such conditions as detailed herein.

Thus in one aspect the invention provides a method of aiding the diagnosis of acute brain damage in a subject, said method comprising

(i) assaying the concentration of at least one oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1 in a sample from said subject; and

(ii) assaying the concentration of at least one further polypeptide selected from Panel A;

(iii) comparing the concentrations of (i) and (ii) to the concentrations of the polypeptides in a reference standard and determining quantitative ratios for said polypeptides;

(iv) wherein a finding of a quantitative ratio of each of the assayed polypeptides in the sample to the polypeptides in the reference standard of greater than 1.3 indicates an increased likelihood of acute brain damage having occurred in said subject.

The oxidative stress polypeptide may be referred to as an oxidative stress related polypeptide.

Optionally the at least one oxidative stress polypeptide of (i) may be assayed in combination with the oxidative stress protein S100B.

The polypeptide is suitably an oxidative stress polypeptide. The polypeptide is suitably selected from the group consisting of PRDX1, PRDX6 and GSTP1. This group shares the common property of being oxidative stress proteins. These proteins are antioxidative enzymes. They are each connected by their involvement in the elimination of reactive oxygen species. Thus these polypeptides are conceptually related. Moreover, they are functionally related. These polypeptides are taught as a group for the first time as diagnostic of stroke. Thus one contribution made to the art by the current invention is to place this biologically connected group of polypeptides together into a single group being diagnostic indicators of stroke.

Optionally the group of PRDX1, PRDX6 and GSTP1 may include other protein(s) induced by oxidative stress. For example the group may include the protein S100B which is induced in oxidative stress. These polypeptides are taught as a group for the first time as diagnostic of stroke.

In addition to the common properties noted above, and in addition to the specific common utility taught here for the first time for this group, and in addition to the small and defined size of this cluster of polypeptides, it is important to note that they are also connected by virtue of being evidenced as direct interactors with one another. For example, these proteins have been demonstrated to be part of a single biological complex.

For example, PRDX1 and GSTP1 are implicated in similar redox protective mechanisms. Furthermore, they have been evidenced to interact together (Krapfenbauer 2003 Brain Res. 967 p 152). In addition, GSTP1 has been shown to reactivate oxidized PRDX6 (Schreibelt 2008 Free Radic. Biol. Med. 45 p 256). In addition, the formation of a complex has been biochemically demonstrated (Kim 2006 Cancer Res. 66 p 7136).

In addition to these powerful indications of common biological function, GSTP1 has been shown to reactivate oxidised PRDX6 (Manevich 2004 PNAS 101 p 3780). Moreover, complex formation between these polypeptides has also been proved (Ralat 2006 Biochemistry (Mosc.) 45 p 360).

Thus for at least these reasons the group consisting of: PRDX1, PRDX6 and GSTP1 forms a single invention, each member of this very small group being linked so as to form a single inventive concept. This concept may be characterised as the assay of oxidative stress proteins as an indicator of stroke. Alternatively this concept may be characterised as the teaching that assaying for a single biological assembly (i.e. the above described peroxiredoxin complex) can aid in the diagnosis of stroke. In order to define the invention in the most definite terms, the individual different molecular members of the complex are individually recited. However, it should be noted that these individual polypeptides share a technical relationship for the reasons given above. Thus each of the individual proteins mentioned share the special technical features of being in the same biological complex, contributing the same biological function, being in the same in vivo macromolecular assembly and other common properties as described. Thus the application relates to a single invention characterised

by the new teaching connecting the members of this complex to the diagnosis of stroke.

Suitably step (i) comprises assaying the concentration of at least two oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1.

Suitably step (i) comprises assaying the concentration of each of the oxidative stress polypeptides PRDX1, PRDX6 and GSTP1

Suitably step (ii) may comprise measurement(s) of one or more of the panel of oxidative stress-related proteins described above as part of a larger panel in combination with proteins with other functions. For example this includes other proteins discovered in brain microdialysates.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel B.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel C.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from enlarged panel ABC

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1H.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1C.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1A.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1B.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2A.

Suitably step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2B.

Suitably step (ii) comprises assaying the concentration of at least two further polypeptides selected from said Panel.

Suitably step (ii) comprises assaying the concentration of at least four further polypeptides selected from said Panel.

Suitably assaying the concentration of at least one further marker from said panel is carried out.

Suitably the acute brain injury is stroke.

Suitably the sample is brain microdialysate fluid, cerebrospinal fluid, or blood.

Most suitably the sample is blood.

Suitably step (i) comprises assaying the concentration of PRDX1 in a sample from said subject.

Suitably the protein is detected by western blotting.

Suitably the protein is detected by bead suspension array or by planar array.

Suitably the protein is detected by isobaric protein tagging or by isotopic protein tagging.

Suitably the protein is detected by mass spectrometer-based assay.

In another aspect, the invention relates to use for diagnostic or prognostic applications relating to acute brain damage of a material which recognises, binds to or has affinity

for a first and a second polypeptide or a fragment, variant or mutant thereof, wherein the first polypeptide is selected from PRDX1, PRDX6 and GSTP1 and the second polypeptide is selected from Panel A.

In another aspect, the invention relates to use for diagnostic or prognostic applications relating to stroke of a material which recognises, binds to or has affinity for a polypeptide or a fragment, variant or mutant thereof, wherein the polypeptide is selected from Panel 2.

In another aspect, the invention relates to use as described above of a combination of materials, each of which respectively recognises, binds to or has affinity for one or more of said polypeptide(s), or a fragment, variant or mutant thereof.

In another aspect, the invention relates to use as described above, in which the or each material is an antibody or antibody chip.

In another aspect, the invention relates to use as described above, in which the material is an antibody with specificity for one or more of said polypeptide(s), or a fragment, variant or mutant thereof.

In another aspect, the invention relates to an assay device for use in the diagnosis of acute brain damage, which comprises a solid substrate having a location containing a material, which recognizes, binds to or has affinity for a first and a second polypeptide or a fragment, variant or mutant thereof, wherein the first polypeptide is selected from PRDX1, PRDX6 and GSTP1 and the second polypeptide is selected from Panel A.

In another aspect, the invention relates to an assay device for use in the diagnosis of stroke, which comprises a solid substrate having a location containing a material, which recognizes, binds to or has affinity for a polypeptide, or a fragment, variant or mutant thereof, wherein the polypeptide is selected from Panel 2.

In an assay device as described above, suitably the material is an antibody or antibody chip.

Suitably the assay device has a unique addressable location for each antibody, thereby to permit an assay readout for each individual polypeptide or for any combination of polypeptides.

In another aspect, the invention relates to a kit for use in the diagnosis of stroke, comprising an assay device as described above, and means for detecting the amount of one or more of the polypeptides in a sample of body fluid taken from a subject.

More suitably the polypeptide is a peroxiredoxin. More suitably the polypeptide is PRDX1.

In another aspect, the invention relates to a method of diagnosis or prognostic monitoring of acute brain damage in a subject, said method comprising

(a) obtaining and extracting the proteins from a relevant tissue sample from an individual;

(b) digesting said proteins to produce a population of peptides;

(c) determining the abundance of one or more of said peptides listed in Table 14 using Selected Reaction Monitoring of one or more of the transitions listed in Table 15;

(d) comparing the abundance of said one or more peptides with a pre-determined peptide abundance associated with a diagnosis of acute brain damage; and

(e) determining whether the subject has suffered acute brain damage and/or that the acute brain damage is worsening or improving based on the differences in abundance of said one or more peptides.

Suitably the pre-determined peptide abundance is determined using a known amount of corresponding synthetic peptide selected from Table 14.

In another aspect, the invention relates to a preparation for making a diagnosis of acute brain damage or prognostic monitoring of a subject with acute brain damage comprising one or more synthetic peptides selected from the group listed in Table 14.

Suitably said one or more synthetic peptides are selected from:

GSTP1	TFIVGDQISFADYNLLDLLLIHEVLAPGCLDAFPLLSAYVGR
	MPPYTVVYFPVR
	DDYVK
	DQQEAALVDMVNDGVEDLR
	FQDGDLTLYQSNTILR
	ASCLYGQLPK
	AFLASPEYVNLPINGNGK

		MLLADQGQSWK
		LSARPK
		TLGLYGK
		EEVVTVETWQEGSLK
		ALPGQLKPFETLLSQNQGGK
		YISLIYTNYEAGK
	PRDX1	HGEVCPAGWKPGSDTIKPDVQK
		QGGLGPMNIPLVSDPK
		ADEGISFR
		DISLSDYK
		LVQAFQFTDK
		IGHPAPNFK
		LNCQVIGASVDSHFCHLAWVNTPK
		YVVFFFYPLDFTFVCPTEIIAFSDR
		MSSGNAK
		TIAQDYGVLK
		ATAVMPDGQFK
	PRDX6	GMPVTAR
		MPGGLLLGDVAPNFEANTTVGR
		DFTPVCTTELGR
		VVFVFGPDK
		LIALSIDSVEDHLAWSK
		ELAILLGMLDPAEK
		LSILYPATTGR
		VATPVDWK
		NFDEILR
		LPFPIIDDR
		VVISLQLTAEK
		DINAYNCEEPTEK
		LAPEFAK
		DGDSVMVLPTIPEEEAK
		FHDFLGDSWGILFSHPR
	DDAH1	ALPESLGQHALR
		DENATLDGGDVLFTGR
		DYAVSTVPVADGLHLK

	GAEILADTFK
	GEEVDVAR
	QHQLYVGVLGSK
	TPEEYPESAK
CYTB	HDELTYF
	SQVVAGTNYFIK
	VFQSLPHENKPLTLSNYQTNK
	VHVGDEDFVHLR
ACBP	MSQAEFEK
	AAEEVR
	QATVGDINTERPGMLDFTGK
	TKPSDEEMLFIYGHYK
	WDAWNELK
	MWGDLWLLPPASANPGTGTEAEFEK
	MPAFAEFEK
CSRP1	GFGFGQGAGALVHSE
	GLESTTLADK
	GYGYGQGAGTLSTDK
MT3	GGEAAEAEAEK
	MDPETCPCPSGGSCTCADSCK
	SCCSCCPAECEK
PEPB1	GNDISSGTVLSDYVGSGPPK
	LYEQLSGK
	LYTLVLTDPDAPSR
	NRPTSISWDGLDSGK
	VLTPTQVK
	YVWLVYEQDRPLK

In another aspect, the invention relates to a preparation as described above wherein each peptide contains one or more stable heavy isotopes selected from hydrogen, carbon, oxygen, nitrogen or sulphur.

In another aspect, the invention relates to a preparation as described above wherein said synthetic peptides are labelled with an isotopic or isobaric tag.

In another aspect, the invention relates to a preparation as described above for the diagnosis or prognostic monitoring of acute brain damage.

In another aspect, the invention relates to a preparation as described above wherein the acute brain damage is ischaemic stroke or transient ischaemic attack.

In another aspect, the invention relates to a method for aiding the diagnosis of stroke in a subject, said method comprising

(i) assaying the concentration of at least one oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1 in a sample from said subject;

(ii) comparing the concentration of (i) to the concentration of the polypeptide in a reference standard and determining a quantitative ratio for said polypeptide;

(iii) wherein a finding of a quantitative ratio of the polypeptide in the sample to the polypeptide in the reference standard of greater than 1.3 indicates an increased likelihood of stroke having occurred in said subject.

Certain method steps discussed herein require the assay of one or more 'further polypeptide(s)' in addition to other requirements of the methods. A further polypeptide is one which is different to the one or more polypeptide(s) already required to be assayed. This is important because some of the groups of polypeptides presented herein contain members which are common to other groups presented herein. Clearly the mention of a 'further polypeptide' is intended to impose the assay of an additional polypeptide in addition to any which are or have been already assayed according to an earlier part of the method. Thus if a method requires one of A/B/C to be assayed and requires the assay of a further polypeptide selected from A/B/D/E/F/G, then merely assaying A twice or B twice does not constitute assaying a 'further' polypeptide as set out herein; assaying A then B would constitute the assay of a further polypeptide; assaying A then D would constitute the assay of a further polypeptide and so on. Thus suitably the further polypeptide is an additional polypeptide; suitably the further polypeptide is different from each other polypeptide assayed in the same method.

Acute Brain Damage embraces any rapid onset insults or injuries to the brain. Acute brain damage may include traumatic brain injury. Acute brain damage may include the effects resulting from stroke such as ischemic stroke. Acute brain damage may

include any other acute brain injury. In a preferred embodiment the acute brain injury is stroke; most preferably ischemic stroke.

The sample may be any suitable biological sample from a subject to be tested. The sample may be microdialysate fluid gathered from microdialyis of the brain. This has the advantage of being most closely associated with the site of possible injury.

The sample may be cerebrospinal fluid. This has the advantage of being more easily collected than microdialysate. This is therefore less demanding on the patient and on the skilled operator performing the collection.

The sample may be blood. This has the advantage of being easily collected in a minimally invasive manner. The collection of blood requires only ordinary commonly available equipment and modest training of the medical staff performing the collection.

The sample may be cleared blood (i.e. plasma or cleared plasma), where the red and white blood cells have been removed for example by centrifugation. These offer advantages of stabilising the sample and making it easier to store or handle, or even easier to analyse/assay.

Suitably the method(s) described do not involve the actual step of collection of the sample from the subject. Suitably the step of sample collection is omitted from the methods of the invention. Suitably the sample is previously collected. Suitably the methods are *in vitro* methods. Suitably the methods do not require the physical presence of the subject from whom the sample has been previously collected. Suitably the sample is an *in vitro* sample.

Plasma can be obtained relatively easily and may reflect the sub-proteomes of other organs, including the brain. Both candidate protein panels and gel based proteomics have previously been used in plasma and serum to identify possible biomarkers with some success.

One of the problems with the proteomic analysis of blood plasma with mass spectrometry, is the huge dynamic range of plasma proteins. Protein levels span an extraordinary 10 orders of magnitude, which makes the investigation of low(er) abundant proteins nearly impossible (Anderson and Anderson, 2002, Jacobs et al., 2005). The instrumental settings in the LC/MS/MS, where the most prominent peaks in a short period of time are chosen for fragmentation, do not allow for the identification and quantitation of low abundant proteins in unfractionated plasma due to the high abundance of serum albumin and other proteins. This is reflected in a low number of

proteins identified. One approach to reduce the dynamic range is to deplete samples of the highest abundant proteins and in this case we exemplify this approach using an immunoaffinity column to remove albumin, transferrin, IgG, IgA, antitrypsin, and haptoglobin. The number of identifiable and quantifiable proteins could be increased considerably and relative protein levels were compared between different samples.

For certain assay formats, the sample according to the invention may be a processed plasma. This is advantageous when the sample is to be analysed by mass spectrometry. For example, plasma may be processed to remove highly abundant proteins, and thereby to increase the number of detectable proteins, or to increase the detectability of proteins present in low absolute concentrations. Techniques for depletion of highly abundant proteins from plasma are well-known in the art. In particular, a multiple affinity removal system may conveniently be used to process plasma for analysis.

Furthermore, the sample may suitably comprise plasma proteins such as enriched plasma proteins. In this embodiment, plasma may be processed as described herein, and may then be subjected to size exclusion chromatography, buffer exchange, or other such treatments in order to arrive at a sample comprising the proteins from said plasma, which may offer advantages such as superior performance in analytical instruments.

Moreover, it is a specific advantage of embodiments of the invention when the sample is blood or a blood product that many of the biomarkers taught herein to be associated with acute brain injury such as stroke are amenable to detection or monitoring from blood from extant subjects for the first time; known techniques have relied on assay of cerebrospinal fluid, often from deceased subjects, and therefore have not previously amounted to a disclosure of aiding diagnosis in a living subject as is taught herein.

Reference Standard

The reference standard typically refers to a sample from a healthy individual i.e. one who has not suffered acute brain damage, cerebrovascular accident or related injury.

The reference standard can an actual sample analysed in parallel. Alternatively the reference standard can be one or more values previously derived from a comparative sample e.g. a sample from a healthy subject. In such embodiments a mere numeric comparison may be made by comparing the value determined for the sample from

the subject to the numeric value of a previously analysed reference sample. The advantage of this is not having to duplicate the analysis by determining concentrations in individual reference samples in parallel each time a sample from a subject is analysed.

Suitably the reference standard is matched to the subject being analysed e.g. by gender e.g. by age e.g. by ethnic background or other such criteria which are well known in the art. The reference standard may be a number such as an absolute concentration drawn up by one or more previous studies.

Reference standards may suitably be matched to specific patient sub-groups e.g. elderly subjects, or those with a previous relevant history such as a predisposition to stroke or having experienced one or more stroke(s) earlier in life.

Suitably the reference standard is matched to the sample type being analysed. For example the concentration of the biomarker polypeptide(s) being assayed may vary depending on the type or nature of the sample. It will be immediately apparent to the skilled worker that the concentration value(s) for the reference standard should be for the same or a comparable sample to that being tested in the method(s) of the invention. For example, if the sample being assayed is blood then the reference standard value should be for blood to ensure that it is capable of meaningful cross-comparison and therefore a meaningful quantitative ratio being calculated. In particular, extreme care must be taken if inferences are attempted by comparison between concentrations determined for a subject of interest and concentrations determined for reference standards where the nature of the sample is non-identical between the two. Suitably the sample type for the reference standard and the sample type for the subject of interest are the same.

It should be noted that for some embodiments of the invention, the polypeptide concentrations determined may be compared to a previous sample from the same subject. This can be beneficial in monitoring the progress of brain damage in a subject. This can be beneficial in monitoring the course and/or effectiveness of a treatment of a subject. In this embodiment the method may comprise further step(s) of comparing the quantitative ratio(s) determined for the sample of interest to one or more quantitative ratio(s) determined for the same polypeptide(s) from different samples such as samples taken at different time points for the same subject. By making such a comparison, information can be gathered about whether a particular polypeptide marker is increasing or decreasing in a particular subject. This information may be useful in

diagnosing or predicting changes over time, or changes inhibited or stimulated by a particular treatment or therapy regime, or any other variable of interest. Thus if a polypeptide biomarker of acute brain damage is elevated, or elevated further, in a sample from a later time point from the same subject then this indicates a likelihood of brain damage progressing or worsening in said subject. Equally, if a polypeptide biomarker of acute brain damage is decreased in a sample from a later time point from the same subject then this indicates a likelihood of improvement or lessening of acute brain damage in said subject. Clearly if these effects are observed in a subject undergoing treatment for the brain damage, then corresponding inferences regarding the effectiveness of the treatment may equally be drawn according to the present invention. In other words, when a subject is undergoing treatment, if a polypeptide biomarker of acute brain damage is decreased in a sample from a later time point from the same subject then this indicates a likelihood that the treatment is effective; if a polypeptide biomarker of acute brain damage is elevated, or elevated further, in a sample from a later time point from the same subject then this indicates a likelihood that the treatment is ineffective.

In this way, the invention can be used to determine whether, for example after treatment of the patient with a drug or candidate drug, the disease has progressed or not, or that the rate of disease progression has been modified. The result can lead to a prognosis of the outcome of the disease.

Combinations

The invention may be applied as part of a panel of biomarkers in order to provide a more robust diagnosis or prognosis. Moreover, the invention may be applied as part of a panel of biomarkers in order to provide a more complete picture of the disease state or possible outcomes for a given patient.

Of course, the skilled reader will appreciate that the specific biomarkers of the present invention may be advantageously combined with other markers known in the art. Such extended groups which comprise the specific biomarkers or panels of biomarkers discussed herein are of course intended to be embraced by the invention. Selection of further known markers for testing in such an embodiment may be accomplished by the skilled reader according to the appropriate sources. In this context additional biomarkers may relate to stroke, to other acute brain damage disorders from which a differential diagnosis of stroke is required, or to other diseases commonly associated with patients with stroke or whose symptoms mimic those of stroke.

Suitably said subject is a human.

Suitably said subject is a non-human mammal.

Suitably said subject is a rodent.

Positional Information

Marker polypeptides of the present invention may show a gradient of concentration in microdialysis fluids directly related to their proximity to the site of brain injury or insult. It should be noted that for some embodiments of the invention, the polypeptide concentrations determined may be compared from different regions of the brain. In particular the polypeptide concentrations in a brain region immediately adjacent to the site of insult or injury may be compared to more distal regions within the same brain hemisphere and/or with the unaffected contralateral hemisphere.

More suitably where the type of brain injury is ischaemic stroke the adjacent region is the infarct core and the more distant region within the same hemisphere is the penumbra.

Detection

A marker protein may have its expression modulated, i.e. quantitatively increased or decreased, in patients with acute brain damage such as stroke. The degree to which expression differs in normal versus affected states need only be large enough to be visualised via standard characterisation techniques, such as silver staining of 2D-electrophoretic gels, measurement of representative peptide ions using isobaric mass tagging and mass spectrometry or immunological detection methods including Western blotting, enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay. Other such standard characterisation techniques by which expression differences may be visualised are well known to those skilled in the art. These include successive chromatographic separations of fractions and comparisons of the peaks, capillary electrophoresis, separations using micro-channel networks, including on a micro-chip, and mass spectrometry methods including multiple reaction monitoring (MRM) and TMTcalibrator (Dayon et al 2009).

The extent to which the protein level is modulated will typically vary in inverse relationship to the distance from the site of brain damage. In the case of brain microdialysates the modulations seen will be relatively large and typically a ratio >2 is

indicative of a disease-related change in expression. In more distal sites such as cerebrospinal fluid and/or plasma the extent of modulation (changes in concentration of protein detected) may be lower than in brain microdialysates yet still provide diagnostically or prognostically useful information. In such materials (e.g. cerebrospinal fluid and/or plasma) typically a ratio >1.3 would be considered representative of brain damage.

Chromatographic separations can be carried out by high performance liquid chromatography as described in Pharmacia literature, the chromatogram being obtained in the form of a plot of absorbance of light at 280 nm against time of separation. The material giving incompletely resolved peaks is then re-chromatographed and so on.

Capillary electrophoresis is a technique described in many publications, for example in the literature "Total CE Solutions" supplied by Beckman with their P/ACE 5000 system. The technique depends on applying an electric potential across the sample contained in a small capillary tube. The tube has a charged surface, such as negatively charged silicate glass. Oppositely charged ions (in this instance, positive ions) are attracted to the surface and then migrate to the appropriate electrode of the same polarity as the surface (in this instance, the cathode). In this electroosmotic flow (EOF) of the sample, the positive ions move fastest, followed by uncharged material and negatively charged ions. Thus, proteins are separated essentially according to charge on them.

Micro-channel networks function somewhat like capillaries and can be formed by photoablation of a polymeric material. In this technique, a UV laser is used to generate high energy light pulses that are fired in bursts onto polymers having suitable UV absorption characteristics, for example polyethylene terephthalate or polycarbonate. The incident photons break chemical bonds with a confined space, leading to a rise in internal pressure, mini-explosions and ejection of the ablated material, leaving behind voids which form micro-channels. The micro-channel material achieves a separation based on EOF, as for capillary electrophoresis. It is adaptable to micro-chip form, each chip having its own sample injector, separation column and electrochemical detector: see J.S.Rossier et al., 1999, Electrophoresis 20: pages 727-731.

Other methods include performing a binding assay for the marker protein. Any reasonably specific binding agent can be used. Preferably the binding agent is labelled. Preferably the assay is an immunoassay, especially between the biomarker and an antibody that recognises the protein, especially a labelled antibody. It can be

an antibody raised against part or all of the marker protein, for example a monoclonal antibody or a polyclonal anti-human antiserum of high specificity for the marker protein.

Where the binding assay is an immunoassay, it may be carried out by measuring the extent of the protein/antibody interaction. Any known method of immunoassay may be used. A sandwich assay is preferred. In an exemplary sandwich assay, a first antibody to the marker protein is bound to the solid phase such as a well of a plastics microtitre plate, and incubated with the sample and with a labelled second antibody specific to the protein to be assayed. Alternatively, an antibody capture assay can be used. Here, the test sample is allowed to bind to a solid phase, and the anti-marker protein antibody is then added and allowed to bind. After washing away unbound material, the amount of antibody bound to the solid phase is determined using a labelled second antibody, anti- to the first.

In another embodiment, a competition assay is performed between the sample and a labelled marker protein or a peptide derived therefrom, these two antigens being in competition for a limited amount of anti-marker protein antibody bound to a solid support. The labelled marker protein or peptide thereof can be pre-incubated with the antibody on the solid phase, whereby the marker protein in the sample displaces part of the marker protein or peptide thereof bound to the antibody.

In yet another embodiment, the two antigens are allowed to compete in a single co-incubation with the antibody. After removal of unbound antigen from the support by washing, the amount of label attached to the support is determined and the amount of protein in the sample is measured by reference to standard titration curves established previously.

The binding agent in the binding assay may be a labelled specific binding agent, which may be an antibody or other specific binding agent. The binding agent will usually be labelled itself, but alternatively it may be detected by a secondary reaction in which a signal is generated, e.g. from another labelled substance.

The label may be an enzyme. The substrate for the enzyme may be, for example, colour-forming, fluorescent or chemiluminescent.

An amplified form of assay may be used, whereby an enhanced "signal" is produced from a relatively low level of protein to be detected. One particular form of amplified

immunoassay is enhanced chemiluminescent assay. Conveniently, the antibody is labelled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

Another form of amplified immunoassay is immuno-PCR. In this technique, the antibody is covalently linked to a molecule of arbitrary DNA comprising PCR primers, whereby the DNA with the antibody attached to it is amplified by the polymerase chain reaction. See E. R. Hendrickson et al., Nucleic Acids Research 23: 522-529 (1995). The signal is read out as before.

The time required for the assay may be reduced by use of a rapid microparticle-enhanced turbidimetric immunoassay such as the type embodied by M. Robers et al., "Development of a rapid microparticle-enhanced turbidimetric immunoassay for plasma fatty acid-binding protein, an early marker of acute myocardial infarction", Clin. Chem. 1998;44:1564-1567.

The full automation of any immunoassay contemplated in a widely used clinical chemistry analyser such as the COBAS™ MIRA Plus system from Hoffmann-La Roche, described by M.Robers et al. supra, or the AxSYM™ system from Abbott Laboratories, should be possible and applied for routine clinical diagnosis.

It is also contemplated within the invention to use (i) an antibody array or 'chip', or a bead suspension array capable of detecting one or more proteins that interact with that antibody.

An antibody chip, antibody array or antibody microarray is an array of unique addressable elements on a continuous solid surface whereby at each unique addressable element an antibody with defined specificity for an antigen is immobilised in a manner allowing its subsequent capture of the target antigen and subsequent detection of the extent of such binding. Each unique addressable element is spaced from all other unique addressable elements on the solid surface so that the binding and detection of specific antigens does not interfere with any adjacent such unique addressable element.

A "bead suspension array" is an aqueous suspension of one or more identifiably distinct particles whereby each particle contains coding features relating to its size and colour

or fluorescent signature and to which all of the beads of a particular combination of such coding features is coated with an antibody with a defined specificity for an antigen in a manner allowing its subsequent capture of the target antigen and subsequent detection of the extent of such binding. Examples of such arrays can be found at www.luminexcorp.com where application of the xMAP® bead suspension array on the Luminex® 100™ System is described.

Alternatively, the diagnostic sample can be subjected to isobaric mass tagging and LC-MS/MS as described herein. An example of preferred ways of carrying out isobaric protein tagging are set out in the examples section of this application.

Isobaric protein tagging using tandem mass tags has been shown before to be able to determine relative proteins levels in a highly accurate manner (Thompson et al., 2003, Dayon et al., 2008). In addition, numerous reports have been published in the last few years using iTRAQ for protein tagging in various tissues and fluids (Aggarwal et al., 2006). Especially for the discovery of biomarkers in various conditions, iTRAQ has been proved to be a highly suitable tool and has been used in cancer (Maurya et al., 2007, Garbis et al., 2008, Matta et al., 2008, Ralhan et al., 2008) and diabetes research (Lu et al., 2008) as well as in the quest for biomarkers in neurodegenerative disorders (Abdi et al., 2006) albeit in CSF.

Multiple Selected Reaction Monitoring (SRM or MRM)

MRM/SRM is the scan type with the highest duty cycle and is used for monitoring one or more specific ion transition(s) at high sensitivity. Here, Q1 is set on the specific parent m/z (Q1 is not scanning), the collision energy is set to produce the optimal diagnostic charged fragment of that parent ion, and Q3 is set to the specific m/z of that fragment. Only ions with this exact transition will be detected. Historically used to quantify small molecules such as drug metabolites, the same principle can be applied to peptides, either endogenous moieties or those produced from enzymatic digestion of proteins. Again historically experiments were performed using triple quadrupole mass spectrometers but the recent introduction of hybrid instrument designs, which combine quadrupoles with ion traps, enables similar and improved experiments to be undertaken. The 4000QTRAP instrument therefore allows peptide and biomolecule quantitation to be performed at very high specificity and sensitivity using Multiple Reaction Monitoring (MRM). This is largely due to the use of the LINAC® Collision Cell, which subsequently enables many MRM scans to be looped together into one experiment to detect the presence of many specific ions (up to 100 different ions) in a

complex mixture. Consequently it is now feasible to measure and quantify multiple peptides from many proteins in a single chromatographic separation. The area under the MRM LC peak is used to quantitate the amount of the analyte present. In a typical quantitation experiment, a standard concentration curve is generated for the analyte of interest. When the unknown sample is then run under identical conditions, the concentration for the analyte in the unknown sample can be determined using the peak area and the standard concentration curve.

The diagnostic sample can be subjected to analysis by MRM on an ion-trap mass spectrometer. Based on the mass spectrometry profiles of the marker proteins described below single tryptic peptides with specific known mass and amino acid sequences are identified that possess good ionising characteristics. The mass spectrometer is then programmed to specifically survey for peptides of the specific mass and sequence and report their relative signal intensity. Using MRM it is possible to survey for up to 5, 10, 15, 20, 25, 30, 40, 50 or 100 different marker proteins in a single LC-MS run. The intensities of the MRM peptides of the specific biomarkers of the present invention in the diagnostic sample are compared with those found in samples from subjects without disease allowing the diagnosis or prognosis to be made.

The MRM assay can be made more truly quantitative by the use of internal reference standards consisting of synthetic absolute quantification (AQUA) peptides corresponding to the MRM peptide of the marker protein wherein one or more atoms have been substituted with a stable isotope such as carbon-13 or nitrogen-15 and wherein such substitutions cause the AQUA peptide to have a defined mass difference to the native, lighter form of the MRM peptide derived from the diagnostic sample. By comparing the relative ion intensity of the native MRM and AQUA peptides the true concentration of the parent protein in the diagnostic sample can thus be determined. General methods of absolute quantitation by such isotope dilution methods are provided in Gerber, Scott A, et al. "Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS" PNAS, June 10, 2003. Vol 100. No 12. p 6940-6945.

In some cases, whilst it is desirable to use isotope-doped standards to provide absolute quantitation in an SRM experiment it is not possible to use the AQUA approach described above. In such cases it is possible to use a pair of isotopic mass tags i.e. two tags with identical chemical structure but different levels of isotopic substitutions giving each a unique mass. Using two forms of the Tandem Mass Tags® (TMT®)that differ in mass by 5 Da it is possible to label standard synthetic reference SRM peptides with a

light tag prior to mixing to form a universal reference for all targeted peptides in an assay. Each patient sample is then subjected to trypsin digestion and the resulting peptides labelled with the heavy TMT tag. An aliquot of the TMT-labelled reference peptides is then added to the sample to give a final concentration of reference peptides that is relevant to the target range to be measured in the patient sample. The spiked sample is then subjected to a standard isotope dilution SRM assay and the concentrations of the SRM peptides from the patient sample are calculated by comparing ion intensites of the heavy form against those of the known concentrations of the lighter form.

An alternative form of MS-based assay for the relative or absolute quantitation of regulated peptides identified as biomarker candidates is the TMTcalibrator method developed by Proteome Sciences plc, Known amounts of synthetic peptides representing tryptic fragments of the candidate biomarker(s) with good MS/MS behaviour are labelled with four of the six reagents of the TMT6 set of isobaric mass tags (TMT6-128 to TMT6-131) and mixed in certain ratios. This allows a multi-point calibration curve reflecting physiological and/or disease-modified concentrations to be designed and implemented quickly. Subsequently, a diagnostic sample taken from a patient suffering from or suspected of suffering from acute brain injury such as stroke is labelled with TMT6-126 and the calibration mix is added to the study sample. During MS/MS of individual peptides, the TMT6-reporter ions of the calibrant peptides are produced and used to establish a calibration curve. The absolute amount of the peptide in the study sample is then readily derived by reading the TMT6126 ion intensity against the calibration curve. Further information on TMTcalibrator assays can be obtained from the Proteome Sciences website (www.proteomics.com).

A preferred method of diagnosis comprises performing a binding assay for the marker protein. Any reasonably specific binding partner can be used. Preferably the binding partner is labelled. Preferably the assay is an immunoassay, especially between the marker and an antibody that recognises the protein, especially a labelled antibody. It can be an antibody raised against part or all of it, most preferably a monoclonal antibody or a polyclonal anti-human antiserum of high specificity for the marker protein.

Thus, the marker proteins described above are useful for the purpose of raising antibodies thereto which can be used to detect the increased or decreased concentration of the marker proteins present in a diagnostic sample. Such antibodies can be raised by any of the methods well known in the immunodiagnostics field.

The antibodies may be anti- to any biologically relevant state of the protein. Thus, for example, they can be raised against the unglycosylated form of a protein which exists in the body in a glycosylated form, against a more mature form of a precursor protein, e.g. minus its signal sequence, or against a peptide carrying a relevant epitope of the marker protein.

The sample can be taken from any valid body tissue, especially body fluid, of a mammalian or non-mammalian subject, but preferably blood, plasma, serum or urine. Other usable body fluids include cerebrospinal fluid (CSF), semen and tears. Preferably the subject is a mammalian species such as a mouse, rat, guinea pig, dog or primate. Most preferably the subject is human.

The preferred immunoassay is carried out by measuring the extent of the protein/antibody interaction. Any known method of immunoassay may be used. A sandwich assay is preferred. In this method, a first antibody to the marker protein is bound to the solid phase such as a well of a plastic microtitre plate, and incubated with the sample and with a labelled second antibody specific to the protein to be assayed. Alternatively, an antibody capture assay can be used. Here, the test sample is allowed to bind to a solid phase, and the anti-marker protein antibody is then added and allowed to bind. After washing away unbound material, the amount of antibody bound to the solid phase is determined using a labelled second antibody, anti- to the first.

In another embodiment, a competition assay is performed between the sample and a labelled marker protein or a peptide derived therefrom, these two antigens being in competition for a limited amount of anti-marker protein antibody bound to a solid support. The labelled marker protein or peptide thereof can be pre-incubated with the antibody on the solid phase, whereby the marker protein in the sample displaces part of the marker protein or peptide thereof bound to the antibody.

In yet another embodiment, the two antigens are allowed to compete in a single co-incubation with the antibody. After removal of unbound antigen from the support by washing, the amount of label attached to the support is determined and the amount of protein in the sample is measured by reference to standard titration curves established previously.

The label is preferably an enzyme. The substrate for the enzyme may be, for example, colour-forming, fluorescent or chemiluminescent.

The binding partner in the binding assay is preferably a labelled specific binding partner, but not necessarily an antibody. The binding partner will usually be labelled itself, but alternatively it may be detected by a secondary reaction in which a signal is generated, e.g. from another labelled substance.

It is highly preferable to use an amplified form of assay, whereby an enhanced "signal" is produced from a relatively low level of protein to be detected. One particular form of amplified immunoassay is enhanced chemiluminescent assay. Conveniently, the antibody is labelled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

The use of a rapid microparticle-enhanced turbidimetric immunoassay such as the type embodied by M. Robers et al., "Development of a rapid microparticle-enhanced turbidimetric immunoassay for plasma fatty acid-binding protein, an early marker of acute myocardial infarction", Clin. Chem. 1998;44:1564-1567, significantly decreases the time of the assay. Thus, the full automation of any immunoassay contemplated in a widely used clinical chemistry analyser such as the COBAS™ MIRA Plus system from Hoffmann-La Roche, described by M.Robers et al. supra, or the AxSYM™ system from Abbott Laboratories, should be possible and applied for routine clinical diagnosis.

Alternatively, the diagnostic sample can be subjected to two dimensional gel electrophoresis to yield a stained gel in which the position of the marker proteins is known and the relative intensity of staining at the appropriate spots on the gel can be determined by densitometry and compared with a corresponding control or comparative gel.

In a yet further embodiment the diagnostic sample can be subjected to analysis by a mass-spectrometer-based assay such as multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer or on certain types of ion-trap mass spectrometer. For each differentially expressed protein it is possible to identify a set of tryptic peptides with specific known mass (parent mass) and amino acid sequence and which upon fragmentation release fragments of specific mass (fragment mass) that are unique to

each protein. The detection of a fragment mass from a defined parent mass ion is known as a transition.

Identification of such proteotypic peptides can be made based on the mass spectrometry profiles of the differentially expressed proteins seen during biomarker discovery, or may be designed in silico using predictive algorithms known to the skilled practitioner. The mass spectrometer is then programmed to specifically survey only for the specific parent mass and fragment mass transitions selected for each protein and reports their relative signal intensity. Using MRM it is possible to survey for up to 5, 10, 15, 20, 25, 30, 40, 50 or 100 different marker proteins in a single LC-MS run. The relative abundances of the proteotypic peptides for each marker protein in the diagnostic sample are compared with those found in samples from subjects without acute brain injury such as stroke allowing the diagnosis to be made. Alternatively comparison may be made with levels of the proteins from earlier samples from the same patient thus allowing prognostic assessment of the stage and/or rate of progression of acute brain injury such as stroke in said patient.

In a further embodiment of the invention the MRM assay can be made more truly quantitative by the use of internal reference standards consisting of synthetic absolute quantification (AQUA) peptides corresponding to the proteotypic peptide of the marker protein wherein one or more atoms have been substituted with a stable isotope such as carbon-13 or nitrogen-15 and wherein such substitutions cause the AQUA peptide to have a defined mass difference to the native proteotypic peptide derived from the diagnostic sample. Once AQUA peptides equivalent to each proteotypic peptide from the differentially expressed biomarkers have been produced, they can be mixed to form a reference standard that is then spiked into the tryptic digest of the patient sample. The combined sample is then subjected to a programmed mass spectrometer-based assay where the intensity of the required transitions from the native and AQUA peptides is detected. By comparing the relative ion intensity of the native peptides from the sample and the spiked AQUA reference peptides the true concentration of the parent protein in the diagnostic sample can thus be determined. General methods of absolute quantitation are provided in Gerber, Scott A, et al. "Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS" PNAS, June 10, 2003. Vol 100. No 12. p 6940-6945 which is incorporated herein by reference.

In a yet further embodiment of the invention an absolute quantitation can be made by using a TMT-SRM assay. Standard synthetic reference SRM peptides corresponding to

the prototypic peptide of the marker protein are labelled with a light TMT tag having no isotope substitutions (light tag) prior to mixing to form a universal reference for all marker proteins in an assay. Each patient sample is then subjected to trypsin digestion and the resulting peptides labelled with the TMT tag having five isotopic substitution (heavy tag). An aliquot of the light TMT-labelled reference peptides is then added to the heavy TMT-labelled sample to give a final concentration of reference peptides that is relevant to the target range to be measured in the patient sample. The spiked sample is then subjected to a standard isotope dilution SRM assay and the concentrations of the SRM peptides from the patient sample are calculated by comparing ion intensities of the heavy form against those of the known concentrations of the lighter form.

The invention further includes the use for a diagnostic (and thus possibly prognostic) or therapeutic purpose of a partner material which recognises, binds to or has affinity for a marker protein specified above. Thus, for example, antibodies to the marker proteins, appropriately humanised where necessary, may be used in treatment. The partner material will usually be an antibody and used in any assay-compatible format, conveniently an immobilised format, e.g. as beads or a chip. Either the partner material will be labelled or it will be capable of interacting with a label.

The invention further includes a kit for use in a method of diagnosis and prognostic monitoring of acute brain injury such as stroke, which comprises a partner material, as described above, in an assay-compatible format, as described above, for interaction with a marker protein present in the diagnostic sample.

It is further contemplated within the invention to use (i) an antibody chip or array of chips, or a bead suspension array capable of detecting one or more proteins differentially expressed in acute brain injury such as stroke.

The method may further comprise determining an effective therapy for treating acute brain injury such as stroke.

In a further aspect, the present invention provides a method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the acute brain injury such as stroke state towards that found in the normal state in order to prevent the development or progression of acute brain injury such as stroke. Preferably, the expression of the protein is restored to that of the normal state.

In a further aspect, the present invention provides a method whereby the pattern of differentially expressed proteins in a tissue sample or body fluid sample of an individual with acute brain injury such as stroke is used to predict the most appropriate and effective therapy to alleviate the acute brain injury such as stroke.

Also provided is a method of screening an agent to determine its usefulness in treating acute brain injury such as stroke, the method comprising:

(a) obtaining a sample of relevant tissue taken from, or representative of, a subject having acute brain injury such as stroke symptoms, who or which has been treated with the agent being screened;

(b) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subject; and,

(c) selecting or rejecting the agent according to the extent to which it changes the expression, activity or amount of the differentially expressed protein or proteins in the treated subject having acute brain injury such as stroke symptoms.

Preferably, the agent is selected if it converts the expression of the differentially expressed protein towards that of a normal subject. More preferably, the agent is selected if it converts the expression of the protein or proteins to that of the normal subject.

Also provided is a method of screening an agent to determine its usefulness in treating acute brain injury such as stroke, the method comprising:

(a) obtaining over time samples of relevant tissue or body fluid taken from, or representative of, a subject having acute brain injury such as stroke symptoms, who or which has been treated with the agent being screened;

(b) determining the presence, absence or degree of expression of a differentially expressed protein or proteins in said samples; and,

(c) determining whether the agent affects the change over time in the expression of the differentially expression protein in the treated subject having acute brain injury such as stroke symptoms.

Samples taken over time may be taken at intervals of weeks, months or years. For example, samples may be taken at monthly, two-monthly, three-monthly, four-monthly, six-monthly, eight-monthly or twelve-monthly intervals.

A change in expression over time may be an increase or decrease in expression, compared to the initial level of expression in samples from the subject and/or compared to the level of expression in samples from normal subjects. The agent is selected if it slows or stops the change of expression over time.

In the screening methods described above, subjects having differential levels of protein expression comprise:

(a) normal subjects and subjects having acute brain injury such as stroke; and,

(b) subjects having acute brain injury such as stroke symptoms which have not been treated with the agent and subjects having acute brain injury such as stroke which have been treated with the agent.

Diagnosis and prognosis

The term "diagnosis", as used herein, includes the provision of any information concerning the existence, non-existence or probability of acute brain injury such as stroke in a patient. It further includes the provision of information concerning the type or classification of the disorder or of symptoms which are or may be experienced in connection with it. It encompasses prognosis of the medical course of the condition. It further encompasses information concerning the age of onset.

Treatment

It will be understood that where treatment is concerned, treatment includes any measure taken by the physician to alleviate the effect of acute brain injury such as stroke on a patient. Thus, although reversal of the damage or elimination of the damage or effects of acute brain injury such as stroke is a desirable goal, effective treatment will also include any measures capable of achieving reduction in the degree of damage or severity of the effects or progression.

In one aspect, the invention provides a method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the acute brain injury such as stroke state towards that found in the normal state in order to prevent the development or progression of acute brain injury such as stroke. Preferably, the expression of the protein is restored to that of the normal state.

In a further aspect, the present invention provides a method whereby the pattern of differentially expressed proteins in a sample from an individual with acute brain injury such as stroke is used to predict the most appropriate and effective therapy to alleviate the neurological damage.

Antibodies

Antibodies against the marker proteins disclosed herein can be produced using known methods. These methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a protein, an antibody specific for the protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with the protein, or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

The antibodies may bind or be raised against any biologically relevant state of the protein. Thus, for example, they can be raised against the unglycosylated form of a protein which exists in the body in a glycosylated form, against a more mature form of a precursor protein, e.g. minus its signal sequence, or against a peptide carrying a relevant epitope of the marker protein.

Antibodies may be polyclonal or monoclonal, and may be multispecific (including bispecific), chimeric or humanised antibodies. Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus, the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Examples of antibody fragments, capable of binding an antigen or other binding partner, are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Antibody fragments, which recognise specific epitopes, may be generated by known techniques. For example, such fragments include, but are not limited to, the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternative, Fab expression libraries may be constructed (Huse, et al., 1989, Science 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a substantially homogenous population of antibodies, i.e. the individual antibodies comprising the population are identical apart from possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies can be produced by the method first described by Kohler and Milstein, Nature, 256:495, 1975 or may be made by recombinant methods, see Cabilly et al, US Patent No. 4,816,567, or Mage and Lamoyi in Monoclonal Antibody Production Techniques and Applications, pages 79-97, Marcel Dekker Inc, New York, 1987.

In the hybridoma method, a mouse or other appropriate host animal is immunised with the antigen by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the nanoparticles used for immunisation. Alternatively, lymphocytes may be immunised in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell, see Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986).

The hybridoma cells thus prepared can be seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine,

aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody producing cells, and are sensitive to a medium such as HAT medium.

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the protein. Preferably, the binding specificity is determined by enzyme-linked immunoabsorbance assay (ELISA). The monoclonal antibodies of the invention are those that specifically bind to the protein.

In a preferred embodiment of the invention, the monoclonal antibody will have an affinity which is greater than micromolar or greater affinity (i.e. an affinity greater than 10-6 mol) as determined, for example, by Scatchard analysis, see Munson & Pollard, Anal. Biochem., 107:220, 1980.

After hybridoma cells are identified that produce neutralising antibodies of the desired specificity and affinity, the clones can be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include Dulbecco's Modified Eagle's Medium or RPM1-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumours in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Nucleic acid encoding the monoclonal antibodies of the invention is readily isolated and sequenced using procedures well known in the art, e.g. by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies. The hybridoma cells of the invention are a preferred source of nucleic acid encoding the antibodies or fragments thereof. Once isolated, the nucleic acid is ligated into expression or cloning vectors, which are then transfected into host cells, which can be cultured so that the monoclonal antibodies are produced in the recombinant host cell culture.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies, humanised antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP 0 184 187 A, GB 2 188 638 A or EP 0 239 400 A. Cloning and expression of chimeric antibodies are described in EP 0 120 694 A and EP 0 125 023 A.

An antibody against a marker protein described herein will bind to said protein. Preferably, said antibody specifically binds said protein. By "specific" is meant that the antibody binds to said protein with an affinity significantly higher than it displays for other molecules.

The term "antibody" includes polyclonal antiserum, monoclonal antibodies, fragments of antibodies such as single chain and Fab fragments, and genetically engineered antibodies. The antibodies may be chimeric or of a single species.

The term "marker protein" or "biomarker" includes all biologically relevant forms of the protein identified, including post-translational modification. For example, the marker protein can be present in the body tissue in a glycosylated, phosphorylated, multimeric or precursor form.

The term "control" refers to a normal human subject, i.e. one not suffering from acute brain injury such as stroke.

The terminology "increased/decreased concentration.. ..compared with a control sample" does not imply that a step of comparing is actually undertaken, since in many cases it will be obvious to the skilled practitioner that the concentration is abnormally high or low. Further, when the stages of acute brain injury such as stroke are being monitored progressively, or when a course of treatment is being monitored, the comparison made can be with the concentration previously seen in the same subject at an earlier stage of progression of the disease, or at an earlier stage of treatment or before treatment has commenced.

The term "valid body tissue" or "relevant tissue" means any tissue in which it may reasonably be expected that a marker protein would accumulate in relation to acute brain injury such as stroke. It may be a cerebrospinal fluid sample or a sample of blood or a blood derivative such as plasma or serum.

The term "antibody array" or "antibody microarray" means an array of unique addressable elements on a continuous solid surface whereby at each unique addressable element an antibody with defined specificity for an antigen is immobilised in a manner allowing its subsequent capture of the target antigen and subsequent detection of the extent of such binding. Each unique addressable element is spaced from all other unique addressable elements on the solid surface so that the binding and detection of specific antigens does not interfere with any adjacent such unique addressable element.

The term "bead suspension array" means an aqueous suspension of one or more identifiably distinct particles whereby each particle contains coding features relating to its size and colour or fluorescent signature and to which all of the beads of a particular combination of such coding features is coated with an antibody with a defined specificity for an antigen in a manner allowing its subsequent capture of the target antigen and subsequent detection of the extent of such binding. Examples of such arrays can be found at www.luminexcorp.com where application of the xMAP® bead suspension array on the Luminex® 100™ System is described.

Mass spectrometry assay" means any quantitative method of mass spectrometery including but not limited to selected reaction monitoring (SRM), multiple reaction monitoring (MRM), absolute quantitation using isotopedoped peptides (AQUA), Tandem Mass Tags with SRM (TMTSRM) and TMTcalibrator.

The term 'mutant' of a biomarker such as a polypeptide biomarker of the invention should have its normal meaning in the art. Mutants are sometimes referred to as 'variants' or 'alleles'. The key is to detect biomarkers as have been set out herein. The biomarkers may possess individual variations in the form of mutations or allelic variants between individuals being studied. Therefore there may be some degree of deviation from the exemplary SEQ ID NOs provided herein. The SEQ ID NOs provided herein are to assist the skilled reader in identifying and working with the polypeptides/biomarkers of the invention and are not intended as a restricted and inflexible definition of the individual polypeptides being assayed. Thus minor sequence differences between the SEQ ID NOs provided and the actual sequences of the polypeptide biomarkers being

detected will be expected within the boundaries of normal variation between subjects. This should not affect the working of the invention.

The term 'comprises' (comprise, comprising) should be understood to have its normal meaning in the art, i.e. that the stated feature or group of features is included, but that the term does not exclude any other stated feature or group of features from also being present.

Fragments/Peptides

It will be appreciated by the skilled worker that the details of the biomarkers discussed herein and in particular the sequences presented for them are given to facilitate their detection. The important information being gathered is the presence or absence (or particular level) of the biomarker in the sample being studied. There is no particular requirement that the full length polypeptide be scored. Indeed, via many of the suitable mass spectrometry based modes of detection set out herein, detection takes place by assaying particular fragments of the polypeptide of interest being present which are thus taken to indicate the presence of the overall biomarker polypeptide in the sample. Therefore the invention embraces the detection of fragments of the polypeptide biomarkers. Moreover, the kits and peptides of the invention may comprise fragments of the polypeptides and need not comprise the full length sequences exemplified herein. Suitably the fragment is sufficiently long to enable its unique identification by mass spectrometry.

Thus a fragment is suitably at least 6 amino acids in length, suitably at least 7 amino acids in length, suitably at least 8 amino acids in length, suitably at least 9 amino acids in length, suitably at least 10 amino acids in length, suitably at least 15 amino acids, suitably at least 25 amino acids, suitably at least 50 amino acids, suitably at least 100 amino acids, or suitably the majority of the biomarker polypeptide of interest. Suitably a fragment comprises a small fragment of the biomarker polypeptide of interest, whilst being long enough to retain an identifiable mass.

For any given polypeptide or set of polypeptides being detected by mass spectrometry based assay, the assay may be conducted via MRM techniques mentioned herein. In this embodiment, certain unique peptides and in particular certain transitions are especially advantageous to detect the peptides of interest. These are typically selected to give the highest representation (or combinations may be used such as any or all peptides giving a particular level of representation if multiple fragments/transitions

give similar levels). Especially preferred transitions used for monitoring are those mentioned in the accompanying examples and/or figures.

Sequence Homology/Identity

Although sequence homology can also be considered in terms of functional similarity (i.e., amino acid residues having similar chemical properties/functions), in the context of the present document it is preferred to express homology in terms of sequence identity.

Sequence comparisons can be conducted by eye or, more usually, with the aid of readily available sequence comparison programs. These publicly and commercially available computer programs can calculate percent homology (such as percent identity) between two or more sequences.

Percent identity may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids). For comparison over longer sequences, gap scoring is used to produce an optimal alignment to accurately reflect identity levels in related sequences having insertion(s) or deletion(s) relative to one another. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA (Altschul et al., 1990, J. Mol. Biol. 215:403-410) and the GENEWORKS suite of comparison tools.

In the context of the present document, a homologous amino acid sequence is taken to include an amino acid sequence which is at least 40, 50, 60, 70, 80 or 90% identical. Most suitably a polypeptide having at least 90% sequence identity to the biomarker of interest will be taken as indicative of the presence of that biomarker; more suitably a polypeptide which is 95% or more suitably 98% identical at the amino acid level will be taken to indicate presence of that biomarker. Suitably said comparison is made over at least the length of the polypeptide or fragment which is being assayed to determine the presence or absence of the biomarker of interest. Most suitably the comparison is made across the full length of the polypeptide of interest. The same considerations apply to nucleic acid nucleotide sequences.

Alternate Methods

It will be understood by the skilled reader that specific techniques exemplified herein may be varied if desired using readily available alternatives to achieve the same effect. For example, assay of the biomarker levels in a blood sample may be carried out by western blot or by isobaric protein tagging or by ELISA or by any other suitable means known in the art.

Quantitative Ratios

It will be appreciated that there are a number of biomarkers disclosed herein which are significantly decreased in subjects having suffered acute brain damage such as stroke. These are scientifically equally valid as is discussed in the accompanying examples section. However, in practical terms it is more technically challenging to determine an absence or decrease in a particular biomarker in a sample being analysed. In particular it is difficult to control for the genuine detection of a decreased amount of a marker versus a problem in detection. For this reason, in preferred embodiments of the invention the biomarkers used are those which are elevated or increased in acute brain damage such as stroke. These have the advantage that positive identification of the biomarker(s) of interest can positively aid diagnosis.

Thus is should be noted that the quantitative ratios determined herein describe the ratio of the concentration in the sample of the subject being analysed to the concentration in the reference standard. Thus a ratio of 1.3 is achieved when the concentration in the sample is 1.3 times the concentration in the reference standard. Clearly the ratios could be expressed in another manner (e.g. in reverse) but for consistency the ratios are discussed herein as sample:standard such that a ratio of 1.3 means a concentration in the sample being 30% greater than that of the concentration in the standard.

Biomarkers

There are advantages to using more than one biomarker in the methods of the invention. The advantages include increased specificity and/or sensitivity to the methods of the invention. We present panels of biomarkers which are particularly advantageous in the method(s) of the invention.

GSTP-1 and Peroxiredoxins 1 & 6 represent useful markers for management of stroke. For the reasons noted above, we also present larger panels of proteins. These panels have technical advantages such as further improving diagnostic sensitivity and/or specificity.

Certain panels disclosed also have the advantage of providing prognostic information. Accordingly the inventors performed a review of literature relating to stroke and cardiovascular biomarkers and pathway analysis for all 53 proteins found differentially expressed in infarct and penumbra compared to contralateral brain microdialysates. Following this comprehensive bioinformatic approach three groups of biomarkers were selected, Panel A, Panel B and Panel C. These are shown below in descending priority order.

PANEL A	ID	Description
N°1	ACBP_HUMAN	Acyl-CoA-binding protein
N°2	CSRP1_HUMAN	Cysteine and glycine-rich protein 1
N°3	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1
N°4	DDAH1_HUMAN	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
N°5	MT3_HUMAN	Metallothionein-3 (MT-3)
N°6	CYTB_HUMAN	Cystatin-B

PANEL B	ID	Description
N°1	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A
N°2	NFM_HUMAN	Neurofilament medium polypeptide
N°3	UBIQ_HUMAN	Ubiquitin.
N°4	B2MG_HUMAN	Beta-2-microglobulin precursor
N°5	CYTC_HUMAN	Cystatin-C precursor (Cystatin-3)
N°6	SH3L1_HUMAN	SH3 domain-binding glutamic acid-rich-like protein.
N°7	TPIS_HUMAN	Triosephosphate isomerase
N°8	MBP_HUMAN	Myelin basic protein (MBP)
N°9	MT2_HUMAN	Metallothionein-2 (MT-2)

PANEL C	ID	Description
N°1	NFM_HUMAN	Neurofilament medium polypeptide
N°2	COTL1_HUMAN	Coactosin-like protein.
N°3	THY1_HUMAN	Thy-1 membrane glycoprotein precursor
N°4	PROF1_HUMAN	Profilin-1
N°5	TYB4_HUMAN	Thymosin beta-4
N°6	MT1E_HUMAN	Metallothionein-1E
N°7	FABPB_HUMAN	Fatty acid-binding protein, brain (B-FABP)

N°8	GFAP_HUMAN	Glial fibrillary acidic protein (GFAP).
N°9	CAH2_HUMAN	Carbonic anhydrase 2
N°10	CERU_HUMAN	Ceruloplasmin precursor
N°11	DCD_HUMAN	Dermcidin precursor
N°12	DEF1_HUMAN	Neutrophil defensin 1 precursor (HNP-1

In some embodiments, Panels A, B and C may be considered as a single cohesive group of biomarkers which may be referred to as the enlarged panel ABC.

In addition to the defined panels A-C larger panels of biomarker proteins can be used in the method of the invention.

Panel 1

Biomarker Polypeptide	Further Details
Acyl-CoA-binding protein Apolipoprotein A-II precursor Apolipoprotein A-IV precursor Carbonic anhydrase 1 Carbonic anhydrase 2 Chitinase-3-like protein 1 precursor Cofilin-1 Cystatin-B Fibrinogen alpha chain precursor Flavin reductase Glial fibrillary acidic protein Hemoglobin subunit alpha Histone H1.2 Histone H1.5 Lysozyme C precursor N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 Neurofilament medium polypeptide Neutrophil defensin 1 precursor Peptidyl-prolyl cis-trans isomerase A Phosphatidylethanolamine-binding protein 1 Thymosin beta-10 Thymosin beta-4 Triosephosphate isomerase Tropomyosin alpha-3 chain	IC v CT Panel 1A
Acyl-CoA-binding protein Beta-2-microglobulin precursor Coactosin-like protein Complement C4-A precursor Cystatin-B Cysteine and glycine-rich protein 1 Fatty acid-binding protein, brain Fibrinogen alpha chain precursor Glutathione S-transferase P Heterogeneous nuclear ribonucleoprotein G	IC v P Panel 1B

Metallothionein-3 Myelin basic protein [ISOFORM 3] Neutrophil defensin 1 precursor Paralemmin Peptidyl-prolyl cis-trans isomerase A Peroxiredoxin-2 Peroxiredoxin-6 Phosphatidylethanolamine-binding protein 1 Plasma retinol-binding protein precursor Plasminogen precursor Platelet basic protein precursor Profilin-1 SH3 domain-binding glutamic acid-rich-like protein Thioredoxin Ubiquitin	
Aquaporin-4 Coactosin-like protein Cystatin-B Cysteine and glycine-rich protein 1 Diazepam binding inhibitor, splice form 1c Fibrinogen alpha chain precursor Glial fibrillary acidic protein Hydroxyacylglutathione hydrolase Kininogen-1 precursor Lysozyme C precursor Metallothionein-2 Metallothionein-3 Myoglobin N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 Neurofilament medium polypeptide Peptidyl-prolyl cis-trans isomerase A Phosphatidylethanolamine-binding protein 1 Plasminogen precursor Platelet basic protein precursor Profilin-1 Prothrombin precursor SH3 domain-binding glutamic acid-rich-like protein Spectrin beta chain, brain 1 Stathmin Ubiquitin Ubiquitin carboxyl-terminal hydrolase isozyme L1	P v CT Panel 1C

An advantage of the markers in Panel 1 is that they are all increased in an affected subject. In other words, an increase in the level of such biomarker(s) is indicative of an increased likelihood of acute brain damage. This facilitates positive detection and helps to eliminate potential problems arising from false negatives due to technical problems of detection being mistaken for an indication that particular biomarker is

decreased in a subject. In particular, the markers in Panel 1 share the advantage that the quantitative ratio for said polypeptides is each above 1.3. This is evidenced in the examples section. This has the advantage of providing statistically significant confidence in each marker used from this panel in a method according to the present invention.

Panel 1 also defines subgroups of markers according to the particular type of analysis in which their statistically significant increased expression was detected. Thus the designations "IC vs CT", "IC vs P" and "P vs CT" in the 'further details' column provide three further sub-groups of markers:

Panel 1A - "IC vs CT"

Panel 1B - "IC vs P"

Panel 1C - "P vs CT"

Panel 1 also defines subgroups of markers which are found to be elevated to a statistically significant level in more than one type of analysis. Thus, individual biomarkers shown to be underlined are shown to occur at elevated levels in affected subjects in at least two of the three types of analysis undertaken ("IC vs CT", "IC vs P" and "P vs CT"). Moreover, there are a smaller number of markers which are shown to occur at elevated levels in affected subjects in all three of the three types of analysis undertaken ("IC vs CT" and "IC vs P" and "P vs CT"). These may be easily identified by comparing the underlined biomarkers in the three treatments and noting those which occur in each of those three treatments in Panel 1 above. Thus, four further subgroups of marker are defined ([Panel 1D - "IC vs CT" and "IC vs P"]; [Panel 1E "IC vs CT" and "P vs CT"]; [Panel 1F "IC vs P" and "P vs CT"]; [Panel 1G "IC vs CT" and "IC vs P" and "P vs CT"]).

Panel 1 also defines a further subgroup which can be described as "X vs CT" where X is P or IC. In other words, this subgroup comprises any marker which is in either IC vs CT (Panel 1A) or P vs CT (Panel 1C) (or both). Thus Panel 1H is defined as "any vs CT". This has the advantage of collating all markers which show an increase in an affected sample compared to the control.

Panel 2

Acyl-CoA-binding protein	Apolipoprotein A-II precursor	Apolipoprotein A-IV precursor	Aquaporin-4
Beta-2-microglobulin precursor	Carbonic anhydrase 2	Chitinase-3-like protein 1 precursor	Coactosin-like protein

Cofilin-1	Complement C4-A precursor	Cystatin-B	Cysteine and glycine-rich protein 1
Diazepam binding inhibitor, splice form 1c	Fibrinogen alpha chain precursor	Flavin reductase	Hemoglobin subunit alpha
Heterogeneous nuclear ribonucleoprotein G	Histone H1.2	Histone H1.5	Hydroxyacylglutathione hydrolase
Kininogen-1 precursor	Lysozyme C precursor	Metallothionein-2	Metallothionein-3
Myelin basic protein [ISOFORM 3]	Myoglobin	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	Neurofilament medium polypeptide
Neutrophil defensin 1 precursor	Paralemmin	Peptidyl-prolyl cis-trans isomerase A	Peroxiredoxin-2
Phosphatidylethanolamine-binding protein 1	Plasma retinol-binding protein precursor	Plasminogen precursor	Platelet basic protein precursor
Profilin-1	Prothrombin precursor	SH3 domain-binding glutamic acid-rich-like protein	Stathmin
Thymosin beta-10	Thymosin beta-4	Triosephosphate isomerase	Ubiquitin
Ubiquitin carboxyl-terminal hydrolase isozyme L1			

Panel 2 presents biomarker polypeptides which are disclosed herein for the first time to have a connection to any kind of brain damage, particularly to acute brain damage such as stroke. Thus it is an advantage of individual markers of panel 2 that they are disclosed for the first time in connection with brain damage.

Panel 2A

Acyl-CoA-binding protein	Coactosin-like protein	Cystatin-B	Cysteine and glycine-rich protein 1

Fibrinogen alpha chain precursor	Lysozyme C precursor	Metallothionein-3	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
Neurofilament medium polypeptide	Neutrophil defensin 1 precursor	Peptidyl-prolyl cis-trans isomerase A	Phosphatidylethanolamine-binding protein 1
Plasminogen precursor	Platelet basic protein precursor	Profilin-1	SH3 domain-binding glutamic acid-rich-like protein
Ubiquitin			

Panel 2A biomarkers are a sub-group of Panel 2 and have the further property that they are increased in at least two out of the three microdialysis studies (IC:P, IC:CT and P:CT) presented in the examples section, suggesting an association with the site of brain damage.

Panel 2B

Cystatin-B	Fibrinogen alpha chain precursor	Peptidyl-prolyl cis-trans isomerase A	Phosphatidylethanolamine-binding protein 1

Panel 2B biomarkers are a sub-group of Panel 2A and have the further property that they are increased in each of the three microdialysis studies (IC:P, IC:CT and P:CT) presented in the examples section, representing a close association with the site of brain damage.

Numerous markers are demonstrated herein such as in the examples section. Some markers show strong associations in more than one patient/experiment in the tables of data and figures. Those markers showing associations for two or more patients/exp.'s in herein are preferred.

References to Metallothionein-1E (MT1E_HUMAN) suitably refer to the protein having the sequence of accession number P04732.

Definitions

The term 'comprises' (comprise, comprising) should be understood to have its normal meaning in the art, i.e. that the stated feature or group of features is included, but that the term does not exclude any other stated feature or group of features from also being present.

The following abbreviations may be used herein: 1-D PAGE, one-dimensional polyacrylamide gel electrophoresis; CT, contralateral; CSF, cerebrospinal fluid; ECF, extracellular fluid; ELISA, enzyme-linked immunosorbent assay; GSTP1; glutathione S-transferase P; IC, infarct core; HUG, Geneva University Hospitals; IEF, isoelectric focusing; LACB, β-lactoglobulin; MALDI, matrix-assisted laser desorption ionization; MCA, middle cerebral artery; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PRDX, peroxiredoxin; P, penumbra; RP-LC, reversed-phase liquid chromatography; SAH, subarachnoid hemorrhage; S100B, protein S100-B; TBI, traumatic brain injury; TMT, tandem mass tag; TMT2, duplex TMT; TMT6, sixplex TMT; TOF/TOF, tandem time-of-flight. Exploring brain microdialysates of stroke patients with ms/ms-based quantitative proteomics is described.

Brief Description of the Figures

Figure 1: Gel images of 4 of the microdialysis samples under study (i.e., CTd, ICd, Pe, and CTe) after separation with 1-D PAGE (home-made 15% Tris-glycine gels) and silver staining. Ten µL of each microdialysate was loaded on the gels.

Figure 2: Immunoblot validation of increased level of GSTP1 in IC with respect to CT microdialysates. Pooled microdialysis samples (n = 3; i.e., ICa-c and CTd-f; 1.5 µg) were separated with 1-D PAGE (home-made 15% Tris-glycine gel). The recombinant GSTP1 (31.25 ng) and post-mortem CSF (10 µL) were taken as a positive control whereas ante-mortem CSF (20 µL) served as a negative control.

Figure 3: ELISA measurement of proteins GSTP1 (a), PRDX1 (b), and S100B (c) in the sera of control and stroke patients.

Figure 4 shows Distribution of relative abundance of TMT2 reporter-ions for Expa-f before and after final normalization steps. Basically, a translation was operated on the relative abundances for both data for reporter-ions at m/z = 126.1 (distribution in red) and 127.1 (distribution in green) in order that the common area between both distribution was maximal (i.e., to minimize the quantitative differences between both populations).

Figure 5 shows Silver-stained 1-D PAGE images of microdialysis samples relative to Expa-c, and Expf. Ten µL of each microdialysate was loaded on home-made 15% Tris-glycine gels. These images were used for designing the TMT2-based quantitative study.

Figure 6 shows Experimental evaluation of the cut-off values to set for the TMT2-based quantitative experiments of the human brain microdialysates. Following the experimental procedure detailed in the article, TMT6 were used to tag identical samples of IC, P, and CT microdialysates. Because no difference was expected between identical samples (e.g., the two IC samples), deviations from 1:1 ratio were evaluated in term of false positive. The mean cut-off values were averaged from the IC,

P, and CT results. To have symmetrical cut-off values at 1% FDR, 1.68 and 0.59 (instead of 0.61) cut-off ratios had to be chosen.

Figure 7 shows bar charts of progression of the levels of several proteins in the MDs. The displayed results correspond to the proteins reported in the corresponding tables for which an evolution could be determined from the ratios obtained by MS.

Figure 8 shows a bar chart of the evolution of the levels of PRDX1 and PRDX6 in the MDs; these trends were determined from the ratios obtained by MS.

Figure 9 shows a diagram of proteomic quantitative workflow used for the analysis of human brain MDs of stroke patients.

Figure 10 shows an example of tandem mass spectrum (a) and tandem mass spectrum zoomed on the TMT reporter-ion area (b) obtained when comparing IC and P MDs.

Figure 11 shows iSRM chromatogram of DENATLDGGDVLFTGR peptide (DDAH1 protein) in human plasma digested with trypsin.

Figure 12 shows iSRM chromatogram of TPEEYPESAK peptide (DDAH1 protein) in human plasma digested with trypsin.

Figure 13 shows iSRM chromatogram of SQVVAGTNYFIK peptide (CYTB protein) in human plasma digested with trypsin.

Figure 14 shows iSRM chromatogram of GYGYGQGAGTLSTDK peptide (CSRP1 protein) in human plasma digested with trypsin.

Figure 15 shows iSRM chromatogram of GLESTTLADK peptide (CSRP1 protein) in human plasma digested with trypsin.

Figure 16 shows iSRM chromatogram of LYEQLSGK peptide (PEBP1 protein) in human plasma digested with trypsin.

The invention is now described by way of example. These examples are intended to be illustrative, and are not intended to limit the appended claims.

Examples

Summary of Examples

In vivo human cerebral microdialysis fluids of stroke patients were investigated for the discovery of potential protein biomarkers associated with cerebrovascular disorders. Microdialysates from the infarct core (IC), the penumbra (P) and the unaffected contralateral (CT) brain regions of patients suffering an ischemic stroke were compared qualitatively and quantitatively using a shotgun proteomic approach. The changes in protein amounts were assessed in several cases; e.g., IC vs. P (n = 2), IC vs. CT (n = 2), and P vs. CT (n = 2). Tandem mass tags (TMTs) were used to label the content of microdialysis fluids after reduction, alkylation and digestion with trypsin. After TMT labeling, the pooled samples were fractionated with off-gel electrophoresis and the

resulting fractions were analyzed with RP-LC MALDI TOF/TOF. One hundred and fifty six proteins were identified in the whole brain microdialysates. MS/MS quantitative analysis showed 43 proteins with increased amounts in the IC with respect to the P and CT samples. Twenty six proteins were increased in the P with respect to the CT. Glutathione S-transferase P (GSTP1), peroxiredoxin-1 (PRDX1) and protein S100-B (S100B) changes were validated with immunoblot on pooled microdialysis samples and/or ELISA on blood of unrelated control and stroke patients (n = 28). In conclusion, the correlation between proteomic quantitative data of the human brain microdialysis and early validations on blood samples from stroke patients demonstrate the value of the methods and biomarker panels described herein.

EXPERIMENTAL PROCEDURES:

Materials

β-Lactoglobulin (LACB) from bovine milk (~ 90%), trypsin from porcine pancreas, iodoacetamide (IAA, ≥ 99%), recombinant GSTP1 (from human, expressed in Escherichia coli) , tris(2-carboxyethyl) phosphine hydrochloride (TCEP) 0.5 M, and a-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Triethylammonium hydrogen carbonate buffer (TEAB) 1 M pH = 8.5, sodium dodecyl sulphate (SDS, ≥ 98%), and trifluoroacetic acid (TFA, ≥ 99.5%) were from Fluka (Büchs, Switzerland). Hydroxylamine solution 50 wt. % in H2O (99.999%) was from Aldrich (Milwaukee, WI, USA). Hydrochloric acid (25%) and ammonium dihydrogen phosphate ((NH4)H2PO4) were from Merck (Darmstadt, Germany). Water for chromatography LiChrosolv® and acetonitrile Chromasolv® for HPLC (≥ 99.9%) were respectively from Merck and Sigma-Aldrich (Büchs, Switzerland). Duplex and sixplex TMTs (TMT2 and TMT6) were provided by Proteome Sciences (Frankfurt am Main, Germany). Oasis® HLB 1 cc (10 and 30 mg) extraction cartridges were from Waters (Milford, MA, USA). ImmobilineTM DryStryp pH 3-10, 13 cm and IPG buffer pH 3-10 were from GE Healthcare (Uppsala, Sweden). Glycerol 50% and mineral oil were from Agilent Technologies (Wilmington, DE, USA).

Sample collection

Microdialysates

Patients with a massive, so-called malignant infarction in the middle cerebral artery (MCA) territory were treated in the Neurointensive Care Unit of Vall d'Hebron University Hospital according to an institutional protocol which combines induced moderate hypothermia (32.5 °C) with decompressive craniotomy. Six malignant MCA infarction patients were included (mean age 50 ± 9.3 years; malignant MCA infarction side: 2 lefts, 4 rights; sex: 4 females, 2 males).

Malignant MCA infraction patients were monitored with high-cut-off (100 kDa) cerebral microdialysis catheters (CMA-71, CMA Microdialysis, Stockholm, Sweden) which were inserted at different brain regions (CT, P, and IC). Computed tomography scan was used to confirm brain microdialysis catheter location. Microdialysate samples were obtained hourly for 5 days after perfusion with an artificial CSF solution (i.e., NaCl 147 mM, KCl 2.7 mM, CaCl2 1.2 mM, and MgCl2 0.85 mM) by using a CMA 106 micropump (CMA microdialysis).

Prior to freezing and storage at –80 °C a routine analysis for glucose, lactate, pyruvate and glycerol/glutamate/urea concentrations in microdialysis samples was performed with the CMA600 analyser (CMA microdialysis). Proteomic analysis was performed on pooled brain microdialysates obtained during the first 24 h of brain monitoring. Table 1 summarizes the patients, the different brain regions sampled and the experimental labels that were used.

Table 1. Overview of the brain microdialysis samples under study.

Experiment	*Patient*	*Infarct Core (IC)*	*Penumbra (P)*	*Contralateral (CT)*
Exp_a	Patient a	IC_a	P_a	
Exp_b	Patient b	IC_b	P_b	
Exp_c	Patient c	IC_c		CT_c
Exp_d	Patient d	IC_d		CT_d
Exp_e	Patient e		P_e	CT_e
Exp_f	Patient f		P_f	CT_f

CSF samples

Ante- and post-mortem CSF collection and clinical data of deceased and living patients have been reported previously (15). Briefly, control ante-mortem CSF samples were collected by routine diagnostic lumbar puncture from living healthy patients. Post-mortem CSF samples were collected by ventricular puncture at autopsy.

Blood samples

The blood samples of control and stroke patients were collected between October 2005 and January 2008 at the Geneva University Hospitals (HUG). During this period, all patients exhibiting unambiguous symptoms and signs of an acute or sub-acute stroke, who were hospitalized at the HUG, were enrolled in the study. Exclusion criteria were defined as follows: (1) a stroke onset time superior to 3 days or occurring after a previous stroke in the preceding 3 months; (2) extra-cerebral hemorrhage or trauma, such SAH, subdural hematoma or traumatic brain injury (TBI); (3) presence of other, potentially confounding pathologies such as cancer, kidney or liver failure, myocardial infarction, and psychiatric conditions. Each patient included in the study underwent a

standardized protocol of clinical and neuroradiological assessments, and therapeutic interventions that was supervised by trained neurologists from the Department of Neurology of the HUG.

Controls were defined as patient's family relatives, as patients suffering from various types of medical and surgical conditions or even from non-cerebrovascular neurological conditions. They were required not to have a past or present history of stroke, cerebrovascular, or thrombotic diseases.

Blood samples were collected according to Standard Operating Procedures (SOP) described by the SOP Internal Working Group (16). Briefly, blood samples were drawn into red top blood collection tubes (silica coated tubes, 6mL, 13* 100mm, ref 368815, BD vacutainers, Plymouth, UK) and kept at room temperature during 45 min to allow the clot to form. No additive (anti-coagulant, protease inhibitor or preservative) was used. At the end of the clotting time, samples were centrifuged (1000 × g for 10 min at room temperature) to discard the cell pellet. Immediately after, each serum sample was aliquoted and stored at -80 °C until use. For the studies reported here, 14 controls and 14 stroke patients, age and gender matched were randomly selected among all the participants collected. Table 2 summarizes the characteristics of the stroke patients and controls.

Table 2. General characteristics of the studied population (blood samples).

	Controls	Stroke
n	14	14
Age (years)		
Mean ± standard deviation	69.3 ± 14.5	69.5 ± 15.6
Median (minimum-maximum)	72.5 (40-88)	72.0 (39-89)
Gender		
Female *n* (%)	5 (25.7)	5 (25.7)
Male *n* (%)	9 (64.3)	9 (64.3)
Time onset of symptoms (min)		
Mean ± standard deviation		486.1 ± 542.8
Median (minimum-maximum)		150 (90-1440)

The local ethical committees approved these studies, and written consent was obtained from patients (or relatives) in accordance with the Helsinki declaration.

SDS PAGE

Ten µL of brain microdialysates was separated with one-dimensional (1-D) SDS polyacrylamide gel electrophoresis (PAGE) on a home-made 15% Tris-glycine gel (8 × 7 × 0.1 cm). Twenty µL of ante- and post-mortem CSF samples (respective concentrations of 172 and 359 µg·mL-1 was determined with the Bradford assay (17)) were also loaded, and taken as controls. Gels were stained with silver nitrate (18). The gel images were

analyzed with the ImageQuant TL software (GE Healthcare). Signal of each lane was integrated and relatively quantified with respect to the other lane signals obtained on the same gel.

Reduction, alkylation, digestion, and TMT labeling

Appropriate volumes of microdialysis samples were taken according to the 1-D PAGE analyses, in order to compare equal protein amounts (i.e., weights) in each quantitative experiment (i.e., Expa-f in Table 1). LACB was spiked in equal quantity in each sample pairs at 1/50 of the expected protein amount (i.e., weight). The 6 × 2 samples were dried.

The samples were dissolved in 100 µL of TEAB 100 mM adjusted to pH = 8 with diluted HCl. One µL of SDS 1% and 2 µL TCEP 50 mM were added to each tube. The reduction was carried out at 60 °C for 1 h. Alkylation was performed (addition of 1 µL of IAA 400 mM) during 30 min in the dark. Ten µL trypsin 0.2 µg · µL-1 freshly prepared in the TEAB solution was added. The digestion was carried out overnight at 37 °C.

TMT2 labeling was achieved for 1 h, after addition of 40.3 µL of TMT2 reagent in CH3CN (i.e. 0.83 mg, 2.42 × 10-6 mol). The tags were used as described in Table 3.

Table 3. Overview of TMT^2 experiments.

Experiment	*Reporter 126.1*	*Reporter 127.1*	*LACB (reporter 126.1)[ς]*	*LACB (reporter 127.1)[ς]*
Exp_a	P_a	IC_a	0.58 ± 0.04	0.42 ± 0.04
Exp_b	IC_b	P_b	0.45 ± 0.07	0.55 ± 0.07
Exp_c	CT_c	IC_c	0.51 ± 0.07	0.49 ± 0.07
Exp_d	IC_d	CT_d	0.45 ± 0.06	0.55 ± 0.06
Exp_e	CT_e	P_e	0.54 ± 0.04	0.46 ± 0.04
Exp_f	P_f	CT_f	0.47 ± 0.08	0.53 ± 0.08

[ς] Normalized mean abundance; the isotopic correction was done. These data were used for subsequent normalization to reduce the manipulation bias.

The quantities of peptides to label varied from a microdialysate sample pair to another because of the different available protein amounts. These quantities were estimated to range from 1.5 to 27 µg according to the used microdialysate volumes and the estimated concentrations determined with respect to ante-mortem CSF (see above).

Eight µL of hydroxylamine 5% was added for 15 min reaction. The differentially TMT2-

labeled samples were pooled in a new tube. The pooled samples were dried. TMT6 experiments were carried out with the same protocol.

Off-gel electrophoresis

The samples were desalted with Oasis® HLB 1 cc (30 mg) extraction cartridges. After drying, the samples were dissolved in 1616.4 µL H2O with 172.8 µL glycerol 50% and 10.8 µL of carrier ampholytes IPG buffer pH 3-10. The IPG strips (pH 3-10, 13 cm) were assembled on the off-gel trays and rehydrated for 30 min with a solution of 89.8% H2O, 9.6% glycerol 50%, and 0.6% of carrier ampholytes. The samples were loaded on the 12 off-gel wells. The isoelectric focusing (IEF) separations were carried out using the 3100 OFFGEL Fractionator (Agilent Technologies) with a limiting current of 50 µA, and a limit of 20 kV·h before holding the voltage to 500 V. The fractions were collected and their pH was measured (744 pH Meter and Biotrode from Metrohm (Herisau, Switzerland)). The fractions were dried, cleaned with Oasis® HLB 1 cc (10 mg) extraction cartridges, and dried again.

RP-LC MALDI TOF/TOF

Matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight (TOF/TOF) MS was performed on a 4800 Proteomics Analyzer from Applied Biosystems (Foster City, CA, USA). The off-gel fractions were first separated with reversed-phase liquid chromatography (RP-LC) using an Alliance system from Waters equipped with a flow splitter. A home-packed 5 µm 200 Å Magic C18 AQ 0.1 × 100 mm column was used. The separation was run for 60 min using a gradient of H2O/CH3CN/TFA 97%/3%/0.1% (solvent A) and H2O/CH3CN/TFA 5%/95%/0.1% (solvent B). The gradient was run as follows: 0–10 min 98% A and 2% B, then to 90% A and 10% B at 12 min, 50% A and 50% B at 55 min, and 98% B at 60 min at a flow rate estimated to 400 nL·min−1. One minute fractions were deposited onto the MALDI plates using a home-made LC-robot. The matrix (α-cyano-4-hydroxycinnamic acid in H2O/CH3CN/TFA 50%/50%/0.1% with 10 mM NH4H2PO4) was then spotted onto the plates. All mass spectra were acquired in positive-ionization mode with an m/z scan range of 800–4000 (1000 shoots with laser intensity of 4000 a.u.). After selection of 20 most-intense precursors at the maximum,

MS/MS experiments (1500 shoots with laser intensity of 4500 a.u.) were performed at medium collision energy.

Protein identification and quantitation

Peak lists were generated using the 4000 Series Explorer software from Applied Biosystems. For each sample, the mgf files resulting from the analysis of the 12 off-gel fractions were combined and searched against UniProt-Swiss-Prot/TrEMBL database (12.6_04-Dec-2007, 5610855 protein entries) using Phenyx 2.6 (GeneBio, Geneva, Switzerland). Homo sapiens taxonomy (93005 protein entries) (and separately Bos taurus (17268 protein entries) to search for the spiked LACB) was specified for database searching. Variable amino acid modifications were oxidized methionine.

TMT2-labeled peptide amino terminus and TMT2-labeled lysine (+225.1558 Da) were set as fixed modifications, as well as carbamidomethylation of cysteines. When using TMT6, a mass increment of +229.1629 Da was specified for TMT6-labeled peptide amino termini and TMT6-labeled lysines. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. Only one search round was used with selection of "turbo" scoring. The peptide p value was 1 E-6 for all runs. The AC and peptide scores were set to control the peptide false peptide discovery rate below 1% (the scores varied from 7.0 to 7.5). The parent ion tolerance was 1.1 Da. Only proteins matching two different peptide sequences were selected and extracted into an excel file using the dedicated Phenyx export. Further filters were applied. Only proteins identified with two different unique peptides were finally kept. When a mass spectrum was attributed to several peptide sequences, all the matched peptides were removed.

The areas of the reporter-ions were extracted from the tandem mass spectra using the analysis tool of the 4000 Series Explorer software. Quantitation was carried out only with peptides which were unique to a protein; at least two peptides with different sequences were needed to quantify a protein. The processing of the data was carried out as already described (13). The processing included an isotopic correction and a normalization with the spiked LACB standard. For each peptide, the relative

abundance of each reporter-ion was calculated as the ratio of the reporter-ion abundance by the sum of all reporter-ion abundances. The protein ratios were then calculated as the ratios of the arithmetic averages of their peptide relative abundances (corresponding to each reporter-ion channel), according to the Libra module used in the Trans-Proteomic Pipeline. A final normalization step was performed assuming that most peptides were in equal quantities in the compared samples; i.e., the common areas between the relative abundance frequency distributions of both TMT2-labeled groups had to be maximal (shown in Figure 4). The normalization coefficients were obtained on the entire reporter-ion dataset (i.e., even when peptides were not matched to any sequences).

Quantitative cut-off values were determined by comparison of identical microdialysis samples analyzed with the protocol described previously. Basically, TMT6 reagents were used to tag identical samples of IC, P, and CT microdialysates. Because no difference was expected between identical samples (e.g., the two IC samples), deviations from 1:1 ratio were considered as falsely positive. The relative abundances provided in each TMT channel were mixed randomly. Ratios were then calculated between identical samples, and geometrical means were obtained from clusters of 10 ratio data points. These mean ratios were then used to evaluate the cut-off values at a given false positive rate. The final cut-off values were averaged from the IC, P, and CT results.

Immunoblot analysis of pooled IC and CT microdialysates

One and a half µg of pooled IC (n = 3; i.e., ICa-c) and 1.5 µg of pooled CT microdialysates (n = 3; i.e., CTd-f) were separated with 1-D SDS PAGE. Twenty µL of ante-mortem CSF, 10 µL of post-mortem CSF, and 31.25 ng of recombinant GSTP1 were also separated. Separated proteins were electroblotted onto a nitrocellulose membrane as described by Towbin et al. (19). Membranes were incubated 1 h with 5% milk-PBS-Tween 0.05% for blocking. Immunodetection was performed with the anti-human GSTP rabbit polyclonal antibody (MBL International Corp., Woburn, MA, USA) diluted 1/2000 in 1% milk-PBS-Tween 0.05%. After several washing steps, appropriate secondary antibody HRP (Dako, Glostrup, Denmark) was incubated 1 h at 1/2000. ECL

plus Western Blotting detection system Kit (GE Healthcare) was used for detection. The membrane was finally scanned with the Typhoon 9400 (GE Healthcare).

ELISA

S100B and PRDX1 were validated using commercial enzyme-linked immunosorbent assay (ELISA) kits from Abnova Corp. (Taipei city, Taiwan) and Biovendor GmbH (Heidelberg, Germany), respectively, according to manufacturer's recommendations. Concerning GSTP1, as no commercial assay is currently available, a sandwich immunoassay was developed in house and used as previously described (20, 21).

Statistical analyses and graphs were performed using GraphPad Prism software (version 4.03, GraphPad software Inc., San Diego, CA, USA).

Example 1: Microdialysis Analysis

Tandem mass tags (TMTs) (13, 14) were used herein to compare brain microdialysis samples of ischemic stroke patients. TMTs comprise a set of isobaric labels. These isobaric labels are synthesized with heavy and light isotopes to present the same total mass but to provide reporter-ions at different masses after activation with collision-induced dissociation and subsequent tandem mass spectrometry (MS/MS). The reporter-ion abundances are used to perform relative quantitation of the peptides labeled with different versions of the TMTs, and by extension determine relative protein amounts.

Samples from the infarct core (IC), the penumbra (P), and the contralateral (CT) brain regions of patients suffering a stroke were investigated. This proteomic study highlighted 43 proteins with increased amount in the IC with respect to the P and the CT microdialysates. Twenty six proteins were increased in the P compared to the CT samples. As candidate markers, glutathione S-transferase P (GSTP1), peroxiredoxin-1 (PRDX1), and S100B were further assessed with immunoassays on microdialysis samples and/or blood of stroke patients that finally confirmed their increased levels in stroke cases.

The human brain microdialysates were sampled in pairs from 2 brain regions of six stroke patients. Six quantitative MS/MS-based comparisons with TMT2, with reporter-ions at m/z = 126.1 and 127.1, were carried out in experiences Expa-f (Table 3).

Concentration of IC, P and CT microdialysis samples

In Figure 1 are displayed brain microdialysates ICd, CTd, Pe, and CTe separated with 1-D PAGE (1-D PAGE images of others samples are shown in Figure 5). The protein amounts in 10 µL of microdialysate were heterogeneous from sample to sample.

Determination of quantitative cut-offs for TMT-based experiments

The quantitative cut-offs that reflected significant increase and decrease in protein amount for the TMT2 experiments were evaluated experimentally (shown in Figure 6). TMT6 were used to label two identical samples of IC, P, and CT microdialysates following the same protocol used for the TMT2 experiments that is detailed in the Experimental Procedures (i.e., reduction, alkylation, digestion, differential TMT6 labeling, off-gel electrophoresis (22, 23), RP-LC MS/MS, identification, and quantitation). After random mixing of the quantitative data, the rate of false positive at a given cut-off was assessed. For instance, 1% of false positive was found at cut-off ratios of 1.68 and 0.59 (see Experimental Procedures, and shown in figure 6). Finally, 0.5 and 2.0 cut-off values were chosen. These values actually corresponded to a larger interval with respect to the experimentally evaluated cut-offs, decreasing further the risk to find false positives. In addition, such differences could be assessed during validation with immunoblot.

Qualitative and quantitative MS analysis

Protein samples were reduced, alkylated, digested with trypsin, and the resulting peptides were labeled with TMT2 as reported in Table 3. Off-gel electrophoresis was performed. The 12 collected off-gel fractions were analyzed with RP-LC MALDI TOF/TOF MS. The quantitative workflow was previously characterized (13, 24). The quality control of the quantitative data was evaluated with the spiked LACB protein standard (Table 3). The mean and maximum relative standard deviation of 12.1% and 17.0% (Expf) correlated with the isobaric tagging technique performances (Table 3) (13).

From these proteomic analyses, 156 proteins were identified with 939 unique peptides. More precisely, 108 proteins were identified in the IC, 137 in the P, and 134 in the CT microdialysates.

The six comparisons carried out with TMT2 showed 94 proteins, which were either increased (ratios > 2.0; 53 proteins) or decreased (ratios < 0.5; 47 proteins) within the compared sample pairs (Table 3). To summarize, 25 proteins were increased in IC with respect to P samples, 24 proteins were increased in IC with respect to CT samples, and 26 proteins were increased in P with respect to CT samples (Tables 7-9). The entire lists of regulated proteins between each brain region are provided in Tables 4-6.

Table 4: List of regulated proteins in infarct core relative to penumbra. Ratio>1 increased in IC; Ratio <1 decreased in IC

Nr.	databank	AC	ID	MW	PI	nb uniqe pept	description	Corr. Ratio IC/P
29	uniprotKB_sptr	P07108	ACBP_HUMAN	11,793.38	5.71	5	Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) (Endozepine) (EP).	1.95
32	uniprotKB_sptr	P07108	ACBP_HUMAN	11,793.38	5.71	5	Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) (Endozepine) (EP).	2.67
20	uniprotKB_sptr	P02763	A1AG1_HUMAN	23,539.62	5.12	4	Alpha-1-acid glycoprotein 1 precursor (AGP 1) (Orosomucoid-1) (OMD 1).	0.48
25	uniprotKB_sptr	P02763	A1AG1_HUMAN	21,560.13	5.10	3	Alpha-1-acid glycoprotein 1 precursor (AGP 1) (Orosomucoid-1) (OMD 1).	0.47
13	uniprotKB_sptr	P01009_CHAIN_0	A1AT_HUMAN	44,324.56	5.44	8	Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (Alpha-1-antiproteinase). [CHAIN 0]	0.61
30	uniprotKB_sptr	P01009_CHAIN_0	A1AT_HUMAN	44,324.56	5.44	5	Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (Alpha-1-antiproteinase). [CHAIN 0]	0.40
74	uniprotKB_sptr	P04217	A1BG_HUMAN	51,940.72	5.72	2	Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein).	0.46
71	uniprotKB_sptr	P02765_CHAIN_0	FETUA_HUMAN	30,221.95	4.57	2	Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Alpha-2-Z-globulin) (Ba- alpha-2-glycoprotein) [Contains: Alpha-2-HS-glycoprotein chain A; Alpha-2-HS-glycoprotein chain B]. [CHAIN 0]	1.07
8	uniprotKB_sptr	P01023	A2MG_HUMAN	160,796.89	5.98	11	Alpha-2-macroglobulin precursor (Alpha-2-M).	0.90
6	uniprotKB_sptr	P01023	A2MG_HUMAN	160,796.89	5.98	16	Alpha-2-macroglobulin precursor (Alpha-2-M).	0.69
47	uniprotKB_sptr	P02647	APOA1_HUMAN	30,777.84	5.68	3	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) [Contains: Apolipoprotein A-I(1-242)].	1.41
8	uniprotKB_sptr	P02647_CHAIN_0	APOA1_HUMAN	27,950.50	5.36	12	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) [Contains: Apolipoprotein A-I(1-242)]. [CHAIN 0]	0.79
31	uniprotKB_sptr	P02652	APOA2_HUMAN	8,579.77	5.39	4	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II) [Contains: Apolipoprotein A-II(1-76)].	1.03
38	uniprotKB_sptr	P61769	B2MG_HUMAN	11,731.17	6.46	3	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3].	1.49
65	uniprotKB_sptr	P61769	B2MG_HUMAN	11,731.17	6.46	2	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3].	2.09
66	uniprotKB_sptr	P62158	CALM_HUMAN	16,706.39	4.12	2	Calmodulin (CaM).	1.53

59	uniprotKB_sptr	P00915	CAH1_HUMAN	28,870.21	6.83	2	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	0.45
38	uniprotKB_sptr	P00915	CAH1_HUMAN	28,739.02	6.92	4	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	1.60
42	uniprotKB_sptr	P00918	CAH2_HUMAN	29,246.06	7.22	4	Carbonic anhydrase 2 (EC 4.2.1.1) (Carbonic anhydrase II) (Carbonate dehydratase II) (CA-II) (Carbonic anhydrase C).	1.89
67	uniprotKB_sptr	P13987	CD59_HUMAN	8,961.10	5.70	2	CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory protein) (MAC-IP) (Protectin) (MEM43 antigen) (Membrane inhibitor of reactive lysis) (MIRL) (20 kDa homologous restriction factor) (HRF-20) (HRF20) (1F5 antigen).	1.50
29	uniprotKB_sptr	P00450	CERU_HUMAN	122,205.23	5.46	5	Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase).	0.44
11	uniprotKB_sptr	P36222	CH3L1_HUMAN	40,488.87	8.80	8	Chitinase-3-like protein 1 precursor (Cartilage glycoprotein 39) (GP- 39) (39 kDa synovial protein) (HCgp-39) (YKL-40).	0.66
54	uniprotKB_sptr	P36222	CH3L1_HUMAN	40,488.87	8.80	3	Chitinase-3-like protein 1 precursor (Cartilage glycoprotein 39) (GP- 39) (39 kDa synovial protein) (HCgp-39) (YKL-40).	1.31
70	uniprotKB_sptr	Q14019	COTL1_HUMAN	15,944.98	5.86	2	Coactosin-like protein.	1.72
73	uniprotKB_sptr	Q14019	COTL1_HUMAN	15,944.98	5.86	2	Coactosin-like protein.	2.04
17	uniprotKB_sptr	P02452	CO1A1_HUMAN	94,766.11	9.36	7	Collagen alpha-1(I) chain precursor (Alpha-1 type I collagen).	1.71
37	uniprotKB_sptr	P08123	CO1A2_HUMAN	91,754.77	10.10	3	Collagen alpha-2(I) chain precursor (Alpha-2 type I collagen).	1.97
19	uniprotKB_sptr	P01024	CO3_HUMAN	187,148.13	6.05	6	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complement C3g fragment; Complement C3d fragment; Complement C3f fragment; Complement C3c alpha' chain fragment 2].	1.45
3	uniprotKB_sptr	P01024_CHAIN_0	CO3_HUMAN	184,951.41	6.04	24	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complement C3g fragment; Complement C3d fragment; Complement C3f fragment; Complement C3c alpha' chain fragment 2]. [CHAIN 0]	0.74
40	uniprotKB_sptr	P0C0L4	CO4A_HUMAN	84,183.35	5.38	3	Complement C4-A precursor (Acidic complement C4) [Contains: Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain].	2.50

24	uniprotKB_sptr	P0C0L4	CO4A_HUMAN	192,742.53	6.72	6	Complement C4-A precursor (Acidic complement C4) [Contains: Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain].	1.10
53	uniprotKB_sptr	P08603	CFAH_HUMAN	137,052.63	6.17	3	Complement factor H precursor (H factor 1).	0.59
60	uniprotKB_sptr	P04080	CYTB_HUMAN	11,139.59	7.91	2	Cystatin-B (Stefin-B) (Liver thiol proteinase inhibitor) (CPI-B).	2.68
22	uniprotKB_sptr	P01034	CYTC_HUMAN	15,799.22	9.21	4	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin).	1.73
62	uniprotKB_sptr	P01034	CYTC_HUMAN	15,799.22	9.21	2	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin).	1.86
48	uniprotKB_sptr	P21291	CSRP1_HUMAN	20,436.21	8.99	2	Cysteine and glycine-rich protein 1 (Cysteine-rich protein 1) (CRP1) (CRP).	3.33
57	uniprotKB_sptr	P21291	CSRP1_HUMAN	20,372.30	9.39	2	Cysteine and glycine-rich protein 1 (Cysteine-rich protein 1) (CRP1) (CRP).	2.88
58	uniprotKB_sptr	O15540	FABPB_HUMAN	14,888.91	5.70	2	Fatty acid-binding protein, brain (B-FABP) (Brain lipid-binding protein) (BLBP) (Mammary-derived growth inhibitor related).	2.65
12	uniprotKB_sptr	P02671	FIBA_HUMAN	94,973.07	5.75	7	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	2.97
4	uniprotKB_sptr	P02671	FIBA_HUMAN	94,973.07	5.75	20	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	0.61
13	uniprotKB_sptr	P02675	FIBB_HUMAN	55,928.17	8.64	8	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B].	0.46
27	uniprotKB_sptr	P02679	FIBG_HUMAN	49,496.55	5.81	6	Fibrinogen gamma chain precursor.	0.41
37	uniprotKB_sptr	P14136	GFAP_HUMAN	49,880.22	5.47	3	Glial fibrillary acidic protein (GFAP).	1.76
63	uniprotKB_sptr	P09211	GSTP1_HUMAN	23,355.84	5.72	2	Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-1).	2.79
7	uniprotKB_sptr	P00738	HPT_HUMAN	43,349.02	6.25	12	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.46
5	uniprotKB_sptr	P00738	HPT_HUMAN	45,205.32	6.24	16	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.36
31	uniprotKB_sptr	P69905	HBA_HUMAN	15,126.36	9.07	4	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin).	0.34
23	uniprotKB_sptr	P69905	HBA_HUMAN	15,126.36	9.07	5	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin).	0.70
30	uniprotKB_sptr	P68871	HBB_HUMAN	15,998.41	7.13	4	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) [Contains: LVV-hemorphin-7].	0.37
7	uniprotKB_sptr	P68871	HBB_HUMAN	15,867.22	7.26	9	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) [Contains: LVV-hemorphin-7].	0.80

25	uniprotKB_sptr	P02790	HEMO_HUMAN	51,676.39	6.67	4	Hemopexin precursor (Beta-1B-glycoprotein).	0.68
16	uniprotKB_sptr	P02790	HEMO_HUMAN	51,676.39	6.67	7	Hemopexin precursor (Beta-1B-glycoprotein).	0.57
61	uniprotKB_sptr	P38159	HNRPG_HUMAN	40,846.19	10.08	2	Heterogeneous nuclear ribonucleoprotein G (hnRNP G) (RNA-binding motif protein, X chromosome) (Glycoprotein p43) [Contains: Processed heterogeneous nuclear ribonucleoprotein G].	2.35
57	uniprotKB_sptr	Q86YZ3	HORN_HUMAN	282,390.06	10.06	2	Hornerin.	1.08
16	uniprotKB_sptr	P01876	IGHA1_HUMAN	52,865.03	7.06	7	Ig alpha-1 chain C region.	0.76
36	uniprotKB_sptr	P01834	KAC_HUMAN	25,772.87	6.31	3	Ig kappa chain C region.	0.51
26	uniprotKB_sptr	P01834	KAC_HUMAN	13,152.70	7.70	5	Ig kappa chain C region.	0.28
9	uniprotKB_sptr	Q6PI81	Q6PI81_HUMAN	52,666.63	7.87	4	IGHM protein.	0.62
75	uniprotKB_sptr	Q14624	ITIH4_HUMAN	103,325.40	6.64	2	Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-inhibitor heavy chain 4) (Inter-alpha-trypsin inhibitor family heavy chain-related protein) (IHRP) (Plasma kallikrein sensitive glycoprotein 120) (PK-120) (GP120) [Contains: 70 kDa inter-alpha-trypsin inhibitor heavy chain H4; 35 kDa inter-alpha- trypsin inhibitor heavy chain H4].	0.83
26	uniprotKB_sptr	Q92876	KLK6_HUMAN	26,855.73	7.57	5	Kallikrein-6 precursor (EC 3.4.21.-) (Protease M) (Neurosin) (Zyme) (SP59) (Serine protease 9) (Serine protease 18).	0.79
4	uniprotKB_sptr	P13645	K1C10_HUMAN	56,561.89	5.13	13	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.72
20	uniprotKB_sptr	P13645	K1C10_HUMAN	56,561.89	5.13	6	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.39
5	uniprotKB_sptr	P35527	K1C9_HUMAN	62,129.47	5.24	11	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9).	0.64
3	uniprotKB_sptr	P04264	K2C1_HUMAN	66,017.70	8.45	20	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.60
14	uniprotKB_sptr	P04264	K2C1_HUMAN	66,017.70	8.45	5	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.51
6	uniprotKB_sptr	P35908	K22E_HUMAN	65,865.35	8.35	9	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2).	0.68
21	uniprotKB_sptr	A2NUT2	A2NUT2_HUMAN	24,960.80	5.40	5	Lambda-chain precursor (AA -20 to 215).	0.74
28	uniprotKB_sptr	A2NUT2	A2NUT2_HUMAN	25,020.97	8.45	2	Lambda-chain precursor (AA -20 to 215).	0.66

41	uniprotKB_sptr	P61626	LYSC_HUMAN	16,537.02	9.50	4	Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C).	1.42
35	uniprotKB_sptr	P25713	MT3_HUMAN	6,926.97	5.00	3	Metallothionein-3 (MT-3) (Metallothionein-III) (MT-III) (Growth inhibitory factor) (GIF) (GIFB).	2.10
44	uniprotKB_sptr	P25713	MT3_HUMAN	6,926.97	5.00	3	Metallothionein-3 (MT-3) (Metallothionein-III) (MT-III) (Growth inhibitory factor) (GIF) (GIFB).	2.79
51	uniprotKB_sptr	P02686_ISOFORM_3	MBP_HUMAN	21,493.21	11.45	2	Myelin basic protein (MBP) (Myelin A1 protein) (Myelin membrane encephalitogenic protein). [ISOFORM 3]	1.71
36	uniprotKB_sptr	P02686_ISOFORM_3	MBP_HUMAN	20,245.79	11.27	2	Myelin basic protein (MBP) (Myelin A1 protein) (Myelin membrane encephalitogenic protein). [ISOFORM 3]	3.11
43	uniprotKB_sptr	O94760	DDAH1_HUMAN	31,121.78	5.64	3	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (EC 3.5.3.18) (Dimethylargininase-1) (Dimethylarginine dimethylaminohydrolase 1) (DDAHI) (DDAH-1).	1.26
62	uniprotKB_sptr	P07197	NFM_HUMAN	102,316.84	4.90	2	Neurofilament medium polypeptide (NF-M) (Neurofilament triplet M protein) (160 kDa neurofilament protein) (Neurofilament 3).	1.54
66	uniprotKB_sptr	P59665	DEF1_HUMAN	6,350.36	8.32	2	Neutrophil defensin 1 precursor (HNP-1) (HP-1) (HP1) (Defensin, alpha 1) [Contains: HP 1-56; Neutrophil defensin 2 (HNP-2) (HP-2) (HP2)].	2.45
24	uniprotKB_sptr	P10451	OSTP_HUMAN	33,016.56	4.59	4	Osteopontin precursor (Bone sialoprotein 1) (Secreted phosphoprotein 1) (SPP-1) (Urinary stone protein) (Nephropontin) (Uropontin).	1.50
54	uniprotKB_sptr	O75781	PALM_HUMAN	37,157.28	4.96	2	Paralemmin.	3.52
15	uniprotKB_sptr	P62937	PPIA_HUMAN	18,012.50	8.34	7	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosporin A-binding protein).	2.45
34	uniprotKB_sptr	P62937	PPIA_HUMAN	18,012.50	8.34	5	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosporin A-binding protein).	1.60
72	uniprotKB_sptr	Q06830	PRDX1_HUMAN	22,110.36	8.59	2	Peroxiredoxin-1 (EC 1.11.1.15) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) (Proliferation-associated gene protein) (PAG) (Natural killer cell-enhancing factor A) (NKEF-A).	1.93
60	uniprotKB_sptr	P32119	PRDX2_HUMAN	16,102.26	6.74	2	Peroxiredoxin-2 (EC 1.11.1.15) (Thioredoxin peroxidase 1) (Thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA) (PRP) (Natural killer cell-enhancing factor B) (NKEF-B).	2.72
45	uniprotKB_sptr	P30041	PRDX6_HUMAN	25,034.99	6.34	3	Peroxiredoxin-6 (EC 1.11.1.15) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aiPLA2) (Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx) (24 kDa protein) (Liver 2D page spot 40) (Red blood cells page spot 12).	2.15

56	uniprotKB_sptr	P30041	PRDX6_HUMAN	25,034.99	6.34	3	Peroxiredoxin-6 (EC 1.11.1.15) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aiPLA2) (Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx) (24 kDa protein) (Liver 2D page spot 40) (Red blood cells page spot 12).	2.16
10	uniprotKB_sptr	P30086	PEBP1_HUMAN	21,056.79	7.76	7	Phosphatidylethanolamine-binding protein 1 (PEBP-1) (Prostatic-binding protein) (HCNPpp) (Neuropolypeptide h3) (Raf kinase inhibitor protein) (RKIP) [Contains: Hippocampal cholinergic neurostimulating peptide (HCNP)].	2.06
21	uniprotKB_sptr	P30086	PEBP1_HUMAN	21,056.79	7.76	7	Phosphatidylethanolamine-binding protein 1 (PEBP-1) (Prostatic-binding protein) (HCNPpp) (Neuropolypeptide h3) (Raf kinase inhibitor protein) (RKIP) [Contains: Hippocampal cholinergic neurostimulating peptide (HCNP)].	1.60
59	uniprotKB_sptr	P05155	IC1_HUMAN	52,843.38	6.10	2	Plasma protease C1 inhibitor precursor (C1 Inh) (C1Inh) (C1 esterase inhibitor) (C1-inhibiting factor).	0.79
72	uniprotKB_sptr	P02753	RETBP_HUMAN	21,071.60	5.48	2	Plasma retinol-binding protein precursor (PRBP) (RBP) [Contains: Plasma retinol-binding protein(1-182); Plasma retinol-binding protein(1-181); Plasma retinol-binding protein(1-179); Plasma retinol- binding protein(1-176)].	2.83
77	uniprotKB_sptr	P02753	RETBP_HUMAN	22,933.85	6.73	2	Plasma retinol-binding protein precursor (PRBP) (RBP) [Contains: Plasma retinol-binding protein(1-182); Plasma retinol-binding protein(1-181); Plasma retinol-binding protein(1-179); Plasma retinol- binding protein(1-176)].	1.63
55	uniprotKB_sptr	P00747	PLMN_HUMAN	15,443.72	8.91	2	Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide; Angiostatin; Plasmin heavy chain A, short form; Plasmin light chain B].	2.27
49	uniprotKB_sptr	P02775	SCYB7_HUMAN	9,105.57	8.74	2	Platelet basic protein precursor (PBP) (C-X-C motif chemokine 7) (Small-inducible cytokine B7) (Leukocyte-derived growth factor) (LDGF) (Macrophage-derived growth factor) (MDGF) [Contains: Connective tissue-activating peptide III (CTAP-III) (Low-affinity platelet factor IV) (LA-PF4); TC-2; Connective tissue-activating peptide III(1-81) (CTAP-III(1-81)); Beta-thromboglobulin (Beta-TG); Neutrophil- activating peptide 2(74) (NAP-2(74)); Neutrophil-activating peptide 2(73) (NAP-2(73)); Neutrophil-activating peptide 2 (NAP-2); TC-1; Neutrophil-activating peptide 2(1-66) (NAP-2(1-66)); Neutrophil- activating peptide 2(1-63) (NAP-2(1-63))].	2.51

49	uniprotKB_sptr	P02775	SCYB7_HUMAN	9,105.57	8.74	3	Platelet basic protein precursor (PBP) (C-X-C motif chemokine 7) (Small-inducible cytokine B7) (Leukocyte-derived growth factor) (LDGF) (Macrophage-derived growth factor) (MDGF) [Contains: Connective tissue-activating peptide III (CTAP-III) (Low-affinity platelet factor IV) (LA-PF4); TC-2; Connective tissue-activating peptide III(1-81) (CTAP-III(1-81)); Beta-thromboglobulin (Beta-TG); Neutrophil- activating peptide 2(74) (NAP-2(74)); Neutrophil-activating peptide 2(73) (NAP-2(73)); Neutrophil-activating peptide 2 (NAP-2); TC-1; Neutrophil-activating peptide 2(1-66) (NAP-2(1-66)); Neutrophil- activating peptide 2(1-63) (NAP-2(1-63))].	0.85
34	uniprotKB_sptr	P07602	SAP_HUMAN	26,308.66	5.88	3	Proactivator polypeptide precursor [Contains: Saposin-A (Protein A); Saposin-B-Val; Saposin-B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin-C (Co-beta-glucosidase) (A1 activator) (Glucosylceramidase activator) (Sphingolipid activator protein 2) (SAP-2); Saposin-D (Protein C) (Component C)].	1.60
58	uniprotKB_sptr	P07602	SAP_HUMAN	26,308.66	5.88	3	Proactivator polypeptide precursor [Contains: Saposin-A (Protein A); Saposin-B-Val; Saposin-B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin-C (Co-beta-glucosidase) (A1 activator) (Glucosylceramidase activator) (Sphingolipid activator protein 2) (SAP-2); Saposin-D (Protein C) (Component C)].	1.25
53	uniprotKB_sptr	P07737	PROF1_HUMAN	15,054.23	8.79	2	Profilin-1 (Profilin I).	2.40
46	uniprotKB_sptr	P07737	PROF1_HUMAN	15,054.23	8.79	3	Profilin-1 (Profilin I).	0.91
28	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18,698.03	8.68	4	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	0.84
50	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18,698.03	8.68	3	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	1.63
64	uniprotKB_sptr	A0N5G5	A0N5G5_HUMAN	12,766.37	9.45	2	Rheumatoid factor D5 light chain (Fragment).	0.96
67	uniprotKB_sptr	A0N5G5	A0N5G5_HUMAN	12,766.37	9.45	2	Rheumatoid factor D5 light chain (Fragment).	0.75
2	uniprotKB_sptr	P02787	TRFE_HUMAN	76,959.79	7.16	29	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin).	0.42

2	uniprotKB_sptr	P02787	TRFE_HUMAN	77,049.89	6.97	24	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin).	0.50
1	uniprotKB_sptr	P02768	ALBU_HUMAN	66,472.22	5.73	46	Serum albumin precursor.	0.40
1	uniprotKB_sptr	P02768	ALBU_HUMAN	69,366.70	5.98	49	Serum albumin precursor.	0.33
33	uniprotKB_sptr	O75368	SH3L1_HUMAN	12,774.25	5.53	3	SH3 domain-binding glutamic acid-rich-like protein.	2.17
48	uniprotKB_sptr	O75368	SH3L1_HUMAN	12,774.25	5.53	3	SH3 domain-binding glutamic acid-rich-like protein.	1.92
32	uniprotKB_sptr	P00441	SODC_HUMAN	16,122.00	6.04	4	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1).	1.18
71	uniprotKB_sptr	P10599	THIO_HUMAN	9,451.99	6.56	2	Thioredoxin (Trx) (ATL-derived factor) (ADF) (Surface-associated sulphydryl protein) (SASP).	2.17
46	uniprotKB_sptr	P04216	THY1_HUMAN	12,553.22	9.33	2	Thy-1 membrane glycoprotein precursor (Thy-1 antigen) (CD90 antigen) (CDw90).	1.29
68	uniprotKB_sptr	P62328	TYB4_HUMAN	7,312.18	8.84	2	Thymosin beta-4 (T beta 4) (Fx) [Contains: Hematopoietic system regulatory peptide (Seraspenide)].	1.69
41	uniprotKB_sptr	P60174	TPIS_HUMAN	26,669.50	6.90	3	Triosephosphate isomerase (EC 5.3.1.1) (TIM) (Triose-phosphate isomerase).	0.82
51	uniprotKB_sptr	P60174	TPIS_HUMAN	27,126.45	8.70	3	Triosephosphate isomerase (EC 5.3.1.1) (TIM) (Triose-phosphate isomerase).	0.68
27	uniprotKB_sptr	P62988	UBIQ_HUMAN	61,523.80	7.25	4	Ubiquitin.	2.09
39	uniprotKB_sptr	P62988	UBIQ_HUMAN	25,761.65	7.78	4	Ubiquitin.	1.50
15	uniprotKB_sptr	A6NGU3	A6NGU3_HUMAN	57,019.61	8.48	2	Uncharacterized protein IGHG3 (Fragment).	0.61
65	uniprotKB_sptr	Q9GZP4	CA128_HUMAN	24,177.85	5.58	2	UPF0424 protein C1orf128.	1.76
43	uniprotKB_sptr	P02774	VTDB_HUMAN	52,963.66	5.47	4	Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDB).	0.63
69	uniprotKB_sptr	P04004_CHAIN_0	VTNC_HUMAN	43,029.62	5.64	2	Vitronectin precursor (Serum-spreading factor) (S-protein) (V75) [Contains: Vitronectin V65 subunit; Vitronectin V10 subunit; Somatomedin-B]. [CHAIN 0]	1.47
76	uniprotKB_sptr	P25311	ZA2G_HUMAN	34,244.70	5.85	2	Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein) (Zn- alpha-2-GP).	0.56

Table 5: List of regulated proteins in infarct core relative to contralateral hemisphere. Ratio>1 increased in IC; Ratio <1 decreased in IC

Nr.	databank	AC	ID	MW	PI	nb uniqe pept	description	Corr. Ratio IC/P
20	uniprotKB_sptr	P60709	ACTB_HUMAN	41,004.97	5.67	4	Actin, cytoplasmic 1 (Beta-actin).	1.65
26	uniprotKB_sptr	P07108	ACBP_HUMAN	11793.375	5.71	3	Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) (Endozepine) (EP).	12.53
26	uniprotKB_sptr	P01009	A1AT_HUMAN	22,828.35	6.35	4	Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (Alpha-1-antiproteinase).	0.49
50	uniprotKB_sptr	P01023	A2MG_HUMAN	160,796.89	5.98	2	Alpha-2-macroglobulin precursor (Alpha-2-M).	0.36
40	uniprotKB_sptr	P02760	AMBP_HUMAN	38,999.49	6.18	3	AMBP protein precursor [Contains: Alpha-1-microglobulin (Protein HC) (Complex-forming glycoprotein heterogeneous in charge) (Alpha-1 microglycoprotein); Inter-alpha-trypsin inhibitor light chain (ITI-LC) (Bikunin) (HI-30)].	1.82
27	uniprotKB_sptr	Q5EFE5	Q5EFE5_HUMAN	52362.454	8.73	2	Anti-RhD monoclonal T125 gamma1 heavy chain precursor.	0.82
24	uniprotKB_sptr	P02647	APOA1_HUMAN	27,950.50	5.36	4	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) [Contains: Apolipoprotein A-I(1-242)].	0.80
59	uniprotKB_sptr	P02652	APOA2_HUMAN	8,579.77	5.39	2	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II) [Contains: Apolipoprotein A-II(1-76)].	2.28
60	uniprotKB_sptr	P06727	APOA4_HUMAN	28,157.53	5.45	2	Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV).	3.79
33	uniprotKB_sptr	P61769	B2MG_HUMAN	11731.167	6.46	2	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3].	0.80
53	uniprotKB_sptr	P61769	B2MG_HUMAN	11,731.17	6.46	2	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3].	0.44
25	uniprotKB_sptr	P62158	CALM_HUMAN	16706.394	4.12	3	Calmodulin (CaM).	1.06
19	uniprotKB_sptr	P00915	CAH1_HUMAN	28739.022	6.92	4	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	0.29
12	uniprotKB_sptr	P00915	CAH1_HUMAN	28,739.02	6.92	5	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	4.68
28	uniprotKB_sptr	P00918	CAH2_HUMAN	29,246.06	7.22	4	Carbonic anhydrase 2 (EC 4.2.1.1) (Carbonic anhydrase II) (Carbonate dehydratase II) (CA-II) (Carbonic anhydrase C).	3.18

56	uniprotKB_sptr	P49913	CAMP_HUMAN	19,301.39	9.59	2	Cathelicidin antimicrobial peptide precursor (18 kDa cationic antimicrobial protein) (CAP-18) (hCAP-18) [Contains: Antibacterial protein FALL-39 (FALL-39 peptide antibiotic); Antibacterial protein LL-37].	1.53
9	uniprotKB_sptr	P36222	CH3L1_HUMAN	40488.867	8.80	6	Chitinase-3-like protein 1 precursor (Cartilage glycoprotein 39) (GP- 39) (39 kDa synovial protein) (HCgp-39) (YKL-40).	2.44
39	uniprotKB_sptr	P10645	CMGA_HUMAN	48960.312	4.57	2	Chromogranin-A precursor (CgA) (Pituitary secretory protein I) (SP-I) [Contains: Vasostatin-1 (Vasostatin I); Vasostatin-2 (Vasostatin II); EA-92; ES-43; Pancreastatin; SS-18; WA-8; WE-14; LF-19; AL-11; GV-19; GR-44; ER-37].	0.29
63	uniprotKB_sptr	P23528	COF1_HUMAN	18,502.50	8.53	2	Cofilin-1 (Cofilin, non-muscle isoform) (18 kDa phosphoprotein) (p18).	2.00
16	uniprotKB_sptr	P01024	CO3_HUMAN	184,951.41	6.04	5	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphylatoxin; Complement C3b alpha' chain; Complement C3c alpha' chain fragment 1; Complement C3dg fragment; Complement C3g fragment; Complement C3d fragment; Complement C3f fragment; Complement C3c alpha' chain fragment 2].	0.43
49	uniprotKB_sptr	P04080	CYTB_HUMAN	11,139.59	7.91	2	Cystatin-B (Stefin-B) (Liver thiol proteinase inhibitor) (CPI-B).	2.10
24	uniprotKB_sptr	P01034_CHAIN_0	CYTC_HUMAN	13347.14	9.00	3	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin). [CHAIN 0]	0.62
31	uniprotKB_sptr	P01034	CYTC_HUMAN	15,799.22	9.21	3	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin).	0.03
20	uniprotKB_sptr	P81605	DCD_HUMAN	11283.862	6.72	4	Dermcidin precursor (Preproteolysin) [Contains: Survival-promoting peptide; DCD-1].	0.85
61	uniprotKB_sptr	P81605	DCD_HUMAN	4,705.34	5.61	2	Dermcidin precursor (Preproteolysin) [Contains: Survival-promoting peptide; DCD-1].	0.35
14	uniprotKB_sptr	P02671	FIBA_HUMAN	94973.068	5.75	5	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	0.46
11	uniprotKB_sptr	P02671	FIBA_HUMAN	94,973.07	5.75	5	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	2.33
47	uniprotKB_sptr	P02675	FIBB_HUMAN	55,928.17	8.64	2	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B].	0.38
48	uniprotKB_sptr	P30043	BLVRB_HUMAN	22,119.35	7.86	2	Flavin reductase (EC 1.5.1.30) (FR) (NADPH-dependent diaphorase) (NADPH-flavin reductase) (FLR) (Biliverdin reductase B) (EC 1.3.1.24) (BVR-B) (Biliverdin-IX beta-reductase) (Green heme-binding protein) (GHBP).	2.22
15	uniprotKB_sptr	P14136	GFAP_HUMAN	49880.22	5.47	4	Glial fibrillary acidic protein (GFAP).	4.13
38	uniprotKB_sptr	P14136	GFAP_HUMAN	49,505.89	5.95	2	Glial fibrillary acidic protein (GFAP).	0.37

13	uniprotKB_sptr	P00738	HPT_HUMAN	45205.318	6.24	5	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.83
41	uniprotKB_sptr	P00738	HPT_HUMAN	45,205.32	6.24	3	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.14
42	uniprotKB_sptr	P69905	HBA_HUMAN	15,126.36	9.07	2	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin).	3.21
40	uniprotKB_sptr	P68871	HBB_HUMAN	15867.217	7.26	2	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) [Contains: LVV-hemorphin-7].	0.81
13	uniprotKB_sptr	P16403	H12_HUMAN	21,233.57	11.02	2	Histone H1.2 (Histone H1d).	2.97
14	uniprotKB_sptr	P16401	H15_HUMAN	22,448.99	10.98	4	Histone H1.5 (Histone H1a).	2.03
15	uniprotKB_sptr	P01859	IGHG2_HUMAN	35,884.65	8.01	2	Ig gamma-2 chain C region.	0.18
16	uniprotKB_sptr	Q92876	KLK6_HUMAN	24499.85	7.26	5	Kallikrein-6 precursor (EC 3.4.21.-) (Protease M) (Neurosin) (Zyme) (SP59) (Serine protease 9) (Serine protease 18).	0.60
4	uniprotKB_sptr	P13645	K1C10_HUMAN	59510.708	5.18	16	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.99
5	uniprotKB_sptr	P13645	K1C10_HUMAN	59,510.71	5.18	9	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.48
7	uniprotKB_sptr	P02533	K1C14_HUMAN	51490.331	5.13	6	Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14).	0.91
10	uniprotKB_sptr	P08779	K1C16_HUMAN	51,267.84	5.03	6	Keratin, type I cytoskeletal 16 (Cytokeratin-16) (CK-16) (Keratin-16) (K16).	1.28
5	uniprotKB_sptr	P35527	K1C9_HUMAN	62129.473	5.24	14	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9).	0.84
4	uniprotKB_sptr	P35527	K1C9_HUMAN	62,129.47	5.24	10	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9).	0.67
2	uniprotKB_sptr	P04264	K2C1_HUMAN	66017.701	8.45	23	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.96
2	uniprotKB_sptr	P04264	K2C1_HUMAN	66,017.70	8.45	16	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.52
3	uniprotKB_sptr	P35908	K22E_HUMAN	65865.354	8.35	13	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2).	0.97
3	uniprotKB_sptr	P35908	K22E_HUMAN	65,865.35	8.35	7	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2).	0.43
8	uniprotKB_sptr	P13647	K2C5_HUMAN	62378.367	8.14	2	Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) (58 kDa cytokeratin).	0.75

6	uniprotKB_sptr	P13647	K2C5_HUMAN	62,378.37	8.14	4	Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) (58 kDa cytokeratin).	0.63
8	uniprotKB_sptr	P02538	K2C6A_HUMAN	60,044.97	8.38	2	Keratin, type II cytoskeletal 6A (Cytokeratin-6A) (CK 6A) (K6a keratin) (Cytokeratin-6D) (CK 6D).	1.07
22	uniprotKB_sptr	A2NUT2	A2NUT2_HUMAN	25,020.97	8.45	2	Lambda-chain precursor (AA -20 to 215).	0.21
9	uniprotKB_sptr	P61626	LYSC_HUMAN	16,537.02	9.50	4	Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C).	2.75
36	uniprotKB_sptr	P25713	MT3_HUMAN	6926.967	5.00	2	Metallothionein-3 (MT-3) (Metallothionein-III) (MT-III) (Growth inhibitory factor) (GIF) (GIFB).	1.39
54	uniprotKB_sptr	P02686	MBP_HUMAN	17,343.36	11.29	2	Myelin basic protein (MBP) (Myelin A1 protein) (Myelin membrane encephalitogenic protein).	0.30
41	uniprotKB_sptr	O94760	DDAH1_HUMAN	31121.782	5.64	2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (EC 3.5.3.18) (Dimethylargininase-1) (Dimethylarginine dimethylaminohydrolase 1) (DDAHI) (DDAH-1).	6.31
11	uniprotKB_sptr	P07197	NFM_HUMAN	102316.835	4.90	7	Neurofilament medium polypeptide (NF-M) (Neurofilament triplet M protein) (160 kDa neurofilament protein) (Neurofilament 3).	4.68
44	uniprotKB_sptr	P59665	DEF1_HUMAN	6,350.36	8.32	2	Neutrophil defensin 1 precursor (HNP-1) (HP-1) (HP1) (Defensin, alpha 1) [Contains: HP 1-56; Neutrophil defensin 2 (HNP-2) (HP-2) (HP2)].	4.23
43	uniprotKB_sptr	P80188	NGAL_HUMAN	22,902.42	9.15	2	Neutrophil gelatinase-associated lipocalin precursor (NGAL) (p25) (25 kDa alpha-2-microglobulin-related subunit of MMP-9) (Lipocalin-2) (Oncogene 24p3).	1.85
18	uniprotKB_sptr	P10451	OSTP_HUMAN	33016.56	4.59	3	Osteopontin precursor (Bone sialoprotein 1) (Secreted phosphoprotein 1) (SPP-1) (Urinary stone protein) (Nephropontin) (Uropontin).	0.88
22	uniprotKB_sptr	P62937	PPIA_HUMAN	18012.497	8.34	3	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosporin A-binding protein).	8.25
55	uniprotKB_sptr	P62937	PPIA_HUMAN	18,012.50	8.34	2	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosporin A-binding protein).	1.69
21	uniprotKB_sptr	P30086	PEBP1_HUMAN	21056.788	7.76	3	Phosphatidylethanolamine-binding protein 1 (PEBP-1) (Prostatic-binding protein) (HCNPpp) (Neuropolypeptide h3) (Raf kinase inhibitor protein) (RKIP) [Contains: Hippocampal cholinergic neurostimulating peptide (HCNP)].	4.88
51	uniprotKB_sptr	P00747	PLMN_HUMAN	9,019.20	6.78	2	Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide; Angiostatin; Plasmin heavy chain A, short form; Plasmin light chain B].	1.22

44	uniprotKB_sptr	P07602	SAP_HUMAN	26308.661	5.88	2	Proactivator polypeptide precursor [Contains: Saposin-A (Protein A); Saposin-B-Val; Saposin-B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin-C (Co-beta-glucosidase) (A1 activator) (Glucosylceramidase activator) (Sphingolipid activator protein 2) (SAP-2); Saposin-D (Protein C) (Component C)].	1.93
12	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18698.032	8.68	6	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	0.27
30	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18,698.03	8.68	3	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	0.46
29	uniprotKB_sptr	P05109	S10A8_HUMAN	10,834.51	6.96	3	Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-related protein 8) (MRP-8) (Cystic fibrosis antigen) (CFAG) (P8) (Leukocyte L1 complex light chain) (Calprotectin L1L subunit) (Urinary stone protein band A).	0.98
19	uniprotKB_sptr	Q6P5S8	Q6P5S8_HUMAN	25,772.87	6.31	2	Putative uncharacterized protein.	0.15
62	uniprotKB_sptr	A0N5G5	A0N5G5_HUMAN	12,766.37	9.45	2	Rheumatoid factor D5 light chain (Fragment).	0.67
6	uniprotKB_sptr	P02787	TRFE_HUMAN	77049.89	6.97	17	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin).	1.47
7	uniprotKB_sptr	P02787	TRFE_HUMAN	77,049.89	6.97	8	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin).	0.42
1	uniprotKB_sptr	P02768	ALBU_HUMAN	69366.701	5.98	33	Serum albumin precursor.	1.17
1	uniprotKB_sptr	P02768	ALBU_HUMAN	69,366.70	5.98	32	Serum albumin precursor.	0.38
18	uniprotKB_sptr	Q9UP60	Q9UP60_HUMAN	53,088.18	6.70	3	SNC73 protein.	0.30
29	uniprotKB_sptr	P00441	SODC_HUMAN	16122.002	6.04	2	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1).	1.83
43	uniprotKB_sptr	P63313	TYB10_HUMAN	5025.673	6.21	2	Thymosin beta-10.	4.16
37	uniprotKB_sptr	P62328	TYB4_HUMAN	7312.177	8.84	2	Thymosin beta-4 (T beta 4) (Fx) [Contains: Hematopoietic system regulatory peptide (Seraspenide)].	2.39

17	uniprotKB_sptr	P60174	TPIS_HUMAN	26669.495	6.90	5	Triosephosphate isomerase (EC 5.3.1.1) (TIM) (Triose-phosphate isomerase).	3.50
57	uniprotKB_sptr	P06753	TPM3_HUMAN	28,809.25	4.75	2	Tropomyosin alpha-3 chain (Tropomyosin-3) (Tropomyosin gamma) (hTM5).	2.04
32	uniprotKB_sptr	P62988	UBIQ_HUMAN	25761.65	7.78	2	Ubiquitin.	1.71

Table 6: List of regulated proteins in penumbra relative to contralateral hemisphere. Ratio>1 increased in P; Ratio <1 decreased in P

Nr.	databank	AC	ID	MW	PI	nb unique pept	description	Cor Rati IC/I
38	uniprotKB_sptr	P63104	1433Z_HUMAN	27,745.11	4.77	3	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1).	1.51
57	uniprotKB_sptr	P60709	ACTB_HUMAN	41,004.97	5.67	2	Actin, cytoplasmic 1 (Beta-actin).	1.15
25	uniprotKB_sptr	Q13747	Q13747_HUMAN	22,828.35	6.35	8	Alpha-1 antitrypsin (Fragment).	1.05
27	uniprotKB_sptr	Q13747	Q13747_HUMAN	22,828.35	6.35	7	Alpha-1 antitrypsin (Fragment).	0.35
39	uniprotKB_sptr	P02763	A1AG1_HUMAN	21,560.13	5.10	2	Alpha-1-acid glycoprotein 1 precursor (AGP 1) (Orosomucoid-1) (OMD 1).	1.38
53	uniprotKB_sptr	P02763	A1AG1_HUMAN	23,539.62	5.12	2	Alpha-1-acid glycoprotein 1 precursor (AGP 1) (Orosomucoid-1) (OMD 1).	0.38
42	uniprotKB_sptr	P04217	A1BG_HUMAN	51,940.72	5.72	3	Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein).	1.11
24	uniprotKB_sptr	P01023	A2MG_HUMAN	160,796.89	5.98	7	Alpha-2-macroglobulin precursor (Alpha-2-M).	0.68
47	uniprotKB_sptr	P01023	A2MG_HUMAN	160,796.89	5.98	4	Alpha-2-macroglobulin precursor (Alpha-2-M).	0.36
55	uniprotKB_sptr	P06733	ENOA_HUMAN	47,037.78	7.55	3	Alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Non- neural enolase) (NNE) (Enolase 1) (Phosphopyruvate hydratase) (C-myc promoter-binding protein) (MBP-1) (MPB-1) (Plasminogen-binding protein).	0.45
92	uniprotKB_sptr	P51693	APLP1_HUMAN	72,176.41	5.58	2	Amyloid-like protein 1 precursor (APLP) (APLP-1) [Contains: C30].	0.86
91	uniprotKB_sptr	P01019	ANGT_HUMAN	53,777.91	6.07	2	Angiotensinogen precursor (Serpin A8) [Contains: Angiotensin-1 (Angiotensin I) (Ang I); Angiotensin-2 (Angiotensin II) (Ang II); Angiotensin-3 (Angiotensin III) (Ang III) (Des-Asp[1]-angiotensin II)].	0.7
51	uniprotKB_sptr	P01008	ANT3_HUMAN	52,691.56	6.32	3	Antithrombin-III precursor (ATIII).	1.1
73	uniprotKB_sptr	P02652	APOA2_HUMAN	8,579.77	5.39	2	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II) [Contains: Apolipoprotein A-II(1-76)].	1.2
96	uniprotKB_sptr	P02652	APOA2_HUMAN	8,579.77	5.39	2	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II) [Contains: Apolipoprotein A-II(1-76)].	1.0
61	uniprotKB_sptr	P02649_CHAIN_0	APOE_HUMAN	34,236.69	5.65	2	Apolipoprotein E precursor (Apo-E). [CHAIN 0]	0.3

WO 2011/144914 PCT/GB2011/000784

87	uniprotKB_sptr	P55087	AQP4_HUMAN	34,829.70	8.09	2	Aquaporin-4 (AQP-4) (WCH4) (Mercurial-insensitive water channel) (MIWC).	2.0
68	uniprotKB_sptr	A6XND9	A6XND9_HUMAN	11,748.41	6.28	2	Beta-2-microglobulin.	0.9
88	uniprotKB_sptr	A6XND9	A6XND9_HUMAN	11,748.41	6.28	2	Beta-2-microglobulin.	0.2
70	uniprotKB_sptr	Q96KN2	CNDP1_HUMAN	56,734.20	5.24	2	Beta-Ala-His dipeptidase precursor (EC 3.4.13.20) (Carnosine dipeptidase 1) (CNDP dipeptidase 1) (Serum carnosinase) (Glutamate carboxypeptidase-like protein 2).	0.3
80	uniprotKB_sptr	Q96GW7	PGCB_HUMAN	92,811.83	4.58	2	Brevican core protein precursor (Brain-enriched hyaluronan-binding protein) (Protein BEHAB).	0.72
75	uniprotKB_sptr	Q9BRL5	Q9BRL5_HUMAN	16,506.53	4.38	3	CALM3 protein.	1.53
36	uniprotKB_sptr	P00915	CAH1_HUMAN	28,739.02	6.92	4	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	1.09
39	uniprotKB_sptr	P00915	CAH1_HUMAN	28,870.21	6.83	5	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I) (Carbonate dehydratase I) (CA-I).	0.71
71	uniprotKB_sptr	P00918	CAH2_HUMAN	29,246.06	7.22	3	Carbonic anhydrase 2 (EC 4.2.1.1) (Carbonic anhydrase II) (Carbonate dehydratase II) (CA-II) (Carbonic anhydrase C).	1.77
60	uniprotKB_sptr	P13987	CD59_HUMAN	14,177.29	6.38	2	CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory protein) (MAC-IP) (Protectin) (MEM43 antigen) (Membrane inhibitor of reactive lysis) (MIRL) (20 kDa homologous restriction factor) (HRF-20) (HRF20) (1F5 antigen).	1.54
95	uniprotKB_sptr	P13987	CD59_HUMAN	8,961.10	5.70	2	CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory protein) (MAC-IP) (Protectin) (MEM43 antigen) (Membrane inhibitor of reactive lysis) (MIRL) (20 kDa homologous restriction factor) (HRF-20) (HRF20) (1F5 antigen).	1.19
16	uniprotKB_sptr	A8K866	A8K866_HUMAN	30,777.84	5.68	11	cDNA FLJ75790, highly similar to Homo sapiens apolipoprotein A-I (APOA1), mRNA (Apolipoprotein A-I, isoform CRA_a).	0.85
22	uniprotKB_sptr	A8K866	A8K866_HUMAN	30,777.84	5.68	8	cDNA FLJ75790, highly similar to Homo sapiens apolipoprotein A-I (APOA1), mRNA (Apolipoprotein A-I, isoform CRA_a).	0.76
17	uniprotKB_sptr	P00450	CERU_HUMAN	122,205.23	5.46	9	Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase).	0.8
35	uniprotKB_sptr	P00450	CERU_HUMAN	122,205.23	5.46	5	Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase).	0.4
64	uniprotKB_sptr	Q2TU75	Q2TU75_HUMAN	52,494.59	5.97	2	Clusterin.	0.4
89	uniprotKB_sptr	Q14019	COTL1_HUMAN	15,944.98	5.86	2	Coactosin-like protein.	2.6

WO 2011/144914 PCT/GB2011/000784

32	uniprotKB_sptr	P0C0L4	CO4A_HUMAN	84,183.35	5.38	5	Complement C4-A precursor (Acidic complement C4) [Contains: Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain].	1.1
72	uniprotKB_sptr	P0C0L4	CO4A_HUMAN	84,183.35	5.38	3	Complement C4-A precursor (Acidic complement C4) [Contains: Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain].	0.7
8	uniprotKB_sptr	A7E236	A7E236_HUMAN	187,148.13	6.05	4	Complement component 3 (Complement component 3, isoform CRA_b).	0.72
12	uniprotKB_sptr	A7E236	A7E236_HUMAN	187,148.13	6.05	12	Complement component 3 (Complement component 3, isoform CRA_b).	0.48
72	uniprotKB_sptr	P00751	CFAB_HUMAN	83,000.83	6.80	2	Complement factor B precursor (EC 3.4.21.47) (C3/C5 convertase) (Properdin factor B) (Glycine-rich beta glycoprotein) (GBG) (PBF2) [Contains: Complement factor B Ba fragment; Complement factor B Bb fragment].	1.15
93	uniprotKB_sptr	P00751	CFAB_HUMAN	83,000.83	6.80	2	Complement factor B precursor (EC 3.4.21.47) (C3/C5 convertase) (Properdin factor B) (Glycine-rich beta glycoprotein) (GBG) (PBF2) [Contains: Complement factor B Ba fragment; Complement factor B Bb fragment].	0.36
63	uniprotKB_sptr	P04080	CYTB_HUMAN	11,139.59	7.91	2	Cystatin-B (Stefin-B) (Liver thiol proteinase inhibitor) (CPI-B).	2.16
79	uniprotKB_sptr	P04080	CYTB_HUMAN	11,139.59	7.91	2	Cystatin-B (Stefin-B) (Liver thiol proteinase inhibitor) (CPI-B).	1.81
49	uniprotKB_sptr	P01034	CYTC_HUMAN	15,799.22	9.21	2	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin).	0.17
57	uniprotKB_sptr	P01034	CYTC_HUMAN	15,799.22	9.21	3	Cystatin-C precursor (Cystatin-3) (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin).	0.38
67	uniprotKB_sptr	P21291	CSRP1_HUMAN	20,436.21	8.99	2	Cysteine and glycine-rich protein 1 (Cysteine-rich protein 1) (CRP1) (CRP).	4.58
56	uniprotKB_sptr	P81605_PEPT_1	DCD_HUMAN	4,705.34	5.61	4	Dermcidin precursor (Preproteolysin) [Contains: Survival-promoting peptide; DCD-1]. [PEPTIDE 1])	0.34
33	uniprotKB_sptr	Q4VWZ6	Q4VWZ6_HUMAN	10,144.58	6.74	6	Diazepam binding inhibitor, splice form 1c.	2.2
102	uniprotKB_sptr	Q16555	DPYL2_HUMAN	62,270.61	6.00	2	Dihydropyrimidinase-related protein 2 (DRP-2) (Collapsin response mediator protein 2) (CRMP-2) (N2A3).	1.1
44	uniprotKB_sptr	Q13822	ENPP2_HUMAN	105,210.85	8.54	4	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 precursor (EC 3.1.4.39) (E-NPP 2) (Extracellular lysophospholipase D) (LysoPLD) (Autotaxin).	0.4
30	uniprotKB_sptr	Q53HR3	Q53HR3_HUMAN	47,140.92	7.57	2	Enolase (EC 4.2.1.11) (Fragment).	1.8

WO 2011/144914 PCT/GB2011/000784

26	uniprotKB_sptr	P02671	FIBA_HUMAN	94,973.07	5.75	7	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	2.9
23	uniprotKB_sptr	P02671	FIBA_HUMAN	94,973.07	5.75	7	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A].	1.0
68	uniprotKB_sptr	P04075	ALDOA_HUMAN	39,420.03	8.49	3	Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Lung cancer antigen NY-LU-1).	1.4
61	uniprotKB_sptr	P09104	ENOG_HUMAN	47,268.59	4.95	2	Gamma-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Neural enolase) (Neuron-specific enolase) (NSE) (Enolase 2).	1.19
38	uniprotKB_sptr	P14136	GFAP_HUMAN	49,505.89	5.95	4	Glial fibrillary acidic protein (GFAP).	0.31
16	uniprotKB_sptr	P14136	GFAP_HUMAN	49,880.22	5.47	9	Glial fibrillary acidic protein (GFAP).	2.33
13	uniprotKB_sptr	P00738	HPT_HUMAN	45,205.32	6.24	12	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.86
10	uniprotKB_sptr	P00738	HPT_HUMAN	27,265.07	6.53	14	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain].	0.45
31	uniprotKB_sptr	P69905_	HBA_HUMAN	15,126.36	9.07	5	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin).	0.11
41	uniprotKB_sptr	P69905	HBA_HUMAN	15,126.36	9.07	4	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin).	0.55
34	uniprotKB_sptr	P68871	HBB_HUMAN	15,867.22	7.26	5	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) [Contains: LVV-hemorphin-7].	0.11
40	uniprotKB_sptr	P68871	HBB_HUMAN	15,998.41	7.13	5	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) [Contains: LVV-hemorphin-7].	0.59
20	uniprotKB_sptr	P02790	HEMO_HUMAN	51,676.39	6.67	8	Hemopexin precursor (Beta-1B-glycoprotein).	0.90
25	uniprotKB_sptr	P02790	HEMO_HUMAN	51,676.39	6.67	6	Hemopexin precursor (Beta-1B-glycoprotein).	0.52
83	uniprotKB_sptr	Q16775	GLO2_HUMAN	28,860.02	7.19	2	Hydroxyacylglutathione hydrolase (EC 3.1.2.6) (Glyoxalase II) (Glx II).	2.64
37	uniprotKB_sptr	P01876	IGHA1_HUMAN	37,654.65	6.26	4	Ig alpha-1 chain C region.	0.66
82	uniprotKB_sptr	P01877	IGHA2_HUMAN	53,776.22	6.91	2	Ig alpha-2 chain C region.	0.51
27	uniprotKB_sptr	P01859	IGHG2_HUMAN	35,884.65	8.01	2	Ig gamma-2 chain C region.	0.87
24	uniprotKB_sptr	P01859	IGHG2_HUMAN	35,884.65	8.01	2	Ig gamma-2 chain C region.	0.22
67	uniprotKB_sptr	P01871	IGHM_HUMAN	65,290.86	8.57	2	Ig mu chain C region.	0.5
29	uniprotKB_sptr	Q6GMW0	Q6GMW0_HUMAN	25,772.87	6.31	4	IGKV1-5 protein.	0.7
35	uniprotKB_sptr	Q16270	IBP7_HUMAN	26,443.00	8.25	4	Insulin-like growth factor-binding protein 7 precursor (IGFBP-7) (IBP- 7) (IGF-binding protein 7) (MAC25 protein) (Prostacyclin-stimulating factor) (PGI2-stimulating factor)	0.0

WO 2011/144614 PCT/GB2011/000784

							(IGFBP-rP1).	
31	uniprotKB_sptr	Q92876	KLK6_HUMAN	26,855.73	7.57	5	Kallikrein-6 precursor (EC 3.4.21.-) (Protease M) (Neurosin) (Zyme) (SP59) (Serine protease 9) (Serine protease 18).	0.4
11	uniprotKB_sptr	P13645	K1C10_HUMAN	59,510.71	5.18	10	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.7
11	uniprotKB_sptr	P13645	K1C10_HUMAN	59,510.71	5.18	8	Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10).	0.53
19	uniprotKB_sptr	P02533	K1C14_HUMAN	51,490.33	5.13	3	Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14).	0.59
26	uniprotKB_sptr	P02533	K1C14_HUMAN	51,621.52	5.13	2	Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14).	0.68
12	uniprotKB_sptr	P08779	K1C16_HUMAN	51,267.84	5.03	5	Keratin, type I cytoskeletal 16 (Cytokeratin-16) (CK-16) (Keratin-16) (K16).	0.60
10	uniprotKB_sptr	P35527	K1C9_HUMAN	62,129.47	5.24	12	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9).	0.76
8	uniprotKB_sptr	P35527	K1C9_HUMAN	62,129.47	5.24	12	Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9).	0.79
5	uniprotKB_sptr	P04264	K2C1_HUMAN	66,017.70	8.45	18	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.73
7	uniprotKB_sptr	P04264	K2C1_HUMAN	66,017.70	8.45	15	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).	0.63
14	uniprotKB_sptr	P35908	K22E_HUMAN	65,865.35	8.35	5	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2).	1.08
9	uniprotKB_sptr	P35908	K22E_HUMAN	65,865.35	8.35	7	Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e) (keratin-2).	0.33
18	uniprotKB_sptr	P13647	K2C5_HUMAN	62,378.37	8.14	4	Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) (58 kDa cytokeratin).	0.69
20	uniprotKB_sptr	P13647	K2C5_HUMAN	62,378.37	8.14	3	Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) (58 kDa cytokeratin).	0.58
15	uniprotKB_sptr	P02538	K2C6A_HUMAN	60,044.97	8.38	5	Keratin, type II cytoskeletal 6A (Cytokeratin-6A) (CK 6A) (K6a keratin) (Cytokeratin-6D) (CK 6D).	0.58
65	uniprotKB_sptr	P01042	KNG1_HUMAN	69,896.73	6.29	2	Kininogen-1 precursor (Alpha-2-thiol proteinase inhibitor) [Contains: Kininogen-1 heavy chain; Bradykinin (Kallidin I); Lysyl-bradykinin (Kallidin II); Kininogen-1 light chain; Low molecular weight growth- promoting factor].	3.9
30	uniprotKB_sptr	A2NUT2	A2NUT2_HUMAN	25,020.97	8.45	3	Lambda-chain precursor (AA -20 to 215).	0.8
46	uniprotKB_sptr	A2NUT2	A2NUT2_HUMAN	24,960.80	5.40	2	Lambda-chain precursor (AA -20 to 215).	0.3
43	uniprotKB_sptr	P61626	LYSC_HUMAN	16,537.02	9.50	3	Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C).	2.2
81	uniprotKB_sptr	P40925	MDHC_HUMAN	36,426.13	7.61	2	Malate dehydrogenase, cytoplasmic (EC 1.1.1.37) (Cytosolic malate dehydrogenase).	1.1

62	uniprotKB_sptr	P02795	MT2_HUMAN	6,042.16	8.38	2	Metallothionein-2 (MT-2) (Metallothionein-II) (MT-II) (Metallothionein-2A).	6.0
60	uniprotKB_sptr	P02795	MT2_HUMAN	6,042.16	8.38	3	Metallothionein-2 (MT-2) (Metallothionein-II) (MT-II) (Metallothionein-2A).	4.7
62	uniprotKB_sptr	P25713	MT3_HUMAN	6,926.97	5.00	3	Metallothionein-3 (MT-3) (Metallothionein-III) (MT-III) (Growth inhibitory factor) (GIF) (GIFB).	4.5
76	uniprotKB_sptr	P78559	MAP1A_HUMAN	######### #	4.86	2	Microtubule-associated protein 1A (MAP 1A) (Proliferation-related protein p80) [Contains: MAP1 light chain LC2].	1.56
58	uniprotKB_sptr	P46821	MAP1B_HUMAN	192,380.44	4.50	3	Microtubule-associated protein 1B (MAP 1B) [Contains: MAP1 light chain LC1].	1.83
70	uniprotKB_sptr	P02686_ISOFORM_3	MBP_HUMAN	21,493.21	11.45	3	Myelin basic protein (MBP) (Myelin A1 protein) (Myelin membrane encephalitogenic protein). [ISOFORM 3]	1.60
90	uniprotKB_sptr	P20916	MAG_HUMAN	69,040.49	4.97	2	Myelin-associated glycoprotein precursor (Siglec-4a).	0.98
53	uniprotKB_sptr	P02144	MYG_HUMAN	17,052.62	8.02	2	Myoglobin.	3.09
29	uniprotKB_sptr	O94760	DDAH1_HUMAN	31,121.78	5.64	6	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (EC 3.5.3.18) (Dimethylargininase-1) (Dimethylarginine dimethylaminohydrolase 1) (DDAHI) (DDAH-1).	2.21
44	uniprotKB_sptr	P07197	NFM_HUMAN	102,316.84	4.90	5	Neurofilament medium polypeptide (NF-M) (Neurofilament triplet M protein) (160 kDa neurofilament protein) (Neurofilament 3).	2.62
47	uniprotKB_sptr	P59665	DEF1_HUMAN	10,244.95	6.54	2	Neutrophil defensin 1 precursor (HNP-1) (HP-1) (HP1) (Defensin, alpha 1) [Contains: HP 1-56; Neutrophil defensin 2 (HNP-2) (HP-2) (HP2)].	0.17
98	uniprotKB_sptr	P10451	OSTP_HUMAN	33,016.56	4.59	2	Osteopontin precursor (Bone sialoprotein 1) (Secreted phosphoprotein 1) (SPP-1) (Urinary stone protein) (Nephropontin) (Uropontin).	0.52
15	uniprotKB_sptr	P62937	PPIA_HUMAN	18,012.50	8.34	10	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosporin A-binding protein).	3.28
74	uniprotKB_sptr	Q06830	PRDX1_HUMAN	22,110.36	8.59	3	Peroxiredoxin-1 (EC 1.11.1.15) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) (Proliferation-associated gene protein) (PAG) (Natural killer cell-enhancing factor A) (NKEF-A).	1.2
51	uniprotKB_sptr	P30041	PRDX6_HUMAN	208,794.31	5.37	4	Peroxiredoxin-6 (EC 1.11.1.15) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aiPLA2) (Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx) (24 kDa protein) (Liver 2D page spot 40) (Red blood cells page spot 12).	1.6
19	uniprotKB_sptr	P30086	PEBP1_HUMAN	21,056.79	7.76	7	Phosphatidylethanolamine-binding protein 1 (PEBP-1) (Prostatic-binding protein) (HCNPpp) (Neuropolypeptide h3) (Raf kinase inhibitor protein) (RKIP) [Contains:	3.3

							Hippocampal cholinergic neurostimulating peptide (HCNP)].	
28	uniprotKB_sptr	P36955	PEDF_HUMAN	46,342.31	6.12	6	Pigment epithelium-derived factor precursor (PEDF) (Serpin-F1) (EPC- 1).	0.1
54	uniprotKB_sptr	P00747	PLMN_HUMAN	63,245.42	6.99	2	Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide; Angiostatin; Plasmin heavy chain A, short form; Plasmin light chain B].	2.5
40	uniprotKB_sptr	P02775	SCYB7_HUMAN	6,982.36	9.64	3	Platelet basic protein precursor (PBP) (C-X-C motif chemokine 7) (Small-inducible cytokine B7) (Leukocyte-derived growth factor) (LDGF) (Macrophage-derived growth factor) (MDGF) [Contains: Connective tissue-activating peptide III (CTAP-III) (Low-affinity platelet factor IV) (LA-PF4); TC-2; Connective tissue-activating peptide III(1-81) (CTAP-III(1-81)); Beta-thromboglobulin (Beta-TG); Neutrophil- activating peptide 2(74) (NAP-2(74)); Neutrophil-activating peptide 2(73) (NAP-2(73)); Neutrophil-activating peptide 2 (NAP-2); TC-1; Neutrophil-activating peptide 2(1-66) (NAP-2(1-66)); Neutrophil-activating peptide 2(1-63) (NAP-2(1-63))].	3.13
46	uniprotKB_sptr	P20742	PZP_HUMAN	140,363.79	5.95	2	Pregnancy zone protein precursor.	0.78
45	uniprotKB_sptr	P07602	SAP_HUMAN	26,308.66	5.88	3	Proactivator polypeptide precursor [Contains: Saposin-A (Protein A); Saposin-B-Val; Saposin-B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin-C (Co-beta-glucosidase) (A1 activator) (Glucosylceramidase activator) (Sphingolipid activator protein 2) (SAP-2); Saposin-D (Protein C) (Component C)].	1.54
50	uniprotKB_sptr	P07602	SAP_HUMAN	26,308.66	5.88	4	Proactivator polypeptide precursor [Contains: Saposin-A (Protein A); Saposin-B-Val; Saposin-B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin-C (Co-beta-glucosidase) (A1 activator) (Glucosylceramidase activator) (Sphingolipid activator protein 2) (SAP-2); Saposin-D (Protein C) (Component C)].	1.70
66	uniprotKB_sptr	P07737	PROF1_HUMAN	15,054.23	8.79	2	Profilin-1 (Profilin I).	3.81
33	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18,698.03	8.68	5	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	0.2
17	uniprotKB_sptr	P41222_CHAIN_0	PTGDS_HUMAN	18,698.03	8.68	9	Prostaglandin-H2 D-isomerase precursor (EC 5.3.99.2) (Lipocalin-type prostaglandin-D synthase) (Glutathione-independent PGD synthetase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS2) (PGDS) (Beta-trace protein) (Cerebrin-28). [CHAIN 0]	0.6
56	uniprotKB_sptr	P00734	THRB_HUMAN	70,008.83	5.69	2	Prothrombin precursor (EC 3.4.21.5) (Coagulation factor II) [Contains: Activation peptide fragment 1; Activation peptide fragment 2; Thrombin light chain; Thrombin heavy chain].	2.6

WO 2011/144914 PCT/GB2011/000784

18	uniprotKB_sptr	Q6N030	Q6N030_HUMAN	57,019.61	8.48	2	Putative uncharacterized protein DKFZp686I15212.	0.3
34	uniprotKB_sptr	P14618_ISOFORM_M1	KPYM_HUMAN	58,062.08	7.99	5	Pyruvate kinase isozymes M1/M2 (EC 2.7.1.40) (Pyruvate kinase muscle isozyme) (Pyruvate kinase 2/3) (Cytosolic thyroid hormone-binding protein) (CTHBP) (THBP1). [ISOFORM M1]	1.6
59	uniprotKB_sptr	A0N5G5	A0N5G5_HUMAN	12,766.37	9.45	2	Rheumatoid factor D5 light chain (Fragment).	1.06
101	uniprotKB_sptr	A0N5G5	A0N5G5_HUMAN	12,766.37	9.45	2	Rheumatoid factor D5 light chain (Fragment).	0.53
85	uniprotKB_sptr	P05060	SCG1_HUMAN	78,246.25	5.03	2	Secretogranin-1 precursor (Secretogranin I) (SgI) (Chromogranin-B) (CgB) [Contains: GAWK peptide; CCB peptide].	1.29
69	uniprotKB_sptr	Q13228	SBP1_HUMAN	52,390.99	6.03	2	Selenium-binding protein 1 (56 kDa selenium-binding protein) (SP56).	1.42
6	uniprotKB_sptr	P02787	TRFE_HUMAN	77,049.89	6.97	23	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin).	0.91
5	uniprotKB_sptr	P02787_CHAIN_0	TRFE_HUMAN	76,959.79	7.16	29	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal- binding globulin). [CHAIN 0]	0.36
48	uniprotKB_sptr	Q9H299	SH3L3_HUMAN	10,437.72	5.03	3	SH3 domain-binding glutamic acid-rich-like protein 3 (SH3 domain- binding protein 1) (SH3BP-1).	1.88
84	uniprotKB_sptr	Q9H299	SH3L3_HUMAN	23,838.08	9.43	2	SH3 domain-binding glutamic acid-rich-like protein 3 (SH3 domain- binding protein 1) (SH3BP-1).	1.76
52	uniprotKB_sptr	O75368	SH3L1_HUMAN	12,774.25	5.53	4	SH3 domain-binding glutamic acid-rich-like protein.	2.77
48	uniprotKB_sptr	Q01082	SPTB2_HUMAN	274,609.33	5.40	4	Spectrin beta chain, brain 1 (Spectrin, non-erythroid beta chain 1) (Beta-II spectrin) (Fodrin beta chain).	2.25
42	uniprotKB_sptr	P16949	STMN1_HUMAN	17,336.53	5.94	4	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18) (Op18) (Leukemia-associated phosphoprotein p18) (pp17) (Prosolin) (Metablastin) (Protein Pr22).	2.15
49	uniprotKB_sptr	P00441	SODC_HUMAN	16,122.00	6.04	4	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1).	1.88
94	uniprotKB_sptr	P10599	THIO_HUMAN	9,451.99	6.56	2	Thioredoxin (Trx) (ATL-derived factor) (ADF) (Surface-associated sulphydryl protein) (SASP).	1.8
77	uniprotKB_sptr	P04216	THY1_HUMAN	12,553.22	9.33	2	Thy-1 membrane glycoprotein precursor (Thy-1 antigen) (CD90 antigen) (CDw90).	1.3
45	uniprotKB_sptr	P60174	TPIS_HUMAN	26,669.50	6.90	4	Triosephosphate isomerase (EC 5.3.1.1) (TIM) (Triose-phosphate isomerase).	1.4
99	uniprotKB_sptr	P09936	UCHL1_HUMAN	24,824.35	5.45	2	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (EC 3.4.19.12) (EC 6.-.-.-) (UCH-L1) (Ubiquitin thioesterase L1) (Neuron cytoplasmic protein 9.5) (PGP 9.5) (PGP9.5).	2.0
69	uniprotKB_sptr	P62988	UBIQ_HUMAN	25,761.65	7.78	2	Ubiquitin.	2.1

WO 2011/144914 PCT/GB2011/000784

36	uniprotKB_sptr	P62988	UBIQ_HUMAN	61,523.80	7.25	5	Ubiquitin.	2.7
74	uniprotKB_sptr	P02774	VTDB_HUMAN	52,963.66	5.47	2	Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDB).	0.8
73	uniprotKB_sptr	P25311	ZA2G_HUMAN	34,244.70	5.85	3	Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein) (Zn- alpha-2-GP).	0.6

Several proteins such as S100B, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP), have been already reported to be associated with stroke or other brain pathologies (25-27). The S100B protein was actually identified in Expb but with only one unique peptide (Phenyx peptide score of 11.67). Its IC/P ratio was 3.38. GSTP1, a protein which was initially found increased in post-mortem CSF (15, 20), exhibited an IC/P ratio of 2.79 in Expa. Several peroxiredoxins were also increased in IC samples as reported in Table 7.

Table 7: Increased ratios IC/P in microdialysis samples.

Protein description	*Ratio IC/P (Exp_a)*	*Ratio IC/P (Exp_b)*
Acyl-CoA-binding protein	1.95	2.67
Beta-2-microglobulin precursor	1.49	2.09
Coactosin-like protein	1.72	2.04
Complement C4-A precursor	2.50	1.10
Cystatin-B		2.68
Cysteine and glycine-rich protein 1	**3.33**	**2.88**
Fatty acid-binding protein, brain	2.65	
Fibrinogen alpha chain precursor	2.97	0.61
Glutathione S-transferase P	2.79	
Heterogeneous nuclear ribonucleoprotein G	2.35	
Metallothionein-3	**2.10**	**2.79**
Myelin basic protein [ISOFORM 3]	1.71	3.11
Neutrophil defensin 1 precursor		2.45
Paralemmin	3.52	
Peptidyl-prolyl cis-trans isomerase A	2.45	1.60
Peroxiredoxin-2	2.72	
Peroxiredoxin-6	**2.15**	**2.16**
Phosphatidylethanolamine-binding protein 1	2.06	1.60
Plasma retinol-binding protein precursor	2.83	1.63
Plasminogen precursor	2.27	
Platelet basic protein precursor	2.51	0.85
Profilin-1	2.40	0.91
SH3 domain-binding glutamic acid-rich-like protein	2.17	1.92
Thioredoxin	2.17	
Ubiquitin	2.09	1.50

Empty cases derive from the lack of the protein identification/quantitation in the studied sample. The values reported in bold indicate increased ratios (*i.e.*, superior to 2) for both patients (*i.e.*, patient a and b).

Although it was not reported in the table because of ratio value below the cut-off, PRDX1 was measured at a ratio of 1.93 in Expb. In the comparison of P and CT microdialysis samples, PRDX1 and peroxiredoxin-6 (PRDX6) were respectively measured with ratios of 1.24 and 1.69 in Expf.

Table 8. Increased ratios IC/CT in microdialysis samples.

Protein description	*Ratio IC/CT (Exp_c)*	*Ratio IC/CT (Exp_d)*
Acyl-CoA-binding protein	12.53	
Apolipoprotein A-II precursor		2.28
Apolipoprotein A-IV precursor		3.79
Carbonic anhydrase 1	0.29	4.78
Carbonic anhydrase 2		3.18
Chitinase-3-like protein 1 precursor	2.44	
Cofilin-1		2.00
Cystatin-B		2.10
Fibrinogen alpha chain precursor	0.46	2.33
Flavin reductase		2.22
Glial fibrillary acidic protein	4.13	0.37
Hemoglobin subunit alpha		3.21
Histone H1.2		2.97
Histone H1.5		2.03
Lysozyme C precursor		2.75
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	6.31	
Neurofilament medium polypeptide	4.68	
Neutrophil defensin 1 precursor		4.23
Peptidyl-prolyl cis-trans isomerase A	8.25	1.69
Phosphatidylethanolamine-binding protein 1	4.88	
Thymosin beta-10	4.16	
Thymosin beta-4	2.39	
Triosephosphate isomerase	3.50	
Tropomyosin alpha-3 chain		2.04

Empty cases derive from the lack of the protein identification/quantitation in the studied sample.

Table 9. Increased ratios P/CT in microdialysis samples.

Protein description	*Ratio P/CT (Exp_e)*	*Ratio P/CT (Exp_f)*
Aquaporin-4		2.00
Coactosin-like protein		2.64
Cystatin-B	2.16	1.81
Cysteine and glycine-rich protein 1		4.58
Diazepam binding inhibitor, splice form 1c		2.21
Fibrinogen alpha chain precursor	2.98	1.05
Glial fibrillary acidic protein	0.31	2.33
Hydroxyacylglutathione hydrolase		2.64
Kininogen-1 precursor	3.97	
Lysozyme C precursor	2.21	
Metallothionein-2	**6.02**	**4.73**
Metallothionein-3		4.57
Myoglobin	3.09	
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1		2.21
Neurofilament medium polypeptide		2.62
Peptidyl-prolyl cis-trans isomerase A		3.28
Phosphatidylethanolamine-binding protein 1		3.35
Plasminogen precursor	2.52	
Platelet basic protein precursor	3.13	
Profilin-1	3.81	
Prothrombin precursor	2.64	
SH3 domain-binding glutamic acid-rich-like protein		2.77
Spectrin beta chain, brain 1		2.25
Stathmin		2.15

Ubiquitin carboxyl-terminal hydrolase isozyme L1		2.06
Ubiquitin	**2.16**	**2.76**

Empty cases derive from the lack of the protein identification/quantitation in the studied sample. The values reported in bold indicate increased ratios (*i.e.*, superior to 2) for both patients (*i.e.*, patient e and f).

The proteins with ratio inferior to 0.5 are reported in Tables 10-12:

Table 10: Decreased ratios IC/P in microdialysis samples.

Protein description	*Ratio IC/P (Exp_a)*	*Ratio IC/P (Exp_b)*
Alpha-1-acid glycoprotein 1 precursor	**0.48**	**0.47**
Alpha-1-antitrypsin precursor	0.61	0.40
Alpha-1B-glycoprotein precursor		0.46
Carbonic anhydrase 1	0.45	1.60
Ceruloplasmin precursor		0.44
Fibrinogen beta chain precursor		0.46
Fibrinogen gamma chain precursor		0.41
Haptoglobin precursor	**0.46**	**0.36**
Hemoglobin subunit alpha	0.34	0.70
Hemoglobin subunit beta	0.37	0.80
Ig kappa chain C region	0.51	0.28
Keratin, type I cytoskeletal 10	0.72	0.39
Serotransferrin precursor	**0.42**	**0.50**
Serum albumin precursor	**0.40**	**0.33**

Empty cases derive from the lack of the protein identification/quantitation in the studied sample. The values reported in bold indicate increased ratios (*i.e.*, inferior to 0.5) for both patients (*i.e.*, patients a and b).

Table 11: Decreased ratios IC/CT in microdialysis samples.

Protein description	*Ratio IC/CT (Exp_c)*	*Ratio IC/CT (Exp_d)*
Alpha-1-antitrypsin precursor		0.49
Alpha-2-macroglobulin precursor		0.36
Beta-2-microglobulin precursor	0.80	0.44
Carbonic anhydrase 1	0.29	4.68
Chromogranin-A precursor	0.29	
Complement C3 precursor		0.43
Cystatin-C precursor	0.62	0.03
Dermcidin precursor	0.85	0.35
Fibrinogen alpha chain precursor	0.46	2.33
Fibrinogen beta chain precursor		0.38
Glial fibrillary acidic protein	4.13	0.37
Haptoglobin precursor	0.83	0.14
Ig gamma-2 chain C region		0.18
Keratin, type I cytoskeletal 10	0.99	0.48
Keratin, type II cytoskeletal 2 epidermal	0.97	0.43
Lambda-chain precursor		0.21
Myelin basic protein		0.30
Prostaglandin-H2 D-isomerase precursor	**0.27**	**0.46**
Putative uncharacterized protein		0.15
Serotransferrin precursor	1.47	0.42
Serum albumin precursor	1.17	0.38

SNC73 protein	0.30

Empty cases derive from the lack of the protein identification/quantitation in the studied sample. The values reported in bold indicate increased ratios (*i.e.*, inferior to 0.5) for both patients (*i.e.*, patients c and d).

Table 12: Decreased ratios P/CT in microdialysis samples.

Protein description	*Ratio P/CT (Exp_e)*	*Ratio P/CT (Exp_f)*
Alpha-1-antitrypsin	1.05	0.35
Alpha-1-acid glycoprotein 1 precursor	1.38	0.38
Alpha-2-macroglobulin precursor	0.68	0.36
Alpha-enolase	0.45	
Apolipoprotein E precursor	0.30	
Beta-2-microglobulin	0.92	0.25
Beta-Ala-His dipeptidase precursor	0.32	
Ceruloplasmin precursor		0.45
Clusterin	0.49	
Complement component 3	0.72	0.48
Complement factor B precursor	1.15	0.36
Cystatin-C precursor	**0.17**	**0.38**
Dermcidin precursor		0.34
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 precursor	0.46	
Glial fibrillary acidic protein	0.31	2.33
Haptoglobin precursor		0.45
Hemoglobin subunit alpha	0.11	0.55
Hemoglobin subunit beta	0.11	0.59
Ig gamma-2 chain C region	0.87	0.22
Insulin-like growth factor-binding protein 7 precursor	0.02	
Kallikrein-6 precursor		0.41
Keratin, type II cytoskeletal 2 epidermal	1.08	0.33
Lambda-chain precursor	0.89	0.35
Neutrophil defensin 1 precursor	0.17	
Pigment epithelium-derived factor precursor	0.15	
Prostaglandin-H2 D-isomerase precursor	0.20	0.64
Putative uncharacterized protein DKFZp686I15212		0.37
Serotransferrin precursor	0.91	0.36

Empty cases derive from the lack of the protein identification/quantitation in the studied sample. The values reported in bold indicate increased ratios (*i.e.*, inferior to 0.5) for both patients (*i.e.*, patients e and f).

Example 2: Validation of candidate biomarkers

Immunoassay experiments were carried out to confirm the quantitative measurements obtained with MS/MS. The choice of candidate biomarkers to be assessed was essentially based on the availability of commercial and in-house developed immunoassays.

GSTP1 protein (MW = 23 kDa) was probed with immunoblot analysis in pooled microdialysates samples (n = 3) as illustrated in Figure 2. The increase in the IC microdialysate with respect to the CT was undisputable, and corroborated the TMT-

based discovery results as well as previous studies of post- and ante-mortem CSF (15, 20).

Second, ELISAs were performed for GSTP1, PRDX1, and S100B on sera of control and stroke patients (n = 28). The ELISA results are given in Figure 3 and summarized in Table 13.

Table 13. Result summary for GSTP1, PRDX1, and S100B levels in sera samples quantified with ELISA.

	Control (n = 14)	*Stroke (n = 14)*	$p^{\S}$
GSTP1 ($\mu g \cdot L^{-1}$)			
Mean ± standard deviation	16.9 ± 5.1	143.1 ± 67.5	0.0002
Median (minimum-maximum)	15.7 (8.6-29.0)	119.0 (63.5-320.0)	
PRDX1 ($ng \cdot L^{-1}$)			
Mean ± standard deviation	7.3 ± 12.7	145.7 ± 144.0	0.0001
Median (min-max)	0.0 (0.0- 41.2)	81.9 (26.7-519.5)	
S100B ($\mu g \cdot L^{-1}$)			
Mean ± standard deviation	7.5 ± 10.0	78.9 ± 113.1	0.0093
Median (minimum-maximum)	1.8 (0.0-28.7)	23.1 (0.0-369.2)	

§ Wilcoxon matched pairs test.

GSTP1 was found significantly elevated in the blood of stroke patients compared to controls (p = 0.0002, Wilcoxon matched pairs test). The mean ratio in blood between stroke patients and controls was 8.47 (Table 13); i.e., three-times more than the ratio IC/P found in brain microdialysis samples (Expa, Table 7). Among the peroxiredoxin family, blood PRDX1 enabled to differentiate control from stroke patients at the p = 0.0001 level of significance. An increase of its levels of almost 20-times was observed in the stroke population. In accordance with results previously described in the literature (28, 29), the concentration measurements of blood S100B were significantly higher in stroke patients than controls (p = 0.0093).

Thus we disclose protein markers of stroke which we have illustrated by comparisons of microdialysis samples from the IC, P, and CT of ischemic stroke patients. Human brain microdialysates were analysed using an isobaric tagging technology coupled to peptide isoelectric focusing fractionation, and RP-LC MS/MS analysis. Increased levels

of GSTP1, PRDX1 and S100B in the IC microdialysates were further verified with immunoblot on pooled microdialysis samples and/or ELISA on blood of control and stroke patients. Thus we have clearly established the utility and applicability of the markers and methods presented herein.

Example 3: Protein amounts in microdialysis samples

Analyses with 1-D PAGE of the different microdialysis samples under study revealed slightly different patterns as well as large variations in the total concentration of proteins between samples (Figure 1 and Figure 5). These variations may possibly result from recovery issues for instance through the microdialysis membrane in addition to the biological variations and/or the severity of stroke. In a previous study of CT microdialysate of stroke patients, the protein concentration in 18 samples was determined to range from 0.083 to 0.395 g·L-1 with a mean content of 0.21 ± 0.11 g·L-1 (11). Smaller molecules such as glutamate, glycerol, lactate, and pyruvate were measured in 50 patients over the same sampling period respectively at 3.9 ± 0.3 µmol·L-1, 38.9 ± 1.9 µmol·L-1, 2.0 ± 0.1 mmol·L-1, and 58.4 ± 3.3 µmol·L-1 in the CT microdialysates, whereas in the IC the median for the same molecules was respectively 196.5 µmol·L-1 (ranging from 126.0 to 453.0), 600.5 µmol·L-1 (ranging from 464.2 to 1187.1), 6.1 mmol·L-1 (ranging from 0.1 to 12.0), and 17.4 µmol·L-1 (ranging from 4.2 to 591.7) (30). In brief, the protein concentration varied considerably from one sample to another in the CT microdialysates, whereas the concentrations of small molecules were uniform in the CT but more heterogeneous in the IC. Thus, a large protein concentration variation might be expected in the IC samples, as well as in the P microdialysates. Protein concentration variations were confirmed here.

Because of the differences in the total protein concentration, equalization of the samples was needed to carry out the quantitative proteomic study.

The samples to compare were equalized according to their protein amount (i.e., weight) before the quantitative analysis.

1-D PAGE images were used to compare the sample concentrations with densitometry (see Experimental Procedures). According to this relative protein quantitation, equal protein amounts between pairs to compare were taken for TMT2-based quantitative assessment.

As a consequence, a further normalization was performed on the TMT2 quantitative data. We hypothesized that most proteins, and therefore most peptides and reporter-ion signals, should be equal among samples (shown in Figure 4). Accordingly, the common areas between the frequency distribution of the peptides relative abundance

of both TMT2-labeled pairs had to be maximal. Besides, this processing was coherent with the first normalization performed from 1-D PAGE images.

Example 4: Increased and decreased proteins

To the best of our knowledge, this is the most-extensive proteomic study of human brain microdialysates, and the first one targeting brain ischemia (6). Through the study we have obtained a quantitative map of human brain microdialysates, as a monitoring of ECF in the brain of stroke patients. Depending on the brain region probed with microdialysis, relevant protein markers of stroke were discovered.

Many of the found proteins were identified previously in CSF (13, 20, 31). More precisely, several proteins with increased amount within the compared pairs (Tables 7-9) were previously identified in a comparative study of ante- and post-mortem CSF (13). This was the case for instance for cystatin-B, GFAP, S100B, PRDX1, and peroxiredoxin-2 (PRDX2). In that previous study, PRDX1 was increased with a ratio of 14.74 in post-mortem CSF compared to ante-mortem CSF. The correlation of many of the quantitative results between both studies not only validated the post-mortem CSF as a model of massive brain injury, but also highlighted the value of the quantitative proteome map obtained with the microdialysis samples.

In Tables 7-9, some proteins exhibited increased and decreased amounts. This was the case for fibrinogen alpha chain (FIBA), platelet basic protein, profilin-1, carbonic anhydrase 1, and GFAP. With a molecular weight of 95 kDa, FIBA might have been recovered inefficiently through the dialysis membrane. The variations could not be directly explained for the other proteins, yet, for instance, GFAP (50 kDa) can dimerize and oligomerize, as well as co-polymerize with other protein like vimentin, desmin, and annexin (32). Its recovery might have then been altered. When the sample in the methods of the invention is other than microdialysate, e.g. when the sample is CSF or blood, such problem(s) are advantageously avoided since there is no molecular weight cut-off when using such samples.

Ischemic stroke is caused by the disturbance of blood flow supplied to the brain. Cerebral blood flow was shown to be decreased in penumbra, and even more in infarct core (33). Interestingly, most of the decreased proteins found in the IC vs. P, IC vs. CT, and P vs. CT studies (Tables 10-12) were blood proteins (e.g., serum albumin, serotransferrin, haptoglobin, hemoglobins), somehow reflecting the regional variation of altered blood flow in these distinct brain areas.

Figure 7 displays the evolution of the protein levels that was observed in the MDs for proteins reported in the Tables above. Most of these proteins were found to increase from CT to P and from P to IC MDs. As shown in Figure 8, PRDX1 and PRDX6 were found

to increase from CT, P, and IC MDs. In most of the cases, the progression/elevation of the protein levels from the CT to the IC, as displayed in Figures 7 and 8, reflects a direct relationship with the severity of the cerebral damage. These results show a relevant biological trend to reinforce the medically relevant aspects of the invention.

Example 5: Further Validation of Biomarkers

Several identified proteins were selected to demonstrate the validity of our discovery approach, based on the availability of an alternative diagnostic tool (i.e., ELISA) and/or a strong scientific rationale for involvement in brain ischemia. S100B, a well-documented biomarker of brain damage (34), is a calcium binding and growth-regulating secretory protein that is highly expressed in brain tissues (9). The concentration of S100B has been assessed in many brain insults and dysfunctions. S100B was increased in stroke (28, 29), SAH (35), and TBI (36). S100B was previously measured in the brain ECF of two patients with acute brain injury using the microdialysis technique (37). The detection and increased level determination of S100B in one IC microdialysate compared to a P sample, as well as its validation in the blood of stroke patients, confirmed the findings reported here, and demonstrated the great value of the studied samples.

GSTP1 protein is an enzyme that is able to inactivate many toxic, electrophiles and organic peroxides (38). GSTP1 is one the three glutathione S-transferases described in the central nervous system (39). Several studies suggested its association with Parkinson's disease (40). High levels of GSTP1 were recently reported in CSF of late stage patients suffering human African trypaniosomiasis (21). The protein is known to be associated with early brain cell death because it was found with increased concentration in CSF of deceased patient compared to alive ones (20). High correlation of the increase of GSTP1 in microdialysis and blood samples stressed the relevance of the obtained quantitative proteome maps of the brain microdialysates of stroke patients as a pertinent model for the discovery of brain markers.

Peroxiredoxins are ubiquitous antioxidant enzymes involved in the degradation of oxygen peroxide and other reactive oxygen species (41, 42). These thiol-specific antioxidant proteins are also termed thioredoxin peroxidases. The family of peroxiredoxins is composed of six distinct groups that can be classified in two categories, the 1-Cys and 2-Cys peroxiredoxins, according to the number of cysteine residues involved in the reduction process. Peroxiredoxin-6 (PRDX6) is actually the sole 1-Cys member. In the brain, PRDX1 and PRDX6 were shown to be primarily expressed in astrocytes whereas PRDX2 was expressed exclusively in neurons (43, 44). PRDX2 was significantly increased in the substantia nigra from Parkinson's disease patients (45), and in the frontal cortex and cerebellum of patients with Down syndrome, Alzheimer's

disease, and Pick's disease (46). PRDX1 was demonstrated to be part of an adaptive response to oxidative stress in brain endothelial cells and have protective effects at the injured blood-brain barrier (47). Herein, the increased amounts and increased concentrations of PRDX1 in respectively the microdialysates of the injured parts of the brain, and the blood of stroke patients appeared therefore highly relevant for further investigation in cerebrovascular diseases. Very interestingly, PRDX1 and GSTP1 are implicated in similar redox protective mechanisms, and were evidenced to interact together (48). As well, GSTP1 was shown to reactivate oxidized PRDX6 (49) through the formation of a complex (50).

Malignant MCA infarction patients as those included in our study are severely impaired patients that receive several treatments at the neurointensive care units, such as moderate hypothermia, that might modify the expression pattern of some of the described proteins. Another limitation is that the recovery rates through the 100 kDa microdialysis probes are unknown for most of the discovered proteins. It may advantageously be possible to alleviate these limitations by choosing a sample which is not collected through a molecular weight-limited route e.g. by using CSF or blood as the sample.

In conclusion, the present study explored the brain microdialysates of stroke patients through proteomic analysis. Qualitative results offered an extensive proteome map of microdialysates, and extracellular fluid from the human brain. Moreover, quantitative comparisons of microdialysates of several areas of the human ischemic brain were shown to provide a valuable source of biomarkers for cerebrovascular diseases. Several of the increased proteins were verified on blood samples of a small cohort of control and stroke patients. The correlation between discovery and early validation data demonstrated that many of the discovered proteins represent biomarkers for the diagnosis and/or prognosis of stroke, as well as other acute brain damage related disorders.

Example 6: Changes in protein levels associated with decreased cerebral blood flow

In vivo human brain extracellular fluids (ECF) of acute ischemic stroke patients were investigated to assess the changes in protein levels associated to decreased cerebral blood flow. Microdialysates (MDs) from the infarct core (IC), the penumbra (P), and the unaffected contralateral (CT) brain regions of patients suffering an ischemic stroke were compared using a shotgun proteomic approach based on isobaric tagging and mass spectrometry (MS). Quantitative analysis showed 53 proteins with increased amounts in the IC or P with respect to the CT samples. Glutathione S-transferase P (GSTP1), peroxiredoxin-1 (PRDX1), and protein S100-B (S100B) were further assessed with

ELISA on the blood of unrelated control and stroke patients (n = 28). Significant increases of 8, 20, and 11-fold were found respectively. Taken together, these results demonstrated clear differences in ECF protein levels between P and IC associated to ischemic damages. In addition, the evaluation of PRDX1 highlighted the value of ECF as an efficient source to further discover blood stroke markers.

Microdialysis sampling of stroke patients was approved by the local institutional ethical committee. Malignant middle cerebral artery infraction patients were monitored with high-cut-off (100 kDa) cerebral microdialysis catheters. Computed tomography scan was used to confirm brain microdialysis catheter location. MDs were obtained hourly for 5 days after perfusion with an artificial CSF solution. Proteomic analysis was performed on brain MDs obtained during the first 24 h of brain monitoring. The 2-plex isobaric Tandem Mass Tag (TMT) technology (Dayon et al 2008) was used to label trypsin-digested extracts from two brain regions of six patients suffering stroke (Figure 9). Following the labeling, the pooled samples were first fractionated by off-gel electrophoresis (OGE). The fractions were then analyzed by reversed-phase liquid chromatography (RP-LC) and matrix-assisted laser desorption tandem time-of-flight (MALDI TOF/TOF) MS (Figure 10). The identification and quantitation of proteins were assessed with stringent criteria using Phenyx search in the Swiss-Prot human database.

Immunoblot validation was carried out for GSTP1. Pooled IC and CT MDs (n = 3) were separated with 1-D SDS PAGE (15%). Immunodetection was performed with the anti-human GSTP1 rabbit polyclonal antibody. S100B, GSTP1, and PRDX1 were further validated with ELISA of blood of control and stroke patients (n =28). S100B and PRDX1 were validated using commercial ELISA kits. Concerning GSTP1, no commercial assay being currently available, a sandwich home-made was developed as previously described in (Burgess et al 2006; Hainard et al 2009).

Microdialysis is a bioanalytical sampling tool to continuously monitor events occurring in living tissues. It is based on probing ECF and allows collecting endogenous substances from the extracellular space, which can diffuse through the semi-permeable membrane at the tip of the microdialysis probe. Such a technique is quite appropriate to search and follow biochemical markers in real-time in many organs. The proteomic comparisons of human brain MDs showed significantly over-represented proteins (with a ratio superior to 2) in the IC compared to the CT and P counterparts (Figure 10). Proteins such as glial fibrillary acidic protein (GFAP) and S100B have already been reported to be associated to brain damage and appeared to be increased in one or several IC MDs. Figure 8 displays an example of the evolution of the protein levels that

was observed in the MDs for 2 peroxiredoxin proteins. Many proteins were found to increase from CT to P and from P to IC MDs.

Similarly, the increase in GSTP1 was validated with immunoblot experiments in pooled MDs (n = 3) as illustrated in Figure 2.

The level of S100B, GSTP1, and PRDX1 in serum was also measured by ELISA in the serum of 14 stroke patients and 14 controls (Figure 3) demonstrating their utility as peripheral markers of brain damage caused by reduced blood flow in ischaemic stroke.

The GSTP1 concentration was found significantly elevated in the blood of stroke patients compared to controls ($p = 0.0002$, Wilcoxon matched pairs test). The mean ratio in blood between stroke patients and controls was 8.47. Blood PRDX1 level enabled to differentiate control from stroke patients at the $p = 0.0001$ level of significance. An increase of its levels of almost 20-times was observed in the stroke population. In accordance with previous results (Buttner et al 1997; Missler et al 1997), the concentration measurements of blood S100B were significantly higher in stroke patients than controls ($p = 0.0093$).

This example explored the brain MDs of stroke patients with proteomic analysis. Qualitative results offered an extensive proteome map of microdialysates and ECF from the human brain. Moreover, quantitative comparisons of MDs of the IC, P and CT parts of the human brain were shown to provide a valuable source of biomarkers for cerebrovascular diseases. Several of the increased proteins were verified on a small cohort of control and stroke patients. The correlation between discovery and early validation data demonstrated the industrial application of the invention for the diagnosis and/or prognosis of stroke, as well as other brain damage related disorders.

Example 7: Selection of enlarged panel proteins

In vivo human brain extracellular fluids (ECF) of acute ischemic stroke patients were previously investigated to assess the changes in protein levels associated to decreased cerebral blood flow as described herein. Microdialysates (MDs) from the infarct core (IC), the penumbra (P), and the unaffected contralateral (CT) brain regions of patients suffering an ischemic stroke ($n = 6$) were compared using a shotgun proteomic approach based on isobaric tagging and mass spectrometry (MS). Quantitative analysis showed 53 proteins with increased amounts in the IC or P with respect to the CT samples. These results demonstrated clear differences in ECF protein levels between CT,

P and IC associated to ischemic damage. Glutathione S-transferase P (GSTP1), peroxiredoxin-1 (PRDX1), and protein S100-B (S100B) were further assessed with ELISA on the blood of unrelated control (n = 14) and stroke (n = 14) patients. Significant increases of 8 (p = 0.0002), 20 (p = 0.0001), and 11-fold (p = 0.0093) were found respectively. These highlighted the value of ECF as an efficient source to further discover blood stroke markers.

Whilst GSTP-1 and Peroxiredoxins 1 and 6 represent useful markers for management of stroke, we wished to construct larger panels of proteins to further improve diagnostic sensitivity and/or specificity and/or provide prognostic information. We therefore undertook the verification and validation of the stroke biomarker candidates found previously in MDs.

Following a comprehensive bioinformatic analysis of candidate proteins, three groups of biomarkers were selected in descending priority order:

PANEL A	**ID**	**Description**
N°1	ACBP_HUMAN	Acyl-CoA-binding protein
N°2	CSRP1_HUMAN	Cysteine and glycine-rich protein 1
N°3	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1
N°4	DDAH1_HUMAN	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
N°5	MT3_HUMAN	Metallothionein-3 (MT-3)
N°6	CYTB_HUMAN	Cystatin-B

PANEL B	**ID**	**Description**
N°1	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A
N°2	NFM_HUMAN	Neurofilament medium polypeptide
N°3	UBIQ_HUMAN	Ubiquitin.
N°4	B2MG_HUMAN	Beta-2-microglobulin precursor
N°5	CYTC_HUMAN	Cystatin-C precursor (Cystatin-3)
N°6	SH3L1_HUMAN	SH3 domain-binding glutamic acid-rich-like protein.
N°7	TPIS_HUMAN	Triosephosphate isomerase
N°8	MBP_HUMAN	Myelin basic protein (MBP)
N°9	MT2_HUMAN	Metallothionein-2 (MT-2)

PANEL C	ID	Description
N°1	NFM_HUMAN	Neurofilament medium polypeptide
N°2	COTL1_HUMAN	Coactosin-like protein.
N°3	THY1_HUMAN	Thy-1 membrane glycoprotein precursor
N°4	PROF1_HUMAN	Profilin-1
N°5	TYB4_HUMAN	Thymosin beta-4
N°6	MT1E_HUMAN	Metallothionein-1E
N°7	FABPB_HUMAN	Fatty acid-binding protein, brain (B-FABP)
N°8	GFAP_HUMAN	Glial fibrillary acidic protein (GFAP).
N°9	CAH2_HUMAN	Carbonic anhydrase 2
N°10	CERU_HUMAN	Ceruloplasmin precursor
N°11	DCD_HUMAN	Dermcidin precursor
N°12	DEF1_HUMAN	Neutrophil defensin 1 precursor (HNP-1

Together, Panels A, B and C form an enlarged panel, referred to as enlarged panel ABC.

Among the 53 biomarker candidates reported above, N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1_HUMAN), cystatin-B (CYTB_HUMAN), acyl-CoA-binding protein (ACBP_HUMAN), cysteine and glycine-rich protein 1 (CSRP1_HUMAN), metallothionein-3 (MT3_HUMAN), and phosphatidylethanolamine-binding protein 1 (PEPB1_HUMAN) (Panel A) have been prioritised.

Example 8: Selected Reaction Monitoring Mass Spectrometry Method for Measuring Signature-Peptides of Stroke Biomarker Candidates

To provide further validation of the enlarged panel proteins single protein and multiplex protein assays are developed using immunoassay (ELISA) and mass spectrometry (MRM) methods.

This example demonstrates the rapid ability of MRM to develop a multiplex panel. In this example we selected proteins from Panel A of example 7 to use in order to illustrate the method. However, it is not intended that this method be limited to that specific panel of biomarkers. This panel of biomarkers is being used as a convenient panel to help understand how to carry out one advantageous mode of detection. The same mode of detection can be used for any other group of markers disclosed herein, simply

by following the method set out here but instead using the markers of a different panel or group as disclosed.

Thus, this example shows the development and evaluation of a method based on selected reaction monitoring (SRM) MS to detect selectively signature-peptides of the prioritised stroke biomarker candidates of Panel A.

DESIGN OF THE METHOD

Design of an MRM method first requires selection of target peptides representative of each marker protein (proteotypic peptides). The second step involves selection of specific peptide fragments that will arise in collision-induced dissociation of the parent peptide during tandem mass spectrometry. The difference in the mass-to-charge (m/z) ratio of the parent and daughter ions are known as transitions.

An *in silico* approach was used to select proteotypic tryptic signature-peptides representative of each stroke biomarker candidate. A total of 7, 4, 7, 3, 3 and 6 proteotypic signature-peptides were selected for DDAH1, CYTB, ACBP (3 isoforms), CSRP1, MT3 and PEPB1 respectively.

The signature-peptide selection was based on i) uniqueness of the peptide sequence in the human protein database (UniProt Swiss-Prot) determined with the home-made Proteotype software, ii) *m/z* value of the peptide precursor-ion for relevant MS detection, and iii) absence of cysteine and methionine residues in the sequence when possible (Table 14 below).

Table 14: Proteotypic peptides of proteins useful in the diagnosis and/or prognostic monitoring of a subject with acute brain damage

accession	description	prev.res	sequence	next.res	observation in the Peptide Atlas (http://www.peptideatlas.org/ accessed on 05/04/2011)	observation in discovery (*J. Proteome Res.*, 2011, 10 (3), pp 1043–1051)	position	frequency in human_uniprot_sprot_varsplic	mass	predicted charge	m/z	rt factor
sp|O94760|DDAH1_HUMAN	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	R	***ALPESLGQHALR***	S	3	3	20:31	1	1291.712	3	431.2	42.3
		K	***DENATLDGGDVLFTGR***	E	6	0	121:136	1	1679.787	2	840.4	55.2
		K	***DYAVSTVPVADGLHLK***	S	55	0	160:175	1	1684.89	3	562.3	56.2
		R	***GAEILADTFK***	D	4	2	150:159	1	1064.562	2	532.8	56.1
		K	***GEEVDVAR***	A	0	0	35:42:00	1	874.4265	2	437.7	26.6
		R	***QHQLYVGVLGSK***	L	0	0	46:57:00	1	1328.732	3	443.6	42.9
		R	***TPEEYPESAK***	V	4	2	238:247	1	1150.526	2	575.8	17.8
sp|P04080|CYTB_HUMAN	Cystatin-B	K	***HDELTYF***	H	0	0	92:98	1	924.4098	2	462.7	49.6
		K	***SQVVAGTNYFIK***	V	56	0	45:56:00	1	1326.705	2	663.9	51.7
		R	***VFQSLPHENKPLTLSNYQTNK***	A	367	0	69:89	1	2458.272	4	615.3	47.1
		K	VHVGDEDFVHLR	V	564	4	57:68	1	1422.712	4	356.4	38.4
sp|P07108|ACBP_HUMAN	Acyl-CoA-binding protein	-	MSQAEFEK	A	0	0	01:08	1	969.4346	2	485.2	23.3
		K	AAEEVR	H	0	1	09:14	3	674.3468	2	337.7	19.0
		K	QATVGDINTERPGMLDFTGK	A	342	0	34:53:00	3	2150.055	3	717.4	55.0
		K	TKPSDEEMLFIYGHYK	Q	63	6 (3 × oxidised)	18:33	3	1957.936	4	490.2	58.1
		K	***WDAWNELK***	G	12	7	56:63	3	1061.505	2	531.3	41.1
sp|P07108-2|ACBP_HUMAN	Isoform 2 of Acyl-CoA-binding protein	-	MWGDLWLLPPASANPGTGTEAEFEK	A	0	0	7108:7132	1	2717.292	2	1359	88.9
sp|P07108-3|ACBP_HUMAN	Isoform 3 of Acyl-CoA-binding protein	-	MPAFAEFEK	A	0	0	7108:7116	1	1069.502	2	535.3	38.9
sp|P21291|CSRP1_HUMAN	Cysteine and glycine-rich protein 1	K	***GFGFGQGAGALVHSE***	G	30	3	179:193	1	1433.681	2	717.3	59.2

		K	***GLESTTLADK***	D	2	0	152:161	1	1034.536	2	517.8	41.9
		K	***GYGYGQGAGTLSTDK***	G	4	0	70:84	1	1474.681	2	737.8	31.1
sp|P25713|MT3_HUMAN	Metallothionein-3	K	***GGEAAEAEAEK***	C	0	8	53:63	1	1061.475	2	531.2	20.0
		-	MDPETCPCPSGGSCTCADSCK	C	0	0	01:21	1	2376.833	2	1189	-5.4
		K	SCCSCCPAECEK	C	0	6	33:44:00	1	1547.505	2	774.3	3.5
sp|P30086|PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	K	***GNDISSGTVLSDYVGSGPPK***	G	412	7	94:113	1	1949.945	2	975.5	45.9
		K	***LYEQLSGK***	-	10	11	180:187	1	937.4989	2	469.3	27.8
		K	***LYTLVLTDPDAPSR***	K	159	10	63:76	1	1560.827	2	780.9	48.1
		K	***NRPTSISWDGLDSGK***	L	467	5	48:62	1	1632.798	3	544.9	36.7
		K	***VLTPTQVK***	N	319	1	40:47:00	1	885.5404	2	443.3	29.4
		R	***YVWLVYEQDRPLK***	C	47	6	120:132	1	1708.906	3	570.3	59.1
sp|P09211|GSTP1_HUMAN	Glutathione S-transferase P	K	TFIVGDQISFADYNLLDLLLIHEVLAPGC LDAFPLLSAYVGR	L	ND	ND	142:183	1	4649.436	3	1550	223.3
		-	MPPYTVVYFPVR	G	ND	ND	01:12	1	1468.766	2	734.9	44.04
		K	DDYVK	A	ND	ND	117:121	1	639.2984	2	320.2	24.38
		K	DQQEAALVDMVNDGVEDLR	C	ND	ND	83:101	1	2116.982	2	1059	70.67
		K	FQDGDLTLYQSNTILR	H	ND	ND	56:71	1	1883.95	2	942.5	62.89
		K	ASCLYGQLPK	F	ND	ND	46:55:00	1	1136.577	2	568.8	37.34
		K	AFLASPEYVNLPINGNGK	Q	ND	ND	192:209	1	1903.991	2	952.5	65.59
		R	MLLADQGQSWK	E	ND	ND	20:30	1	1276.635	2	638.8	47.63
		R	LSARPK	L	ND	ND	184:189	1	671.4199	3	224.5	1.119
		R	TLGLYGK	D	ND	ND	76:82	1	751.4349	2	376.2	39.09
		K	EEVVTVETWQEGSLK	A	ND	ND	31:45:00	1	1733.859	2	867.4	58.34
		K	ALPGQLKPFETLLSQNQGGK	T	ND	ND	122:141	1	2126.16	3	709.4	60.47
		K	YISLIYTNYEAGK	D	ND	ND	104:116	1	1534.779	2	767.9	55.73
sp|P30041|PRDX6_HUMAN	Peroxiredoxin-6	K	GMPVTAR	V	ND	ND	126:132	1	731.3869	2	366.2	30.53
		-	MPGGLLLGDVAPNFEANTTVGR	I	ND	ND	01:22	1	2229.133	2	1115	63.65

		R	DFTPVCTTELGR	A	ND	ND	42:53:00	1	1395.657	2	698.3	40.89
		R	VVFVFGPDK	K	ND	ND	133:141	1	1007.556	2	504.3	49.98
		K	LIALSIDSVEDHLAWSK	D	ND	ND	68:84	1	1897.007	3	633	79.81
		R	ELAILLGMLDPAEK	D	ND	ND	109:122	1	1512.834	2	756.9	91.41
		K	LSILYPATTGR	N	ND	ND	145:155	1	1191.673	2	596.3	37.97
		R	VATPVDWK	D	ND	ND	175:182	1	915.4934	2	458.3	34.03
		R	NFDEILR	V	ND	ND	156:162	1	906.4679	2	453.7	49.47
		K	LPFPIIDDR	N	ND	ND	98:106	1	1085.599	2	543.3	47.47
		R	VVISLQLTAEK	R	ND	ND	163:173	1	1200.72	2	600.9	56.15
		K	DINAYNCEEPTEK	L	ND	ND	85:97	1	1582.669	2	791.8	26.56
		K	LAPEFAK	R	ND	ND	57:63	1	775.4348	2	388.2	28.4
		K	DGDSVMVLPTIPEEEAK	K	ND	ND	183:199	1	1829.884	2	915.4	63.4
		R	FHDFLGDSWGILFSHPR	D	ND	ND	25:41:00	1	2030.987	4	508.5	76.44
sp|Q06830|PRDX1_HUMAN	Peroxiredoxin-1	K	HGEVCPAGWKPGSDTIKPDVQK	S	ND	ND	169:190	1	2406.187	5	482	22
		K	QGGLGPMNIPLVSDPK	R	ND	ND	94:109	1	1622.857	2	811.9	58.44
		K	ADEGISFR	G	ND	ND	121:128	1	894.4316	2	447.7	35.7
		K	DISLSDYK	G	ND	ND	28:35:00	1	940.4622	2	470.7	42.1
		R	LVQAFQFTDK	H	ND	ND	159:168	1	1196.631	2	598.8	47.24
		K	IGHPAPNFK	A	ND	ND	08:16	1	980.5312	3	327.5	16.05
		K	LNCQVIGASVDSHFCHLAWVNTPK	K	ND	ND	69:92	1	2753.329	4	689.1	63.37
		K	YVVFFFYPLDFTFVCPTEIIAFSDR	A	ND	ND	38:62	1	3093.511	2	1547	153.1
		-	MSSGNAK	I	ND	ND	01:07	1	694.3189	2	347.7	4.153
		R	TIAQDYGVLK	A	ND	ND	111:120	1	1107.604	2	554.3	46.78
		K	ATAVMPDGQFK	D	ND	ND	17:27	1	1164.572	2	582.8	44.71

To aid the selection of the most appropriate transitions for each peptide, previous empirical observations of the peptides in a public repository of tandem mass spectra (the Peptide Atlas [http://www.peptideatlas.org/]) and/or during the preceding discovery exercise (described above) were reviewed.

As a preferred but not limiting method an intelligent SRM (iSRM) method was set up consisting of a combination of so-called primary and secondary transitions. In such an approach, when all primary transitions relative to a given peptide are detected above a defined threshold, secondary transitions are then triggered to help confirming the identity of the targeted molecule. Here, the approach aimed to reduce the number of transitions to be continuously monitored in the assay and evaluate the peptide detection level in a particular matrix.Two primary and 6 secondary transitions were selected as reported in Table 15.

Table 15: List of transitions for use in SRM methods for diagnosis and/or prognostic monitoring of a subject with acute brain damage

Q1 (m/z)	Q3 (m/z)	Collision Energy (V)	S-lens (V)	Primary (0) / Secondary (1)	Peptide sequence	Protein description	Precursor charge state	Product charge state	Ion type
337.677	272.124	15	68	1	AAEEVR	sp|P07108|ACBP_HUMAN Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2	2	1	b3
337.677	274.187	15	68	1			2	1	y2
337.677	302.158	15	68	1			2	2	y5
337.677	401.166	15	68	0			2	1	b4
337.677	403.229	15	68	0			2	1	y3
337.677	500.235	15	68	1			2	1	b5
337.677	532.272	15	68	0			2	1	y4
337.677	603.309	15	68	0			2	1	y5
437.717	345.224	18	98	1	GEEVDVAR	sp|O94760|DDAH1_HUMAN N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	y3
437.717	415.182	18	98	1			2	1	b4
437.717	460.251	18	98	0			2	1	y4
437.717	530.209	18	98	0			2	1	b5
437.717	559.319	18	98	0			2	1	y5
437.717	629.277	18	98	1			2	1	b6
437.717	688.362	18	98	0			2	1	y6
437.717	700.314	18	98	1			2	1	b7
443.274	411.26	18	100	0	VLTPTQVK	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	b4
443.274	475.287	18	100	1			2	1	y4
443.274	512.307	18	100	1			2	1	b5
443.274	572.34	18	100	0			2	1	y5
443.274	640.366	18	100	1			2	1	b6
443.274	673.387	18	100	0			2	1	y6
443.274	739.434	18	100	0			2	1	b7

443.274	786.471	18	100	1			2	1	y7
462.708	382.135	19	105	1	HDELTYF	sp|P04080|CYTB_HUMAN Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	2	1	b3
462.708	430.197	19	105	1			2	1	y3
462.708	495.219	19	105	0			2	1	b4
462.708	543.281	19	105	1			2	1	y4
462.708	596.267	19	105	0			2	1	b5
462.708	672.323	19	105	0			2	1	y5
462.708	759.33	19	105	0			2	1	b6
462.708	787.35	19	105	1			2	1	y6
469.253	277.154	19	107	1	LYEQLSGK	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	b2
469.253	291.166	19	107	1			2	1	y3
469.253	534.255	19	107	1			2	1	b4
469.253	647.339	19	107	1			2	1	b5
469.253	661.351	19	107	0			2	1	y6
469.253	734.371	19	107	1			2	1	b6
469.253	791.393	19	107	1			2	1	b7
469.253	824.414	19	107	0			2	1	y7
485.221	347.138	20	112	1	MSQAEFEK	sp|P07108|ACBP_HUMAN Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2	2	1	b3
485.221	419.7	20	112	1			2	2	y7
485.221	423.223	20	112	0			2	1	y3
485.221	547.218	20	112	1			2	1	b5
485.221	552.266	20	112	1			2	1	y4
485.221	623.303	20	112	0			2	1	y5
485.221	751.362	20	112	0			2	1	y6
485.221	838.394	20	112	0			2	1	y7
517.772	446.26	21	122	1	GLESTTLADK	sp|P21291|CSRP1_HUMAN Cysteine and glycine-rich protein 1 OS=Homo sapiens GN=CSRP1 PE=1 SV=3	2	1	y4
517.772	488.235	21	122	1			2	1	b5

517.772	547.308	21	122	1			2	1	y5
517.772	648.356	21	122	1			2	1	y6
517.772	702.366	21	122	1			2	1	b7
517.772	735.388	21	122	0			2	1	y7
517.772	773.403	21	122	1			2	1	b8
517.772	864.43	21	122	0			2	1	y8
531.241	476.235	21	126	1			2	1	y4
531.241	515.209	21	126	1			2	1	b6
531.241	547.272	21	126	0			2	1	y5
531.241	586.246	21	126	1	GGEAAEAEAEK	sp|P25713|MT3_HUMAN Metallothionein-3 OS=Homo sapiens GN=MT3 PE=1 SV=1	2	1	b7
531.241	676.314	21	126	0			2	1	y6
531.241	747.351	21	126	0			2	1	y7
531.241	818.389	21	126	0			2	1	y8
531.241	915.369	21	126	1			2	1	b10
531.256	503.282	21	126	1			2	1	y4
531.256	559.229	21	126	1			2	1	b4
531.256	673.272	21	126	1			2	1	b5
531.256	689.361	21	126	0	WDAWNELK	sp|P07108|ACBP_HUMAN Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2	2	1	y5
531.256	760.398	21	126	0			2	1	y6
531.256	802.315	21	126	1			2	1	b6
531.256	875.425	21	126	1			2	1	y7
531.256	915.399	21	126	1			2	1	b7
532.785	555.313	21	126	1			2	1	b6
532.785	581.292	21	126	1			2	1	y5
532.785	670.34	21	126	1	GAEILADTFK	sp|O94760|DDAH1_HUMAN N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	b7
532.785	694.377	21	126	1			2	1	y6
532.785	771.388	21	126	0			2	1	b8

532.785	807.461	21	126	0			2	1	y7
532.785	918.456	21	126	1			2	1	b9
532.785	936.503	21	126	1			2	1	y8
535.255	423.223	22	127	1			2	1	y3
535.255	447.206	22	127	1			2	1	b4
535.255	469.734	22	127	1			2	2	y8
535.255	552.266	22	127	1	MPAFAEFEK	sp|P07108-3|ACBP_HUMAN Isoform 3 of Acyl-CoA-binding protein OS=Homo sapiens GN=DBI	2	1	y4
535.255	623.303	22	127	0			2	1	y5
535.255	770.371	22	127	0			2	1	y6
535.255	841.409	22	127	0			2	1	y7
535.255	938.461	22	127	0			2	1	y8
575.767	457.192	23	139	1			2	1	b4
575.767	531.277	23	139	1			2	1	y5
575.767	620.256	23	139	1			2	1	b5
575.767	694.34	23	139	0	TPEEYPESAK	sp|O94760|DDAH1_HUMAN N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	y6
575.767	823.383	23	139	0			2	1	y7
575.767	846.351	23	139	1			2	1	b7
575.767	933.383	23	139	1			2	1	b8
575.767	952.425	23	139	1			2	1	y8
646.359	359.24	25	160	1			2	1	y3
646.359	441.251	25	160	1			2	2	y8
646.359	554.299	25	160	0			2	2	y10
646.359	624.357	25	160	1	ALPESLGQHALR	sp|O94760|DDAH1_HUMAN N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	y5
646.359	681.379	25	160	0			2	1	y6
646.359	881.495	25	160	1			2	1	y8
646.359	933.478	25	160	1			2	1	b9
646.359	1107.59	25	160	1			2	1	y10

663.856	485.271	26	165	1	SQVVAGTNYFIK	sp|P04080|CYTB_HUMAN Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	2	1	b5
663.856	785.419	26	165	1			2	1	y6
663.856	842.44	26	165	0			2	1	y7
663.856	913.477	26	165	0			2	1	y8
663.856	1012.546	26	165	1			2	1	y9
663.856	1067.515	26	165	1			2	1	b10
663.856	1111.614	26	165	1			2	1	y10
663.856	1180.599	26	165	1			2	1	b11
664.87	507.267	26	165	1	QHQLYVGVLGSK	sp|O94760|DDAH1_HUMAN N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	b4
664.87	560.34	26	165	1			2	1	y6
664.87	659.408	26	165	1			2	1	y7
664.87	670.33	26	165	1			2	1	b5
664.87	769.399	26	165	1			2	1	b6
664.87	822.471	26	165	0			2	1	y8
664.87	935.556	26	165	1			2	1	y9
664.87	1063.614	26	165	0			2	1	y10
711.86	752.32	28	179	1	VHVGDEDFVHLR	sp|P04080|CYTB_HUMAN Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	2	1	b7
711.86	786.425	28	179	1			2	1	y6
711.86	915.468	28	179	1			2	1	y7
711.86	998.457	28	179	1			2	1	b9
711.86	1030.495	28	179	1			2	1	y8
711.86	1087.516	28	179	0			2	1	y9
711.86	1135.516	28	179	1			2	1	b10
711.86	1186.585	28	179	0			2	1	y10
717.344	783.399	28	181	1	GFGFGQGAGALVHSE	sp|P21291|CSRP1_HUMAN Cysteine and glycine-rich protein 1 OS=Homo sapiens GN=CSRP1 PE=1 SV=3	2	1	y8
717.344	840.421	28	181	0			2	1	y9
717.344	850.384	28	181	1			2	1	b10

717.344	963.468	28	181	1			2	1	b11
717.344	968.479	28	181	1			2	1	y10
717.344	1025.5	28	181	0			2	1	y11
717.344	1062.536	28	181	1			2	1	b12
717.344	1199.595	28	181	1			2	1	b13
717.356	561.292	35	181	1			3	2	y10
717.356	676.337	35	181	1			3	2	y12
717.356	789.901	35	181	1			3	2	y14
717.356	847.414	35	181	1	QATVGDINTERPGMLDFTGK	sp|P07108|ACBP_HUMAN Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2	3	2	y15
717.356	875.925	35	181	0			3	2	y16
717.356	925.459	35	181	1			3	2	y17
717.356	965.476	35	181	1			3	1	y9
717.356	975.983	35	181	0			3	2	y18
737.844	721.372	28	187	1			2	1	y7
737.844	754.315	28	187	1			2	1	b8
737.844	792.409	28	187	0			2	1	y8
737.844	849.431	28	187	1	GYGYGQGAGTLSTDK	sp|P21291|CSRP1_HUMAN Cysteine and glycine-rich protein 1 OS=Homo sapiens GN=CSRP1 PE=1 SV=3	2	1	y9
737.844	977.489	28	187	1			2	1	y10
737.844	1034.511	28	187	0			2	1	y11
737.844	1254.596	28	187	1			2	1	y13
737.844	1328.575	28	187	1			2	1	b14
774.256	436.186	30	198	1			2	1	y3
774.256	650.725	30	198	1			2	2	y10
774.256	733.318	30	198	0	SC[Carboxyamidomethyl]C[Carboxyamidomethyl]SC[Carboxyamidomethyl]C[Carboxyamidomethyl]PAEC[Carboxyamidomethyl]EK	sp|P25713|MT3_HUMAN Metallothionein-3 OS=Homo sapiens GN=MT3 PE=1 SV=1	2	1	y6
774.256	893.349	30	198	0			2	1	y7
774.256	1053.379	30	198	1			2	1	y8
774.256	1112.326	30	198	1			2	1	b9

774.256	1140.411	30	198	1			2	1	y9
774.256	1300.442	30	198	1			2	1	y10
780.917	757.347	30	200	1	LYTLVLTDPDAPSR	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	y7
780.917	804.486	30	200	1			2	1	b7
780.917	858.395	30	200	0			2	1	y8
780.917	919.513	30	200	1			2	1	b8
780.917	971.479	30	200	0			2	1	y9
780.917	1070.547	30	200	1			2	1	y10
780.917	1131.593	30	200	1			2	1	b10
780.917	1202.63	30	200	1			2	1	b11
816.902	942.479	31	211	1	NRPTSISWDGLDSGK	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	b8
816.902	964.437	31	211	1			2	1	y9
816.902	1057.506	31	211	0			2	1	b9
816.902	1227.611	31	211	1			2	1	b11
816.902	1342.638	31	211	0			2	1	b12
816.902	1362.653	31	211	1			2	1	y13
816.902	1429.67	31	211	1			2	1	b13
816.902	1486.692	31	211	1			2	1	b14
840.397	921.478	32	217	1	DENATLDGGDVLFTGR	sp|O94760|DDAH1_HUMAN N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	y9
840.397	1036.505	32	217	0			2	1	y10
840.397	1087.453	32	217	1			2	1	b11
840.397	1149.589	32	217	0			2	1	y11
840.397	1200.537	32	217	1			2	1	b12
840.397	1250.637	32	217	1			2	1	y12
840.397	1347.606	32	217	1			2	1	b13
840.397	1448.653	32	217	1			2	1	b14
842.949	932.472	32	218	1	DYAVSTVPVADGLHLK	sp|O94760|DDAH1_HUMAN N(G);N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Homo sapiens GN=DDAH1 PE=1 SV=3	2	1	b9

842.949	949.546	32	218	0			2	1	y9
842.949	1003.509	32	218	1			2	1	b10
842.949	1048.614	32	218	1			2	1	y10
842.949	1118.536	32	218	1			2	1	b11
842.949	1149.662	32	218	1			2	1	y11
842.949	1236.694	32	218	0			2	1	y12
842.949	1335.763	32	218	1			2	1	y13
854.956	513.35	32	222	1			2	1	y4
854.956	756.436	32	222	1			2	1	y6
854.956	885.478	32	222	1			2	1	y7
854.956	1048.542	32	222	0	YVWLVYEQDRPLK	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	y8
854.956	1147.61	32	222	0			2	1	y9
854.956	1196.562	32	222	1			2	1	b9
854.956	1260.694	32	222	1			2	1	y10
854.956	1352.663	32	222	1			2	1	b10
906.435	423.223	43	237	1			3	1	y3
906.435	490.175	43	237	1			3	1	b4
906.435	623.303	43	237	0			3	1	y5
906.435	789.338	43	237	0	MWGDLWLLPPASANPGTGTEAEFEK	sp|P07108-2|ACBP_HUMAN Isoform 2 of Acyl-CoA-binding protein OS=Homo sapiens GN=DBI	3	1	b6
906.435	910.415	43	237	0			3	1	y8
906.435	1058.023	43	237	1			3	2	y20
906.435	1165.537	43	237	1			3	1	y11
906.435	1200.589	43	237	0			3	2	y23
975.476	542.293	36	258	0			2	1	y6
975.476	919.451	36	258	1	GNDISSGTVLSDYVGSGPPK	sp|P30086|PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	2	1	y9
975.476	1006.483	36	258	1			2	1	y10
975.476	1119.568	36	258	0			2	1	y11

975.476	1218.636	36	258	1			2	1	y12
975.476	1376.705	36	258	1			2	1	y14
975.476	1408.659	36	258	1			2	1	b14
975.476	1463.737	36	258	1			2	1	y15
979.472	864.9	37	259	0	TKPSDEEMLFIYGHYK	sp|P07108|ACBP_HUMAN Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2	2	2	y14
979.472	927.472	37	259	0			2	1	y7
979.472	1031.471	37	259	1			2	1	b9
979.472	1171.596	37	259	1			2	1	y9
979.472	1178.539	37	259	1			2	1	b10
979.472	1291.623	37	259	1			2	1	b11
979.472	1429.682	37	259	1			2	1	y11
979.472	1454.687	37	259	1			2	1	b12
1188.92	509.202	44	316	1	MDPETC[Carboxyamidomethyl]PC[Carboxyamidomethyl]PSGGSC[Carboxyamidomethyl]TC[Carboxyamidomethyl]ADSC[Carboxyamidomethyl]K	sp|P25713|MT3_HUMAN Metallothionein-3 OS=Homo sapiens GN=MT3 PE=1 SV=1	2	1	y4
1188.92	740.27	44	316	1			2	1	y6
1188.92	831.301	44	316	0			2	1	b7
1188.92	841.317	44	316	1			2	1	y7
1188.92	1001.348	44	316	0			2	1	y8
1188.92	1202.423	44	316	1			2	1	y11
1188.92	1289.455	44	316	0			2	1	y12
1188.92	1386.508	44	316	0			2	1	y13
1229.64	854.4	45	316	1	VFQSLPHENKPLTLSNYQTNK	sp|P04080|CYTB_HUMAN Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	2	1	y7
1229.64	938.473	45	316	1			2	1	b8
1229.64	942.484	45	316	0			2	2	y16
1229.64	1068.531	45	316	1			2	1	y9
1229.64	1099.565	45	316	1			2	2	b19
1229.64	1156.587	45	316	1			2	2	b20
1229.64	1180.61	45	316	1			2	1	b10

105

When no data was available, prediction from SRM Atlas or Pinpoint software (Thermo Scientific) was used to choose the transitions. In that case, 4 primary and 4 secondary transitions were selected as reported in Table 15. The S-lens parameters for each precursor-ion were set-up according to *m/z* values and previous experimental data. Collision energies were determined by Pinpoint using a pre-defined calculation. The chosen cycle time was 1.6 s to monitor 80 primary transitions. A total of 240 transitions were used to monitor the 30 signature-peptides. The scan time of the triggered transitions was 0.2 s. A TSQ Vantage mass spectrometer (Thermo Scientific) was used using Q1 peak width (FWHM) of 0.7 and argon pressure in the collision cell of 1.2 mTorr. Positive ionisation was used. Capillary temperature, vaporizer, sheath gas and auxiliary gas were optimized for maximal ion sensitivities.

A reversed-phase liquid chromatography (RP-LC) separation was implemented before MS. Peptide separation occurred on a 50 X 1 mm column at 100 µL/min with a 13.25 min gradient of 30% CH_3CN. A Finnigan Surveyor MS Pump Plus LC system (Thermo Scientific) was used.

TESTING OF THE METHOD

To demonstrate the presence of the target proteins in a more readily accessible sample, the developed MRM method was evaluated on human plasma sample digested with trypsin. Briefly, a volume of 30 µL plasma (Dade Behring) was added to 1680 µL triethylammonium hydrogen carbonate buffer (TEAB) 100 mM and 90 µL sodium dodecyl sulfate 1%. Reduction was performed at 55 °C for 1 h with tris(2-carboxyethyl) phosphine hydrochloride 20 mM (95.4 µL). A volume of 90 µL iodoacetamide 150 mM was then added for 1 h reaction in the dark at room temperature. A volume of 180 µL trypsin (Promega) 0.4 µg/ µL in TEAB was added. Digestion was performed overnight at 37 °C. Sample purification was first performed with Hypersep C18 500 mg (Thermo Scientific). Strong cation-exchange cartridges were used for further purification. The sample was divided into three aliquots. Aliquots were re-suspended in 500 µL 3% CH_3CN, 0.2% formic acid, 0.2 mg/mL glucagon before RP-LC SRM analysis. Twenty µL were used per RP-LC iSRM analysis. Data analysis was carried out using Pinpoint.

Figures 11 to 16 show chromatograms of the iSRM signals of transitions for signature-peptides DENATLDGGDVLFTGR, TPEEYPESAK, SQVVAGTNYFIK, GYGYGQGAGTLSTDK, GLESTTLADK and LYEQLSGK representative of proteins DDAH1, CYTB, CSRP1 and PEBP1.

Thus it is demonstrated that the SRM method developed herein allows for the monitoring of 30 signature-peptides representative of 6 stroke biomarker candidates.

Proof-of-principle of the method applicability was demonstrated in a plasma sample digested with trypsin. The method could be applied to several sample matrixes.

The demonstrations in this example were carried out using the markers of Panel A. As noted above, this is illustrative of this mode of detection. This mode of detection may be applied equally to any other of the markers or groups of markers disclosed in this document. To work the invention using those other marker(s) according to this mode of detection, the skilled worker simply follows the guidance given above but substitutes their selected other marker(s) for those of Panel A.

REFERENCES

1. Foerch, C., Montaner, J., Furie, K. L., Ning, M. M., and Lo, E. H. (2009) Invited Article: Searching for oracles? Blood biomarkers in acute stroke. *Neurology* 73, 393-399.
2. Poca, M. A., Sahuquillo, J., Vilalta, A., De los Rios, J., Robles, A., and Exposito, L. (2006) Percutaneous implantation of cerebral microdialysis catheters by twist-drill craniostomy in neurocritical patients: Description of the technique and results of a feasibility study in 97 patients. *J. Neurotrauma* 23, 1510-1517.
3. Hutchinson, P. J., O'Connell, M. T., Kirkpatrick, P. J., and Pickard, J. D. (2002) How can we measure substrate, metabolite and neurotransmitter concentrations in the human brain? *Physiol. Meas.* 23, R75-R109.
4. Reinstrup, P., Stahl, N., Mellergard, P., Uski, T., Ungerstedt, U., and Nordstrom, C. H. (2000) Intracerebral microdialysis in clinical practice: Baseline values for chemical markers during wakefulness, anesthesia, and neurosurgery. *Neurosurgery* 47, 701-709.
5. Tisdall, M. M., and Smith, M. (2006) Cerebral microdialysis: research technique or clinical tool. *Br. J. Anaesth.* 97, 18-25.
6. Maurer, M. H. (2008) Proteomics of brain extracellular fluid (ECF) and cerebrospinal fluid (CSF). *Mass Spectrom. Rev.*, DOI:10.1002/mas.20213.
7. Maurer, M. H., Haux, D., Unterberg, A. W., and Sakowitz, O. W. (2008) Proteomics of human cerebral microdialysate: From detection of biomarkers to clinical application. *Proteomics Clin. Appl.* 2, 437-443.
8. Helmy, A., Carpenter, K. L. H., Skepper, J. N., Kirkpatrick, P. J., Pickard, J. D., and Hutchinson, P. J. (2009) Microdialysis of Cytokines: Methodological Considerations, Scanning Electron Microscopy, and Determination of Relative Recovery. *J. Neurotrauma* 26, 549-561.
9. Donato, R. (2001) S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int. J. Biochem. Cell Biol.* 33, 637-668.
10. Afinowi, R., Tisdall, M., Keir, G., Smith, M., Kitchen, N., and Petzold, A. (2009) Improving the recovery of S100B protein in cerebral microdialysis: Implications for multimodal monitoring in neurocritical care. *J. Neurosci. Methods* 181, 95-99.
11. Maurer, M. H., Berger, C., Wolf, M., Futterer, C. D., Feldmann, R. E., Jr., Schwab, S., and Kuschinsky, W. (2003) The proteome of human brain microdialysate. *Proteome Science* 1, 7.
12. Maurer, M. H., Haux, D., Sakowitz, O. W., Unterberg, A. W., and Kuschinsky, W. (2007) Identification of early markers for symptomatic vasospasm in human cerebral microdialysate after subarachnoid hemorrhage: Preliminary results of a proteome-wide screening. *J. Cereb. Blood Flow Metab.* 27, 1675-1683.
13. Dayon, L., Hainard, A., Licker, V., Turck, N., Kuhn, K., Hochstrasser, D. F., Burkhard, P. R., and Sanchez, J. C. (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal. Chem.* 80, 2921-2931.
14. Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., and Hamon, C. (2003) Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* 75, 1895-1904.
15. Lescuyer, P., Allard, L., Zimmermann-Ivol, C. G., Burgess, J. A., Hughes-Frutiger, S., Burkhard, P. R., Sanchez, J. C., and Hochstrasser, D. F. (2004) Identification of post-mortem cerebrospinal fluid proteins as potential biomarkers of ischemia and neurodegeneration. *Proteomics* 4, 2234-2241.
16. Tuck, M. K., Chan, D. W., Chia, D., Godwin, A. K., Grizzle, W. E., Krueger, K. E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W., and Brenner, D. E. (2009) Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J Proteome Res* 8, 113-117.
17. Bradford, M. M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
18. Blum, H., Beier, H., and Gross, H. J. (1987) Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8, 93-99.
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets - procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350-4354.

20. Burgess, J. A., Lescuyer, P., Hainard, A., Burkhard, P. R., Turck, N., Michel, P., Rossier, J. S., Reymond, F., Hochstrasser, D. F., and Sanchez, J. C. (2006) Identification of brain cell death associated proteins in human post-mortem cerebrospinal fluid. *J. Proteome Res.* 5, 1674-1681.

21. Hainard, A., Tiberti, N., Robin, X., Lejon, V., Ngoyi, D. M., Matovu, E., Enyaru, J. C., Fouda, C., Ndung'u, J. M., Lisacek, F., Muller, M., Turck, N., and Sanchez, J. C. (2009) A Combined CXCL10, CXCL8 and H-FABP Panel for the Staging of Human African Trypanosomiasis Patients. *PLoS Neglected Tropical Diseases* 3, e459.

22. Horth, P., Miller, C. A., Preckel, T., and Wenz, C. (2006) Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. *Mol. Cell. Proteomics* 5, 1968-1974.

23. Ros, A., Faupel, M., Mees, H., van Oostrum, J., Ferrigno, R., Reymond, F., Michel, P., Rossier, J. S., and Girault, H. H. (2002) Protein purification by Off-Gel electrophoresis. *Proteomics* 2, 151-156.

24. Dayon, L., Turck, N., Kienle, S., Schulz-Knappe, P., Hochstrasser, D. F., Scherl, A., and Sanchez, J. C. (2010) Isobaric tagging-based selection and quantitation of cerebrospinal fluid tryptic peptides with reporter calibration curves. *Anal. Chem.*, DOI 10.1021/ac901854k.

25. Herrmann, M., Vos, P., Wunderlich, M. T., de Bruijn, C., and Lamers, K. J. B. (2000) Release of glial tissue-specific proteins after acute stroke - A comparative analysis of serum concentrations of protein S-100B and glial fibrillary acidic protein. *Stroke* 31, 2670-2677.

26. Jauch, E. C., Lindsell, C., Broderick, J., Fagan, S. C., Tilley, B. C., Levine, S. R., and Grp, N. r.-P. S. S. (2006) Association of serial biochemical markers with acute ischemic stroke - The National Institute of Neurological Disorders and Stroke recombinant tissue plasminogen activator Stroke Study. *Stroke* 37, 2508-2513.

27. Lamers, K. J. B., Vos, P., Verbeek, M. M., Rosmalen, F., van Geel, W. J. A., and van Engelen, B. G. M. (2003) Protein S-100B, neuron-specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) and blood of neurological patients. *Brain Res. Bull.* 61, 261-264.

28. Buttner, T., Weyers, S., Postert, T., Sprengelmeyer, R., and Kuhn, W. (1997) S-100 protein: Serum marker of focal brain damage after ischemic territorial MCA infarction. *Stroke* 28, 1961-1965.

29. Missler, U., Wiesmann, M., Friedrich, C., and Kaps, M. (1997) S-100 protein and neuron-specific enolase concentrations in blood as indicators of infarction volume and prognosis in acute ischemic stroke. *Stroke* 28, 1956-1960.

30. Berger, C., Dohmen, C., Maurer, M. H., Graf, R., and Schwab, S. (2004) Cerebral microdialysis in stroke. *Nervenarzt* 75, 113-123.

31. Zougman, A., Pilch, B., Podtelejnikov, A., Kiehntopf, M., Schnabel, C., Kurnar, C., and Mann, M. (2008) Integrated analysis of the cerebrospinal fluid peptidome and proteome. *J. Proteome Res.* 7, 386-399.

32. da Silva, S. F., Correa, C. L., Tortelote, G. G., Einicker-Lamas, M., Martinez, A. M. B., and Allodi, S. (2004) Glial fibrillary acidic protein (GFAP)-like immunoreactivity in the visual system of the crab Ucides cordatus (Crustacea, Decapoda). *Biol. Cell* 96, 727-734.

33. Kaufmann, A. M., Firlik, A. D., Fukui, M. B., Wechsler, L. R., Jungries, C. A., and Yonas, H. (1999) Ischemic core and penumbra in human stroke. *Stroke* 30, 93-99.

34. Rothermundt, M., Peters, M., Prehn, J. H. M., and Arolt, V. (2003) S100B in brain damage and neurodegeneration. *Microsc. Res. Tech.* 60, 614-632.

35. Wiesmann, M., Missler, U., Hagenstrom, H., and Gottmann, D. (1997) S-100 protein plasma levels after aneurysmal subarachnoid haemorrhage. *Acta Neurochir. (Wien).* 139, 1155-1160.

36. Romner, B., Ingebrigtsen, T., Kongstad, F., and Borgesen, S. E. (2000) Traumatic brain damage: Serum S-100 protein measurements related to neuroradiological findings. *J. Neurotrauma* 17, 641-647.

37. Sen, J., Belli, A., Petzold, A., Russo, S., Keir, G., Thompson, E. J., Smith, M., and Kitchen, N. (2005) Extracellular fluid S100B in the injured brain: a future surrogate marker of acute brain injury? *Acta Neurochir. (Wien).* 147, 897-900.

38. Salinas, A. E., and Wong, M. G. (1999) Glutathione S-transferases - A review. *Curr. Med. Chem.* 6, 279-309.

39. Theodore, C., Singh, S. V., Hong, T. D., and Awasthi, Y. C. (1985) Glutathione S-transferases of human brain. Evidence for two immunologically distinct types of 26500-Mr subunits. *Biochem. J.* 225, 375-382.

40. Shi, M., Bradner, J., Bammler, T. K., Eaton, D. L., Zhang, J. P., Ye, Z. C., Wilson, A. M., Montine, T. J., Pan, C., and Zhang, J. (2009) Identification of Glutathione S-Transferase Pi as a Protein Involved in Parkinson Disease Progression. *Am. J. Pathol.* 175, 54-65.

41. Rhee, S. G., Yang, K. S., Kang, S. W., Woo, H. A., and Chang, T. S. (2005) Controlled elimination of intracellular H2O2: Regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. *Antioxidants & Redox Signaling* 7, 619-626.

42. Wood, Z. A., Schroder, E., Harris, J. R., and Poole, L. B. (2003) Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* 28, 32-40.

43. Power, J. H. T., Asad, S., Chataway, T. K., Chegini, F., Manavis, J., Temlett, J. A., Jensen, P. H., Blumbergs, P. C., and Gai, W. P. (2008) Peroxiredoxin 6 in human brain: molecular forms, cellular distribution and association with Alzheimer's disease pathology. *Acta Neuropathol. (Berl).* 115, 611-622.

44. Sarafian, T. A., Verity, M. A., Vinters, H. V., Shih, C. C. Y., Shi, L. R., Ji, X. D., Dong, L. P., and Shau, H. Y. (1999) Differential expression of peroxiredoxin subtypes in human brain cell types. *J. Neurosci. Res.* 56, 206-212.

45. Basso, M., Giraudo, S., Corpillo, D., Bergamasco, B., Lopiano, L., and Fasano, M. (2004) Proteome analysis of human substantia nigra in Parkinson's disease. *Proteomics* 4, 3943-3952.

46. Krapfenbauer, K., Engidawork, E., Cairns, N., Fountoulakis, M., and Lubec, G. (2003) Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. *Brain Res.* 967, 152-160.

47. Schreibelt, G., van Horssen, J., Haseloff, R. F., Reijerkerk, A., van der Pol, S. M. A., Nieuwenhuizen, O., Krause, E., Blasig, I. E., Dijkstra, C. D., Ronken, E., and de Vries, H. E. (2008) Protective effects of peroxiredoxin-1 at the injured blood-brain barrier. *Free Radic. Biol. Med.* 45, 256-264.

48. Kim, Y. J., Lee, W. S., Ip, C., Chae, H. Z., Park, E. M., and Park, Y. M. (2006) Prx1 suppresses radiation-induced c-Jun NH2-terminal kinase signaling in lung cancer cells through interaction with the glutathione S-transferase Pi/c-Jun NH2-terminal kinase complex. *Cancer Res.* 66, 7136-7142.

49. Manevich, Y., Feinstein, S. I., and Fisher, A. B. (2004) Activation of the antioxidant enzyme 1-CYS peroxiredoxin requires glutathionylation mediated by heterodimerization with pi GST. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3780-3785.

50. Ralat, L. A., Manevich, Y., Fisher, A. B., and Colman, R. F. (2006) Direct evidence for the formation of a complex between 1-cysteine peroxiredoxin and glutathione S-transferase pi with activity changes in both enzymes. *Biochemistry (Mosc).* 45, 360-372.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described aspects and embodiments of the present invention will be apparent to those skilled in the art without departing from the scope of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the art are intended to be within the scope of the following claims.

CLAIMS

1. A method of aiding the diagnosis of acute brain damage in a subject, said method comprising

(i) assaying the concentration of at least one oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1 in a sample from said subject; and

(ii) assaying the concentration of at least one further polypeptide selected from Panel A;

(iii) comparing the concentrations of (i) and (ii) to the concentrations of the polypeptides in a reference standard and determining quantitative ratios for said polypeptides;

(iv) wherein a finding of a quantitative ratio of each of the assayed polypeptides in the sample to the polypeptides in the reference standard of greater than 1.3 indicates an increased likelihood of acute brain damage having occurred in said subject.

2. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel B.

3. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel C.

4. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1.

5. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1H.

6. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1C.

7. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1A.

8. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 1B.

9. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2.

10. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2A.

11. A method according to claim 1 wherein step (ii) comprises assaying the concentration of at least one further polypeptide selected from Panel 2B.

12. A method according to any of claims 1 to 11 wherein step (i) comprises assaying the concentration of at least two oxidative stress polypeptide selected from the group consisting of: PRDX1, PRDX6 and GSTP1.

13. A method according to any of claims 1 to 11 wherein step (i) comprises assaying the concentration of each of the oxidative stress polypeptides PRDX1, PRDX6 and GSTP1.

14. A method according to any of claims 1 to 11 wherein step (ii) comprises assaying the concentration of at least two further polypeptides selected from said Panel.

15. A method according to claim 14 wherein step (ii) comprises assaying the concentration of at least four further polypeptides selected from said Panel.

16. A method according to any of claims 1 to 15 wherein the acute brain injury is stroke.

17. A method according to any of claims 1 to 16 wherein the sample is brain microdialysate fluid, cerebrospinal fluid, or blood.

18. A method according to claim 17 wherein the sample is blood.

19. A method according to any preceding claim wherein step (i) comprises assaying the concentration of PRDX1 in a sample from said subject.

20. A method according to any of claims 1 to 19 wherein the protein is detected by western blotting.

21. A method according to any of claims 1 to 19 wherein the protein is detected by bead suspension array or by planar array.

22. A method according to any of claims 1 to 19 wherein the protein is detected by isobaric protein tagging or by isotopic protein tagging.

23. A method according to any of claims 1 to 19 or claim 22 wherein the protein is detected by mass spectrometer-based assay.

24. Use for diagnostic or prognostic applications relating to acute brain damage of a material which recognises, binds to or has affinity for a first and a second polypeptide or a fragment, variant or mutant thereof, wherein the first polypeptide is selected from PRDX1, PRDX6 and GSTP1 and the second polypeptide is selected from Panel A.

25. Use for diagnostic or prognostic applications relating to stroke of a material which recognises, binds to or has affinity for a polypeptide or a fragment, variant or mutant thereof, wherein the polypeptide is selected from Panel 2.

26. Use according to claim 24 or claim 25 of a combination of materials, each of which respectively recognises, binds to or has affinity for one or more of said polypeptide(s), or a fragment, variant or mutant thereof.

27. Use according to any of claims 24 to 26, in which the or each material is an antibody or antibody chip.

28. Use according to claim 22, in which the material is an antibody with specificity for one or more of said polypeptide(s), or a fragment, variant or mutant thereof.

29. An assay device for use in the diagnosis of acute brain damage, which comprises a solid substrate having a location containing a material, which recognizes, binds to or has affinity for a first and a second polypeptide or a fragment, variant or mutant thereof, wherein the first polypeptide is selected from PRDX1, PRDX6 and GSTP1 and the second polypeptide is selected from Panel A.

30. An assay device for use in the diagnosis of stroke, which comprises a solid substrate having a location containing a material, which recognizes, binds to or has affinity for a polypeptide, or a fragment, variant or mutant thereof, wherein the polypeptide is selected from Panel 2.

31. An assay device according to claim 29 or 30, in which the material is an antibody or antibody chip.

32. An assay device according to claim 31, which has a unique addressable location for each antibody, thereby to permit an assay readout for each individual polypeptide or for any combination of polypeptides.

33. A kit for use in the diagnosis of stroke, comprising an assay device according to any of claims 29 to 32, and means for detecting the amount of one or more of the polypeptides in a sample of body fluid taken from a subject.

34. A method of diagnosis or prognostic monitoring of acute brain damage in a subject, said method comprising

(a) obtaining and extracting the proteins from a relevant tissue sample from an individual;

(b) digesting said proteins to produce a population of peptides;

(c) determining the abundance of one or more of said peptides listed in Table 14 using Selected Reaction Monitoring of one or more of the transitions listed in Table 15;

(d) comparing the abundance of said one or more peptides with a pre-determined peptide abundance associated with a diagnosis of acute brain damage; and

(e) determining whether the subject has suffered acute brain damage and/or that the acute brain damage is worsening or improving based on the differences in abundance of said one or more peptides.

35. A method according to claim 34 wherein the pre-determined peptide abundance is determined using a known amount of corresponding synthetic peptide selected from Table 14.

36. A preparation for making a diagnosis of acute brain damage or prognostic monitoring of a subject with acute brain damage comprising one or more synthetic peptides selected from the group listed in Table 14.

37. A preparation according to claim 36 wherein said one or more synthetic peptides are selected from

GSTP1	TFIVGDQISFADYNLLDLLLIHEVLAPGCLDAFPLLSAYVGR
	MPPYTVVYFPVR
	DDYVK
	DQQEAALVDMVNDGVEDLR
	FQDGDLTLYQSNTILR
	ASCLYGQLPK
	AFLASPEYVNLPINGNGK
	MLLADQGQSWK
	LSARPK
	TLGLYGK
	EEVVTVETWQEGSLK
	ALPGQLKPFETLLSQNQGGK
	YISLIYTNYEAGK
PRDX1	HGEVCPAGWKPGSDTIKPDVQK
	QGGLGPMNIPLVSDPK
	ADEGISFR
	DISLSDYK
	LVQAFQFTDK
	IGHPAPNFK
	LNCQVIGASVDSHFCHLAWVNTPK
	YVVFFFYPLDFTFVCPTEIIAFSDR
	MSSGNAK
	TIAQDYGVLK
	ATAVMPDGQFK
PRDX6	GMPVTAR
	MPGGLLLGDVAPNFEANTTVGR
	DFTPVCTTELGR
	VVFVFGPDK
	LIALSIDSVEDHLAWSK
	ELAILLGMLDPAEK
	LSILYPATTGR
	VATPVDWK
	NFDEILR
	LPFPIIDDR
	VVISLQLTAEK
	DINAYNCEEPTEK

		LAPEFAK
		DGDSVMVLPTIPEEEAK
		FHDFLGDSWGILFSHPR
	DDAH1	ALPESLGQHALR
		DENATLDGGDVLFTGR
		DYAVSTVPVADGLHLK
		GAEILADTFK
		GEEVDVAR
		QHQLYVGVLGSK
		TPEEYPESAK
	CYTB	HDELTYF
		SQVVAGTNYFIK
		VFQSLPHENKPLTLSNYQTNK
		VHVGDEDFVHLR
	ACBP	MSQAEFEK
		AAEEVR
		QATVGDINTERPGMLDFTGK
		TKPSDEEMLFIYGHYK
		WDAWNELK
		MWGDLWLLPPASANPGTGTEAEFEK
		MPAFAEFEK
	CSRP1	GFGFGQGAGALVHSE
		GLESTTLADK
		GYGYGQGAGTLSTDK
	MT3	GGEAAEAEAEK
		MDPETCPCPSGGSCTCADSCK
		SCCSCCPAECEK
	PEPB1	GNDISSGTVLSDYVGSGPPK
		LYEQLSGK
		LYTLVLTDPDAPSR
		NRPTSISWDGLDSGK
		VLTPTQVK

YVWLVYEQDRPLK

38. A preparation according to any one of claims 36 to 37 wherein each peptide contains one or more stable heavy isotopes selected from hydrogen, carbon, oxygen, nitrogen or sulphur.

39. A preparation according to any one of claims 36 to 37 wherein said synthetic peptides are labelled with an isotopic or isobaric tag.

40. A preparation according to any one of claims 36 to 39 for the diagnosis or prognostic monitoring of acute brain damage.

41. A preparation according to claim 40 wherein the acute brain damage is ischaemic stroke or transient ischaemic attack.

42. A method, use, device or kit substantially as described herein.

Figure 1

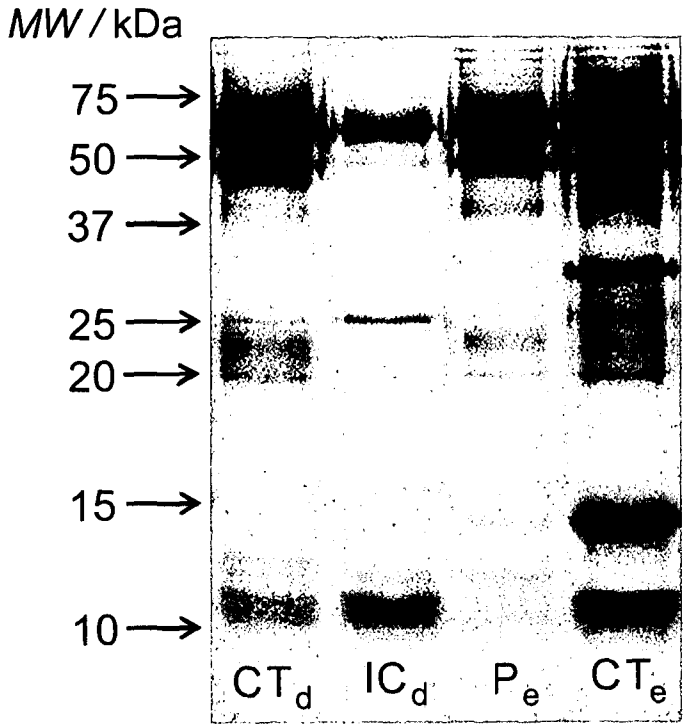


Figure 2

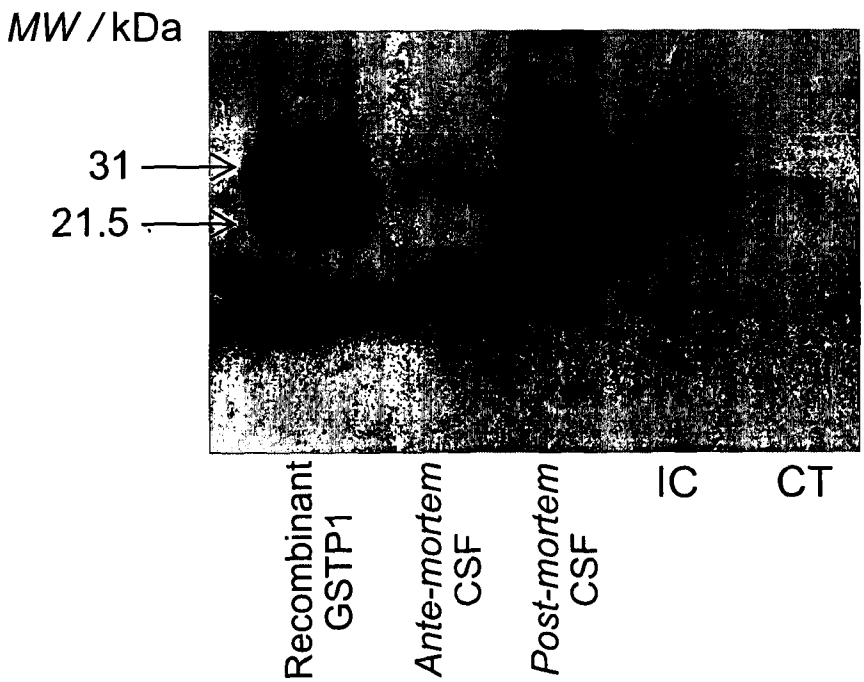
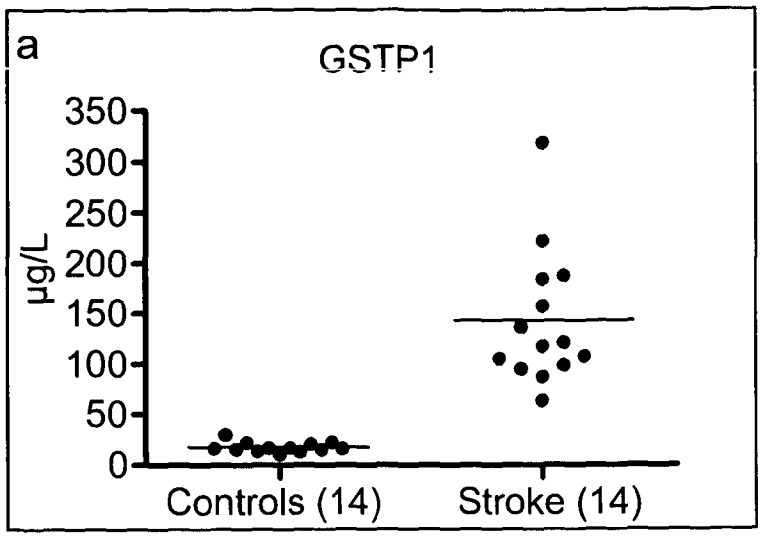
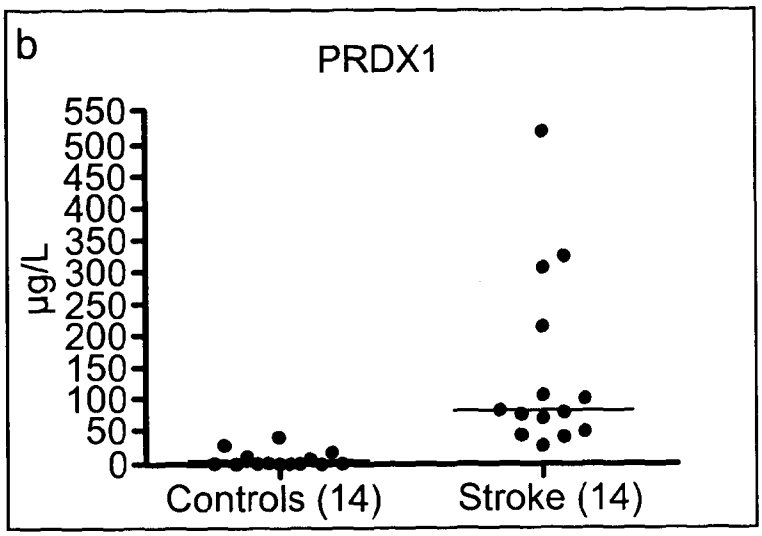


Figure 3


a
GSTP1
350
300
250
200
150
100
50
0
µg/L
Controls (14)
Stroke (14)


b
PRDX1
550
500
450
400
350
300
250
200
150
100
50
0
µg/L
Controls (14)
Stroke (14)

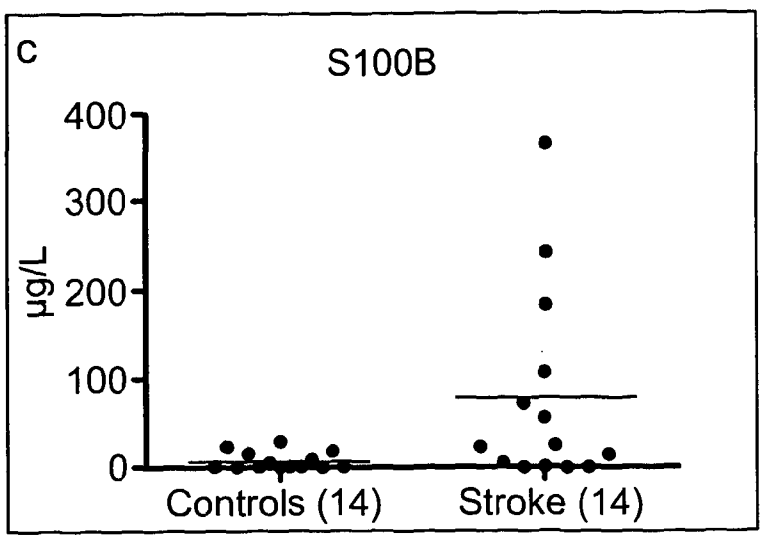

c
S100B
400
300
200
100
0
µg/L
Controls (14)
Stroke (14)

Figure 4 - SD1

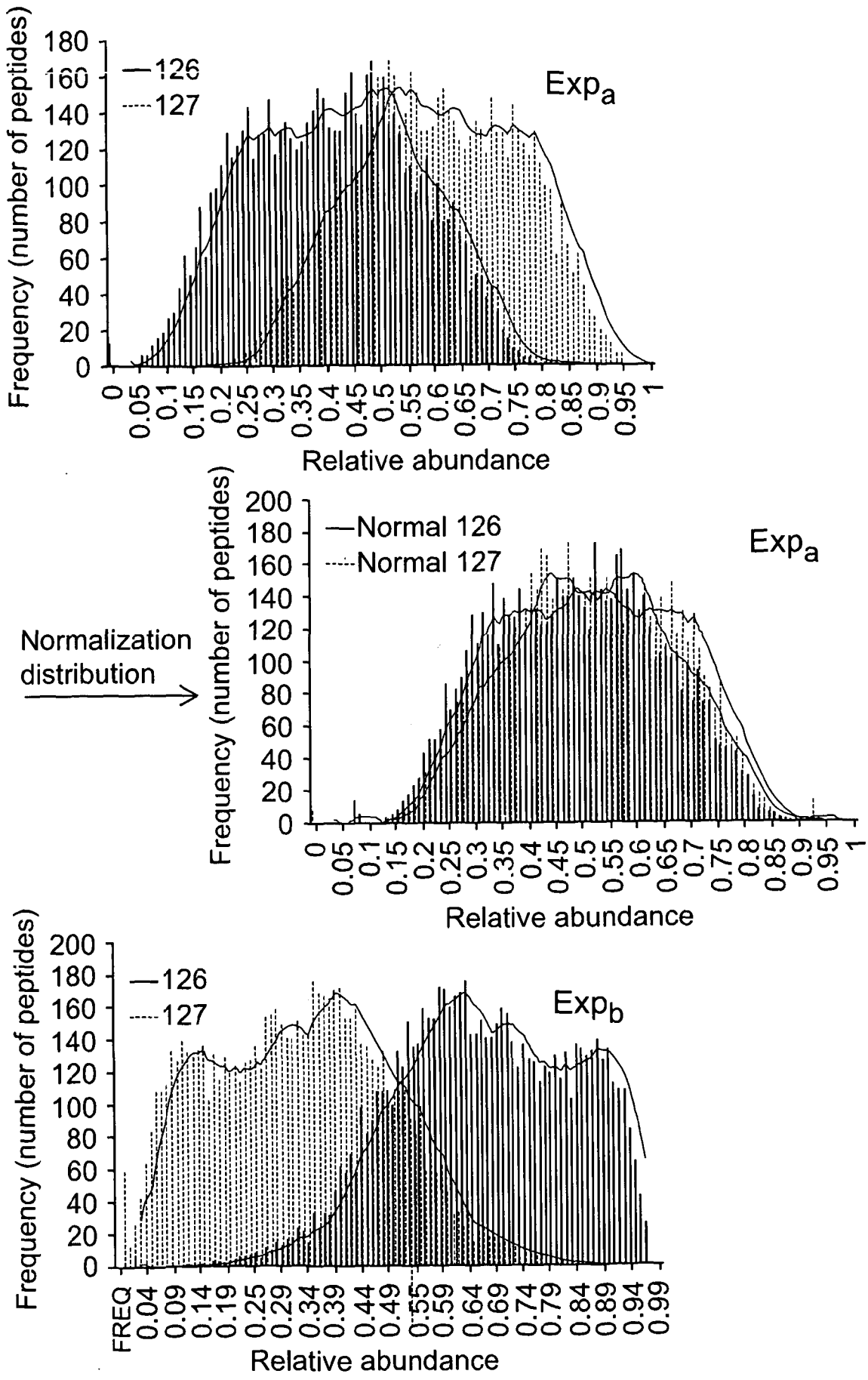

Frequency (number of peptides)
180
160
140
120
100
80
60
40
20
0
—126
----127
Exp$_a$
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5 0.55 0.6 0.65 0.7 0.75 0.8 0.85 0.9 0.95 1
Relative abundance
Normalization distribution
Frequency (number of peptides)
200
180
160
140
120
100
80
60
40
20
0
—Normal 126
----Normal 127
Exp$_a$
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5 0.55 0.6 0.65 0.7 0.75 0.8 0.85 0.9 0.95 1
Relative abundance
Frequency (number of peptides)
200
180
160
140
120
100
80
60
40
20
0
—126
----127
Exp$_b$
FREQ 0.04 0.09 0.14 0.19 0.25 0.29 0.34 0.39 0.44 0.49 0.55 0.59 0.64 0.69 0.74 0.79 0.84 0.89 0.94 0.99
Relative abundance

Figure 4 - SD1

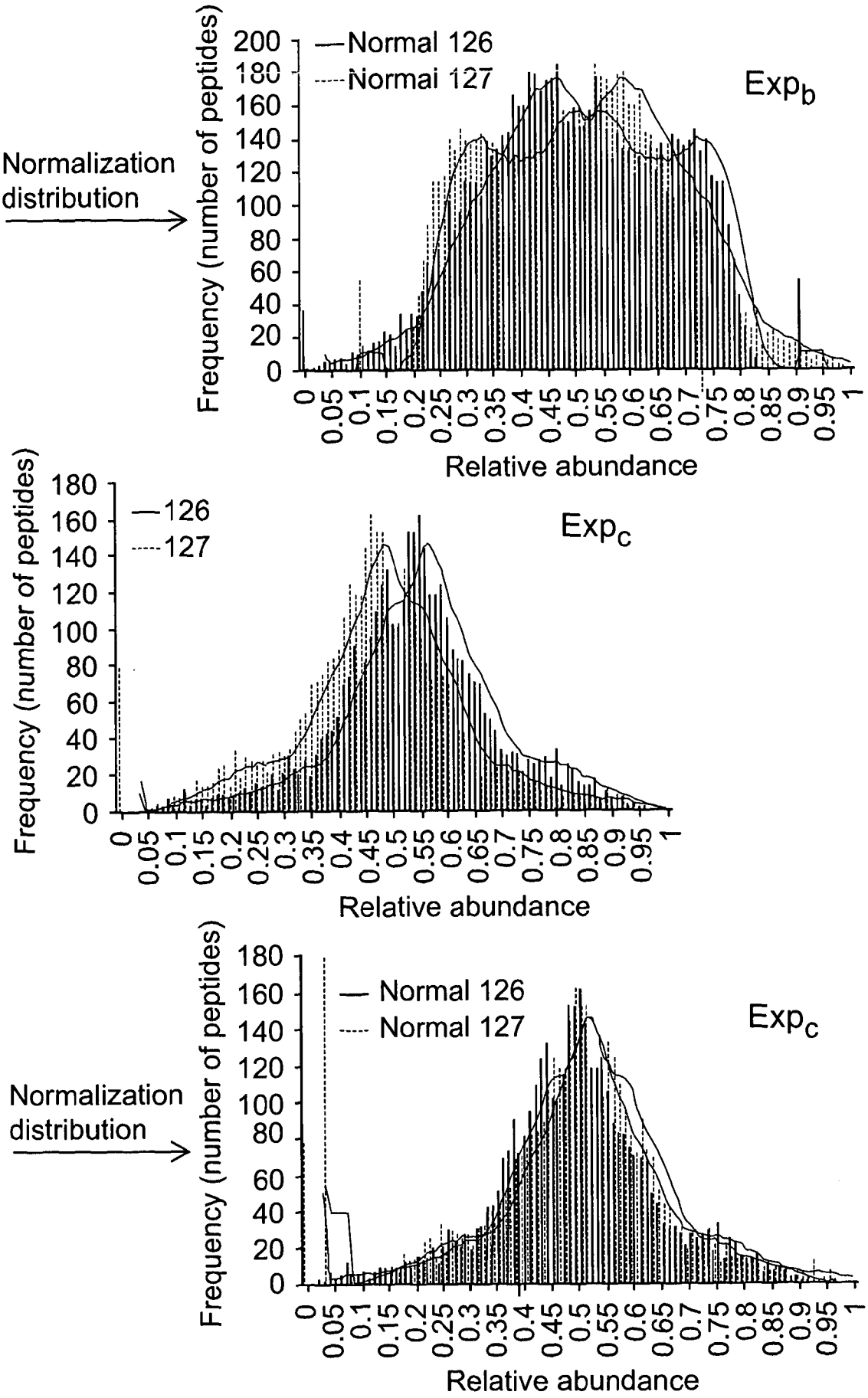

Normalization distribution
Frequency (number of peptides)
Normal 126
Normal 127
Exp$_b$
Relative abundance
Frequency (number of peptides)
126
127
Exp$_c$
Relative abundance
Normalization distribution
Frequency (number of peptides)
Normal 126
Normal 127
Exp$_c$
Relative abundance

Figure 4 - SD1

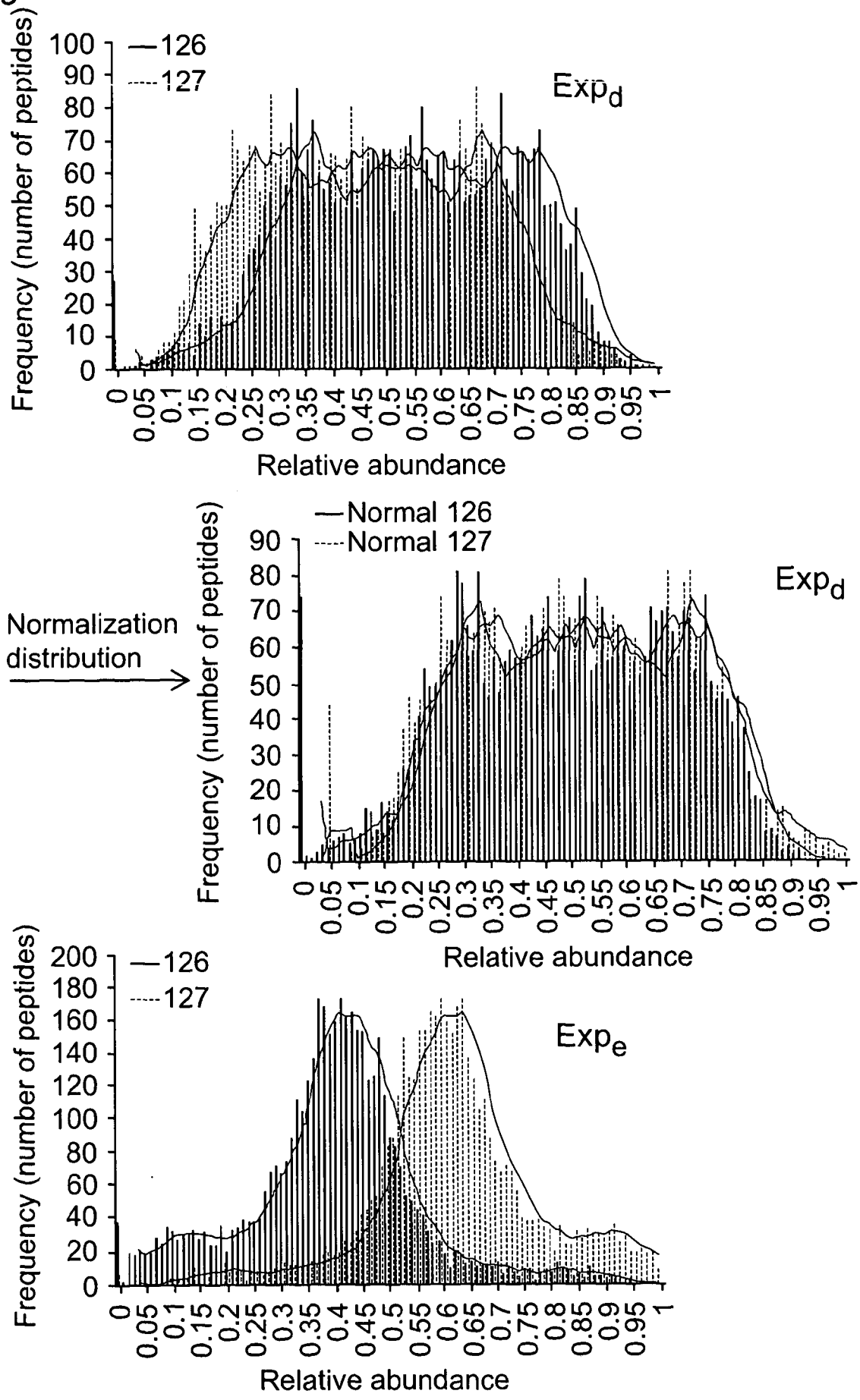

Frequency (number of peptides)
—126
----127
Exp$_d$
Relative abundance
Normalization distribution
—Normal 126
----Normal 127
Exp$_d$
Relative abundance
Frequency (number of peptides)
—126
----127
Exp$_e$
Relative abundance

Figure 4 - SD1

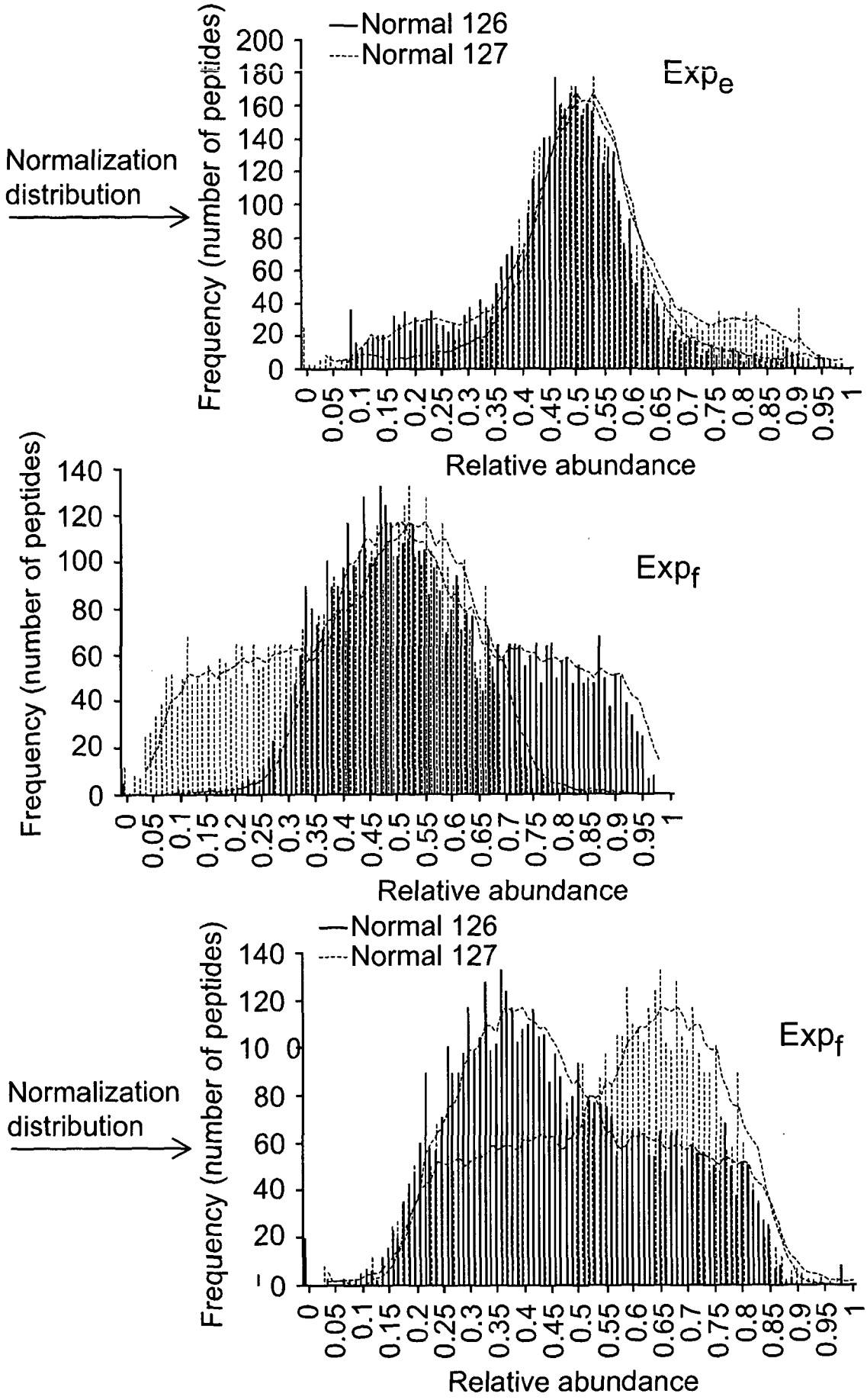

—Normal 126
----Normal 127
Exp$_e$
Normalization distribution
Frequency (number of peptides)
Relative abundance
Exp$_f$
Frequency (number of peptides)
Relative abundance
—Normal 126
----Normal 127
Exp$_f$
Normalization distribution
Frequency (number of peptides)
Relative abundance

Figure 5 - SD2

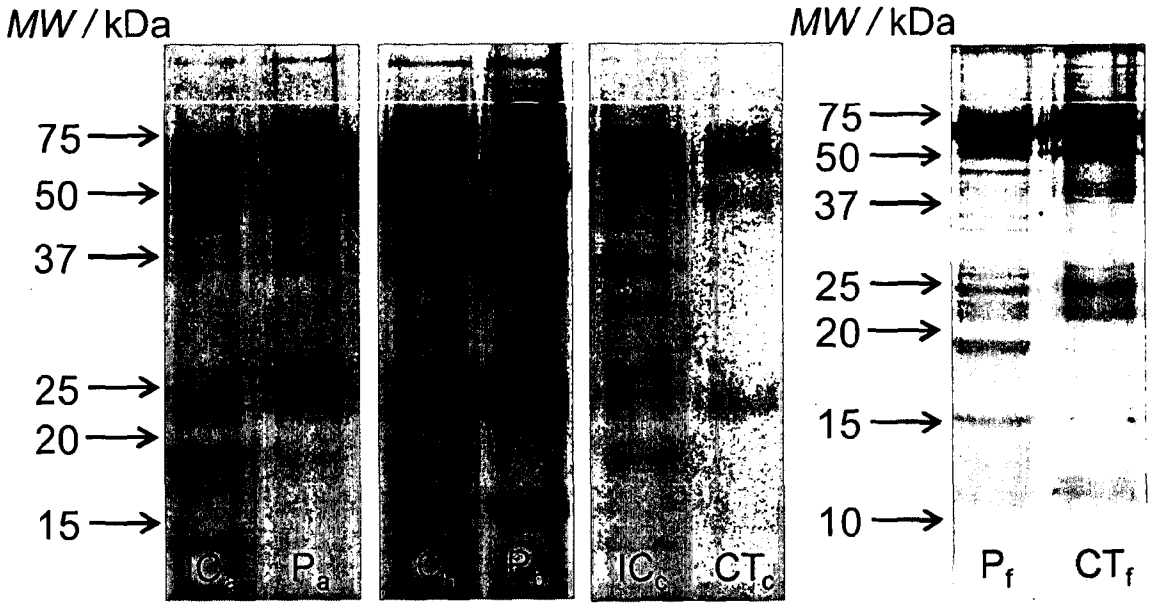
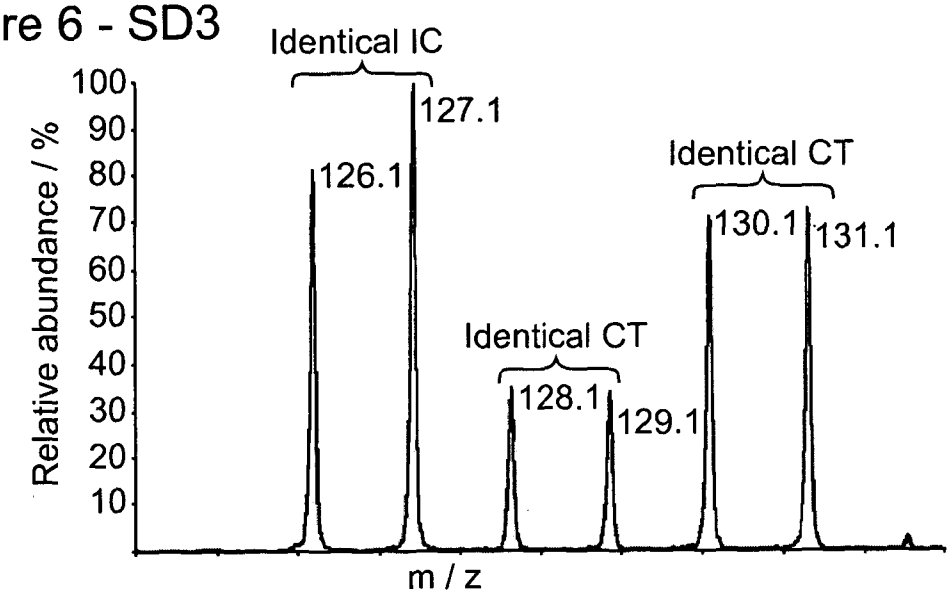


Figure 6 - SD3



	Average on 10 peptides	
False discovery rate	1%	5%
Lower cut-off	0.61	0.74
Higher cut-off	1.68	1.48

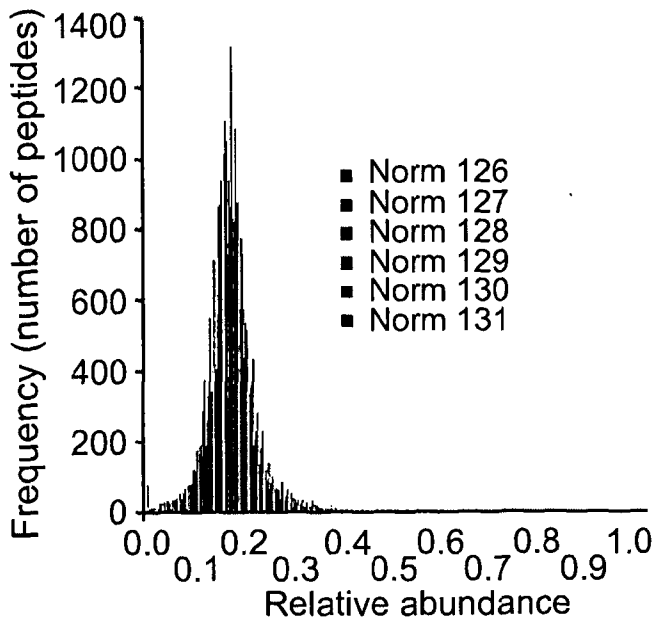


Figure 7

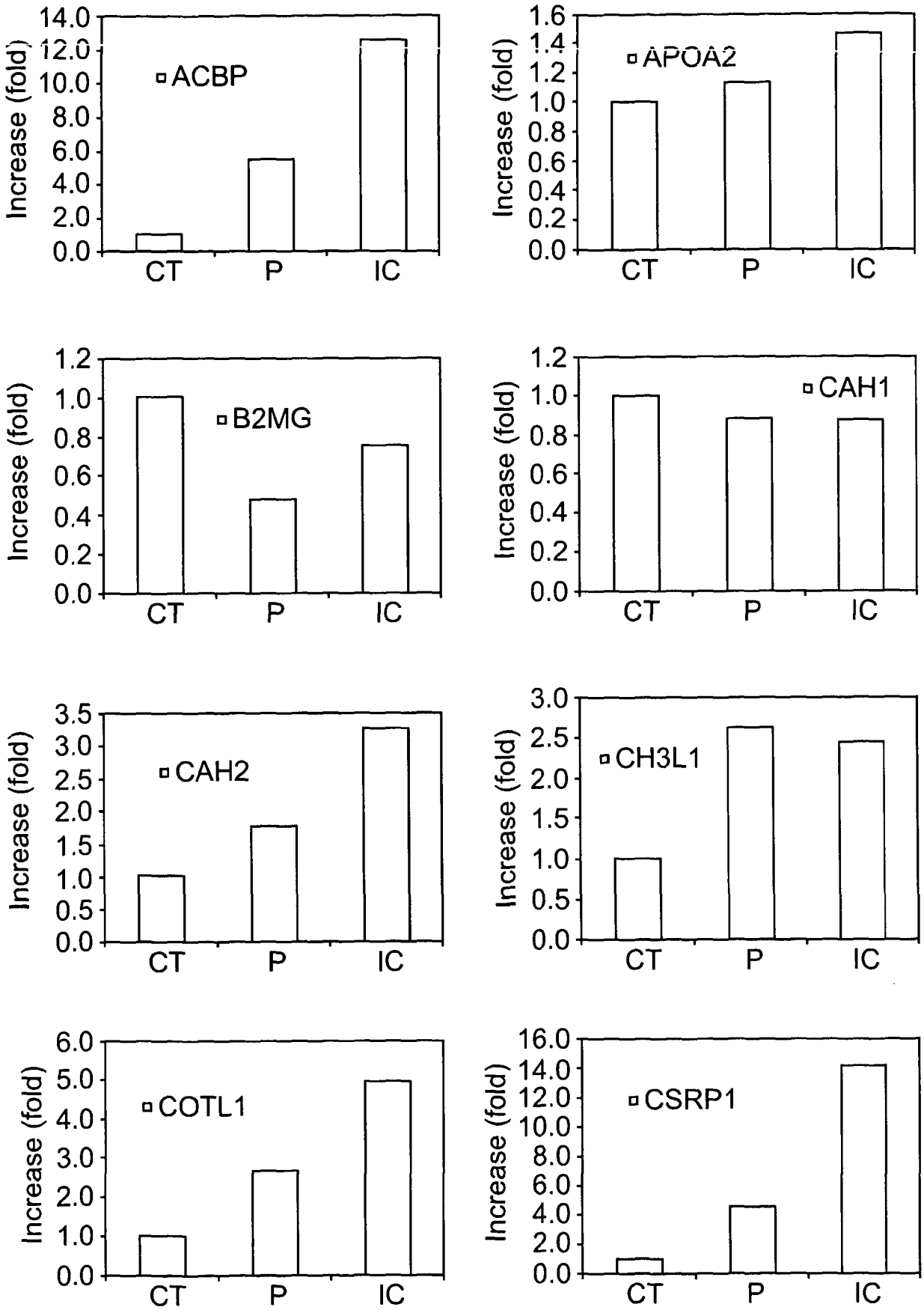

ACBP
Increase (fold)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CT
P
IC
APOA2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
B2MG
CAH1
CAH2
3.5
3.0
2.5
2.0
1.5
1.0
0.5
CH3L1
COTL1
6.0
5.0
4.0
3.0
2.0
1.0
CSRP1
16.0

Figure 7 Continued

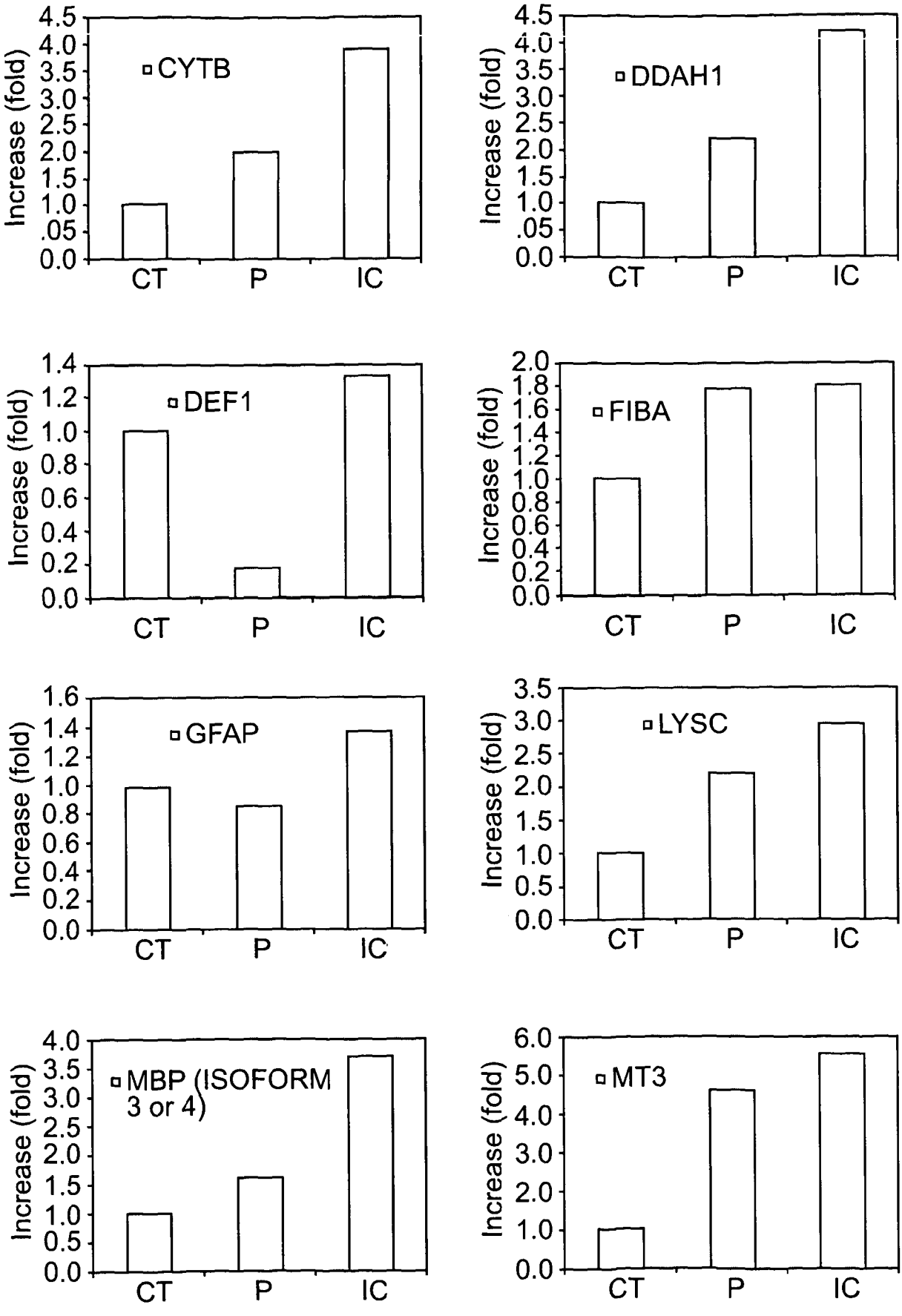


Figure 7 Continued

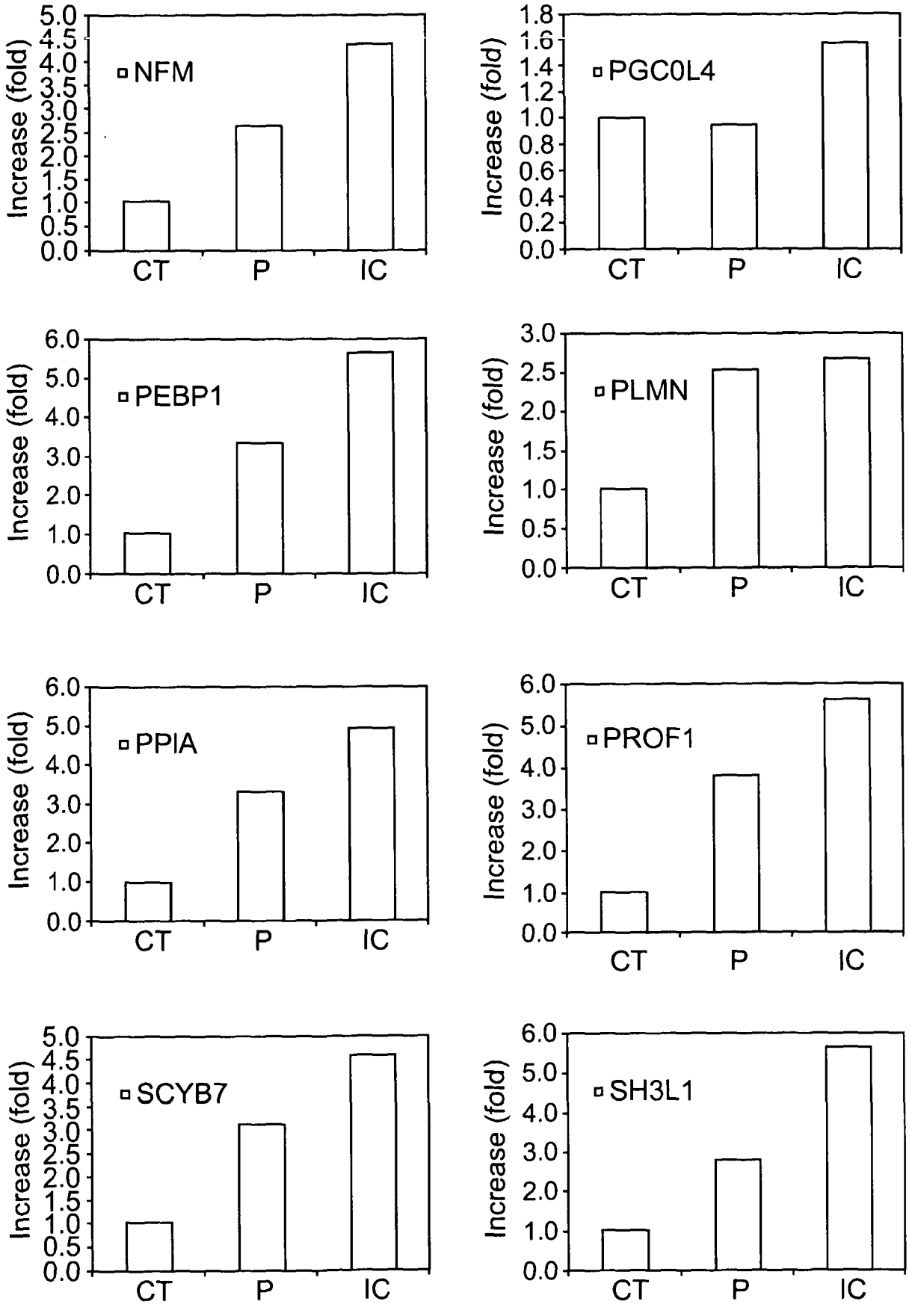


Figure 7 Continued

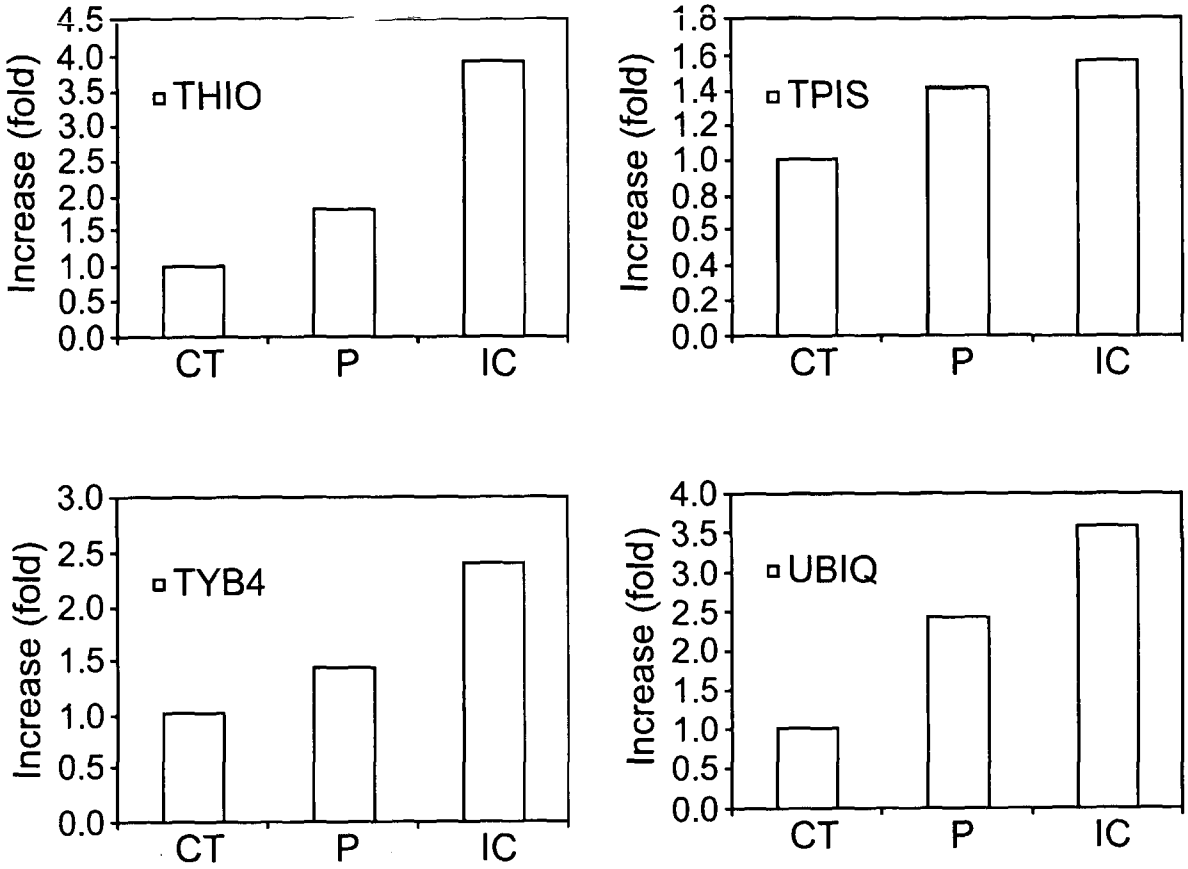


Figure 8

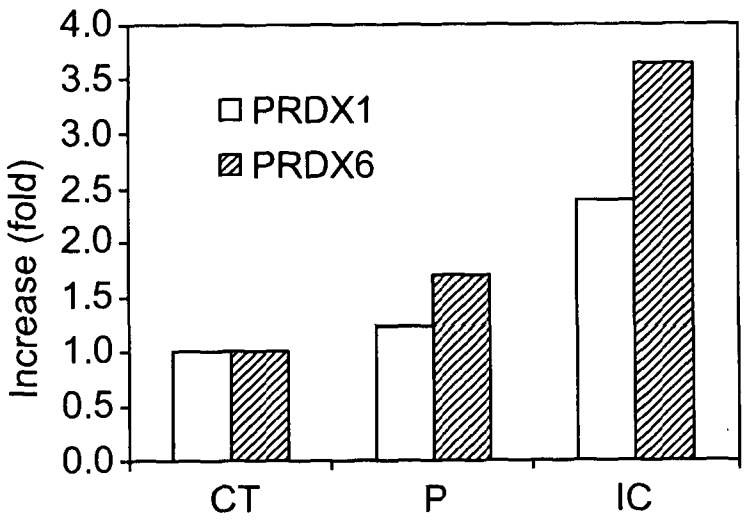


Figure 9

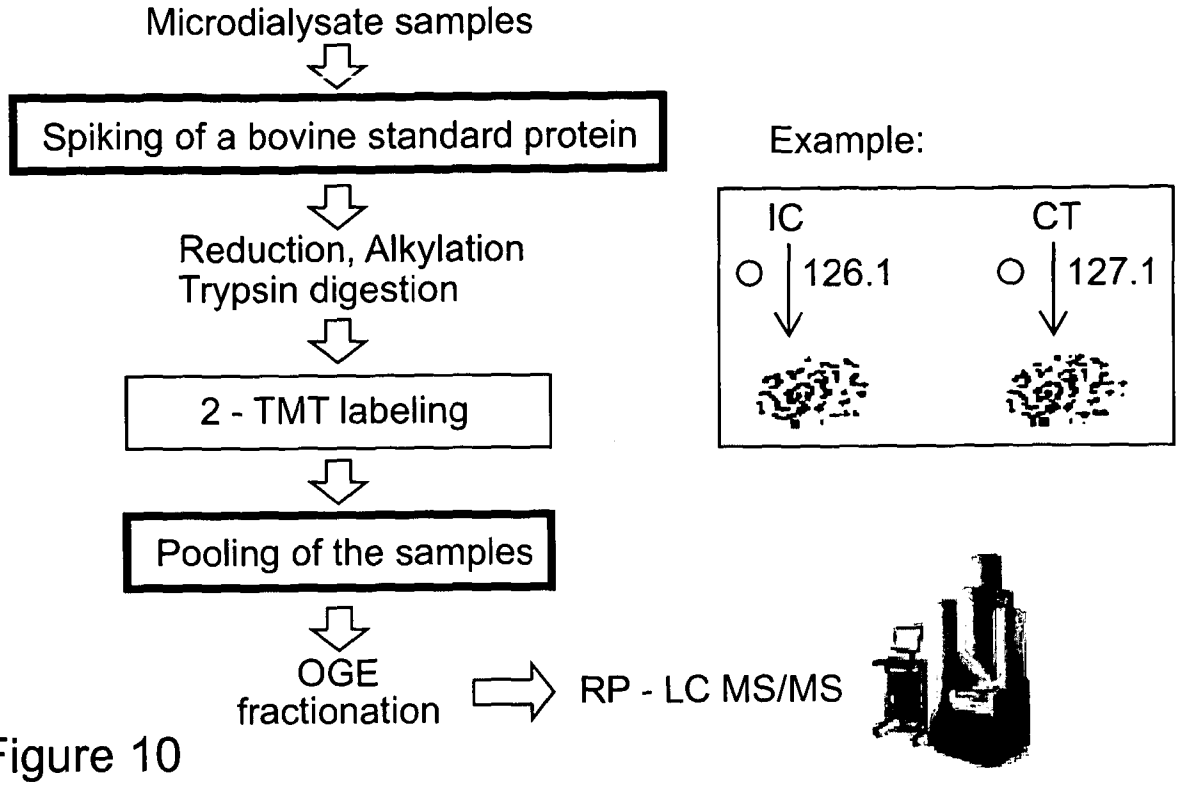


Figure 10

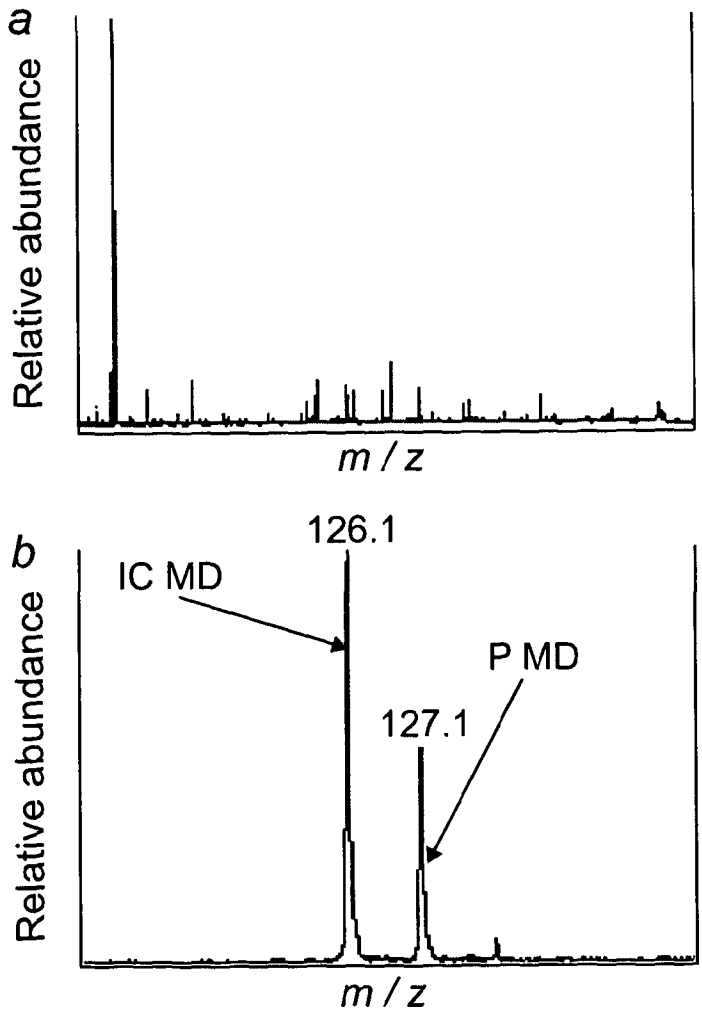


Figure 11

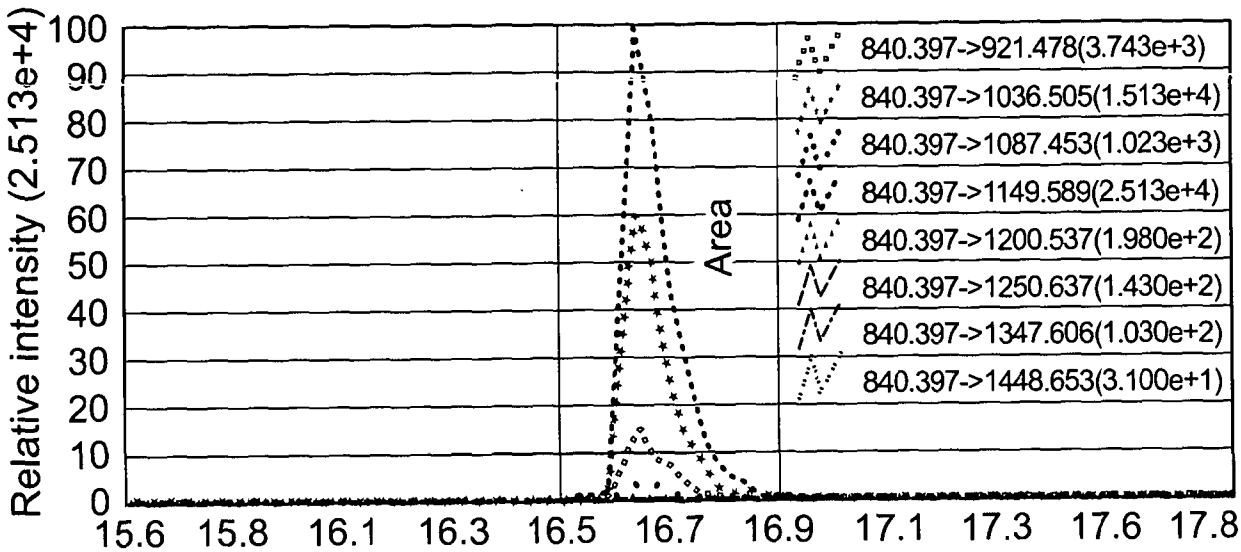


Figure 12

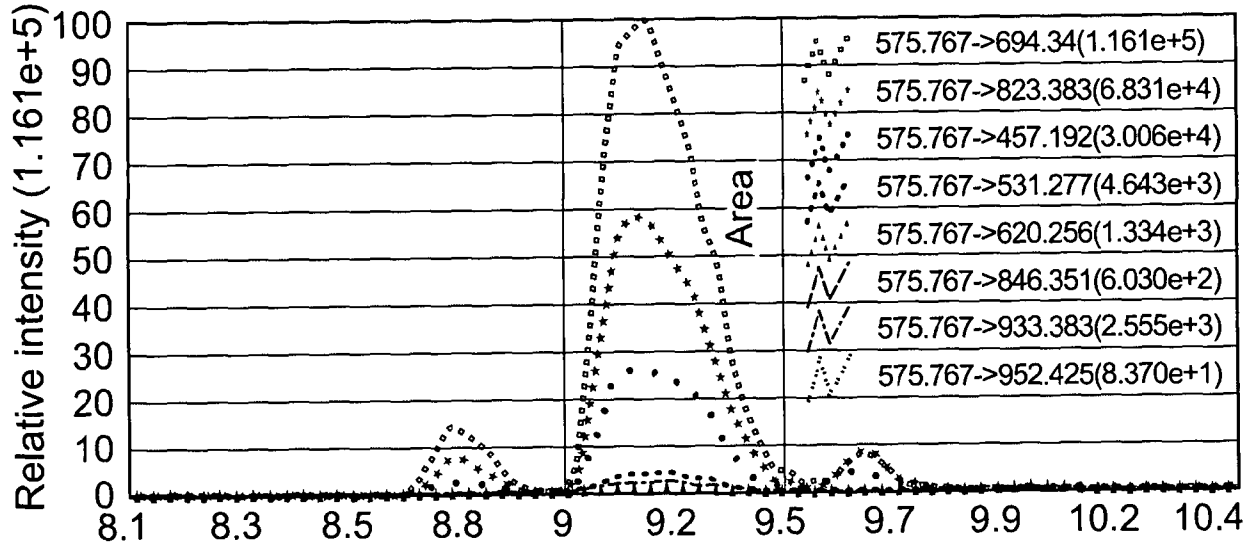


Figure 13

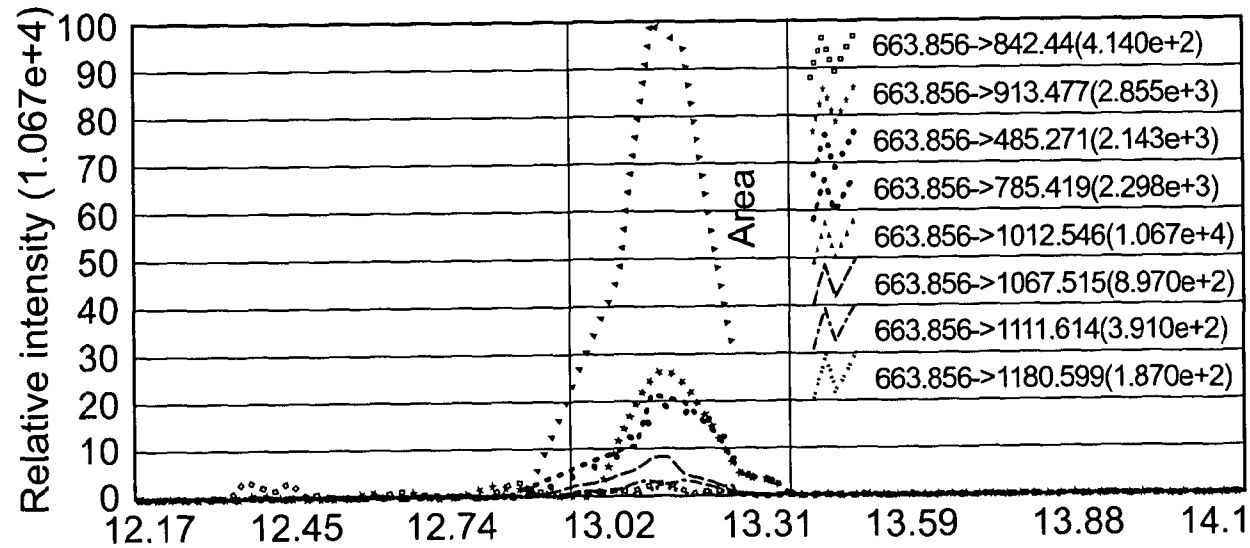


Figure 14

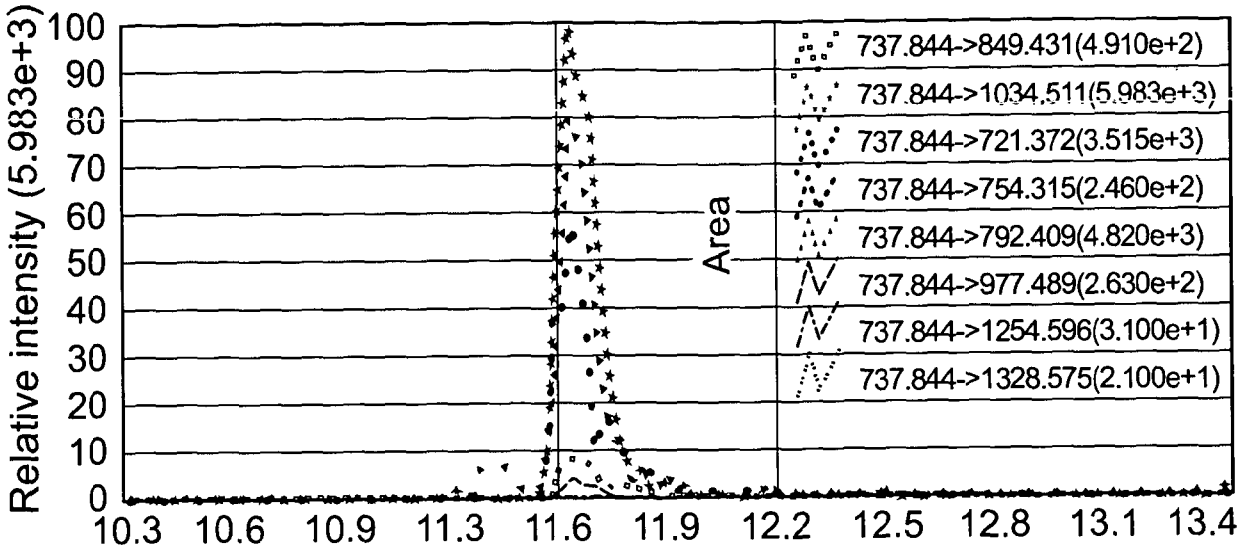


Figure 15

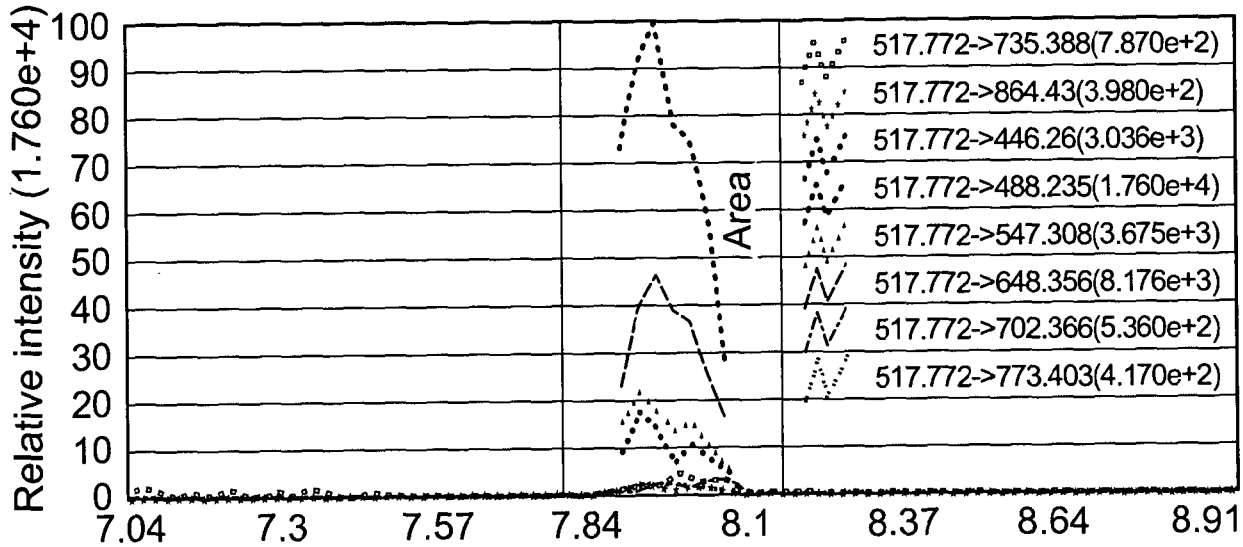


Figure 16