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(54) Title: BIOMARKER MOLECULES FOR SARCOPENIA AND USES THEREOF

(57) Abstract: The present invention relates to the identification of novel protein biomarkers for the detection and monitoring of sarcopenia in humans, in the absence of any underlying disease which could affect muscle mass or muscular strength. It is in particular demonstrated that specific forms of the non- mature Cathepsin D protein are significantly overexpressed in sarcopenia patients. The present invention additionally describes methods and devices to assay said biomarker molecules in human samples, and provide means for generating binding agents against said biomarker molecules.

Biomarker molecules for sarcopenia and uses thereof

Field of the invention

The present invention relates to the identification of novel protein biomarkers for the detection and monitoring of sarcopenia in humans. It additionally describes methods and devices to assay said biomarker molecules in human samples.

Background art

Sarcopenia is a recently identified syndrome linked to loss of muscular strength, function, and mass (Cruz-Jentoft AJ, et al. *Age Ageing* 2010; 39: 412-23.; Sayer AA, et al. *Age Ageing* 2013; 42: 145-150). It is classified as a pathology since 2016.

Sarcopenia is linked with aging, and according to WHO reports on aging and lifestyle, it can be estimated that sarcopenia currently affects over 50 million people worldwide and is likely to affect ca. 200 million individuals in 40 years' time. Sarcopenia affects only skeletal muscle and then, appears without chronic disease.
It is however thought that sarcopenia is largely under-diagnosed (Brotto M. *Lessons from the FNIH-NIA-FDA sarcopenia consensus summit*. IBMS BoneKEy 2012; 9: 210).

It is important to realize that while cachexia is defined as weight loss due to an underlying illness, sarcopenia concerns the loss of muscle mass and function associated with aging. A detailed review of the two conditions can be found in the publication by Ali and Garcia (Gerontology, 2014; 60(4), p.294-305) who report that sarcopenia assessment should include muscle mass study (by methods such as DEXA), muscle strength study (handgrip strength), and muscle function study (e.g. gait speed over 6 meters) (see Table 2 in Ali and Garcia).

The current diagnosis of sarcopenia, performed by a mix of medical imaging and assessment of clinical physical parameters, is costly, slow to deliver results and burdensome for the patient.

A number of publications have reported potential biomarkers for sarcopenia, for example the reviews of Brotto et al., 2012 (*supra*, citing creatinuria as a potential biomarker); Cesari et al., 2012 (J. Cachexia Sarcopenia Muscle 2012; 3: 181-90) and Pahor et al., 2009 (The journal of Nutrition Health and Aging 2009; 13: 8) (citing inflammatory biomarkers such as C-reactive protein, IL-6, IL-1 or TNF-alpha as potentially useful in the diagnosis of sarcopenia); and Scharf G et al., 2012 (J Cachexia Sarcopenia Muscle 2012; 3: 145-8).

As of today there is however no sensitive, simple and reliable markers for the diagnosis and/or prognosis of sarcopenia which could be put into practice through a rapid, cost effective method using readily available biological samples.

A number of previous approaches have focussed on the identification of biomarkers of cachexia, rather than of sarcopenia. For the reasons given above on the differences between these two conditions (and summarized for example in Table 3 of Ali and Garcia, *supra*), there can be no inference that a biomarker identified in the context of cachexia can be useful in the context of sarcopenia.

One such study is reported in WO2015/111008 by Reinker et al., who searched for urine biomarkers most significantly associated with cancer patients' weight loss. As apparent from a review of Table 3 of Ali and Garcia (*supra*), weight loss used in the Reinker at al. study is different from muscle mass loss in the context of sarcopenia. By studying biomarkers which are statistically associated with loss of body weight, Reinker et al. thus report biomarkers characteristic of cachexia.

In order to assess sarcopenia in absence of cachexia, it is understood that the test and control populations should differ only in muscle mass and function. As reported in Table 3 of Ali and Garcia (*supra*), while cachexia is defined as a weight loss greater than 5% in 6 months, it is the case that loss of muscle mass in sarcopenia is compensated by an increase in fat mass, such that there may be no loss of body weight.

For these reasons, the results reported by Reinker et al. are reflective of changes occurring during cachexia, but the population they studied is not suited to assess sarcopenia, which is defined as a loss of muscle mass and function in the absence of any underlying disease.

There is thus a need in the art for easily accessible and measurable biomarkers for sarcopenia. Ideally such biomarker(s) would give access to diagnostic methods to assess disease severity and prognosis. Such a biomarker(s) would additionally provide means for monitoring the effects of therapeutic interventions on the management of sarcopenia.

Summary of the invention

The present invention provides novel biomarker molecules of sarcopenia which are quantifiable in readily available biological fluids, especially in serum samples.

The present invention additionally discloses a method for the diagnosis or prognosis of a subject suspected to have or having sarcopenia.

The present invention also concerns a method for determining the efficacy of a treatment for sarcopenia in a patient.

The invention also provides a compound for use in the quantification of a novel biomarker molecule of the invention.

In another embodiment, the invention proposes a kit for practicing the diagnostic/prognostic method of the invention.

The invention additionally discloses a method of raising antibodies to a biomarker molecule of the invention for use in the methods and kits of the invention.

Detailed description of the invention

Definitions

Sarcopenia is a syndrome linked to loss of muscular strength, function, and mass.

"Sarcopenia", as used herein, requires the presence of low muscle mass and either low muscular strength or low physical performance, as further clinically defined by the European Working Group on Sarcopenia in Older People (EWGSOP; Cruz-Jentoft AJ, et al. Age Ageing 2010; 39: 412-23) and further requires that the subject does not have any underlying disease which could affect muscle mass or muscular strength. In a particular embodiment, "sarcopenia", as used herein, does not include patients suffering from cachexia, from cancer, from renal diseases, from liver diseases, from chronic inflammatory disease, or from an infectious disease. Patients suffering from sarcopenia may, however, concomitantly suffer from osteoporosis.

"Severe sarcopenia", as used herein, requires the presence of all three of low muscle mass, low muscular strength and low physical performance.

Sarcopenia is classified as M62.84 in the ICD-10 Clinical Modification.

As used herein, "treatment for sarcopenia" refers to an approach to limit, reduce or suppress the symptoms generated by sarcopenia. These approaches comprise but are not limited to: physical exercises and training, diet (modification of protein intake, food supplementation, vitamin D…) or drugs (for example targeting molecules involved in mitochondrial regulation or in myokines signalling, selective androgen receptor modulator (SARMs), growth hormones, myostatin inhibitors,…). The skilled person will find further guidance on the available treatments for sarcopenia, for example in the publication by JE Morley from 2016 ("Pharmacologic Options for the Treatment of Sarcopenia", *Calcif. Tissue Int.*, 98(4), p.319-33).

As used herein, "prepro Cathepsin D" refers to the full length sequence of the unprocessed protein which comprises 412 amino acids. This full length sequence is presented herein as Seq ID N°1. It is referenced as P07339 in the UniProt knowledgebase (www.uniprot.org).

Both prepro Cathepsin D and pro Cathepsin D (after removal of amino acids 1-20 of Seq ID N°1 which correspond to the secretion signal peptide) are inactive forms of the protein devoid of enzymatic activity, with a molecular weight greater than 48kDa.

Pro Cathepsin D is processed into an "active intermediate form" (of about 48 kDa, corresponding to amino acids 65 to 412 of Seq ID N°1).

Finally the active intermediate form is transformed into the mature active form composed of both a heavy and a light chain. The heavy chain has a theoretical molecular weight of 34kDa and may be detected by Western Blot at a weight of from about 25 to about 34 kDa. It corresponds to amino acids 169 to 412 of Seq ID N°1 while the light chain has a molecular weight of about 14 kDa and corresponds to amino acids 65 to 162 of Seq ID N°1.

As used herein, “non-mature Cathepsin D protein” thus includes the following forms of the protein: pro Cathepsin D (inactive form) as well as the active intermediate form of the protein.

As used herein, “mature active Cathepsin D protein” thus refers to the heavy and the light chain obtained after processing of the active intermediate form.

As used herein, “active Cathepsin D protein” refers to both the mature active Cathepsin D protein and the active intermediate form of the protein.

“Biological fluid”, as used herein, refers to blood, plasma, serum, synovial fluid, a cell culture supernatant fluid, or a cell extract.

As used herein, "sarcopenia status" refers to any distinguishable manifestation of sarcopenia, as defined above. For example, sarcopenia status includes, without limitation, the presence or absence of sarcopenia in a subject, the risk of a subject developing sarcopenia, the stage of the disease, the progression of the disease, and the effectiveness or response of a patient to an intervention for the prevention or the treatment of sarcopenia.

As used herein, “biomarker molecule” refers to a molecule selected from the peptides of Seq ID N° 2, 3, 4, 5, 6 and 7, the non-mature Cathepsin D protein, and fragments thereof.

“About” as used herein referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/-10% or less, more preferably +/-5% or less, even more preferably +/-1% or less of and from the specified value, in so far such variations are appropriate to perform in the disclosed invention. However, it is to be understood that the value to which the modifier “about” refers is itself also specifically disclosed.

By a careful study of biological samples from a significant number of sarcopenia patients and matched controls, the present inventors have now identified a number of molecules which are useful in the diagnosis and prognosis of sarcopenia.
These molecules include the non-mature Cathepsin D protein and fragments thereof, such as those of Seq ID N°2 to 21. Preferred fragments include the tryptic fragments of Seq ID N° 2, 3, 4, 5, 6 and 7.

Methods

The concentration of the biomarker molecules of the invention in a sample may be determined by suitable assays using the information herein provided. A suitable assay may include one or more of the following methods: an enzyme assay, an immunoassay, mass spectrometry, HPLC (including nano 2D UPLC such as using a nanoAcquity device from Waters, www.waters.com), electrophoresis or an antibody microarray, or any combination thereof. If an immunoassay is used

it may be an enzyme linked immunoassay (ELISA), a sandwich assay, a competitive assay, a radioimmunoassay, a Western Blot, an immunoassay using a biosensor, an immunoprecipitation assay, an agglutination assay, a turbidity assay or a nephlelometric assay. If mass spectrometry is used it may be Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI/TOF), liquid chromatography quadruple ion trap electrospray (LCQ-MS), or surface enhanced laser desorption ionization/time of flight (SELDI/TOF) mass spectrometry.

One preferred method of detection is the use of an immunoassay, utilizing an antibody, which binds to an epitope on non-mature Cathepsin D protein or on fragments thereof.
Assay forms in which such an antibody can be applied include, but are not limited to, ELISA, microarray, radioimmunoassay (RIA), fluorescence activated cell sorting (FACS), Western blotting, chromatography, immunofluorescence and histochemistry.

Another preferred method of detection is based on mass spectrometry. In this context, advanced mass spectrometry techniques such as Multiple Reaction Monitoring may be used. MRM (sometimes called Selected Reaction Monitoring, SRM) is a highly sensitive and selective method for the targeted quantitation of protein/peptide abundances in complex biological samples. Unlike traditional mass spectrometry, which attempts to detect all proteins in a biological sample in an unfocused fashion, Multiple Reaction Monitoring (MRM) is highly selective (targeted), allowing the skilled person to fine tune an instrument to specifically look for peptides, or protein fragments, of interest.
Details on MS techniques for biomarker quantitation can be found in the published literature, such as in Lemoine et al., 2012 ("The current status of clinical proteomics and the use of MRM and MRM^3 for biomarker validation", *Expert Rev. Mol. Diagn.*, 12(4), pages 333–342), in Calderon-Celis et al., 2017 ("Standardization approaches in absolute quantitative proteomics with mass spectrometry", *Mass Spec Rev.* 2017; pages 1–23) or in Boja et al., 2014 ("Analytical Validation Considerations of Multiplex Mass-Spectrometry-Based Proteomic Platforms for Measuring Protein Biomarkers", *J. Proteome Res.*; 13; pages 5325-5332).

In one embodiment, the invention provides a method for detecting the amount of non-mature Cathepsin D protein or fragments thereof in biological fluids. Typically when a biological fluid sample is contacted with a binding agent (as further defined herein) specific towards an epitope within the amino acid sequence of non-mature Cathepsin D protein, essentially all non-mature Cathepsin D protein or fragments thereof in the sample containing this epitope will be bound by such a binding agent. The amount of protein or fragments thereof bound by the binding agent will be detected by methods which the skilled artisan will develop using the information herein provided. Typically, the epitope bound by binding agents reactive with non-mature Cathepsin D protein or fragments thereof may comprise five or more amino acids.

In one aspect, the present invention provides a method of determining the sarcopenia diagnosis of a subject, comprising the steps of:

(i) determining the concentration(s) in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein, and fragments thereof;

(ii) comparing the molecule concentration(s) determined in step (i) with one or more reference values, wherein an increase in the concentration(s) of the molecule(s) compared to the reference values is diagnostic of sarcopenia.

As used herein, “reference values” refer to the biomarker levels that can be measured in samples from control subjects (i.e. not suffering from sarcopenia) by a skilled person, using the same sample type, purification method, and analytical method as those used to determine the sarcopenia status of a subject according to a method of the invention.

Reference herein to "fragment" is intended to refer to a polypeptide, a peptide or otherwise, released from mammalian non-mature Cathepsin D protein by an oxidative or enzymatic processing. A fragment is different from the non-mature Cathepsin D protein by its structure and configuration and may undergo modifications such as phosphorylation, glycosylation or any other post-translational modification including, but not limited to, nitration, chlorination, sumoylation, ubiquitylation and glycation. The fragment according to the present invention contributes to the identification of the sarcopenia status of the patient.

The present invention also provides a method of determining the prognosis of a patient with sarcopenia comprising the steps of:

(i) determining the concentration(s) in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein, and fragments thereof;

(ii) comparing the molecule(s) concentration(s) determined in step (i) with one or more reference value(s), wherein a change in the concentration(s) of the molecule(s) compared to the reference value(s) is indicative of the prognosis of the patient.

The present invention also provides a method of determining the prognosis of sarcopenia incidence or progression in a subject comprising the steps of:

(i) determining the concentration(s) in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein, and fragments thereof;

(ii) determining after a certain amount of time the concentration(s) in a biological sample from the same subject of said one or more molecule(s);

(ii) comparing the molecule(s) concentrations determined in step (i) and step (ii) with one another, and optionally with one or more reference value(s), wherein a change in concentration over time or a change in the time-integrated curve is predictive of the incidence or progression of the disease.

The present invention further provides a method of determining the efficacy of a treatment for sarcopenia in a patient, comprising the steps of:

(i) determining the concentration in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein and fragments thereof;

(ii) administering a treatment for sarcopenia to the patient;

(iii) determining after a certain time of treatment the concentration(s) in a biological sample from the same patient of said one or more molecule(s);

(iv) comparing the molecule(s) concentrations determined in step (i) and step (iii) with one another, and optionally with one or more reference value(s), wherein a stable concentration or a decreasing concentration of said molecule(s) indicates an efficacious treatment.

In this method of the invention, the treatment for sarcopenia may include, by way of non-limiting examples, physical exercises and training, diet (modification of protein intake, food supplementation, vitamin D…) or drugs (for example targeting molecules involved in mitochondrial regulation or in myokines signalling, selective androgen receptor modulator (SARMs), growth hormones, myostatin inhibitors,…). Other approaches for the treatment of sarcopenia are described in JE Morley from 2016 (“Pharmacologic Options for the Treatment of Sarcopenia”, *Calcif. Tissue Int.*, 98(4), p.319-33).

Since the present inventors have shown in the Examples that the levels of the one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein and fragments thereof, are correlated with the sarcopenia status of the patient, the skilled person will directly understand that the efficacy of a given treatment for sarcopenia may be monitored by following the concentration in a biological sample of said molecule(s) over time as the treatment is taking place.

For example, after a treatment time of 1 week, of 2 weeks, of 3 weeks, of 1 month, of 2 months, of 3 months, or of 6 months, a decrease in concentration of one or more of said molecule(s) of 5% or more, of 10% or more, of 20% or more, or of 30% or more, compared to the concentration of said molecule(s) before initiation of the treatment, or at an earlier treatment time, is indicative of efficacy of said treatment.

Efficacy of a treatment may be monitored by analysing samples taken from a patient at various time points following initiation of the treatment. By monitoring changes in the concentration of the biomarker molecule(s) over time and comparing these concentrations to normal and/or reference values, efficacy of the treatment may be determined. In this case, reference concentration(s) may include the concentration of the biomarker molecule in the patient when a sample was first taken and analysed, or the concentration of the biomarker molecule in the patient when a sample was last taken, or both.

The present invention additionally provides a method of treating a patient suffering from sarcopenia, comprising the steps of:

(i) determining the concentration in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein and fragments thereof;
(ii) administering a treatment for sarcopenia to the patient;
(iii) determining after a certain time of treatment the concentration(s) in a biological sample from the same patient of said one or more molecule(s);
(iv) comparing the molecule(s) concentrations determined in step (i) and step (iii) with one another, and optionally with one or more reference value(s); and
(v) adjusting the treatment regimen according to the result of the comparison of step (iv);
wherein the treatment regimen is increased when the molecule level(s) are stable or increasing.

As used herein, "the treatment regimen is increased" refers to a situation where the administered treatment is considered not efficacious enough, and a stronger treatment needs to be administered. By way of example and not limitation, in case the treatment comprises physical exercise, more intense and/or more frequent physical exercise is instructed.

The present invention also provides a means of selecting a subject for treatment, comprising the steps of:
(i) determining the concentration(s) in a biological sample of one or more molecule(s) selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein and fragments thereof;
(ii) comparing the molecule concentration(s) determined in step (i) with one or more reference values, wherein an increase in the concentration(s) of the molecule(s) compared to the reference values is indicative that said subject has to be selected for treatment.

In different embodiments of the invention, the protein, or fragment, sequence, detected comprise a number of modifications, such as post-translational modifications, and/or appear in a non-native form, such as an unfolded form.
In a particular embodiment of the invention the biological sample to be assessed is first processed with trypsin, and the protein fragments to be detected are proteolytic fragments, such as those of Seq ID N°2 to 7.

The present invention thus provides a method of detecting a molecule selected from the peptides of Seq ID N°2 to 7, the non-mature Cathepsin D protein and fragments thereof, wherein the method uses a purposely-designed immunoassay or a specific mass spectrometry assay, such as an MRM-based assay.

Any disclosed method may be used in conjunction with the assessment of clinical symptoms and/or imaging results and/or the concentration of one or more other biomarkers.
Preferably the method of the invention is carried out in vitro.

Preferably the reference value, to which the determined concentration of the biomarker molecule is compared, is the concentration of the same molecule in one or more "normal" subjects that do not have any detectable sarcopenia, or any clinical symptoms of sarcopenia, and have so called "normal values" of the biomarker molecule.

Alternatively, the reference value may be a previous value for the biomarker molecule obtained from a specific subject. This kind of reference value may be used if the method is to be used to monitor progression of sarcopenia, or to monitor the response of a patient to a particular treatment.
When the determined concentration of the biomarker molecule is compared with a reference value, an increase in the concentration of the biomarker molecule may be indicative of the sarcopenia status in the subject.
More specifically an increase in the concentration of the biomarker molecule may be indicative, or diagnostic, of sarcopenia in the subject.
Preferably an at least about 5%, at least about 10%, at least about 20%, at least about 30% at least about 50%, at least about 70% increase in the concentration of a biomarker molecule according to the invention in a sample from a subject compared to a reference sample from a normal subject is diagnostic of sarcopenia. In another embodiment, an increase in the concentration of a biomarker molecule according to the invention of 2 fold or more in a sample from a subject compared to a reference sample from a normal subject is diagnostic of sarcopenia.

Binding agent development:

As used herein, a “binding agent” refers to any molecule capable of specifically binding a biomarker molecule of the invention. As such, “binding agent” includes, but is not limited to, aptamers (both DNA and peptide aptamers), affimers, synthetic binding proteins (such as polypeptides having a specific domain of protein A as a backbone - affibodies™), antibodies or any fragment derived thereof, such as Fab, Fab' and F(ab')2, Fd, single-chain Fvs, single-chain antibodies, disulfide-linked Fvs and fragments comprising either a VT or VFI domain, a heavy chain antibody, a single domain antibody, a minibody, the variable domain derived from camelid heavy chain antibodies, the variable domain of the new antigen receptors derived from shark antibodies, a protein scaffold including an alphabody, protein A, protein G, designed ankyrin-repeat domains, fibronectin type III repeats, anticalins, knottins, or engineered CH2 domains.

In the development of antibodies, the biomarker molecule, or an immunogenic fragment thereof, is used as an antigen for immunisation. The peptide is emulsified in an adjuvant medium, preferably incomplete Freund’s adjuvant and injected subcutaneously or into the peritoneal cavity of a mammalian host, preferably a rodent, more preferably a rabbit, even more preferably a mouse. To enhance immunogenic properties of the antigenic peptide, it may be coupled to a carrier protein before being emulsified in an adjuvant medium. Useful carriers are proteins such as keyhole limpet hemocyanin (KLH), edestin, albumins, such as bovine or human serum albumin (BSA or HSA),

tetanus toxoid, and cholera toxoid, polyaminoacids, such as poly-(D-lysine-D-glutamic acid). Booster injections may be given at regular intervals until an immune response is obtained, the last injection may be given intravenously to ensure maximal B-cell stimulation.
Antisera are subsequently screened for their ability to bind an epitope within the biomarker molecule sequence. Antisera from the most promising hosts may be used in their crude form or purified.

Monoclonal antibodies may be generated from immunised mice or other animals with the most promising antibody titre, by fusing lymphocytes isolated from the spleen or nodes of these mice or animals with a myeloma cell line. The generated hybridoma clones are screened for antibodies with reactivity toward an epitope within the biomarker molecule sequence, and cell lines can be established for production and purification of monoclonal antibodies.

Antibodies may be synthetic, monoclonal, polyclonal, oligoclonal, bispecific, chimeric and/or humanised. The antibody may be complete or a fragment thereof, such as, Fv, Fab and F(ab)2 fragments.

More generally, binding agents may be selected from existing libraries of binding agents by known techniques such as, e.g. phage display.

One or more of the binding agents used may comprise a tag or a label, such as a radioactive, fluorescent, chemiluminescent tag or a label, a dye, an enzyme, or a histidine tag or label, or any other suitable label or tag known in the art.

Samples

Mammalian body fluids, such as blood, plasma, serum, synovial fluid, and subcutaneous interstitial fluid, as well as extracts from cells or tissues or supernatants from cells or tissues cultured in vitro may be used in the methods of the invention. In a preferred embodiment, the biological fluid used is serum.

Samples preparation methods:
The biological fluid may be used as it is, or it may be purified prior to the quantification step. This purification step may be accomplished using a number of standard procedures, including but not limited to, cartridge adsorption and elution, molecular sieve chromatography, dialysis, ion exchange, alumina chromatography, hydroxyapatite chromatography, and combinations thereof. By way of exemplification and not limitation, a suitable purification protocol is illustrated in Example 2.

In specific embodiments of the invention, the biological fluids to be used for measuring biomarkers levels are first prepared by depletion of the most abundant proteins before they are analysed.

Preparation methods include but are not limited to: protein depletion methods, methods to dissociate protein complexes as they can occur in the biological fluid, etc… as these methods are performed by the skilled artisan using the detailed information provided herein.

In one embodiment, the biological sample may be depleted in Human Serum Albumin using a commercial kit, such as the Pierce Albumin Depletion kit (catalog number: 85160; Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA USA 02451). The biological sample may be depleted using the ProteoPrep® Blue Albumin & IgG Depletion Kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA). The biological sample may be depleted in the most abundant proteins using the "ProteoPrep20 Plasma Immunodepletion kit" from Sigma (http://www.sigmaaldrich.com/, Catalog No: PROT20).

Devices and kits

One embodiment of the present invention constitutes a diagnostic kit for use in detection and/or monitoring of sarcopenia. This includes a binding agent recognizing an epitope comprised in the sequence of a biomarker molecule of the invention. Most preferred are antibodies of the present invention, either alone or with a second antibody with specificity towards the first antibody or another part of the epitope-containing biomarker molecule. The kit can be applied on mammalian body fluids or extracts of cells or tissues, preferably derived from humans. For competition detections a peptide between 6 and 20 amino acids, in which a succession of amino acids is equivalent to the binding epitope for one of said antibodies, might be supplied either in a labelled or non-labelled form. In other embodiments, the non-mature Cathepsin D protein, or fragments thereof, is supplied in a labelled or non-labelled form in the kit. The peptide, the non-mature Cathepsin D protein or fragments thereof, may be obtained by a synthetic or a recombinant route or a cell-free system.

The antibodies may be labelled by joining them, either covalently or non-covalently, with a reporter molecule. Suitable reporter molecules or labels, which may be used for ease of detection, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like. One of the non-labelled antibodies or a peptide of the kit might be immobilised, preferably on a solid surface like a microtiter plate, possibly by conjugation to a suitable protein carrier like BSA.

The kit comprises at least one agent for determining the concentration of a biomarker molecule according to the invention, in a biological sample. The agent may be an enzyme, an antibody, a protein probe, a metabolite or any other suitable composition. The agent for determining the concentration of the biomarker molecule is preferably labelled. The kit may also comprise means for detecting the label.

The kit may comprise one or more capture agents for capturing the biomarker molecule in a biological sample. The capture agent may be one or more antibodies.

The kit may comprise two antibodies for use in a sandwich assay to determine the concentration of a biomarker molecule. Preferably the kit comprises two antibodies, each directed to a different epitope on the biomarker molecule.

One antibody is preferably the capture antibody, and the other antibody is preferably a detection antibody and may be labelled to allow its detection.

The capture agent may be attached to a solid support. The solid support may be a chip, a microtiter plate, a bead or a resin.

The kit may comprise instructions for suitable operational parameters in the form of a label or separate insert. The instructions may inform a user about how to collect the sample, and/or how to wash the capture agent.

The kit may comprise samples of the biomarker molecule to be detected, for example in the form of a synthetic peptide or polypeptide. The samples of the biomarker molecule may be used as a standard for calibration and comparison and may optionally be labelled. The kit may also comprise instructions to compare the concentration of the biomarker molecule detected in a sample with a calibration sample or chart. The kit may also include instructions indicating what concentration of the biomarker molecule is diagnostic of sarcopenia.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The invention is further illustrated in the following examples, which are not intended to limit the scope of the invention in any manner. Modifications and alternative implementations of some parts or elements are possible, and are included in the scope of protection as defined in the appended claims.

Examples

Example 1: Recruitment of sarcopenia patients and healthy controls

Patients aged 65 and above were recruited and classified into cohorts, using three parameters measured and assessed as follows:

- muscle strength, using the “handgrip strength” test. The parameter was deemed “normal” if above the cut-off (>= 30kg for men and >= 20kg for women), and “degraded” if below cut-off.
- usual walking speed (six meters walk test - 6MWT), with a cut-off at 0.8 meters/ second: parameter was “normal” is above cut-off, and “degraded” if equal to or below cut-off.

- skeletal muscle mass index (SMI – as measured by Dual energy X-ray Absorptiometry, appendicular lean soft tissue - ALST) with a cut-off of 7.25 kg/m2 for men and 5.67 kg/m2 for women. The parameter was “degraded” if equal to or below the cut-off.

The enrolled patients were thus classified into one of the following groups:

Parameter	Control Group		Sarcopenia group	
	Subgroup 1	Subgroup 2 “pre-sarcopenia”	Subgroup 3 “mild sarcopenia”	Subgroup 4 “severe sarcopenia”
Muscle strength	Normal	Normal	One of these Degraded	Degraded
Walking speed	Normal	Normal		Degraded
Muscle mass	Normal	Degraded	Degraded	Degraded
Number of patients in subgroup	8	11	10	10

Pairs of subjects were matched between the control group and the sarcopenia group in order to eliminate individual differences based on sex (same sex) and age (the age difference between the matched subjects could not exceed 10 years).

In order to focus the study on biomarkers specific to sarcopenia and to exclude as much as possible cachexia, the following exclusion criteria were used:

- any significant chronic disease which is active and not stable (e.g. diabetes, thyroid disorders, …);
- chronic inflammatory disease (e.g. Rheumatoid Arthritis);
- cardiac, respiratory, neurologic or osteoarticular disease likely to affect physical performances;
- neurodegenerative disease in case where it affects muscular function or modifies test measures;
- hematologic or oncologic disease under treatment by chemotherapy and/or radiotherapy – prostate cancer treated by hormonal therapy;
- malabsorption or severe gastro-intestinal disease;
- known anaemia with a significant impact on physical performances,
- known haemoglobin level below 10 g/dL;
- proven undernutrition or cachexia, as assessed by: a score of Mini Nutritional Assessment (MNA) below 17/30 or a Body Mass Index below 17.0 kg/m^2, or a CRP level above 15 mg/L.

A blood sample of approximately 29 mL and a urine sample of approximately 10 mL were taken from each enrolled patient.

It was decided to proceed with the proteomic analysis in Example 2 below on the basis of samples from female patients. A total of 10 samples in the sarcopenia group (7 from subgroup 4 and 3 from subgroup 3) and of 10 samples in the control group (6 from subgroup 1 and 4 from subgroup 2) were used.

Example 2: Proteomics analysis of collected serum samples

Sample processing:

Proteomic analysis was performed with serum samples collected in BD Vacutainer serum tubes (ref. 368815).

The most abundant proteins were depleted using the "ProteoPrep20 Plasma Immunodepletion kit" from Sigma (http://www.sigmaaldrich.com/, Catalog No: PROT20) according to the manufacturer's instructions.

Subsequently the samples were reduced, alkylated and reduced, and processed using the 2D-Clean up kit (GE Healthcare LifeSciences www.gelifesciences.com, Product code 80-6484-51) according to the manufacturer's instructions. The protein pellets after the washing steps were further resolubilized in bicarbonate ammonium 50 mM.

The samples were digested in solution with trypsin (16 hours at 37°C with a trypsin/total proteins ratio (w/w) of 1/10, followed by 3 hours at 37°C with a ratio of 1/20 in 80% ACN). The reaction was stopped by addition of trifluoroacetic acid. The samples were evaporated to dryness in a speed vacuum.

0.7 µg of protein digest for each sample was purified using a Zip-Tip C18 High Capacity (Merck Millipore www.merckmillipore.com - catalogue Number ZTC18S008) according to the manufacturer's instructions. The samples were evaporated to dryness in a speed vacuum.

The samples were re-suspended in 100 mM Ammonium Formiate (pH10) at 0.067µg/µL.

A volume of 9µL per sample, corresponding to 0.6 µg of digested proteins was injected on the nano 2D UPLC – Orbitrap MS System.

Mass spectrometry:

The analyses were performed on a nano-UPLC (nanoAcquiXy, Waters) - ESI-Q-Orbitrap (Q Exactive, Thermo), in positive ion mode.

An internal standard sample ("MPDSMIX" from Waters, at www.waters.com, Part. No. 186002865) containing 4 digested proteins was spiked in each sample at a quantity of 150 fmoles of ADH digest per injection.

The LC method was a 2D LC method with 3 steps of 180 minutes. The 3 steps are made on the column at high pH with increasing percentage of acetonitrile and the peptides eluted from the column at high pH are loaded after dilution on the low pH column. On low pH column, each step consists of a gradient of 5 min from 99% of A (A= water 0.1% formic acid, B = acetonitrile) to 93% of A followed by a gradient of 135 minutes from 93% of A to 65% of A.

The mass spectrometer method is a TopN-MSMS method where N was set to 12, meaning that the spectrometer acquires one Full MS spectrum, selects the 12 most intense peaks in this spectrum (singly charged precursors excluded) and makes a Full MS2 spectrum of each of these 12 compounds.

The parameters for MS spectrum acquisition are: Mass range from 400 to 1750 w/z, Resolution of 70000, AGC target of 1e6 or Maximum injection time of 200 ms.

The parameters for MS2 spectrum acquisition are: Isolation Window of 1.6 w/z, Collision energy (NCE) of 25, Resolution of 17500, AGC target of 1e5 or Maximum injection time of 50 ms.

Mass spectra analysis:

Spectra were processed using the MaxQuant software (version 1.5.2.8 – Cox et al., 2008, Nat. Biotechnol. 26, 1367-72).

Protein identifications are considered significant if proteins are identified with at least 2 peptides per protein taking into account only an FDR<0.01 (False Discovery Rate).

Analysis of differential protein expression between different sample groups:

The Perseus software (available at http://www.perseus-framework.org, and described in Cox, J and Mann, M., BMC Bioinformatics, 2012, 13 Suppl 16:S12; and in Tyanova, S. et al. Nature Methods, 2016) was used to determine the ratio of the protein expression level in the sarcopenia group to the protein expression level in the control group and to calculate the attached statistical significance (p-value).

Results:

Cathepsin D, a 412 amino acids long protein with a UniprotKB (http://www.uniprot.org/) accession number of P07339, was identified through six most represented tryptic sequences which are listed below as Seq ID 2 to 7.

The non-mature Cathepsin D protein, or at least fragments of it, was detected with twice higher an expression level in the sarcopenia group than in the control group. Moreover, the p-value of this finding was $p < 0.001$.

Table 1 – tryptic sequences of Cathepsin D detected by Proteomic analysis

Seq ID N°	Sequence	Mean Intensity Sarco	Mean Intensity Control	Position in Full length protein	Length
2	AIGAVPLIQGEYMIPCEK	4649680	610350	314 -> 331	18 a.a.
3	DPDAQPGGELMLGGTDSK	4684210	2180963	236 -> 253	18 a.a.
4	FDGILGMAYPR	5785330	1560590	195 -> 205	11 a.a.
5	VSTLPAITLK	11417760	0	332 -> 341	10 a.a.
6	TMSEVGGSVEDLIAK	9707110	2273550	35 -> 49	15 a.a.
7	YSQAVPAVTEGPIPEVLK	2230330	161550	55 -> 72	18 a.a.

Of the peptides identified above, Seq ID N°6 was detected in 9 out of 10 samples from the sarcopenia group and in 5 out of 10 samples from the control group. It was quantified at a 4.3 higher level in the serum of the sarcopenia group compared to the level in the serum of the control group. The peptide of Seq ID N°5 has a particular status since it was never detected in the serum of control subjects, but was identified in 7 (out of 10) samples from the sarcopenia group.
The peptide of Seq ID N°3 finally, was detected in 10 out of 10 samples from the sarcopenia group and in 9 out of 10 samples from the control group. It was quantified at a 2.2 higher level in the serum of sarcopenia patients compared to the level in the serum of control patients.

It is interesting to compare the proteomics results above to the findings reported by Reinker et al in WO2015/111008. The approach adopted by Reinker et al was to study cachexia patients with loss of body weight and to detect biomarkers seen in urine. Reinker et al. reported, among others, that the entire sequence of the Cathepsin D protein (i.e. the full sequence of Seq ID N°1) was found in urine samples as significantly associated with body weight loss. These authors selected cancer patients, as reported in the examples of WO2015/111008. By contrast, in the present approach, as detailed in Example 1 above, a particular attention was paid to include only patients with no underlying disease. Under normal conditions, only minute amounts of the Cathepsin D protein are detectable in serum samples. As a consequence, it was surprisingly found that the Cathepsin D protein represents a useful biomarker to detect in serum samples a condition of sarcopenia, in the absence of cachexia and other underlying diseases, such as cancer.

Example 3: Confirmatory Elisa experiments for biomarker molecules identified in Example 2

Cathepsin D is an acid protease active in intracellular protein breakdown. It is synthesized as "prepro-cathepsin D" as a 412 amino acids chain, with a signal peptide of 20 amino acids which, upon cleavage in the endoplasmic reticulum yields pro-cathepsin D. The primary sequence of Cathepsin D is prone to many post-translational modifications. After transportation into the lysosome compartment, an active chain of pro-cathepsin D is formed (MW of 48 kDa), which is in turn transformed into the mature form comprising a heavy chain (34 kDa) and a light chain (14 kDa). Cathepsin D levels were first assessed in the 20 samples from Example 1 using a commercial ELISA test (Abcam, 330 Cambridge Science Park, Cambridge, CB4 0FL, UK, product code: #ab213470), using a 10-times dilution of the samples.

Table 2 – Cathepsin D levels (comprising all the forms detected by the Elisa test used) in 20 samples from sarcopenia or control patients using a commercial ELISA test:

Sample group (concentration multiplied by 10 to compensate for the 10-times dilution)					
Sarcopenia Patient #	Concentration (ng/mL)	%CV	Control Patient #	Concentration (ng/mL)	%CV
101	198.98	1.37	124	156.42	2.17
104	674.28	1.86	127	212.37	8.10
106	413.15	5.17	129	287.72	3.09
108	246.92	2.27	134	245.35	4.76
109	244.98	1.48	135	276.31	0.59
110	246.59	6.71	207	158.18	9.08
117	320.56	1.57	208	220.25	0.37
121	245.49	6.66	209	161.71	2.32
131	330.59	0.26	211	185.03	2.07
133	337.94	0.96	212	159.22	2.54

As can be seen in Table 2, the coefficients of variation, expressed as %, are very satisfactory. Moreover, running a Mann-Whitney test between the two patient groups yields a p=0.0052.

The results from Example 2, reporting Cathepsin D and/or fragments thereof, as a useful biomarker for sarcopenia, are thus nicely confirmed by the Elisa results detailed here.

Example 4: Confirmatory Western Blot experiments for biomarkers identified in Example 2

In this Example, 85 µg of HSA-depleted serum from sarcopenia patients and controls were run on an AnyKD gel (Bio-Rad Laboratories N.V., 3, Winninglaan, 9140 Temse). As a positive control, 40 µg of a MCF-7 cell lysate was used. The primary antibody was obtained from Abcam (330 Cambridge Science Park, Cambridge, CB4 0FL, UK, product code: #ab75852, as used herein, "Abcam antibody #ab75852") and was used at a 1/1000 dilution. The secondary antibody was obtained from Jackson Immunoresearch (Jackson ImmunoResearch Europe Ltd., Unit 7, Acorn Business Centre, Oaks Drive – Newmarket, Suffolk, CB8 7SY, UK, product GAR-HRP) and was used at a 1/5000 dilution.

SuperSignal™ West Pico plus ECL (ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA 02451) was used to reveal by chemiluminescence.

A difference was observed between the sarcopenia and control groups in the form of bands corresponding to non-mature Cathepsin D protein (appearing at from about 45 kDa to about 55 kDa). The active intermediate form, as well as the heavy chain from the mature active form (appearing at from about 25 kDa to about 30 kDa), were not identified by this approach due to a cross-reaction between secondary antibody and serum proteins around 25 kDa (probably light chain of IgG).

Example 5 : Confirmatory Western Blot experiments for biomarkers identified in Example 2

In order to limit the cross-reaction between IgG and secondary antibody, 50 µg of HSA and IgG depleted serum from sarcopenia patients and controls were run on an AnyKD gel (Bio-Rad Laboratories N.V., 3, Winninglaan, 9140 Temse). The depletion of samples was performed using the ProteoPrep® Blue Albumin & IgG Depletion Kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA). As a positive control, 40 µg of a MCF-7 cell lysate was used. The primary antibody was obtained from Abcam (330 Cambridge Science Park, Cambridge, CB4 0FL, UK, product code: #ab75852, as used herein, "Abcam antibody #ab75852") and was used at a 1/1000 dilution. A preadsorbed against human serum protein secondary antibody was selected and obtained from Abcam (Abcam PLC, 330 Cambridge Science Park, Cambridge, UK., product code: ab97080) and used at a 1/10000 dilution.

SuperSignal™ West Pico plus ECL (ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA 02451) was used to reveal by chemiluminescence.

Only bands corresponding to the non-mature Cathepsin D protein (appearing from about 45 kDa to about 55 kDa) were observed with a different intensity between the sarcopenia patients and controls. No signal at from about 25 kDa to about 30 kDa was detected.

These results confirm that the non-mature form of the Cathepsin D could be a key biomarker of sarcopenia.

Example 6: Identification of antigenic peptides from the biomarker molecules of the invention

In order to raise antibodies against the biomarker molecules of the present invention, a search for the best antigenic peptides of Cathepsin D protein by the Kolaskar & Tongaonkar Antigenicity approach was conducted. For details on the method used, please see Kolaskar AS and Tongaonkar PC (FEBS Lett. 1990 Dec 10;276(1-2):172-4).

A total of 16 antigenic peptides of interest within the Cathepsin D protein were found. They are detailed in Table 3 below and correspond to Seq ID N°8 to 23.

Table 3:

Seq ID N°	Start position within Seq ID N°1	End position within Seq ID N°1	Peptide
8	39	45	VGGSVED
9	47	64	IAKGPVSKYSQAVPAVTE
10	68	73	PEVLKN
11	88	98	PPQCFTVVFDT
12	102	122	NLWVPSIHCKLLDIACWIHHK
13	138	145	FDIHYGSG
14	147	177	LSGYLSQDTVSVPCQSASSASALGGVKVERQ
15	187	197	GITFIAAKFDG
16	200	217	GMAYPRISVNNVLPVFDN
17	220	235	QQKLVDQNIFSFYLSR
18	255	267	YKGSLSYLNVTRK
19	269	297	YWQVHLDQVEVASGLTLCKEGCEAIVDTG
20	299	324	SLMVGPVDEVRELQKAIGAVPLIQGE
21	326	342	MIPCEKVSTLPAITLKL
22	347	369	YKLSPEDYTLKVSQAGKTLCLSG
23	376	397	PPPSGPLWILGDVFIGRYYTVF

Claims

1. A method of diagnosis of sarcopenia in a subject who does not have any underlying disease which could affect muscle mass or muscular strength, the method comprising the following steps:
   a. quantifying the level(s) of one or more molecule(s) selected from the peptides of Seq ID N°2 to 23 and the non-mature Cathepsin D protein in a sample of body fluid from the subject, for example a sample of blood, plasma, or serum;
   b. comparing the level(s) obtained in step a) with the level(s) of the same molecule(s) in the same type of sample from a healthy control;
   c. wherein an increased level(s) of the molecule(s) respective to the level(s) observed in the control is indicative of sarcopenia.
2. The method of claim 1 wherein said non-mature Cathepsin D protein is detectable by Western Blot under forms appearing at from about 45 kDa to about 55 kDa.
3. The method of claim 1 wherein said molecule(s) is specifically detected by Abcam antibody #ab75852.
4. A method of determining the efficacy of a treatment for sarcopenia in a patient who does not have any underlying disease which could affect muscle mass or muscular strength, the method comprising the following steps:
   a. quantifying the level(s) of one or more molecule(s) selected from the peptides of Seq ID N°2 to 23 and the non-mature Cathepsin D protein in a sample of body fluid from the patient, for example a sample of blood, plasma, or serum, at a first point in time;
   b. quantifying at a successive second point in time after said treatment is administered, the level(s) in a new biological sample from the same patient of said one or more molecule(s);
   c. comparing the molecule(s) levels determined in step (a) and step (b) with one another, and optionally with one or more reference value(s);
   d. wherein a decreased level(s) of the molecule(s) at said second time point respective to the level(s) at said first time point is indicative of an efficacious treatment.
5. A binding agent for use in the quantifying step a) of the method of claim 1, or in the quantifying steps a) and b) of the method of claim 4, wherein said binding agent specifically binds to at least one molecule selected from the peptides of Seq ID N°2 to 23.
6. The binding agent for use of claim 5 wherein said binding agent is an antibody or an aptamer.
7. A kit for use in the method of claim 1 or claim 4, wherein said kit comprises:
   a. a capture reagent that specifically binds said one or more molecule(s) selected from the peptides of Seq ID N°2 to 23;
   b. means for detecting binding between said capture agent and said one or more molecule(s);
   c. optionally, a sample of said one or more molecule(s) to be used as a standard for calibration.

8. The kit of claim 7, wherein said means for detecting binding is a detection reagent that specifically binds to said of one or more molecule(s).
9. Use of a molecule selected from the peptides of Seq ID N°3 to 23 in the manufacture of an antibody.
10. Use according to Claim 9 wherein the fragment is selected from Seq ID N°8 to 23.
11. Use according to Claim 9 wherein the fragment is selected from Seq ID N°3 to 7.
12. A molecule selected from the peptides of Seq ID N°6 to 23 for use as a standard for calibration in a kit of Claim 7.
13. The molecule of Claim 12 wherein the fragment is selected from Seq ID N°8 to 23.
14. The molecule of claim 12 or 13 wherein the molecule is produced synthetically, recombinantly, or by a cell-free system.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/064328

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, Sequence Search, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 101 735 317 A (BEIJING INST OF GENE SCIENCE C) 16 June 2010 (2010-06-16) Whole document, in particular abstract and SEQ ID: 2	5-8
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2018	26/06/2018

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	US 2016/154006 A1 (HERMANSON GREG [US] ET AL) 2 June 2016 (2016-06-02) SEQ ID:42 in Table 1 of example 6	12,14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/064328

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