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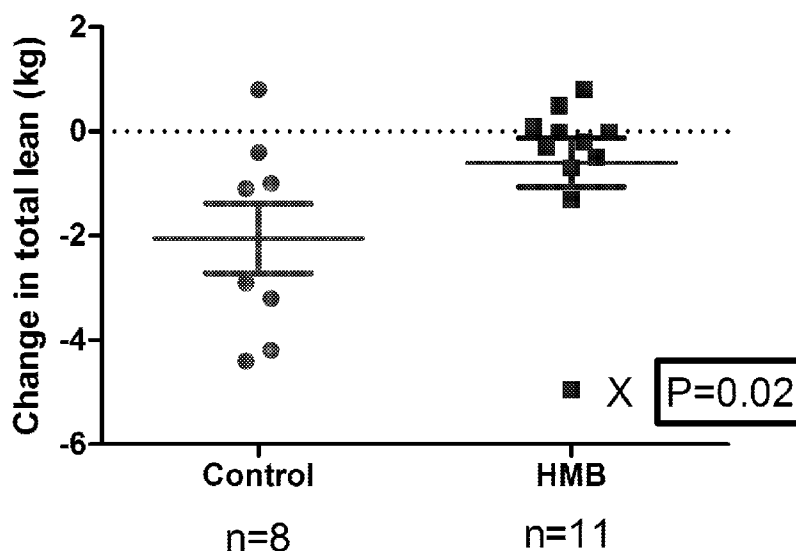


Figure 1

(57) Abstract: Biomarkers and related methods of using the biomarkers to identify subjects at risk of losing lean body mass during a prolonged period of physical inactivity are provided. The biomarkers may be blood biomarkers and include Tissue Inhibitor of Metalloprotease-1 (TIMP-1), Tenascin C (TNC), and Apolipoprotein A2 (ApoA-2). Also provided is a method of treating subjects at risk of losing lean body mass during a prolonged period of physical inactivity.

BIOMARKERS, RELATED METHODS AND SYSTEMS FOR PREDICTING LOSS OF MUSCLE MASS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and any benefit of U.S. Provisional Application No. 61/783,258, filed March 14, 2013, the entire content of which is incorporated herein by reference.

FIELD

[0002] The present disclosure relates to biomarkers. More particularly, the present disclosure relates to biomarkers, methods and systems for characterizing an adult subject's risk of losing lean body mass (LBM) during prolonged physical inactivity. The present disclosure also relates to methods of treating adult subjects who are at risk of losing LBM during prolonged physical inactivity.

BACKGROUND

[0003] Loss of muscle or lean body mass (LBM) can be an undesirable consequence of prolonged physical inactivity. Young adults have been shown to lose significant muscle mass and strength after 14 or 28 days of bed rest (Ferrando AA et al., *Am J. Physiol* 270: E627-E633, 1996) (Paddon-Jones, D. et al., *J Clin Endocrinol Metab* 89: 4351-4358, 2004). An even more dramatic loss of muscle mass occurs in healthy elderly adults after 10 days of bed rest. Elderly adults have been reported to lose approximately 1 kg of lean tissue from lower extremities after 10 days of bed rest (Kortebein P. et al., *J Gerontol A Biol Sci Med Sci* 63: 1076-1081, 2008) (Kortebein P., et al., *JAMA* 297: 1772-1774, 2007).

[0004] A majority of hospitalized patients that are 65 years or older have longer hospital stays (He W. et al., Current Population Reports 65+ in the United States. In: Bureau UC, edited by US Government Printing Office, p 23-209, 2005), in large part due to loss of functional capacity associated with decreases in muscle mass (Hoenig HM and Rubenstein LZ, *J Am Geriatr Soc* 39:220-222, 1991) (Creditor, HM, *Ann Intern Med* 118: 219-223, 1993). Approximately 65% of patients 65 years or older experience a decrease in ambulatory function and between 30% to 55% report a decline in activities

of daily living (Sager MA *et al.*, *Arch Intern Med Sci* 63:1076-1081, 2008) (Hirsch CH *et al.*, *J Am Geriatr Soc* 38: 1296-1303, 1990).

SUMMARY

[0005] Methods of treating a subject at risk of losing lean body mass (LBM) during a prolonged period of physical inactivity are provided herein. In certain embodiments the method comprises the steps of: (a) measuring levels of one or more biomarkers selected from Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject; (b) comparing the levels of the one or more biomarkers in the test subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples from a control population; and (c) administering β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof to the subject if the level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1 and/or if the level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3.

[0006] In certain embodiments the method of treating a subject at risk of losing LBM during prolonged physical inactivity comprises the steps of: (a) measuring levels of one or more sets of biomarkers selected from (i) TIMP-1 and Tenascin C (TNC) and (ii) MMP-3 and Apolipoprotein A2 (ApoA-2) in a biological sample obtained from the subject; (b) comparing the levels of the one or more sets of biomarkers in the subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from a control population; and (c) administering HMB to the subject if (i) the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for each of these biomarkers and/or (ii) the levels of MMP-3 and ApoA-2 in the test subject's biological sample are higher than the control values for each of these biomarkers.

[0007] Provided herein are methods for characterizing an adult human subject's risk of losing LBM during a prolonged period of physical inactivity. In one embodiment the method comprises: (a) measuring the levels of one or more biomarkers selected from the group consisting of Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix

Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject; (b) comparing the levels of the one or more biomarkers in the biological sample to one or more control values based on the levels of the one or more biomarkers in a control population; and (c) characterizing the test subject as being at risk of losing LBM during a prolonged period of physical inactivity if the level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1 and/or if the level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3. In certain embodiments, the method further comprises the steps of: (d) measuring the levels of one or more biomarkers selected from the group consisting of Tenascin C (TNC) and Apolipoprotein A2 (ApoA-2) in the subject's biological sample; (e) comparing the levels of the one or more biomarkers in the subject's biological sample to control values based on the levels of the one or more biomarkers in comparable biological samples from a control population; and (f) characterizing the subject as being at risk of losing LBM during a prolonged period of physical inactivity if (i) the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for TIMP-1 and TNC, respectively; and/or (ii) the levels of MMP-3 and Apo A2 in the test subject's biological sample are higher than the control value for MMP-3 and ApoA-2, respectively. In certain embodiments the method further comprises administering β -hydroxy- β -methylbutyrate (HMB) to the subject if the subject is characterized as being at risk of losing LBM during a prolonged period of physical inactivity.

[0008] Provided herein are systems for identifying and treating an adult human subject needing muscle preservation during a prolonged period of physical inactivity. The system comprises: (a) a subsystem for identifying an adult human subject undergoing physical inactivity or expected to undergo physical inactivity in the near future; (b) a subsystem for taking a biological sample from the subject identified in (a); (c) a subsystem for measuring the levels of one or more biomarkers in said biological sample, the biomarkers selected from the group consisting of (i) TIMP-1, (ii) TIMP-1 and TNC, (iii) MMP-3, and (iv) MMP-3 and ApoA-2; (d) a subsystem for comparing the measured levels of the one or more biomarkers to control values based on the levels of the one or more biomarkers in comparable biological samples obtained from a control population; and (e) a subsystem for characterizing the subject as being at risk of

needing muscle preservation during physical inactivity if (i) the levels of TIMP-1 in the subject's biological sample are higher than the control value of TIMP-1, and/or (b) the levels of MMP-3 in the subject's biological sample are lower than the control value for MMP-3, or (iii) the levels of TIMP-1 and TNC in the subject's biological sample are lower than their respective control values, and/or (iv) the levels of MMP-3 and ApoA-2 in the subject's biological sample are higher than their control values. In certain embodiments the system further comprises a subsystem for administering HMB to a subject characterized as being at risk for needing muscle preservation during the period of physical inactivity. In certain embodiments the system comprises a subsystem for administering one or more nutritional interventions selected from a high protein diet, high doses of leucine, and high doses of essential amino acids to a subject characterized as being at risk of needing muscle growth preservation during the period of physical inactivity.

[0009] In one exemplary embodiment, provided herein is a composition for use in the treatment of a subject in need of lean body mass preservation, wherein the composition comprises β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof, and wherein the subject to be treated is identified by a method comprising the steps of: (a) measuring levels of one or more biomarkers selected from Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject; (b) comparing the levels of the one or more biomarkers in the test subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples from a control population; and (c) determining that the subject is in need of lean body mass preservation when the level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1 and/or when the level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3.

[00010] In one exemplary embodiment, provided herein is a composition for use in the treatment of a subject in need of lean body mass preservation, wherein the composition comprises β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof, and wherein the subject to be treated is identified by a method comprising the steps of: (a) measuring levels of one or more sets of biomarkers selected from (i) TIMP-1 and

Tenascin C (TNC) and (ii) MMP-3 and Apolipoprotein A2 (ApoA-2) in a biological sample obtained from the subject; (b) comparing the levels of the one or more sets of biomarkers in the subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from a control population; and (c) determining that the subject is in need of lean body mass preservation when (i) the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for TIMP-1 and TNC, respectively; and/or (ii) the levels of MMP-3 and Apo A2 in the test subject's biological sample are higher than the control value for MMP-3 and ApoA-2, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

[00011] The foregoing and other features of the present invention will become apparent to those skilled in the art to which the present invention relates upon reading the following description with reference to the accompanying drawings, in which:

[00012] Figure 1 represents the change in total lean mass in individual subjects over 10-day bed rest in Control (●) and β -hydroxy- β -methylbutyrate (HMB)(▪). Lines with error bars represent mean $\pm$ SEM for each group. Values from all subjects (Control n=8; HMB n=10). Values are from all Control subjects (n=8) and HMB subjects (n=10), the difference between treatment groups is statistically significant (p=0.02, ANOVA).

[00013] Figure 2 represents the percent change in total lean mass in individual subjects over 10-day bed rest in Control (●) and HMB (▪). Lines with error bars represent mean $\pm$ SEM for each group. Values are from all subjects (Control n=8; HMB n=10). The difference between treatment groups is statistically significant (p=0.0383, ANOVA).

[00014] Figure 3 represents the change in leg lean body mass in individual subjects over 10-day bed rest in Control (●) and HMB (▪). Lines with error bars represent mean $\pm$ SEM for each group. Values are from all subjects (Control n=8; HMB n=10). The difference between treatment groups is statistically significant (p=0.02, ANOVA).

[00015] Figure 4 represents the percent change in leg lean mass in individual subjects over 10-day bed rest in Control (•) and HMB (▪). Lines with error bars represent mean $\pm$ SEM for each group. Values are from all subjects (Control n=8; HMB n=10). The difference between treatment groups is statistically significant ($p=0.0443$, ANOVA).

[00016] Figure 5 displays the percent loss of total lean body mass (TLBMPCT) in individual subjects (n=18; Control =8, HMB =10) (solid squares) as it correlates to levels of TIMP-1 and TenascinC (TNC) in blood.

[00017] Figure 6 displays the percent loss of total lean body mass (TLBMPCT) in individual female subjects (n=15; Control =7, HMB =8) (solid squares) as it correlates to levels of TIMP-1 and TenascinC (TNC) in blood.

[00018] Figure 7 displays the percent loss of leg lean body mass (LEBMPCT) in individual female subjects (n=15; Control= 7, HMB= 8) (solid squares) as it correlates to levels of MMP-3 and ApoA-2 in blood.

DETAILED DESCRIPTION

[00019] The present invention will now be described by reference to more detailed embodiments, with occasional reference to the accompanying drawings. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather these embodiments are provided so that this disclosure will be thorough and complete, and will convey the scope of the invention to those skilled in the art.

[00020] 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. The terminology used in the description of the invention herein is for describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[00021] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as

used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated, the numerical properties set forth in the following specification and claims are approximations that may vary depending on the desired properties sought to be obtained in embodiments of the present invention. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical values; however, inherently contain certain errors necessarily resulting from error found in their respective measurements.

[00022] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

METHODS AND SYSTEMS

[00023] Provided herein are methods and systems for characterizing a test subject's risk of losing lean body mass (LBM) during prolonged physical inactivity. Also provided are methods for treating subjects who are at risk of losing LBM during a prolonged period of physical inactivity. Also provided are methods of preserving lean body mass in a subject. As used herein the term "prolonged physical inactivity" refers to a condition or situation in which the subject seldom moves his or her limbs or body for 3 or more days. The present methods and systems may be used on a subject who is hospitalized

[00024] The present methods and systems may be used on a subject whose activities are restricted by others, *e.g.*, a subject who is restricted to bed rest by a physician or other health care provider. The present methods and systems may be used on a subject whose activities are limited due to surgery, injury, infirmity, frailty, old age, *etc.* The present methods and systems may be used on a subject whose physical inactivity is self-imposed, *e.g.* the subject is depressed, anxious, or has a phobia, *etc.*

[00025] As used herein, the term "risk of losing LBM during a prolonged period of physical inactivity" refers to a loss of 2% or more of LBM over a period of physical inactivity that lasts 3 to 10 days. Thus, the term refers to a loss of 2% or more of LBM over 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, or 10 days of

physical inactivity. The term also refers to a loss of 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10% or more of LBM over this time period. As used herein a loss of 6% or more over a 10 day period of physical inactivity is a “high loss”. A loss >2% to >6% over a 10 day period of physical inactivity is considered a moderate loss. A loss of 2% or less over a 10 day period of physical inactivity is considered acceptable or a “low loss”.

[00026] In one embodiment the method comprises: (a) measuring the levels of one or more biomarkers selected from the group consisting of Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject; (b) comparing the levels of the one or more biomarkers in the biological sample to a control value based on the levels of the one or more biomarkers in comparable biological samples from a control population; and (c) characterizing the subject as being at risk of losing LBM during a prolonged period of physical inactivity if the levels of TIMP-1 in the subject's biological sample are higher than the control value for TIMP-1 and/or if the levels of MMP-3 in the subject's biological sample are lower than the control value for MMP-3. In certain embodiments the subject is characterized as being at risk of a high loss of LBM during a prolonged period of physical inactivity if the levels of TIMP-1 in the subject's biological sample are higher than the control value for TIMP-1 and/or if the levels of MMP-3 in the subject's biological sample are lower than the control value for MMP-3. In certain embodiments, the method further comprises the step of administering β -hydroxy- β -methylbutyrate (HMB) to the subject if the subject is characterized as being at risk of losing LBM, particularly at risk of a high loss of LBM, during a prolonged period of physical inactivity.

[00027] In certain embodiments, the method further comprises: measuring the levels of one or more biomarkers selected from the group consisting of Tenascin C (TNC) and Apolipoprotein A2 (ApoA-2) in the subject's biological sample; comparing the levels of the one or more biomarkers to control values for the one or more biomarkers, and characterizing the subject as being at risk of losing LBM over a prolonged period of physical inactivity if the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values of TIMP-1 and TNC, respectively, and/or if the levels of MMP-3 and ApoA-2 in the subject's biological sample are higher than the control values of MMP-3 and ApoA-2, respectively. In certain embodiments the subject is

characterized as being at risk of a moderate loss of LBM over a prolonged period of physical inactivity if the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for TIMP-1 and TNC, respectively, and/or if the levels of MMP-3 and ApoA-2 in the subject's biological sample are higher than the control values for MMP-3 and ApoA-2, respectively. In certain embodiments the method further comprises administering HMB to the subject if the subject is characterized as being at risk of losing LBM or at risk of a moderate loss of LBM during a prolonged period of physical inactivity.

[00028] In certain embodiments HMB is first administered to the subject prior to the period of physical inactivity. In certain embodiments HMB is first administered to the subject when the period of physical inactivity begins. In certain embodiments the HMB is first administered to the subject within a few hours after the period of physical inactivity begins. In certain embodiments HMB is first administered to the subject within a day after the period of physical inactivity begins. In certain embodiments HMB is administered throughout the entire period of physical inactivity. In certain embodiments HMB is administered throughout the entire period of physical inactivity and for a period of time thereafter. In certain embodiments HMB is administered to the subject for up to one year. In certain embodiments HMB is administered to the subject for more than one year.

[00029] In certain embodiments the methods further comprise a step of administering one or more nutritional interventions selected from a high protein diet (*e.g.* >1 or 1.2 g protein /kg body weight/day), supplements containing high doses of leucine (~15 g/day) and/or leucine metabolites, and high doses of essential amino acids (~45 g/day containing at least 15 grams of leucine) if the subject has been characterized as being at risk of losing LBM during a prolonged period of physical inactivity. Examples of suitable leucine metabolites include, but are not limited to, HMB, alpha-ketoisocaproate (KIC), and alpha-hydroxyisocaproate (HICA). In certain embodiments, the method further comprises a step of administering an agent that preserves muscle to the subject. Examples of such agents include, but are not limited to, testosterone, growth hormone, selective androgen receptor modulators (SARMs), myostatin inhibitors, and combinations thereof.

[00030] In certain embodiments the nutritional intervention is first administered to the subject prior to the period of physical inactivity. In certain embodiments the nutritional intervention is first administered to the subject when the period of physical inactivity begins. In certain embodiments the nutritional intervention is first administered to the subject within a few hours after the period of physical inactivity begins. In certain embodiments the nutritional intervention is first administered to the subject within a day after the period of physical inactivity begins. In certain embodiments the nutritional intervention is administered throughout the entire period of physical inactivity. In certain embodiments the nutritional intervention is administered throughout the entire period of physical inactivity and for a period of time thereafter. In certain embodiments the nutritional intervention is administered to the subject for up to one year. In certain embodiments the nutritional intervention is administered to the subject for more than one year.

[00031] The systems for characterizing a subject's need for muscle preservation comprise (a) a sub-system for identifying a subject undergoing physical inactivity or expected to undergo physical inactivity in the near future; (b) a sub-system for taking a biological sample from the subject identified in (a); (c) a sub-system for measuring the levels of one or more biomarkers selected from TIMP-1 and MMP-3 in the biological sample obtained in (b); (d) a sub-system for comparing the levels of the one or more biomarkers measured in (c) to control values for the one or more biomarkers; and (e) a sub-system for characterizing a subject as needing muscle preservation during the physical inactivity if the levels of TIMP-1 in the subject's biological sample are higher than the control value for TIMP-1 and/or the levels of MMP-3 in the subject's blood sample are lower than the control value for MMP-3.

[00032] In certain embodiments, the system further comprises (f) a sub-system for measuring the levels of one or more biomarkers selected from TNC and ApoA-2 in the biological samples obtained in (b); (g) a sub-system for comparing the levels of the one or more biomarkers measured in (f) to control levels for the one or more biomarkers; and (h) a sub-system for characterizing a subject as needing muscle preservation during the physical inactivity if the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control levels of TIMP-1 and TNC,

respectively, and/or the levels of MMP-3 and ApoA-2 in the subject's biological sample are higher than the control levels of MMP-3 and ApoA-2, respectively. In certain embodiments, the systems described herein comprise a subsystem for identifying the age of the subject. This subsystem may be the same as or different from the subsystem that identifies the status of the subject, *i.e.*, whether the subject is undergoing physical inactivity or is expected to undergo physical inactivity.

[00033] The present systems may be used on subjects whose period of physical inactivity has already commenced, *e.g.*, the subject has already undergone a procedure that typically results in long periods of physical inactivity. The present systems may also be used on subjects who are expected to undergo a prolonged period of physical inactivity in the near future, *e.g.* the subject is scheduled to undergo a procedure that results in a prolonged period of bed rest within the next few days, the next few weeks or the next few months. For example, the subject may be scheduled to undergo a procedure (*e.g.*, surgery) that may lead to a prolonged period of physical inactivity within the next 1, 2, 3, 4, 5, 6, *etc.* months. As used herein the term a "prolonged period of physical inactivity" refers to a period of inactivity that lasts 3 days or more.

[00034] The present systems may be used by facilities housing subjects who may or are expected to undergo prolonged physical inactivity (*e.g.*, bed rest) for an extended period of time, such as for example, hospitals, rehabilitation centers, nursing homes, *etc.* All of the sub-systems may be used directly by the facility. Alternatively, some of the sub-systems may be used by testing facilities such as laboratories that report to or are directed to perform certain tests by the facility housing the subject. The present systems may also be used by physicians directing the care of subjects who are undergoing or are expected to undergo a prolonged period of physical inactivity at a hospital, rehabilitation facility, home setting, *etc.*

SUBJECTS

[00035] The methods and systems described herein typically are used to characterize an adult mammalian subject's risk of losing LBM during a prolonged period of physical inactivity. In certain embodiments, the subject is an adult human subject.

As used herein the term “adult human subject” refers to a human subject above age 20. Thus, the adult human subject can be 30, 40, or 50 years of age or more. In certain embodiments the subject is an “elderly human subject”, *i.e.*, a human subject 50 years of age, or older.

Biological Samples

[00036] Suitable biological samples useful for determining a subject's risk of losing LBM over a prolonged period of physical inactivity include, but are not limited, to whole blood samples, samples of blood fractions, including but not limited to serum and plasma. The sample may be fresh blood or stored blood (*e.g.* in a blood bank) or blood fractions. The sample may be a blood sample expressly obtained for the assays of this invention or a blood sample obtained for another purpose which can be subsampled for the assays of this invention.

[00037] In one embodiment, the biological sample is whole blood. Whole blood may be obtained from the subject using standard clinical procedures and subsystems. In another embodiment, the biological sample is plasma. Plasma may be obtained from whole blood samples by centrifugation of anti-coagulated blood. Such process provides a buffy coat of white cell components and a supernatant of the plasma. In another embodiment, the biological sample is serum. Serum may be obtained by centrifugation of whole blood samples that have been collected in tubes that are free of anti-coagulant. The blood is permitted to clot prior to centrifugation. The yellowish-reddish fluid that is obtained by centrifugation is the serum.

[00038] The sample may be pretreated as necessary by dilution in an appropriate buffer solution, heparinized, concentrated if desired, or fractionated by any number of methods including but not limited to ultracentrifugation, fractionation by fast performance liquid chromatography (FPLC). Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

[00039] Other biological samples that may be used in the present methods include, but are not limited to, urine and saliva.

Methods and Sub-Systems for Measuring Levels of the Biomarkers

[00040] Levels of TIMP-1, TNC, ApoA-2, or MMP-3 in the biological sample can be determined by various methods such as by using polyclonal or monoclonal antibodies that are immunoreactive with the respective biomarker or by using other binding agents such as aptamers or protein domains suitable for binding target from phase display libraries. For example, antibodies immunospecific for TIMP-1 may be made and labeled using standard procedures and then employed in immunoassays to detect the presence of TIMP-1 in the sample. Suitable immunoassays include, by way of example, radioimmunoassays, both solid and liquid phase, fluorescence-linked assays, competitive immunoassays, or enzyme-linked immunosorbent assays. In certain embodiments, the immunoassays are also used to quantify the amount of the biomarker that is present in the sample.

[00041] Each of the biomarkers can be used as an immunogen to produce antibodies immunospecific for the oxidized protein or peptide fragment. The term "immunospecific" means the antibodies have substantially greater affinity for the immunogen than for other proteins. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, and Fab fragments.

[00042] Antibodies raised against the select biomarker species are produced according to established procedures. Generally, the biomarker is used to immunize a host animal. Suitable host animals, include, but are not limited to, rabbits, mice, rats, goats, and guinea pigs. Various adjuvants may be used to increase the immunological response in the host animal. The adjuvant used depends, at least in part, on the host species. Such animals produce heterogeneous populations of antibody molecules, which are referred to as polyclonal antibodies and which may be derived from the sera of the immunized animals.

[00043] Polyclonal antibodies are generated using conventional techniques by administering the biomarker to a host animal. Depending on the host species, various adjuvants may be used to increase immunological response. Among adjuvants used in humans, Bacilli-Calmette-Guerin (BCG), and *Corynebacterium parvum* are especially preferable. Conventional protocols are also used to collect blood from the immunized animals and to isolate the serum and or the IgG fraction from the blood.

[00044] For preparation of monoclonal antibodies, conventional hybridoma techniques are used. Such antibodies are produced by continuous cell lines in culture. Suitable techniques for preparing monoclonal antibodies include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV hybridoma technique.

[00045] Various immunoassays may be used for screening to identify antibodies having the desired specificity. These include protocols that involve competitive binding or immunoradiometric assays and typically involve the measurement of complex formation between the respective biomarker and the antibody.

[00046] The present antibodies may be used to detect the presence of or measure the amount of biomarker in a biological sample from the subject. The method comprises contacting a sample taken from the individual with one or more of the present antibodies; and assaying for the formation of a complex between the antibody and the biomarker in the sample. For ease of detection, the antibody can be attached to a substrate such as a column, plastic dish, matrix, or membrane, preferably nitrocellulose. In certain embodiments, the method employs an enzyme-linked immunosorbent assay (ELISA) or a Western immunoblot procedure.

[00047] The presence or amount of one or more biomarkers can be determined using antibodies that specifically bind to each marker as well as any additional biomarkers if such additional biomarkers are used. Examples of antibodies that can be used include a polyclonal antibody, a monoclonal antibody, a human antibody, an immunoglobulin molecule, a disulfide linked Fv, a monoclonal antibody, an affinity matured, a scFv, a chimeric antibody, a single domain antibody, a CDR-grafted antibody, a diabody, a humanized antibody, a multispecific antibody, a Fab, a dual specific antibody, a DVD, a Fab', a bispecific antibody, a F(ab')₂, a Fv and combinations thereof. For example, the immunological method may include (a) measuring the levels of a biomarker by: (i) contacting the test sample with at least one capture antibody, wherein the capture antibody binds to an epitope on the biomarker or a fragment thereof to form a capture antibody- antigen complex; (ii) contacting the capture antibody- antigen complex with at least one detection antibody comprising a detectable label, wherein the detection antibody binds to an epitope on the biomarker (antigen) that is not

bound by the capture antibody and forms a capture antibody- antigen-detection antibody complex; and (iii) determining the biomarker level in the test sample based on the signal generated by the detectable label in the capture antibody -antigen-detection antibody complex formed in (a)(ii). Any immunoassay may be utilized. The immunoassay may be an enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), a competitive inhibition assay, such as forward or reverse competitive inhibition assays, a fluorescence polarization assay, or a competitive binding assay, for example. The ELISA may be a sandwich ELISA. Specific immunological binding of the antibody to the marker can be detected via direct labels, such as fluorescent or luminescent tags, metals and radionuclides attached to the antibody or via indirect labels, such as alkaline phosphatase or horseradish peroxidase.

[00048] The use of immobilized antibodies or fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material, and the like. An assay strip can be prepared by coating the antibody or plurality of antibodies in an array on a solid support. This strip can then be dipped into the test biological sample and then processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

[00049] The sandwich ELISA measures the amount of antigen between two layers of antibodies (*i.e.* a capture antibody and a detection antibody (which may be labeled with a detectable label)). The marker to be measured may contain at least two antigenic sites capable of binding to antibody. Either monoclonal or polyclonal antibodies may be used as the capture and detection antibodies in the sandwich ELISA.

[00050] Generally, at least two antibodies are employed to separate and quantify the marker of interest (as well as any additional biomarkers), in a test or biological sample. More specifically, the at least two antibodies bind to certain epitopes of the marker forming an immune complex which is referred to as a "sandwich". One or more antibodies can be used to capture the marker in the test sample (these antibodies are frequently referred to as a "capture" antibody or "capture" antibodies) and one or more antibodies is used to bind a detectable (namely, quantifiable) label to the

sandwich (these antibodies are frequently referred to as the "detection" antibody or "detection" antibodies). In a sandwich assay, both antibodies binding to their epitope may not be diminished by the binding of any other antibody in the assay to its respective epitope. In other words, antibodies may be selected so that the one or more first antibodies brought into contact with a test sample suspected of containing the marker do not bind to all or part of an epitope recognized by the second or subsequent antibodies, thereby interfering with the ability of the one or more second detection antibodies to bind to the marker.

[00051] In a preferred embodiment, a test or biological sample suspected of containing the marker can be contacted with at least one first capture antibody (or antibodies) and at least one second detection antibody, either simultaneously or sequentially. In the sandwich assay format, a test sample suspected of containing the marker is first brought into contact with the at least one first capture antibody that specifically binds to a particular epitope under conditions which allow the formation of a first antibody-marker complex. If more than one capture antibody is used, a first multiple capture antibody-marker complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of marker expected in the test sample.

[00052] Optionally, prior to contacting the test sample with the at least one first capture antibody, the at least one first capture antibody can be bound to a solid support which facilitates the separation of the first antibody-marker complex from the test sample. Any solid support known in the art can be used, including but not limited to, solid supports made out of polymeric materials in the forms of wells, tubes or beads. The antibody (or antibodies) can be bound to the solid support by adsorption, by covalent bonding using a chemical coupling agent or by other means known in the art, provided that such binding does not interfere with the ability of the antibody to bind the marker. Moreover, if necessary, the solid support can be derivatized to allow reactivity with various functional groups on the antibody. Such derivatization requires the use of certain coupling agents such as, but not limited to, maleic anhydride, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

[00053] After the test sample suspected of containing the marker is brought into contact with the at least one first capture antibody, the test sample is incubated in order to allow for the formation of a first capture antibody (or multiple antibody)-marker complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2°C to about 45°C, and for a period from at least about one (1) minute to about eighteen (18) hours, from about 2-6 minutes, or from about 3-4 minutes.

[00054] After formation of the first/multiple capture antibody-marker complex, the complex is then contacted with at least one second detection antibody (under conditions which allow for the formation of a first/multiple antibody-marker second antibody complex). If the first antibody-marker complex is contacted with more than one detection antibody, then a first/multiple capture antibody-marker-multiple antibody detection complex is formed. As with first antibody, when the at least second (and subsequent) antibody is brought into contact with the first antibody-marker complex, a period of incubation under conditions similar to those described above is required for the formation of the first/multiple antibody-marker-second/multiple antibody complex. Preferably, at least one second antibody contains a detectable label. The detectable label can be bound to the at least one second antibody prior to, simultaneously with or after the formation of the first/multiple antibody-marker-second/multiple antibody complex. Any detectable label known in the art can be used.

Kits for Performing the Methods

[00055] Provided herein is a kit, which may be used for performing the methods described above. The kit may provide (1) reagents capable of specifically binding to the marker to quantify the levels of the marker, in a biological sample isolated from a subject (2) a reference standard indicating reference level of the marker, wherein at least one reagent comprises at least one antibody capable of specifically binding the marker; and (3) a reference standard. The kit may further comprise at least one reagent capable of specifically binding (*i.e.*, an antibody) at least one additional biomarker and a reference standard indicating a reference level of the at least one additional biomarker of the condition being assessed, if present.

[00056] The kit may comprise the antibodies and a means for administering the antibodies. The kit can further comprise instructions for using the kit and conducting the analysis, monitoring, or treatment.

[00057] The kit may also comprise one or more containers, such as vials or bottles, with each container containing a separate reagent. The kit may further comprise written instructions, which may describe how to perform or interpret an analysis, monitoring, treatment, or method described herein.

[00058] For example, the kit can comprise instructions for assaying the test sample for one or more biomarkers by immunoassay, *e.g.*, chemiluminescent microparticle immunoassay. The instructions can be in paper form or computer-readable form, such as a disk, CD, DVD, or the like. The antibody can be a detection antibody (meaning an antibody labeled with a detectable label). For example, the kit can contain at least one capture antibody that specifically binds the antigen or biomarker of interest. The kit can also contain a conjugate antibody (such as an antibody labeled with a detectable label) for each capture antibody. Alternatively or additionally, the kit can comprise a calibrator or control, *e.g.*, purified, and optionally lyophilized, and/or at least one container (*e.g.*, tube, microtiter plates or strips, which can be already coated with an anti-biomarker monoclonal antibody) for conducting the assay, and/or a buffer, such as an assay buffer or a wash buffer, either one of which can be provided as a concentrated solution, a substrate solution for the detectable label (*e.g.*, an enzymatic label), or a stop solution. Preferably, the kit comprises all components, *i.e.*, reagents, standards, buffers, diluents, *etc.*, which are necessary to perform the assay. The instructions also can include instructions for generating a standard curve or a reference standard for purposes of quantifying the biomarker of interest.

[00059] As alluded to above, any antibodies, which are provided in the kit, such as recombinant antibodies specific for the biomarker, can incorporate a detectable label, such as a fluorophore, radioactive moiety, enzyme, biotin/avidin label, chromophore, chemiluminescent label, or the like, or the kit can include reagents for labeling the antibodies or reagents for detecting the antibodies (*e.g.*, detection antibodies) and/or for labeling the analytes or reagents for detecting the analyte. The

antibodies, calibrators and/or controls can be provided in separate containers or pre-dispensed into an appropriate assay format, for example, into microtiter plates.

[00060] Optionally, the kit includes quality control components (for example, sensitivity panels, calibrators, and positive controls). Preparation of quality control reagents is well-known in the art and is described on insert sheets for a variety of immunodiagnostic products. Sensitivity panel members optionally are used to establish assay performance characteristics, and further optionally are useful indicators of the integrity of the immunoassay kit reagents, and the standardization of assays.

[00061] The kit can also optionally include other reagents required to conduct a diagnostic assay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), also can be included in the kit. The kit can additionally include one or more other controls. One or more of the components of the kit can be lyophilized, in which case the kit can further comprise reagents suitable for the reconstitution of the lyophilized components.

[00062] The various components of the kit optionally are provided in suitable containers as necessary, e.g., a microtiter plate. The kit can further include containers for holding or storing a sample (e.g., a container or cartridge for a blood sample). Where appropriate, the kit optionally also can contain reaction vessels, mixing vessels, and other components that facilitate the preparation of reagents or the test sample. The kit can also include one or more instrument for assisting with obtaining a test sample, such as a syringe, pipette, forceps, measured spoon, or the like.

[00063] If the detectable label is at least one acridinium compound, the kit can comprise at least one acridinium-9-carboxamide, at least one acridinium-9-carboxylate aryl ester, or any combination thereof. If the detectable label is at least one acridinium compound, the kit also can comprise a source of hydrogen peroxide, such as a buffer, solution, and/or at least one basic solution.

[00064] If desired, the kit can contain a solid phase, such as a magnetic particle, bead, test tube, microtiter plate, cuvette, membrane, scaffolding molecule, film, filter paper, a quartz crystal, disc or chip. The kit may also include a detectable label

that can be or is conjugated to an antibody, such as an antibody functioning as a detection antibody. The detectable label can for example be a direct label, which may be an enzyme, oligonucleotide, nanoparticle, chemiluminophore, fluorophore, fluorescence quencher, chemiluminescence quencher, or biotin. Kits may optionally include any additional reagents needed for detecting the label.

[00065] If desired, the kit can further comprise one or more components, alone or in further combination with instructions, for assaying the test sample for another analyte, which can be a biomarker, such as a biomarker of another condition of interest. A sample, such as a serum sample, can also be assayed for an additional biomarker using TOF-MS and an internal standard.

[00066] The kit (or components thereof), as well as the method of determining the concentration of the biomarker in a test sample by an immunoassay as described herein, can be adapted for use in a variety of automated and semi-automated systems (including those wherein the solid phase comprises a microparticle), as described, *e.g.*, in U.S. Patent Nos. 5,089,424 and 5,006,309, and as commercially marketed, *e.g.*, by Abbott Laboratories (Abbott Park, IL) as ARCHITECT®.

[00067] Some of the differences between an automated or semi-automated system as compared to a non-automated system (*e.g.*, ELISA) include the substrate to which the first specific binding partner (*e.g.*, analyte antibody or capture antibody) is attached (which can impact sandwich formation and analyte reactivity), and the length and timing of the capture, detection and/or any optional wash steps. Whereas a non-automated format such as an ELISA may require a relatively longer incubation time with sample and capture reagent (*e.g.*, about 2 hours), an automated or semi-automated format (*e.g.*, ARCHITECT® and any successor platform, Abbott Laboratories) may have a relatively shorter incubation time (*e.g.*, approximately 18 minutes for ARCHITECT®). Similarly, whereas a non-automated format such as an ELISA may incubate a detection antibody such as the conjugate reagent for a relatively longer incubation time (*e.g.*, about 2 hours), an automated or semi-automated format (*e.g.*, ARCHITECT® and any successor platform) may have a relatively shorter incubation time (*e.g.*, approximately 4 minutes for the ARCHITECT® and any successor platform).

[00068] Other platforms available from Abbott Laboratories include, but are not limited to, AxSYM®, IMx® (see, e.g., U.S. Pat. No. 5,294,404, which is hereby incorporated by reference in its entirety), PRISM®, EIA (bead), and Quantum™ II, as well as other platforms. Additionally, the assays, kits and kit components can be employed in other formats, for example, on electrochemical or other hand-held or point-of-care assay systems. The present disclosure is, for example, applicable to the commercial Abbott Point of Care (i-STAT®, Abbott Laboratories) electrochemical immunoassay system that performs sandwich immunoassays. Immunosensors and their methods of manufacture and operation in single-use test devices are described, for example in, U.S. Patent No. 5,063,081, U.S. Pat. App. Pub. No. 2003/0170881, U.S. Pat. App. Pub. No. 2004/0018577, U.S. Pat. App. Pub. No. 2005/0054078, and U.S. Pat. App. Pub. No. 2006/0160164, which are incorporated in their entireties by reference for their teachings regarding same.

[00069] In particular, with regard to the adaptation of an assay to the I-STAT® system, the following configuration is preferred. A microfabricated silicon chip is manufactured with a pair of gold amperometric working electrodes and a silver-silver chloride reference electrode. On one of the working electrodes, polystyrene beads (0.2 mm diameter) with immobilized capture antibody are adhered to a polymer coating of patterned polyvinyl alcohol over the electrode. This chip is assembled into an I-STAT® cartridge with a fluidics format suitable for immunoassay. On a portion of the wall of the sample-holding chamber of the cartridge there is a layer comprising the detection antibody labeled with alkaline phosphatase (or other label). Within the fluid pouch of the cartridge is an aqueous reagent that includes p-aminophenol phosphate.

[00070] In operation, a sample suspected of containing the biomarker is added to the holding chamber of the test cartridge and the cartridge is inserted into the I-STAT® reader. After the second antibody (detection antibody) has dissolved into the sample, a pump element within the cartridge forces the sample into a conduit containing the chip. Here it is oscillated to promote formation of the sandwich between the first capture antibody, the biomarker, and the labeled second detection antibody. In the penultimate step of the assay, fluid is forced out of the pouch and into the conduit to wash the sample off the chip and into a waste chamber. In the final step of the assay,

the alkaline phosphatase label reacts with p-aminophenol phosphate to cleave the phosphate group and permit the liberated p-aminophenol to be electrochemically oxidized at the working electrode. Based on the measured current, the reader is able to calculate the amount of biomarker in the sample by means of an embedded algorithm and factory-determined calibration curve.

Control Value

[00071] Levels of each of the biomarkers in a biological sample from the test subject used in the present methods are compared to a control value. The control value is based upon levels of the respective biomarker in comparable biological samples obtained from a control population, *e.g.*, the general population or a select population of human subjects. For example, the select population may be comprised of male subjects or female subjects, elderly subjects, and/or subjects with similar body mass indices (BMIs), *etc.* Accordingly, the control values selected may take into account the category into which the test subject falls. Appropriate categories can be selected with no more than routine experimentation by those of ordinary skill in the art.

[00072] The control value can take a variety of forms. The control value can be a single cut-off value, such as a median or mean. The control value can be a range of values. The control value can be established based upon comparative groups such as where the risk in one defined group is double the risk in another defined group. The control values can be divided equally (or unequally) into groups, such as a low risk group, a medium risk group and a high-risk group, or into quadrants, the lowest quadrant being individuals with the lowest risk the highest quadrant being individuals with the highest risk, and the test subject's risk of having losing muscle mass can be based upon which group his or her test value falls.

[00073] Control values of TIMP-1, TNC, ApoA-2, or MMP-3, such as for example, mean levels, median levels, or "cut-off" levels, are established by assaying a large sample of individuals in the general population or the select population and using a statistical model such as the predictive value method for selecting a positivity criterion or receiver operator characteristic curve that defines optimum specificity (highest true negative rate) and sensitivity (highest true positive rate) as described in Knapp, R. G.,

and Miller, M. C. (1992). Clinical Epidemiology and Biostatistics. William and Wilkins, Harual Publishing Co. Malvern, Pa., which is specifically incorporated herein by reference. A "cutoff" value can be determined for each biomarker that is assayed. In addition, reference intervals or expected values for the general population or the select population can be established by following the guidance from the Clinical and Laboratory Standards Institute (CLSI), document C28-A3c (2011).

Comparison of TIMP-1, TNC, ApoA-2, or MMP-3 Levels from the Test Subject to the Control Value

[00074] Levels of each select biomarker, *i.e.*, TIMP-1, TNC, ApoA-2, or MMP-3 in the individual's biological sample may be compared to a single control value or to a range of control values. If the level of TIMP-1 in the test subject's biological sample is greater than the control value or exceeds or is in the upper range of control values for TIMP-1, the subject is at greater risk of losing muscle mass due to prolonged physical inactivity than individuals with TIMP-1 levels comparable to or below the control value or in the lower range of control values. If the level of MMP-3 in the subject's biological sample is lower than the control value or is in the lower range of control values for this biomarker, the test subject is at greater risk of losing muscle mass due to prolonged physical inactivity than individuals with MMP-3 levels comparable to or greater than the control value or in the lower range of control values. If the levels of TIMP-1 and TNC in the subject's biological sample are both below the control value or are both in the lower range of control values for each of these biomarkers, the test subject is at greater risk of losing muscle mass due to prolonged physical inactivity than individuals whose levels of TIMP-1 are lower than the control value for TIMP-1 and whose levels of TNC are higher than the control value for TNC or are in the upper range of control values for TNC. In other words, even if the subject's biological levels of TIMP-1 are lower than the control value for TIMP-1, if that subject's biological levels of TNC are also lower than the control value for TNC, then the individual subject is at a risk of losing muscle mass due to prolonged physical inactivity. If the levels of both MMP-3 and ApoA-2 in the subject's biological samples are both higher than the control values for these biomarkers or are in the higher range of control values for these biomarkers, the subject is at a risk of losing LBM due to prolonged physical inactivity than subjects

whose levels of MMP-3 are higher than the control value for MMP-3 and whose levels of ApoA-2 are lower than the control value for ApoA-2. In other words, even if the test subject's biological levels of MMP-3 are higher than the control value for MMP-3, if that subject's biological levels of ApoA-2 are also higher than the control value for ApoA-2, then the individual subject is at risk of losing muscle mass due to prolonged physical inactivity. The extent of the difference between the test subject's risk predictor levels and control value is also useful for characterizing the extent of the risk and thereby, determining which individuals would most greatly benefit from certain aggressive therapies. In those cases, where the control value ranges are divided into a plurality of groups, such as the control value ranges for individuals at high risk, average risk, and low risk, the comparison involves determining into which group the test subject's level of the relevant risk predictor falls.

Treatment of at Risk Subjects

[00075] In certain embodiments, the present methods comprise a step of administering HMB to a subject that has been characterized as being at risk of losing LBM over a prolonged period of physical inactivity. In certain embodiments HMB is first administered to the subject prior to period of physical inactivity. In certain embodiments the HMB is first administered to the subject on the day the period of physical inactivity begins or within a day thereafter. In certain embodiments HMB is administered to the subject throughout the period of physical inactivity. In certain embodiments HMB is administered to the subject throughout the period of physical inactivity and for a period of time thereafter.

[00076] Alternatively, the present methods may comprise a step of administering nutritional interventions such as one or more of high protein diets (*e.g.* >1 or 1.2 g protein /kg body weight/day), supplements containing high doses of leucine (~15 g/day) or leucine metabolites, and/or high doses of essential amino acids (~45 g/day containing at least 15 grams of leucine). Examples of suitable leucine metabolites include, but are not limited to, HMB, alpha-ketoisocaproate (KIC), and alpha-hydroxyisocaproate (HICA). In certain embodiments the nutritional interventions or supplements are first administered to the subject before the period of physical inactivity commences. In certain embodiments the nutritional intervention is first

administered to the subject on the day the period of physical inactivity begins or within a day thereafter. In certain embodiments the nutritional intervention is administered to the subject throughout the period of physical inactivity and for a period of time thereafter.

[00077] The term HMB, which is also referred to as β -hydroxy- β -methylbutyric acid, or β -hydroxy-isovaleric acid, can be represented in its free acid form as $(\text{CH}_3)_2(\text{OH})\text{CCH}_2\text{COOH}$. HMB is a metabolite of leucine formed by transamination to α -ketoisocaproate (KIC) in muscle followed by oxidation of the KIC in the cytosol of the liver to give HMB. While any suitable form of HMB can be used within the context of the present invention, preferably, HMB is selected from the group consisting of a free acid, a salt, an ester, and a lactone; more preferably, HMB is in the form of a non-toxic, edible salt. Preferably, the HMB salt is water-soluble or becomes water-soluble in the stomach or intestines of a patient. More preferably, the HMB salt is selected from the group consisting of a sodium salt, a potassium salt, a magnesium salt, a chromium salt, and a calcium salt. However, other non-toxic salts, such as other alkali metal or alkaline earth metal salts, can be used.

[00078] Similarly, any pharmaceutically acceptable ester can be used in the context of the present invention. Desirably, the HMB ester is rapidly converted to HMB in its free acid form. Preferably, the HMB ester is a methyl ester or ethyl ester. HMB methyl ester and HMB ethyl ester are rapidly converted to the free acid form of HMB. Likewise, any pharmaceutically acceptable lactone can be used in the context of the present invention. Desirably, the HMB lactone is rapidly converted to HMB in its free acid form. Preferably, the HMB lactone is an isovaleryl lactone or a similar lactone. Such lactones are rapidly converted to the free acid form of HMB.

[00079] Methods for producing HMB and its derivatives are well known in the art. For example, HMB can be synthesized by oxidation of diacetone alcohol. One suitable procedure is described by Coffman et al., J. Am. Chem. Soc. 80: 2882-2887 (1958). As described therein, HMB is synthesized by an alkaline sodium hypochlorite oxidation of diacetone alcohol. The product is recovered in free acid form, which can be converted to the desired salt. For example, 3-hydroxy-3-methylbutyric acid (HMBA) can be synthesized from diacetone alcohol (4-hydroxy-4-methylpentan-2-one) via oxidation using cold, aqueous hypochlorite (bleach). After acidifying the reaction mixture using

HCl, the HMBA product is recovered by extraction using ethyl acetate, and separating and retaining the organic layer from the extraction mixture. The ethyl acetate is removed by evaporation and the residue dissolved in ethanol. After addition of Ca(OH)_2 and cooling, crystalline CaHMB can be recovered by filtration, the crystals washed with ethanol and then dried. Alternatively, the calcium salt of HMB is commercially available from Technical Sourcing International (TSI) of Salt Lake City, Utah.

[00080] The routes for administering HMB include an oral diet, tube feeding and peripheral or total parenteral nutrition. The preferred embodiment for the present methods is by the oral route. An alternate to oral feeding is tube feeding by means of nasogastric, nasoduodenal, esophagostomy, gastrostomy, or jejunostomy tubes.

[00081] The beneficial effects that HMB has on preservation of LBM in a subject who is at risk of losing muscle due to a prolonged period of bed rest or immobilization or age can be achieved in a number of ways. If desired, the HMB may be administered alone, without a carrier. The HMB may simply be dissolved in water and consumed by the patient. Alternatively, the HMB may be sprinkled on food, dissolved in coffee, *etc.* The total daily dose for the patient will vary widely, but typically a patient will benefit from consuming at least 2 g/day of HMB, 2-5 g/day, or, alternatively, from 20 to 40 mg/kg body weight/day.

[00082] In a further embodiment, the HMB may be incorporated into pills, capsules, rapidly dissolved tablets, lozenges, *etc.* The active dose can vary widely, but will typically range from 250 mg to 1 g/dose with the patient consuming from 2 to 8 doses/day to achieve the target of either 2 g/day minimum or 2-5 g/day. Methods for preparing such dosage forms are well known in the art. The reader's attention is directed to the most recent edition of Remingtons Pharmaceutical Sciences for guidance on how to prepare such dosage forms.

[00083] In a further embodiment, the HMB may be combined with other nutritional supplements such as amino acids. One example of such supplement is Juven®, a powder (sachet) containing 1.5 grams of HMB, 5 grams of arginine, and 5 grams of glutamine.

Nutritional Matrices

[00084] While the HMB may be administered as a single entity, it will typically be incorporated into food products and consumed by the patient during their meals or snack. If desired, the patient may simply modify the recipe of foods they normally consume by sprinkling on food, dissolving in coffee, *etc.*

[00085] In a further embodiment, the HMB will be incorporated into beverages, bars, cookies, *etc.* that have been specifically designed to enhance the palatability of the HMB and increase the selection of alternative forms, thereby enhancing patient/consumer acceptance.

[00086] Typically, the HMB will be incorporated into meal replacement beverages such as Ensure®, Boost®, Glucerna®. The HMB may also be incorporated into meal replacement bars. Alternatively, the HMB may be incorporated into juices, carbonated beverages, bottled water, *etc.* Additionally, the HMB may be incorporated into medical nutritionals designed to support specific disease states. Methods for producing any of such food products are well known to those skilled in the art. The following discussion is intended to illustrate such food products and their preparation.

[00087] Most meal replacement products (*i.e.*, bars or liquids) provide calories from fat, carbohydrates, and protein. These products also typically contain vitamins and minerals, because they are intended to be suitable for use as the sole source of nutrition. While these meal replacement products may serve as the sole source of nutrition, they typically don't. Individuals consume these products to replace one or two meals a day, or to provide a healthy snack. The nutritional products of this invention should be construed to include any of these embodiments. In some embodiments, the nutritional products, such as Ensure Clear®, lack fat.

[00088] Suitable protein sources include, but are not limited to, milk, whey and whey fractions, soy, rice, meat (*e.g.*, beef), animal and vegetable (*e.g.*, pea, potato), egg (egg albumin), gelatin and fish. Suitable intact protein sources include, but are not limited to, soy based, milk based, casein protein, whey protein, rice protein, beef collagen, pea protein, potato protein, and mixtures thereof.

[00089] Optionally, the intact protein source is enriched in large neutral amino acids (LNAA) comprising valine, isoleucine, leucine, threonine, tyrosine and

phenylalanine. Typically, about 40% of casein, whey and soy protein sources are large neutral amino acids. For example, caseinate contains about 38 wt/wt % LNAA, whey protein concentrate contains about 39 wt/wt % LNAA and soy protein isolate contains about 34 wt/wt % LNAA. Typically, the meal replacement is formulated with a protein source that will deliver about 1 to 25 grams of LNAA per day, preferably from about 1 to 20 grams of LNAA per day, more preferably from about 4 to 20 grams of LNAA per day. As an example, a meal replacement consumed 3 times a day that contains a protein comprising 4.8 grams LNAA will deliver 14.4 grams LNAA per day.

[00090] The meal replacements preferably also contain vitamins and minerals in an amount designed to supply or supplement the daily nutritional requirements of the person receiving the formula. Those skilled in the art recognize that nutritional formulas often include overages of certain vitamins and minerals to ensure that they meet targeted level over the shelf life of the product. These same individuals also recognize that certain micro ingredients may have potential benefits for people depending upon any underlying illness or disease that the patient is afflicted with. For example, cancer patients benefit from such antioxidants as beta-carotene, vitamin E, vitamin C and selenium. The meal replacements preferably include, but are not limited to, the following vitamins and minerals: calcium, phosphorus, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, chromium, molybdenum, conditionally essential nutrients m-inositol, carnitine and taurine, and Vitamins A, C, D, E, K and the B complex, and mixtures thereof.

[00091] In addition to fiber, the meal replacements may also contain oligosaccharides such as fructooligosaccharides (FOS) or glucooligosaccharides (GOS). Oligosaccharides are rapidly and extensively fermented to short chain fatty acids by anaerobic microorganisms that inhabit the large bowel. These oligosaccharides are preferential energy sources for most Bifidobacterium species, but are not utilized by potentially pathogenic organisms such as Clostridium perfringens, C. difficile, or Escherichia coli.

[00092] Typically, the FOS comprises from 0 to 5 g/serving of the meal replacement, preferably from 1 to 5 g/serving, more preferably from 2 to 4 g/serving of the meal replacement.

[00093] The meal replacements may also contain a flavor to enhance its palatability. Artificial sweeteners may be added to complement the flavor and mask salty taste. Useful artificial sweeteners include saccharin, nutrasweet, sucralose, acesulfane-K (ace-K), *etc.* Meal replacements can be manufactured using techniques well known to those skilled in the art.

[00094] Solid compositions such as bars, cookies, *etc.* may also be manufactured utilizing techniques known to those skilled in the art. For example, they may be manufactured using cold extrusion technology as is known in the art. To prepare such compositions, typically all of the powdered components will be dry blended together. Such constituents typically include the proteins, vitamin premixes, certain carbohydrates, *etc.* The fat-soluble components are then blended together and mixed with the powdered premix above. Finally any liquid components are then mixed into the composition, forming a plastic-like composition or dough.

[00095] The process above is intended to give a plastic mass that can then be shaped, without further physical or chemical changes occurring, by the procedure known as cold forming or extrusion. In this process, the plastic mass is forced at relatively low pressure through a die, which confers the desired shape. The resultant extrudate is then cut off at an appropriate position to give products of the desired weight. If desired the solid product is then coated, to enhance palatability, and packaged for distribution.

[00096] The solid compositions of the instant invention may also be manufactured through a baked application or heated extrusion to produce cereals, cookies, and crackers. One knowledgeable in the arts would be able to select one of the many manufacturing process.

[00097] As noted above, the HMB may also be incorporated into juices, non-carbonated beverages, carbonated beverages, electrolyte solutions, flavored waters (hereinafter collectively "beverage"), *etc.* The HMB will typically comprise from 0.5 to 2 g/serving of the beverages. Methods for producing such beverages are well known in the art. The reader's attention is directed to U. S. Pat. Nos. 6,176,980 and 5,792,502, the contents of each which are hereby incorporated by reference. For example, all of the ingredients, including the HMB are dissolved in an appropriate

volume of water. Flavors, colors, vitamins, *etc.* are then optionally added. The mixture is then pasteurized, packaged and stored until shipment.

EXAMPLES

[00098] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention.

[00099] The present methods and systems are based, at least in part, on the inventors' discovery that the levels in blood of four lead biomarkers, namely TIMP-1, TNC, ApoA-2, and MMP-3, correlate with loss of LBM following 10 days of bed rest in 18 healthy elderly patients. The blood levels of these markers correlate with absolute changes in lean leg mass and total lean mass in these patients. The blood levels of these blood markers also correlate with percent change of total lean mass and percent change of leg lean mass in these patients. These latter outcomes were evaluated to take into account the baseline variability in lean mass of subjects based on body mass index (BMI). These studies showed that baseline levels of these four biomarkers can be used to predict the likelihood for muscle loss over the course of extended immobilization.

SUBJECTS

[000100] The following inclusion criteria were verified at screening: male or female ≥ 60 to ≤ 79 years of age; body mass index (BMI) ≥ 20 but ≤ 35 ; ambulatory with a Short Performance Physical Battery (SPPB) score of ≥ 9 (fully functional with no mobility limitations); compliance with prescribed activity level. Exclusion criteria ruled out subjects who had undergone recent major surgery, had active malignancy (exception basal or squamous cell skin carcinoma or carcinoma in situ of the uterine cervix); history of Deep Vein Thrombosis (DVT) or other hypercoagulation disorders; refractory anemia; history of diabetes or fasting blood glucose value > 126 mg/dL; presence of partial or full artificial limb; kidney disease or serum creatinine > 1.4 mg/dL; evidence of cardiovascular disease assessed during resting or exercise EKG; untreated

hypothyroidism; liver disease; chronic or acute GI disease; uncontrolled severe diarrhea, nausea or vomiting; were actively pursuing weight loss; were enrolled in other clinical trials; could not refrain from smoking over the bed rest study period or could not discontinue anticoagulant therapy over bed rest period. Potential subjects were also excluded if they were taking any medications known to affect protein metabolism (e.g. progestational agents, steroids, growth hormone, dronabinol, marijuana, HMB, free amino acid supplements, dietary supplements to aid weight loss).

[000101] The healthy subjects initially involved in the study were randomized into two groups. Subjects in the treatment group received two β -hydroxy- β -methylbutyrate (HMB) sachets containing 1.5 grams Ca-HMB-monohydrate (TSI, Salt Lake City, Utah), 4 grams maltodextrin and 200 milligrams Calcium with additional sweetener and flavoring groups. Subjects in the control group received two control sachets that were identical to the HMB sachets with the exclusion of Ca-HMB. This study was a double-blinded study. Neither the investigators nor the subjects were informed of the identity of any of the study products during the clinical portion of the study. Subjects were instructed to consume twice daily by mixing each sachet into a non-caloric, non-caffeinated, non-carbonated, non-milk-based beverage of their choice. Treatment with HMB or Control was initiated 5 days prior to bed rest and was continued until the end of the rehabilitation period.

[000102] For diet stabilization over the pre-bed rest and bed rest period, subjects were fed a metabolically controlled diet providing the RDA for protein intake (0.8 g protein/kg body weight per day). Total calorie needs were estimated using the Harris-Benedict equation for resting energy expenditure according to the following equation: For women= $[655 + (9.56 \times \text{body weight in kg}) + (1.85 \times \text{height in cm}) - (4.68 \times \text{age in years})] \times \text{AF}$, or, For men = $[66 + (13.7 \times \text{body weight in kg}) + (5 \times \text{height in cm}) - (608 \times \text{age in years})] \times \text{AF}$, where AF= activity factor of 1.6 for the ambulatory and 1.35 for the bed rest periods. Given the total calorie and protein intakes, the remainder of the diet was manipulated to keep the non-protein calories at about 60% from carbohydrates and 40% from fat. Water was provided *ad libitum*.

[000103] After a diet stabilization of 5 days (ambulatory period), subjects remained in bed continuously for 10 days. While confined to bed rest, subjects were

allowed to use the bedside commode for urination or were taken in a wheelchair for toileting. Subjects were given the option of taking a sponge bath or showering in a wheelchair. Prophylactic measures were taken to detect and prevent deep vein thrombosis including a blood d-dimer test followed by an ultrasound examination if d-dimer test was positive, passive range of motion exercise during bed rest, the use of TED hose and SCD over the bed rest period. Subjects were offered medication to help mitigate reflux problems associated with being supine. Subjects were constantly monitored by nursing staff and received a daily physical examination by the study physician.

[000104] Subjects were exited from study if they permanently discontinued product during the pre-bed rest period (Day 1 to Day 5), or if they discontinued product during the bed rest period and had completed less than 8 days of bed rest. Subjects with a positive D-dimer test or ultrasound for deep vein thrombosis (DVT) diagnosis were also exited from the study.

[000105] A subject's outcome data were classified as unevaluable for the analysis if one or more of the following events occurred: A. Subject received wrong product, contrary to the randomization scheme; B. Subject received excluded concomitant treatment defined as medications or dietary supplements that affect weight or metabolism (e.g. progestational agents, steroids, growth hormone, dronabinol, marijuana, HMB, free amino acid supplements, dietary supplements to aid weight loss, and fish oil supplements); or C. Subject had <67% of total study product consumption at Final Visit/Exit as determined by product consumption records.

Body composition

[000106] Body weight was measured at baseline and after bed rest to the nearest 0.1 kilogram on an Ohaus scale (Ohaus Corporation, model 15S, Florham Park, NJ). Nude body weight was calculated as total body weight minus hospital robe weight. Body height was measured to the nearest 0.1 cm without shoes using a stadiometer. Body mass index (BMI) was calculated as $\text{weight}/\text{height}^2$ (kg/m^2). Measurements of body composition were conducted prior to and at the end of the 10-day bed rest period. DXA (Hologic Delphi W running QDR System Software Version 11.2) was used to

estimate total and lower extremity LBM using a standard protocol (Kortbein, P. *et al.*, *JAMA* 2007; 297 (16): 1772-4.

[000107] Fasted blood samples were collected from subjects on Day 1 of bed rest and at end of bed rest for measurement of biomarkers.

Statistical Analysis

[000108] Analysis of variance (ANOVA) with treatment group in the model was used to test for baseline differences for the following variables: age and described blood values. ANOVA (with treatment group, gender, and their interaction) was used to test for baseline differences for BMI, body weight, total body fat, and bone mineral density. Analysis of covariance (ANCOVA) with treatment group and baseline value in the model was used to test for post-bed rest differences for described blood levels of fasted glucose, cholesterol, and total cholesterol. ANCOVA (treatment group, gender, their interaction, and baseline value) was used to test for post-bed rest differences for the following variables: body weight, total body fat, bone mineral density.

[000109] Paired t-tests by treatment group were used to test for differences from baseline to end of bed rest for variables as described in Results section. All main effects were tested using 2-sided, 0.05 significance level tests. Tests of interactions were 2-sided, 0.10 significance level tests. All values are presented as means $\pm$ standard error of mean (SEM).

RESULTS

Body Composition

[000110] There was no significant change in total body weight over the bed rest. Total lean mass was measured by Dual Energy X-Ray Absorptiometry (DXA). The Control group lost an average of -2.05 ± 0.66 kg total lean mass ($p=0.02$, paired t test), whereas the HMB group lost an average of -0.17 ± 0.19 kg total lean mass ($p=0.42$, paired t-test). Comparison of the change value in total lean mass over the bed rest period between treatment groups was statistically significant ($p=0.02$, ANOVA). Similar results were obtained for leg lean mass with the HMB group showing an average loss of -0.08 ± 0.17 kg ($p=0.65$, paired t-test) versus the Control (-1.01 ± 0.35 kg, $p=0.02$,

paired t-test). There was a statistically significant difference in the change value leg lean mass over the bed rest period between treatment groups ($p=0.02$, ANOVA).

[000111] A sub-analysis was carried out on female subjects, since there was a greater number and more equal distribution of females in the study (Control $n=7$; HMB $n=8$). This sub-analysis revealed that compared to the Control group, HMB significantly attenuated muscle loss (total lean and leg lean mass) over bed rest. For total lean mass, HMB group lost -0.2 ± 0.24 kg and Control group lost -1.89 ± 0.75 kg ($p=0.04$, ANOVA).

Outcomes:

[000112] Two outcomes, absolute change in leg lean mass over bed rest and absolute change in total lean mass over bed rest were investigated. Two additional outcomes, percent change of leg lean mass and percent change of total lean mass were also evaluated in order to take into account the baseline variability in lean mass of subjects based on BMI and gender differences.

Outcome Distributions

[000113] The distributions of the outcomes for absolute and percent change for total lean mass and leg lean mass by treatment groups are presented in Figures 1-4

[000114] As can be seen in Figures 1-4, the distributions for the control group, for both total lean mass and leg lean mass are more variable than the HMB group. On average the HMB group showed a preservation of lean mass over bed rest where as the control group on average lost lean mass.

BIOMARKER STUDIES

[000115] Biomarker data for specimens from eighteen subjects for both pre- and post-bed rest values were generated using RBM's MultiAnalyte Panels (MAPs). (Myriad, TX). Of the 18 subjects, 8 (1 male and 7 females) were in the control group 10 subjects (2 males and 8 females) were in the experimental HMB group.

[000116] Rules Based Medicine (RBM) data consists of $n = 187$ biomarkers that was measured in serum collected at two time points, pre-bed rest and post-bed rest from the 18 elderly subjects. The RBM panel used was Human Discovery Map v1.0.

Marker Distributions

[000117] Initially, the distribution of each of the 187 RBM markers was evaluated. Each antigen marker has a least detectable dose (LDD) value, defined as the mean + 3 standard deviations (SD) of 20 blank samples. For any subject whose marker result was $\leq$ LDD, the LDD value as provided by RBM was imputed. If the marker result was $>$ LDD, the original marker result was used. Any marker in which $\geq 30\%$ of all subject's results were imputed was excluded from further statistical analyses. Of the initial $n = 187$ RBM markers, $n = 63$ markers from the RBM dataset were excluded from further analyses, leaving $n = 124$ markers for evaluation.

Statistical Evaluation

Assessing Statistical Significance: Multiple-Hypotheses Testing

[000118] In order to detect a potentially significant differential response in a large set of variables between the treatment groups, the hypothesis tested for each individual variable was adjusted to reduce the possibility of spurious findings. The multiple-hypotheses testing problem is addressed through the estimation of the false discovery rate (FDR). FDR methods aim to increase the power in statistical testing. The positive FDR (pFDR) method was chosen. This method computes the "q-values", which are adaptive adjusted p-values. These adjusted p-values are smaller than raw (unadjusted) p-values, and by controlling the false discovery rate, hypothesis tests at a level less than the observed p-value can be rejected.

[000119] **Pre-bed rest data:** Prior to the adjustment method, few markers would be considered statistically significant. After adjustment for the multiple tests, however, none of the markers evaluated were significantly different between the control and experiment groups. This confirms that the control and experiment groups are statistically equivalent populations at the pre-bed rest time point.

[000120] **Post-bed rest data:** Prior to adjustment, the outcome measures defined previously were found to be statistically different between the control and experiment groups. On an unadjusted comparison level, statistically significant results are found. After adjustment for the multiple tests, however, none of the outcomes evaluated were significantly different between the control and experiment groups post bed rest.

Partition Analysis

[000121] A partition analysis was performed to identify pre-bed rest markers that are associated with the lean mass outcomes under investigation. The fitting process optimizes the model parameters and thus the model predicts the fitted data very well. However, the model may predict future subjects poorly. As a result, a process of validation should be used. In validation, a part of the data ('training' set) is used to estimate the model parameters, and the other part ('validation' set) is used to assess the predictive ability of the model. A k-fold cross-validation method randomly partitions the data into k subsamples. One of the k samples is used to test the model, and the remaining k-1 subsamples are used to build the model. The process is repeated k times, building k models, which are then averaged to produce a single estimation. A 5-fold cross-validation method was chosen to aid in model selection as it is efficient for smaller sample sizes.

[000122] The outcome of LBM in terms of percent change was used as it takes into account baseline differences in lean mass due to gender or BMI that absolute changes cannot account for. The partition analysis was performed for all 18 subjects in the analytic dataset and then repeated separately for just the 15 females.

RESULTS of PARTITION ANALYSIS:

[000123] Table 1 identifies for each outcome and analysis group (All subjects: n = 18, or females: n = 15) the markers that were found to be associated with that outcome. The first row indicates the first partition, and if another partitioning of the data was necessary, the name of the second marker. The table also includes the R^2

value of the model after the 5-fold cross validation. Bolded data indicates high correlations.

Table 1.

		Total Body Lean Mass			Leg Lean Mass		
		R^2			R^2		
Percent Change	All	TIMP-1	TIMP-1	0.71	TRT	TRT	0.42
			TNC			ANGPT2	
	Females	TIMP-1	TIMP-1	0.76	MMP-3	MMP-3	0.59
			TNC			ApoA-2	

Partition Plots

[000124] Figure 5 displays the percent loss of total lean body mass (TLBMPCT) in individual subjects (n=18; Control =8, HMB =10) (solid squares) as it correlates to levels of TIMP-1 and TenascinC (TNC) in blood. Subjects with baseline levels of TIMP-1 $\geq$ 141 had the highest loss of total LBM. Subjects with TIMP1 <141 and TNC $\geq$ 461 did not display loss of muscle over bed rest. Subjects with TIMP1 <141 and TNC <461 showed some loss of total lean body mass over bed rest.

[000125] Figure 6 displays the percent loss of total lean body mass (TLBMPCT) in individual female subjects (n=15; Control =7, HMB =8) (solid squares) as it correlates to levels of TIMP-1 and TenascinC (TNC) in blood. Female subjects with baseline levels of TIMP-1 $\geq$ 141 at bed rest had the highest loss of total LBM. Subjects with TIMP1 <141 and TNC $\geq$ 356 did not display loss of muscle over bed rest. Subjects with TIMP1 <141 and TNC <356 showed some loss of total lean mass over bed rest.

[000126] Figure 7 displays the percent loss of leg lean body mass (LEBMPCT) in female subjects (n=15; Control= 7, HMB= 8) (solid squares) as it correlates to levels of MMP-3 and ApoA-2 in blood. Subjects with baseline levels of MMP<6.93 had the highest loss of total LBM. Subjects with MMP-3 ≥ 6.93 and ApoA-2<276 did not display loss of muscle over bed rest. Subjects with MMP-3 ≥ 6.93 and ApoA-2 ≥ 276 showed some loss of total lean mass over bed rest.

CONCLUSIONS:

[000127] A consistent model which identified TIMP-1 and TNC was produced for the outcome of total body lean percent change for the entire population and for females only. These models produced the highest R^2 value after cross-validation. In addition, MMP-3 and ApoA-2 were identified as markers that correlate with leg lean mass outcomes in females.

WHAT IS CLAIMED IS:

1. A method of treating a subject at risk of losing lean body mass (LBM) during a prolonged period of physical inactivity, comprising:
 - (a) measuring levels of one or more biomarkers selected from Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject;
 - (b) comparing the levels of the one or more biomarkers in the test subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from a control population; and
 - (c) administering β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof to the subject if the level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1 and/or the level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3.
2. The method of claim 1, wherein the subject is an adult human subject and is undergoing or is expected to undergo bed rest in the near future.
3. The method of claim 1, wherein the subject is an elderly human subject.
4. The method of claim 1, wherein the biological sample is a blood sample.
5. A method of treating a subject at risk of losing lean body mass during a prolonged period of physical inactivity, comprising:
 - (a) measuring levels of one or more sets of biomarkers selected from (i) TIMP-1 and Tenascin C (TNC) and (ii) MMP-3 and Apolipoprotein A2 (ApoA-2) in a biological sample obtained from the subject;
 - (b) comparing the levels of the one or more sets of biomarkers in the subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from a control population;
 - (c) administering HMB to the subject if

- (i) the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for each of said biomarkers and/or
 - (ii) the levels of MMP-3 and ApoA-2 in the test subject's biological sample are higher than the control values for each of said biomarkers.
- 6. The method of claim 5, wherein the subject is an adult human subject and is undergoing or is expected to undergo bed rest in the near future.
- 7. The method of claim 5, wherein the subject is an elderly human subject.
- 8. The method of claim 5, wherein the biological sample is a blood sample.
- 9. A method of characterizing a test subject's risk of losing LBM during a prolonged period of physical inactivity, wherein the test subject is an adult human subject, the method comprising:
 - (a) measuring the levels of one or more biomarkers selected from Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the test subject;
 - (b) comparing the levels of the one or more biomarkers in the test subject's blood sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from subjects in a control population; and
 - (c) characterizing the test subject as being at risk of losing LBM during a prolonged period of physical inactivity if the level of TIMP-1 in the test subject's blood sample is higher than the control value for TIMP-1 and/or the level of MMP-3 in the test subject's biological sample is lower than the control value for MMP-3.
- 10. The method of claim 9, wherein the biological sample is a blood sample.
- 11. The method of claim 9, wherein the subject is an elderly human subject.

12. The method of claim 9, further comprising the step of administering one or more nutritional interventions selected from the group consisting of HMB, a high protein diet, high doses of leucine, high doses of leucine metabolites, and high doses of essential amino acids to the subject if the subject has been characterized as being at risk of losing LBM during a prolonged period of physical inactivity.

13. The method of claim 12, wherein the leucine metabolites are selected from HMB, alpha-ketoisocaproate (KIC), and alpha-hydroxyisocaproate (HICA).

14. The method of claim 9, further comprising the steps of:

(d) measuring the levels of one or more biomarkers selected from the group consisting of Tenascin C (TNC) and Apolipoprotein A2 (ApoA-2) in the test subject's biological sample;

(e) comparing the levels of TNC and/or ApoA3 in the test subject's biological sample to control values based on the levels of each of said biomarkers in comparable biological samples from a population of control subjects; and

(f) characterizing the test subject as being at risk of losing lean body mass during a prolonged period of physical inactivity if (i) the levels of TIMP-1 and TNC in the test subject's biological sample are lower than the control values for TIMP-1 and TNC respectively; and/or (ii) the levels of MMP-3 and ApoA-2 in the test subject's biological sample are higher than the control values for MMP-3 and ApoA-2, respectively.

15. The method of claim 14, further comprising the step of administering one or more nutritional interventions selected from the group consisting of HMB, a high protein diet, high doses of leucine, high doses of leucine metabolites, and high doses of essential amino acids to the subject if the subject has been characterized as being at risk of losing LBM during a prolonged period of physical inactivity.

16. The method of claim 15, wherein the leucine metabolites are selected from HMB, KIC, and HICA.

17. The method of claim 14, wherein the test subject is an elderly human subject.
18. The method of claim 14, wherein the biological sample is a blood sample.
19. A system for identifying an adult human subject in need of muscle preservation during a prolonged period of physical inactivity, the system comprising:
 - (a) a sub-system for identifying an adult human subject undergoing physical inactivity or expected to undergo physical inactivity in the near future;
 - (b) a sub-system for taking a biological sample from the subject identified in (a);
 - (c) a sub-system for measuring the levels of one or more sets of biomarkers selected from the group consisting of (i) TIMP-1, (ii) TIMP-1 and TNC, (iii) MMP-3, and (iv) MMP-3 and ApoA-2 in the biological sample obtained in (b);
 - (d) a sub-system for comparing the measured levels of the one or more biomarkers to control values based on the levels of the one or more biomarkers in comparable biological samples obtained from a control population; and
 - (e) a sub-system for characterizing the subject as needing muscle preservation during a prolonged period of physical inactivity if one or more of the following is met:
 - (i) the measured level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1;
 - (ii) the measured level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3;
 - (iii) the measured levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for TIMP and TNC, respectively; or
 - (iv) the measured levels of MMP-3 and ApoA-2 in the subject's biological sample are greater than the control values for MMP-3 and ApoA-2, respectively.
20. A system according claim 19, wherein the physical inactivity is bed rest.
21. A system according to claim 19, further comprising a subsystem for administering HMB to a subject characterized as needing muscle preservation during a prolonged period of physical inactivity.

22. A system according to claim 19, further comprising a subsystem for administering one or more nutritional interventions selected from a high protein diet, high doses of leucine, high doses of leucine metabolites, and high doses of essential amino acids to a subject identified as needing muscle preservation during a prolonged period of physical inactivity.

23. The system of claim 22, wherein the leucine metabolites are selected from KIC and HICA.

24. A system according to claim 19, wherein the biological sample is a blood sample.

25. A composition for use in the treatment of a subject in need of lean body mass preservation, wherein the composition comprises β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof, and wherein the subject to be treated is identified by a method comprising the steps of:

(a) measuring levels of one or more biomarkers selected from Tissue Inhibitor of Metalloprotease-1 (TIMP-1) and Matrix Metalloprotease-3 (MMP-3) in a biological sample obtained from the subject;

(b) comparing the levels of the one or more biomarkers in the test subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples from a control population; and

(c) determining that the subject is in need of lean body mass preservation when the level of TIMP-1 in the subject's biological sample is higher than the control value for TIMP-1 and/or when the level of MMP-3 in the subject's biological sample is lower than the control value for MMP-3.

26. A composition for use in the treatment of a subject in need of lean body mass preservation, wherein the composition comprises β -hydroxy- β -methylbutyric acid (HMB) or a salt thereof, and wherein the subject to be treated is identified by a method comprising the steps of:

(a) measuring levels of one or more sets of biomarkers selected from (i) TIMP-1 and Tenascin C (TNC) and (ii) MMP-3 and Apolipoprotein A2 (ApoA-2) in a biological sample obtained from the subject;

(b) comparing the levels of the one or more sets of biomarkers in the subject's biological sample to one or more control values based on the levels of the one or more biomarkers in comparable biological samples taken from a control population; and

(c) determining that the subject is in need of lean body mass preservation when

(i) the levels of TIMP-1 and TNC in the subject's biological sample are lower than the control values for TIMP-1 and TNC, respectively; and/or

(ii) the levels of MMP-3 and Apo A2 in the test subject's biological sample are higher than the control value for MMP-3 and ApoA-2, respectively.

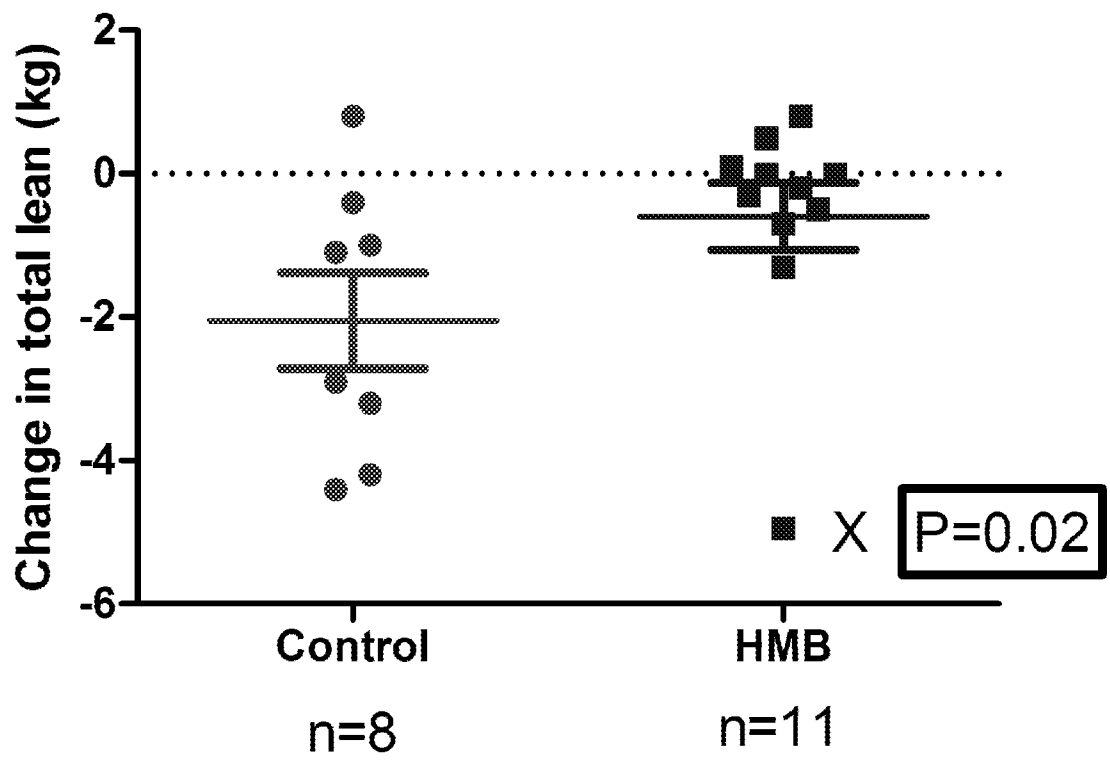


Figure 1

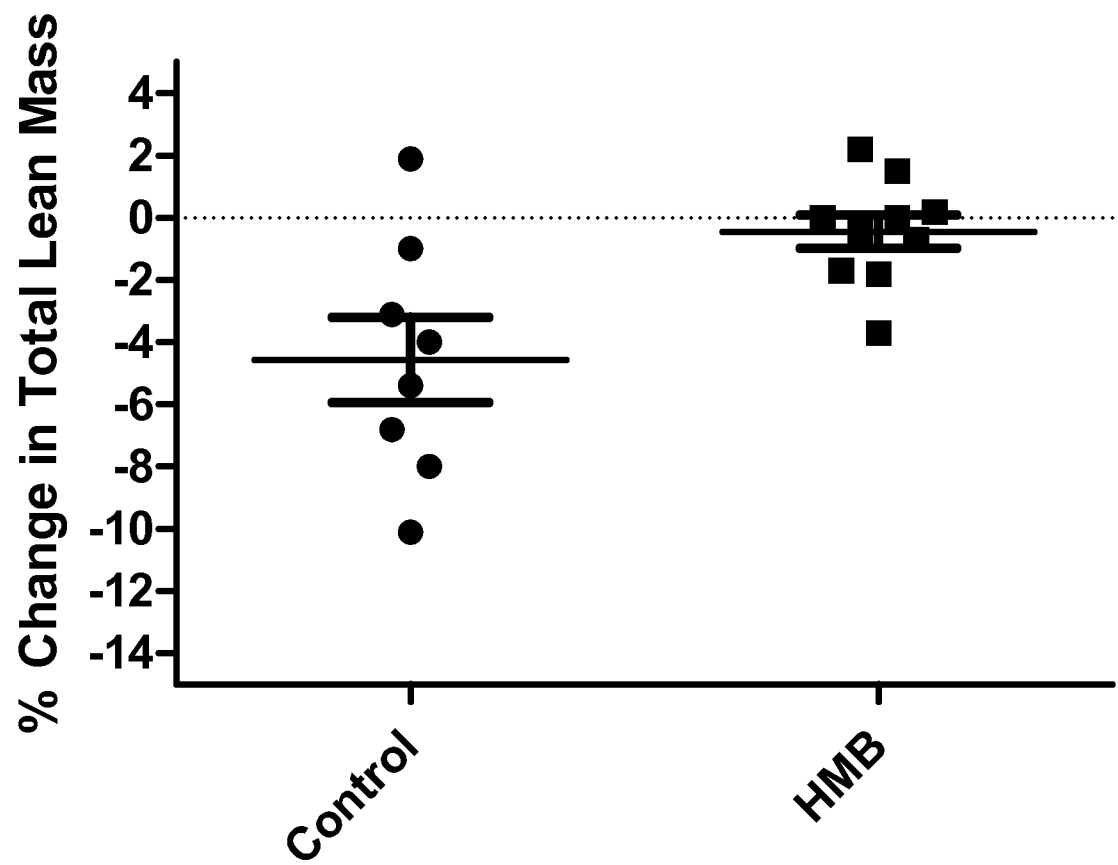


Figure 2

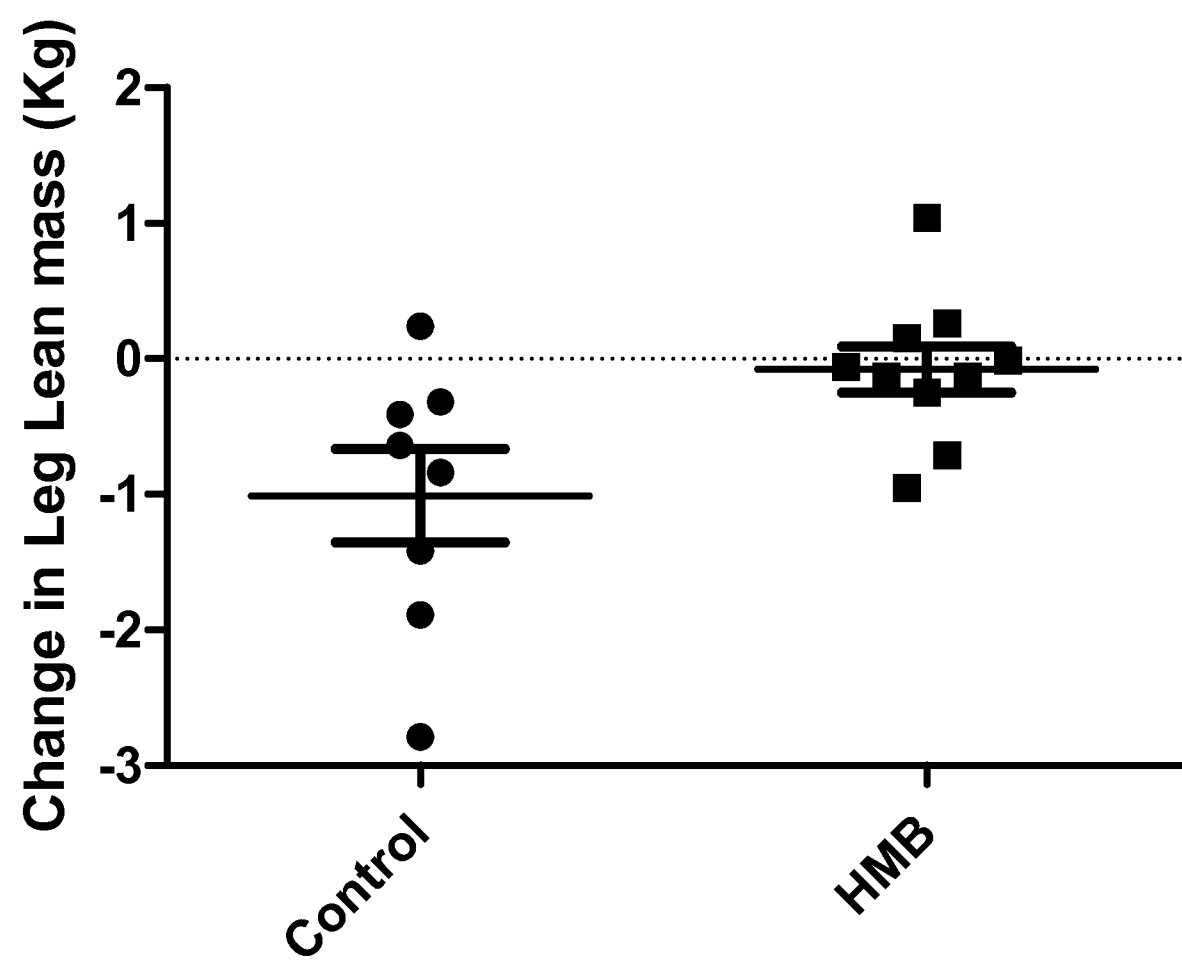


Figure 3

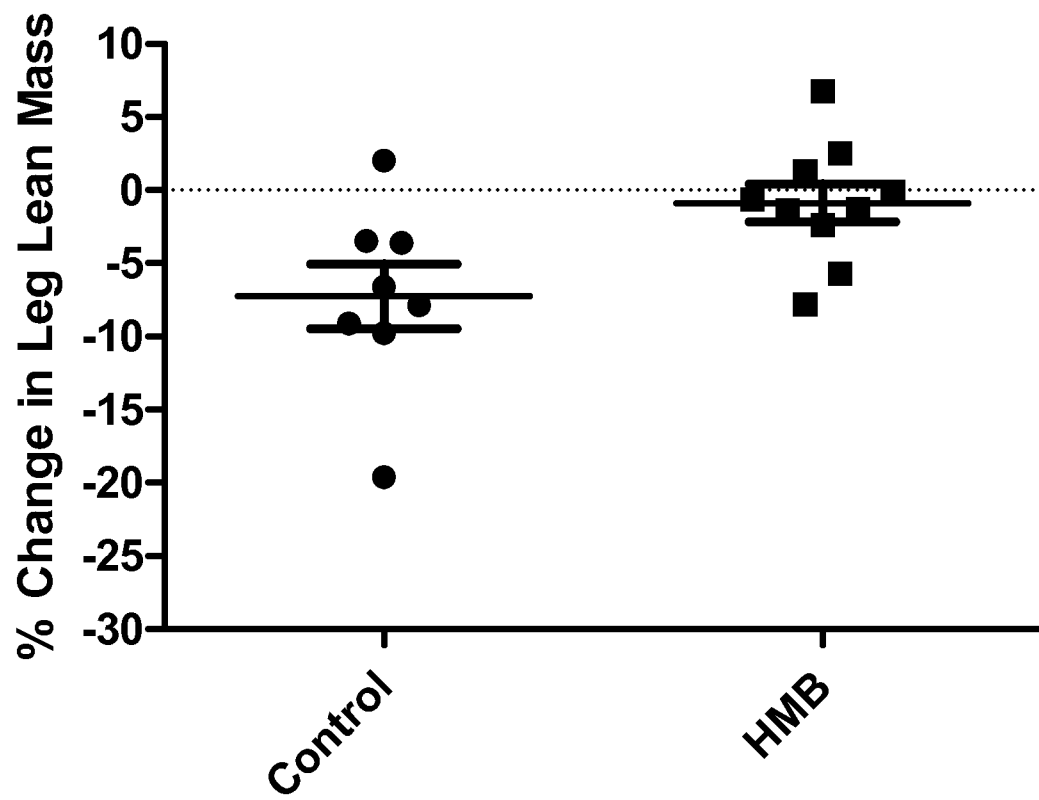


Figure 4

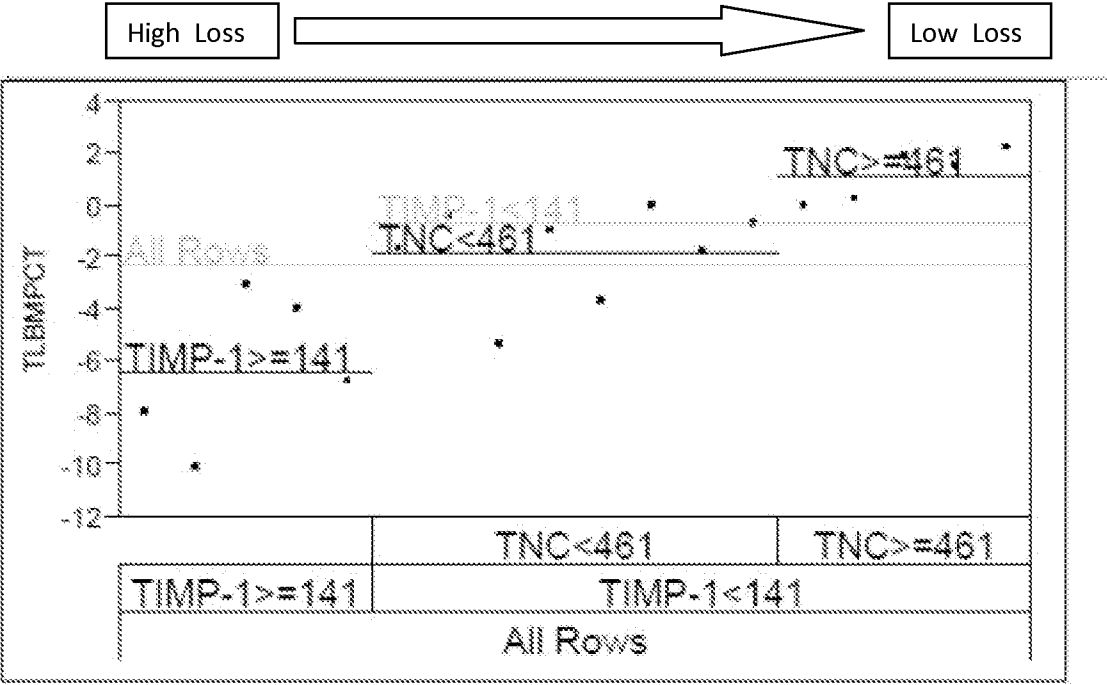


Figure 5

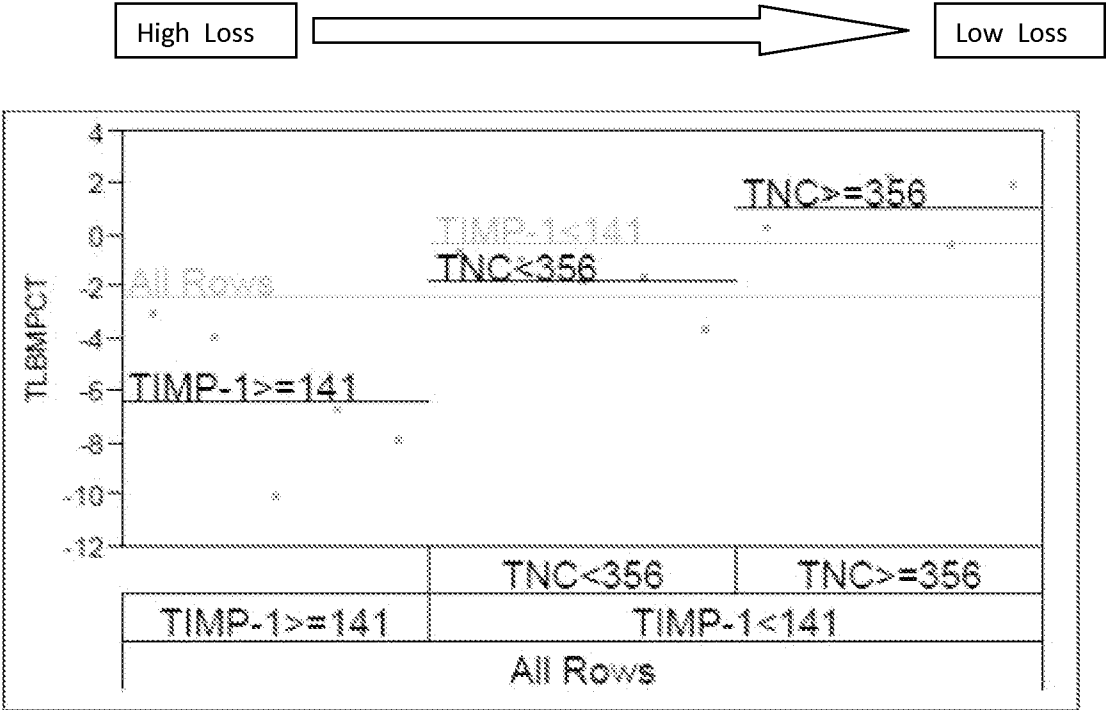


Figure 6

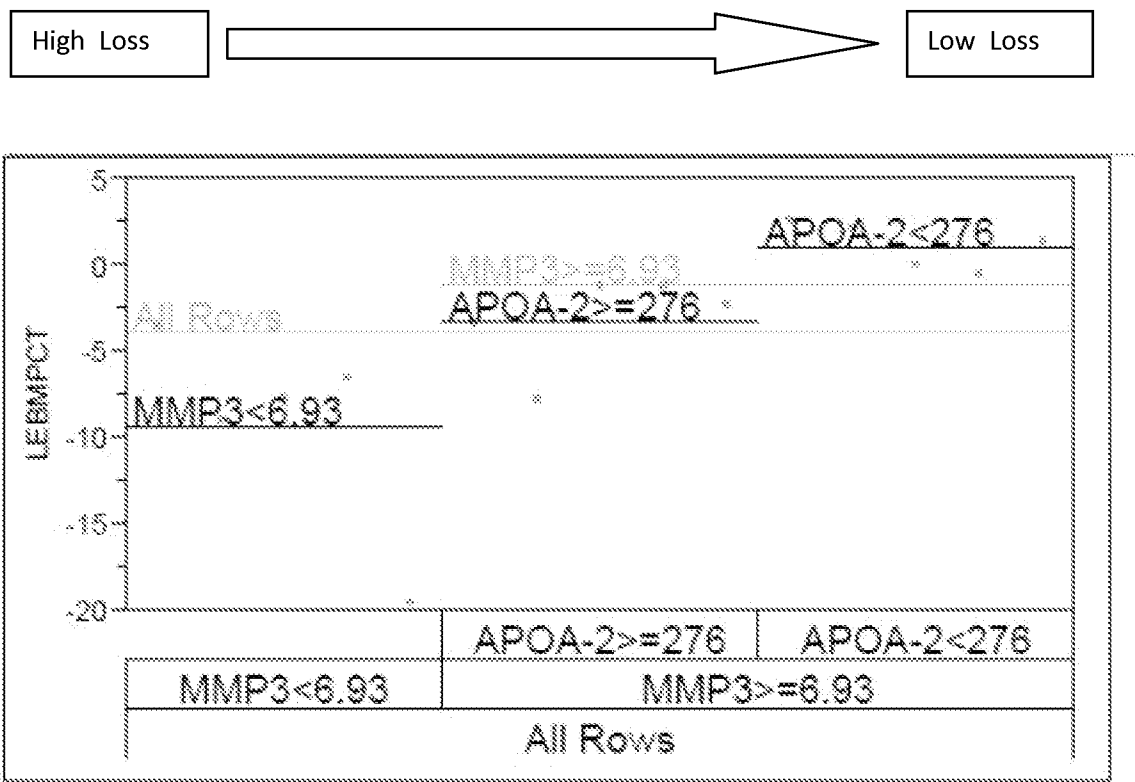


Figure 7

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/027528

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	GAWEL S H ET AL: "LB014-SUN SERUM BIOMARKERS THAT PREDICT LEAN BODY MASS (LBM) LOSS OVER BEDREST (BR) IN OLDER ADULTS", CLINICAL NUTRITION, vol. 32, 1 September 2013 (2013-09-01), XP028699377, ISSN: 0261-5614, DOI: 10.1016/S0261-5614(13)60592-0 abstract	1-26
X	WO 2012/092035 A1 (ABBOTT LAB [US]; PEREIRA SUZETTE L [US]; EDENS NEILE K [US]) 5 July 2012 (2012-07-05)	25,26
Y	claim 1	1-24
	----- -/-	



Further documents are listed in the continuation of Box C.



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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

25 June 2014

Date of mailing of the international search report

08/07/2014

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INTERNATIONAL SEARCH REPORT

International application No
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