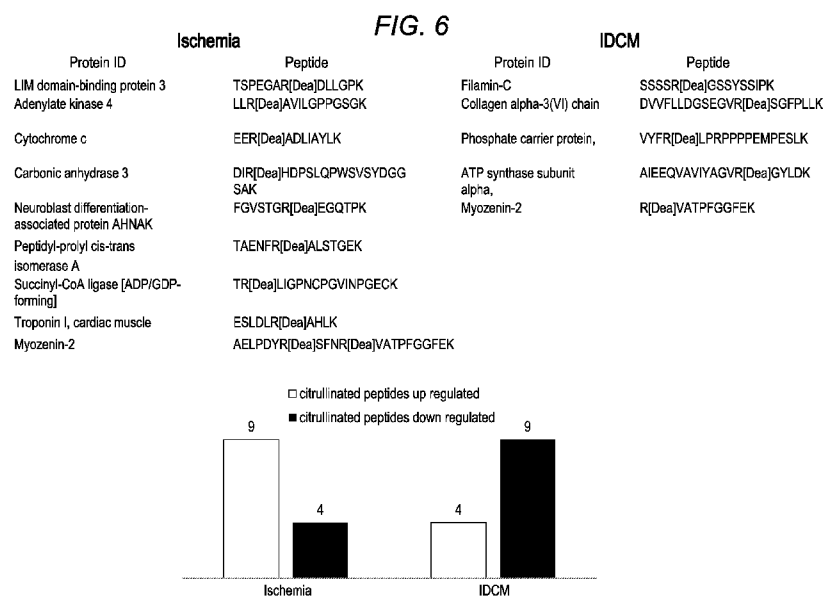




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[Continued on next page]

(54) **Title:** ROLE OF CITRULLINATION IN DIAGNOSING DISEASES

(57) **Abstract:** Provided herein are methods and markers for diagnosing cardiovascular disease and/or neurodegenerative diseases in a subject. The methods include obtaining a biological sample from a subject in need of diagnosis and detecting the amount of a citrullinated protein or a citrullinated peptide in the biological sample obtained from said subject.



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ROLE OF CITRULLINATION IN DIAGNOSING DISEASES

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH

[0001] This invention was made with Government support under Grant Nos. HL112586-01 and HHSN268201000032C awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0002] All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[0003] Citrullination, the irreversible post-translational modification (PTM) involving the conversion of arginine to citrulline by the family of enzymes peptidylarginine deiminase (PAD), is associated with several diseases. Citrullination appears to be a generalized process; autoantibodies targeting citrullinated proteins are relatively specific for rheumatoid arthritis (RA) and, although occasionally observed in other autoimmune conditions, are uncommonly observed in healthy individuals. In our previous study, elevated levels of autoantibodies to citrullinated protein antigens were found in the myocardium of RA patients. PADs were detected in cardiomyocytes, resident inflammatory cells, endothelial cells and vascular smooth muscle cells.

[0004] Citrullination results in a small increase in molecular mass (+0.984 Da) but converts the positively charged guanidine group on an arginine residue into the neutrally charged ureido group on the citrulline amino acid. The loss of charge from an arginine to a citrulline can have dramatic consequences on protein structure, proteolytic susceptibility, protein-protein interactions and intracellular signaling. Since citrullination can lead to profound changes in protein structure and function, it is not surprising that citrullination and the PAD enzymes are found in numerous chronic diseases. Furthermore, the conversion of arginine to citrulline is catalyzed in a Ca^{2+} -dependent manner with relatively high intracellular concentration of calcium. Because the cytosolic and nucleoplasmic calcium concentrations

are relatively low, PADs should be inactive under normal conditions. However PADs become activated in injured and dying cells, when calcium concentrations increase because of the influx of calcium ions from the extracellular environment and release from intracellular calcium stores.

[0005] The actual proteins that are citrullinated in myocardium is unknown as is whether i) myocardial citrullinated proteins are immune targets for circulating autoantibodies, ii) myocardial citrullinated proteins can themselves induce an autoimmune response, and iii) citrullinated proteins directly mediate phenotypic modifications to cardiac structure or function. There is precedent for PTMs of myocardial proteins leading to changes in cardiac contractility and structure in heart failure (HF). Phosphorylation, oxidation, and acetylation of sarcomeric proteins cause morphologic changes to proteins that lead to decreased contractile performance and adverse cardiac remodeling with HF. However, it is unknown whether citrullination of myocardial proteins plays a similar role.

[0006] The framework of health information on neurodegenerative diseases sometimes includes brain diseases, defined as pathologic conditions also affecting the brain (composed of the intracranial components of the central nervous system). This includes (but is not limited to) the cerebral cortex, intracranial white matter, basal ganglia, thalamus, hypothalamus, brain stem, and cerebellum (MeSH definition). It is important to note that not all individuals with a family history of neurodegenerative disease will have an identifiable gene mutation. This could be because the responsible gene has not yet been identified, or because the disease is not actually due to a gene mutation.

[0007] There have been challenges in identification of citrullinated proteins and the modified amino acid residues. Here, the inventors provide examples of citrullinated proteins and peptides showing modified amino acid residues which may serve as markers for cardiovascular diseases and neurodegenerative diseases.

SUMMARY

[0008] The following embodiments and aspects thereof are described and illustrated in conjunction with systems, compositions and methods which are meant to be exemplary and illustrative, not limiting in scope

[0009] Provided herein are method for diagnosing and predicting cardiovascular diseases. The methods include obtaining a biological sample from a subject in need of diagnosing

cardiovascular disease and detecting the amount of a citrullinated protein or a citrullinated peptide in the biological sample obtained from said subject. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increase risk of cardiovascular disease. In one embodiment, the change in citrullination levels is an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of cardiovascular disease. In another embodiment, the change in citrullination levels is a decrease in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of cardiovascular disease. In exemplary embodiments, presence of one or more citrullinated proteins or peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E is indicative of increased risk of cardiovascular disease. In exemplary embodiments, a change in the levels of citrullinated proteins or peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E is indicative of increased risk of cardiovascular diseases. In exemplary embodiments, the change in the levels is an increase in the levels of citrullination of proteins or peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E which is indicative of increased risk of cardiovascular diseases. In exemplary embodiments, the change in the levels is a decrease in the levels of citrullination of proteins or peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E which is indicative of increased risk of cardiovascular diseases. In some embodiments, an increase in citrullination levels of some peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E and a decrease in citrullination of some peptides shown in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E is indicative of increased risk of cardiovascular disease. In various embodiments, citrullination is at the Arginine residues, as indicated as “Dea” in Table 1A, Table 1B, Table 1C, Table 1D and/or Table 1E. In exemplary embodiments, cardiovascular diseases are any one or more of ischemic heart disease (ISHD), idiopathic myopathy (IDCM) or combinations thereof.

[0010] Also provided herein are methods for treating cardiovascular diseases in a subject in need thereof. The methods include diagnosing cardiovascular diseases by the methods set forth herein and administering an effective amount of a therapeutic agent to the subject diagnosed with cardiovascular diseases so as to treat cardiovascular diseases in the subject. In exemplary embodiments, cardiovascular diseases are any one or more of ischemic heart disease (ISHD), idiopathic myopathy (IDCM) or combinations thereof.

[0011] Provided herein are methods for diagnosing and/or predicting amyotrophic lateral sclerosis (ALS). The methods include obtaining a biological sample from a subject in need of diagnosing ALS and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of increased risk of ALS. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of ALS. In one embodiment, an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of ALS. In another embodiment, a decrease in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of ALS. In an exemplary embodiment, presence of citrullinated peptides shown in Table 2 is indicative of ALS. In exemplary embodiments, presence of one or more citrullinated peptides shown in Tables 2, 3 or 4 is indicative of increased risk of ALS. In exemplary embodiments, a change in the levels of citrullinated peptides shown in Tables 2, 3 or 4 is indicative of increased risk of ALS. In exemplary embodiments, a change in the amount of citrullination of peptides shown in Tables 2, 3 or 4 is indicative of increased risk of neurodegenerative diseases. In various embodiments, citrullination is at the Arginine residues, as indicated as “Dea” in Table 2, 3 or 4.

[0012] Also provided herein are methods for treating ALS in a subject in need thereof. The methods include diagnosing ALS by the methods set forth herein and administering an effective amount of a therapeutic agent to the subject diagnosed with ALS so as to treat ALS in the subject.

[0013] Provided herein are methods for diagnosing and/or predicting chronic neurodegenerative disease. The methods include obtaining a biological sample from a subject in need of diagnosing chronic neurodegenerative disease and detecting citrullinated proteins or citrullinated peptides in the biological sample obtained from said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of increased risk of chronic neurodegenerative disease. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of chronic neurodegenerative disease. In one embodiment, an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of chronic neurodegenerative disease. In another embodiment, a decrease in the levels of the citrullinated protein or the citrullinated

peptide relative to a reference value is indicative of increased risk of chronic neurodegenerative disease. In exemplary embodiments, a change in the levels of one or more citrullinated proteins or citrullinated peptides shown in Tables 5A-5B is indicative of increased risk of neurodegenerative diseases. In exemplary embodiments, the change in the citrullination levels is an increase in the levels of citrullination of one or more peptides or proteins shown in Tables 5A-5B which is indicative of increased risk of neurodegenerative diseases. In exemplary embodiments, the change in the citrullination levels is a decrease in the levels of citrullination of one or more peptides or proteins shown in Tables 5A-5B which is indicative of increased risk of chronic neurodegenerative diseases. In some embodiments, an increase in citrullination levels of some peptides shown in Table 5A-5B and a decrease in citrullination of some peptides shown in Tables 5A-5B is indicative of increased risk of chronic neurodegenerative disease. In exemplary embodiments, examples of neurodegenerative diseases include but are not limited to acquired brain injury, chronic neurodegenerative disease Alzheimer disease, Parkinson disease, Lewy body dementia, frontotemporal dementia, amyotrophic lateral sclerosis, Huntington disease, Friedreich's ataxia, spinal muscular atrophy, prion diseases, multiple sclerosis, stroke, degenerative nerve diseases, encephalitis, spinal muscular dystrophy and/or Creutzfeld-Jakob disease.

[0014] Also provided herein are methods for treating chronic neurodegenerative diseases in a subject in need thereof. The methods include diagnosing chronic neurodegenerative diseases by the methods set forth herein and administering an effective amount of a therapeutic agent to the subject diagnosed with chronic neurodegenerative disease so as to treat chronic neurodegenerative disease in the subject. In exemplary embodiments, examples of neurodegenerative diseases include but are not limited to acquired brain injury, chronic neurodegenerative disease Alzheimer disease, Parkinson disease, Lewy body dementia, frontotemporal dementia, amyotrophic lateral sclerosis, Huntington disease, Friedreich's ataxia, spinal muscular atrophy, prion diseases, multiple sclerosis, stroke, degenerative nerve diseases, encephalitis, spinal muscular dystrophy and/or Creutzfeld-Jakob disease.

[0015] Provided herein are methods for diagnosing and/or predicting acquired brain injury. The methods include obtaining a biological sample from a subject in need of diagnosing acquired brain injury and detecting citrullinated proteins or citrullinated peptides in the biological sample obtained from said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of increased risk of acquired brain injury. In some embodiments a change in the level of the citrullinated protein or the

citrullinated peptide relative to a reference value is indicative of increased risk of acquired brain injury. In one embodiment, an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of acquired brain injury. In another embodiment, a decrease in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of acquired brain injury. In exemplary embodiments, presence of one or more citrullinated proteins or citrullinated peptides shown in Tables 6A-6B is indicative of increased risk of acquired brain injury. In exemplary embodiments, a change in the levels of one or more citrullinated proteins or citrullinated peptides shown in Tables 6A-6B is indicative of increased risk of acquired brain injury. In exemplary embodiments, the change in the levels of citrullination is an increase in the levels of one or more peptides or proteins shown in Tables 6A-6B which is indicative of increased risk of acquired brain injury. In exemplary embodiments, the change in the citrullination levels is a decrease in the levels of citrullination of one or more peptides or proteins shown in Tables 6A-6B which is indicative of increased risk of acquired brain injury. In some embodiments, an increase in citrullination levels of some peptides shown in Table 6A-6B and a decrease in citrullination of some peptides shown in Tables 6A-6B is indicative of increased risk of acquired brain injury. In exemplary embodiments, acquired brain injury includes but is not limited to traumatic brain injuries (TBI's), strokes, brain illness, tumor, hemorrhage, and any other kind of brain injury acquired after birth.

[0016] Also provided herein are methods for treating acquired brain injury in a subject in need thereof. The methods include diagnosing acquired brain injury by the methods set forth herein and administering an effective amount of a therapeutic agent to the subject diagnosed with acquired brain injury so as to treat acquired brain injury in the subject. In exemplary embodiments, acquired brain injury includes but is not limited to traumatic brain injuries (TBI's), strokes, brain illness, tumor, hemorrhage, and any other kind of brain injury acquired after birth.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive

[0018] Figure 1 depicts in accordance with various embodiments of the invention, the cardiac citrullinated proteome. Diagrams show citrullinated proteins with significant p value group

by (A) cellular component and by (B) molecular function. Details can be found in Tables 1A-F. (C) Detection of citrullinated proteins in heart obtained from control and HF patients (ISHD, IDCM) using 4-12% SDS PAGE. Densitometry analysis revealed that there was no significant change between groups.

[0019] Figure 2 depicts in accordance with various embodiments of the invention, 2DE DIGE analysis with samples treated with PAD2. (A) Samples labeled with Cy2 (internal control), Cy3 (untreated) and Cy5 (treated) as described in methods section. (B) Proteins described by the network: contractility and energy production. Citrullinated proteins up and down regulated in ischemia were mapped onto the human STRING database of known human protein interactions. Red, protein marked as down regulated, green, proteins up-regulated and blue = protein-protein interacting partner. The following abbreviations were used: FLNC: Filamin C; LDB3: LIM domain-binding protein 3; MYBPC: Myosin-binding protein C, cardiac-type; MYOZ2: Myozenin-2; TNNI3: Troponin I; MYH: Myosin; CRYAB: Alpha-crystallin B chain; VIM: ATP synthase subunit alpha; AHNAK: Neuroblast differentiation-associated protein; HSP90AA1: Heat shock protein HSP 90-alpha; HSPD1: 60 kDa heat shock protein; PPIA: Peptidyl-prolyl cis-trans isomerase A; FABP3: Fatty acid-binding protein.

[0020] Figure 3 depicts in accordance with various embodiments of the invention, actin-binding studies. Increasing concentrations of A) TM (0.5uM to 2 uM) were incubated with F-actin or B) actin-HMM and C) TnI (0.5uM to 2uM) in buffer containing 40mM Tris-HCl (pH 7.6), 100mM NaCl, 5 mM MgCl₂, and 1 mM DTT. Binding of TM to F-actin was carried out at 25°C for 30 min and ultracentrifuged at 60,000 rpm for 25 min, 20°C, in a Beckman model TL-100.2. Both pellet and supernatant (unbound protein) were analyzed. Representative silver-stained gels show proteins composition of the supernatants and pellets. A triplicate set of gels, were analyzed by densitometry. Each data point is an average (and range) of the values obtained from the three sets of gels.

[0021] Figure 4 depicts in accordance with various embodiments of the invention, citrullination of sarcomeric proteins, biochemical and physiological effects. A) Regulation of the actomyosin HMM-ATPase activity by citrullinated F-actin and/or citrullinated HMM. B) Inhibition of actomyosin HMM-ATPase activity by TM. ATPase activity was measured as a function of TM concentration. The results are the average of four independent experiments for each protein at each TM concentration. Assay conditions: 0.2mg/ml F-actin, 0.02mg/ml

HMM, 0–2.0 μ M TM in 10 mM Hepes, pH 7.5, 30 mM NaCl, 5 mM MgCl₂, 4 mM ATP. PAD2 treatment reduced myofilament calcium sensitivity. C) Force-calcium relationships for untreated membrane-permeabilized myocytes from untreated control (n=8 myocytes from 3 mice, gray circles) and PAD2 treated (n=8 myocytes from 3 mice, open circles) groups. D) There was no difference in maximal calcium activated force (F_{max}) between the two groups. E) PAD2 treatment caused a significant (p=0.009) increase in EC₅₀ (calcium required to generate 50% F_{max}), indicating a decrease in calcium sensitivity. F) While the hill coefficient (nH) trended to be decreased by PAD2 treatment (see steepness of curve in panel C), the difference was not significant (p=0.34).

[0022] Figure 5 depicts in accordance with various embodiments of the invention, RT-PCR analysis of expression level of PAD isoforms in A, B) heart from control mouse, C) mouse keratinocytes and D) mouse macrophage cell line activated by lipopolysaccharide. The PCR product PAD2 is seen in all types of samples; PAD4 and PAD1 is seen in cardiac fibroblast, keratinocytes and macrophage cell line. PAD3 has not been detected. (MW: PAD1 285bp; PAD2 390bp; PAD3 200bp; PAD4 550bp).

[0023] Figure 6 depicts in accordance with various embodiments of the invention, a citrullinated protein up/down regulated between heart failure groups, ischemia and ICDM.

[0024] Figure 7 depicts in accordance with various embodiments of the invention, citrullination of the contractile proteins could affect different aspects of regulatory function. It could either trigger a structural change or stabilizes a conformation that is necessary for actin-activated release of Pi and completion of the ATPase cycle.

[0025] Figure 8 depicts in accordance with various embodiments of the invention, the use of SWATH-MS technology to identify citrullination of protein involved in neurodegenerative diseases.

[0026] Figure 9 depicts in accordance with various embodiments of the invention, an overview bioinformatics workflow for processing citrullinated spectra library file.

DETAILED DESCRIPTION

[0027] All references cited herein are incorporated by reference in their entirety as though fully set forth. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

invention belongs. Allen et al., Remington: The Science and Practice of Pharmacy 22nd ed., Pharmaceutical Press (September 15, 2012); Hornyak et al., Introduction to Nanoscience and Nanotechnology, CRC Press (2008); Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology 3rd ed., revised ed., J. Wiley & Sons (New York, NY 2006); Smith, March's Advanced Organic Chemistry Reactions, Mechanisms and Structure 7th ed., J. Wiley & Sons (New York, NY 2013); Singleton, Dictionary of DNA and Genome Technology 3rd ed., Wiley-Blackwell (November 28, 2012); and Green and Sambrook, Molecular Cloning: A Laboratory Manual 4th ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2012), provide one skilled in the art with a general guide to many of the terms used in the present application. For references on how to prepare antibodies, see Greenfield, Antibodies A Laboratory Manual 2nd ed., Cold Spring Harbor Press (Cold Spring Harbor NY, 2013); Köhler and Milstein, Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion, Eur. J. Immunol. 1976 Jul, 6(7):511-9; Queen and Selick, Humanized immunoglobulins, U. S. Patent No. 5,585,089 (1996 Dec); and Riechmann et al., Reshaping human antibodies for therapy, Nature 1988 Mar 24, 332(6162):323-7.

[0028] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Other features and advantages of the invention will become apparent from the following detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, various features of embodiments of the invention. Indeed, the present invention is in no way limited to the methods and materials described. For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[0029] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0030] The terms "marker" or "biomarker" are used interchangeably herein, and in the context of the present invention refer to a protein or peptide that has specific citrullinated amino acid residues or the enzyme itself, PAD 1, PAD2, PAD3 or PAD4 (of a particular specific identity or apparent molecular weight) which is differentially present in a sample taken from patients having a specific disease or disorder as compared to a control value, the control value consisting of, for example, average or mean values in comparable samples taken from control subjects (e.g., a person with a negative diagnosis, normal or healthy subject). Biomarkers may be determined as specific peptides or proteins (Tables 1-10) which may be detected by antibodies or mass spectroscopy. In some applications, for example, a mass spectroscopy or other profile or multiple antibodies may be used to determine multiple biomarkers, and differences between individual biomarkers and/or the partial or complete profile may be used for diagnosis. This can include detection of the enzyme or a protein it has citrullinated, alone or in combination.

[0031] The term "differentially present" or "change in level" refers to differences in the quantity and/or the frequency of a marker present in a sample taken from patients having a specific disease or disorder as compared to a control subject. For example, a marker can be present at an elevated level or at a decreased level in samples of patients with the disease or disorder compared to a control value (e.g. determined from samples of control subjects). Alternatively, a marker can be detected at a higher frequency or at a lower frequency in samples of patients compared to samples of control subjects. A marker can be differentially present in terms of quantity, frequency or both as well as a ratio of differences between two or more specific modified amino acid residues and/or the enzyme itself. In one embodiment, an increase in the ratio of modified to unmodified proteins and peptides described herein is diagnostic of any one or more of the diseases described herein. In an embodiment, an increase in citrullination of proteins and peptides as described herein refers to an increase in ratio of modified (citrullinated) to unmodified (non-citrullinated) proteins and/or peptides described herein. In one embodiment, a decrease in the ratio of modified to unmodified proteins and peptides described herein is diagnostic of any one or more of the diseases described herein. In an embodiment, a decrease in citrullination of proteins and peptides as described herein refers to a decrease in ratio of modified (citrullinated) to unmodified (non-citrullinated) proteins and/or peptides described herein.

[0032] A marker, compound, composition or substance is differentially present in a sample if the amount of the marker, compound, composition or substance in the sample is statistically

significantly different from the amount of the marker, compound, composition or substance in another sample, or from a control value. For example, a compound is differentially present if it is present at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% greater or less than it is present in the other sample (e.g. control), or if it is detectable in one sample and not detectable in the other.

[0033] Alternatively or additionally, a marker, compound, composition or substance is differentially present between samples if the frequency of detecting the marker, etc. in samples of patients suffering from a particular disease or disorder, is statistically significantly higher or lower than in the control samples or control values obtained from healthy individuals. For example, a biomarker is differentially present between the two sets of samples if it is detected at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 100% more frequently or less frequently observed in one set of samples than the other set of samples. These exemplary values notwithstanding, it is expected that a skilled practitioner can determine cut-off points, etc. that represent a statistically significant difference to determine whether the marker is differentially present.

[0034] "Diagnostic" means identifying the presence or nature of a pathologic condition and includes identifying patients who are at risk of developing a specific disease or disorder. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[0035] The terms "detection", "detecting" and the like, may be used in the context of detecting biomarkers, or of detecting a disease or disorder (e.g. when positive assay results are obtained). In the latter context, "detecting" and "diagnosing" are considered synonymous.

[0036] By "indicative of" or "at risk of" is intended to mean at increased risk of, compared to a normal subject, or compared to a control group, e.g. a patient population. Thus a subject

carrying a particular marker may have an increased risk for a specific disease or disorder, and be identified as needing further testing. "Increased risk" or "elevated risk" mean any statistically significant increase in the probability, e.g., that the subject has the disorder. The risk is preferably increased by at least 10%, more preferably at least 20%, and even more preferably at least 50% over the control group with which the comparison is being made.

[0037] A "test amount" of a marker refers to an amount of a marker present in a sample being tested. A test amount can be either in absolute amount (e.g., $\mu\text{g/ml}$) or a relative amount (e.g., relative intensity of signals).

[0038] A "diagnostic amount" of a marker refers to an amount of a marker in a subject's sample that is consistent with a diagnosis of a particular disease or disorder. A diagnostic amount can be either in absolute amount (e.g., $\mu\text{g/ml}$) or a relative amount (e.g., relative intensity of signals).

[0039] A "control amount" of a marker can be any amount or a range of amount which is to be compared against a test amount of a marker. For example, a control amount of a marker can be the amount of a marker in a person who does not suffer from the disease or disorder sought to be diagnosed. A control amount can be either in absolute amount (e.g., $\mu\text{g/ml}$) or a relative amount (e.g., relative intensity of signals).

[0040] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of α -amino acid residues, in particular, of naturally-occurring α -amino acids. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally-occurring amino acid, as well as to naturally-occurring amino acid polymers. Polypeptides can be modified, e.g., by the addition of carbohydrate residues to form glycoproteins, phosphorylation to form phosphoproteins, and a large number of chemical modifications (oxidation, deamidation, amidation, methylation, formylation, hydroxymethylation, guanidination, for example) as well as degraded, reduced, or crosslinked. The terms "polypeptide," "peptide" and "protein" include all unmodified and modified forms of the protein. A peptide would have a citrullinated residue or is part of the PAD enzyme.

[0041] "Detectable moiety" or a "label" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , ^{35}S , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly

used in an ELISA), biotin-streptavidin, digoxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantify the amount of bound detectable moiety in a sample. Quantitation of the signal is achieved by, e.g., scintillation counting, densitometry, flow cytometry, or direct analysis by mass spectrometry of intact protein or peptides (one or more peptide can be assessed) that has a potential citrullinated residue or part of the PAD enzyme. Citrullinated Arg as part of a protein or peptide can be detected directly by MS or via chemical derivatization. Any capture reagent including but not limited to antibodies and aptamers may be used to detect citrullinated peptides described herein.

[0042] "Antibody" refers to a polypeptide ligand substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an epitope (e.g., an antigen). The recognized immunoglobulin genes include the kappa and lambda light chain constant region genes, the alpha, gamma, delta, epsilon and mu heavy chain constant region genes, and the myriad immunoglobulin variable region genes. Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. This includes, e.g., Fab' and F(ab)'.sub.2 fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. It also includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, or single chain antibodies. "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH1, CH2 and CH3, but does not include the heavy chain variable region.

[0043] By "binding assay" is meant a biochemical assay wherein the biomarkers are detected by binding to an agent, such as an antibody, through which the detection process is carried out. The detection process may involve radioactive or fluorescent labels, and the like. The assay may involve immobilization of the biomarker, or may take place in solution. Further, chemical binding to the citrullinated residue can occur directly.

[0044] "Immunoassay" is an assay that uses an antibody to specifically bind an antigen (e.g., a marker). The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

[0045] The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, *Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

[0046] Methods for detecting citrullination refer to the mass spectrometry (MS) based methods used to detect citrullinated peptides, polypeptides and proteins. The methods include but are not restricted to neutral loss of 1 Da when deimination occurs on Arg; neutral loss of isocyanic acid from unmodified citrulline and used this ion as a diagnostic marker for detecting protein citrullination; derivatization when chemical modification of 238 Da or 239 Da occurs on Cit residue (can be monitored at the peptide and protein level); enrichment of citrullinated peptides (or proteins) that is based on the specific reaction of glyoxal derivatives that is immobilized on beads/column/matrix reacts exclusively with the ureido group of the citrulline residue at low pH. As well, MS using a targeted method like multiple or selective reaction monitoring can be used to quantify the modified peptide directly. In some embodiments, a labeled (e.g. labeled with ^{15}N or chemically labeled with additional stable isotopes) peptide of known concentration is added to the sample and compared directly to the endogenous (unlabeled) corresponding peptide allowing for quantification (based on a ratio of the endogenous peptide to the isotopic labeled peptides or absolute concentration if the exact amount of the isotopic labeled peptide is known).

[0047] The terms "subject", "patient" or "individual" generally refer to a human, although the methods of the invention are not limited to humans, and should be useful in other animals (e.g. birds, reptiles, amphibians, mammals), particularly in mammals, since albumin is homologous among species.

[0048] "Sample" is used herein in its broadest sense. A sample may comprise a bodily fluid including blood, serum, plasma, tears, aqueous and vitreous humor, spinal fluid; a soluble fraction of a cell or tissue preparation, or media in which cells were grown; or membrane isolated or extracted from a cell or tissue; polypeptides, or peptides in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, skin or hair; fragments and derivatives thereof. Subject samples usually comprise derivatives of blood products, including blood, plasma and serum.

[0049] The term "modulation of specific PAD isoforms" includes but is not limited to, increasing or decreasing the activity of endogenous PAD isoforms using gene therapy, siRNA, known inhibitors of PADs, or site-directed mutagenesis.

[0050] The term "peptide" as used herein refers to a polymer of amino acid residues typically ranging in length from 2 to about 30, or to about 40, or to about 50, or to about 60, or to about 70 residues. In certain embodiments the peptide ranges in length from about 2, 3, 4, 5, 7, 9, 10, or 11 residues to about 60, 50, 45, 40, 35, 30, 25, 20, or 15 residues. In certain embodiments the peptide ranges in length from about 8, 9, 10, 11, or 12 residues to about 15, 20 or 25 residues. In certain embodiments the amino acid residues comprising the peptide are "L-form" amino acid residues, however, it is recognized that in various embodiments, "D" amino acids can be incorporated into the peptide. Peptides also include amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. In addition, the term applies to amino acids joined by a peptide linkage or by other, "modified linkages" (e.g., where the peptide bond is replaced by an α -ester, a β -ester, a thioamide, phosphonamide, carbamate, hydroxylate, and the like (see, e.g., Spatola, (1983) *Chem. Biochem. Amino Acids and Proteins* 7: 267-357), where the amide is replaced with a saturated amine (see, e.g., Skiles et al., U.S. Pat. No. 4,496,542, which is incorporated herein by reference, and Kaltenbronn et al., (1990) Pp. 969-970 in *Proc. 11th American Peptide Symposium*, ESCOM Science Publishers, The Netherlands, and the like)).

[0051] Citrullination (also called deimination) is a post-translational protein modification catalyzed by PADs that involves conversion of the amino acid arginine to citrulline within proteins. The involvement of citrullinated proteins in the pathogenesis of a number of autoimmune diseases has been established. For example, protein citrullination has been directly linked to the generation of autoantibodies contributing to rheumatoid arthritis (RA). However, the exact circumstances and underlying purpose of PAD activation and consequent protein citrullination within cells are not fully understood. In addition, the production of autoantibodies targeting citrullinated proteins has been implicated in many other autoimmune diseases such as multiple sclerosis, psoriasis, sporadic Creutzfeld–Jakob disease, Parkinson's disease and Alzheimer's disease (AD). The knowledge of citrullinated proteins and citrullinated sites in protein sequences can provide invaluable information about the etiological importance and function of these citrullinated proteins. Provided herein are new biomarkers predictive of cardiovascular diseases and neurodegenerative diseases which include citrullinated proteins and peptides.

[0052] Citrullination is linked to autoantigen and autoantibody response which may be used for detection or measurement of ratios of autoantigen (unmodified/not-citrullinated) to autoantigen (modified/citrullinated) to detect heart disease (citrullinated autoantibody/autoantigens as biomarker). Further, detection of citrullinated autoantibody can be used as risk stratification factor. Risk of developing heart failure (HF) and succumbing to myocardial infarction increases with age. Citrullination is known to increase with age and an increase in citrullination may be predictive of heart failure. Inhibition (eg. drug inhibition) of PAD enzyme responsible for citrullination may be a therapeutic target.

[0053] Protein citrullination plays a role in the generation of autoantibodies during the pathogenesis of specific neurodegenerative diseases such as Alzheimer's disease (AD). Since citrullination alters the overall charge distribution within a protein, potentially modifying tertiary structure, many citrullinated proteins in these PAD-expressing cells may be in a non-native conformation that is potentially immunogenic, especially if they are released from the cell and thus become accessible to immune surveillance.

[0054] Citrullination is linked to autoantigen and autoantibody response which may be used for detection or measurement of ratios of autoantigen (unmodified/not-citrullinated) to autoantigen (modified/citrullinated) to detect neurodegenerative disease (citrullinated autoantibody/autoantigens as biomarker). Citrullination is known to increase with age and an

increase in citrullination may be predictive of neurodegenerative diseases. Inhibition (eg. drug inhibition) of PAD enzyme responsible for citrullination may be a therapeutic target.

Detection of peptidyl-citrulline - Comparison with other methods

[0055] Current research on citrullination focuses on its role in auto-immune diseases such as multiple sclerosis and rheumatoid arthritis (RA) [Baka Z, et al. Citrullination under physiological and pathological conditions. *Joint Bone Spine* 2012,79:431-436; De Ceuleneer M, et al. In vivo relevance of citrullinated proteins and the challenges in their detection. *Proteomics* 2012,12:752-760]. In this context, the exact knowledge of citrullination sites in a protein can provide invaluable information about the etiological importance of these citrullinated proteins. However, few techniques exist that can accurately detect citrullination at the peptide level in a high-throughput format.

[0056] In general analysis of PTMs by MS is a difficult task and dependent on PTM profound effect on local peptide characteristics, like: (i) the mass shift in the peptide molecular weight induced by the PTM, (ii) the overall abundance of the modified peptide, (iii) the stability of the modification during MS and MS/MS analysis, and (iv) the effect of the modification on the peptide's ionization efficiency. Citrullination is complicated as citrullinated proteins / peptides are difficult to discern from their non-PTM forms because citrullination leads to an additional mass of nominally 0.9802 Dalton compared to regular arginine residues, which prone to false positives. The commonly applied shotgun type liquid chromatography tandem MS (LC-MS/MS) methods depend on automated database screening which in this case may cause misidentification of ^{13}C isotopes or misidentification of other modifications that lead to a similar mass increase (in particular the commonly occurring deamidation of glutamine or asparagine residues). Misidentification of ^{13}C isotopes can be avoided by searching the database that has a small enough parent mass tolerance (<5 ppm), because ^{13}C leads to a slightly larger mass increase (1.0036 Da) than the conversion of arginine into citrulline (0.9802 Da) [De Ceuleneer M, et al. In vivo relevance of citrullinated proteins and the challenges in their detection. *Proteomics* 2012,12:752-760; Raijmakers R, et al. Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res Ther* 2012,14:R114]. Deamidation, however, cannot be discriminated from citrullination based on mass alone. Deamidation does not change the number of charged residues in the peptide (under the acidic conditions used for LC-MS), whereas the conversion of arginine to citrulline neutralizes a positive charge.

Citrullination results in a loss of positive charge that causing a shift in the isoelectric point from 11.41 for arginine to 5.91 for citrulline. Therefore, citrullination increases the overall hydrophobicity of a peptide compared to the non-modified and/or deamidated peptide resulting in a shift in retention time, which is a proof for a peptide being citrullinated rather than deamidated [Raijmakers R, *et al.* Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res Ther* 2012,14:R114.]. However, with the current methods it remains challenging to rapidly, consistently, reproducibly, accurately, and sensitively detect and quantify citrullinated peptides across multiple samples.

Advantages of SWATH MS over other strategies for citrullinated-targeted protein detection

[0057] The most commonly used mass spectrometry-based method for high-throughput protein citrullination analysis is data-dependent acquisition (DDA) [Taheri DC, Set al. Data-controlled automation of liquid chromatography/tandem mass spectrometry analysis of peptide mixtures. *J Am Soc Mass Spectrom* 1996,7:532-540; Yates JR, 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem* 1995,67:1426-1436]. However, stochastic precursor selection and dependence on MS1 ions for quantification impede comprehensive. To overcome this limitation a few years ago Aebersold's group came up with SWATH MS as a targeting method similar to multiple reaction monitoring (MRM). SWATH MS is a technique that combines data-independent acquisition (DIA) with targeted data extraction, which together gives quantitative accuracy, reproducibility, dynamic range, and extends the number of quantifiable peptides [Gillet LC1 NP TS, Röst H, Selevsek N, Reiter L, Bonner R, Aebersold R. . Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. . *Mol Cell Proteomics* 2012; Liu Y, et al. Mass spectrometric protein maps for biomarker discovery and clinical research. *Expert Rev Mol Diagn* 2013,13:811-825]. Creation of highly citrullinated library improves the sensitivity and quantitative accuracy.

Cardiovascular Diseases

[0058] Provided herein are method for diagnosing and/or predicting cardiovascular diseases. The methods include obtaining a biological sample from a subject in need of diagnosing cardiovascular disease and detecting the amount of a citrullinated protein or a citrullinated peptide in the biological sample obtained from said subject. In some embodiments a change

in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of cardiovascular disease. In one embodiment, an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of cardiovascular disease. In another embodiment, a decrease in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of cardiovascular disease. In exemplary embodiments, change in citrullination levels of one or more proteins or peptides shown in Tables 1A, 1B, 1C, 1D and/or 1E is indicative of increased risk of cardiovascular diseases. In some embodiments, an increase in citrullination of the proteins shown in Table 1C, 1D, 1E and 1F as indicated by “up” in Table 1C, 1D, 1E and 1F is indicative of increased risk of cardiovascular disease. In some embodiments, a decrease in citrullination of the proteins shown in Table 1C, 1D, 1E and 1F as indicated by “down” in Table 1C, 1D, 1E and 1F is indicative of increased risk of cardiovascular disease. In some embodiments, change in citrullination refers to ‘differentially present’ citrullination as defined herein

[0059] In some embodiments, a subject has ischemic heart disease (ISHD) or is at an increased risk of ISHD if the sample from the subject has an increase in citrullination in any one or more of the proteins in Table 1C, as indicated by ‘up’ in ischemic heart disease column (‘I’). In some embodiments, the subject has or is at increased risk of ISHD if the sample from the subject has the presence of citrullination or an increase in citrullination of any one or more of the peptides in Table 1A. In some embodiments, the subject has or is at an increased risk of ISHD if the sample from the subject has the absence of citrullination or a decrease in citrullination of any one or more of the peptides in Table 1A. In some embodiments, a subject has or is at an increased risk of ISHD if the sample from the subject has an increase in citrullination in any one or more of ES1 protein homolog, aconitate hydratase or citrullinated fragments thereof, glyceraldehyde-3-phosphate dehydrogenase or citrullinated fragments thereof, delta-1-pyrroline-5-carboxylate dehydrogenase or citrullinated fragments thereof, vimentin or citrullinated fragments thereof, neuroblast differentiation-associated protein AHNK or citrullinated fragments thereof, serum deprivation-response protein or citrullinated fragments thereof, enoyl-CoA hydratase or citrullinated fragments thereof, aconitate hydratase or citrullinated fragments thereof, or combinations thereof.

[0060] In some embodiments, a subject has idiopathic cardiomyopathy (IDCM) or is at increased risk of IDCM if the sample from the subject has an increase in citrullination in any

one or more of the proteins in Table 1D, as indicated by ‘up’ in IDCM column (‘ID’). In some embodiments, the subject has or is at increased risk of IDCM if the sample from the subject has the presence of citrullination or an increase in citrullination of any one or more of the peptides in Table 1A. In some embodiments, the subject has or is at an increased risk of IDCM if the sample from the subject has the absence of citrullination or a decrease in citrullination of any one or more of the peptides in Table 1A. In some exemplary embodiments, a subject has or is at an increased risk of IDCM if the sample from the subject has an increase in citrullination in any one or more of ATP synthase subunit alpha or citrullinated fragments thereof, Myozenin-2 or citrullinated fragments thereof, Glyceraldehyde-3-phosphate dehydrogenase or citrullinated fragments thereof, ES1 protein homolog or citrullinated fragments thereof and/or combinations thereof.

[0061] In some embodiments, the subject has or is at increased risk of having cardiovascular disease (ISHD, IDCM or both ISHD and IDCM) if the sample from the subject has an increase in citrullination in any one or more of the proteins or peptides set forth in Tables 1A-1F. In some embodiments, the subject has or is at increased risk of having cardiovascular disease (ISHD, IDCM or both ISHD and IDCM) if the sample from the subject has an increase in citrullination in any one or more of ATP synthase subunit alpha or citrullinated fragments thereof, glyceraldehyde-3-phosphate dehydrogenase or citrullinated fragments thereof, ES1 protein homolog or citrullinated fragments thereof, delta-1-pyrroline-5-carboxylate dehydrogenase or citrullinated fragments thereof, enoyl-CoA hydratase or citrullinated fragments thereof, voltage-dependent anion-selective channel protein 1 or citrullinated fragments thereof, aconitate hydratase or citrullinated fragments thereof, pyruvate dehydrogenase E1 component subunit alpha, somatic form or citrullinated fragments thereof, vimentin or citrullinated fragments thereof, trifunctional enzyme subunit beta or citrullinated fragments thereof, myosin-binding protein C, cardiac-type or citrullinated fragments thereof, chloride intracellular channel protein 4 or citrullinated fragments thereof, heat shock protein HSP 90-alpha or citrullinated fragments thereof, serum deprivation-response protein or citrullinated fragments thereof, alcohol dehydrogenase 1B or citrullinated fragments thereof, succinate dehydrogenase [ubiquinone] iron-sulfur subunit or citrullinated fragments thereof, dual specificity protein phosphatase 3 or citrullinated fragments thereof or combinations thereof (Table 1E). In some embodiments, the subject has or is at increased risk of having cardiovascular disease (ISHD, IDCM or both ISHD and IDCM) if the sample from the subject has a decrease in citrullination in any one or more of Troponin I, Alpha-

crystalline B chain, ATP-synthase-coupling factor 6, beta-enolase, adenylate kinase isoenzyme I or combination thereof (Table 1F). In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0062] Also provided herein is a method comprising obtaining a biological sample from a subject in need of diagnosing cardiovascular disease, using a peptide to assay the biological sample to detect the presence or absence of an antibody capable of binding specifically to the peptide, determining the presence of cardiovascular disease when the presence of the antibody capable of binding specifically to the peptide is detected and directing the subject to treatment of cardiovascular disease when the presence of the antibody capable of binding specifically to the peptide is detected. In various embodiments, presence of the antibody capable of binding specifically to the peptide is indicative of any one or more of ISHD, ICDM, heart failure or a combination thereof. In some embodiments, the peptide is a fragment of Troponin I, Vimentin, Myosin-7 or a combination thereof. In various embodiments, the peptide is citrullinated. In exemplary embodiments, the peptide comprises, consists of or consists essentially of the amino acid sequence NIDALSGMEGRK (SEQ ID NO: 18), PRSFMPNLVPPK (SEQ ID NO: 20), ESLDLRAHLK (SEQ ID NO: 19), AEETQRSVNDLTSQRAK (SEQ ID NO: 24) or FADLSEAANRNNDALRQAK (SEQ ID NO: 22). In exemplary embodiments, the peptide sequence of the citrullinated peptides in the sample from the subject is at least 95%, 90%, 80%, 70% or 60% identical to peptides set forth in any of Table 1A-1F. In exemplary embodiments, the peptide sequence of the citrullinated peptides in the sample from the subject is at least 95%, 90%, 80%, 70% or 60% identical to NIDALSGMEGRK (SEQ ID NO: 18), PRSFMPNLVPPK (SEQ ID NO: 20), ESLDLRAHLK (SEQ ID NO: 19), AEETQRSVNDLTSQRAK (SEQ ID NO: 24) or FADLSEAANRNNDALRQAK (SEQ ID NO: 22). In various embodiments, the length of the peptide is optimized to bind the antibody in the sample. In various embodiments, citrullinated peptides are detected using mass spectrometry, high resolution mass spectrometry, tandem mass spectrometry, binding assay, immunoassay, SDS page electrophoresis, Western blot analysis, conformation on mass spectrometry, including SWATH.

[0063] In various embodiments, the methods further comprise prescribing treatment to the subject diagnosed with cardiovascular disease. In various embodiments, treatments include but are not limited to any one or more of therapeutic lifestyle changes, therapeutic agents, surgical treatments or a combination thereof. In exemplary embodiments, lifestyle changes

include any one or more of healthy diet, physical activity, weight management, stress management, not smoking or a combination thereof. In exemplary embodiments, therapeutic agents include any one or more of Angiotensin-converting enzyme (ACE) inhibitors, Angiotensin II receptor blockers (ARBs), Beta-blockers, Aldosterone antagonists, Cardiac glycosides, Diuretics, Vasodilators, Antiarrhythmics, Human B-type natriuretic peptide, Inotropic agent, Anticoagulants, inhibitors of peptidyl arginine deiminase (PAD) enzymes or a combination thereof. In exemplary embodiments, surgical treatments include any one or more of use of left ventricular assist devices, cardiac resynchronization therapy (biventricular pacing), automatic implantable cardioverter-defibrillators, ventricular restoration surgery, heart transplantation, angioplasty, stents, atherectomy, radiation therapy, coronary artery bypass grafting or a combination thereof. Appropriate therapies will be apparent to one skilled in the art.

[0064] In various embodiments of the processes, assays and methods described herein, the reference value is based on the change in the level of citrullinated proteins or citrullinated peptides, as set forth in, for example, Tables 1A, 1B, 1C, 1D, 1E and/or 1F. In one embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in a cardiomyocyte. In another embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in a non-cardiac cell. In an additional embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in any cell. In an additional embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in any bodily fluid. In some embodiments, the reference value is the mean or median change in level of citrullinated proteins or citrullinated peptides in a population of subjects that do not have cardiovascular disease. In other embodiments, the reference value is the mean or median change in level of citrullinated proteins or citrullinated peptides in a population of subjects that have cardiovascular diseases but have undergone treatment and show no or reduced expression of citrullinated proteins or citrullinated peptides. In additional embodiments, the reference value is the change in expression level of citrullinated proteins or citrullinated peptides in a sample obtained from the subject from a different (for example, an earlier) time point, such as during diagnosis, before treatment, after treatment or a combination thereof. In some embodiments, cardiovascular disease is any one or more of ISHD, IDCM, heart failure or a combination thereof. In some embodiments, change in citrullination refers to ‘differentially present’ citrullination as defined herein.

[0065] In various embodiments, the change in the level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having cardiovascular disease compared to the reference value is increased by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. In various embodiments, the change in the level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having cardiovascular disease compared to the reference value is increased by at least or about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, 100-fold or a combination thereof.

[0066] In various embodiments, the change in the level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having cardiovascular disease compared to the reference value is decreased by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. In various embodiments, the change in the level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having cardiovascular disease compared to the reference value is decreased by at least or about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, 100-fold or a combination thereof.

Neurodegenerative Diseases

[0067] Provided herein are methods for diagnosing and/or predicting chronic neurodegenerative diseases and/or acquired brain injury and/or acute brain disease. The methods include obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of neurodegenerative diseases. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of neurodegenerative disease and/or acquired brain injury and/or acute brain disease. In one embodiment, an increase in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of neurodegenerative disease and/or acquired brain injury and/or acute brain disease. In another embodiment, a decrease in the levels of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of neurodegenerative disease and/or acquired brain injury and/or acute brain

disease. In exemplary embodiments, a change in the levels of one or more citrullinated peptides or citrullinated proteins shown in Tables 2-10 is indicative of neurodegenerative diseases. In exemplary embodiments, a change in the amount of citrullination of one or more peptides or proteins shown in Tables 2-10 is indicative of neurodegenerative diseases. In some embodiments, the change in the level is an increase in the level of one or more citrullinated peptides or citrullinated proteins shown in Tables 2-10 which is indicative of chronic neurodegenerative disease. In some embodiments, the change in the level is a decrease in the level of one or more citrullinated peptides or citrullinated proteins shown in Tables 2-10 which is indicative of chronic neurodegenerative disease. In some embodiments, change in citrullination refers to ‘differentially present’ citrullination as defined herein.

[0068] In exemplary embodiments, examples of neurodegenerative diseases include but are not limited to Alzheimer disease, Parkinson disease, Lewy body dementia, frontotemporal dementia, amyotrophic lateral sclerosis, Huntington disease, Friedreich’s ataxia, spinal muscular atrophy, prion diseases, multiple sclerosis, stroke, degenerative nerve diseases, encephalitis, Motor neurone diseases (MND, Spinocerebellar ataxia (SCA), Spinal muscular atrophy (SMA) or Creutzfeld-Jacob disease.

[0069] In some embodiments, the biological sample is any one or more of blood, plasma, serum, urine or tissue (tissue biopsy).

[0070] In various embodiments, the level of the citrullinated protein or citrullinated peptide is detected using mass spectrometry, high resolution mass spectrometry, tandem mass spectrometry, binding assay, immunoassay, antibody binding or immunohistochemistry.

[0071] Provided herein is a method for diagnosing ALS in a subject in need thereof. The method includes obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from the said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of ALS. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of ALS. In one embodiment, an increase in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of ALS. In one embodiment, the subject has ALS or is at increased risk of having ALS if the sample from the subject has an increase in citrullination in any one or more of the proteins in Table 2, Table 3 or Table 4. In another embodiment, the subject has ALS or is at increased risk of having ALS if the sample from the

subject has an increase in citrullination in any one or more of the peptides PSGVQMDPCCRALYDFEPENEGELGFK (SEQ ID NO: 306), QYMRRSTCTINYSK (SEQ ID NO: 307), LILIARNK (SEQ ID NO: 308), VIVVWVGTTNNHENTAEVAGGIEAIVQLINTRQPQAK (SEQ ID NO: 309), EVDVGLAADVGTLLQRLPK (SEQ ID NO: 310), HEEAPGHRPTTNPASK (SEQ ID NO: 311), GETPVNSTMSIGQARK (SEQ ID NO: 312), VRIQTQPGYANTLRDAAPK (SEQ ID NO: 313), NVGCLQEALQLATSFAQLRLGDVK (SEQ ID NO: 314) or combinations thereof. In exemplary embodiments, the citrullination sites in peptides having the sequence set forth in SEQ ID Nos. 306-314 are shown in Table 2. In a further embodiment, the subject has ALS or is at increased risk of having ALS if the sample from the subject has an increase in citrullination in any one or more of the peptides having the sequence set forth in any one or more of SEQ ID NO.: 315, SEQ ID NO.: 316, SEQ ID NO.: 317, SEQ ID NO.: 318, SEQ ID NO.: 319, SEQ ID NO.: 320, SEQ ID NO.: 321, SEQ ID NO.: 322, SEQ ID NO.: 323, SEQ ID NO.: 324, SEQ ID NO.: 325, SEQ ID NO.: 326, SEQ ID NO.: 327, SEQ ID NO.: 328, SEQ ID NO.: 329, SEQ ID NO.: 330, SEQ ID NO.: 331, SEQ ID NO.: 332, SEQ ID NO.: 333, SEQ ID NO.: 334, SEQ ID NO.: 335, SEQ ID NO.: 336, SEQ ID NO.: 337, SEQ ID NO.: 338, or combinations thereof. In exemplary embodiments, the citrullination sites in peptides having the sequence set forth in SEQ ID Nos. 315-338 are shown in Table 3. In exemplary embodiments, each of the citrullinated peptide sequences in the sample obtained from the subject is at least 100%, 95%, 90%, 80%, 70% or 60% identical to the sequences set forth in any of SEQ ID Nos. 306-338. In some embodiments, the subject has ALS or is at increased risk of having ALS if the sample from the subject has an increase in citrullination in any one or more of the peptides having the sequence set forth in any one or more of SEQ ID Nos. 339-474. In exemplary embodiments, each of the peptide sequence in the sample obtained from the subject is at least 100%, 95%, 90%, 80%, 70% or 60% identical to the sequences set forth in any of SEQ ID Nos. 339-474. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0072] Provided herein is a method for diagnosing chronic neurodegenerative disease in a subject in need thereof. The method includes obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from the said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of chronic neurodegenerative disease. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide

relative to a reference value is indicative of chronic neurodegenerative disease. In one embodiment, an increase in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of chronic neurodegenerative disease. In one embodiment, a decrease in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of chronic neurodegenerative disease. In one embodiment, the subject has chronic neurodegenerative disease or is at increased risk of having chronic neurodegenerative disease if the sample from the subject has a change in citrullination in any one or more of the proteins in Table 5A. In some embodiments, the citrullinated peptides are brain specific. In some embodiments, the citrullinated peptides are not brain specific. In a further embodiment, the subject has chronic neurodegenerative disease or is at increased risk of having chronic neurodegenerative disease if the sample from the subject has a change in citrullination in any one or more of the peptides having the sequence set forth in any one or more of SEQ ID NO.: 355, SEQ ID NO.: 402, SEQ ID NO.: 1, SEQ ID NO.: 348, SEQ ID NO.: 363, SEQ ID NO.: 428, SEQ ID NO.: 373, SEQ ID NO.: 315, SEQ ID NO.: 316, SEQ ID NO.: 340, SEQ ID NO.: 377, SEQ ID NO.: 331, SEQ ID NO.: 332, SEQ ID NO.: 325, SEQ ID NO.: 375, SEQ ID NO.: 429, SEQ ID NO.: 414, SEQ ID NO.: 313, SEQ ID NO.: 366, SEQ ID NO.: 360, SEQ ID NO.: 423, SEQ ID NO.: 405, SEQ ID NO.: 439, SEQ ID NO.: 321, SEQ ID NO.: 475, SEQ ID NO.: 476, SEQ ID NO.: 477, SEQ ID NO.: 478, SEQ ID NO.: 479, SEQ ID NO.: 480, SEQ ID NO.: 481, SEQ ID NO.: 482, SEQ ID NO.: 483, SEQ ID NO.: 484, SEQ ID NO.: 485, SEQ ID NO.: 486, SEQ ID NO.: 487, SEQ ID NO.: 488, SEQ ID NO.: 489, SEQ ID NO.: 490, SEQ ID NO.: 491, SEQ ID NO.: 492, SEQ ID NO.: 493, SEQ ID NO.: 494, SEQ ID NO.: 495, SEQ ID NO.: 496, SEQ ID NO.: 497, SEQ ID NO.: 498, SEQ ID NO.: 499, SEQ ID NO.: 500, or combinations thereof (Table 5A). In exemplary embodiments, the citrullination sites in peptides having the sequence set forth in SEQ ID Nos. above are shown in Table 5A. In exemplary embodiments, each of the citrullinated peptide sequences in the sample from the subject is at least 95%, 90%, 80%, 70% or 60% identical to the sequences set forth Table 5A. In exemplary embodiments, peptides that may be used for diagnosing chronic neurodegenerative disease are brain specific and comprises, consists of or consists essentially of any one or more of the sequences shown in Table 5A. In exemplary embodiments, peptides that may be used for diagnosing chronic neurodegenerative disease are not brain specific and comprises, consists of or consists essentially of sequences set forth in SEQ ID Nos. 501, 502, 503, 506 or combinations thereof (Table 5B). In exemplary embodiments, chronic neurodegenerative disease include but are not limited to any one or more of

Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD) and PD-related disorders, Prion disease, Motor neurone diseases (MND), Huntington's Disease (HD), Spinocerebellar ataxia (SCA), spinal muscular atrophy (SMA) or combinations thereof. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0073] Provided herein is a method for diagnosing acquired brain injury in a subject in need thereof. The method includes obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from the said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of acquired brain injury. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of acquired brain injury. In one embodiment, an increase in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of acquired brain injury. In one embodiment, a decrease in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of acquired brain injury. In a further embodiment, the subject has acquired brain injury or is at increased risk of having acquired brain injury if the sample from the subject has a change in citrullination in any one or more of the peptides having the sequence set forth in any one or more of SEQ ID NO.: 125, SEQ ID NO.: 265, SEQ ID NO.: 285, SEQ ID NO.: 505, SEQ ID NO.: 506, SEQ ID NO.: 507, SEQ ID NO.: 508, SEQ ID NO.: 509, SEQ ID NO.: 510, SEQ ID NO.: 511, SEQ ID NO.: 512, SEQ ID NO.: 513, SEQ ID NO.: 514, SEQ ID NO.: 515, SEQ ID NO.: 516, SEQ ID NO.: 517, SEQ ID NO.: 518, SEQ ID NO.: 519, SEQ ID NO.: 520, SEQ ID NO.: 521, SEQ ID NO.: 522, SEQ ID NO.: 523, SEQ ID NO.: 524, SEQ ID NO.: 525, SEQ ID NO.: 526, SEQ ID NO.: 527, SEQ ID NO.: 528, SEQ ID NO.: 529, SEQ ID NO.: 530, SEQ ID NO.: 531, SEQ ID NO.: 532, SEQ ID NO.: 533, SEQ ID NO.: 534, SEQ ID NO.: 535, SEQ ID NO.: 536, SEQ ID NO.: 537, SEQ ID NO.: 538, SEQ ID NO.: 539, SEQ ID NO.: 540, SEQ ID NO.: 541, SEQ ID NO.: 542, SEQ ID NO.: 543, SEQ ID NO.: 544, SEQ ID NO.: 545, SEQ ID NO.: 546, SEQ ID NO.: 547 or combinations thereof. In a further embodiment, the subject has acquired brain injury or is at increased risk of having acquired brain injury if the sample from the subject has a change in citrullination in any one or more of the peptides having the sequence set forth in Tables 6A and/or 6B. In exemplary embodiments, each of the citrullinated peptide sequence in the sample obtained from the subject is at least 95%, 90%, 80%, 70% or 60% identical to the sequences set forth in any of

Tables 6A and/or 6B. In exemplary embodiments, acquired brain injury includes but is not limited to traumatic brain injuries (TBI's), strokes, brain illness, tumor, hemorrhage, and any other kind of brain injury acquired after birth. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0074] Provided herein is a method for diagnosing chronic brain disease in a subject in need thereof. The method includes obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from the said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of chronic brain disease. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of chronic brain disease. In one embodiment, an increase in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of chronic brain disease. In one embodiment, a decrease in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of chronic brain disease. In one embodiment, the subject has chronic brain disease or is at increased risk of having chronic brain disease if the sample from the subject has a change in citrullination in any one or more of the secreted proteins in Table 7. In one embodiment, the subject has chronic brain disease or is at increased risk of having chronic brain disease if the sample from the subject has a change in citrullination in any one or more of the non-secreted proteins in Table 8. In a further embodiment, the subject has chronic brain disease or is at increased risk of having chronic brain disease if the sample from the subject has a change in citrullination in any one or more of secreted proteins selected from Syntaxin-binding protein 1 or citrullinated fragments thereof, Histone H4 or citrullinated fragments thereof, Cytoplasmic dynein 1 heavy chain 1 or citrullinated fragments thereof, Dynamin-1 or citrullinated fragments thereof, Gamma-enolase or citrullinated fragments thereof, Endoplasmic precursor or citrullinated fragments thereof, Heat shock 70 kDa protein 12A or citrullinated fragments thereof, Serine/threonine-protein phosphatase PP1-alpha catalytic subunit or citrullinated fragments thereof, Calreticulin precursor or citrullinated fragments thereof, Coronin-1A or citrullinated fragments thereof, Electron transfer flavoprotein subunit beta or citrullinated fragments thereof, Hemoglobin subunit beta or citrullinated fragments thereof, Mitogen-activated protein kinase 1 or citrullinated fragments thereof, Mitogen-activated protein kinase 3 or citrullinated fragments thereof, 26S proteasome non-ATPase regulatory subunit 12 or citrullinated fragments thereof, 60S ribosomal protein L4 or citrullinated fragments thereof,

60S acidic ribosomal protein P2 or citrullinated fragments thereof, Septin-7 or citrullinated fragments thereof, Alanine-tRNA ligase, cytoplasmic or citrullinated fragments thereof or combinations thereof. In a further embodiment, the subject has chronic brain disease or is at increased risk of having chronic brain disease if the sample from the subject has a change in citrullination in any one or more of non-secreted proteins selected from Synapsin-1 or citrullinated fragments thereof, Aconitate hydratase or citrullinated fragments thereof, Complexin-1 or citrullinated fragments thereof, Complexin-2 or citrullinated fragments thereof, Band 4.1-like protein 3 or citrullinated fragments thereof, Synapsin-2 or citrullinated fragments thereof, AP2-associated protein kinase 1 or citrullinated fragments thereof, Alpha-adducin or citrullinated fragments thereof, AP-2 complex subunit beta or citrullinated fragments thereof, Serine/threonine-protein kinase DCLK1 or citrullinated fragments thereof, Glutaminase kidney isoform or citrullinated fragments thereof, Isocitrate dehydrogenase [NAD] subunit alpha or citrullinated fragments thereof, Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform or citrullinated fragments thereof or combinations thereof. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0075] Provided herein is a method for diagnosing acute brain disease in a subject in need thereof. The method includes obtaining a biological sample from a subject in need of a diagnosis and detecting citrullinated protein or citrullinated peptide in the biological sample obtained from the said subject. In some embodiments, presence of citrullinated proteins or citrullinated peptides is indicative of acute brain disease. In some embodiments a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of acute brain disease. In one embodiment, an increase in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of acute brain disease. In one embodiment, a decrease in the levels of citrullination of protein or citrullination of peptide relative to a reference value is indicative of acute brain disease. In one embodiment, the subject has acute brain disease or is at increased risk of having acute brain disease if the sample from the subject has a change in citrullination in any one or more of the secreted proteins in Table 9. In one embodiment, the subject has acute brain disease or is at increased risk of having acute brain disease if the sample from the subject has a change in citrullination in any one or more of the non-secreted proteins in Table 10. In a further embodiment, the subject has acute brain disease or is at increased risk of having acute brain disease if the sample from the subject has a change in citrullination in any one or more of

secreted proteins selected from Cullin-associated NEDD8-dissociated protein 1 or citrullinated fragments thereof, Peroxiredoxin-5 or citrullinated fragments thereof or combinations thereof. In a further embodiment, the subject has acute brain disease or is at increased risk of having acute brain disease if the sample from the subject has an increase in citrullination Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-3 precursor or citrullinated fragments thereof. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0076] Also provided herein are methods for treating neurodegenerative diseases in a subject in need thereof. The methods include diagnosing a neurodegenerative disease by the methods set forth herein and administering an effective amount of a therapeutic agent to the subject diagnosed with the neurodegenerative disease so as to treat neurodegenerative disease in the subject. In exemplary embodiments, examples of neurodegenerative diseases include but are not limited to Alzheimer disease, Parkinson disease, Lewy body dementia, frontotemporal dementia, amyotrophic lateral sclerosis, Huntington disease, Friedreich's ataxia, spinal muscular atrophy, prion diseases, multiple sclerosis, stroke, degenerative nerve diseases, encephalitis or Creutzfeldt-Jacob disease. In various embodiments, treatments include but are not limited to any one or more of therapeutic lifestyle changes, therapeutic agents, surgical treatments or a combination thereof. In exemplary embodiments, lifestyle changes include any one or more of healthy diet, physical activity, weight management, stress management, not smoking or a combination thereof.

[0077] In exemplary embodiments, therapeutic agents for Alzheimer's disease include but are not limited to cholinesterase inhibitors (such as Donepezil, Rivastigmine, Galantamine), memantine (Namenda), Vitamin E, alternative treatments (for example, Caprylic acid and coconut oil, Concerns, Coenzyme Q10, Coral calcium, Ginkgo biloba, Huperzine A, Omega-3 fatty acids, Phosphatidylserine, Tramiprosate), or combinations thereof. Appropriate therapies and dosages will be apparent to one skilled in the art.

[0078] In exemplary embodiments, therapeutic agents for Parkinson's disease include but are not limited to carbidopa/levodopa, dopamine agonists, monoamine oxidase inhibitors, anticholinergics, surgery, lifestyle changes, alternative treatments (for example, Antioxidants Vitamin C and E, the Mediterranean Diet, Calcium and Vitamin D, Coenzyme Q10, Folate (Folic Acid), Ginger (*Zingiber Officinalis*), Ginkgo Biloba, Green Tea Polyphenols (GTPs), Milk Thistle (*Silybum Marianum*), St. John's Wort (*Hypericum Perforatum*), Vitamin B12) or

combinations thereof. Appropriate therapies and dosages will be apparent to one skilled in the art.

[0079] In exemplary embodiments, treatments for Huntington's disease include therapeutic agents and therapies including psychotherapy, speech therapy, physical therapy, occupation therapy or combinations thereof. Exemplary, therapeutic agents for Huntington's disease include but are not limited to Tetrabenazine (Xenazine), Antipsychotic drugs, (such as haloperidol (Haldol)), amantadine, levetiracetam (Keppra), clonazepam (Klonopin) or combinations thereof. Appropriate therapies and dosages will be apparent to one skilled in the art.

[0080] In exemplary embodiments, therapeutic agents for ataxia include but are not limited to riluzole, antiglutaminergic medication, nicotine receptor agonists, serotonergic therapy, GABAergic therapy, cholinergic therapy, channel stabilizing treatments (such as carbonic anhydrase inhibitors), insulin-like growth factors, supplements (such as Vitamin E) or combinations thereof. Appropriate therapies and dosages will be apparent to one skilled in the art.

[0081] In exemplary embodiments, treatments for spinal muscular atrophy include proper nutrition, therapeutic agents and therapies including psychotherapy, speech therapy, physical therapy, occupation therapy or combinations thereof. Therapeutic agents for spinal muscular atrophy include but are not limited to muscle relaxants such as baclofen, tizanidine, and the benzodiazepines to reduce spasticity, Botulinum toxin to treat jaw spasms or drooling, amitriptyline, glycopyolate, and atropine or by botulinum injections into the salivary glands to treat excessive saliva, antidepressants to treat depression. Appropriate therapies and dosages will be apparent to one skilled in the art.

[0082] In exemplary embodiments, therapeutic agents for prion disease include but are not limited to quinacrine, pentosan polysulfate (PPS), tetracyclic compounds or combinations thereof.

[0083] In various embodiments of the processes, assays and methods described herein, the reference value is based on the change in the level of citrullinated proteins or citrullinated peptides as set forth in, for example Tables 2-10. In one embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in a neuronal cell. In another embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in a non-neuronal

cell. In an additional embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in any cell. In an additional embodiment, the change in the level of citrullinated proteins or citrullinated peptides is in any bodily fluid. In some embodiments, the reference value is the mean or median expression level of citrullinated proteins or citrullinated peptides in a population of subjects that do not have neurodegenerative disease. In other embodiments, the reference value is the mean or median expression level of citrullinated proteins or citrullinated peptides in a population of subjects that have neurodegenerative diseases but have undergone treatment and show no or reduced expression of citrullinated proteins or citrullinated peptides. In additional embodiments, the reference value is the expression level of citrullinated proteins or citrullinated peptides in a sample obtained from the subject from a different (for example, an earlier) time point, such as during diagnosis of neurodegenerative disease, before treatment of neurodegenerative disease, after treatment of neurodegenerative disease or a combination thereof. In exemplary embodiments, neurodegenerative diseases include but are not limited to ALS, Alzheimer's disease, brain stroke, chronic brain disease, acute brain disease or combinations thereof. In some embodiments, change in citrullination refers to 'differentially present' citrullination as defined herein.

[0084] In various embodiments, the expression level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having neurodegenerative disease compared to the reference value is increased by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. In various embodiments, the expression level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having neurodegenerative disease compared to the reference value is increased by at least or about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, 100-fold or a combination thereof. In exemplary embodiments, neurodegenerative diseases include but are not limited to ALS, Alzheimer's disease, brain stroke, chronic brain disease, acute brain disease or combinations thereof.

[0085] In various embodiments, the expression level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having neurodegenerative disease compared to the reference value is decreased by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. In various embodiments, the expression level of citrullinated proteins or citrullinated peptides in the subject having or suspected of having neurodegenerative disease

compared to the reference value is decreased by at least or about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, 100-fold or a combination thereof. In exemplary embodiments, neurodegenerative diseases include but are not limited to ALS, Alzheimer's disease, brain stroke, chronic brain disease, acute brain disease or combinations thereof.

Example 1: *Experimental Methods*

Reagents and materials

[0086] The following reagents were obtained: rabbit skeletal muscle PAD cocktail (PAD) (SignalChem); PAD2 (Sigma), heavy meromyosin (HMM), tropomyosin (TM) (Sigma); F-actin (Cytoskeleton, Inc), cardiac troponin (TnI) (Abcam), anti-modified citrulline antibody (Millipore); sequencing grade Lys-C protease (WAKO), and protease inhibitor cocktail (Roche).

Human heart tissue

[0087] Left ventricular tissue samples were obtained from Cris Dos Remedios, University of Sydney, Australia after informed consent and with approval of the local Ethical Committee. The samples were acquired during heart transplantation surgery, from patients with HF (Ischemic Heart Disease (ISHD) and Idiopathic Cardiomyopathy (IDCM), n=10 each) and non-failing donor hearts (n=10) as previously described [Zhang P et al., Multiple reaction monitoring to identify site-specific troponin I phosphorylated residues in the failing human heart. *Circulation* 2012, 126:1828-1837].

Mouse heart tissue and neonatal myocytes

[0088] Male C57BL/6 mice (n=3) (5 days old neonatal mice, Jackson Laboratories) were obtained. Animal study was approved by The Johns Hopkins University Animal Care and Use Committee and followed established NIH guidelines. Briefly, Primary cultured ventricular myocytes were isolated from neonatal mice. Collagenase-digested isolated myocytes were incubated in buffer with increasing concentrations of Ca^{2+} , achieving a final concentration of 1.2 mM Ca^{2+} as in the MEM culture media. Cells were seeded at 25,000 rod-shaped myocytes/ml on 6-well plates or 60-mm dishes coated with laminin. After 1 hour incubation in 37°C, 5% CO_2 , the culture media was replaced to remove unattached cells.

Protein extraction and SWATH MS

[0089] Hearts were fractionated into myofilament- and cytosolic-enriched fractions using the IN Sequence protocol [Kane LA et al. Subfractionation of heart tissue: the "in sequence" myofilament protein extraction of myocardial tissue. *Methods Mol Biol* 2007, 357:87-90]. Protein extraction and generation of LysC peptides from subfractions was performed using a filter-aided sample preparation (FASP) protocol [24]. When needed recombinant proteins or the In Sequence fractions were incubated with PAD's cocktail at a ratio of 1:20 for 2 hr. at 37°C in 100mM Tris, pH 7.6, 5mM DTT, 10mM CaCl₂. The reaction was stopped by addition of 5 mM EDTA prior to digestion. A TripleTOF 6600 mass spectrometer (Sciex) was used for both data dependent acquisition to build peptide spectral ion library and SWATH-MS (data independent acquisition) for each individual sample analysis. The raw data was searched with ProteinPilot™ Software 5.0 to create a spectral ion library. Individual SWATH-MS runs were matched against the spectral library created in the presence or absence of PAD (plus and minus PAD) for both the myofilament- and cytosolic-enriched protein fractions (see Data Supplement).

Preparation of citrullinated samples

[0090] Recombinant proteins and the fractions obtained from IN Sequence were incubated with PAD2 at a ratio of 1:20 for 2 hr. at 37°C in working buffer (100mM Tris, pH 7.6, 5mM DTT, 10mM CaCl₂). The reaction was stopped by addition of 5 mM EDTA.

Statistical validation peptides, proteins and citrullination residues: Acceptance Criteria.

[0091] Bioinformatics Analysis was performed with the workflow described in data supplement. The peptide normalization used in this study was based on the iRT peptide retention time [Escher C, et al. Using iRT, a normalized retention time for more targeted measurement of peptides. *Proteomics* 2012, 12:1111-1121] and normalized values were used for downstream analysis. Ensemble protein ID accession numbers were mapped back to their associated encoding Ensemble gene entries. Data analysis and mining were performed using iProXpress (<http://proteininformationresource.org/iproxpress2>) [Natale DA et al. Protein Ontology: a controlled structured network of protein entities. *Nucleic Acids Res* 2014, 42:D415-421] and Cytoscape [Saito R et al. A travel guide to Cytoscape plugins. *Nat Methods* 2012, 9:1069-1076]. The Kruskal-Wallis test (non-parametric one way ANOVA) for each peptide was used to calculate p-values. The significance of the biochemical changes was

determined by performing a t-test ($p \leq 0.05$) on the differences for all paired data. Unless otherwise stated all biochemical assays were replicated three times.

SDS-PAGE immunoblot for citrullination

[0092] A 1:2000 diluted of the anti-citrulline (Modified) antibody was used for the 1 DE western blot (see Data Supplement).

Two-Dimensional Gel Electrophoresis (2DE)

[0093] The independent verification of proteomics data was performed with fluorescence 2DE gel electrophoresis (2D-DIGE, pI range and SDS PAGE range) as reported previously [Matt P et al. Biomarker discovery: proteome fractionation and separation in biological samples. *Physiol Genomics* 2008, 33:12-17]. The treatment of sample with PAD2 enzyme, which removes a guanidino group from specific arginine residues within the modified protein, can be used to identify citrullinated proteins based on the change in charge of the protein after treatment (see Data Supplement).

Membrane-permeabilized myocytes

[0094] Left ventricular tissue from C57BL/6 mice was flash frozen in liquid nitrogen and stored at -80 °C. For analysis, tissue was homogenized in the presence of 0.3% Triton X-100, and protease and phosphatase inhibitors, as described [Kirk JA et al. Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3beta. *J Clin Invest.* 2014, 1:129-38]. Myocytes were washed without Triton X-100 to remove the detergent, and resuspended in isolation buffer. PAD2 was activated in 10 mM Ca^{2+} and 50 mM DTT for 60 minutes at 37° C. PAD2 treated myocytes were then exposed to a 1:10 dilution of activated PAD2 in isolation buffer for 30 minutes at room temperature. Myocytes were then glued with silicone to the tips of 150 μm diameter minutia pins attached to a force transducer and motor arm (Aurora Scientific Inc., Aurora, ON, Canada). Sarcomere length was monitored by video camera (Imperx, Boca Raton, FL) and calculated by the High-speed Video Sarcomere Length Program (Aurora Scientific Inc.). Myocyte sarcomere length was set at 2.1 μm . A complete activation of the myocyte occurred at the beginning and end of the experiment, and the myocyte discarded if there was >10% rundown, as described [Kirk JA et al. Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3beta. *J Clin Invest.* 2014, 1:129-38].

ATPase activity

[0095] The HMM-ATPase activity was analyzed at three separate experiments described previously [White HD. Special instrumentation and techniques for kinetic studies of contractile systems. *Methods Enzymol* 1982, 85:698-708; Tauhata SB, et al. High affinity binding of brain myosin-Va to F-actin induced by calcium in the presence of ATP. *J Biol Chem* 2001, 276:39812-39818]. First experiment was carried at constant HMM and F-actin concentration with citrullinated or non-citrullinated HMM and/or F-actin. Second, HMM-ATPase activity was determined at increasing TM concentrations (citrullinated or non-citrullinated). Third experiment HMM-ATPase activity was determined at constant HMM, F-actin and TM concentration with citrullinated or non-citrullinated TnI. Each experiment was done in triplicate and three separate times.

Actin Binding Experiments

[0096] Various concentrations of citrullinated and non-citrullinated HMM, TM and/or TnI were added to F-actin and centrifuged to determine extent of binding. Pellet and supernatant were analyzed using 10% SDS PAGE, and amount of each protein quantified by densitometry as previously described [Skorzewski R et al. Effect of actin C-terminal modification on tropomyosin isoforms binding and thin filament regulation. *Biochim Biophys Acta* 2009, 2: 237-243; Nascimento AA et al. Enzymatic characterization and functional domain mapping of brain myosin-V. *J Biol Chem* 1996, 271:17561-17569]. Each assay was carried out in triplicate.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

[0097] Total mRNA from mouse neonatal cardiac myocytes was extracted using TRI-reagent (Sigma) according to the manufacturer's protocols. Complementary DNA (cDNA) was generated using the SuperScript III First-Standard Synthesis System (Invitrogen) according to the instructions of the manufacturer. RT-PCR was performed using primers specific for the PAD1, PAD2, PAD3, PAD4 and β -actin. The PCR products were separated by electrophoresis on a 1.8% agarose gel and visualized under UV light. Each assay was done in duplicate.

1D-PAGE and Western Blot

[0098] Proteins 10ug per well were separated by 1D-PAGE using 12-well 4-12% NuPAGE Bis-Tris gels and blotted onto PVDF membranes according to manufacturer's instruction. After staining the blotted membrane with Direct Blue followed by scanning images, membranes were incubated with modification reagent [1 vol of 1% diacetyl monoxime/0.5% antipyrine/1 N acetic acid, and 2 vol of a mixture of 85% H₃PO₄/98% H₂SO₄/H₂O (20/25/55) containing 0.025% FeCl₃] overnight at 37°C, protected from light. The membrane was blocked, probed overnight at 4°C with an anti-modified citrulline antibody at 1:2000 (Millipore, Billerica, MA) and followed by washes incubated with the secondary antibody for 1 hour (goat polyclonal antirabbit IgG, 1:10000 dilution). Bound antibodies were visualized by chemiluminescent substrate as described by the manufacturer (Amersham Biosciences, Piscataway, NJ). When developed, films were scanned with Epson Expression 10000 XL scanner (Epson, US) and imported into GraphPad Prism tools. A student's t-test was used to determine statistical difference between the groups. A p-value < 0.05 was considered statistically significant.

Sample deimination, Cy labeling, 2-DE separation

[0099] Heart samples, weighing approximately 150 mg, were subfractionated as described earlier. For deimination of each fraction was treated with 1.5mg bacterial PAD2 in 200mM Tris-HCl pH 7.5/10mM dithiothreitol (DTT)/20mM CaCl₂ in a total volume of 100uL. The same amount of each sample was treated at the same condition but without enzyme. After incubating the samples at 50°C for 2 hours samples were mixed with DIGE labeling buffer (7 M urea/2 M thiourea/4% CHAPS/30 mM Tris pH 8.5) at a final concentration of 70ug/110uL. Cy dyes were added (3200 pmol/400 mg aliquot) and aliquots were incubated at RT in the dark for 30 min. Reactions were stopped by adding 1 mL 10mM lysine per 400 pmol dye. Pooled standard was labeled with Cy2, samples no PAD2 treated were labeled with Cy3, and samples treated with PAD2 were labeled with Cy5. A 150ug aliquot of pooled standard, sample +PAD2 and sample – PAD2, labeled as stated above, were mixed together and diluted in IEF buffer (7 M urea/2 M thiourea/4%CHAPS/1% DTT/1% 3–10 ampholytes) to final volume 350ul. Prepared samples were applied to 18 cm immobilized linear pH gradients (4–7) strips (IPG, BioRad), with active rehydration for 12 h at 20°C. For isoelectrofocusing (IEF), the BioRad Protean IEF cell was used with the following conditions at 20°C: Step 1:1hr with end voltage at 200 V; Step 2: 1h ramping with end voltage at 500 V;

Step 3: 1 hr with end voltage 500V; Step 4: 1 hr ramping with end voltage at 1000 V; Step 5: 2 hr with end voltage 1000V; Step 6: 2 hrs. ramping with end voltage at 1000 V; Step 7: 50000V/h (approximately 6hrs). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2-DE was then carried out with 10% gels and electrophoresed at constant voltage (80 V) for 30–60 min, followed by constant power (200V, 200 mA) for 6 hours. The DIGE gels were scanned using a Typhoon 9410 (GE Healthcare) at different emission wavelengths, which allows the differentially expressed proteins to be viewed as changed in color. Preparative gels were stained with silver staining and scanned with the Typhoon 9410.

Image Analysis

[0100] Small gels were scanned using an Epson Expression 10000 XL (Epson America), and large gels were scanned using an Amersham Typhoon 9410 (GE Healthcare). Differentially expressed proteins (treated vs. no treated with PAD2) were identified using Ludesi REDFIN analysis (Ludesi, <http://www.ludesi.com>) for spot detection, matching, and analysis. Ludesi REDFIN analysis was performed in two steps: differential in-gel analysis (DIA) followed by biological variation analysis (BVA). DIA quantifies protein volume from fluorescence intensity for Cy2, Cy3 and Cy5 and calculates the ratios Cy3/Cy2 (no PAD2 treated to internal standard) and Cy5/Cy2 (PAD2 treated to internal standard). Values are then normalized based on the assumption that the amount of protein per image is the same. 2-DE gel images were analyzed in REDFINA (<http://www.ludesi.com>) using student's t-test to determine statistical difference between the groups. A p-value < 0.05 was considered statistically significant. Spots of interest were then excised from gel, destained and in-gel digested with trypsin. Digested samples were used for LC-MS/MS analysis as described below.

In-gel protein digestion and peptide identification

[0101] Spots of interest were picked out from the gels with a shortened pipette tip, placed in small eppendorf tubes and destained by using a 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. After a further washing with bi-distilled water, gel plugs were dried and rehydrated directly in reducing and alkylate reagent follow by digestion of 0.1 µg/µl trypsin in 50 mM NH₄HCO₃ pH 8.8 solution (Trypsin ultra grade sequencing grade, Promega, WI, USA). Digestion was performed 1hr on ice; next the extra solution was removed and gel plugs were cover with 100ul 50 mM NH₄HCO₃ and incubate overnight at 37°C. Peptides

were desalted by ZipTip and analyzed by nanoflow LC–MS/MS by using an LTQ ion trap MS (Thermo Fisher Scientific) interfaced with an Agilent 1200 nanoLC system.

Mass Spectrometry

[0102] Each human heart was fractionated into myofilament- and cytosolic-enriched fractions using IN Sequence protocol [Kane LA, et al. Subfractionation of heart tissue: the "in sequence" myofilament protein extraction of myocardial tissue. *Methods Mol Biol* 200, 357:87-90]. Protein extracts were denatured, reduced and alkylated prior to digestion with LysC (1:20) using a filter-aided sample preparation (FASP) protocol (<https://www.biochem.mpg.de/226356/FASP>). Exogenous internal retention time standards (IRT, company and reference [Escher C, et al. Using iRT, a normalized retention time for more targeted measurement of peptides. *Proteomics* 2012, 12:1111-1121]) were added to each peptide fraction. To maximize the citrullinated proteome depth in vitro citrullination, using PADs cocktail, of cardiac tissue lysates was performed followed by the proteins digestion with LysC. Spectral ion libraries were constructed by data dependent acquisition on an AB Sciex 6600 TripleTOF and the raw data was searched with ProteinPilot™ Software 5.0 to create a spectral ion library. The two fractions from each individual were sequentially analyzed using data independent acquisition (SWATH-MS) on the 6600 Triple TOF. The peptide data were kept for further analysis if they contained at least one high confidence citrulline-peptide/spectrum assignment (>95% confidence) and the confidence level of the protein to which it mapped was >95%. Peptide and ProteinProphet thresholds, the global false discovery rates for this study, obtained from a search of the reversed human database, were estimated to be on the order of <0.1% at the peptide and protein levels respectively. Importantly, transitions of each citrullinated peptide were selected and verified to provide accurate sensitivity and specificity for these targeted proteomics experiments. SWATH-MS fragment ion maps were created (from the endogenous samples) between 400-1250 m/z using 200 variable SWATH windows with a dwell time of 20 ms for each window. Source conditions were as follows for DDA and SWATH experiments: Spray voltage was set to 2.4 kV, source gas was set to 5, curtain gas was set to 30, interface heater temperature was set to 80, and declustering potential was set to 100. Rolling collision energy was used for MS2 experiments in the DDA runs. For both DDA and SWATH runs peptides were separated using an Eksigent ekspert™ 415 nanoLC equipped with ekspert™ cHiPLC and ekspert™ nanoLC 400 autosampler. Samples were loaded onto a trap column (nano cHiPLC Trap 200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å) for 10 minutes at a flow rate of 2 µL/min in

100% solvent A (0.1% formic acid in water) after which point they were separated using a nano cHiPLC 75 μm x 15 cm ChromXP C18-CL 3 μm 120 Å column using a flow rate of 300 nL/min and a linear gradient of 5-35% solvent B (0.1% formic acid in acetonitrile) for 120 min, 35-85% B for 2 minutes, holding at 85% for 5 minutes, then re-equilibration at 5% B for 17 minutes.

Construction of a verified citrullinated spectral ion library

[0103] A verified citrullinated peptide ion library was constructed based on the retention time (RT) difference between pairs of peptides that had un-modified (non-citrullinated) and citrullinated forms present in the ion library and between peptides that had N/Q deaminated and citrullinated forms. Based on our data we observed that the difference in RT between the unmodified and citrullinated form of the same peptide sequence (in the absence of any other modifications) was on average 10 minutes, with the citrullinated peptide having the longer retention time, whereas N/Q deamidation resulted in a RT shift on average of about 2.5 minutes. Therefore, for citrullinated peptide sequences that had paired unmodified forms or paired N/Q deamidation forms and the RT difference was greater than 5 minutes we did not alter the selected transition ions in the ion library. For citrullinated peptides where the RT was less than 5 minutes between the pairs or if there was no unmodified form to use as a reference we manually selected transition ions that would unambiguously distinguish the citrullinated peptide from the unmodified or N/Q deaminated form and any transitions that did not unambiguously distinguish the citrullination site were removed from the ion library. For this subset of peptides five unique transitions were required for a citrullinated peptide to be included in the library. In addition to the verified citrullinated peptides for each protein, a maximum of 10 unmodified peptides were incorporated into the library for total protein quantification.

SWATH data analysis

[0104] The verified ion library was imported into PeakView v2.1 and SWATH files were loaded into the SWATH microapp version 2.0. Extraction settings were as follows: 30 peptides per protein, 5 transitions per peptide, 95% peptide confidence cutoff, an FDR threshold of 1 %, an XIC extraction window of 5 min and tolerance of 0.05 Da.

Co-sedimentation assay

[0105] Binding assays were performed by TM cosedimentation (0-2 μ M) with 5 μ M F-actin essentially as described earlier [Skorzewski R, et al. Effect of actin C-terminal modification on tropomyosin isoforms binding and thin filament regulation. *Biochim Biophys Acta*, 2009, 2:237–243], with the following modifications. Binding of TM alone was assayed in 40mM Tris HCl, pH 7.6, 1 mM DTT, 5 mM MgCl₂, and 100 mM NaCl. After mixing of all assay components, samples were incubated at room temperature for ~0.5 h to ensure attainment of the steady-state. Protein mixtures were ultracentrifuged for 25 min at 60,000 rpm in Beckman rotor TLA 100.2. The composition of the proteins in pellets and the amount of free TM left in the supernatant were examined on 4-12% SDS-PAGE. The gels stained with Coomassie Blue were scanned and quantified. Intensities of SDS-gel bands of pelleted F-actin were similar. TM bound to actin was calculated as TM/actin band intensity ratio normalized to the maximum ratio reached at saturation. The concentration of unbound TM was calculated from band intensities of TM left in the supernatant.

Myosin HMM-induced binding of TM to F-actin

[0106] F-actin (5 μ M) and TM (1 μ M, citrullinated or control) in 30 mM NaCl, 0.5 mM MgCl₂, 1 mM DTT, 5 mM imidazole, pH 7.0, were mixed with HMM (0–3.5 μ M). The mixture was incubated at room temperature for 30 min and then centrifuged in a TLA-100.2 rotor for 25 min, at 60,000 rpm at 15°C. The composition of proteins sedimented in pellets was analyzed by SDS-PAGE and densitometry [Sliwinska M, et al. Role of actin C-terminus in regulation of striated muscle thin filament. *Biophys J*. 2008, 4:1341-].

[0107] HMM-ATPase assays. The assays were performed as described by White [White HD. Special instrumentation and techniques for kinetic studies of contractile systems. *Methods Enzymol* 1982, 85:698-708] with several modifications. All assays were carried out at 22° C in a buffer containing 30 mM NaCl, 5mM MgCl₂, and 10mM Hepes, pH 7.5 with F-actin 0.2 mg/ml and HMM 0.02 mg/ml final concentrations. When present, non-citrullinated or citrullinated TM was added to F-actin to a final molar concentrations between 0-2 μ M TM. HMM-Actin-TM-TnI assay was carried as above with citrullinated or not citrullinated TnI at F-actin:TM:TnI molar ration 7:1:1 respectively. To assess the sensitivity of the regulated acto-S1 system, all assays were done in parallel both in the presence (0.5 mM CaCl₂,) and the absence (2 mM EGTA) of calcium. All reactions were initiated by addition of 4 mM

ATP. The amount of liberated phosphate was determined calorimetrically at 650 nm. All assays were carried out within the linear range and each assay was done in triplicate.

Example 2

Identification of myocardial citrullinated proteins.

[0108] To identify citrullinated targets in the heart we assessed the citrullinome in three groups, ISHD, IDCM and non-failing donor hearts (n=10 per group) using SWATH-MS [34]. SWATH-MS allowed for the quantification. 53 citrullinated sites were altered with HF compared to the non-failing controls ($p < 0.05$) and are listed in Table 1A.

[0109] Table 1A: List of citrullinated protein ($p = 0.05$) with citrullinated peptides sequence. “Dea” is Citrullination or deamination. “CAM” is carbamidomethylation. Citrullinated proteins were grouped by cellular component. UP identifier = Universal Protein (UP) Resource protein ID; p_{kw} = p value for Kruskal-Wallis test (statistic)

Protein	Peptide	UniProtKB Accessions	p_{kw}
Adenylate kinase isoenzyme 1	RGETSGR[Dea]VDDNEETIK (SEQ ID NO: 1)	Q5T9B7	0.03
Alcohol dehydrogenase 1B	AAGAAR[Dea]IIAVDINK (SEQ ID NO: 2)	ADH1B	0.04
Beta-enolase	SPDDPAR[Dea]HITGEK (SEQ ID NO: 3)	ENOB	0.00
Carbonic anhydrase 3	DIR[Dea]HDPSLQPWSVSYDGGSAK (SEQ ID NO: 4)	CAH3	0.01
Fatty acid-binding protein, heart	LILTLTHGTAVC[CAM]TR[Dea]TYEK (SEQ ID NO: 5)	FABPH	0.04
Peptidyl-prolyl cis-trans isomerase A	TAENFR[Dea]ALSTGEK (SEQ ID NO: 6)	PPIA	0.01
Serum deprivation-response protein	FQHPGSDMR[Dea]QEK (SEQ ID NO: 7)	SDPR	0.01
Serum deprivation-response protein	VSPLTFGR[Dea]K (SEQ ID NO: 8)	SDPR	0.01
Heat shock protein beta-7	RHPHTEHVQQTFR[Dea]TEIK (SEQ ID NO: 9)	HSPB7	0.05
Alpha-crystallin B chain	GLSEMR[Dea]LEK (SEQ ID NO: 10)	CRYAB	0.01
Actin *	C[CAM]DIDIR[Dea]K (SEQ ID NO: 11)	ACTA1	>0.05
Actin *	QEYDEAGPSIVHR[Dea]K (SEQ ID NO: 12)	ACTA2	>0.05
Filamin-C	SSSSR[Dea]GSSYSSIPK (SEQ ID NO: 13)	FLNC	0.03
LIM domain-binding protein 3	TSPEGAR[Dea]DLLGPK (SEQ ID NO: 14)	LDB3	0.00
Myosin-binding protein C, cardiac-type	EPVFIPR[Dea]PGITYEPPNYK (SEQ ID NO: 15)	A8MXZ9	0.03
Myozenin-2	R[Dea]VATPFGGFKEK (SEQ ID NO: 16)	MYOZ2	0.01
Myozenin-2	AELPDYR[Dea]SFNR[Dea]VATPFGGFKEK (SEQ ID NO: 17)	MYOZ2	0.02

Protein	Peptide	UniProtKB Accessions	p_kw
Troponin I, cardiac muscle	NIDALSGMEGR[Dea]K (SEQ ID NO: 18)	TNNI3	0.02
Troponin I, cardiac muscle	ESLDLR[Dea]AHLK (SEQ ID NO: 19)	TNNI3	0.02
Troponin T, cardiac muscle	PR[Dea]SFMPNLVPPK (SEQ ID NO: 20)	E7EPW4	0.01
Tropomyosin **	ETR[Dea]AEFAERSVTKLEK (SEQ ID NO: 21)	TPM1	0.002
Vimentin	FADLSEAAN[Dea]RNNDALR[Dea]QAK (SEQ ID NO: 22)	VIME	0.03
Glyceraldehyde-3-phosphate dehydrogenase	LWR[Dea]D[Dhy]GRGALQN[Oxi]IIPASTGAAK (SEQ ID NO: 23)	G3P	0.01
Myosin-7	AEETQR[Dea]SVNDLTSQR[Dea]AK (SEQ ID NO: 24)	MYH7	0.05
Aconitate hydratase,	SYLR[Dea]LRPDRVAMQDATAQ[Dea]M[Oxi]A MLQFISSGLSK (SEQ ID NO: 25)	ACON	0.01
Aconitate hydratase,	ANSVR[Dea]NAVTQEFQVPDPTAR[Dea]YYK (SEQ ID NO: 26)	ACON	0.03
Aconitate hydratase,	IVYGHLLDDPASQEIER[Dea]GK (SEQ ID NO: 27)	ACON	0.03
Adenylate kinase 4,	LLR[Dea]AVILGPPGSGK (SEQ ID NO: 28)	KAD4	0.00
Alcohol dehydrogenase class-3	VAGASR[Dea]IIGVDINK (SEQ ID NO: 29)	ADHX	0.05
ATP synthase subunit alpha,	R[Dea]TGAIVDVPVGEELLGR[Dea]VVDALGN AIDGK (SEQ ID NO: 30)	ATPA	0.00
ATP synthase subunit alpha,	AIEEQVAVIYAGVR[Dea]GYLDK (SEQ ID NO: 31)	ATPA	0.01
ATP synthase subunit alpha,	GIRPAINVGLSVSR[Dea]VGSAAQ[Dea]TRAMK (SEQ ID NO: 32)	ATPA	0.01
ATP synthase subunit alpha,	QGQYSPMAIEEQVAVIYAGVR[Dea]GYLDK (SEQ ID NO: 33)	ATPA	0.03
ATP synthase-coupling factor 6,	SGGPVDASSEYQQELER[Dea]ELFK (SEQ ID NO: 34)	ATP5J	0.00
Chloride intracellular channel protein 4	YR[Dea]NFDIPK (SEQ ID NO: 35)	CLIC4	0.04
Cytochrome c (Fragment)	EER[Dea]ADLIAYLK (SEQ ID NO: 36)	C9JFR7	0.01
Delta-1-pyrroline-5-carboxylate dehydrogenase,	VLR[Dea]NAAGNFYINDK (SEQ ID NO: 37)	AL4A1	0.00
Delta-1-pyrroline-5-carboxylate dehydrogenase,	AADMLSGPR[Dea]R[Dea]AEILAK (SEQ ID NO: 38)	AL4A1	0.01
Delta-1-pyrroline-5-carboxylate dehydrogenase,	VANEPVLAFTQGSPER[Dea]DALQK (SEQ ID NO: 39)	AL4A1	0.03
Enoyl-CoA hydratase,	EMVLTGDR[Dea]ISAQDAK (SEQ ID NO: 40)	ECHM	0.01
ES1 protein homolog,	VLR[Dea]GVEVTVGHEQEEGGK (SEQ ID NO: 41)	ES1	0.00
Heat shock protein HSP 90-alpha	HLEINPDHSIIETLR[Dea]QK (SEQ ID NO: 42)	HS90A	0.04
NAD(P) transhydrogenase,	AATITPFR[Dea]K (SEQ ID NO: 43)	NNTM	0.05

Protein	Peptide	UniProtKB Accessions	p_kw
Phosphate carrier protein,	VYFR[Dea]LPRPPPEMPESLK (SEQ ID NO: 44)	MPCP	0.00
Pyruvate dehydrogenase E1 component subunit alpha, somatic form,	C[CAM]DLHR[Dea]LEEGPPVTTVLTR[Dea]EDGLK (SEQ ID NO: 45)	ODPA	0.01
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit,	FAIYR[Dea]WDPDK (SEQ ID NO: 46)	SDHB	0.05
Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha,	TR[Dea]LIGPNC[CAM]PGVINPGEC[CAM]K (SEQ ID NO: 47)	SUCA	0.02
Succinyl-CoA:3-ketoacid coenzyme A transferase 1,	ADR[Dea]AGNVIFR[Dea]K (SEQ ID NO: 48)	SCOT1	0.04
Voltage-dependent anion-selective channel protein 1	SR[Dea]VTQSNFAVGKY (SEQ ID NO: 49)	VDAC1	0.02
60 kDa heat shock protein,	FDR[Dea]GYISPYFINTSK (SEQ ID NO: 50)	CH60	0.04
Trifunctional enzyme subunit beta,	PNIR[Dea]NVVVVDGVR[Dea]TPFLLSGTSYK (SEQ ID NO: 51)	ECHB	0.02
Aconitate hydratase,	TGR[Dea]EDIANLADEFK (SEQ ID NO: 52)	ACON	0.00
Aconitate hydratase,	R[Dea]LQLLEPFDK (SEQ ID NO: 53)	ACON	0.01
Dual specificity protein phosphatase 3	LGITHVLNAAEGR[Dea]SFMHVNTNANFYK (SEQ ID NO: 54)	DUS3	0.01
Neuroblast differentiation-associated protein AHNAK	FGVSTGR[Dea]EGQTPK (SEQ ID NO: 55)	AHNK	0.01
Collagen alpha-3(VI) chain	DVVFLLDGSEGVVR[Dea]SGFPLLK (SEQ ID NO: 56)	E7ENL6	0.03

[0110] Table 1B shows all citrullinated peptides found in this study. These were proteins with diverse cellular functions, including the regulators of transcription and chromatin structure, cytoskeletal and contraction, cellular signaling processes and metabolism (Figure 1).

[0111] Table 1B: all citrullinated peptides found in this study. “Dea” is Citrullination or deamination. “CAM” is carbamidomethylation.

Protein	Peptide	UniProtKB Accessions	p_kw
Cysteine-rich protein 2	GVNTGAVGSYIYDR[Dea]DPEGK (SEQ ID NO: 57)	P52943	0.58
Adenylate kinase isoenzyme 1	RGETSGR[Dea]VDDNEETIK (SEQ ID NO: 1)	Q5T9B7	0.03
Adenylate kinase isoenzyme 1	R[Dea]GETSGR[Dea]VDDNEETIK (SEQ ID NO: 58)	Q5T9B7	0.15
Adenylate kinase isoenzyme 1	YGYTHLSTGDLLR[Dea]SEVSSGSAR[Dea]GK (SEQ ID NO: 59)	Q5T9B7	0.57

Protein	Peptide	UniProtKB Accessions	p_kw
Adenylate kinase isoenzyme 1	LLR[Dea]SEVSSGSAR[Dea]GK (SEQ ID NO: 60)	Q5T9B7	0.68
Adenylate kinase isoenzyme 1	YGYTHLSTGDLLR[Dea]SEVSSGSARGK (SEQ ID NO: 61)	Q5T9B7	0.74
Alcohol dehydrogenase 1B	AAGAAR[Dea]IIAVDINK (SEQ ID NO: 2)	P00325	0.04
Aldose reductase	PEDPSLLEDPR[Dea]IK (SEQ ID NO: 62)	P15121	0.18
Alpha-crystallin B chain	GLSEMR[Dea]LEK (SEQ ID NO: 10)	P02511	0.01
Alpha-crystallin B chain	QVSGPER[Dea]TIPITREEK (SEQ ID NO: 63)	P02511	0.23
Alpha-crystallin B chain	YR[Dea]IPADVDP LTTSSSLSDGVLTVNGPR[Dea]JK (SEQ ID NO: 64)	P02511	0.41
Alpha-crystallin B chain	SPFYLR[Dea]PPSFLR[Dea]APSWFDTGLSEMRLEK (SEQ ID NO: 65)	P02511	0.51
Alpha-crystallin B chain	SPFYLRPPSFLR[Dea]APSWFDTGLSEMRLEK (SEQ ID NO: 66)	P02511	0.55
Alpha-crystallin B chain	QVSGPER[Dea]TIPITR[Dea]EEK (SEQ ID NO: 67)	P02511	0.97
Aspartate aminotransferase,	HIYLLPSGR[Dea]INVSGLTTK (SEQ ID NO: 68)	P17174	0.84
ATP-dependent 6-phosphofructokinase	LLAHVR[Dea]PPVSK (SEQ ID NO: 69)	P08237	0.53
ATP-dependent 6-phosphofructokinase	NVLGHMQQGGSPFPDR[Dea]NFATK (SEQ ID NO: 70)	P08237	0.80
ATP-dependent 6-phosphofructokinase	[1Ac]-MDADDSR[Dea]APK (SEQ ID NO: 71)	Q01813	0.55
Beta-enolase	SPDDPAR[Dea]HITGEK (SEQ ID NO: 3)	P13929	0.00
Carbonic anhydrase 3	DIR[Dea]HDP SLQPWSVSYDGGSAK (SEQ ID NO: 4)	P07451	0.01
Creatine kinase M-type	ELFDPIISDR[Dea]HGGYK (SEQ ID NO: 72)	P06732	0.20
Creatine kinase M-type	GGDDLDPNYVLSSSR[Dea]VR[Dea]TGR[Dea]SIK (SEQ ID NO: 73)	P06732	0.36
Creatine kinase M-type	PIISDR[Dea]HGGYKPTDK (SEQ ID NO: 74)	P06732	0.47
Creatine kinase M-type	ELFDPIISDR[Dea]HGGYKPTDK (SEQ ID NO: 75)	P06732	0.51
Creatine kinase M-type	GGDDLDPNYVLSSSRVR[Dea]TGRSIK (SEQ ID NO: 76)	P06732	0.66
Creatine kinase M-type	FEEILTR[Dea]LRLQK (SEQ ID NO: 77)	P06732	0.82
Fatty acid-binding protein	LILTLTHGTAVC[CAM]TR[Dea]TYEK (SEQ ID NO: 5)	P05413	0.04
Glyceraldehyde-3-phosphate dehydrogenase	LWR[Dea]D[Dhy]GRGALQN[Oxi]IIPASTGAAK (SEQ ID NO: 23)	P04406	0.01
Glycogen phosphorylase	VAIQLNDTHPALSIPELMR[Dea]ILVDVEK (SEQ ID NO: 78)	P11216	0.39
Glycogen phosphorylase	QISVR[Dea]GLAGLG DVAEVRK (SEQ ID NO: 79)	P11216	0.63
Hemoglobin subunit alpha	LR[Dea]VDPVN[Dea]FK (SEQ ID NO: 80)	P69905	0.91
Hemoglobin subunit alpha	VGAHAGEYGAEALER[Dea]MFLSFPT (SEQ ID NO: 81)	P69905	0.93
Hemoglobin subunit alpha	LER[Dea]MFLSFPTTK (SEQ ID NO: 82)	P69905	0.93
Hemoglobin subunit alpha	R[Dea]VDPVNFK (SEQ ID NO: 83)	P69905	0.93
Hemoglobin subunit beta	VNVDEVGGEALGRLLVVYPWTQ[Dea]R[Dea]FF	P68871	0.40

Protein	Peptide	UniProtKB Accessions	p_kw
	ESFGDLSTPDVAM[Oxi]GNPK (SEQ ID NO: 84)		
Hemoglobin subunit beta	VNVDEVGGALGR[Dea]LLV (SEQ ID NO: 85)	P68871	0.46
Hemoglobin subunit beta	VNVDEVGGALGR[Dea]LLVVYPWTQ[Dea]RFF ESFGDLSTPDVAMGNPK (SEQ ID NO: 86)	P68871	0.67
Hemoglobin subunit beta	R[Dea]FFESFGDLSTPDVAMGNPK (SEQ ID NO: 87)	P68871	0.82
Hemoglobin subunit beta	VNVDEVGGALGR[Dea]L (SEQ ID NO: 88)	P68871	0.90
Peptidyl-prolyl cis-trans isomerase A	TAENFR[Dea]ALSTGEK (SEQ ID NO: 6)	P62937	0.01
Phosphatidylethanolamine-binding protein 1	VLTDPDAPSRL[Dea]KDPK (SEQ ID NO: 89)	P30086	0.12
Phosphatidylethanolamine-binding protein 1	C[CAM]DEPILSN[Dea]RSGDHR[Dea]GK (SEQ ID NO: 90)	P30086	0.55
Phosphoglycerate kinase 1	SVVLMShLGR[Dea]PDGVPMPPK (SEQ ID NO: 91)	P00558	0.26
Phosphoglycerate kinase 1	R[Dea]VVMR[Dea]VDFNVPMK (SEQ ID NO: 92)	P00558	0.27
Serum albumin	YL YEIARR[Dea]H[AAS]P[Oxi]YFYAPELLFFAK (SEQ ID NO: 93)	P02768	0.06
Serum albumin	VPQVSTPTLVEVSR[Dea]N (SEQ ID NO: 94)	P02768	0.31
Serum albumin	WAVAR[Dea]LSQR[Dea]FPK (SEQ ID NO: 95)	P02768	0.40
Serum albumin	FGER[Dea]AFK (SEQ ID NO: 96)	P02768	0.46
Serum albumin	TESLVNR[Dea]RPC[CAM] (SEQ ID NO: 97)	P02768	0.49
Serum albumin	AWAVAR[Dea]LSQR[Dea]FPK (SEQ ID NO: 98)	P02768	0.64
Serum albumin	DDNPNLRLVR[Dea]PEVDVMC[CAM]TAFHDN EETFLK (SEQ ID NO: 99)	P02768	0.66
Serum albumin	TPVSDR[Dea]VTK (SEQ ID NO: 100)	P02768	0.69
Serum albumin	ALLVR[Dea]YTK (SEQ ID NO: 101)	P02768	0.70
Serum albumin	SEVAHR[Dea]FK (SEQ ID NO: 102)	P02768	0.72
Serum albumin	LVEVSR[Dea]NLGK (SEQ ID NO: 103)	P02768	0.74
Serum albumin	R[Dea]MPC[CAM]AEDYLSVVLNQLC[CAM]VLH EK (SEQ ID NO: 104)	P02768	0.82
Serum albumin	DDNPNLPR[Dea]LVR[Dea]PEVDVMC[CAM]TAF HDNEETFLK (SEQ ID NO: 105)	P02768	0.84
Serum albumin	[CRM]-YL YEIAR[Dea]RHPYFYAPELLFFAK (SEQ ID NO: 106)	P02768	0.86
Serum albumin	C[CAM]C[CAM]TESLVNR[Dea]RPC[CAM] (SEQ ID NO: 107)	P02768	0.93
Serum deprivation-response protein	FQHPGSDMR[Dea]QEK (SEQ ID NO: 7)	O95810	0.01
Serum deprivation-response protein	VSPLTFGR[Dea]K (SEQ ID NO: 8)	O95810	0.01
Transgelin	R[Dea]EFTESQLQEGK (SEQ ID NO: 108)	Q01995	0.22
Translationally-controlled tumor protein	MIYR[Dea]DLISHDEMFSDIYK (SEQ ID NO: 109)	Q5W0H4	0.14
Tumor protein D54	TSAALSTVGSAISR[Dea]K (SEQ ID NO: 110)	O43399	0.08
Malate dehydrogenase	FVEGLPINDFSR[Dea]EK (SEQ ID NO: 111)	P40925	0.17

Protein	Peptide	UniProtKB Accessions	p_kw
Actin, alpha skeletal muscle	C[CAM]DIDIR[Dea]K (SEQ ID NO: 11)	Q5T8M8	0.11
Actin, alpha skeletal muscle	QEYDEAGPSIVHR[Dea]K (SEQ ID NO: 12)	Q5T8M8	0.16
Desmin	EEAENNLAAFR[Dea]ADVDAATLAR (SEQ ID NO: 112)	P17661	0.06
Desmin	SR[Dea]LGTTTR[Dea]TPSSYGAGELLDFSLADAV NQEFLTTRTNEK (SEQ ID NO: 113)	P17661	0.08
Desmin	NFRETSPEQR[Dea]GSEVHTK (SEQ ID NO: 114)	P17661	0.23
Desmin	MALDVEIATYR[Dea]K (SEQ ID NO: 115)	P17661	0.23
Desmin	SR[Dea]LGTTTRTPSSYGAGELLDFSLADAVNQEFLTTRTNEK (SEQ ID NO: 116)	P17661	0.31
Desmin	VDVER[Dea]DNLLDDLQRLK (SEQ ID NO: 117)	P17661	0.39
Desmin	NFR[Dea]ETSPEQR[Dea]GSEVHTK (SEQ ID NO: 118)	P17661	0.43
Desmin	TIETR[Dea]DGEVVSEATQQQHEVL (SEQ ID NO: 119)	P17661	0.55
Desmin	SSPVFPR[Dea]AGFGSK (SEQ ID NO: 120)	P17661	0.88
Destrin	[1Ac]-ASGVQVADEV[CAM]R[Dea]IFYDMK (SEQ ID NO: 121)	P60981	0.78
Filamin-C	SSSSR[Dea]GSSYSSIPK (SEQ ID NO: 13)	Q14315	0.03
Four and a half LIM domains protein 2	KPITTGGVTYR[Dea]EQPWHK (SEQ ID NO: 122)	Q14192	0.08
Fructose-bisphosphate aldolase A	ELSDIAHR[Dea]IVAPGK (SEQ ID NO: 123)	P04075	0.55
Glyceraldehyde-3-phosphate dehydrogenase	LVIN[Dea]GNPITIFQER[Dea]DPSK (SEQ ID NO: 124)	P04406	0.66
Glyceraldehyde-3-phosphate dehydrogenase	R[Dea]VIISAPSADAPMFVMGVNHEK (SEQ ID NO: 125)	P04406	0.68
Glyceraldehyde-3-phosphate dehydrogenase	AFRVPTAN[Dea]VSVVDLTC[CAM]R[Dea]LEK (SEQ ID NO: 126)	P04406	0.88
Glyceraldehyde-3-phosphate dehydrogenase	VSVVDLTC[CAM]R[Dea]LEKPAK (SEQ ID NO: 127)	P04406	0.93
Glyceraldehyde-3-phosphate dehydrogenase	LWR[Dea]DGR[Dea]GALQNIIPASTGAAK (SEQ ID NO: 128)	P04406	0.99
LIM domain-binding protein 3	TSPEGAR[Dea]DLLGPK (SEQ ID NO: 14)	O75112	0.00
Moesin	DR[Dea]SEEER[Dea]TTEAEK (SEQ ID NO: 129)	P26038	0.28
Myomesin-1	HVSGITDTEER[Dea]IK (SEQ ID NO: 130)	P52179	0.07
Myomesin-1	SPR[Dea]FALFDLAEGK (SEQ ID NO: 131)	P52179	0.57
Myosin light chain 3	LGQNPTQAEVLR[Dea]VLGKPRQEELNTK (SEQ ID NO: 132)	P08590	0.09
Myosin light chain 3	LGKPR[Dea]QEELNTK (SEQ ID NO: 133)	P08590	0.16
Myosin light chain 3	[CRM]-EAFMLFDR[Dea]TPK (SEQ ID NO: 134)	P08590	0.39
Myosin light chain 3	EAFMLFDR[Dea]TPK (SEQ ID NO: 135)	P08590	0.41
Myosin light chain 3	VLGKPR[Dea]QEELNTK (SEQ ID NO: 136)	P08590	0.42
Myosin light chain 3	ALGQNPTQAEVLR[Dea]VLGK (SEQ ID NO: 137)	P08590	0.59
Myosin light chain 3	LGQNPTQAEVLR[Dea]VLGKPRQ[Dea]EELNTK	P08590	0.70

Protein	Peptide	UniProtKB Accessions	p_kw
	(SEQ ID NO: 138)		
Myosin light chain 3	LGQNPTQAEVLR[Dea]VLGK (SEQ ID NO: 139)	P08590	0.71
Myosin light chain 3	EAFM[Oxi]LFDR[Dea]TPK (SEQ ID NO: 140)	P08590	0.86
Myosin light chain 4	MLDFETFLPILQHISR[Dea]NK (SEQ ID NO: 141)	P12829	0.28
Myosin light chain 4	EQGTYEDFVEGLR[Dea]VFDK (SEQ ID NO: 142)	P12829	0.74
Myosin regulatory light chain 2	NDLRDTFAALGR[Dea]VNVK (SEQ ID NO: 143)	P10916	0.71
Myosin regulatory light chain 2	EAFIMDQNR[Dea]DGFIDK (SEQ ID NO: 144)	P10916	0.77
Myosin-7	AEETQR[Dea]SVNDLTSQR[Dea]AK (SEQ ID NO: 24)	P12883	0.05
Myosin-7	[1Ac]-GDSEMAVFGAAAPYLR[Dea]K (SEQ ID NO: 145)	P12883	0.18
Myosin-7	VR[Dea]ELENELEAEQK (SEQ ID NO: 146)	P12883	0.27
Myosin-7	R[Dea]YR[Dea]ILNPAAIPEGQFIDSRK (SEQ ID NO: 147)	P12883	0.28
Myosin-7	RNHLR[Dea]VVDLQT (SEQ ID NO: 148)	P12883	0.31
Myosin-7	R[Dea]VR[Dea]ELENELEAEQK (SEQ ID NO: 149)	P12883	0.40
Myosin-7	AVFGAAAPYLR[Dea]K (SEQ ID NO: 150)	P12883	0.42
Myosin-7	LEAR[Dea]VR[Dea]ELENELEAEQK (SEQ ID NO: 151)	P12883	0.43
Myosin-7	YR[Dea]ILNPAAIPEGQFIDSRK (SEQ ID NO: 152)	P12883	0.51
Myosin-7	RYR[Dea]ILNPAAIPEGQFIDSRK (SEQ ID NO: 153)	P12883	0.52
Myosin-7	DFELNALNAR[Dea]IEDEQALGSQLQK (SEQ ID NO: 154)	P12883	0.58
Myosin-7	AEETQRSVNDLTSQR[Dea]AK (SEQ ID NO: 155)	P12883	0.60
Myosin-7	RSVNDLTSQR[Dea]AK (SEQ ID NO: 156)	P12883	0.74
Myosin-7	R[Dea]VRELENELEAEQK (SEQ ID NO: 157)	P12883	0.76
Myosin-7	YRILNPAAIPEGQFIDSR[Dea]K (SEQ ID NO: 158)	P12883	0.81
Myosin-7	RVIQYFAVIAAIGDR[Dea]SK (SEQ ID NO: 159)	P12883	0.86
Myosin-7	RYR[Dea]ILNPAAIPEGQFIDSR[Dea]K (SEQ ID NO: 160)	P12883	0.96
Myosin-binding protein C	EPVFIPR[Dea]PGITYEPPNYK (SEQ ID NO: 15)	A8MXZ9	0.03
Myosin-binding protein C	VRWQR[Dea]GGSDISASNK (SEQ ID NO: 161)	A8MXZ9	0.09
Myosin-binding protein C	PR[Dea]PQVTWTK (SEQ ID NO: 162)	A8MXZ9	0.21
Myozenin-2	R[Dea]VATPFGGFEEK (SEQ ID NO: 16)	Q9NPC6	0.01
Myozenin-2	AELPDYR[Dea]SFNR[Dea]VATPFGGFEEK (SEQ ID NO: 17)	Q9NPC6	0.02
Phosphoglucomutase-1	SGEHDFGAADFDDGDR[Dea]NMILGK (SEQ ID NO: 163)	P36871	0.86
Phosphoglucomutase-1	DLEALMFDR[Dea]SFVGK (SEQ ID NO: 164)	P36871	0.87
Plastin-2	VNDDIIVNWVNETLR[Dea]EAKK (SEQ ID NO: 165)	P13796	0.45

Protein	Peptide	UniProtKB Accessions	p_kw
Profilin-1	C[CAM]SVIR[Dea]DS[Dhy]LLQDGEFSMDLRTK (SEQ ID NO: 166)	P07737	0.18
Titin	C[CAM]DVS[Dhy]R[Dea]GDWVTALASVTK (SEQ ID NO: 167)	Q8WZ42	0.17
Tropomyosin*	ETR[Dea]AEFAERSVTKLEK (SEQ ID NO: 21)	B7Z596	0.002*
Troponin I	NIDALSGMEGR[Dea]K (SEQ ID NO: 18)	P19429	0.02
Troponin I	ESLCLR[Dea]AHLK (SEQ ID NO: 19)	P19429	0.02
Troponin I	YR[Dea]AYATEPHAK (SEQ ID NO: 168)	P19429	0.09
Troponin I	[PGQ]-QELEREAEER[Dea]R[Dea]GEK (SEQ ID NO: 169)	P19429	0.49
Troponin T	PR[Dea]SFMPNLVPPK (SEQ ID NO: 20)	E7EPW4	0.01
Troponin T	GERVDFDDIHR[Dea]K (SEQ ID NO: 170)	E7EPW4	0.06
Troponin T	EAEDGPMEEKPKPR[Dea] (SEQ ID NO: 171)	E7EPW4	0.23
Troponin T	AEAETEETR[Dea]AEDEEEEEAK (SEQ ID NO: 172)	E7EPW4	0.66
Tubulin alpha-1B chain	HVPR[Dea]AVFVDLEPTVIDEVR[Dea]TGTYR[Dea]QLFHPEQLITGK (SEQ ID NO: 173)	P68363	0.43
Tubulin alpha-4A chain	HVPR[Dea]AVFVDLEPTVIDEIRN[Dea]GPYRQ[Dea]LFHPEQLITGK (SEQ ID NO: 174)	P68366	0.26
Tubulin alpha-4A chain	EIIDPVLDR[Dea]IR[Dea]K (SEQ ID NO: 175)	P68366	0.72
Tubulin alpha-8 chain	EDAANNYAR[Dea]GHYTVGK (SEQ ID NO: 176)	C9J2C0	0.37
Vimentin	FADLSEAAN[Dea]RNNDALR[Dea]QAK (SEQ ID NO: 22)	P08670	0.03
Vimentin	TVETR[Dea]DGQVINETSQHDDLE (SEQ ID NO: 177)	P08670	0.09
Heat shock protein beta-1	LATQSNEITIPVTFESR[Dea]AQLGGPEAAK (SEQ ID NO: 178)	P04792	0.34
Heat shock protein beta-7	RHPHTEHVQQTFR[Dea]TEIK (SEQ ID NO: 9)	Q9UBY9	0.05
Leucine-rich PPR motif-containing protein	IPENIYRGIR[Dea]N[Dea]LLESYHVPELIK (SEQ ID NO: 179)	P42704	0.55
Troponin C	SEEELSDLFR[Dea]MFDK (SEQ ID NO: 180)	P63316	0.69
Alpha-1-antitrypsin	ELDR[Dea]DTVFALVN[Dea]YIFFK (SEQ ID NO: 181)	P01009	0.61
Apolipoprotein A-I	[PGE]-EN[Dea]GGAR[Dea]LAEYHAK (SEQ ID NO: 182)	P02647	0.58
Apolipoprotein A-I	DSGR[Dea]DYVSQFEGSALGK (SEQ ID NO: 183)	P02647	0.90
Apolipoprotein A-I	AKPALEDLR[Dea]QGLLPVLESFK (SEQ ID NO: 184)	P02647	0.96
Calreticulin	PR[Dea]QIDNPDYK (SEQ ID NO: 185)	P27797	0.17
Calumenin	TEREQFVEFR[Dea]DK (SEQ ID NO: 186)	O43852	0.06
Sarcalumenin	IER[Dea]AITQELPGLLGSLGLGK (SEQ ID NO: 187)	Q86TD4	0.24
Sarcalumenin	TIEGIVMAADSAR[Dea]SFSPLEK (SEQ ID NO: 188)	Q86TD4	0.60
Decorin	ISR[Dea]VDAASLK (SEQ ID NO: 189)	P07585	0.25

Protein	Peptide	UniProtKB Accessions	p_kw
Beta-2-glycoprotein 1	ATVVYQGER[Dea]VK (SEQ ID NO: 190)	P02749	0.55
Cathepsin D	VER[Dea]QVFGAETK (SEQ ID NO: 191)	P07339	0.14
Lumican	YLYLR[Dea]NNQIDHIDEK (SEQ ID NO: 192)	P51884	0.66
Myoglobin	ALELFR[Dea]K (SEQ ID NO: 193)	P02144	0.11
Ubiquitin carboxyl-terminal hydrolase 5	EEDPATGTGDPPR[Dea]K (SEQ ID NO: 194)	P45974	0.50
2,4-dienoyl-CoA reductase	GAFSR[Dea]LDPTGTFEK (SEQ ID NO: 195)	B7Z6B8	0.26
28S ribosomal protein S36	PNVSEALR[Dea]SAGLPSSSVISQHSK (SEQ ID NO: 196)	P82909	0.29
3-ketoacyl-CoA thiolase	YALQSQR[Dea]WK (SEQ ID NO: 197)	P42765	0.55
3-ketoacyl-CoA thiolase	VSPETVDSVIMGNVLQSSSDAIYLAR[Dea]HVGLR[Dea]VGIPK (SEQ ID NO: 198)	P42765	0.82
Acetyl-CoA acetyltransferase	EVVIVSATR[Dea]TPIGSFLGSLSLLPATK (SEQ ID NO: 199)	P24752	0.07
Aconitate hydratase	SYLR[Dea]LRPDRVAMQDAT[Dhy]AQ[Dea]M[Oxi]AMLQFISSGLSK (SEQ ID NO: 25)	Q99798	0.01
Aconitate hydratase,	ANSVR[Dea]NAVTQEFGPVPDTR[Dea]YYK (SEQ ID NO: 26)	Q99798	0.03
Aconitate hydratase,	IVYGHLLDDPASQEIER[Dea]GK (SEQ ID NO: 27)	Q99798	0.03
Aconitate hydratase	SYLRLR[Dea]PDRVAMQDATAQMAMLQFISSGLSK (SEQ ID NO: 200)	Q99798	0.14
Aconitate hydratase	FR[Dea]GHLDNISNNLLIGAINIEN[Dea]GK (SEQ ID NO: 201)	Q99798	0.19
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain, isoform CRA_a	AFTGFIVEADTPGIQIGR[Dea]K (SEQ ID NO: 202)	Q5T4U5	0.15
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain, isoform CRA_a	FAR[Dea]EEIIPVAAEYDK (SEQ ID NO: 203)	Q5T4U5	0.26
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain, isoform CRA_a	TR[Dea]PVVAAGAVGLAQRALDEATK (SEQ ID NO: 204)	Q5T4U5	0.93
Adenylate kinase 4	LLR[Dea]AVILGPPGSGK (SEQ ID NO: 28)	P27144	0.00
Adenylate kinase isoenzyme 1	KR[Dea]GETSGR[Dea]VDDNEETIK (SEQ ID NO: 205)	Q5T9B7	0.95
Alcohol dehydrogenase class-3	VAGASR[Dea]IIGVDINK (SEQ ID NO: 29)	P11766	0.05
Aldehyde dehydrogenase	VAFTGSTIIGR[Dea]VIQVAAGSSNLK (SEQ ID NO: 206)	P05091	0.08
Aldehyde dehydrogenase	SR[Dea]VVGNPFDISK (SEQ ID NO: 207)	P05091	0.55
Aspartate aminotransferase	PYVLPSVR[Dea]K (SEQ ID NO: 208)	P00505	0.22
ATP synthase subunit alpha	R[Dea]TGAIVDVPVGEELLGR[Dea]VVDALGNAIDGK (SEQ ID NO: 30)	P25705	0.00
ATP synthase subunit alpha	AIEEQVAVIYAGVR[Dea]GYLDK (SEQ ID NO: 31)	P25705	0.01
ATP synthase subunit alpha	GIRPAINVGLSVSR[Dea]VGSAAQ[Dea]TRAMK	P25705	0.01

Protein	Peptide	UniProtKB Accessions	p_kw
	(SEQ ID NO: 32)		
ATP synthase subunit alpha	QGQYSPMAIEEQVAVIYAGVR[Dea]GYLDK (SEQ ID NO: 33)	P25705	0.03
ATP synthase subunit beta	IPVGPETLGRIMNVIGEPIDER[Dea]GPIK (SEQ ID NO: 209)	P06576	0.26
ATP synthase subunit beta	IPVGPETLGR[Dea]IMNVIGEPIDER[Dea]GPIK (SEQ ID NO: 210)	P06576	0.51
ATP synthase subunit beta	IQR[Dea]FLSQPFQVAEVFTGHMGK (SEQ ID NO: 211)	P06576	0.70
ATP synthase subunit beta	AHGGYSVFAGVGERTR[Dea]EGN[Dea]DLYHEM IESGVINLK (SEQ ID NO: 212)	P06576	0.87
ATP synthase subunit e	PRAEEER[Dea]R[Dea]IAAEEK (SEQ ID NO: 213)	P56385	0.32
ATP synthase-coupling factor 6,	SGGPVDASSEYQQELER[Dea]ELFK (SEQ ID NO: 34)	P18859	0.00
Chloride intracellular channel protein 4	YR[Dea]NFDIPK (SEQ ID NO: 35)	Q9Y696	0.04
Citrate synthase	SQLSAAVTALNSES[Dea]FARAYAQGISR[Dea] TK (SEQ ID NO: 214)	B4DJV2	0.34
Citrate synthase	TVVGQITVDDMMYGGMR[Dea]GMK (SEQ ID NO: 215)	B4DJV2	0.75
Creatine kinase B-type	LR[Dea]FPAEDEFPDLSAHNNHMAK (SEQ ID NO: 216)	P12277	0.33
Creatine kinase B-type	EVFTR[Dea]FC[CAM]TGLTQIETLFK (SEQ ID NO: 217)	P12277	0.82
Creatine kinase S-type,	C[CAM]TR[Dea]AER[Dea]R[Dea]EVENVAITALE GLK (SEQ ID NO: 218)	P17540	0.31
Creatine kinase S-type,	TR[Dea]AER[Dea]R[Dea]EVENVAITALEGLK (SEQ ID NO: 219)	P17540	0.59
Creatine kinase S-type,	EVREQ[Dea]PR[Dea]LFPPSADYPDLRK (SEQ ID NO: 220)	P17540	0.72
Cytochrome c	EER[Dea]ADLIAYLK (SEQ ID NO: 36)	C9JFR7	0.01
Cytochrome c	KEER[Dea]ADLIAYLK (SEQ ID NO: 221)	C9JFR7	0.01
Cytochrome c oxidase subunit 5A,	EIYPYVIQELR[Dea]PTLNELGISTPEELGLDKV (SEQ ID NO: 222)	P20674	0.07
Cytochrome c oxidase subunit 5A,	SHGSQETDEEFDARWVTYFN[Dea]KPDIDAWEL R[Dea]K (SEQ ID NO: 223)	P20674	0.18
Cytochrome c oxidase subunit 5A,	ASGGGVPTDEEQATGLER[Dea]EIMLAKE (SEQ ID NO: 224)	P10606	0.49
Delta-1-pyrroline-5- carboxylate dehydrogenase	VLR[Dea]NAAGNFYINDK (SEQ ID NO: 37)	P30038	0.00
Delta-1-pyrroline-5- carboxylate dehydrogenase	AADMLSGPR[Dea]R[Dea]AEILAK (SEQ ID NO: 38)	P30038	0.01
Delta-1-pyrroline-5- carboxylate dehydrogenase	VANEPVLAFTQGSPER[Dea]DALQK (SEQ ID NO: 39)	P30038	0.03
Delta-1-pyrroline-5- carboxylate dehydrogenase	TVIQAEIDAAAELIDFFR[Dea]FNAK (SEQ ID NO: 225)	P30038	0.12
Dihydrolipoyl dehydrogenase	NFQR[Dea]ILQK (SEQ ID NO: 226)	P09622	0.87
Electron transfer flavoprotein subunit alpha	VVVSGGR[Dea]GLK (SEQ ID NO: 227)	P13804	0.18

Protein	Peptide	UniProtKB Accessions	p_kw
Electron transfer flavoprotein subunit alpha	VFSVR[Dea]GTSFDAAATSGGSASSEK (SEQ ID NO: 228)	P13804	0.18
Electron transfer flavoprotein subunit alpha,	LLYDLADQLHAAVGASR[Dea]AAVDAGFVPND MQVGQTGK (SEQ ID NO: 229)	P13804	0.48
Electron transfer flavoprotein subunit beta	VEREIDGGLETLR[Dea]LK (SEQ ID NO: 230)	P38117	0.17
Electron transfer flavoprotein subunit beta	LSVISVEDPPQR[Dea]TAGVK (SEQ ID NO: 231)	P38117	0.70
Elongation factor Tu,	PFLLPVEAVYSVPGR[Dea]GTVVTGTLER[Dea]GI LK (SEQ ID NO: 232)	P49411	0.34
Elongation factor Tu,	SLERAEAGDN[Dea]LGALVR[Dea]GLK (SEQ ID NO: 233)	P49411	0.53
Elongation factor Tu,	NIR[Dea]TVVTGIEMFHK (SEQ ID NO: 234)	P49411	0.58
Elongation factor Tu,	PFLLPVEAVYSVPGR[Dea]GTVVTGTLERGILK (SEQ ID NO: 235)	P49411	0.80
Elongation factor Tu,	SLERAEAGDNLGALVR[Dea]GLK (SEQ ID NO: 236)	P49411	0.80
Enoyl-CoA hydratase,	EMVLTGDR[Dea]ISAQDAK (SEQ ID NO: 40)	P30084	0.01
Enoyl-CoA hydratase,	AQFAQPEILIGTIPGAGGTQ[Dea]RLTR[Dea]AVG K (SEQ ID NO: 237)	P30084	0.11
ES1 protein homolog,	VLR[Dea]GVEVTVGHEQEEGGK (SEQ ID NO: 41)	P30042	0.00
Fumarate hydratase,	IANDIRFLGSGPR[Dea]SGLGELILPEN[Dea]EPGS SIMPGK (SEQ ID NO: 238)	P07954	0.19
Fumarate hydratase,	YYGAQTVR[Dea]STMNFK (SEQ ID NO: 239)	P07954	0.55
Glutathione S-transferase kappa 1	DSGNKPPGLLPR[Dea]K (SEQ ID NO: 240)	Q9Y2Q3	0.95
Glutathione S-transferase P	DQQEAAALVDMVNDGVEDLR[Dea]C[CAM]K (SEQ ID NO: 241)	P09211	0.63
Heat shock 70 kDa protein 1A/1B	AQIHDLVLVGGSTR[Dea]IPK (SEQ ID NO: 242)	P08107	0.68
Heat shock 70 kDa protein 1A/1B	DAGVIAGLNLVRIIN[Dea]EPTAAAIAYGLDR[De a]TGK (SEQ ID NO: 243)	P08107	0.74
Heat shock protein HSP 90-alpha	HLEINPDHSIETLR[Dea]QK (SEQ ID NO: 42)	P07900	0.04
Heat shock protein HSP 90-beta	EQVANSFAVERVR[Dea]K (SEQ ID NO: 244)	P08238	0.60
Isocitrate dehydrogenase [NAD] subunit alpha,	ANIMR[Dea]MSDGLFLQK (SEQ ID NO: 245)	P50213	0.84
Isocitrate dehydrogenase [NADP],	IWYEHR[Dea]LIDDMVAQVLK (SEQ ID NO: 246)	P48735	0.13
Isocitrate dehydrogenase [NADP],	SNLDR[Dea]ALGRQ (SEQ ID NO: 247)	P48735	0.19
Isocitrate dehydrogenase [NADP],	ATDFVADR[Dea]AGTFK (SEQ ID NO: 248)	P48735	0.35
Isocitrate dehydrogenase [NADP],	PITIGR[Dea]HAHGDQYK (SEQ ID NO: 249)	P48735	0.58
Isocitrate dehydrogenase [NADP],	TIEAEAAHGTVTR[Dea]HYREHQK (SEQ ID NO: 250)	P48735	0.73
Ketimine reductase mu-crystallin	HR[Dea]GYLGVMPPAYSAAEDALTTK (SEQ ID NO: 251)	Q14894	0.21

Protein	Peptide	UniProtKB Accessions	p_kw
Ketimine reductase mu-crystallin	PGAHINAVGASR[Dea]PDWR[Dea]ELDDELMK (SEQ ID NO: 252)	Q14894	0.49
L-lactate dehydrogenase A chain	GEMMDLQHGSFLR[Dea]TPK (SEQ ID NO: 253)	P00338	0.23
L-lactate dehydrogenase B chain	HR[Dea]VIGSGC[CAM]NLDSAR[Dea]FR[Dea]YLM AEK (SEQ ID NO: 254)	P07195	0.87
L-lactate dehydrogenase B chain	IVVVTAGVRQQEGESR[Dea]LNLVQRN[Dea]VNVFK (SEQ ID NO: 255)	P07195	0.87
Lon protease homolog,	LQQ[Dea]RLGR[Dea]EVEEK (SEQ ID NO: 256)	K7EKE6	0.97
Malate dehydrogenase,	GC[CAM]DVVVIPAGVPR[Dea]K (SEQ ID NO: 257)	P40926	0.49
Malate dehydrogenase,	NSPLVSR[Dea]LTLYDIAHTPGVAADLSHIETK (SEQ ID NO: 258)	P40926	0.97
Methylcrotonoyl-CoA carboxylase subunit alpha,	QEGIIFIGPPSAIR[Dea]DMGIK (SEQ ID NO: 259)	Q96RQ3	0.31
Methylmalonate-semialdehyde dehydrogenase [acylating],	NLR[Dea]VNAGDQPGADLGPLITPQAK (SEQ ID NO: 260)	Q02252	0.70
NAD(P) transhydrogenase	AATITPFR[Dea]K (SEQ ID NO: 43)	Q13423	0.05
NAD(P) transhydrogenase,	SMGAIVR[Dea]GFDTR[Dea]AAALEQFK (SEQ ID NO: 261)	Q13423	0.11
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	PIFSR[Dea]DMNEAK (SEQ ID NO: 262)	P56556	0.16
Peroxiredoxin-1	R[Dea]TIAQDYGV LK (SEQ ID NO: 263)	Q06830	0.21
Peroxiredoxin-2	EGGLGPLNIPLADVTR[Dea]R[Dea]LSEDYGV LK (SEQ ID NO: 264)	P32119	0.16
Peroxiredoxin-5,	VR[Dea]LLADPTGAF GK (SEQ ID NO: 265)	P30044	0.16
Peroxiredoxin-6	LPFPIIDDR[Dea]NR[Dea]ELAILLGMLDPAEK (SEQ ID NO: 266)	P30041	0.68
Phosphate carrier protein,	VYFR[Dea]LPRPPPEMPESLK (SEQ ID NO: 44)	Q00325	0.00
Pyruvate dehydrogenase E1 component subunit alpha, somatic form,	C[CAM]DLHR[Dea]LEEGPPVTTVLTR[Dea]EDGLK (SEQ ID NO: 45)	P08559	0.01
Pyruvate dehydrogenase E1 component subunit alpha, somatic form,	DR[Dea]MVNSNLASVEELK (SEQ ID NO: 267)	P08559	0.60
Pyruvate dehydrogenase protein X component,	HSLDASQGTATGPR[Dea]GIFTK (SEQ ID NO: 268)	O00330	0.37
Short-chain specific acyl-CoA dehydrogenase,	SAAVVFASTDR[Dea]ALQNK (SEQ ID NO: 269)	P16219	0.54
Stress-70 protein,	SDIGEVLVGGMTR[Dea]MPK (SEQ ID NO: 270)	P38646	0.11
Stress-70 protein,	IVR[Dea]ASN[Dea]GDAWVEAHGK (SEQ ID NO: 271)	P38646	0.16
Succinate dehydrogenase [ubiquinone] flavoprotein subunit,	VTLEYR[Dea]PVIDK (SEQ ID NO: 272)	P31040	0.07
Succinate dehydrogenase [ubiquinone] flavoprotein subunit,	IYQR[Dea]AFGGQSLK (SEQ ID NO: 273)	P31040	0.40
Succinate dehydrogenase	FAIYR[Dea]WDPDK (SEQ ID NO: 46)	P21912	0.05

Protein	Peptide	UniProtKB Accessions	p_kw
[ubiquinone] iron-sulfur subunit,			
Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha,	TR[Dea]LIGPNC[CAM]PGVINPGEC[CAM]K (SEQ ID NO: 47)	P53597	0.02
Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha,	PVVSFIAGLTAPPGR[Dea]R[Dea]MGHAGAIAG GK (SEQ ID NO: 274)	P53597	0.09
Succinyl-CoA ligase [ADP-forming] subunit beta,	INFDSNSAYR[Dea]QK (SEQ ID NO: 275)	Q9P2R7	0.27
Succinyl-CoA ligase [ADP-forming] subunit beta,	GRIC[CAM]N[Dea]QVLVC[CAM]ER[Dea]K (SEQ ID NO: 276)	Q9P2R7	0.39
Succinyl-CoA ligase [ADP-forming] subunit beta,	IFDLQDWTQEDER[Dea]DK (SEQ ID NO: 277)	Q9P2R7	0.73
Succinyl-CoA:3-ketoacid coenzyme A transferase 1,	ADR[Dea]AGNVIFR[Dea]K (SEQ ID NO: 48)	P55809	0.04
Succinyl-CoA:3-ketoacid coenzyme A transferase 1,	GLTAVSNNAGVDNFGLLLR[Dea]SK (SEQ ID NO: 278)	P55809	0.93
Thioredoxin-dependent peroxide reductase,	HLSVNDLPVGR[Dea]SVEETLRLVK (SEQ ID NO: 279)	P30048	0.18
Thioredoxin-dependent peroxide reductase,	HLSVNDLPVGR[Dea]SVEETLR[Dea]LVK (SEQ ID NO: 280)	P30048	0.68
Trifunctional enzyme subunit alpha,	ALTSFER[Dea]DSIFSNLTGQLDYQGFEK (SEQ ID NO: 281)	P40939	0.99
Very long-chain specific acyl-CoA dehydrogenase,	SDSHPSDALTR[Dea]K (SEQ ID NO: 282)	P49748	0.32
Very long-chain specific acyl-CoA dehydrogenase,	ITAFVVER[Dea]GFGGITHGPPEK (SEQ ID NO: 283)	P49748	0.55
Very long-chain specific acyl-CoA dehydrogenase,	ALVER[Dea]GGVVTSNPLGF (SEQ ID NO: 284)	P49748	0.87
Voltage-dependent anion-selective channel protein 1	SR[Dea]VTQSNFAVGYK (SEQ ID NO: 49)	P21796	0.02
60 kDa heat shock protein,	FDR[Dea]GYISPYFINTSK (SEQ ID NO: 50)	P10809	0.04
61 kDa heat shock protein,	VVR[Dea]TALLDAAGVASLLTTAEVVVTEIPK (SEQ ID NO: 285)	P10809	0.07
62 kDa heat shock protein,	LVQDVANNTNEEAGDGTATVTLAR[Dea]SIK (SEQ ID NO: 286)	P10809	0.14
63 kDa heat shock protein,	GANPVEIR[Dea]R[Dea]GVMLAVDAVIAELK (SEQ ID NO: 287)	P10809	0.22
64 kDa heat shock protein,	GR[Dea]TVIEQSWGSPK (SEQ ID NO: 288)	P10809	0.55
65 kDa heat shock protein,	GANPVEIRR[Dea]GVMLAVDAVIAELK (SEQ ID NO: 289)	P10809	0.58
66 kDa heat shock protein,	FGADAR[Dea]ALMLQGVDLLADAVAVTMGPK (SEQ ID NO: 290)	P10809	0.93
Cytoplasmic aconitate hydratase	PAR[Dea]VILQDFTGVPVVDFAAMR[Dea]DAV K (SEQ ID NO: 291)	P21399	0.77
Polymerase I and transcript release factor	TVRGSRLER[Dea]QAGQIK (SEQ ID NO: 292)	Q6NZI2	0.09
Polymerase I and transcript release factor	TVR[Dea]GSLER[Dea]QAGQIK (SEQ ID NO: 293)	Q6NZI2	0.34
Trifunctional enzyme subunit beta,	PNIR[Dea]NVVVVDGVR[Dea]TPFLSGTSYK (SEQ ID NO: 51)	P55084	0.02

Protein	Peptide	UniProtKB Accessions	p_kw
Aconitate hydratase,	TGR[Dea]EDIANLADEFK (SEQ ID NO: 52)	Q99798	0.00
Aconitate hydratase,	R[Dea]LQLLEPFDK (SEQ ID NO: 53)	Q99798	0.01
Dual specificity protein phosphatase 3	LGITHVLNAAEGR[Dea]SFMHVNTNANFYK (SEQ ID NO: 54)	P51452	0.01
Elongation factor 1-alpha 2	ASGVSLLEALDTILPPTR[Dea]PTDK (SEQ ID NO: 294)	Q05639	0.45
Elongation factor 1-alpha 2	IGGIGTVPVGR[Dea]VETGILR[Dea]PGMVVTFAPVNITTEVK (SEQ ID NO: 295)	Q05639	0.80
Elongation factor 2	VNFTVDQIR[Dea]AIMDK (SEQ ID NO: 296)	P13639	0.09
Eukaryotic translation initiation factor 4B	VAPAQPSEEGPGR[Dea]K (SEQ ID NO: 297)	E7EX17	0.95
Histone H2B type 1-M	AVR[Dea]LLLPGELAK (SEQ ID NO: 298)	Q99879	0.99
Histone H3 (Fragment)	LPFQR[Dea]LVR[Dea]EIAQDFK (SEQ ID NO: 299)	K7EK07	0.36
Histone H4	RISGLIYEETR[Dea]GVLK (SEQ ID NO: 300)	P62805	0.65
Histone H4	VFLENVIR[Dea]DAVITYTEHAK (SEQ ID NO: 301)	P62805	0.74
Neuroblast differentiation-associated protein AHNAK	FGVSTGR[Dea]EGQTPK (SEQ ID NO: 55)	Q09666	0.01
Fibrinogen alpha chain	ESSSHHPGIAEFPSR[Dea]GK (SEQ ID NO: 302)	P02671	0.39
Fibrinogen gamma chain	R[Dea]LDGSVDFK (SEQ ID NO: 303)	C9JEU5	0.62
Haptoglobin	LR[Dea]TEGDGVYTLNNEK (SEQ ID NO: 304)	P00738	0.40
Serotransferrin	DYELLC[CAM]LDGTR[Dea]K (SEQ ID NO: 305)	P02787	0.10
Collagen alpha-3(VI) chain	DVVFLLDGSEGVVR[Dea]SGFPLLK (SEQ ID NO: 56)	E7ENL6	0.03

[0112] Table 1C: Citrullinated peptides that are upregulated or downregulated in healthy versus ischemic and healthy versus IDCM subjects. I: Ischemic Heart Disease (ISHD); I/N = ischemia vs. normal (healthy); ID= Idiopathic Cardiomyopathy (IDCM); ID/N = IDCM vs. control (healthy).

Protein	I/N	I
ES1 protein homolog	10.32617915	up
Aconitate hydratase	8.871956482	up
Glyceraldehyde-3-phosphate dehydrogenase	7.914843955	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	7.552745345	up
Vimentin	6.886890573	up
Neuroblast differentiation-associated protein AHNAK	6.631800512	up
Serum deprivation-response protein	5.310526396	up
Enoyl-CoA hydratase	5.304039413	up
Aconitate hydratase	5.18248378	up

Protein	I/N	I
Peptidyl-prolyl cis-trans isomerase A	4.691862761	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	4.531765219	up
Chloride intracellular channel protein 4	4.143020683	up
Alcohol dehydrogenase 1B	3.979284116	up
Adenylate kinase 4	3.954001114	up
Fatty acid-binding protein	3.891772332	up
Myosin-binding protein C	3.770886194	up
Aconitate hydratase	3.740779581	up
Aconitate hydratase,\	3.54332942	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	3.484011583	up
Dual specificity protein phosphatase 3	3.348178139	up
Pyruvate dehydrogenase E1 component subunit alpha, somatic form	3.336292196	up
Voltage-dependent anion-selective channel protein 1	3.075867039	up
Heat shock protein HSP 90-alpha	3.018162117	up
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	2.797967329	up
Aconitate hydratase	2.646902712	up
Carbonic anhydrase 3	2.393105549	up
Serum deprivation-response protein	2.181113303	up
ATP synthase subunit alpha	2.125964715	up
Succinyl-CoA:3-ketoacid coenzyme A transferase 1	2.02609809	up
Alcohol dehydrogenase class-3	1.955112799	up
Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha	1.813434217	up
Troponin I, cardiac muscle	1.770629108	up
ATP synthase subunit alpha	1.757665967	up
ATP synthase subunit alpha	1.716317069	up
Filamin-C	1.707322518	up
60 kDa heat shock protein	1.691322503	up
Heat shock protein beta-7	1.659329245	up
Myozenin-2	1.640224333	up
Cytochrome c (Fragment)	1.516000483	up
LIM domain-binding protein 3	1.456615429	up
Trifunctional enzyme subunit beta	1.262914035	up
Phosphate carrier protein	0.90684035	down
ATP synthase subunit alpha	0.821046528	down
Collagen alpha-3(VI) chain	0.787448747	down
Myozenin-2	0.750016161	down
Troponin I	0.707050891	down
NAD(P) transhydrogenase	0.689499324	down
Alpha-crystallin B chain	0.574071929	down
ATP synthase-coupling factor 6	0.558912444	down

Protein	I/N	I
Troponin T, cardiac muscle (Fragment)	0.474388613	down
Beta-enolase	0.324050919	down
Adenylate kinase isoenzyme 1	0.134886108	down

[0113] Table 1D: Citrullinated peptides that are upregulated or downregulated in healthy versus ischemic and healthy versus IDCM subjects. I: Ischemic Heart Disease (ISHD); I/N = ischemia vs. normal (healthy); ID= Idiopathic Cardiomyopathy (IDCM); ID/N = IDCM vs. control (healthy)

Protein	ID/N	ID
ATP synthase subunit alpha	18.47537571	up
ATP synthase subunit alpha	15.31631115	up
Myozenin-2	14.27029851	up
Glyceraldehyde-3-phosphate dehydrogenase	12.08226474	up
ATP synthase subunit alpha	9.229219757	up
ATP synthase subunit alpha	7.956015709	up
ES1 protein homolog	6.450987219	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	4.442955018	up
Enoyl-CoA hydratase	3.92858654	up
Voltage-dependent anion-selective channel protein 1	3.872789427	up
Aconitate hydratase	3.868674325	up
Phosphate carrier protein	3.29840839	up
Pyruvate dehydrogenase E1 component subunit alpha, somatic form	3.241986763	up
Aconitate hydratase	3.202560876	up
Vimentin	3.18977634	up
NAD(P) transhydrogenase	3.087350045	up
Trifunctional enzyme subunit beta	3.078371104	up
Aconitate hydratase	3.033531051	up
Myosin-binding protein C	2.938608028	up
Chloride intracellular channel protein 4	2.768865949	up
Aconitate hydratase	2.733376766	up
Heat shock protein HSP 90-alpha	2.673523729	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	2.570795078	up
Collagen alpha-3(VI) chain	2.444430998	up
Serum deprivation-response protein	2.396582653	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	2.194292783	up
Alcohol dehydrogenase 1B	1.513050932	up
Aconitate hydratase	1.359569141	up

Protein	ID/N	ID
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	1.182430989	up
Dual specificity protein phosphatase 3	1.089131195	up
Serum deprivation-response protein	1.05734493	up
60 kDa heat shock protein	0.676500721	down
Heat shock protein beta-7	0.569844647	down
Filamin-C	0.543903731	down
Alcohol dehydrogenase class-3	0.539343446	down
Adenylate kinase 4	0.516646378	down
Fatty acid-binding protein	0.500998082	down
Myozenin-2	0.483486835	down
Neuroblast differentiation-associated protein AHNAK	0.440092248	down
Cytochrome c (Fragment)	0.256014197	down
Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha	0.234602593	down
Peptidyl-prolyl cis-trans isomerase A	0.231427358	down
Carbonic anhydrase 3	0.230059909	down
LIM domain-binding protein 3	0.21853459	down
Troponin I	0.188118204	down
Troponin I	0.184373163	down
Troponin T	0.153543871	down
Alpha-crystallin B chain	0.145172906	down
Succinyl-CoA:3-ketoacid coenzyme A transferase 1	0.121733077	down
ATP synthase-coupling factor 6	0.076789945	down
Adenylate kinase isoenzyme 1	0.023786955	down
Beta-enolase	0.013231973	down

[0114] Table 1E: Proteins that were up in both I and ID groups. I: Ischemic Heart Disease (ISHD); I/N = ischemia vs. normal (healthy); ID= Idiopathic Cardiomyopathy (IDCM); ID/N = IDCM vs. control (healthy).

Protein	I/N	I	ID/N	ID
ATP synthase subunit alpha	2.125964715	up	18.47537571	up
ATP synthase subunit alpha	1.757665967	up	15.31631115	up
Glyceraldehyde-3-phosphate dehydrogenase	7.914843955	up	12.08226474	up
ATP synthase subunit alpha	1.716317069	up	7.956015709	up
ES1 protein homolog	10.32617915	up	6.450987219	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	4.531765219	up	4.442955018	up
Enoyl-CoA hydratase	5.304039413	up	3.92858654	up
Voltage-dependent anion-selective channel protein 1	3.075867039	up	3.872789427	up

Protein	I/N	I	ID/N	ID
Aconitate hydratase	3.54332942	up	3.868674325	up
Pyruvate dehydrogenase E1 component subunit alpha, somatic form	3.336292196	up	3.241986763	up
Aconitate hydratase	8.871956482	up	3.202560876	up
Vimentin	6.886890573	up	3.18977634	up
Trifunctional enzyme subunit beta	1.262914035	up	3.078371104	up
Aconitate hydratase	5.18248378	up	3.033531051	up
Myosin-binding protein C	3.770886194	up	2.938608028	up
Chloride intracellular channel protein 4	4.143020683	up	2.768865949	up
Aconitate hydratase	3.740779581	up	2.733376766	up
Heat shock protein HSP 90-alpha	3.018162117	up	2.673523729	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	7.552745345	up	2.570795078	up
Serum deprivation-response protein	2.181113303	up	2.396582653	up
Delta-1-pyrroline-5-carboxylate dehydrogenase	3.484011583	up	2.194292783	up
Alcohol dehydrogenase 1B	3.979284116	up	1.513050932	up
Aconitate hydratase	2.646902712	up	1.359569141	up
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	2.797967329	up	1.182430989	up
Dual specificity protein phosphatase 3	3.348178139	up	1.089131195	up
Serum deprivation-response protein	5.310526396	up	1.05734493	up

[0115] Table 1F: Proteins that were down in both I and ID groups. I: Ischemic Heart Disease (ISHD); I/N = ischemia vs. normal (healthy); ID= Idiopathic Cardiomyopathy (IDCM); ID/N = IDCM vs. control (healthy).

Protein	I/N	I	ID/N	ID
Troponin I	0.707051	down	0.188118	down
Alpha-crystallin B chain	0.574072	down	0.145173	down
ATP synthase-coupling factor 6	0.558912	down	0.07679	down
Troponin T, cardiac muscle (Fragment)	0.474389	down	0.153544	down
Beta-enolase	0.324051	down	0.013232	down
Adenylate kinase isoenzyme 1	0.134886	down	0.023787	down

[0116] Western blotting of myofilament- and cytosolic-enriched fractions obtained from ISHD, IDCM and non-failing donor hearts (n=10/group) using an anti-modified citrulline antibody confirmed that citrullination occurs to intracellular proteins. Although there was no difference (Figure 1B) in the overall immunoreactivity between groups with regard to the number of bands or band density per blot, in-gel digestion and subsequent mass spectrometry

of the immuno-reactive bands identified the major sarcomeric proteins at their expected molecular weight (e.g. myosin heavy and light chains and actin. It must be noted that quantitative assessment by immuno-1DE was confounded by the presence of other proteins in the gel bands and challenges of direct site-specific assessment using mass spectrometry.

[0117] To further validate citrullination of the high abundant sarcomeric proteins, DIGE 2D gel electrophoresis (pH 4-7, 10% SDS PAGE) was carried out. Myofilament- and cytosolic-enriched fractions of ISHD, IDCM and non-failing donor hearts (n=4/group) were pretreated with PAD2 to induce maximum citrullination, combined at a 1:1 ratio with the matching untreated samples and simultaneously resolved by 2D gel electrophoresis. The sarcomeric proteins, including actin, TM and myosin light chains were shown to be citrullinated with ectopic treatment of PAD2 (Figure 2).

Biochemical assessment of modified sarcomeric proteins.

[0118] To test whether citrullination can affect sarcomeric protein function, actin, HMM, TM and troponin were citrullinated by ectopic PAD2 and then compared to respective unmodified recombinant proteins to determine if citrullination affects their biochemical, structural, or enzymatic properties. The binding of citrullinated or untreated HMM, TM or TM-TnI to F-actin was determined using classical co-sedimentation assays (Figure 3A-C). Citrullination of actin did not alter F-actin formation and over 95% of the actin was pelleted upon centrifugation. Binding of citrullinated TM to F-actin was enhanced compared to unmodified TM (Figure 3A). Since TM and HMM can affect binding of each other to F-actin in a cooperative manner [Galinska A et al. The C terminus of cardiac troponin I stabilizes the Ca²⁺-activated state of tropomyosin on actin filaments. *Circ Res* 2010, 106:705-711], we tested for cooperativity under conditions in which binding of TM alone to F-actin is poor, but increased upon the binding of myosin heads to F-actin [Eaton BL. Tropomyosin binding to F-actin induced by myosin heads. *Science* 1976, 192:1337-1339]. As illustrated in Figure 3B, at low salt concentration citrullinated TM in the presence of HMM was able to bind to F-actin.

[0119] The binding of cardiac TnI to F-actin in the presence of TM was performed by co-sedimenting citrullinated TnI in the presence of F-actin and TM. It was found that both citrullinated and non-citrullinated forms of cardiac TnI bound to F-actin equally well (Figure 3C)

Inhibition of HMM-ATPase activity.

[0120] To verify a possible modulation of the actomyosin HMM-ATPase activity by citrullination of sarcomeric proteins, experiments were performed in the presence of citrullinated HMM, F-actin and TM. The data summarized in Figure 4A show that, in the presence of citrullinated HMM (0.5 μ mol/L HMM and 7 μ mol/L F-actin), the actomyosin HMM-ATPase activity decreased from 0.32 ± 0.01 nmol Pi to 0.22 ± 0.002 nmol Pi, corresponding to 30% decrease in the enzyme activity. In contrast, citrullination of F-actin enhanced actomyosin HMM-ATPase activity up to 55% on the enzyme activity (Figure 4A). When both F-actin and HMM were citrullinated, the rate of ATP hydrolysis remained greater than control (0.368 ± 0.005 nmol Pi to 0.32 ± 0.01 nmol Pi). Figure 4B showed that actomyosin HMM-ATPase activity was affected by TM citrullination. The inhibition of actomyosin HMM-ATPase conferred by non-citrullinated and citrullinated TnI was compared. Addition of non-citrullinated TnI to HMM-F-actin-TM caused a decrease in the ATPase rate at 37 °C. Citrullinated TnI acted similar to non-citrullinated TnI and caused a decrease in the ATPase with no significant deference to non-citrullinated form.

PAD2 reduces calcium sensitivity in skinned myocytes

[0121] Chemically skinned cardiomyocytes isolated from the left ventricle of wild-type C57Bl6 male mice were exposed to varying concentrations of calcium (n=8 myocytes from 3 mice per group, Figure 4C-F). PAD2 treatment had no effect either maximal calcium activated force ($F_{\max}$) or hill coefficient (nH). However, PAD2 caused a rightward shift in the force-calcium relationship that indicates an increase in EC_{50} , or a decrease in calcium-sensitivity (p=0.009, Figure 4C). This suggests that citrullination of myofilament proteins causes a loss-of-function phenotype, reducing its ability to generate force in response to intracellular calcium.

Analysis of PAD mRNA expression.

[0122] To determine the cell specificity of the PAD isoform expression in the myocardium, mRNA expression level was determined using isoform specific primers by Nested-PCR on isolated cardiac myocytes and fibroblasts (Figure 5). PAD2 was the primary isoform expressed in both cardiomyocytes and cardiac fibroblasts, although fibroblasts also had significant expression of PAD1 and PAD4 mRNA. In contrast, PAD3 mRNA was not

detected in any of the cell types and conditions tested (Figure 5, note no band at 200 bp, see online supplement for more details).

Example 3

[0123] Our experimental findings characterize citrullinated proteins in the normal and HF myocardium. The analysis revealed that citrullination is enriched in the mitochondria and sarcomere subproteomes. Protein citrullination has broad cellular distribution (Figure 1) but is highly enriched in the mitochondria and sarcomeric subproteome. A close connection between these two subproteomes is not unexpected due to the high energetic requirements of the sarcomere. We speculated that citrullination, similar to phosphorylation and acetylation, could potentially regulate muscle-contraction proteins in a coordinated manner. Interestingly, the multiple enzymes involved in metabolic pathways/metabolism were up regulated in ischemia but down regulated in IDCM (Figure 6). To understand the topology and functional annotation of citrullinated protein-protein interaction in the heart system the protein network was constructed using the STRING database (Figure 2B). The entire protein network consisted of high scoring interaction partners (STRING relevant confidence score ≥ 0.5 , ischemia/IDCM vs. control). The visual analysis of this sub-network showed that citrullinated proteins are interacted to each other and are involved in the metabolism and respiratory chain targets, contraction and signal transduction systems. As an initial assessment to understand potential functional consequences of the citrullination, recombinant sarcomere proteins HMM, F-actin, TM and TnI were used as model proteins to study the interactions that govern the thick and thin filament function and by measured the contractile properties of single skinned myocytes treated with PAD2.

[0124] Ca^{2+} -dependent alterations to Tn alter an azimuthal movement of TM on the actin surface, which allows myosin binding and cross-bridge isomerization to strong binding, force-producing states and muscle contraction. At low intracellular Ca^{2+} , Tn-TM sterically blocks myosin binding sites on actin (blocked state), while in presence of elevated Ca^{2+} Tn-TM moves and partially exposes the myosin binding sites on F-actin (closed state). Myosin binding in the presence of Ca^{2+} is required for full activation. It is a combination of Ca^{2+} -induced TnI conformational change which is, in part, transmitted via TnT to TM (especially the T1 region that binds along TM) as well as myosin binding that influence the exact positioning of TM on the actin filament. Based on our MS data, citrullination sites on myosin, actin, TM, TnI and TnT are in regions what can influence these interactions and thus,

the actomyosin HMM-ATPase activity and contraction. Below outlines the potential impact based on the biochemical and physiological experiments presented in this manuscript.

[0125] First, intrinsic actomyosin HMM-ATPase was inhibited by citrullination, but this was overcome in the presence of F-actin regardless of whether F-actin is citrullinated or not. Citrullinated F-actin was a more potent modulator of HMM enzymatic activity and increased actomyosin HMM-ATPase rate by 55% compared to the unmodified F-actin. This suggests that citrullination of F-actin changes the conformation of the actin filament to alter the ease of ATP hydrolysis by the myosin once it is bound. The actin-HMM interaction was also affected by the citrullination of TM. Citrullinated TM displayed enhanced binding to F-actin compared to unmodified TM. This was also observed in the presence of HMM (based on the centrifugation assays). This correlated to an inhibition of the citrullinated TM-actin-HMM ATPase activity compared to the non-citrullinated TM, suggesting that citrullinated TM altered the ability of HMM to bind to the actin filament (Figure 7). Intriguingly, citrullinated TnI, like the unmodified protein complex, bound tightly to actin-TM based on centrifugation studies. Figure 3C shows that >95% of the unmodified and modified TnI was present in the pellet.

[0126] Biochemical results were related to our physiological finding and showed that citrullination of the sarcomeric proteins caused a decrease in Ca^{2+} -sensitivity in the skinned cardiac myocyte (Figure 4C). On TnI, C-terminus residues 191 to 210, which contains the citrullinated residue 203, is primarily responsible for maintaining the TM conformation that prevents cross bridge cycling. Thus, not only does TnI promote the blocked state, but also contributes to the stabilization of TM in the closed state. In mice, cardiac TnI containing cardiomyopathy mutations, R192H or R204H (which are equivalent to the human sequence of 191 and 203 the latter being the same citrullinatable residue as discussed above) increases the binding affinity of Tn for actin-TM. Perhaps this is augmented by the citrullination of TnT at residue 77, which is located in the N-terminal tail (TnT1) that lies along TM and has been shown to be involved in cooperativity of the actin-TM-Tn filament. In addition, binding of Ca^{2+} or myosin to actin-TM-Tn can displace TnI residues 151 and 188, which flank the second citrullinated residue 169, away from the outer domain of F-actin. This is consistent with an azimuthal displacement to TnI-TM by Ca^{2+} that can expose the high affinity-binding site on F-actin for myosin. Studies on familial hypertrophic cardiomyopathic mutations occurring on thick filament proteins (TM, MHC, MyBP-C, ELC, and RLC) show that a change in one amino acid side chain can have an enormous effect on cardiac morphology and

function. Furthermore, factors that lead to abnormal contraction and relaxation in the failing heart include metabolic pathway abnormalities that result in decreased energy production, energy transfer and energy utilization.

[0127] Finally, to better understand the involvement of citrullinated proteins in the heart, it is important to obtain insight about cell specificity. Previous data acquired by the immunohistochemistry showed that PADs 1-3 and to some degree, PAD6 were detected in cardiomyocytes with PADs 2 and 4 found in endothelial cells and vascular smooth muscle cells. In the present study, we relied not on antibodies and tissue slices, but rather examined the mRNA expression of PAD family members in isolated mouse cardiomyocytes and cardiac fibroblasts. The data showed that PAD2 was primarily expressed in both cardiomyocytes and cardiac fibroblasts whereas PAD1 and PAD4 mRNA were the major forms in the cardiac fibroblasts.

[0128] We have presented previously unexplored roles for citrullination in the heart. Ultimately, identification of citrullination of the majority of the sarcomeric proteins and alterations in their biochemical properties suggest that there is potentially a new PTM regulation of cardiac contractility. Since citrullination at some of these residues were increased in the myocardium of individuals with HF compared to the controls, suggest that citrullination could play a role, in the decrease contractile dysfunction in HF.

Example 4: Citrullinated peptides in neurodegenerative diseases

[0129] Citrullination is a PTM of arginine residues that is characterized by the oxidization of the side chain. This modification is catalyzed by Peptidyl Arginine Deiminase enzymes (PAD) that causes the conversion of arginine to citrulline. Citrullination, also known as deimination occurs when there is a loss of a positive charge and reduction in hydrogen-bonding ability, ultimately causing a change in the protein's chemical structure. This modification plays both a physiological role, for instance during apoptosis and epigenetics, and a pathological role in cancer or diseases of the central nervous system [3]. Furthermore, anti-citrullinated protein antibodies (ACPAs) are present in ~60% of patients with rheumatoid arthritis (RA) and are associated with aggressive disease and a poor prognosis [4, 5]. Genetic studies of RA have identified many disease-prone SNPs at >100 non-HLA gene loci [6]. Therefore, the investigation to associate citrullination, SNPs and disease can provide an insight to many pathological disorders.

Protocol overview

[0130] The purpose of this protocol is to build citrullinated assay libraries that support accurate detection of citrullinated proteins/peptides in vivo. The protocol covers the sample preparation, the acquisition of high-quality fragment ion spectra in DIA mode, the spectral citrullinated library generation and bioinformatics pipeline for binning the citrullinated peptides based on quality. Furthermore, additional tools were developed to translate the large datasets into knowledge by further annotating the peptide data with known vs. novel, and linkage to disease associated genomic alteration information.

Materials

[0131] Reagents: Rabbit skeletal muscle PAD cocktail (PAD) (SignalChem); Sequencing grade Lys-C protease (WAKO); iRT retention time peptides [13]; FASP™ Protein Digestion Kit (Expedeon),

[0132] Human brain tissue. Healthy control post-mortem human brain tissues corresponding to superior frontal gyrus (SFG) (n=10) obtained from NIH NeuroBioBank (NBB). The cases had neither a clinical history nor a neuropathology diagnosis of neurologic disease. Human ALS motor cortex(n=1) and ALS thoracic spinal cord (n=4) with corresponding healthy samples obtained from occipital lobe (n=3). Mouse brains: Alzheimer's model (1. 9 months old, lots of beta-amyloid, memory deficit and onset of neurodegeneration, n=5; 2. 9 months old, normal/healthy mouse, with intact memory, n=5; 3. 2 months old, little to no detectable beta-amyloid and intact memory, n=2; 4. 2 months old, healthy mouse, n=2). Stroke mouse (a middle cerebral artery occlusion stroke, n=5 Left side of brain where stroke occurred, right side of brain, corresponding healthy control); Healthy mouse (n=5, brain, aorta, skeletal muscle)..

[0133] Brain buffer A: Ice-cold low salt buffer: 10mM Tris, pH 7.5, 5mM EDTA, 1mM DTT, 10% sucrose, 10mM b-glycerophosphate, 10mM sodium orthovanadate, 10mM tetrasodium pyrophosphate, 50mM sodium fluoride, 1 × Roche complete protease inhibitor cocktail).

[0134] Brain buffer B: 1% Triton, 10mM Tris, pH 7.5, 5mM EDTA, 1mM DTT, 10% sucrose, 10mM b-glycerophosphate, 10mM sodium orthovanadate, 10mM tetrasodium pyrophosphate, 50mM sodium fluoride, 1 × Roche complete protease inhibitor cocktail

[0135] PAD Buffer: 0.1M tris-HCl, pH7.4, 10 mM CaCl₂, 5mM DTT, 10ug/ul aprotinin, 10μg/ul leupeptin, 10ug/ul pepstatin.

[0136] Equipment: High Speed Mixer Mill(MM400) (Retsch); Mixer Mill jar (# 22.008.0005 Retsch) and 7mm stainless steel ball (#22.455.0008 Retsch); Refrigerated Bench-top centrifuge (Eppendorf 5415R), temperature 4°C and 20°C; Beckman Coulter Optima Ultracentrifuge with TLA 100.2 rotor; TripleTof 5600+ mass spectrometer (AB Sciex); TripleTof 6600+ mass spectrometer (AB Sciex); iProXpress (<http://proteininformationresource.org/iproxpress2>) [14]; Computer: PC with Microsoft Windows 7 (Microsoft), ≥4 GB of RAM, sufficient hard disk space (for the protocol case study ≥40 GB).

[0137] Software: Microsoft Windows 7 (Microsoft); MS Excel (Microsoft); Cytoscape Plugin BiNGO A Biological Network Gene Ontology Tool (<http://baderlab.org/Software/EnrichmentMap/BingoTutorial>).

[0138] Equipment Setup: Detailed instructions, including screenshots, for the installation of each software module are provided in Supplementary material.

[0139] Methods: 1. Brain sample preparation. TIMING 90 min to a few days (depends on number of samples); 2. Weigh tissue and record wet tissue weight. 3. Cut the tissue into small pieces with a scalpel or a tissue slice blade. 4. Transfer the tissue pieces into 3 volumes (w/v) of ice cold Buffer A. 5. Homogenize the tissue on ice using a homogenizer (30 s at 20 Hz using a single 7mm stainless-steel ball). 6. Centrifuge the sample for 15 min, 30800 rpm, 4°C using a TLA 100.2 rotor in a Beckman Coulter Optima Ultracentrifuge. 7. Transfer the supernatant into a clean tube. 8. To remaining pellet add buffer B (1:3 tissue to buffer ratio) and homogenized at 30 Hz for 30 s in a cold Mixer Mill MM400. 9. Centrifuge at 100,000 rpm for 20 min in a TLA-100.2 rotor (Beckman). 10. Transfer the supernatant into a clean tube and determine the protein concentration by the Bradford method.

[0140] Protein citrullination: Peptides can only be identified by targeted MS if they are included in the assay library. To cover proteins that are expressed in specific biological conditions we needed to prepare the pool of citrullinated peptides that theoretically represents the entire biological space. Most PAD isoforms are exclusively expressed in the cytoplasm, except for PAD4, which contains a nuclear localization signal and is indeed found to reside at least in part in the nucleus. It would therefore be reasonable to assume that the substrate specificity differs between the PAD isotypes [15, 16]. A key step in the process is the treatment of pooled tissue/sample(s) in vitro with a PAD cocktail to induce hypo-citrullination of accessible arginine residues. (1) Pool samples in each fraction in order to

obtain 200 ug sample pool. (2) Mix up to 30µl of a protein pool with 150µl of PAD Buffer. (3) Add PAD cocktail (Catalog #P312-37C) in an enzyme/substrate (w/w) ratio of 1:25, and incubate the reaction for 2 h at 37°C, gentle shaking. Stop citrullination by adding EDTA until 50 mM final concentration. (4) Prepare the negative control the same way but add water instead of PAD cocktail. (5) Keep on ice until use.

[0141] Protein digestion. Protein Discovery's FASP Protein Digestion Kit was used in order to digest all samples with LysC in an enzyme/substrate (w/w) ratio of 1:30, and incubate the reaction for 20h at 37°C, gentle shaking.

[0142] SWATH-MS Data Acquisition. Generation of SWATH-MS maps: Triple-TOF MS analysis in DDA and SWATH mode. A TripleTOF 6600 mass spectrometer (Sciex) was used for both data dependent acquisition to build peptide spectral ion library and SWATH-MS (data independent acquisition) for each individual sample analysis. The raw data was searched with ProteinPilot™ Software 5.0 to create a spectral ion library. Individual SWATH-MS runs were matched against the spectral library created in the presence or absence of PAD (plus and minus PAD).

[0143] Extensive identification and analysis of conserved citrullinated peptides. The blast on the peptide level was performed to find human homology (sequence-similar) equivalent of the mouse data obtained from brain stroke mouse model, Alzheimer's and healthy mouse brain, as well as from mouse aorta and mouse skeletal muscle. The matching list only includes the top blastp-short hit, meaning >80% of peptide homology between mouse and human was found. In most cases, if the top hit was an isoform, the other isoforms and canonical protein was also conserved. Figure 9 shows the overview bioinformatics workflow for processing citrullinated spectra library file. Two different types of tissue sample, heart and brain, were used to develop the presented methodologies. The final SWATH assay library generated from the human brain consists of 360,202 peptides (16,275 unique). The bioinformatics pipeline extracted 1,530 unique peptides that were considered to be high confident and valid citrullinated peptides (Table 11). An additional 1,571 unique peptides were identified as potentially high confident valid citrullinated peptides, and the other remaining peptides were considered to be low confident (Table 11). It is important to note that a peptide may have more than 1 citrullinated R, and some peptides share the exact citrullinated R site on the full length protein. Therefore, the number of unique peptides does

not equal the number of unique citrullinated sites. The total number of unique citrullinated sites (on full length protein) in Output1, 2, and 3 is 2413.

[0144] Table 11. Summary of output files for brain sample plus and minus PAD treatment for brain samples (confidence threshold used was 0.995) (JF_150402_5600-plus/minusPAD).

Output File	Description	NG Rule	Total Peptides		Total Unique Peptides	
			minus PAD	plus/minus PAD	minus PAD	plus/minus PAD
1	delta $\Rightarrow$ 5 min conf >0.95	Not checked	1,637	36,202	58	1,530
2	delta < 5 min OR conf < 0.95 OR no matched control	pass	3,238	30,382	152	1,571
3	delta < 5 min OR conf < 0.95 OR no matched control	fail	1,370	19,450	55	1,006
4	every line without R[Dea]	Not checked	375,717	431,816	17,147	20,702

[0145] The citrullinated peptides are binned into 4 different output files based. Output 1 contains the high confidence peptides, which have a > 5 min delta in retention time between the modified peptide and the unmodified peptide, plus a confidence score (user input). Output 2 and 3 contains peptides that had a citrullinated R residue; however, no unmodified peptide was found in the dataset limiting the ability to calculate the retention time difference. However, for Output 2 an N/Q residue is not within 5 amino acids on either side of the citrullinated R residue; whereas in Output 3 an N/Q residue is located within 5 amino acids on either side the citrullinated R residue. Output 4 contains all peptides in the original file that did not meet the criteria for Outputs 1-3.

[0146] Herein we describe a step-by-step protocol and an integrated, openly accessible computational pipeline to generate high-quality assay libraries for analysis of citrullinated peptides in complex biological samples. Using the high resolution of the time-of-flight mass analyzer ($>30,000$) that allowing for confident identification of the peptide elution profile and discrimination between near isobaric parent mass tolerance and bioinformatics pipeline that rules the outcomes we were able to identified citrullinated peptides over a dynamic range of 6 orders of magnitude and quantified those with relative abundance.

[0147] There are some chemical, proteomic and antibody-based procedures that are currently available for the analysis of citrullination in complex samples [15-17]. However, with the current methods it remains challenging to rapidly, consistently, reproducibly, accurately, and

sensitively detect and quantify citrullinated peptides across multiple samples. Here we present a new strategy that systematically queries sample sets for the presence and quantity of citrullinated proteins with exact site of modified residues. It consists of using the information available in fragment ion spectral libraries, like relative intensities, chromatographic concurrence to mine the complete citrullinated fragment ion maps generated using DIA method.

[0148] In essence, our tools identify peak groups that uniquely associate with the targeted citrullinated peptide within the comprehensive SWATH MS signal map, and then compute a probability that the targeted citrullinated peptide has been correctly identified by following the set up rules in bioinformatics pipeline. In order to increase sensitivity a high-quality library of citrullinated assays is a prerequisite for SWATH MS and similar targeting MS methods. Ideally, the citrullinated peptides in the assay library cover all citrullinated proteins of interest for a particular study, or even an entire proteome. In order to make sure it is true samples on based the library is built on are in vitro citrullinated by PAD cocktail. This step helps improve detection of citrullinated proteins present in lower amounts as well as cover potentially new sites.

[0149] We show that SWATH™-MS can be successfully adopted for PTM analysis. Bioinformatics pipeline added for analysis of citrullinated peptide makes the analysis reliable, robust and quick. We believe that this methodology will increase popularity of analysis of citrullination in the field and shortly will be invaluable to elucidate the importance of this posttranslational modification in vivo.

[0150] As described herein, citrullination, the post-translational (PTM) conversion of arginine to citrulline by the family of peptidylarginine deiminase (PADs), has been commonly implicated as abnormal pathological features in neurodegenerative diseases such as prion diseases, multiple sclerosis, and Alzheimer's disease. This PTM could be a target for novel diagnostic or therapeutic agents. Therefore, an unambiguous and efficient method to identify citrullinated proteins and their modified residues is of extreme importance.

[0151] ALS, or amyotrophic lateral sclerosis, is a progressive neurodegenerative disease that affects nerve cells in the brain and the spinal cord. The inventors investigated the role of citrullination in ALS disorders, with a focus on identifying a potentially clinically relevant PTM biomarker(s).

[0152] Fig. 8B depicts construction of citrullinated ion library. Figure 8C depicts delta RT for R[Dea] and N/Q[Dea] compared to unmodified sequence. Respectively, peptide containing citrullinated Arg eluted later compared to unmodified peptides (>5 min difference). The same trend was observed between peptides with citrullinated R and peptides with N and Q deamination.

[0153] As shown in Fig. 8F, alteration in citrullination of any one or more of Glial fibrillary acidic protein, Glucose-6-phosphate isomerase, Vimentin, Endophilin-A1, Myelin basic protein, Delta-1-pyrroline-5-carboxylate dehydrogenase, Peroxiredoxin-5, Neurogranin, Dual specificity mitogen-activated protein kinase kinase 1, Tubulin polymerization-promoting protein family member 3, Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, Cysteine and glycine-rich protein 1, Cellular retinoic acid-binding protein 1 GMP reductase 2 and/or Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-3 may be indicative of ALS.

[0154] Table 2: Citrullinated peptides as biomarkers for ALS. These peptides detected in subject with ALS but not healthy humans. “Dea” is Citrullination or deamination. “CAM” is carbamidomethylation.

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
Q99962	SH3 Endophilin-A1 G2	PSGVQMDPC[CAM]C[CAM]R[Dea]ALYDFEPENEGEL GFK (SEQ ID NO: 306)	296
P49419	Alpha-amino adipic semialdehyde dehydrogenase	QYMR[Dea]R[Dea]STC[CAM]TINYSK (SEQ ID NO: 307)	518, 519
H3BRN4	4-aminobutyrate aminotransferase	LILIAR[Dea]NK (SEQ ID NO: 308)	465
P68402	Platelet-activating factor acetylhydrolase IB subunit beta	VIVVWVGTTNNHENTAEVAGGIEAIVQLINTR[Dea]QP QAK (SEQ ID NO: 309)	128
Q13011	ECH Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	EVDVGLAADVGTLLQR[Dea]LPK (SEQ ID NO: 310)	211
P21291	CSRP1 Cysteine and glycine-rich protein 1	HEEAPGHR[Dea]PTTNPASK (SEQ ID NO: 311)	109
P63215	GBG Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-33	GETPVNSTMSIGQAR[Dea]K (SEQ ID NO: 312)	17
F8VVM2	F Phosphate carrier protein,	VRIQTQPGYANTLR[Dea]DAAPK (SEQ ID NO: 313)	200

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
P49588	SYA Alanine--tRNA ligase	NVGC[CAM]LQEALQLATSFAQLR[Dea]LGDVK (SEQ ID NO: 314)	962

[0155] Table 3: Citrullinated peptides (from humans) as biomarkers for ALS. “Dea” is Citrullination or deamination. “CAM” is carbamidomethylation.

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
P14136	Glial fibrillary acidic protein	FADLTDAAR[Dea]NAELLR[Dea]QAK (SEQ ID NO: 315)	270, 276
P14136	Glial fibrillary acidic protein	EIR[Dea]TQYEAMASSNMHEAEEWYRSK (SEQ ID NO: 316)	239
P14136	Glial fibrillary acidic protein	MASSNMHEAEEWYR[Dea]SK (SEQ ID NO: 317)	258
P14136	Glial fibrillary acidic protein	ASSNMHEAEEWYR[Dea]SK (SEQ ID NO: 318)	258
P14136	Glial fibrillary acidic protein	LAR[Dea]LEEEGQSLK (SEQ ID NO: 319)	330
P06744	Glucose-6-phosphate isomerase	ILLANFLAQTEALMR[Dea]GK (SEQ ID NO: 320)	438
P08670	Vimentin	TVETR[Dea]DGQVINETSQHHDDLE (SEQ ID NO: 321)	450
P08670	Vimentin	VELQELNDR[Dea]FANYIDK (SEQ ID NO: 322)	113
P08670	Vimentin	SSVPGVR[Dea]LLQDSVDFSLADAINTEFK (SEQ ID NO: 323)	78
P08670	Vimentin	ALR[Dea]DVR[Dea]QQYESVAAK (SEQ ID NO: 324)	270, 273
P02686	Myelin basic protein	GAEGQRPGFGYGGR[Dea]ASDYK (SEQ ID NO: 325)	264
P02686	Myelin basic protein	SHGR[Dea]TQDENPVVHFF (SEQ ID NO: 326)	213
P02686	Myelin basic protein	SRFSWGAEGQRPGFGYGGR[Dea]ASDYK (SEQ ID NO: 327)	264
P02686	Myelin basic protein	SR[Dea]FSWGAEGQR[Dea]PGFGYGGR[Dea]ASDYK (SEQ ID NO: 328)	247, 256, 264
P02686	Myelin basic protein	SWGAEGQR[Dea]PGFGYGGR[Dea]ASDYK (SEQ ID NO: 329)	256, 264
P02686	Myelin basic protein	SHGR[Dea]TQDENPVVHF (SEQ ID NO: 330)	213
P02686	Myelin basic protein	LGGR[Dea]DSRSGSPMARR[Dea] (SEQ ID NO: 331)	293
P02686	Myelin basic protein	EGQRPGFGYGGR[Dea]ASDYK (SEQ ID NO: 332)	264
P30038	Delta-1-pyrroline-5-carboxylate dehydrogenase	AIEAALAAR[Dea]K (SEQ ID NO: 333)	113
P30044	Peroxiredoxin-5	VR[Dea]LLADPTGAFGK (SEQ ID NO: 334)	148
Q9BW30	Tubulin polymerization-promoting protein family member 3	TGGAVDR[Dea]LTDTSR[Dea]YTGSHK (SEQ ID NO: 335)	124, 130

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	VIGNQSLVNELAFTAR[Dea]K (SEQ ID NO: 336)	230
P29762	Cellular retinoic acid-binding protein 1	MR[Dea]SSENFDELLK (SEQ ID NO: 337)	11
Q9P2T1	GMP reductase 2	YAGGVAEYR[Dea]ASEGK (SEQ ID NO: 338)	286

[0156] Table 4: Correlated citrullinated LysC peptides based on matching mouse sequence to human sequence. “Dea” is Citrullination or deamination. “CAM” is carbamidomethylation.

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
P69905	Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2	R[Dea]MFLSFPTTK (SEQ ID NO: 339)	32,
P69905	Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=3	VGAHAGEYGAELER[Dea]MFL (SEQ ID NO: 340)	32,
P46821	Microtubule-associated protein 1B OS=Homo sapiens GN=MAP1B PE=1 SV=2	TTR[Dea]TSDVGGYYEYK (SEQ ID NO: 341)	1897,
Q15149	Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3	VPQR[Dea]AGEVER[Dea]DLDK (SEQ ID NO: 342)	571, 577,
Q15149	Plectin OS=Homo sapiens GN=PLEC PE=1 SV=4	LHVAILER[Dea]EK (SEQ ID NO: 343)	517,
Q14204	Cytoplasmic dynein 1 heavy chain 1 OS=Homo sapiens GN=DYNC1H1 PE=1 SV=5	R[Dea]VEPLR[Dea]NELQK (SEQ ID NO: 344)	3408, 3413,
Q01484	Ankyrin-2 OS=Homo sapiens GN=ANK2 PE=1 SV=4	EGHVGLVQELLGR[Dea]GSSVDSATK (SEQ ID NO: 345)	86,
J3KPX8	MAP1 light chain LC2 OS=Homo sapiens GN=MAP1A PE=1 SV=1	AIVFEIMEAGEPTGPILGAEALPGGLR[Dea]TLPQEPGKPK (SEQ ID NO: 346)	1123,
Q13885	Tubulin beta-2A chain OS=Homo sapiens GN=TUBB2A PE=1 SV=1	IREEYPDR[Dea]IMNTFSVMPSK (SEQ ID NO: 347)	162,
Q00610	Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC PE=1 SV=5	GILR[Dea]TPDTIR[Dea]R[Dea]FQSVPAQPGQTSPLLQYFGILLDQGQLNK (SEQ ID NO: 348)	393, 399, 400,
Q00610	Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC PE=1 SV=6	YESLELC[CAM]R[Dea]PVLQQGR[Dea]K (SEQ ID NO: 349)	436, 437, 444,
Q99798	Aconitate hydratase, OS=Homo sapiens GN=ACO2 PE=1 SV=2	SYLR[Dea]LR[Dea]PDR[Dea]VAMQD ATAQMAMLQFISSGLSK (SEQ ID NO: 350)	90, 92, 95,
E7ESP9	Neurofilament medium polypeptide OS=Homo sapiens GN=NEFM PE=1 SV=1	SIELESVR[Dea]GTK (SEQ ID NO: 351)	335,
Q9NQC3	Reticulon-4 OS=Homo sapiens GN=RTN4 PE=1 SV=2	AESAIVANPR[Dea]EEIIVK (SEQ ID NO: 352)	320,
Q9NQC3	Reticulon-4 OS=Homo sapiens GN=RTN4 PE=1 SV=2	EAQIR[Dea]ETETFSDDSPIIIDEFPTLISSK (SEQ ID NO: 353)	854,
Q9NQ66	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 OS=Homo	EWTNEVFSLATNLLAQNMSR[Dea]DAFLEK (SEQ ID NO: 354)	148,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
	sapiens GN=PLCB1 PE=1 SV=1		
P04075	Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2	ADDGR[Dea]PFPQVIK (SEQ ID NO: 355)	92,
P10809	60 kDa heat shock protein, OS=Homo sapiens GN=HSPD1 PE=1 SV=2	LVQDVANNTNEEAGDGTATVLA R[Dea]SIK (SEQ ID NO: 356)	121,
P00558	Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3	ALESPER[Dea]PFLAILGGAK (SEQ ID NO: 357)	206,
P00558	Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3	R[Dea]VVMR[Dea]VDFNVPMK (SEQ ID NO: 358)	18, 22,
P13639	Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4	YEWDVAEAR[Dea]K (SEQ ID NO: 359)	647,
P38646	Stress-70 protein, OS=Homo sapiens GN=HSPA9 PE=1 SV=2	VLENAEGAR[Dea]TTPSVVAFTADG ER[Dea]LVGMPAK (SEQ ID NO: 360)	85, 99,
P06744	Glucose-6-phosphate isomerase OS=Homo sapiens GN=GPI PE=1 SV=4	NLVTEDVMR[Dea]MLVDLAK (SEQ ID NO: 361)	66,
P12277	Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1	DLFDPIIEDR[Dea]HGGYKPSDEHK (SEQ ID NO: 362)	96,
P12277	Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=2	DLFDPIIEDR[Dea]HGGYK (SEQ ID NO: 363)	96,
P11216	Glycogen phosphorylase, brain form OS=Homo sapiens GN=PYGB PE=1 SV=5	QISVR[Dea]GLAGLGDVAEVR[Dea] K (SEQ ID NO: 364)	17, 29,
P49588	Alanine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=AARS PE=1 SV=2	DVQR[Dea]EIADLGEALATAVIPQW QK (SEQ ID NO: 365)	793,
Q01814	Plasma membrane calcium-transporting ATPase 2 OS=Homo sapiens GN=ATP2B2 PE=1 SV=2	GIIDSTHTEQR[Dea]QVVAVTGDGT NDGPALK (SEQ ID NO: 366)	812,
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase OS=Homo sapiens GN=CNP PE=1 SV=2	ITPGAR[Dea]GAFSEEEK (SEQ ID NO: 367)	93,
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase OS=Homo sapiens GN=CNP PE=1 SV=2	TLFILR[Dea]GLPGSGK (SEQ ID NO: 368)	56,
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase OS=Homo sapiens GN=CNP PE=1 SV=2	ELR[Dea]QFVPGDEPREK (SEQ ID NO: 369)	224,
P19367	Hexokinase-1 OS=Homo sapiens GN=HK1 PE=1 SV=3	MVSGMYLGELVR[Dea]LILVK (SEQ ID NO: 370)	307,
Q9ULC6	Protein-arginine deiminase type-1 OS=Homo sapiens GN=PADI1 PE=1 SV=2	R[Dea]ELGLAESDIVDIPQLFFLK (SEQ ID NO: 371)	556,
Q8N573	Oxidation resistance protein 1 OS=Homo sapiens GN=OXR1 PE=1 SV=2	TLYR[Dea]TMTGLDTPVLMVIK (SEQ ID NO: 372)	754,
P15311	Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4	EELER[Dea]QAVDQIK (SEQ ID NO: 373)	405,
P08238	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4	IDIIPNPQER[Dea]TLTLVDTGIGMTK (SEQ ID NO: 374)	82,
P29966	Myristoylated alanine-rich C-kinase substrate OS=Homo sapiens GN=MARCKS PE=1 SV=4	GEAAAER[Dea]PGEEAAVASSPSK (SEQ ID NO: 375)	18,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
P49418	Amphiphysin OS=Homo sapiens GN=AMPH PE=1 SV=1	AEEPLAAVTPAVGLDLGMDTR[Dea] AEEPVEEAVIIPGADADA AVGTLVS AAEGAPGEEAEAEK (SEQ ID NO: 376)	457,
P10636	Microtubule-associated protein tau OS=Homo sapiens GN=MAPT PE=1 SV=5	SR[Dea]LQTAPVPMPLDK (SEQ ID NO: 377)	559,
P52306	Rap1 GTPase-GDP dissociation stimulator 1 OS=Homo sapiens GN=RAP1GDS1 PE=1 SV=3	LLGTLR[Dea]MLIDAQAEAAEQLGK (SEQ ID NO: 378)	427,
Q05639	Elongation factor 1-alpha 2 OS=Homo sapiens GN=EEF1A2 PE=1 SV=1	YYITIIDAPGHR[Dea]DFIK (SEQ ID NO: 379)	96,
Q02252	Methylmalonate-semialdehyde dehydrogenase [acylating], OS=Homo sapiens GN=ALDH6A1 PE=1 SV=2	YAHLLVDVGQVGVNVPVPLPMFSF TGSR[Dea]SSFR[Dea]GDTNFYGK (SEQ ID NO: 380)	488, 492,
P09972	Fructose-bisphosphate aldolase C OS=Homo sapiens GN=ALDOC PE=1 SV=2	R[Dea]AEVNGLA AQGK (SEQ ID NO: 381)	331,
P40926	Malate dehydrogenase, OS=Homo sapiens GN=MDH2 PE=1 SV=3	GLDPA[Dea]VNVPIVGGHAGK (SEQ ID NO: 382)	191,
P48735	Isocitrate dehydrogenase [NADP], OS=Homo sapiens GN=IDH2 PE=1 SV=2	ATDFVADR[Dea]AGTFK (SEQ ID NO: 383)	188,
F5GXC8	Succinyl-CoA ligase [ADP-forming] subunit beta, OS=Homo sapiens GN=SUCLA2 PE=1 SV=1	IFDLQDWTQEDER[Dea]DK (SEQ ID NO: 384)	242,
P09104	Gamma-enolase OS=Homo sapiens GN=ENO2 PE=1 SV=3	LAMQEFMILPVGAESFR[Dea]DAMR LGAEVYHTLK (SEQ ID NO: 385)	179,
Q9BPU6	Dihydropyrimidinase-related protein 5 OS=Homo sapiens GN=DPYSL5 PE=1 SV=1	VR[Dea]GVDR[Dea]TPYLGDVAVVV HPGK (SEQ ID NO: 386)	485, 489,
Q9BPU6	Dihydropyrimidinase-related protein 5 OS=Homo sapiens GN=DPYSL5 PE=1 SV=2	MDENR[Dea]FVAVTSSNAAK (SEQ ID NO: 387)	372,
Q14195	Dihydropyrimidinase-related protein 3 OS=Homo sapiens GN=DPYSL3 PE=1 SV=1	SAADLISQAR[Dea]K (SEQ ID NO: 388)	268,
P09471	Guanine nucleotide-binding protein G(o) subunit alpha OS=Homo sapiens GN=GNAO1 PE=1 SV=4	PVVYSNTIQSLAAIVR[Dea]AMDTL GIEYGDK (SEQ ID NO: 389)	86,
Q9ULU8	Calcium-dependent secretion activator 1 OS=Homo sapiens GN=CADPS PE=1 SV=3	ELGR[Dea]VILHPTPNPK (SEQ ID NO: 390)	479,
P49419	Alpha-aminoadipic semialdehyde dehydrogenase OS=Homo sapiens GN=ALDH7A1 PE=1 SV=5	DLGR[Dea]IFR[Dea]WLGPK (SEQ ID NO: 391)	466, 469,
P38606	V-type proton ATPase catalytic subunit A OS=Homo sapiens GN=ATP6V1A PE=1 SV=2	TVGMLSNMIAFYDMAR[Dea]R[Dea] AVETTAQSDNK (SEQ ID NO: 392)	552, 553,
H3BRN4	4-aminobutyrate aminotransferase, OS=Homo sapiens GN=ABAT PE=1 SV=1	EEFR[Dea]PNAPYR[Dea]IFNTWLGD PSK (SEQ ID NO: 393)	386, 392,
P07237	Protein disulfide-isomerase OS=Homo sapiens GN=P4HB PE=1 SV=3	R[Dea]TGPAATTLPDGAAAESLVES SEVAVIGFFK (SEQ ID NO: 394)	132,
O94760	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	IMQQMSDHR[Dea]YDK (SEQ ID NO: 395)	208,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
Q16181	Septin-7	DR[Dea]LPLAVVGSNTIIEVNGK (SEQ ID NO: 396)	243,
O75781	Paralemmmin-1 OS=Homo sapiens GN=PALM PE=1 SV=2	R[Dea]RQLEDER[Dea]RQLQHLK (SEQ ID NO: 397)	32, 39,
P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform OS=Homo sapiens GN=PPP2R1A PE=1 SV=4	HMLPTVLR[Dea]MAGDPVANVR[Dea]FNVAK (SEQ ID NO: 398)	527, 537,
P15121	Aldose reductase OS=Homo sapiens GN=AKR1B1 PE=1 SV=3	PEDPSLLEDPR[Dea]IK (SEQ ID NO: 400)	233,
P07954	Fumarate hydratase OS=Homo sapiens GN=FB PE=1 SV=3	IGR[Dea]THTQDAVPLTLGQEFSGY VQQVK (SEQ ID NO: 401)	233,
P24752	Acetyl-CoA acetyltransferase, OS=Homo sapiens GN=ACAT1 PE=1 SV=1	EVVIVSATR[Dea]TPIGSFLGSLSLLP ATK (SEQ ID NO: 402)	49,
E9PFW3	AP-2 complex subunit mu OS=Homo sapiens GN=AP2M1 PE=1 SV=1	DIILPFR[Dea]VIPLVR[Dea]EVGR TK (SEQ ID NO: 403)	313, 319,
P30086	Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1 PE=1 SV=3	LYTLVL TDPDAPSR[Dea]K (SEQ ID NO: 404)	76,
P04350	Tubulin beta-4A chain OS=Homo sapiens GN=TUBB4A PE=1 SV=2	GHYTEGAELVDAVLDVVR[Dea]K (SEQ ID NO: 405)	121,
Q9UI15	Transgelin-3 OS=Homo sapiens GN=TAGLN3 PE=1 SV=2	AAETYGVR[Dea]TTDIFQTVDLWEG K (SEQ ID NO: 406)	106,
O43175	D-3-phosphoglycerate dehydrogenase OS=Homo sapiens GN=PHGDH PE=1 SV=4	VLISDSLDP[CAM]C[CAM]R[Dea]K (SEQ ID NO: 407)	18, 19, 20,
P00915	Carbonic anhydrase 1 OS=Homo sapiens GN=CA1 PE=1 SV=2	ESISVSSEQLAQFRSLLSN[Dea]VEG DNAVPMQHNNR[Dea]PTQPLK (SEQ ID NO: 408)	233, 247,
P61981	14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2	MKGDYYR[Dea]YLAEVATGEK (SEQ ID NO: 409)	132,
P61604	10 kDa heat shock protein, OS=Homo sapiens GN=HSPE1 PE=1 SV=2	DYFLFR[Dea]DGDILGK (SEQ ID NO: 410)	92,
P21281	V-type proton ATPase subunit B, brain isoform OS=Homo sapiens GN=ATP6V1B2 PE=1 SV=3	DIGWQLLR[Dea]IFPK (SEQ ID NO: 411)	485,
B4DKF8	PH and SEC7 domain-containing protein 3 OS=Homo sapiens GN=PSD3 PE=1 SV=1	FSR[Dea]PLLPATTTK (SEQ ID NO: 412)	245,
P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5	EGGLGPLNIPLADVTR[Dea]R[Dea] LSEDYGV LK (SEQ ID NO: 413)	109, 110,
P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5	TDEGIAYR[Dea]GLFIIDGK (SEQ ID NO: 414)	127,
P02647	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	VEPLR[Dea]AELQEGARQK (SEQ ID NO: 415)	147,
Q12765	Secernin-1 OS=Homo sapiens GN=SCRN1 PE=1 SV=2	NSAR[Dea]PR[Dea]DEVQEVVYFSA ADHEPESK (SEQ ID NO: 416)	30, 32,
Q12765	Secernin-1 OS=Homo sapiens GN=SCRN1 PE=1 SV=2	AHEWAR[Dea]AIIESDQEQGR[Dea]K (SEQ ID NO: 417)	355, 366,
P00918	Carbonic anhydrase 2 OS=Homo sapiens GN=CA2 PE=1 SV=2	LNFNGEPEPEELMVDNWR[Dea]PA QPLK (SEQ ID NO: 418)	245,
E9PDE8	Heat shock 70 kDa protein 4L OS=Homo sapiens GN=HSPA4L PE=1 SV=1	VR[Dea]YLEEER[Dea]PFAIEQVTGM LLA K (SEQ ID NO: 419)	78, 84,
Q9H115	Beta-soluble NSF attachment protein	ER[Dea]EAVQLMAEAEK (SEQ ID	8,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
	OS=Homo sapiens GN=NAPB PE=1 SV=2	NO: 420)	
Q9UPY8	Microtubule-associated protein RP/EB family member 3 OS=Homo sapiens GN=MAPRE3 PE=1 SV=1	LIGTAVPQR[Dea]TSPTGPK (SEQ ID NO: 421)	160,
P00505	Aspartate aminotransferase, OS=Homo sapiens GN=GOT2 PE=1 SV=3	FSR[Dea]DVFLPK (SEQ ID NO: 422)	153,
Q13509	Tubulin beta-3 chain OS=Homo sapiens GN=TUBB3 PE=1 SV=2	FWEVISDEHGIDPSGNYVGDSLQLER[Dea]ISVYYNEASSHK (SEQ ID NO: 423)	46,
O15144	Actin-related protein 2/3 complex subunit 2 OS=Homo sapiens GN=ARPC2 PE=1 SV=1	DR[Dea]VTVVVFSTVFK (SEQ ID NO: 424)	160,
P09211	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1 PE=1 SV=2	FQDGDLTLYQSNTILR[Dea]HLGR[Dea]TLGLYGK (SEQ ID NO: 425)	71, 75,
O95292	Vesicle-associated membrane protein-associated protein B/C OS=Homo sapiens GN=VAPB PE=1 SV=3	FR[Dea]GPFTDVVTNLK (SEQ ID NO: 426)	19,
P42765	3-ketoacyl-CoA thiolase, OS=Homo sapiens GN=ACAA2 PE=1 SV=2	QTMQVDEHAR[Dea]PQTTLEQLQK (SEQ ID NO: 427)	224,
Q8TAM6	Ermin OS=Homo sapiens GN=ERMN PE=2 SV=1	GHQAAEIEWLGFR[Dea]K (SEQ ID NO: 428)	163,
Q92686	Neurogranin OS=Homo sapiens GN=NRGN PE=1 SV=1	GPGPGGPGGAGVAR[Dea]GGAGGGPSGD (SEQ ID NO: 429)	68,
K7ELL7	Glucosidase 2 subunit beta OS=Homo sapiens GN=PRKCSH PE=1 SV=1	MPPYDEQTQAFIDAAQEAR[Dea]NK (SEQ ID NO: 430)	381,
Q9BY11	Protein kinase C and casein kinase substrate in neurons protein 1 OS=Homo sapiens GN=PACSIN1 PE=1 SV=1	GPQYGSRLER[Dea]AWGAIMTEADK (SEQ ID NO: 431)	82,
O15075	Serine/threonine-protein kinase DCLK1 OS=Homo sapiens GN=DCLK1 PE=1 SV=2	TTSASR[Dea]AVSSLATAK (SEQ ID NO: 432)	161,
P09622	Dihydrolipoyl dehydrogenase, OS=Homo sapiens GN=DLD PE=1 SV=2	NLGLLEELGIELDPR[Dea]GR[Dea]IPVNTRFQ[Dea]TK (SEQ ID NO: 433)	334, 336, 344,
P25705	ATP synthase subunit alpha, OS=Homo sapiens GN=ATP5A1 PE=1 SV=1	R[Dea]TGAIVDVPVGEELLGR[Dea]VDALGNAIDGK (SEQ ID NO: 434)	133, 149,
Q02750	Dual specificity mitogen-activated protein kinase kinase 1 OS=Homo sapiens GN=MAP2K1 PE=1 SV=2	AGR[Dea]IPEQILGK (SEQ ID NO: 435)	160,
Q01995	Transgelin OS=Homo sapiens GN=TAGLN PE=1 SV=4	GPSYGMSR[Dea]EVQSK (SEQ ID NO: 436)	12,
Q16352	Alpha-internexin OS=Homo sapiens GN=INA PE=1 SV=2	SEMAR[Dea]HLR[Dea]EYQDLLNVK (SEQ ID NO: 437)	374, 377,
Q92752	Tenascin-R OS=Homo sapiens GN=TNR PE=1 SV=3	R[Dea]HAVLMGLQPATEYIVNLVAVHGTVTSEPIVGSITTGIDPPK (SEQ ID NO: 438)	827,
O94811	Tubulin polymerization-promoting protein OS=Homo sapiens GN=TPPP PE=1 SV=1	AISSPTVSR[Dea]LTDTTK (SEQ ID NO: 439)	165,
P30048	Thioredoxin-dependent peroxide reductase, OS=Homo sapiens GN=PRDX3 PE=1 SV=3	HLSVNDLPVGRSVEETLR[Dea]LVK (SEQ ID NO: 440)	214,
Q9Y570	Protein phosphatase methylesterase 1 OS=Homo sapiens GN=PPME1 PE=1 SV=3	R[Dea]DFSPVPWSQYFESMEDVEVENETGK (SEQ ID NO: 441)	39,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
P69891	Hemoglobin subunit gamma-1 OS=Homo sapiens GN=HBG1 PE=1 SV=2	MVTAVASALSSR[Dea]YH (SEQ ID NO: 442)	145,
Q5T9B7	Adenylate kinase isoenzyme 1 OS=Homo sapiens GN=AK1 PE=1 SV=1	GQLVPLETVLDMLR[Dea]DAMVAK (SEQ ID NO: 443)	93,
F5GXJ9	CD166 antigen OS=Homo sapiens GN=ALCAM PE=1 SV=1	PDGSPVFIAFR[Dea]SSTK (SEQ ID NO: 444)	20,
Q6PUV4	Complexin-2 OS=Homo sapiens GN=CPLX2 PE=1 SV=2	AALEQPC[CAM]EGSLTR[Dea]PK (SEQ ID NO: 445)	90, 96,
Q5T6W5	Heterogeneous nuclear ribonucleoprotein K OS=Homo sapiens GN=HNRNPK PE=1 SV=1	R[Dea]PAEDMEEEQAFK (SEQ ID NO: 446)	22,
Q9NQR4	Omega-amidase NIT2 OS=Homo sapiens GN=NIT2 PE=1 SV=1	R[Dea]SDLYAVEMK (SEQ ID NO: 447)	265,
P48426	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha OS=Homo sapiens GN=PIP4K2A PE=1 SV=2	R[Dea]FLDFIGHILT (SEQ ID NO: 448)	396,
Q4J6C6	Prolyl endopeptidase-like OS=Homo sapiens GN=PREPL PE=1 SV=1	LMR[Dea]TAADTPAIMNWDLFFTMK (SEQ ID NO: 449)	326,
P61106	Ras-related protein Rab-14 OS=Homo sapiens GN=RAB14 PE=1 SV=4	ADLEAQR[Dea]DVTYEEAK (SEQ ID NO: 450)	132,
Q99447	Ethanolamine-phosphate cytidyltransferase OS=Homo sapiens GN=PCYT2 PE=1 SV=1	GPPVFTQEER[Dea]YK (SEQ ID NO: 451)	76,
P48506	Glutamate--cysteine ligase catalytic subunit OS=Homo sapiens GN=GCLC PE=1 SV=2	SR[Dea]YDSIDSYLSK (SEQ ID NO: 452)	328,
P27361	Mitogen-activated protein kinase 3 OS=Homo sapiens GN=MAPK3 PE=1 SV=4	R[Dea]ITVEEALAHPLYEQYYDPTD EPVAEEPFTFAMELDDLK (SEQ ID NO: 453)	318,
B1AKZ3	Astrocytic phosphoprotein PEA-15 OS=Homo sapiens GN=PEA15 PE=1 SV=1	LTR[Dea]IPSAK (SEQ ID NO: 454)	122,
P12036	Neurofilament heavy polypeptide OS=Homo sapiens GN=NEFH PE=1 SV=4	EQLQALNDR[Dea]FAGYIDK (SEQ ID NO: 455)	107,
P62333	26S protease regulatory subunit 10B OS=Homo sapiens GN=PSMC6 PE=1 SV=1	IHIDL PNEQAR[Dea]LDILK (SEQ ID NO: 456)	309,
P36578	60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=5	GHR[Dea]IEEVPELPLVVEDK (SEQ ID NO: 457)	143,
P13861	cAMP-dependent protein kinase type II-alpha regulatory subunit OS=Homo sapiens GN=PRKAR2A PE=1 SV=2	NLDQEQLSQVLDAMFER[Dea]IVK (SEQ ID NO: 4589)	158,
Q8WW12	PEST proteolytic signal-containing nuclear protein OS=Homo sapiens GN=PCNP PE=1 SV=2	R[Dea]SAEEEEAADLPTKPTK (SEQ ID NO: 459)	52,
P50897	Palmitoyl-protein thioesterase 1 OS=Homo sapiens GN=PPT1 PE=1 SV=1	ETIPLQETSLYTQDR[Dea]LGLK (SEQ ID NO: 460)	268,
Q9UJU6	Drebrin-like protein OS=Homo sapiens GN=DBNL PE=1 SV=1	ESGR[Dea]FQDVGPQAPVGSVYQK (SEQ ID NO: 461)	148,
Q14019	Coactosin-like protein OS=Homo sapiens GN=COTL1 PE=1 SV=3	FALITWIGENVSGLQR[Dea]AK (SEQ ID NO: 462)	91,
Q8TEA8	D-tyrosyl-tRNA(Tyr) deacylase 1	ILNLR[Dea]VFEDESGK (SEQ ID NO: 463)	53,

UniProtKB Accessions	Protein Name	Modified Sequence	Modification position
	OS=Homo sapiens GN=DTD1 PE=1 SV=2	463)	
H0Y7Q1	Arf-GAP with dual PH domain-containing protein 1 (Fragment) OS=Homo sapiens GN=ADAP1 PE=1 SV=1	AVDR[Dea]PMLPQEYAVEAHFK (SEQ ID NO: 464)	340,
O00401	Neural Wiskott-Aldrich syndrome protein OS=Homo sapiens GN=WASL PE=1 SV=2	AALLDQIR[Dea]EGAQLK (SEQ ID NO: 465)	414,
O94772	Lymphocyte antigen 6H OS=Homo sapiens GN=LY6H PE=2 SV=1	R[Dea]HFFSDYLMGFINSGLK (SEQ ID NO: 466)	82,
A8MX49	Protein phosphatase 1F OS=Homo sapiens GN=PPM1F PE=1 SV=2	APGTVLSQEEVEGELAELAMGFLGS R[Dea]K (SEQ ID NO: 467)	69,
O95336	6-phosphogluconolactonase OS=Homo sapiens GN=PGLS PE=1 SV=2	IVAPISDSPKPPQR[Dea]VTLTLPVL NAAR[Dea]TVIFVATGEGK (SEQ ID NO: 468)	185, 197,
O43708	Maleylacetoacetate isomerase OS=Homo sapiens GN=GSTZ1 PE=1 SV=3	R[Dea]ASVR[Dea]MISDLIAGGIQPLQ NLSVLK (SEQ ID NO: 469)	96, 100,
Q9UII2	ATPase inhibitor, OS=Homo sapiens GN=ATPIF1 PE=1 SV=1	GSDQSENVDR[Dea]GAGSIR[Dea]EAGGAFGK (SEQ ID NO: 470)	35, 41,
Q9P1F3	Costars family protein ABRACL OS=Homo sapiens GN=ABRACL PE=1 SV=1	FGVLFR[Dea]DDK (SEQ ID NO: 471)	35,
P62851	40S ribosomal protein S25 OS=Homo sapiens GN=RPS25 PE=1 SV=1	IR[Dea]GSLAR[Dea]AALQELLSK (SEQ ID NO: 472)	80, 85,
C9J0K6	Sorcin OS=Homo sapiens GN=SRI PE=1 SV=1	ALTTMGFR[Dea]LSPQAVNSIAK (SEQ ID NO: 473)	92,
Q9NVJ2	ADP-ribosylation factor-like protein 8B OS=Homo sapiens GN=ARL8B PE=1 SV=1	R[Dea]DLPNALDEK (SEQ ID NO: 474)	132,

[0157] Table 5A: Citrullinated peptides observed in chronic neurodegenerative disease. These citrullinated peptides are brain specific.

Peptide	UniProtKB Accessions	Name
ADDGR[Dea]PFPQVIK (SEQ ID NO: 355)	sp P04075-2 ALDOA_HUMAN	Isoform 2 of Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA
EVVIVSATR[Dea]TPIGSFLGSLSL PATK (SEQ ID NO: 402)	sp P24752 THIL_HUMAN	Acetyl-CoA acetyltransferase
RGETSGR[Dea]VDDNEETIK (SEQ ID NO: 1)	sp P00568 KAD1_HUMAN	Adenylate kinase isoenzyme 1 OS=Homo sapiens GN=AK1 PE=1 SV=3
PGVVHVIDIDR[Dea]GEEK (SEQ ID NO: 475)	sp P55087 AQP4_HUMAN	Aquaporin-4 OS=Homo sapiens GN=AQP4 PE=1 SV=2
IPVGPETLGR[Dea]IMNVIGEPIDER GPIK (SEQ ID NO: 476)	sp P06576 ATPB_HUMAN	ATP synthase subunit beta, OS=Homo sapiens GN=ATP5B PE=1 SV=3
SVHR[Dea]GEVPCTVTTASPLDDA VLSELK (SEQ ID NO: 477)	sp P48047 ATPO_HUMAN	ATP synthase subunit O, OS=Homo sapiens GN=ATP5O PE=1 SV=1
GILR[Dea]TPDTIR[Dea]R[Dea]FQS VPAQPGQTSPLLQYFGILLDQGQL NK (SEQ ID NO: 348)	sp Q00610 CLH1_HUMAN	Clathrin heavy chain 1
ELEEDFIR[Dea]SELK (SEQ ID NO: 478)	sp Q14019 COTL1_HUMAN	Coactosin-like protein OS=Homo sapiens GN=COTL1 PE=1 SV=3

DLFDPIIEDR[Dea]HGGYK (SEQ ID NO: 363)	sp P12277 KCRB_HUMAN	Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1
EGNASGVSLLEALDTILPPTR[Dea]PTDK (SEQ ID NO: 479)	sp Q05639 EF1A2_HUMAN	Elongation factor 1-alpha 2 OS=Homo sapiens GN=EEF1A2 PE=1 SV=1
GHQAAEIEWLGFR[Dea]K (SEQ ID NO: 428)	sp Q8TAM6 ERMIN_HUMAN	Ermin
EELER[Dea]QAVDQIK (SEQ ID NO: 373)	sp P15311 EZRI_HUMAN	Ezrin
EIR[Dea]TQYEAMASSNMHEAEEWYRSK (SEQ ID NO: 316)	sp P14136 GFAP_HUMAN	Glial fibrillary acidic protein
FADLTDAAR[Dea]NAELLR[Dea]QAK (SEQ ID NO: 315)	sp P14136 GFAP_HUMAN	Glial fibrillary acidic protein
GYFEDRR[Dea]PSANCDPYAVTEAIVRTCLLNETGDEPFQYK (SEQ ID NO: 480)	sp P15104 GLNA_HUMAN	Glutamine synthetase OS=Homo sapiens GN=GLUL PE=1 SV=4
RLIGRR[Dea]FDDAVVQSDMK (SEQ ID NO: 481)	sp P11142 HSP7C_HUMAN	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1
VGAHAGEYGAEALER[Dea]MFL (SEQ ID NO: 340)	sp P69905 HBA_HUMAN	Hemoglobin subunit alpha
LGRLLVVYPWTQR[Dea]YFDSFGDLSSASAIMGNAK (SEQ ID NO: 482)	sp P69892 HBG2_HUMAN	Hemoglobin subunit gamma-2 OS=Homo sapiens GN=HBG2 PE=1 SV=2
ESLLFEGR[Dea]ITPELLTRGK (SEQ ID NO: 483)	sp P19367 HXK1_HUMAN	Hexokinase-1 OS=Homo sapiens GN=HK1 PE=1 SV=3
RSTITSR[Dea]EVQTA VR[Dea]LLLPGELAK (SEQ ID NO: 484)	sp Q8N257 H2B3B_HUMAN	Histone H2B type 3-B OS=Homo sapiens GN=HIST3H2BB PE=1 SV=3
CR[Dea]EVAENCK (SEQ ID NO: 485)	sp P50213 IDH3A_HUMAN	Isocitrate dehydrogenase [NAD] subunit alpha, OS=Homo sapiens GN=IDH3A PE=1 SV=1
CR[Dea]APEVSQYIYQVYDSILK (SEQ ID NO: 486)	sp P63010-2 AP2B1_HUMAN	Isoform 2 of AP-2 complex subunit beta OS=Homo sapiens GN=AP2B1
GILR[Dea]TPDTIR[Dea]RFQSVPAQPGQTSPLLQYFGILLDQGQLNK (SEQ ID NO: 487)	sp Q00610-2 CLH1_HUMAN	Isoform 2 of Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC
PITIFQE[Dea]RDPTNIK (SEQ ID NO: 488)	sp P04406-2 G3P_HUMAN	Isoform 2 of Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH
GCDVVVIPAGVPR[Dea]K (SEQ ID NO: 489)	sp P40926-2 MDHM_HUMAN	Isoform 2 of Malate dehydrogenase, OS=Homo sapiens GN=MDH2
HR[Dea]DTGILDSIGRFFSGDR[Dea]GAPK (SEQ ID NO: 490)	sp P02686-2 MBP_HUMAN	Isoform 2 of Myelin basic protein OS=Homo sapiens GN=MBP
ER[Dea]LDQPMTEIVSR[Dea]VSK (SEQ ID NO: 491)	sp P22314-2 UBA1_HUMAN	Isoform 2 of Ubiquitin-like modifier-activating enzyme 1 OS=Homo sapiens GN=UBA1
LLVSSDYGR[Dea]DLTGQVQLRK (SEQ ID NO: 492)	sp Q13813-3 SPTN1_HUMAN	Isoform 3 of Spectrin alpha chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTAN1
VAVTPPGLAR[Dea]EDWK (SEQ ID NO: 493)	sp P28331-4 NDUS1_HUMAN	Isoform 4 of NADH-ubiquinone oxidoreductase 75 kDa subunit, OS=Homo sapiens GN=NDUFS1
VR[Dea]IQTQPGYAN (SEQ ID NO: 494)	sp Q00325-2 MPCP_HUMAN	Isoform B of Phosphate carrier protein, OS=Homo sapiens GN=SLC25A3
RIER[Dea]ELAEAQDDSILK (SEQ ID NO: 495)	sp P30530-2 UFO_HUMAN	Isoform Short of Tyrosine-protein kinase receptor UFO OS=Homo sapiens GN=AXL
SR[Dea]LQTAPVPMPDLK (SEQ ID NO: 377)	sp P10636 TAU_HUMAN	Microtubule-associated protein tau
HRDTGILDSIGRFFGGDR[Dea]GAPK (SEQ ID NO: 496)	sp P02686 MBP_HUMAN	Myelin basic protein
LGGR[Dea]DSRSGSPMARR[Dea] (SEQ ID NO: 331)	sp P02686 MBP_HUMAN	Myelin basic protein
SHGR[Dea]TQDENPVVHF (SEQ ID NO: 330)	sp P02686 MBP_HUMAN	Myelin basic protein
EGQRPFGYGGGR[Dea]ASDYK (SEQ ID NO: 332)	sp P02686 MBP_HUMAN	Myelin basic protein

GAEGQRPFGYGGGR[Dea]ASDYK (SEQ ID NO: 325)	sp P02686 MBP_HUMAN	Myelin basic protein
GEAAAER[Dea]PGEAAVASSPSK (SEQ ID NO: 375)	sp P29966 MARCS_HUMAN	Myristoylated alanine-rich C-kinase substrate
GPGPGPGGAGVAR[Dea]GGAGG GPSGD (SEQ ID NO: 429)	sp Q92686 NEUG_HUMAN	Neurogranin
TDEGIAYR[Dea]GLFIIDGK (SEQ ID NO: 414)	sp P32119 PRDX2_HUMAN	Peroxiredoxin-2
VRIQTQPGYANTLR[Dea]DAAPK (SEQ ID NO: 313)	sp F8VVM2 F8VVM2_HUMAN	Phosphate carrier protein,
GIIDSTHTEQR[Dea]QVVAVTGDG TNDGPALK (SEQ ID NO: 366)	sp Q01814 AT2B2_HUMAN	Plasma membrane calcium-transporting ATPase 2
EAFQNAYLELGLGER[Dea]VLGF CHYYLP EEQFPK (SEQ ID NO: 497)	sp P13637 AT1A3_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-3 OS=Homo sapiens GN=ATP1A3 PE=1 SV=3
VLENAEGAR[Dea]TTPSVVAFTAD GER[Dea]LVGMPAK (SEQ ID NO: 360)	sp P38646 GRP75_HUMAN	Stress-70 protein, OS=Homo sapiens GN=HSPA9 PE=1 SV=2
RIHVLPIDDTVEGITGNLFEVYLK (SEQ ID NO: 498)	sp P55072 TERA_HUMAN	Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4
HVPR[Dea]AVFVDLEPTVIDEIRNG PYRQLFHPEQLITGK (SEQ ID NO: 499)	sp P68366 TBA4A_HUMAN	Tubulin alpha-4A chain OS=Homo sapiens GN=TUBA4A PE=1 SV=1
FWEVISDEHGIDPSGNYVGSDSLQ LER[Dea]ISVYYNEASSHK (SEQ ID NO: 423)	sp Q13509 TBB3_HUMAN	Tubulin beta-3 chain
AILVDLEPGTMDSVRS[Dea]GAFG HLFR[Dea]PDNFI FGQSGAGNNWAK (SEQ ID NO: 500)	sp Q13509 TBB3_HUMAN	Tubulin beta-3 chain OS=Homo sapiens GN=TUBB3 PE=1 SV=2
GHYTEGAELVDAVL DVVR[Dea]K (SEQ ID NO: 405)	sp P04350 TBB4A_HUMAN	Tubulin beta
AISSPTVSR[Dea]LTDTTK (SEQ ID NO: 439)	sp O94811 TPPP_HUMAN	Tubulin polymerization-promoting protein
TVETR[Dea]DGQVINETSQHDDL E (SEQ ID NO: 321)	sp P08670 VIME_HUMAN	Vimentin

[0158] Table 5B. Citrullinated peptides observed in chronic neurodegenerative disease. These citrullinated peptides are not brain specific.

Peptide	UniProtKB Accessions	Protein Name
AVLDVAETGTEAAAATGVIGGI RK (SEQ ID NO: 501)	sp Q96P15-3 SPB11_HUMAN	Isoform 3 of Serpin B11 OS=Homo sapiens GN=SERPINB11
GFPVVLDSPRDGNLK (SEQ ID NO: 502)	sp Q9Y2J8 PADI2_HUMAN	Protein-arginine deiminase type-2 OS=Homo sapiens GN=PADI2 PE=1 SV=2
GPDRLPAGYEIVLYISMSDSK (SEQ ID NO: 503)	sp Q9Y2J8 PADI2_HUMAN	Protein-arginine deiminase type-2 OS=Homo sapiens GN=PADI2 PE=1 SV=2
RFSMVIDNGIVK (SEQ ID NO: 504)	sp P30044 PRDX5_HUMAN	Peroxiredoxin-5, OS=Homo sapiens GN=PRDX5 PE=1 SV=4

[0159] Table 6A Citrullinated peptide detected in acquired brain injury in human brains.
These citrullinated peptides are brain specific

Peptide	UniProtKB Accessions	Protein Name
AQQRDVDGATLARLDLEK (SEQ ID NO: 505)	sp Q16352 AINX_HUMAN	Alpha-internexin OS=Homo sapiens GN=INA PE=1 SV=2
AVTELNEPLSNEDR[Dea]NLLSVA YK (SEQ ID NO: 506)	sp Q04917 1433F_HUMAN	14-3-3 protein eta OS=Homo sapiens GN=YWHAH PE=1 SV=4
AVTEQGAELSNEER[de]NLLSVA YK (SEQ ID NO: 507)	sp P27348 1433T_HUMAN	14-3-3 protein theta OS=Homo sapiens GN=YWHAQ PE=1 SV=1
VVR[Dea]TALLDAAGVASLLTTA EVVVTEIPK (SEQ ID NO: 285)	sp P10809 CH60_HUMAN	60 kDa heat shock protein
LEAPDADELPR[Dea]SDFDPGQDT YQHPPK (SEQ ID NO: 508)	sp Q99798 ACON_HUMAN	Aconitate hydratase, OS=Homo sapiens GN=ACO2 PE=1 SV=2
LSEELSGGR[Dea]LK (SEQ ID NO: 509)	sp P61158 ARP3_HUMAN	Actin-related protein 3 OS=Homo sapiens GN=ACTR3 PE=1 SV=3
ILPWLLSPER[Dea]LDIK (SEQ ID NO: 510)	sp Q8WXF7 ATLA1_HUMAN	Atlastin-1 OS=Homo sapiens GN=ATL1 PE=1 SV=1
LR[Dea]FPAEDEFDPDLSSHNNHMA K (SEQ ID NO: 511)	sp P12277 KCRB_HUMAN	Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1
ITR[Dea]TSFLDDAFRK (SEQ ID NO: 512)	sp Q14204 DYHC1_HUMAN	Cytoplasmic dynein 1 heavy chain 1 OS=Homo sapiens GN=DYNC1H1 PE=1 SV=5
DNFTLIPEGTNGTEER[Dea]MSVI WDK (SEQ ID NO: 513)	sp Q16555 DPYL2_HUMAN	Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1
MVEGFFDR[Dea]GASIVEDK (SEQ ID NO: 514)	sp P49448 DHE4_HUMAN	Glutamate dehydrogenase 2, OS=Homo sapiens GN=GLUD2 PE=1 SV=2
GDYYR[Dea]YLAEVAAGDDK (SEQ ID NO: 515)	sp P63104-2 1433Z_HUMAN	Isoform 2 of 14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ
R[Dea]ALVFQPVTELK (SEQ ID NO: 516)	sp P08237-2 PFKAM_HUMAN	Isoform 2 of ATP-dependent 6-phosphofructokinase, muscle type OS=Homo sapiens GN=PFKM
GVPLYR[Dea]HIADLAGNPEVILP VPAFNVINGGSHAGNK (SEQ ID NO: 517)	sp P09104-2 ENOG_HUMAN	Isoform 2 of Gamma-enolase OS=Homo sapiens GN=ENO2
R[Dea]VIISAPSADAPMFVMGVNH EK (SEQ ID NO: 125)	sp P04406-2 G3P_HUMAN	Isoform 2 of Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH
NSPLVSR[Dea]LTLYDIAHTPGVA ADLSHIETR[Dea]ANVK (SEQ ID NO: 518)	sp P40926-2 MDHM_HUMAN	Isoform 2 of Malate dehydrogenase, OS=Homo sapiens GN=MDH2
HEGFE[Dea]DLAALGDK (SEQ ID NO: 519)	sp Q13813-2 SPTN1_HUMAN	Isoform 2 of Spectrin alpha chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTAN1
LFVTNDAATILR[Dea]ELEVQHPA AK (SEQ ID NO: 520)	sp P50990-2 TCPQ_HUMAN	Isoform 2 of T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8
GR[de]GITGIEDK (SEQ ID NO: 521)	sp P29401-2 TKT_HUMAN	Isoform 2 of Transketolase OS=Homo sapiens GN=TKT
PHSVSLNDTETR[Dea]K (SEQ ID NO: 522)	sp Q9P0L0-2 VAPA_HUMAN	Isoform 2 of Vesicle-associated membrane protein-associated protein A OS=Homo sapiens GN=VAPA

Peptide	UniProtKB Accessions	Protein Name
MDIRG[Dea]AVDAAVPTNIIAAK (SEQ ID NO: 523)	sp Q9UI12-2 VATH_HUMAN	Isoform 2 of V-type proton ATPase subunit H OS=Homo sapiens GN=ATP6V1H
TDDYGR[Dea]DLSSVQTLLTK (SEQ ID NO: 524)	sp Q13813-3 SPTN1_HUMAN	Isoform 3 of Spectrin alpha chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTAN1
R[Dea]LVPGGGATEIELAK (SEQ ID NO: 525)	sp P50990-3 TCPQ_HUMAN	Isoform 3 of T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8
VR[Dea]LLADPTGAFGK (SEQ ID NO: 265)	sp P30044-4 PRDX5_HUMAN	Isoform 4 of Peroxiredoxin-5, OS=Homo sapiens GN=PRDX5
R[Dea]GFGFVTFDDHDPVDK (SEQ ID NO: 526)	sp P22626-2 ROA2_HUMAN	Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1
IATPR[Dea]GAASPAQK (SEQ ID NO: 527)	sp P10636-7 TAU_HUMAN	Isoform Tau-E of Microtubule-associated protein tau OS=Homo sapiens GN=MAPT
RVVAEPVELAQEFR[Dea]K (SEQ ID NO: 528)	sp O75489 NDUS3_HUMAN	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, OS=Homo sapiens GN=NDUFS3 PE=1 SV=1
GADEAALAR[Dea]AELEK (SEQ ID NO: 529)	sp P07196 NFL_HUMAN	Neurofilament light polypeptide OS=Homo sapiens GN=NEFL PE=1 SV=3
QIVWNGPVGVFWEAFAR[Dea]G TK (SEQ ID NO: 530)	sp P00558 PGK1_HUMAN	Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3
PMQFLGDEETVR[Dea]K (SEQ ID NO: 531)	sp P18669 PGAM1_HUMAN	Phosphoglycerate mutase 1 OS=Homo sapiens GN=PGAM1 PE=1 SV=2
R[Dea]GFGFVYFQSHDAADK (SEQ ID NO: 532)	sp Q4VXU2 PAP1L_HUMAN	Polyadenylate-binding protein 1-like OS=Homo sapiens GN=PABPC1L PE=2 SV=1
VPDFSDYR[Dea]RAEVL DSTK (SEQ ID NO: 533)	sp P0C7P4 UCRIL_HUMAN	Putative cytochrome b-c1 complex subunit Rieske-like protein 1 OS=Homo sapiens GN=UQCRFS1P1 PE=5 SV=1
R[Dea]DVAGDASESALLK (SEQ ID NO: 534)	sp P13637 AT1A3_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-3 OS=Homo sapiens GN=ATP1A3 PE=1 SV=3
DLTSVMR[Dea]LLSK (SEQ ID NO: 535)	sp Q01082 SPTB2_HUMAN	Spectrin beta chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTBN1 PE=1 SV=2
FMELLEPLSER[Dea]K (SEQ ID NO: 536)	sp Q01082 SPTB2_HUMAN	Spectrin beta chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTBN1 PE=1 SV=2
ER[Dea]VEAVNMAEGIIHDTETK (SEQ ID NO: 537)	sp P38646 GRP75_HUMAN	Stress-70 protein, OS=Homo sapiens GN=HSPA9 PE=1 SV=2
HVLSGTLGIPEHTYR[Dea]SR[Dea]VTLSNQPYIK (SEQ ID NO: 538)	sp P04216 THY1_HUMAN	Thy-1 membrane glycoprotein OS=Homo sapiens GN=THY1 PE=1 SV=2
MIIYR[Dea]DLISHDELFSDIYK (SEQ ID NO: 539)	sp P13693 TCTP_HUMAN	Translationally-controlled tumor protein OS=Homo sapiens GN=TPT1 PE=1 SV=1
TGGA VDR[Dea]LTDTSK (SEQ ID NO: 540)	sp Q9BW30 TPPP3_HUMAN	Tubulin polymerization-promoting protein family member 3 OS=Homo sapiens GN=TPPP3 PE=1 SV=1
SSEEAVR[Dea]EVHR[Dea]LIEGRA PVISGVTK (SEQ ID NO: 541)	sp O94811 TPPP_HUMAN	Tubulin polymerization-promoting protein OS=Homo sapiens GN=TPPP PE=1 SV=1
LLIIGTTSR[Dea]K (SEQ ID NO: 542)	sp P46459 NSF_HUMAN	Vesicle-fusing ATPase OS=Homo sapiens GN=NSF PE=1 SV=3
WGDPVTR[Dea]VLDDGELLVQQT	sp P46459 NSF	Vesicle-fusing ATPase OS=Homo sapiens GN=NSF

Peptide	UniProtKB Accessions	Protein Name
K (SEQ ID NO: 543)	F_HUMAN	PE=1 SV=3
ITR[Dea]VEMLEIIEAIYK (SEQ ID NO: 544)	sp P62760 VISL1_HUMAN	Visinin-like protein 1 OS=Homo sapiens GN=VSNL1 PE=1 SV=2
FPR[dea]YAEIVHLTLPDGTK (SEQ ID NO: 545)	sp P21281 VATB2_HUMAN	V-type proton ATPase subunit B, brain isoform OS=Homo sapiens GN=ATP6V1B2 PE=1 SV=3

[0160] Table 6B. Citrullinated peptide detected in acquired brain injury in human brains. These citrullinated peptides are not brain specific

Peptide	UniProtKB Accessions	Name
GDARPAEIDSLWEISK (SEQ ID NO: 546)	sp P49821 NDUV1_HUMAN	NADH dehydrogenase [ubiquinone] flavoprotein 1, OS=Homo sapiens GN=NDUFV1 PE=1 SV=4
SVVLMSHLGRPDGVMPDK (SEQ ID NO: 547)	sp P00558-2 PGK1_HUMAN	Isoform 2 of Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1

[0161] Table 7: Secreted citrullinated human proteins in chronic brain disease

UniProtKB Accessions	Protein Name
P61764	Syntaxin-binding protein 1;
P62805	Histone H4
Q14204	Cytoplasmic dynein 1 heavy chain 1;
Q05193	Dynamin-1;
P09104	Gamma-enolase;
P14625	Endoplasmin precursor;
O43301	Heat shock 70 kDa protein 12A
P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit;
P27797	Calreticulin precursor;
P31146	Coronin-1A;
P38117	Electron transfer flavoprotein subunit beta;
P68871	Hemoglobin subunit beta;
P28482	Mitogen-activated protein kinase 1;
P27361	Mitogen-activated protein kinase 3;
O00232	26S proteasome non-ATPase regulatory subunit 12;
P36578	60S ribosomal protein L4;
P05387	60S acidic ribosomal protein P2;
Q16181	Septin-7;
P49588	Alanine--tRNA ligase, cytoplasmic;

[0162] Table 8: Non-secreted citrullinated human proteins in chronic brain disease

UniProtKB Accessions	Protein Name
P17600	Synapsin-1;

Q99798	Aconitate hydratase,
O14810	Complexin-1;
Q6PUV4	Complexin-2;
Q9Y2J2	Band 4.1-like protein 3;
Q92777	Synapsin-2;
Q2M2I8	AP2-associated protein kinase 1;
P35611	Alpha-adducin;
P63010	AP-2 complex subunit beta;
O15075	Serine/threonine-protein kinase DCLK1;
O94925	Glutaminase kidney isoform,
P50213	Isocitrate dehydrogenase [NAD] subunit alpha
Q08209	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform;

[0163] Table 9: Secreted citrullinated human proteins in acute brain disease

UniProtKB Accessions	Protein Name
Q86VP6	Cullin-associated NEDD8-dissociated protein 1;
P30044	Peroxiredoxin-5,

[0164] Table 10: Non-secreted citrullinated human proteins in acute brain disease

UniProtKB Accessions	Protein Name
P63215	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-3 precursor

References for Example 4

1. Ren J, Jiang C, Gao X, Liu Z, Yuan Z, Jin C, et al. PhosSNP for systematic analysis of genetic polymorphisms that influence protein phosphorylation. *Mol Cell Proteomics* 2010, 9:623-634.
2. Ryu GM, Song P, Kim KW, Oh KS, Park KJ, Kim JH. Genome-wide analysis to predict protein sequence variations that change phosphorylation sites or their corresponding kinases. *Nucleic Acids Res* 2009, 37:1297-1307.
3. Baka Z, Gyorgy B, Geher P, Buzas EI, Falus A, Nagy G. Citrullination under physiological and pathological conditions. *Joint Bone Spine* 2012, 79:431-436.
4. Demoruelle MK, Deane K. Antibodies to citrullinated protein antigens (ACPAs): clinical and pathophysiologic significance. *Curr Rheumatol Rep* 2011, 13:421-430.
5. Uysal H, Nandakumar KS, Kessel C, Haag S, Carlsen S, Burkhardt H, et al. Antibodies to citrullinated proteins: molecular interactions and arthritogenicity. *Immunol Rev* 2010, 233:9-33.

6. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature* 2014, 506:376-381.
7. De Ceuleneer M, Van Steendam K, Dhaenens M, Deforce D. In vivo relevance of citrullinated proteins and the challenges in their detection. *Proteomics* 2012, 12:752-760.
8. Raijmakers R, van Beers JJ, El-Azzouny M, Visser NF, Bozic B, Pruijn GJ, et al. Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res Ther* 2012, 14:R114.
9. Stahl DC, Swiderek KM, Davis MT, Lee TD. Data-controlled automation of liquid chromatography/tandem mass spectrometry analysis of peptide mixtures. *J Am Soc Mass Spectrom* 1996, 7:532-540.
10. Yates JR, 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem* 1995, 67:1426-1436.
11. Gillet LC1 NP TS, Röst H, Selevsek N, Reiter L, Bonner R, Aebersold R. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics* 2012.
12. Liu Y, Huttenhain R, Collins B, Aebersold R. Mass spectrometric protein maps for biomarker discovery and clinical research. *Expert Rev Mol Diagn* 2013, 13:811-825.
13. Escher C, Reiter L, MacLean B, Ossola R, Herzog F, Chilton J, et al. Using iRT, a normalized retention time for more targeted measurement of peptides. *Proteomics* 2012, 12:1111-1121.
14. Crowgey EL, Stabley DL, Chen C, Huang H, Robbins KM, Polson SW, et al. An integrated approach for analyzing clinical genomic variant data from next-generation sequencing. *J Biomol Tech* 2015, 26:19-28.
15. Hensen SM, Boelens WC, Bongers KM, van Cruchten RT, van Delft FL, Pruijn GJ. Phenylglyoxal-based visualization of citrullinated proteins on Western blots. *Molecules* 2015, 20:6592-6600.
16. Hensen SM, Pruijn GJ. Methods for the detection of peptidylarginine deiminase (PAD) activity and protein citrullination. *Mol Cell Proteomics* 2014, 13:388-396.
17. Stensland M, Holm A, Kiehne A, Fleckenstein B. Targeted analysis of protein citrullination using chemical modification and tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2009, 23:2754-2762.

[0165] The various methods and techniques described above provide a number of ways to carry out the application. Of course, it is to be understood that not necessarily all objectives or advantages described can be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as taught or suggested herein. A variety of alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several features, while others specifically exclude one, another, or several features, while still others mitigate a particular feature by inclusion of one, another, or several advantageous features.

[0166] Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be employed in various combinations by one of ordinary skill in this art to perform methods in accordance with the principles described herein. Among the various elements, features, and steps some will be specifically included and others specifically excluded in diverse embodiments.

[0167] Although the application has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the application extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

[0168] Preferred embodiments of this application are described herein, including the best mode known to the inventors for carrying out the application. Variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the application can be practiced otherwise than specifically described herein. Accordingly, many embodiments of this application include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the application unless otherwise indicated herein or otherwise clearly contradicted by context.

[0169] All patents, patent applications, publications of patent applications, and other material, such as articles, books, specifications, publications, documents, things, and/or the like, referenced herein are hereby incorporated herein by this reference in their entirety for all purposes, excepting any prosecution file history associated with same, any of same that is inconsistent with or in conflict with the present document, or any of same that may have a limiting affect as to the broadest scope of the claims now or later associated with the present document. By way of example, should there be any inconsistency or conflict between the description, definition, and/or the use of a term associated with any of the incorporated material and that associated with the present document, the description, definition, and/or the use of the term in the present document shall prevail.

[0170] It is to be understood that the embodiments of the application disclosed herein are illustrative of the principles of the embodiments of the application. Other modifications that can be employed can be within the scope of the application. Thus, by way of example, but not of limitation, alternative configurations of the embodiments of the application can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present application are not limited to that precisely as shown and described.

[0171] Various embodiments of the invention are described above in the Detailed Description. While these descriptions directly describe the above embodiments, it is understood that those skilled in the art may conceive modifications and/or variations to the specific embodiments shown and described herein. Any such modifications or variations that fall within the purview of this description are intended to be included therein as well. Unless specifically noted, it is the intention of the inventors that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s).

[0172] The foregoing description of various embodiments of the invention known to the applicant at this time of filing the application has been presented and is intended for the purposes of illustration and description. The present description is not intended to be exhaustive nor limit the invention to the precise form disclosed and many modifications and variations are possible in the light of the above teachings. The embodiments described serve to explain the principles of the invention and its practical application and to enable others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. Therefore, it is intended that

the invention not be limited to the particular embodiments disclosed for carrying out the invention.

[0173] While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this invention.

What is claimed is:

1. A method for diagnosing a disease-state in a subject in need thereof, comprising:
obtaining a biological sample from the subject; and
detecting the amount of a citrullinated protein or a citrullinated peptide in the biological sample obtained from said subject;
wherein a change in the level of the citrullinated protein or the citrullinated peptide relative to a reference value is indicative of increased risk of the disease-state in the subject.
2. The method of claim 1, wherein the citrullinated protein or the citrullinated peptide is increased relative to the reference value.
3. The method of claim 1, wherein the disease-state is any one or more of chronic neurodegenerative disease, acquired brain injury, cardiovascular disease, or a combination thereof.
4. The method of claim 3, wherein the cardiovascular disease is ischemic heart disease or idiopathic cardiomyopathy.
5. The method of claim 3, wherein the chronic neurodegenerative disease is Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD) and PD-related disorders, Prion disease, Motor neurone diseases (MND), Huntington's Disease (HD), Spinocerebellar ataxia (SCA), Spinal muscular atrophy (SMA) or combinations thereof.
6. The method of claim 3, wherein the acquired brain injury is any one or more of traumatic brain injuries (TBI's), strokes, brain illness, tumor, hemorrhage, or combinations thereof.
7. The method of claim 1, wherein the citrullinated protein or the citrullinated peptide is secreted.
8. The method of claim 1, wherein the citrullinated protein or the citrullinated peptide is not secreted.
9. The method of claim 1, wherein the disease-state is cardiovascular disease and the citrullinated peptide is, or is at least 80% identical to, any one or more of NIDALSGMEGRK (SEQ ID NO: 18), PRSFMPNLVPPK (SEQ ID NO: 20),

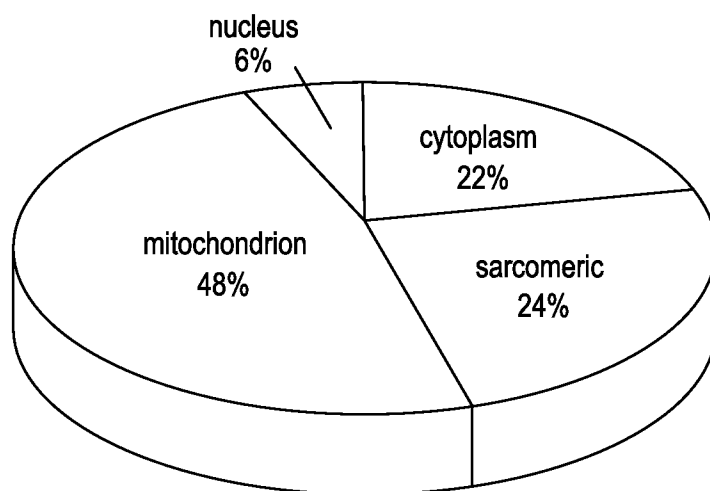
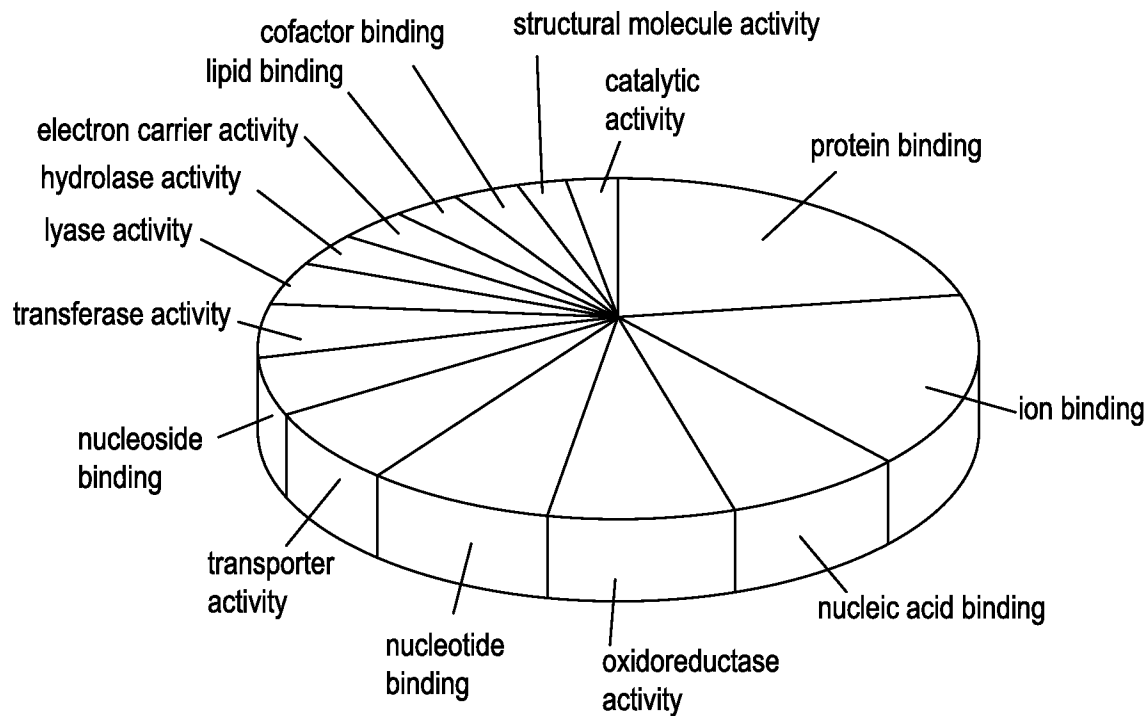
ESLDLRAHLK (SEQ ID NO: 19), AEETQRSVNDLTSQRAK (SEQ ID NO: 24), FADLSEAANRNNDALRQAK (SEQ ID NO: 22) or combinations thereof.

10. The method of claim 1, wherein the disease-state is ALS and the citrullinated peptide is, or is at least 80% identical to, any one or more of PSGVQMDPCCRALYDFEPENEGELGFK (SEQ ID NO: 306), QYMRRSTCTINYSK (SEQ ID NO: 307), LILIARNK (SEQ ID NO: 308), VIVVWVGTTNNHENTAEVAGGIEAIVQLINTRQPQAK (SEQ ID NO: 309), EVDVGLAADVGTQLRLPK (SEQ ID NO: 310), HEEAPGHRPTTNPASK (SEQ ID NO: 311), GETPVNSTMSIGQARK (SEQ ID NO: 312), VRIQTQPGYANTLRDAAPK (SEQ ID NO: 313), NVGCLQEALQLATSFAQLRLGDVK (SEQ ID NO: 314) or combinations thereof.
11. The method of claim 5, wherein the disease-state is chronic neurodegenerative disease and the citrullinated peptide is, or is 80% identical to, any one or more of the peptides having the sequence set forth in SEQ ID NO.: 355, SEQ ID NO.: 402, SEQ ID NO.: 1, SEQ ID NO.: 348, SEQ ID NO.: 363, SEQ ID NO.: 428, SEQ ID NO.: 373, SEQ ID NO.: 315, SEQ ID NO.: 316, SEQ ID NO.: 340, SEQ ID NO.: 377, SEQ ID NO.: 331, SEQ ID NO.: 332, SEQ ID NO.: 325, SEQ ID NO.: 375, SEQ ID NO.: 429, SEQ ID NO.: 414, SEQ ID NO.: 313, SEQ ID NO.: 366, SEQ ID NO.: 360, SEQ ID NO.: 423, SEQ ID NO.: 405, SEQ ID NO.: 439, SEQ ID NO.: 321, SEQ ID NO.: 475, SEQ ID NO.: 476, SEQ ID NO.: 477, SEQ ID NO.: 478, SEQ ID NO.: 479, SEQ ID NO.: 480, SEQ ID NO.: 481, SEQ ID NO.: 482, SEQ ID NO.: 483, SEQ ID NO.: 484, SEQ ID NO.: 485, SEQ ID NO.: 486, SEQ ID NO.: 487, SEQ ID NO.: 488, SEQ ID NO.: 489, SEQ ID NO.: 490, SEQ ID NO.: 491, SEQ ID NO.: 492, SEQ ID NO.: 493, SEQ ID NO.: 494, SEQ ID NO.: 495, SEQ ID NO.: 496, SEQ ID NO.: 497, SEQ ID NO.: 498, SEQ ID NO.: 499, SEQ ID NO.: 500, SEQ ID NO.: 501, SEQ ID NO.: 502, SEQ ID NO.: 503, SEQ ID NO.: 504 or combinations thereof.
12. The method of claim 5, wherein the disease-state is acquired brain injury and the citrullinated peptide is, or is 80% identical to, any one or more of the peptides having the sequence set forth in SEQ ID NO.: 125, SEQ ID NO.: 265, SEQ ID NO.: 285, SEQ ID NO.: 505, SEQ ID NO.: 506, SEQ ID NO.: 507, SEQ ID NO.: 508, SEQ ID NO.: 509, SEQ ID NO.: 510, SEQ ID NO.: 511, SEQ ID NO.: 512, SEQ ID NO.: 513, SEQ ID NO.: 514, SEQ ID NO.: 515, SEQ ID NO.: 516, SEQ ID NO.: 517, SEQ ID NO.: 518,

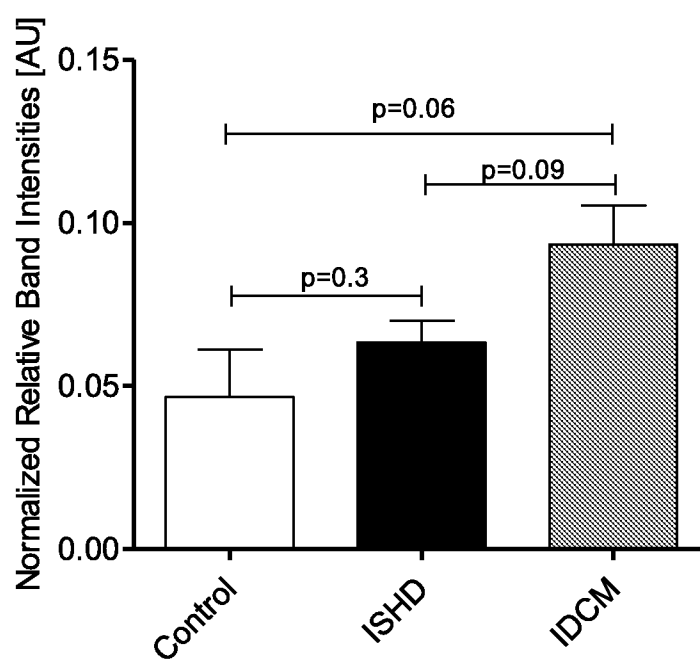
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13. The method of claim 1, wherein the biological sample is any one or more of blood, plasma, serum, tissue biopsy or a combination thereof.
14. The method of claim 1, wherein the level of the citrullinated protein or citrullinated peptide is detected using mass spectrometry, high resolution mass spectrometry, tandem mass spectrometry, binding assay, immunoassay, antibody binding or immunohistochemistry.
15. The method of claim 3, further comprising prescribing treatment to the subject diagnosed any one or more of cardiovascular disease, chronic neurodegenerative disease, acquired brain injury or a combination thereof.
16. The method of claim 12, the treatment comprises any one or more of any one or more of therapeutic lifestyle changes, therapeutic agents, surgical treatments or a combination thereof.
17. The method of claim 3, wherein the presence of the citrullinated protein or the citrullinated peptide is indicative of the generation of autoantibodies during the pathogenesis of the chronic neurodegenerative disease, acquired brain injury or a combination thereof.

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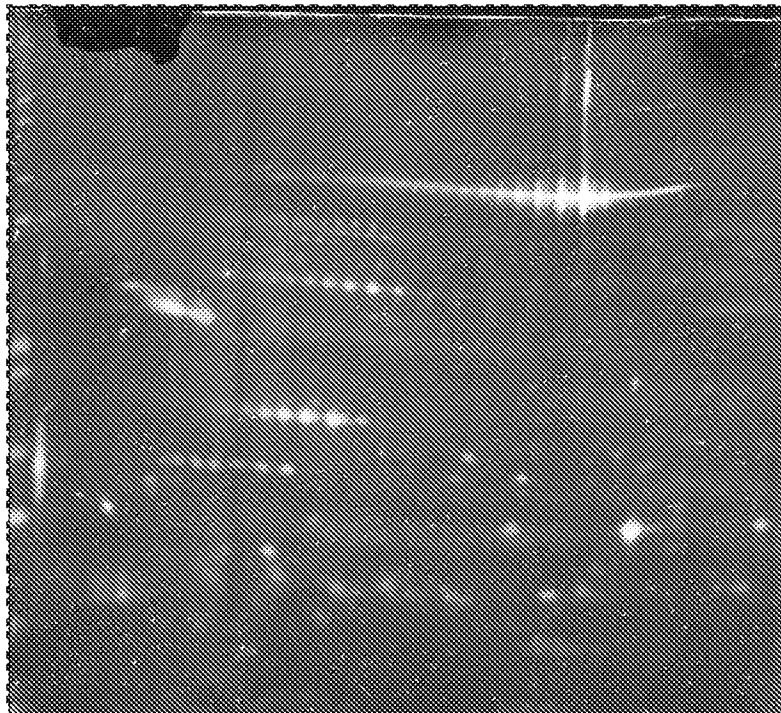
FIG. 1A**FIG. 1B**

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FIG. 1C

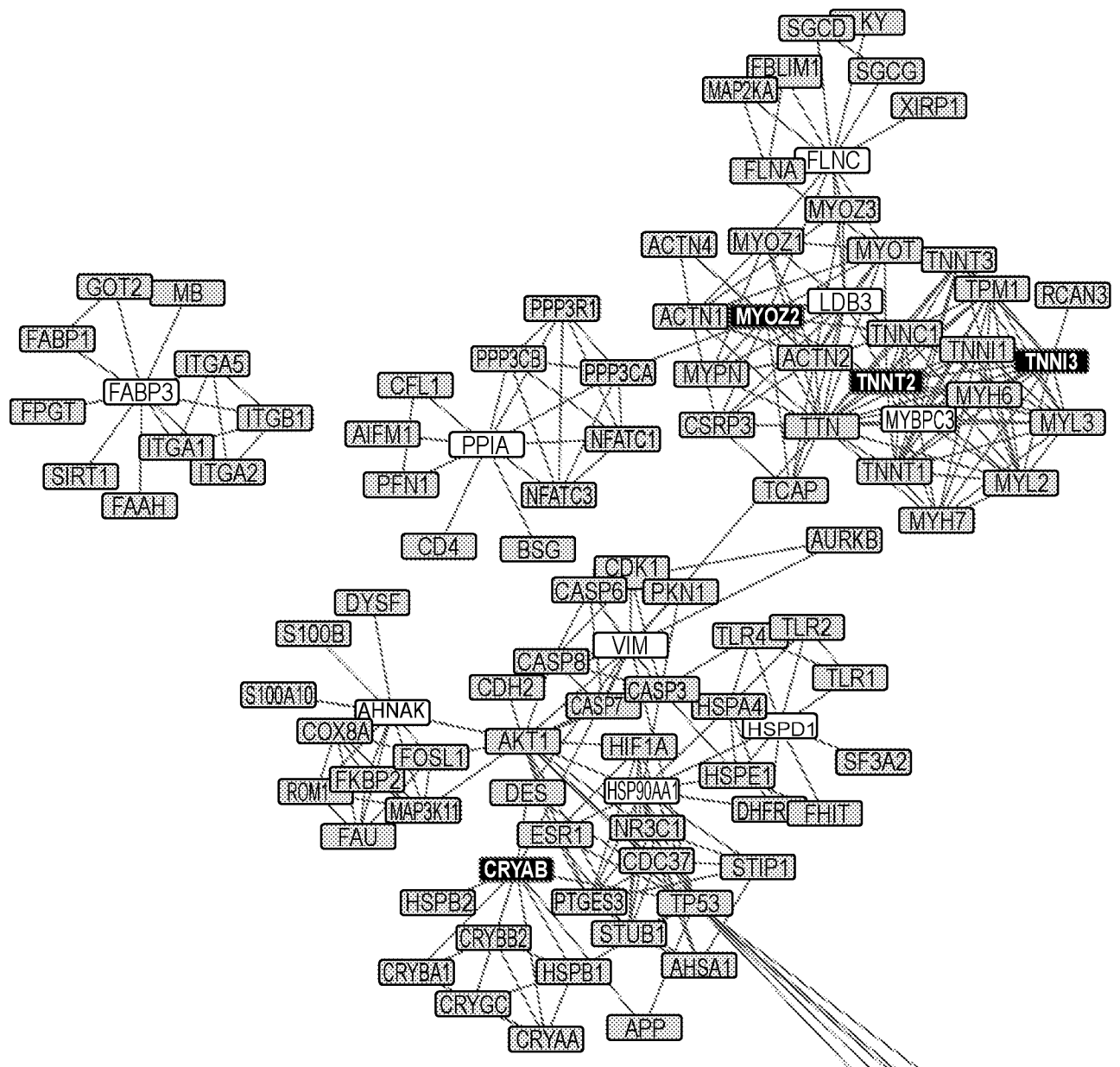
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FIG. 2A



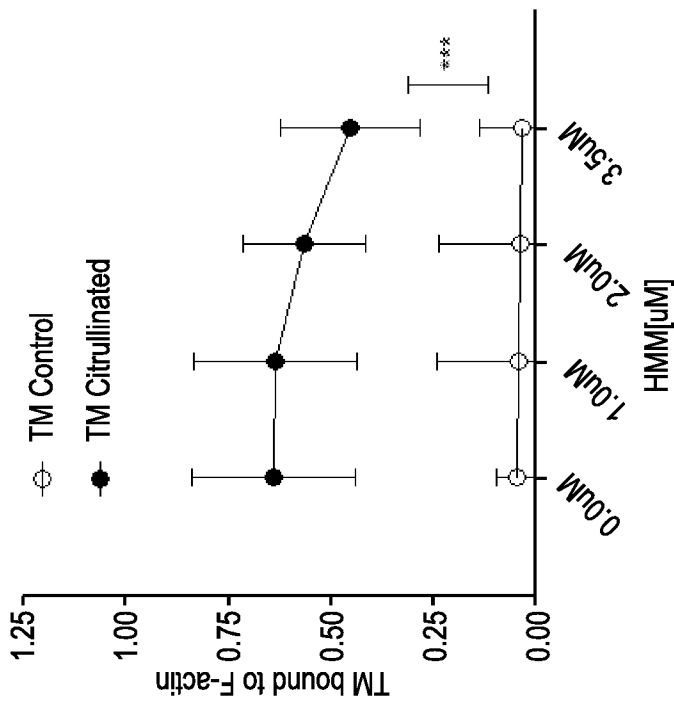
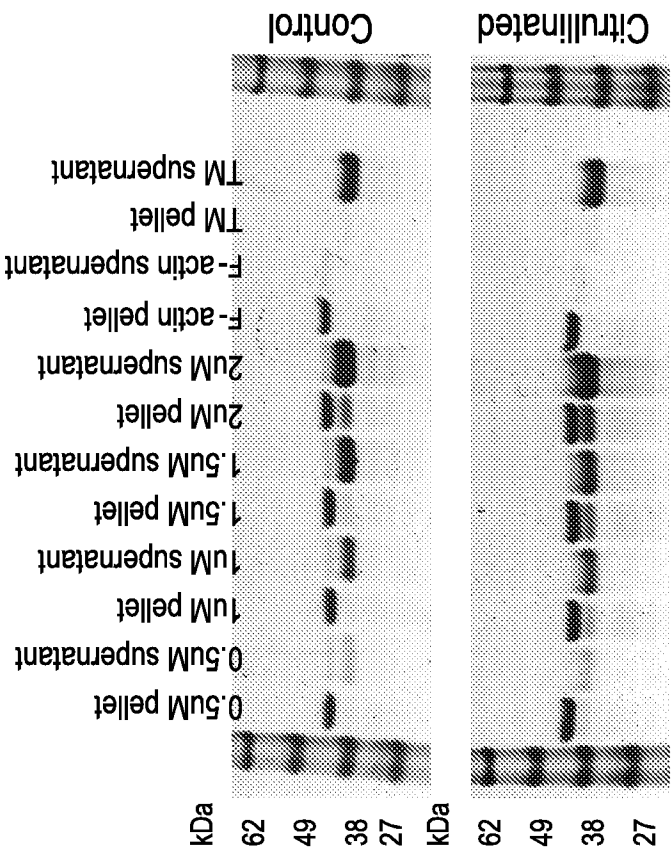
Gel view	MS/MS ID*	Accession Number	Coverage %	Observed pI/MW	Theoretical pI/MW	P<0.05
	Fatty acid binding protein heart	P05413	63.2	6.0/ 14.500	6.29/ 14.858	0.20*
	L-lactate dehydrogenase B chain	P07195	35.9	5.8/ 36.00	5.71/ 36.638	0.13*
	Vinculin	P18206	4.5	6.2/ 170.00	5.83/ 123.799	0.19*
	Actin, alpha cardiac	P62736	30.1	5.5/ 44.00	5.24/ 42.009	0.020
	Tropomyosin	P09493	6.2	4.6/ 38.000	4.69/ 32.709	0.0020
	Myosin light chain	P08590	32	5.0/ 24.000	5.03/ 21.932	0.021
	Myosin regulatory light chain 2	P10916	39.8	4.9/ 18.000	4.92/ 18.789	0.0018

* Not statistical significant



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FIG. 3A



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FIG. 3B

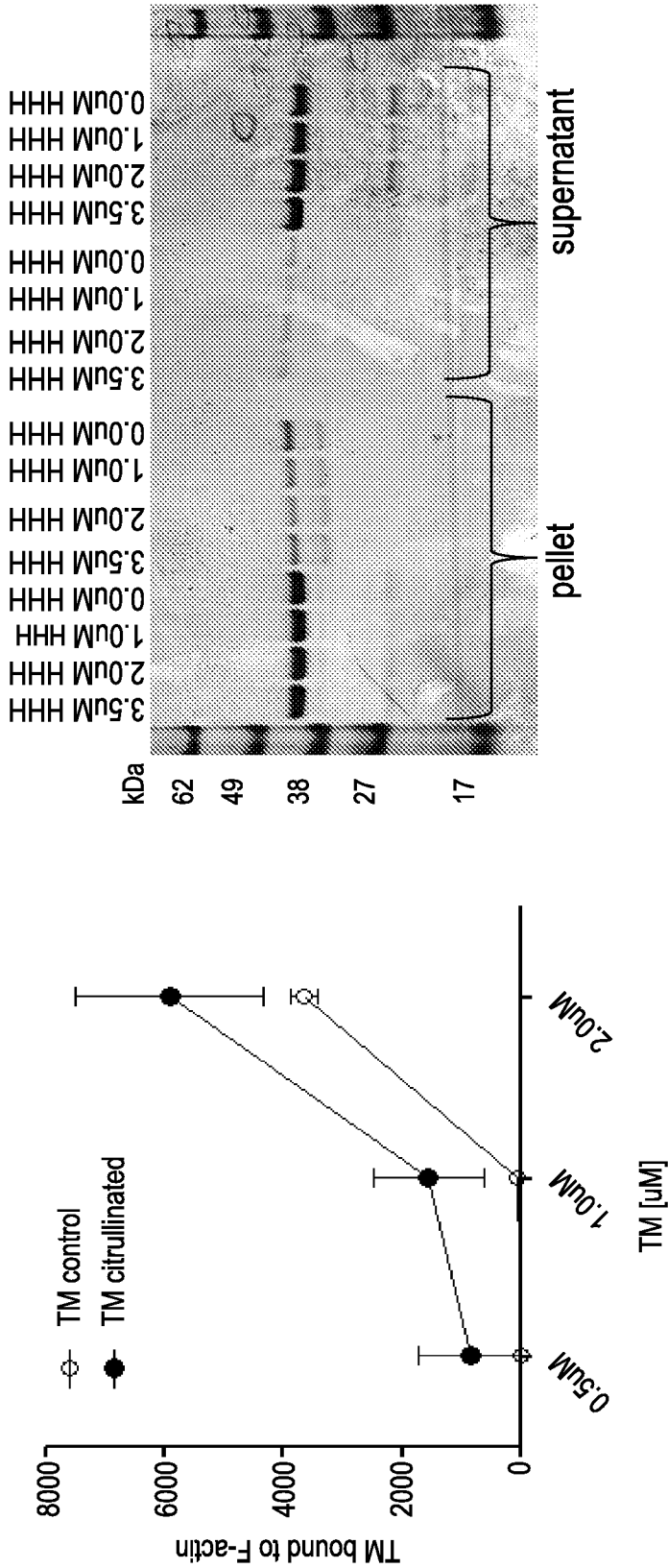


FIG. 3C

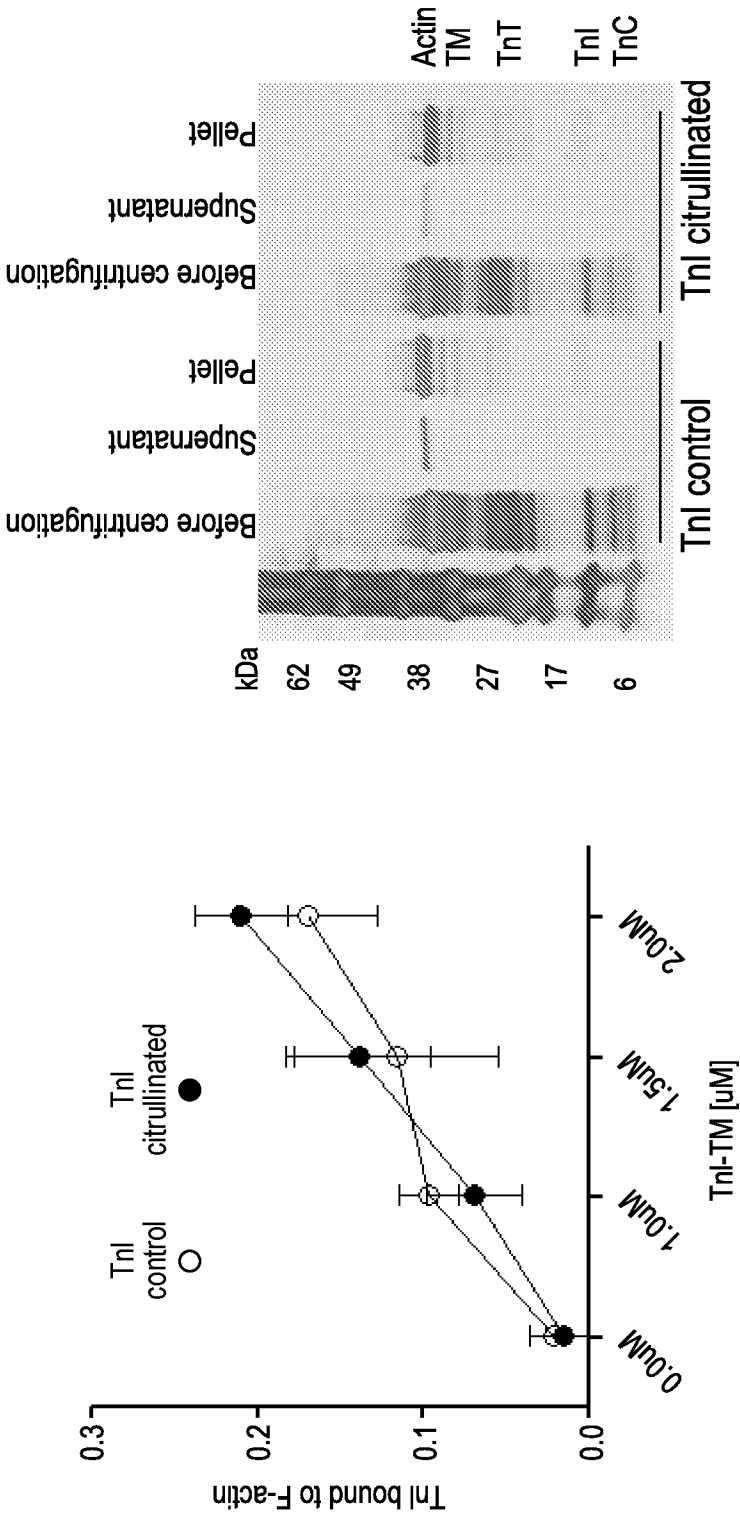


FIG. 4B

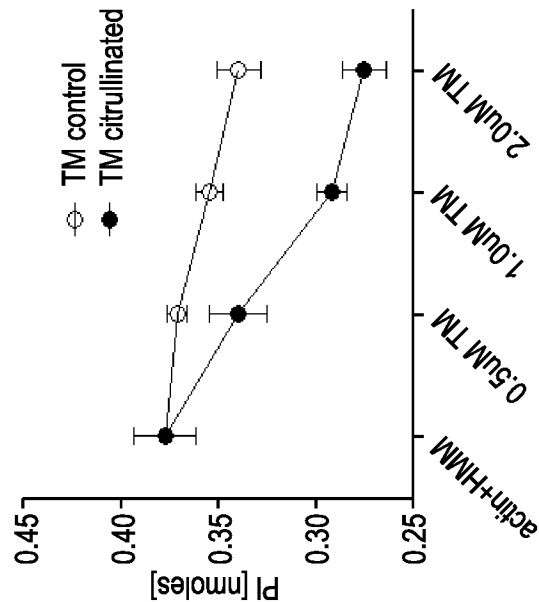


FIG. 4A

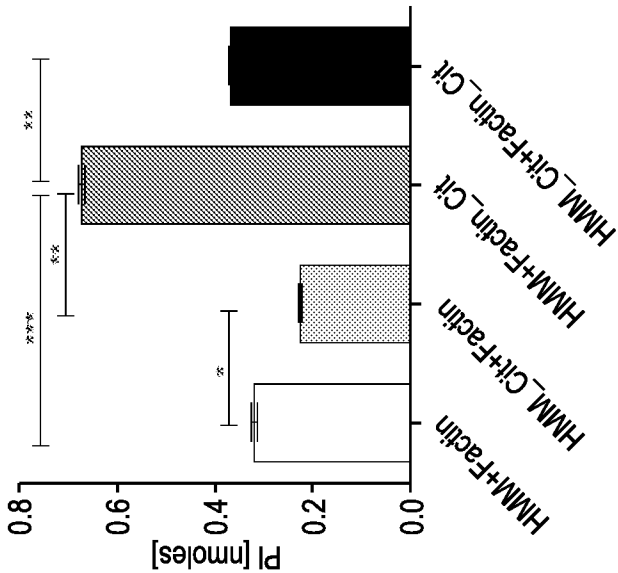
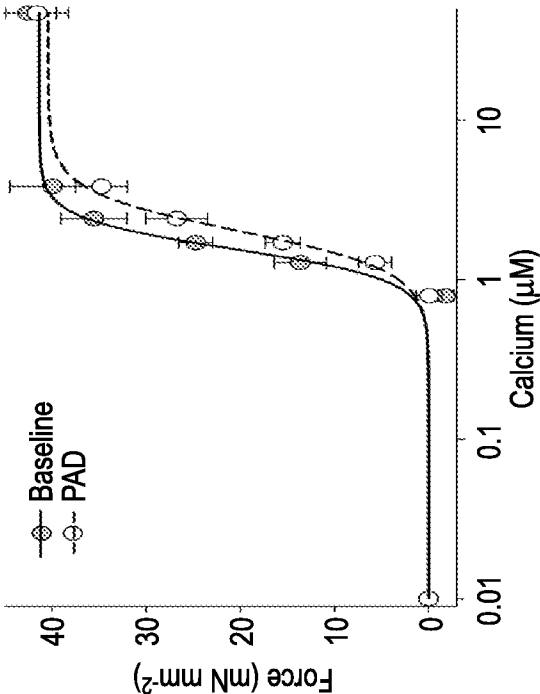


FIG. 4C



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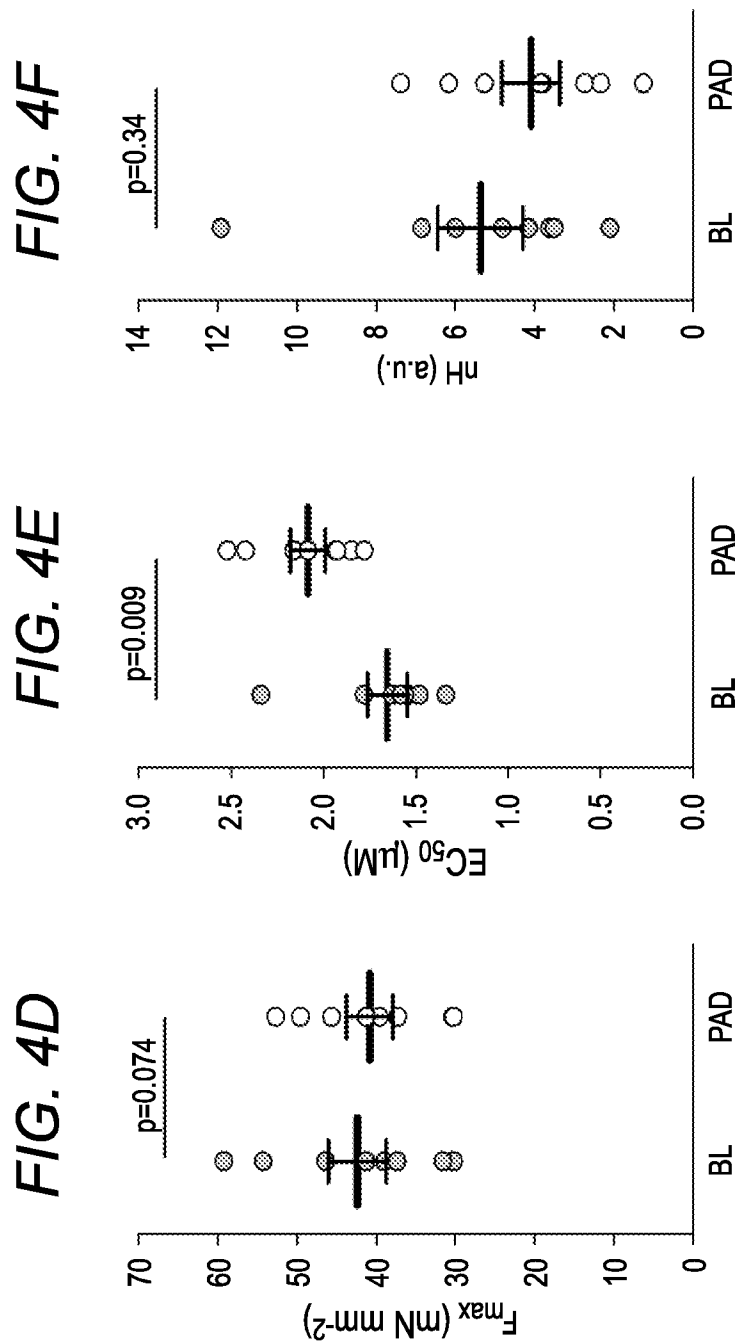


FIG. 5

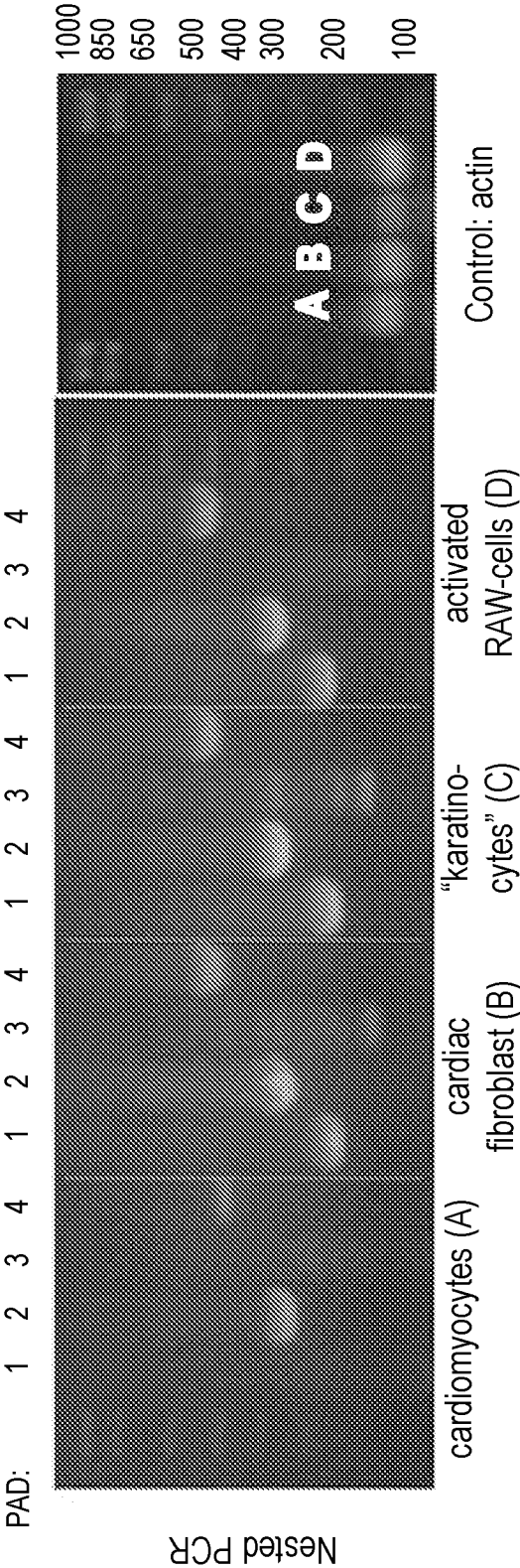
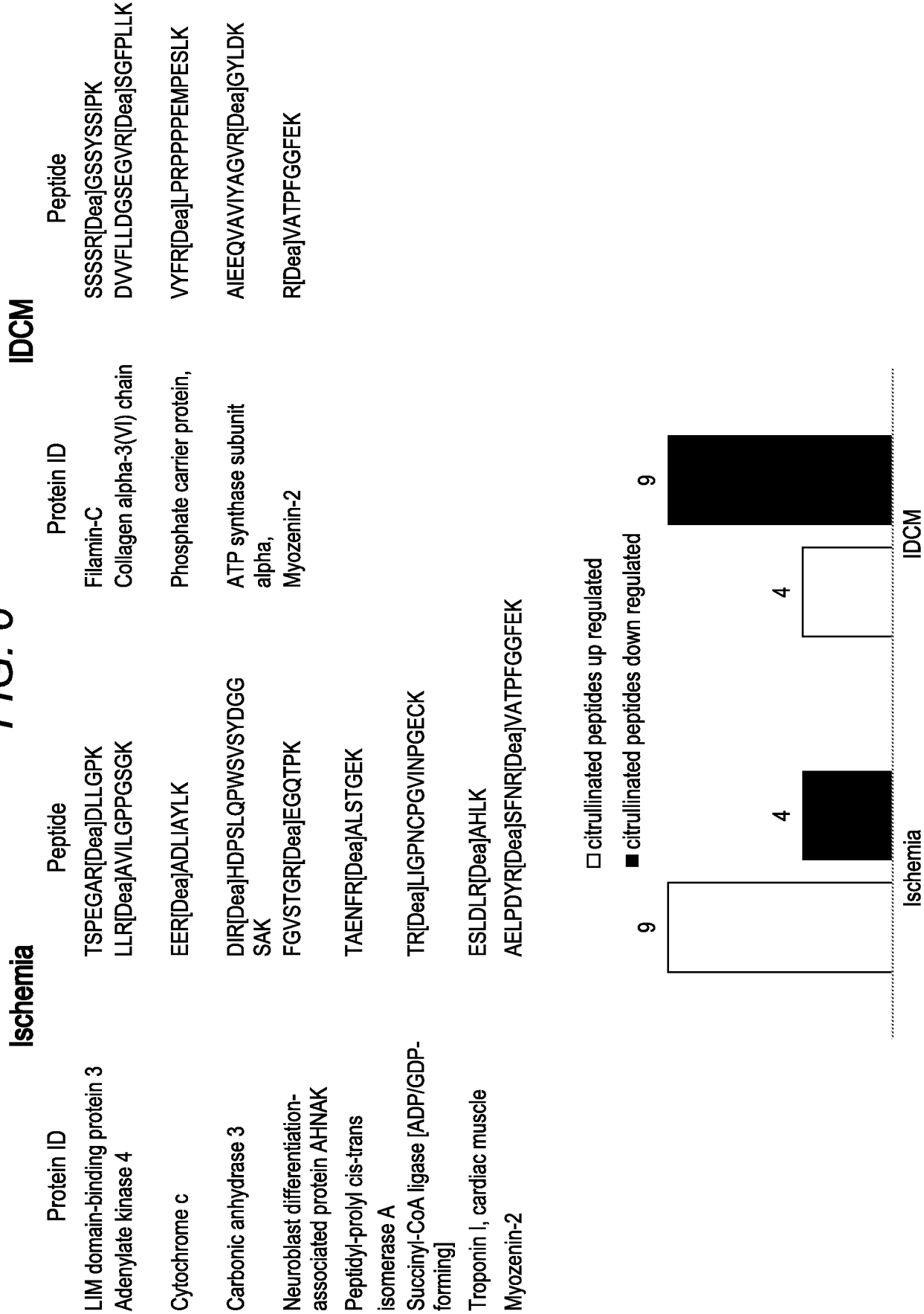


FIG. 6



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FIG. 7

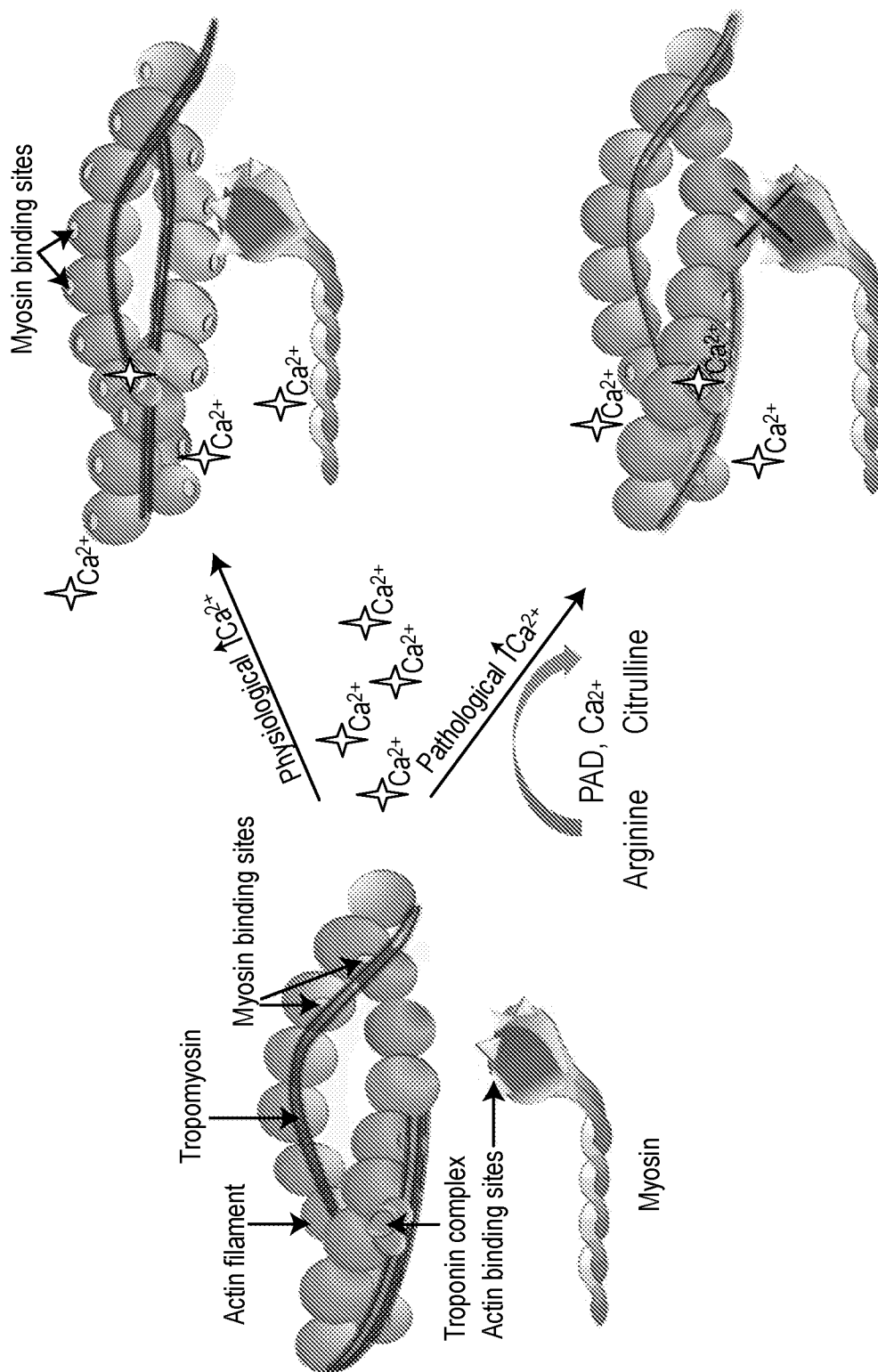
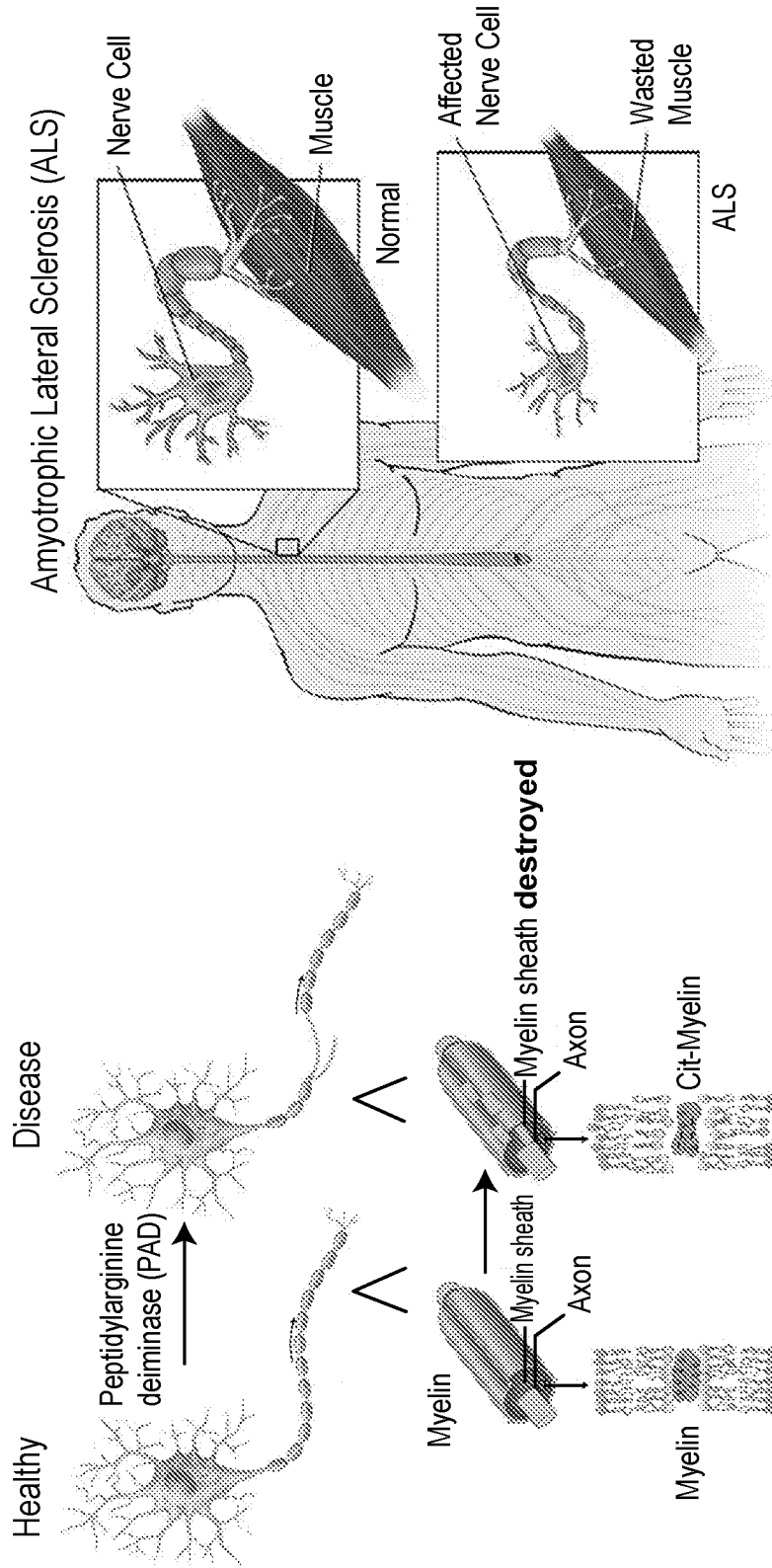


FIG. 8A



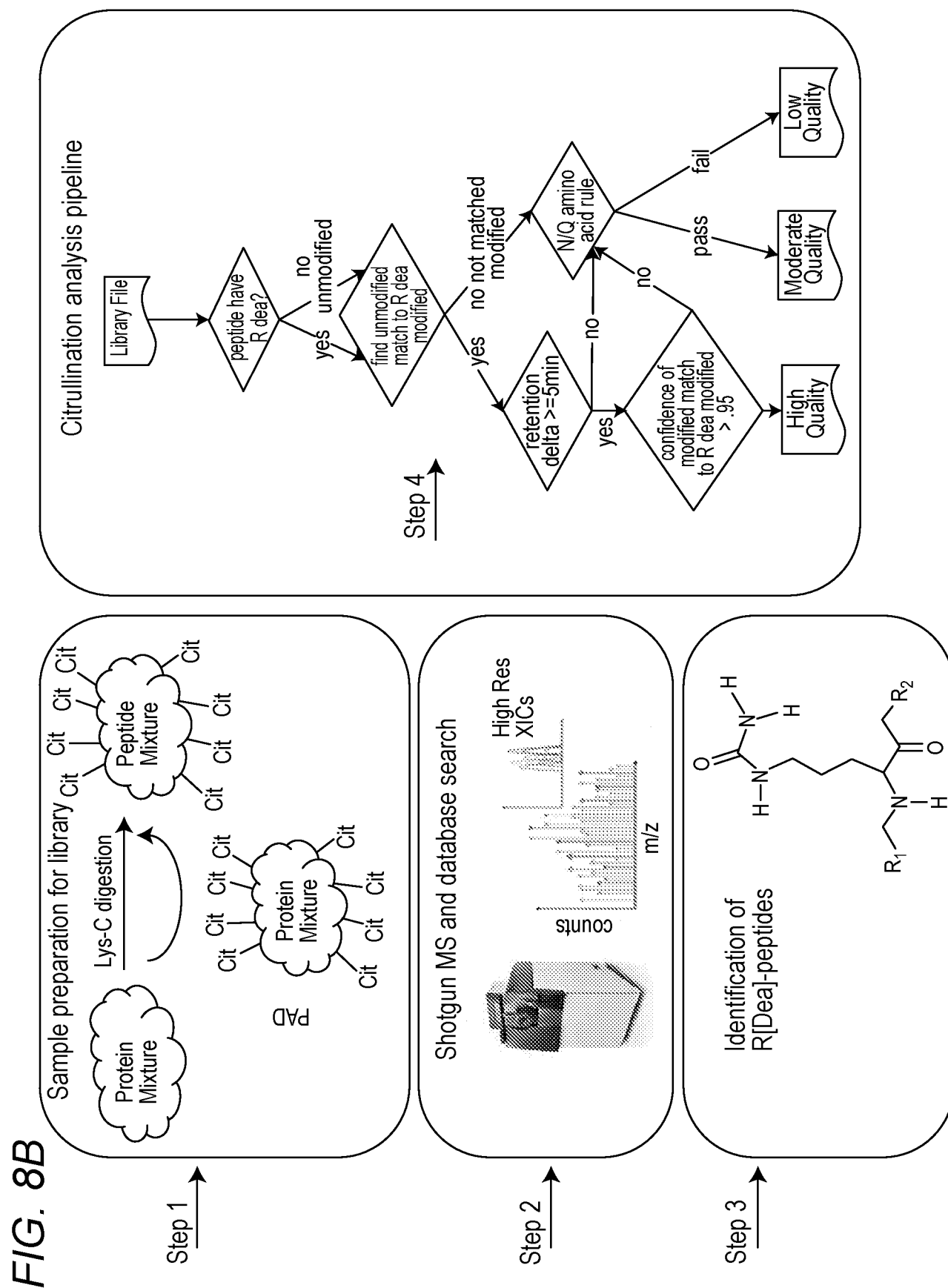


FIG. 8C

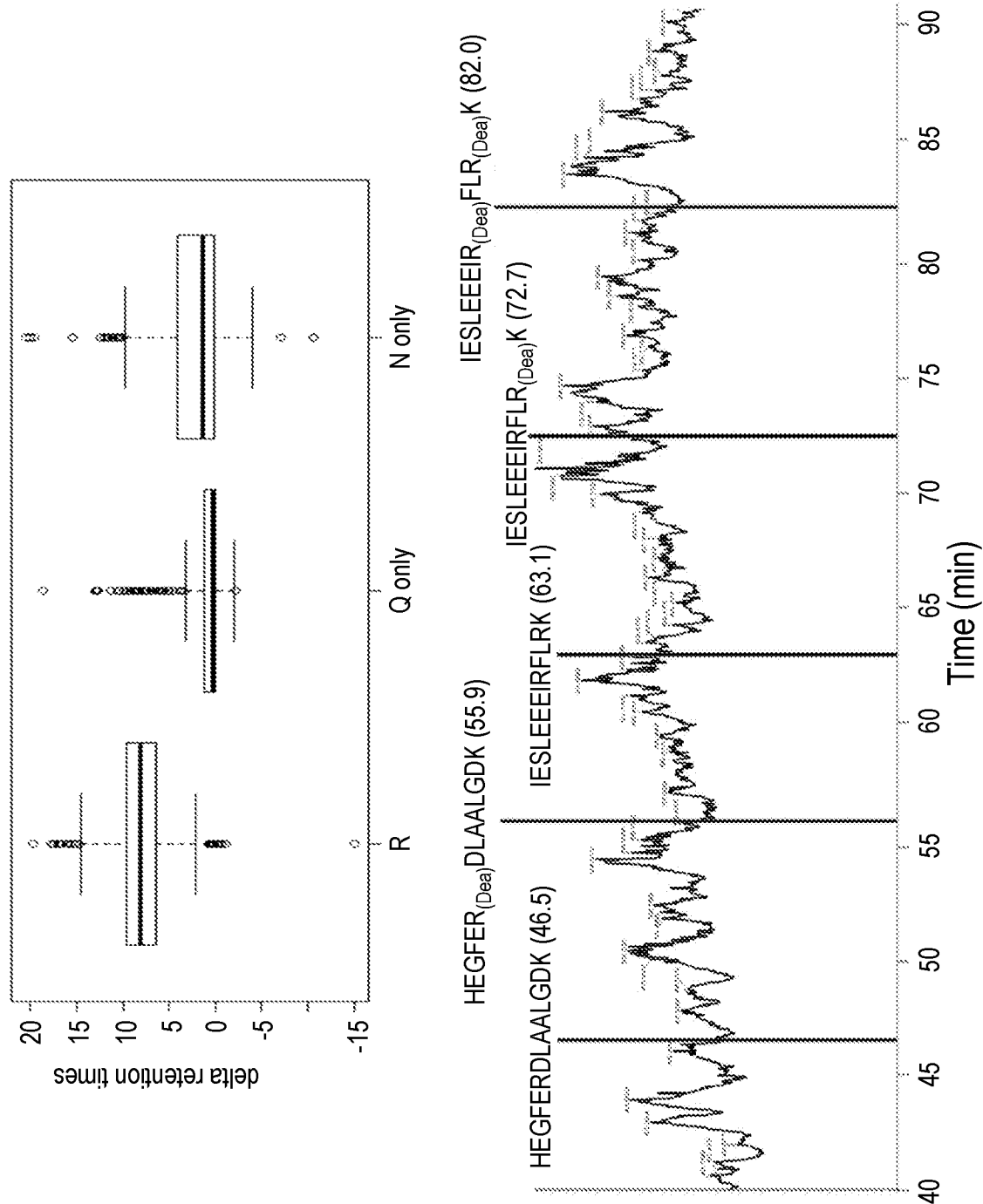


FIG. 8D

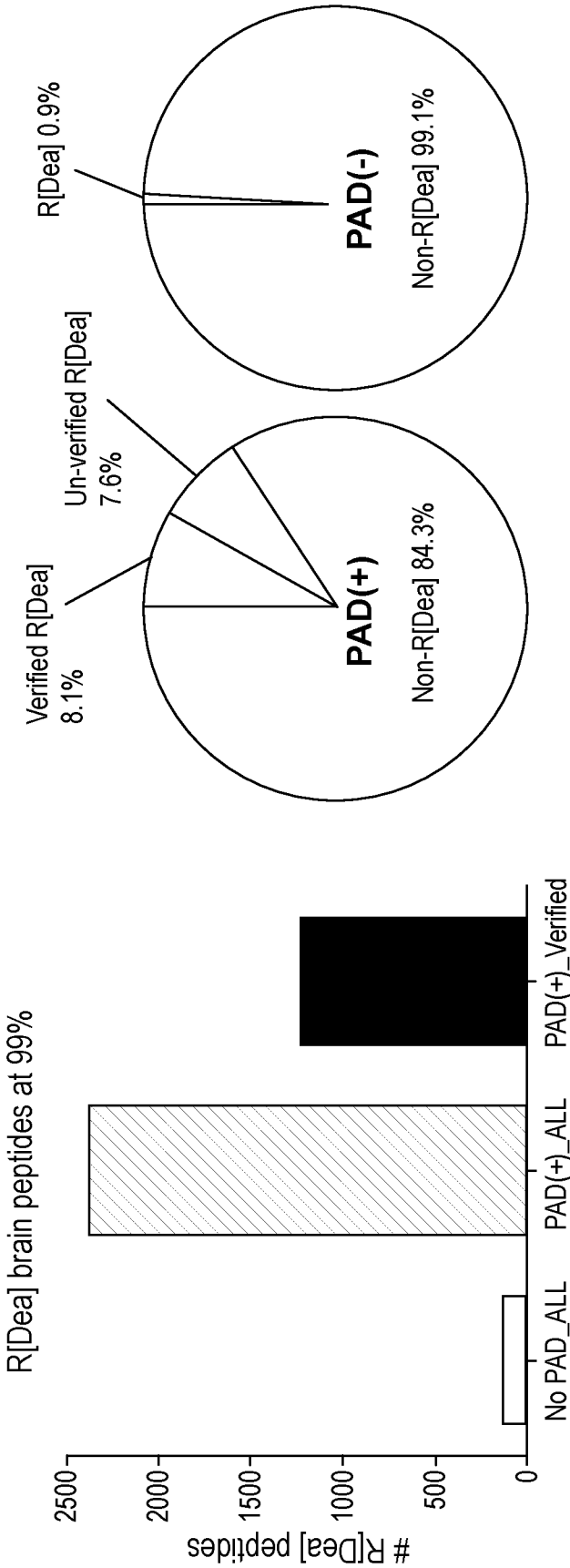
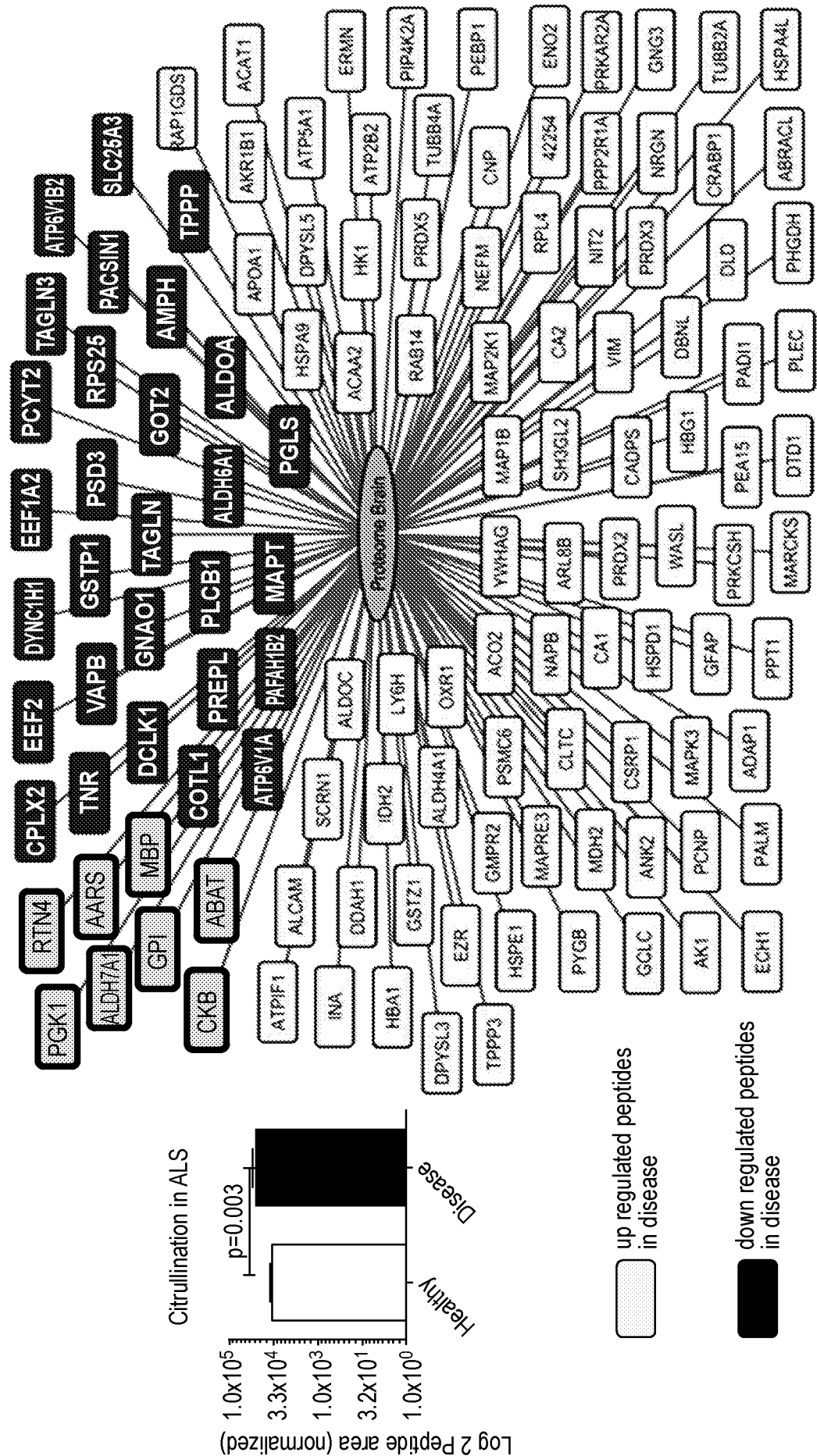


FIG. 8E

Citrullination: A Target for ALS and other neurodegenerative disease



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FIG. 8F

Protein of interest

Protein name	Protein function
Glial fibrillary acidic protein	Class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells.
Glucose-6-phosphate isomerase	GPI is a neurotrophic factor (Neuroleukin) for spinal and sensory neurons.
Vimentin	Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally.
Endophilin-A1	Implicated in synaptic vesicle endocytosis. May recruit other proteins to membranes with high curvature.
Myelin basic protein	Role in formation and stabilization of myelin membrane in the CNS.
Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	Irreversible conversion of delta-1-pyrroline-5-carboxylate(P5C), derived either from proline or ornithine, to glutamate. This is a necessary step in the pathway interconnecting the urea and tricarboxylic acid cycles.
Peroxisredoxin-5, mitochondrial	Reduces hydrogen peroxide and alkyl hydroperoxides with reducing equivalents provided through the thioredoxin system. Involved in intracellular redox signaling.
Neurogranin	Acts as a "third messenger" substrate of protein kinase C-mediated molecular cascades during synaptic development and remodeling. Binds to calmodulin in the absence of calcium.
Dual specificity mitogen-activated protein kinase kinase 1	Dual specificity protein kinase which acts as an essential component of the MAP kinase signal transduction pathway
Tubulin polymerization-promoting protein family member 3	Binds tubulin and has microtubule bundling activity. May play a role in cell proliferation and mitosis
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	This protein is involved in the pathway fatty acid beta-oxidation, which is part of Lipid metabolism.
Cysteine and glycine-rich protein 1	Could play a role in neuronal development.
Cellular retinoic acid-binding protein 1	Cytosolic CRABPs may regulate the access of retinoic acid to the nuclear retinoic acid receptors.
GMP reductase 2	Catalyzes the irreversible NADPH-dependent deamination of GMP to IMP.
Guanine nucleotide-binding protein G(1)/G(S)/G(O) subunit gamma-3	Guanine nucleotide-binding proteins (G proteins) are involved as a modulator or transducer in various transmembrane signaling systems.

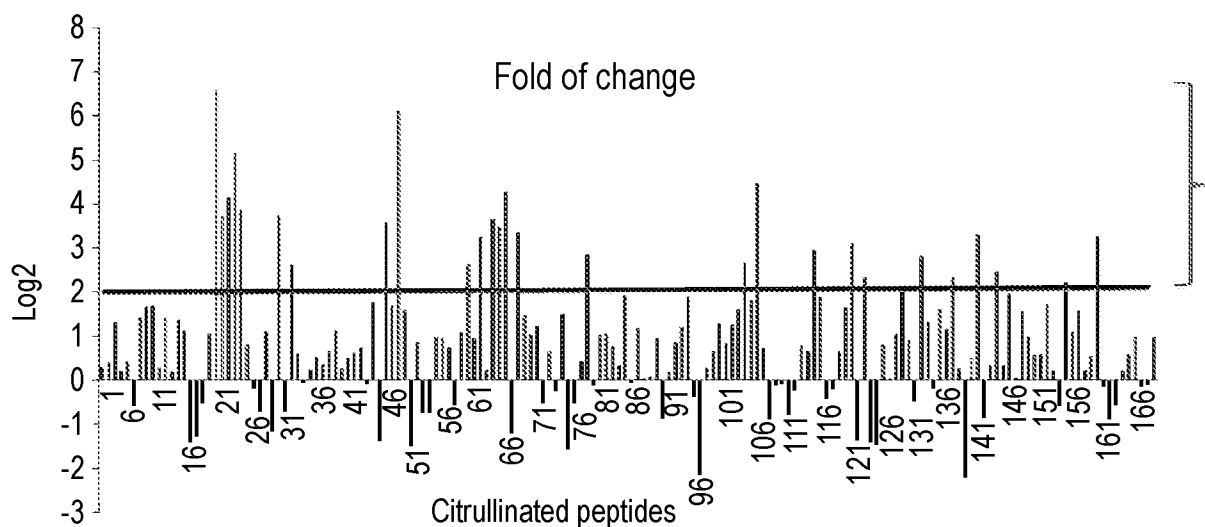


FIG. 8G

Disassembly of intermediate filaments by citrullination

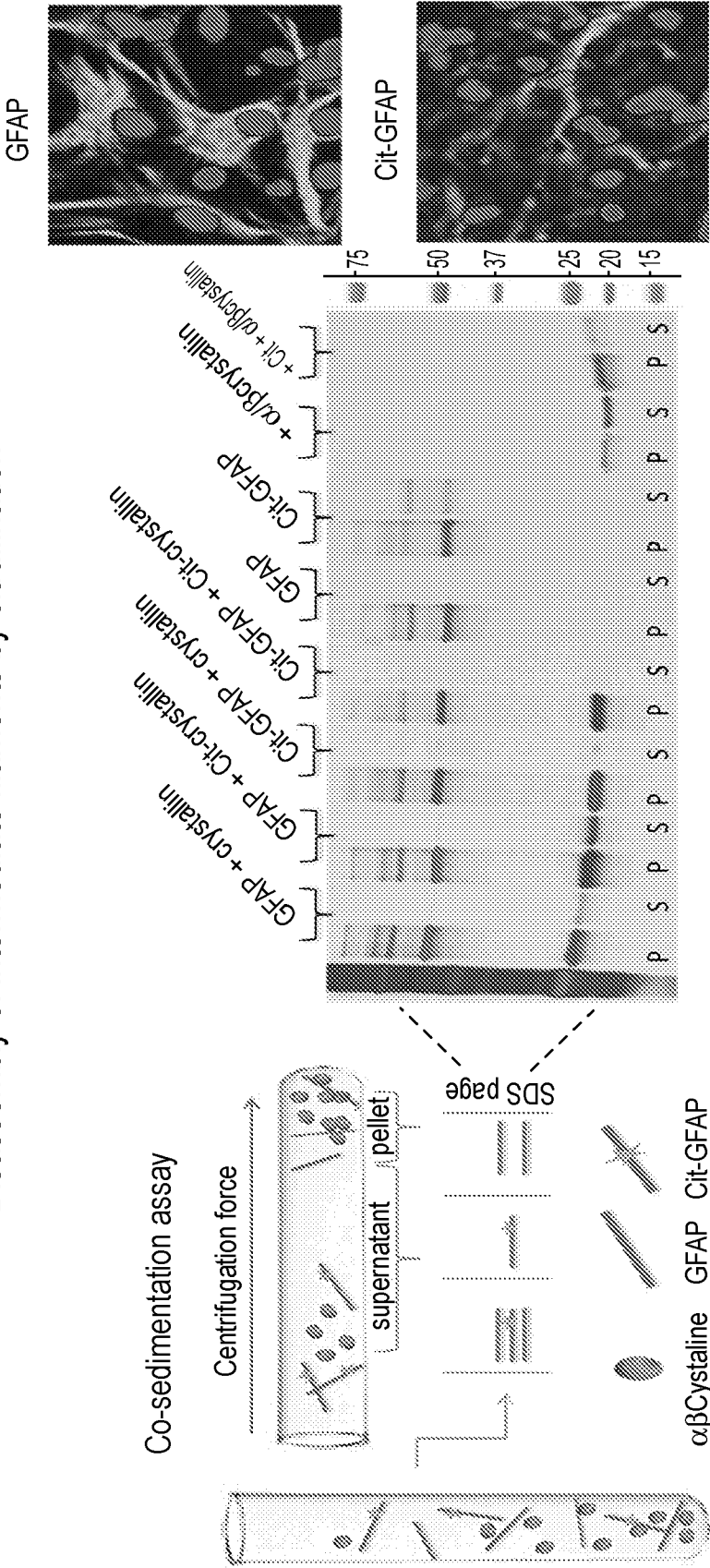


FIG. 9

