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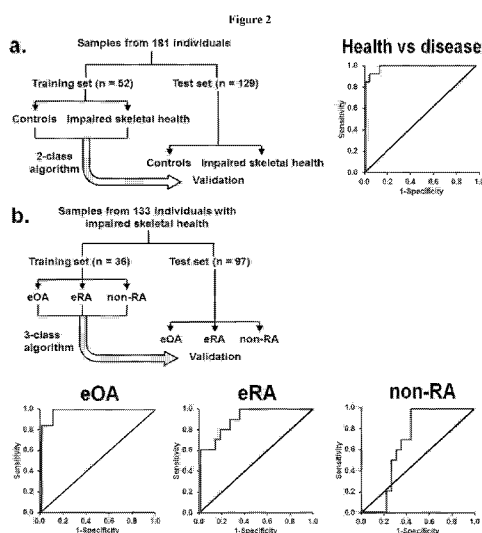
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(54) **Title:** MARKERS FOR SKELETAL DISORDERS



(57) **Abstract:** There is provided a method for determining the skeletal health of an individual comprising: (a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), Nε-(1- carboxyethyl)lysine (CEL), Nω-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp); (b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual.

## MARKERS FOR SKELETAL DISORDERS

The present invention relates to methods for determining the skeletal health of an individual by quantifying oxidised, nitrated and glycated free adducts, methods for determining whether an individual has an early-stage skeletal disorder by quantifying oxidised, nitrated and glycated free adducts, the use of said oxidised, nitrated and glycated free adducts as markers of skeletal health and early-stage skeletal disorder, as well as kits comprising reagents for quantifying said free adducts.

Osteoarthritis (OA), rheumatoid arthritis (RA) and related musculoskeletal disease is the most common cause of chronic disability worldwide and is increasingly important in current Westernised populations of increasing age and obesity (Connelly, L. B., Woolf, A. & Brooks, P. in *Disease Control Priorities in Developing Countries* Vol. 2nd (D. T. Jamieson *et al.*) 963-980 (World Bank, 2006)). Severe life impairment may be prevented if decline in musculoskeletal health and development of OA and RA are identified and treated in the early-stages (Neogi, T. & Zhang, Y. Osteoarthritis Prevention. *Current opinion in rheumatology* 23, 185-191 (2011); Isaacs, J. D. The changing face of rheumatoid arthritis: sustained remission for all? *Nat Rev Immunol* 10, 605-611 (2010)).

Detecting and distinguishing different types of early-stage arthritis is unachievable routinely. There is no established method to detect early-stage OA (eOA); radiography remains the method of choice for staging OA, but shows little or no changes in eOA. When OA is confirmed, significant pain, discomfort and damage to the joint is already present and the opportunity to intervene at the early and likely reversible stages has been lost. Magnetic resonance imaging (MRI) techniques have been developed for evaluation of cartilage damage in eOA. They have approximately 70% sensitivity and 90% specificity compared to reference diagnosis by arthroscopy (Menashe, L. *et al.* The diagnostic performance of MRI in osteoarthritis: a systematic review and meta-analysis. *Osteoarthritis and Cartilage* 20, 13-21 (2012)). However, MRI techniques require expensive instrumentation time and facilities, and they are contraindicated in certain populations who have implanted devices such as pacemakers or aneurysm coils. Detection of early-stage rheumatoid arthritis (eRA) involves measuring rheumatoid factor (RF) by immunochemical tests, and has a sensitivity and specificity of 63% and 94% respectively for established or advanced disease (Raza, K. *et al.* Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis. *J.Rheumatol.* 32, 231-238 (2005)). RF is often negative with eRA. Early-stage diagnosis was improved by the development of the anti-cyclic citrullinated peptide (CCP) antibody test which has a sensitivity of 61% for eRA (Van Venrooij, W. J., Van Beers, J. J. B. C. & Pruijn, G. J. M. Anti-CCP antibody, a marker for the early detection of rheumatoid arthritis. *Annals of the New York Academy of Sciences* 1143, 268-285 (2008)), and a sensitivity 68% and a specificity of 98% for established disease (Van Venrooij, W. J., van Beers, J. J. B. C. & Pruijn, G. J. M. Anti-CCP antibodies: the past, the present and the future. *Nature Reviews Rheumatology* 7, 391-398 (2011)).

Recently, approaches using diagnostic algorithms have been investigated for diagnosis of skeletal disorder. In one example, a 4-class diagnostic algorithm was developed to detect and discriminate eOA, eRA and other inflammatory joint disease which may be self-resolving (non-RA) (WO 2014/016584). This diagnostic algorithm combined measurement of hydroxyproline (Hyp), anti-CCP antibody and citrullinated

protein (CP) in plasma with subject age and gender. Sensitivities and specificities were in the ranges 0.25 – 0.73 and 0.75 – 0.91, respectively; with a random outcome value of 0.25. Increased levels of CP are observed in both eOA and eRA with only autoimmunity in eRA, judged by anti-CCP antibody test positivity (Ahmed, U. *et al.* Biomarkers of early-stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. *Sci. Rep.* 5, 9259 (9251-9257) (2015)). Other studies have investigated using markers of skeletal health that result from damage to proteins by oxidation (eg. methionine sulfoxide), nitration (3-nitrotyrosine) and glycation (N<sub>ε</sub>-fructosyl-lysine derived from glucose and hydroimidazolone derived from methylglyoxal).

Despite these recent developments, there remains a need for an inexpensive and minimally invasive test for determining the skeletal health of individuals with a high degree of specificity and sensitivity. There also remains a need for a sensitive and specific test for early-stage diagnosis of skeletal disorder, and in addition for distinguishing common types of arthritis at the early-stage with high sensitivity and specificity. This need is addressed by the present invention, which provides methods for determining the skeletal health of individuals, and for determining whether an individual has an early-stage skeletal disorder.

In one aspect, the present invention provides a method for determining the skeletal health of an individual comprising:

(a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

(b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual.

This aspect of the invention uses a 2-class diagnostic algorithm (eg. as illustrated in Figure 2(a)) to determine the skeletal health of a test individual, allowing reliable diagnosis of individuals having a skeletal disorder, including those having an early-stage skeletal disorder. By testing a panel of different adduct residue markers the present inventors have identified various subsets of markers that provide highly sensitive and specific determination of skeletal health. As demonstrated in Example 2 and Figures 12 and 14, the method of the invention allows the skeletal health of a test individual to be determined with a high level of sensitivity and specificity.

The invention further provides the use of at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK); dityrosine (DT); 3-nitrotyrosine (3-NT); N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived

hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp) for determining skeletal health.

The invention also provides a method of treating a skeletal disorder in an individual, comprising:

- (a) determining the skeletal health of an individual by performing a method comprising the steps of:
  - (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);
  - (ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and
  - (iii) determining the skeletal health of the test individual;
- (b) selecting an individual identified as having a skeletal disorder; and
- (c) administering a treatment for the skeletal disorder to the individual.

In a further aspect, the invention provides a method of determining whether an individual has an early-stage skeletal disorder comprising:

- (a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;
- (b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

This aspect of the invention uses a 3-class diagnostic algorithm (as illustrated in Figure 2(b)) to reliably determine whether an individual has an early-stage skeletal disorder. As demonstrated by Example 2 and the data presented in Figures 12 and 14, the present inventors have identified various subsets of markers that can be used in a diagnostic algorithm to distinguish different types of early-stage skeletal disorder with a high degree of sensitivity and specificity. In particular, the method permits sensitive and specific distinction between different types of early-stage skeletal disorder, such as early-stage osteoarthritis, early-stage rheumatoid arthritis and inflammatory joint disease which may be self-resolving (see Figures 12 and 14). There are currently no methods available in the art which allow the different types of early-

stage skeletal disorder to be distinguished in a specific and sensitive manner. The method of the invention therefore provides a significant technical contribution to the art.

The invention also provides the use of at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) for determining whether a test individual has an early-stage skeletal disorder.

The invention also provides a method of treating an early-stage skeletal disorder in an individual, comprising:

(a) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder;

(b) selecting an individual diagnosed as having an early-stage skeletal disorder;

(c) administering a treatment for the early-stage skeletal disorder to the individual.

The invention also provides a method of determining whether an individual has an early-stage skeletal disorder comprising:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on

corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

This method provides a highly sensitive way of detecting early-stage skeletal disorder using a combination of 2 diagnostic algorithms. The first algorithm is used to determine the skeletal health of an individual, thereby identifying individuals having a skeletal disorder. The second algorithm is used to determine whether the skeletal disorder is an early-stage skeletal disorder, and may be used to distinguish between specific types of early-stage skeletal disorder such as early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving. This method provides the most sensitive and specific approach to determining whether an individual has an early-stage skeletal disorder (as shown in Figures 12 and 14).

The invention also provides a method of treating an early-stage skeletal disorder in an individual, comprising:

(a) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) determining the skeletal health of an individual by quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp); and classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, so as to determine the skeletal health of the test individual;

- (ii) selecting an individual identified as having a skeletal disorder; and
- (iii) determining whether the individual has an early-stage skeletal disorder by quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody; and classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, so as to determine whether the test individual has an early-stage skeletal disorder;
- (b) selecting an individual diagnosed as having an early-stage skeletal disorder;
- (c) administering a treatment for the early-stage skeletal disorder to the individual.

In a further aspect, the invention provides a method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, comprising:

- (a) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL);
- (b) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health;

and thereby determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis.

If a plurality of markers is selected the method may comprise the step of classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

The invention further provides the use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL) as markers for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis.

These methods and uses of the invention provide a useful way of monitoring the skeletal health of the test individual over time to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

In a further aspect, the invention provides a method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, comprising:

(a) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine;

(b) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health;

and thereby determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis.

The invention further provides the use of the combination of the oxidised free adducts: methionine sulfoxide (MetSO), and dityrosine (DT), and the glycated free adduct pentosidine as markers for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis.

If a plurality of markers is selected the method may comprise the step of classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

These methods (and uses) of the invention provide a useful way of determining whether an individual has an advanced-stage skeletal disorder or an early-stage skeletal disorder. This can be used to assess the overall skeletal health of an individual undergoing treatment for an advanced-stage skeletal disorder.

In a further aspect, the invention provides a kit comprising reagents for quantifying markers of skeletal health, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp), and/or related stable isotype substituted compounds (isotopomers).

The invention also provides a kit comprising reagents for quantifying markers of early-stage skeletal disorder, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane, and/or related stable isotype substituted compounds (isotopomers); and anti-cyclic citrullinated peptide antibody.

The invention also provides a kit comprising reagents for quantification of markers for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, wherein said markers comprise: the combination of the oxidised, nitrated and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL); and/or related stable isotype substituted compounds (isotopomers).

The invention also provides a kit comprising reagents for quantification of markers for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, wherein said markers comprise the combination of the oxidised free adducts: methionine sulfoxide (MetSO), and dityrosine (DT), and the glycated free adduct pentosidine; and/or related stable isotype substituted compounds (isotopomers).

The invention also provides a computational model based on a diagnostic algorithm adapted to classify the skeletal health based on the amount of a plurality of markers quantified in a test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

Said markers may comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

Said markers may comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

The invention also extends to software adapted to produce a computational model as aforementioned. The invention also extends to a processor adapted to produce a computational model as aforementioned.

The invention also provides a method

- for determining the skeletal health of an individual and/or
- of treating a skeletal disorder in an individual and/or
- of determining whether an individual has an early-stage skeletal disorder and/or
- of treating an early-stage skeletal disorder in an individual and/or
- for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis and/or

- for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis;

the method comprising:

(a) optionally determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine and glucosepane; and hydroxyproline (Hyp);

(ii) comparing the amount of each marker quantified in the test sample to corresponding reference values for each marker in a diagnostic algorithm, wherein the reference values are obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) optionally determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) comparing the amount of each marker quantified in the test sample to corresponding reference values for each marker in a second diagnostic algorithm, wherein the reference values are obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder;

(c) optionally determining whether the individual has advanced-stage osteoarthritis or early-stage osteoarthritis by performing a method comprising the steps of:

(i) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL);

(ii) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health, and thereby determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis;

(d) optionally determining whether the individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis by performing a method comprising the steps of:

(i) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine;

(ii) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health, and thereby determining whether the test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis;

(d) optionally selecting an individual identified as having a skeletal disorder, optionally an early-stage skeletal disorder; and

(e) optionally administering a treatment for the skeletal disorder, optionally the early-stage skeletal disorder, to the individual.

#### SPECIFIC DESCRIPTION

The present inventors have conducted extensive experimentation to identify markers of skeletal health. The present inventors have identified various sets of oxidised, nitrated, and glycated free adducts and adduct residues of proteins that can be used to determine the skeletal health of an individual, and to determine whether an individual has an early-stage skeletal disorder, or to distinguish between advanced and early-stage skeletal disorders with a high degree of specificity and sensitivity.

The present invention provides a method for determining the skeletal health of an individual comprising:

(a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

(b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual.

As used herein the phrase “determining the skeletal health of an individual” means determining whether an individual has a skeletal disorder, or does not have a skeletal disorder.

The terms “individual”, “subject” and “patient” are used interchangeably herein to refer to a mammalian subject whose skeletal health requires investigation. The mammal can be a human, or an animal including, but not limited to an equine, porcine, canine, feline, ungulate, and primate animal, or any other load-bearing animal that is known to be susceptible to, or suffer from, a skeletal health disorder. In one embodiment, the individual is a human. The methods and uses of the invention described herein are useful for both medical and veterinary uses.

In the methods of the invention, the individual may not have been previously diagnosed as having a skeletal disorder. The individual may also be one who has been previously diagnosed as having the disorder (ie. for advanced stage skeletal disorders). Alternatively, the individual may be one who does exhibit disease risk factors, or one who is asymptomatic for the disease (ie. for early-stage skeletal disorders). The individual may also be one who is suffering from or a risk of developing a skeletal disorder.

As used herein, the term “skeletal disorder” means any joint or bone disorder, or a condition that gives rise to a lack of skeletal health or integrity. Examples of joint or bone disorders include arthritic conditions, such as non-inflammatory arthritic conditions (“non-inflammatory arthritis”) and inflammatory arthritic conditions (“inflammatory arthritis”). Non-inflammatory arthritic conditions include without limitation osteoarthritis (OA), such as early-stage OA (eOA) or advanced-stage OA (aOA). Inflammatory arthritic conditions include without limitation rheumatoid arthritis, such as early-stage RA (eRA) or advanced-stage RA (aRA). Skeletal disorders may also include inflammatory joint disorders which may be self-resolving (non-RA).

In one embodiment, the method of the invention is used to diagnose the test individual as having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), advanced-stage osteoarthritis (aOA), early-stage rheumatoid arthritis (eRA), advanced-stage rheumatoid arthritis (aRA), or other inflammatory joint disease that may be self-resolving.

In one embodiment, the method of the invention is used to diagnose the test individual as having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

As used herein, the term “early-stage skeletal disorder” refers to a skeletal disorder that is in the early stages of the disease condition. The individual may experience no symptoms of the skeletal disorder. The individual may experience temporary symptoms. The individual may experience mild symptoms.

The early-stage skeletal disorder may be early-stage osteoarthritis. Individuals having eOA may experience no joint pain. Individuals may experience pain after prolonged use of a joint. The pain may be mild. The individuals may experience stiffness after prolonged non-use of the joint. Radiographs of the joints in the individual may appear normal. The individual may have minimal cartilage breakdown. The individual may be classified as grade I or grade II on the Outerbridge scale. The joints commonly affected in OA are of the spine, fingers, thumbs, hips, knees and toes

The early-stage skeletal disorder may be early-stage rheumatoid arthritis. Individuals having eRA may experience temporary joint pain and joint swelling during or after use. Radiographs of the joints in the individual may appear normal. The individual may have minimal cartilage breakdown. The appearance of the joint may be normal. The joints commonly affected in RA are of the hand, wrist, shoulder, elbow, knee, ankle and feet.

Accordingly, the methods of the invention as described herein, permit diagnosis of individuals that may not have been previously diagnosed as having a skeletal disorder. The individual may not exhibit disease risk factors or may be asymptomatic for the disease.

- 5 As used herein, the term “advanced-stage skeletal disorder” refers to a skeletal disorder that is in the later stages of the disease condition. The individual may experience persistent symptoms of the skeletal disorder. The individual may experience severe symptoms.

10 The advanced-stage skeletal disorder may be advanced-stage osteoarthritis. Individuals having aOA may experience frequent joint pain during or after use (eg. when walking, running, bending or kneeling). Individuals may experience joint swelling after prolonged use (eg. when walking, running, bending or kneeling). The pain may be severe. Individuals may experience joint stiffness that worsens after prolonged non-use of the joint (eg. after sitting for a prolonged period or when waking up in the morning). Radiographs of the joints in the individual may indicate breakdown and change in surface morphology of  
15 bone in joints. The individual may be classified as grade III or grade IV on the Outerbridge scale.

The advanced-stage skeletal disorder may be advanced-stage rheumatoid arthritis. Individuals having aRA may experience persistent joint pain. Individuals may experience joint swelling. Individuals may experience a limited range of motion in the joint. Individuals may experience stiffness in the joint  
20 (particularly in the morning). Individuals may experience weakness and malaise. Radiographs of the joints in the individual may indicate breakdown and change in surface morphology of bone in joints. The joints in the individual may be deformed. The individual may have muscle atrophy.

As used herein, the skeletal disorder described as non-RA, an “inflammatory joint disease that is self-resolving” refers to an inflammatory skeletal disorder that is mild in severity and often short in duration,  
25 resolving without major treatment. Examples of etiology are: reactive arthritis (joint pain and swelling triggered by an infection in another part of your body), pseudogout (caused by deposits of crystals of calcium pyrophosphate in and around the joints), and other unclassified conditions.

30 In one embodiment, the method of the invention is used to diagnose the test individual as having no skeletal disorder

As used herein, the term “body fluid sample” includes a sample obtained from eye fluid, urine, whole blood, blood serum, blood plasma, lymphatic fluid, saliva, synovial fluid, seminal fluid, cerebrospinal fluid, sebaceous secretions, or sputum. As will be appreciated by those skilled in the art, said sample may be  
35 pre-treated for analysis, typically, by using conventional techniques as described herein and known by those skilled in the art.

In one embodiment, the body fluid sample is urine. In one embodiment, the body fluid sample is sputum. In one embodiment, the body fluid sample is selected from blood serum, blood plasma and synovial fluid. In one embodiment, the body fluid sample is a synovial fluid sample. In one embodiment, the body fluid  
40 sample is a blood serum sample. In one embodiment, the body fluid sample is a blood plasma sample. A key advantage to using blood plasma or blood serum in the methods of the invention is that these

samples are readily available and can be obtained using minimally invasive techniques. This is particularly advantageous when attempting to diagnose early-stage skeletal disorder.

As used herein, the phrase "quantifying markers of skeletal health in a body fluid sample" means determining the amount of the markers that are present in the body fluid sample of a test individual.

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When determining the amount of the markers that are present in the body fluid sample this means quantifying the marker by determining, for example, the relative or absolute amount of the marker. It will be appreciated that the assay methods do not necessarily require measurement of absolute values of marker, unless it is desired, because relative values are sufficient for many applications of the invention.

10 Accordingly, the "amount" can be the (absolute) total amount of the marker that is detected in a sample, or it can be a "relative" amount, e.g., the difference between the marker detected in a sample and e.g. another constituent of the sample. In some embodiments, the amount of the marker may be expressed by its concentration in a sample, or by the concentration of a reagent that detects the marker.

15 When the amounts of the markers are determined, the methods of the present invention may determine the amount of each marker. Alternatively, the methods of the invention may determine the cumulative amount of all the markers. Alternatively, the amount of the markers can be combined with each other in a formula to form an index value.

20 Oxidised, nitrated and glycated free adducts and adduct residues are markers of impaired skeletal health. Oxidative and nitration damage to proteins (such as those present in joint tissue) in arthritis arises as a consequence of increased reactive oxygen species (ROS) in the phagocytic respiratory burst of phagocytes in cell-mediated inflammatory response, and during mitochondrial dysfunction (Wright, H. L., Moots, R. J., Bucknall, R. C. & Edwards, S. W. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology* **49**, 1618-1631 (2010); Blanco, F. J., Rego, I. & Ruiz-Romero, C. The role of mitochondria in osteoarthritis. *Nature Reviews Rheumatology* **7**, 161-169 (2011)). Protein glycation has been little studied in arthritis (Ahmed, U. *et al.*, N. Possible role of methylglyoxal and glyoxalase in arthritis. *Biochemical Society Transactions* **42**, 538-542 (2014)). It is, however, increased in oxidative stress through: (i) increased advanced glycation endproducts (AGEs) formed by oxidative processes – glycoxidation adducts such as CML, (ii) decreased metabolism of dicarbonyl precursors glyoxal, methylglyoxal and 3-deoxyglucosone (which increase formation of dicarbonyl-derived AGEs, CMA, G-H1, MG-H1, and 3DG-H), and (iii) and increased pentosephosphate pathway activity countering oxidative stress (increasing formation of trace pentose dicarbonyl precursor of pentosidine) (Thornalley, P. J. & Rabbani, N. Detection of oxidised and glycated proteins in clinical samples using mass spectrometry - A user's perspective. *Biochim. Biophys. Acta* **1840**, 818-829 (2014). Rabbani, N. & Thornalley, P. J. Dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease. *Biochem. Biophys. Res. Commun.* **458**, 221-226 (2015)).

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40 These processes thus result in proteins having oxidised, nitrated and glycated adduct residues. When these proteins undergo proteolysis, they release oxidised, nitrated and glycated free adducts, which transit into plasma for clearance in the kidney and eventual excretion in urine. The adduct residues (attached to protein) and free adducts (released from protein) thus provide markers that relate to directly

impaired skeletal health. The oxidised, nitrated and glycated free adducts and adduct residues of proteins therefore provide suitable markers for determining skeletal health of individuals.

As used herein, the phrase “oxidised, nitrated, and glycated free adducts” refers to the proteolytic digestion products that have been released into the body fluid of the test individual following proteolysis of oxidised, nitrated and glycated proteins.

Advantageously, the pre-analytic processing steps required to obtain a sample of oxidised, nitrated and glycated free adducts are simple and rapid. Oxidised, nitrated and glycated free adducts therefore provide ideal markers for use in the methods of the invention.

As an alternative to quantifying “oxidised, nitrated, and glycated free adducts”, “oxidised, nitrated, and glycated adduct residues” can instead be quantified in the method of determining the skeletal health of an individual.

As used herein, the phrase “oxidised, nitrated, and glycated adduct residues” refers to the oxidised, nitrated and glycated adduct residues of proteins that are present in the body fluid of the test individual.

The oxidised, nitrated, and glycated adduct residues may also be used in all methods, uses and kits of the invention. Thus, in all embodiments of the methods, uses and kits of the invention described herein, the reference to “oxidised, nitrated, and glycated free adducts” may be replaced with a reference to “oxidised, nitrated, and glycated adduct residues”.

As illustrated in Example 2, the present inventors have demonstrated that by quantifying specific sub-sets of oxidised, nitrated and glycated free adducts (or adduct residues), it is possible to determine the skeletal health of an individual with a high degree of specificity and sensitivity, which advantageously allows early diagnosis of skeletal disorder. The inventors observed that results could be also be improved by including hydroxyproline (Hyp) in the method of the invention. Hydroxyproline is a known marker of skeletal health (Ahmed, U. *et al.* Biomarkers of early stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. *Sci. Rep.* **5**, 9259 (9251-9257) (2015)). As demonstrated by Example 2 of the application, two sub-sets of markers have been identified as providing excellent results. Good results can, however, still be obtained when using fewer markers.

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health may comprise at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least 11, or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine

sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp). The advantageous results obtained when using this sub-set of markers is described in Example 2 and illustrated in Figures 11 and 12.

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp). The advantageous results obtained when using this sub-set of markers is described in Example 2 and illustrated in Figure 14.

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine and glucosepane; and hydroxyproline (Hyp).

As demonstrated by Example 2, some of the markers in the group have been identified as being particularly useful for ensuring diagnostic performance of the algorithm. When using the first sub-set of markers, the order of utility of the skeletal markers for diagnostic performance of the algorithm is as follows:

MetSO > 3DG-H ≈ 3-NT ≈ pentosidine > CMA ≈ NFK > CEL > MG-H1 > DT > FL ≈ G-H1

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N<sub>ω</sub>-carboxymethylarginine (CMA); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: N-formylkynurenine (NFK), dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N-formylkynurenine (NFK); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: N<sub>ω</sub>-carboxymethylarginine (CMA), dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA) and N-formylkynurenine (NFK); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone (MG-H1), and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), and dityrosine (DT); and optionally further comprise one or more markers selected from: the glycated free adduct glyoxal-derived hydroimidazolone (G-H1) and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and glyoxal-derived hydroimidazolone (G-H1), and optionally further comprise N<sub>ε</sub>-fructosyl-lysine (FL) and/or hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and N<sub>ε</sub>-fructosyl-lysine (FL), and optionally further comprise glyoxal-derived hydroimidazolone (G-H1) and/or hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL), and optionally further comprise hydroxyproline (Hyp).

In view of the results observed in Example 1, all combinations of markers described herein may additionally comprise the oxidised free adducts or adduct residues: AASA and/ or GSA.

When using the second sub-set of markers, the order of utility of the skeletal markers for diagnostic performance of the algorithm is as follows:

MetSO > 3DG-H ≈ 3-NT > CMA ≈ NFK > GSP > CEL > MG-H1 > DT > FL ≈ G-H1

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), and glucosepane (GSP); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-

carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone (MG-H1); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

5 In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), and dityrosine (DT); and optionally further  
10 comprise one or more markers selected from: the oxidised and glycated free adducts: glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

15 In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and N<sub>ε</sub>-fructosyl-lysine (FL); and optionally further comprise glyoxal-derived hydroimidazolone (G-H1) and/or hydroxyproline (Hyp).

20 In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and glyoxal-derived  
25 hydroimidazolone (G-H1); and optionally further comprise N<sub>ε</sub>-fructosyl-lysine (FL) and/or hydroxyproline (Hyp).

30 In one embodiment, the markers of skeletal health that are quantified in the method for determining skeletal health comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and optionally further comprise hydroxyproline  
35 (Hyp).

In view of the results observed in Example 1, all combinations of markers described herein may additionally comprise the oxidised free adducts or adduct residues: AASA and/ or GSA.

40 The above combination of markers may also be used as markers of early-stage skeletal disorder in the method described herein.

Measurement of the markers can be performed by any method that provides satisfactory analytical specificity, sensitivity and precision. The invention thus encompasses the use of those methods known to a person skilled in the art to measure the amount of the free adduct or adduct residues in a body fluid sample.

5 In one embodiment, the free adducts or adduct residues may be quantified by liquid chromatography-tandem mass spectrometry, such as stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS).

10 Advantageously, this technique has high sensitivity and specificity for analytes, is multiplexed for analyte combinations at minimal additional cost and uses instrumentation that is now widely available with increasing extent of automation and availability for non-specialist use. Methods for preparation of analytical standards have been established and some are commercially available. By using stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry quantification techniques, the  
15 methods of the invention may be performed in approximately 30 minutes, thereby providing a rapid and simple approach to diagnosing skeletal health.

When quantifying oxidised, nitrated, and glycated free adducts, the method may further comprise an initial step of isolating the oxidised, nitrated, and glycated free adducts from the body fluid sample by  
20 ultrafiltration. The ultrafiltrate sample (containing the free adducts) is collected and used in the quantification step of the method. In one embodiment, the oxidised, nitrated and glycated free adducts are collected by microspin ultrafiltration. A molecular weight cut-off of at least about 10kDa may be used in the ultrafiltration step. In one embodiment, the molecular weight cut-off may be at least about 5kDa (such as at least about 6kDa, 7kDa, 8kDa, 9kDa, 10kDa, 11kDa, 12kDa, 13kDa, 14kDa or 15kDa). The  
25 ultrafiltration step may be performed at a temperature of between about 2°C and 10°C, such as at about 4°C.

When quantifying the oxidised, nitrated and glycated adduct residues, the method may further comprise an initial step of hydrolysing the adduct residues to release amino acids for quantification. In one  
30 embodiment, the hydrolysis is performed enzymatically. Protein hydrolysis by enzymatic digestion is advantageous because it avoids the severe conditions of acid hydrolysis which may compromise the analyte content of the sample during pre-analytic processing. In one embodiment, enzymatic digestion may involve treatment with pepsin, followed by treatment with pronase E, prolidase and aminopeptidase. Additionally, collagenase may be used, particularly, but not exclusively, where the protein to be assayed  
35 is present in the extracellular matrix. Automated exhaustive enzymatic hydrolysis may be used, thereby avoiding harsh, pre-analytic processing. In one embodiment, prior to hydrolysis, the proteins may be first washed by ultrafiltration to remove free amino acids, and retained protein is collected for hydrolysis. In one embodiment, the retained protein may be delipidated prior to hydrolysis. The oxidised, nitrated and glycated adduct residues may be normalised to their amino acid residue precursors and given as  
40 mmol/mol amino acid modified.

Hydroxyproline may be quantified using routine immunoassays known to the skilled person. Alternatively,

the hydroxyproline (Hyp) marker may be quantified using liquid chromatography-tandem mass spectrometry. In one embodiment, the hydroxyproline (Hyp) marker is quantified using stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described above. In one embodiment, the hydroxyproline (Hyp) quantified in the body fluid is free (dialyzable) hydroxyproline (Hyp).

The body fluid sample used for quantification of hydroxyproline (Hyp) may be the same sample used to quantify the oxidised, nitrated and glycated free adducts or adduct residues, or it may be a different body fluid sample obtained from the test individual. In one embodiment, the body fluid sample used to quantify Hyp may be a urine sample. When quantifying Hyp in a urine sample, the sample may be further assayed for creatinine, and the amount of Hyp in the test sample is normalised, having regard to the amount of creatinine present in said sample.

When hydroxyproline is included as a marker of skeletal health in the method of the invention for determining skeletal health, the method may further comprise an initial step of isolating the hydroxyproline (Hyp) marker from the body fluid sample by ultrafiltration prior to quantification, as described above. The ultrafiltrate sample (containing the hydroxyproline) is collected and used in the quantification step of the method.

The method of the invention for determining the skeletal health of an individual may further comprise the step of quantifying anti-cyclic citrullinated peptide antibody (anti-CCP antibody). By including this additional marker of skeletal health, the overall sensitivity and specificity of the method may be improved. The body fluid sample used for quantification of anti-CCP antibody may be the same sample used to quantify the other markers of skeletal health, or it may be a different body fluid sample obtained from the test individual.

In one embodiment, the method of determining the skeletal health of an individual further comprises quantifying rheumatoid factor (RF) in a body fluid sample obtained from the test individual.

In one embodiment, the method of determining the skeletal health of an individual further comprises quantifying citrullinated proteins in a body fluid sample obtained from the test individual. Further discussion of suitable methods for detecting citrullinated proteins may be found in WO 2014/016584.

In addition to the markers of skeletal health discussed above, the method of the invention for determining the skeletal health of an individual may further comprise including the age and/or gender of the test individual as further markers. Quantification of gender may for example comprise assigning a value of 1 if the test individual is female, and a value of 0 if the test individual is male.

As used herein, the phrase "comparing the amount of each marker quantified in the test sample to corresponding reference values for each marker in a diagnostic algorithm" refers to the comparative process by which the amount of a marker quantified in the test sample is compared to a reference value for the same marker using a diagnostic algorithm. The comparative process may be part of a

classification by a diagnostic algorithm. The comparative process may occur at an abstract level, e.g. in n-dimensional feature space or in a higher dimensional space.

As used herein, the term “reference value” refers to a value obtained from a population of individual(s) whose disease state is known. The reference value may be in n-dimensional feature space and may be defined by a maximum-margin hyperplane. A reference value can be determined for any particular population, subpopulation, or group of individuals according to standard methods well known to those of skill in the art.

As used herein, the phrase “classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm” refers to the statistical or machine learning classification process by which the amount of a marker quantified in the test sample is used to determine a category of skeletal health with a diagnostic algorithm, typically a statistical or machine learning classification algorithm.

Classification by a diagnostic algorithm may include scoring likelihood of a panel of marker values belonging to each possible category, and determining the highest-scoring category. Classification by a diagnostic algorithm may include comparing a panel of marker values to previous observations by means of a distance function. Examples of diagnostic algorithms suitable for classification include random forests, support vector machines, logistic regression (e.g. multiclass or multinomial logistic regression, and/or algorithms adapted for sparse logistic regression). A wide variety of other diagnostic algorithms that are suitable for classification may be used, as known to a person skilled in the art.

As used herein, the phrase “training the diagnostic algorithm” may refer to supervised learning of a diagnostic algorithm on the basis of values for each marker obtained from a population of individuals having known skeletal health. The phrase “training the diagnostic algorithm” may refer to variable selection in a statistical model on the basis of values for each marker obtained from a population of individuals having known skeletal health. Training a diagnostic algorithm may for example include determining a weighting vector in feature space for each category, or determining a function or function parameters.

As used herein, the term “population of individuals” means one or more individuals. In one embodiment, the population of individuals consists of one individual. In one embodiment, the population of individuals comprises multiple individuals. As used herein, the term “multiple” means at least 2 (such as at least 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30) individuals. In one embodiment, the population of individuals comprises at least 10 individuals.

In one embodiment, the reference value is the amount of a marker in a sample or samples derived from one individual. Alternatively, the reference value may be derived by pooling data obtained from multiple individuals, and calculating an average (for example, mean or median) amount for a marker. Thus, the reference value may reflect the average amount of a marker in multiple individuals. Said amounts may be

expressed in absolute or relative terms, in the same manner as described above in relation to the sample that is to be tested using the method of the invention.

The reference value may be derived from the same sample as the sample that is being tested, thus allowing for an appropriate comparison between the two. Thus, by way of example, if the sample is derived from urine, the reference value is also derived from urine. Alternatively, if the sample is a blood sample (e.g. a plasma or a serum sample), then the reference value will also be a blood sample (e.g. a plasma sample or a serum sample, as appropriate).

When comparing between the sample and the reference value, the way in which the amounts are expressed is matched between the sample and the reference value. Thus, an absolute amount can be compared with an absolute amount, and a relative amount can be compared with a relative amount. Similarly, the way in which the amounts are expressed for classification with the diagnostic algorithm is matched to the way in which the amounts are expressed for training the diagnostic algorithm.

When the amounts of the markers are determined, the method may comprise comparing the amount of each marker to its corresponding reference value. When the cumulative amount of one, some or all the markers is determined, the method may comprise comparing the cumulative amount to a corresponding reference value. When the amounts of the markers are combined with each other in a formula to form an index value, the index value can be compared to a corresponding reference index value derived in the same manner.

The reference values may be obtained either within (ie. constituting a step of) or external to the (ie. not constituting a step of) methods of the invention. In one embodiment, the methods of the invention may comprise a step of establishing a reference value for the quantity of the markers. In one embodiment, the reference values are obtained externally to the method of the invention and accessed during the comparison step of the invention.

The training of a diagnostic algorithm may be obtained either within (ie. constituting a step of) or external to (ie. not constituting a step of) the methods of the invention. In one embodiment, the methods of the invention may comprise a step of training of a diagnostic algorithm. In one embodiment, the diagnostic algorithm is trained externally to the method of the invention and accessed during the classification step of the invention.

The reference value may be determined by quantifying the amount of a marker in a sample obtained from a population of healthy individual(s). The diagnostic algorithm may be trained by quantifying the amount of a marker in a sample obtained from a population of healthy individual(s). As used herein, the term "healthy individual" refers to an individual or group of individuals who are in a healthy state, e.g. patients who have not shown any symptoms of the disease, have not been diagnosed with the disease and/or are not likely to develop the disease. Preferably said healthy individual(s) is not on medication affecting the disease and has not been diagnosed with any other disease. The one or more healthy individuals may have a similar sex, age and body mass index (BMI) as compared with the test individual.

The reference value may be determined by quantifying the amount of a marker in a sample obtained from a population of individual(s) suffering from the disease. The diagnostic algorithm may be trained by quantifying the amount of a marker in a sample obtained from a population of individual(s) suffering from the disease. More preferably such individual(s) may have similar sex, age and body mass index (BMI) as compared with the test individual. The reference value may be obtained from a population of individuals suffering from early-stage skeletal disorders (such as early-stage osteoarthritis or early-stage rheumatoid arthritis), or advanced-stage skeletal disorders (such as advanced-stage osteoarthritis or advanced-stage rheumatoid arthritis). The diagnostic algorithm may be trained by quantifying the amount of a marker in a sample obtained from a population of individuals suffering from early-stage skeletal disorders (such as early-stage osteoarthritis or early-stage rheumatoid arthritis), or advanced-stage skeletal disorders (such as advanced-stage osteoarthritis or advanced-stage rheumatoid arthritis). The markers quantities characteristic of early-stage skeletal disorder may, in fact, be determined only by a retrospective analysis of samples obtained from individuals who ultimately manifest clinical symptoms of the skeletal disorder. The known skeletal health may be determined only by a retrospective analysis of samples obtained from individuals who ultimately manifest clinical symptoms of the skeletal disorder. Once the characteristic marker profile of an early-stage skeletal disorder is determined, the profile of markers from a biological sample obtained from an individual may be compared to this reference profile to determine whether the test subject is also at that particular stage of the skeletal disorder. Once the diagnostic algorithm is trained to classify early-stage skeletal disorder, the profile of markers from a biological sample obtained from an individual may be classified by the diagnostic algorithm to determine whether the test subject is also at that particular stage of the skeletal disorder.

In one embodiment, when performing the method for determining skeletal health of an individual, the population of individuals used to obtain reference values for the diagnostic algorithm, and/or the population of individuals used to train the diagnostic algorithm, may comprise: at least one healthy individual having no skeletal disorder, and/or at least one individual having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), advanced-stage osteoarthritis (aOA), early-stage rheumatoid arthritis (eRA), advanced-stage rheumatoid arthritis (aRA), or other inflammatory joint disease that may be self-resolving. In one embodiment, the population of individuals may comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder, and/or multiple (eg. at least 10) individuals having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), advanced-stage osteoarthritis (aOA), early-stage rheumatoid arthritis (eRA), advanced-stage rheumatoid arthritis (aRA), or other inflammatory joint disease that may be self-resolving.

In one embodiment, when performing the method for determining skeletal health of an individual, the population of individuals used to obtain reference values for the diagnostic algorithm, and/or the population of individuals used to train the diagnostic algorithm, may comprise: at least one healthy individual having no skeletal disorder, at least one individual having early-stage osteoarthritis (eOA), at least one individual having advanced-stage osteoarthritis (aOA), at least one individual having early-stage rheumatoid arthritis (eRA), at least one individual having advanced-stage rheumatoid arthritis (aRA), and/or at least one individual having another inflammatory joint disease that may be self-resolving. In one

embodiment, the population of individuals may comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder, multiple (eg. at least 10) individuals having early-stage osteoarthritis (eOA), multiple (eg. at least 10) individuals having advanced-stage osteoarthritis (aOA), multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis (eRA), multiple (eg. at least 10) individuals having advanced-stage rheumatoid arthritis (aRA), and/or multiple (eg. at least 10) individuals having another inflammatory joint disease that may be self-resolving.

In one embodiment, when performing the method for determining skeletal health of an individual, the population of individuals used to obtain reference values for the diagnostic algorithm, and/or the population of individuals used to train the diagnostic algorithm, may comprise: at least one healthy individual having no skeletal disorder, at least one individual having early-stage osteoarthritis (eOA), at least one individual having early-stage rheumatoid arthritis (eRA), and/or at least one individual having another inflammatory joint disease that may be self-resolving. In one embodiment, the population of individuals may comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder, multiple (eg. at least 10) individuals having early-stage osteoarthritis (eOA), multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis (eRA), and/or multiple (eg. at least 10) individuals having another inflammatory joint disease that may be self-resolving.

As shown in Figures 3-8 and 13 and described in Example 1, samples obtained from individuals that have a skeletal disorder (including those having early and advanced stage skeletal disorders) and individuals having no skeletal disorder have different marker profiles (ie. the abundance of the markers quantified varies between the samples). These differences in marker abundance between individuals having a skeletal disorder and those not having a skeletal disorder provides a way to classify individuals as having a skeletal disorder or not having a skeletal disorder by determining which marker profile they display.

By comparing the amount of markers quantified in a sample obtained from a test individual to the amount of markers quantified for a reference value obtained from a population of individuals having known skeletal health, it is possible to determine the skeletal health of the individual. By classifying the skeletal health based on the amount of markers with a diagnostic algorithm trained on corresponding values obtained from a population of individuals having known skeletal health, it is possible to determine the skeletal health of the individual. The method permits classification of the individual as belonging to or not belonging to the reference population (ie. by determining whether the amounts of marker quantified in the individual are statistically similar to the reference population or statistically deviate from the reference population). Hence, classification of the individual's marker profile (ie. the overall pattern of change observed for the markers quantified) as corresponding to the profile derived from a particular reference population is predictive that the patient falls (or does not fall) within the reference population.

In one embodiment, an individual may be diagnosed as having a skeletal disorder when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having a skeletal disorder. In one embodiment, an individual may be diagnosed as having no skeletal disorder when the amount of markers quantified is statistically similar to

the amount determined for the corresponding values obtained from a population of individuals having no skeletal disorder.

As used herein, the term “statistically similar” means that the amounts of marker quantified for the test individual are similar to those quantified for the reference population to a statistically significant level. The term “statistically significant” means that the alteration is greater than what might be expected to happen by chance alone. Statistical significance can be determined by any method known in the art.

In one embodiment, an individual may be diagnosed as having a skeletal disorder when the amount of markers quantified statistically deviates from the amount determined for the corresponding values obtained from a population of individuals having no skeletal disorder. In one embodiment, an individual may be diagnosed as having no skeletal disorder when the amount of markers quantified statistically deviates from the amount determined for the corresponding values obtained from a population of individuals having a skeletal disorder.

As used herein, the term “statistically deviates” means that the amounts of marker quantified for the test individual differs from those quantified for the reference population to a statistically significant level. The deviation in marker abundance may be an increase or decrease.

In one embodiment, comparing the amount of the marker relative to the reference value and determining an increase indicates that the individual has a skeletal disorder. The increase can be, for example, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 110%, at least 120%, at least 130%, at least 140% or at least 150% of the reference value. The increase in the amount of the markers may be statistically significant.

In one embodiment, comparing the amount of the marker relative to the reference value and determining a decrease indicates that the individual has a skeletal disorder. The decrease can be, for example, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the reference value. The decrease in the amount of the marker may be statistically significant.

In one embodiment, comparing the amount of the marker relative to the reference value and determining an increase indicates that the individual does not have a skeletal disorder. The increase can be, for example, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 110%, at least 120%, at least 130%, at least 140% or at least 150% of the reference value. The increase in the amount of the marker may be statistically significant.

In one embodiment, comparing the amount of the marker relative to the reference value and determining a decrease indicates that the individual does not have a skeletal disorder. The decrease can be, for example, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%,

at least 40%, at least 45%, or at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the reference value. The decrease in the amount of the markers may be statistically significant.

Classification of the individual in the methods of the invention is performed using a diagnostic algorithm.

- 5 The diagnostic algorithm used in the method of the invention is a classification algorithm. In one embodiment, the classification algorithm is an ensemble algorithm comprising different types of classification algorithms. In one embodiment, the classification algorithm comprises a decision tree based algorithm. In one embodiment, the classification algorithm comprises a support vector machine algorithm. Other types of algorithms, such as regression algorithms and neural networks, may also be used. In one  
10 embodiment, the classification algorithm comprises a random forest algorithm. The diagnostic algorithm used in the method of the invention for determining the skeletal health of an individual may be a 2-class algorithm (e.g. see Figure 2(a)).

Classification of the individual by the diagnostic algorithm does not require perfect classification.

- 15 Classification may be characterized by its "sensitivity." The "sensitivity" of classification relates to the percentage of individuals who were correctly identified as having a skeletal disorder, or in the case of determining whether an individual has an early-stage skeletal disorder, the percentage of individuals correctly identified as having a particular early-stage skeletal disorder. "Sensitivity" is defined in the art as the number of true positives divided by the sum of true positives and false negatives.

- 20 The sensitivity of the methods of the invention may be at least about 90%, at least about 89%, at least about 88%, at least about 87%, at least about 86%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, or at least about 65%.

The "specificity" of the methods of the invention is defined as the percentage of patients who were  
25 correctly identified as not having a skeletal disorder, or in the in the case of determining whether an individual has an early-stage skeletal disorder, the percentage of individuals correctly identified as not having a particular early-stage skeletal disorder. "Specificity" relates to the number of true negatives divided by the sum of true negatives and false positives. The specificity of the methods of the invention may be at least about 90%, at least about 89%, at least about 88%, at least about 87%, at least about  
30 86%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, or at least about 65%.

The invention further provides the use of at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least  
35 11, or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp) for determining skeletal health.

- 40 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1),

methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp) as markers for determining skeletal health.

- 5 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp) as markers for determining skeletal health.
- 10 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp) as markers for determining skeletal health.
- 15 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N<sub>ω</sub>-carboxymethylarginine (CMA) as markers for determining skeletal health.
- 20 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N-formylkynurenine (NFK) as markers for determining skeletal health.
- 25 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA) and N-formylkynurenine (NFK) as markers for determining skeletal health.
- 30 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL) as markers for determining skeletal health.
- 35 In one embodiment, the invention provides the use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL) and methylglyoxal-derived hydroimidazolone (MG-H1) as markers for determining skeletal health.
- 40 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts of methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), N<sub>ε</sub>-(1-carboxyethyl)lysine

(CEL), methylglyoxal-derived hydroimidazolone (MG-H1) and dityrosine (DT) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT) and glyoxal-derived hydroimidazolone (G-H1) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL) as markers for determining skeletal health.

In view of the results observed in Example 1, the combination of markers described herein may additionally include the oxidised free adducts or adduct residues: AASA and/ or GSA.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), and glucosepane (GSP) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone (MG-H1) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), and dityrosine (DT) as markers for determining skeletal health.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-

NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and glyoxal-derived hydroimidazolone (G-H1) as markers for determining skeletal health.

5 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and N<sub>ε</sub>-fructosyl-lysine (FL) as markers for determining skeletal health.

10 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), glyoxal-  
15 derived hydroimidazolone (G-H1) and N<sub>ε</sub>-fructosyl-lysine (FL) as markers for determining skeletal health.

In view of the results observed in Example 1, the combination of markers described herein may additionally include the oxidised free adducts or adduct residues: AASA and/ or GSA.

20 The above combinations of markers may also be used as markers for determining early stage skeletal disorder.

The invention also provides a method of treating a skeletal disorder in an individual, comprising:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

25 (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-  
30 deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health,  
35 and

(iii) determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) administering a treatment for a skeletal disorder to the individual.

40 All embodiments of the 'method for determining the skeletal health of the individual' described above apply equally to the method for treating an individual having a skeletal disorder.

In one embodiment, the “skeletal disorder” may be early-stage osteoarthritis (eOA), advanced-stage osteoarthritis (aOA), early-stage rheumatoid arthritis (eRA), advanced-stage rheumatoid arthritis (aRA), or other inflammatory joint disease that may be self-resolving. In one embodiment, the “skeletal disorder” may be early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

In one embodiment, the administration of a treatment to the individual improves the skeletal health of the individual. As used herein, the phrase “improve the skeletal health of the individual” refers to the improvement in skeletal health of the individual identified as having a skeletal disorder (such as an early-stage or advanced-stage skeletal disorder) by administering a treatment for that skeletal disorder. In one embodiment, the progression of the skeletal disorder from an early-stage disorder to an advanced-stage disorder is reduced or prevented. In one embodiment, the symptoms of the skeletal disorder are alleviated. In one embodiment, the treatment of the individual results in regression of an advanced-stage skeletal disorder to an early-stage skeletal disorder. In one embodiment, the treatment of the individual results in the regression of an advanced-stage skeletal disorder to no skeletal disorder.

The method of the invention is intended to encompass all known treatments for skeletal disorders. The skilled person will be familiar with treatments for skeletal disorders.

The invention provides a method of determining whether an individual has an early-stage skeletal disorder comprising:

- (a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;
- (b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

The method of the invention is for determining whether an individual has an early-stage skeletal disorder. In one embodiment, the method of the invention is for determining whether an individual has an early-stage skeletal disorder selected from: early-stage osteoarthritis, early-stage rheumatoid arthritis and other inflammatory joint disease that may be self-resolving. In one embodiment, the method of the invention is for distinguishing between early-stage skeletal disorders including early-stage osteoarthritis, early-stage rheumatoid arthritis and other inflammatory joint disease that may be self-resolving. In one embodiment, the method of the invention may be for typing an early-stage skeletal disorder in an individual. The typing may involve determining whether the individual has an early-stage skeletal disorder selected from one of: early-stage osteoarthritis, early-stage rheumatoid arthritis and other inflammatory joint disease that may be self-resolving.

As illustrated in Example 2, the present inventors have demonstrated that by quantifying two specific sub-sets of oxidised, nitrated and glycated free adducts (or adduct residues), it is possible to determine whether an individual has an early-stage skeletal disorder, and in particular to distinguish between different types of early-stage skeletal disorder. The inventors observed that results could be also be improved by including anti-cyclic citrullinated peptide antibody as a marker for early-stage skeletal disorder in the method of the invention. Anti-cyclic citrullinated peptide antibody is a known marker of skeletal health (Raza, K. *et al.* Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis. *J.Rheumatol.* 32, 231-238 (2005)). As demonstrated by Example 2 of the application, excellent results are obtained when either of the sub-sets of markers of skeletal health. Good results can, however, still be obtained when using fewer markers.

All embodiments of the quantification step described above for the 'method of determining the skeletal health of an individual' apply equally to the 'method for determining whether an individual has an early-stage skeletal disorder'.

In one embodiment, the method for determining whether an individual has an early-stage skeletal disorder comprises quantifying markers of skeletal health that comprise: at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least 11, or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

In one embodiment, the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and anti-cyclic citrullinated peptide antibody. The advantageous results obtained when using this sub-set of markers are described in Example 2 and illustrated in Figure 12.

In one embodiment, the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody. The advantageous results obtained when using this sub-set of markers are described in Example 2 and illustrated in Figure 14.

In one embodiment, the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

In view of the results observed in Example 1, the combination of markers described herein may additionally comprise the oxidised free adducts or adduct residues: AASA and/ or GSA.

When quantifying oxidised, nitrated, and glycated free adducts as markers of skeletal health in a method of determining whether an individual has an early-stage skeletal disorder, the method may further comprise an initial step of isolating the oxidised, nitrated, and glycated free adducts from the body fluid sample by ultrafiltration. All embodiments of the ultrafiltration step described above with respect to the method for determining the skeletal health of an individual apply equally to the method for determining whether an individual has an early-stage skeletal disorder.

When quantifying the oxidised, nitrated and glycated adduct residues as markers of skeletal health in a method of determining whether an individual has an early-stage skeletal disorder, the method may further comprise an initial step of hydrolysing the adduct residues to release amino acids for quantification. All embodiments of the hydrolysis step described above with respect to the method for determining the skeletal health of an individual apply equally to the method for determining whether an individual has an early-stage skeletal disorder.

The method of the invention for determining whether an individual has an early-stage skeletal disorder may further comprise the step of quantifying hydroxyproline (Hyp). All embodiments described above with respect to the step of quantifying hydroxyproline (Hyp) in a method of determining the skeletal health of an individual apply equally to the step of quantifying hydroxyproline (Hyp) in a method of determining whether an individual has an early-stage skeletal disorder. By including this additional marker of skeletal health, the overall sensitivity and specificity of the method may be improved. Methods for quantifying hydroxyproline may also involve an initial step of isolating the hydroxyproline by ultrafiltration, as described above.

In one embodiment, the method of determining whether an individual has an early-stage skeletal disorder further comprises quantifying rheumatoid factor (RF) in a body fluid sample obtained from the test individual.

In one embodiment, the method of determining whether an individual has an early-stage skeletal disorder further comprises quantifying citrulline in a body fluid sample obtained from the test individual. Further discussion of suitable methods for detecting citrulline may be found in WO 2014/016584.

In addition to the markers of skeletal health discussed above, the method of the invention for determining whether an individual has an early-stage skeletal disorder may further comprise including the age and/or gender of the test individual as further markers. Quantification of gender may for example comprise assigning a value of 1 if the test individual is female, and a value of 0 if the test individual is male.

The method of determining whether an individual has an early-stage skeletal disorder comprises the step of "classifying the skeletal health based on the amount of each marker quantified in the test sample with a

diagnostic algorithm”, wherein the diagnostic algorithm is trained on “a population of individuals having known early-stage skeletal disorder”.

All embodiments of the “comparison step” and/or the “classification step” described above for the ‘method of determining the skeletal health of an individual’ apply equally to the ‘method for determining whether an individual has an early-stage skeletal disorder’.

The “reference value” and/or the “diagnostic algorithm” used in the method of determining whether an individual has an early-stage skeletal disorder is as defined above for the ‘method for determining whether an individual has an early-stage skeletal disorder’.

In one embodiment, the population of individuals used to obtain reference values for the diagnostic algorithm and/or used to train the diagnostic algorithm may comprise at least one individual having early-stage osteoarthritis, at least one individual having early-stage rheumatoid arthritis, and/ or at least one individual having other inflammatory joint disease that may be self-resolving. In one embodiment, the population of individuals used to obtain reference values for the diagnostic algorithm may comprise multiple (eg. at least 10) individuals having early-stage osteoarthritis, multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis, and/ or multiple (eg. at least 10) individuals having other inflammatory joint disease that may be self-resolving.

In one embodiment, the population of individuals comprises at least one individual having early-stage osteoarthritis, at least one individual having early-stage rheumatoid arthritis, and at least one individual having other inflammatory joint disease that may be self-resolving. In one embodiment, the population of individuals comprises multiple (eg. at least 10) individuals having early-stage osteoarthritis, multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis, and multiple (eg. at least 10) individuals having other inflammatory joint disease that may be self-resolving.

In one embodiment, the population of individuals used as a reference and/or used to train the diagnostic algorithm may additionally comprise at least one individual having no known skeletal disorder. In one embodiment, the population may additionally comprise multiple (eg. at least 10) individuals having no known skeletal disorder. In one embodiment, the population of individuals used as a reference may additionally comprise at least one individual having a known advanced-stage skeletal disorder. In one embodiment, the population may additionally comprise multiple (eg. at least 10) individuals having a known advanced-stage skeletal disorder. The known advanced-stage skeletal disorder may be advanced-stage osteoarthritis. The known advanced-stage skeletal disorder may be advanced-stage rheumatoid arthritis.

As shown in Figures 3-8 and 13 and described in Example 1, samples obtained from individuals that have early-stage skeletal disorders (including those having early-stage OA, early-stage RA and non-RA) have different marker profiles (ie. the abundance of the markers quantified varies between the samples) to those individuals having no skeletal disorder and those individuals having advanced-stage skeletal disorder (including those having advanced-stage OA, and advanced-stage RA). The differences in marker

abundance between these individuals provide a way to determine whether an individual has an early-stage skeletal disorder. In addition, different marker profiles were also observed for individuals having different early-stage skeletal disorders. The differences in marker abundance between these individuals provide a way to classify individuals as having a particular type of early-stage skeletal disorder, such as early-stage OA, early-stage RA and non-RA.

By comparing the amount of markers quantified in a sample obtained from a test individual to the amount of markers quantified for a reference value obtained from a population of individuals having known early-stage disorder, it is possible to determine whether an individual has an early-stage skeletal disorder, and in particular to determine whether an individual has early-stage OA, early-stage RA or non-RA. By classifying the skeletal health based on the amount of markers with a diagnostic algorithm trained on corresponding values obtained from a population of individuals having known skeletal health, it is possible to determine whether an individual has an early-stage skeletal disorder, and in particular to determine whether an individual has early-stage OA, early-stage RA or non-RA. The method permits classification of the individual as belonging to or not belonging to the reference population (ie. by determining whether the amounts of marker quantified in the individual are statistically similar to the reference population or statistically deviate from the reference population). Hence, classification of the individual's marker profile as corresponding to the profile derived from a particular reference population is predictive that the patient falls (or does not fall) within the reference population.

In one embodiment, an individual may be diagnosed as having an early-stage skeletal disorder when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having a known early-stage skeletal disorder.

In one embodiment, an individual may be diagnosed as having early-stage OA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having early-stage OA. In one embodiment, an individual may be diagnosed as having early-stage RA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having early-stage RA. In one embodiment, an individual may be diagnosed as having non-RA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having non-RA.

The diagnostic algorithm used in the method of the invention is a classification algorithm. All embodiments of the classification algorithm described above with respect to the 'method for determining skeletal health of an individual' apply to the 'method for determining whether an individual has an early-stage skeletal disorder'. The diagnostic algorithm used in the method of the invention for determining whether an individual has an early-stage skeletal disorder is a 3-class algorithm (see Figure 2(b)).

The invention further provides the use of at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least 11, or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-

lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) for determining whether a test individual has an early-stage skeletal disorder.

5 In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H),  
10 and pentosidine; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) as markers for determining whether a test individual has an early-stage skeletal disorder.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine  
15 (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) as markers for determining whether a test individual has an early-stage skeletal disorder.

In one embodiment, the use is of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H),  
20 pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) as markers for determining whether a test individual has an early-stage skeletal disorder.

25 In view of the results observed in Example 1, the combination of markers described herein may additionally include the oxidised free adducts or adduct residues: AASA and/ or GSA.

The invention also provides a method of treating an early-stage skeletal disorder in an individual,  
30 comprising:

(a) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and  
35 glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

40 (ii) classifying the skeletal health based on the amount of each marker quantified in the test with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for

- each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder;
- (b) selecting an individual identified as having an early-stage skeletal disorder;
  - (c) administering a treatment for an early-stage skeletal disorder to the individual.

5 All embodiments of the 'method for determining whether an individual has an early-stage skeletal disorder' described above apply equally to the 'method for treating an early-stage skeletal disorder in an individual'.

In one embodiment, the "early-stage skeletal disorder" may be early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

10 The method of the invention is intended to encompass all known treatments for early-stage skeletal disorder. The skilled person will be familiar with treatments for early-stage skeletal disorder, such as those for treating early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

15 In one embodiment, the treatment administered to the individual improves the skeletal health of the individual.

The invention also provides a method of determining whether an individual has an early-stage skeletal disorder comprising:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

20 (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

25 (ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

35 (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-

deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

All embodiments of the 'method for determining the skeletal health of an individual' and the 'method for determining whether an individual has an early-stage skeletal disorder' as described above apply equally to the 'method for determining whether an individual has an early-stage skeletal disorder' in which 2 separate algorithms are used.

In one embodiment, the method of determining whether an individual has an early-stage skeletal disorder may comprise:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

For example, the method of determining whether an individual has an early-stage skeletal disorder may comprise:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

In one embodiment, the method of determining whether an individual has an early-stage skeletal disorder may comprise:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

For example, the method of determining whether an individual has an early-stage skeletal disorder may comprise:

(a) determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp);

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

(b) selecting an individual identified as having a skeletal disorder; and

(c) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and anti-cyclic citrullinated peptide antibody;

(ii) classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the second diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

To avoid unnecessary duplication of the quantification steps in this 2-algorithm method, the values obtained for the markers quantified in step (a) part (i) of the method may be used directly in step (c) part (i), thereby avoiding the need to repeat quantification of overlapping markers. For example, if step (a) part (i) of the method involves quantification of any one of the oxidised, nitrated and glycated free adducts selected from: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), pentosidine, and glucosepane, these values may be used directly in step (c) part (i).

The invention also provides a method of treating an early-stage skeletal disorder in an individual, comprising:

(a) determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

(i) determining the skeletal health of an individual by quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp), and classifying the skeletal health based on the amount of each marker quantified in the test sample with a first diagnostic algorithm, wherein the first diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, so as to determine the skeletal health of the test individual;

(ii) selecting an individual identified as having a skeletal disorder; and

(iii) determining whether the individual has an early-stage skeletal disorder by quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody, and classifying the skeletal health based on the amount of each marker quantified in the test sample with a second diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values are obtained from a population of individuals having known early-stage skeletal disorder, so as to determine whether the test individual has an early-stage skeletal disorder;

(b) selecting an individual diagnosed as having an early-stage skeletal disorder;

(c) administering a treatment for an early-stage skeletal disorder to the individual.

All embodiments of the 'method for determining the skeletal health of an individual' and the 'method for determining whether an individual has an early-stage skeletal disorder' as described above apply equally to the 'method for treating an individual diagnosed as having an early-stage skeletal disorder'.

In one embodiment, the “early-stage skeletal disorder” may be early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

The method of the invention is intended to encompass all known treatments for early-stage skeletal disorder. The skilled person will be familiar with treatments for early-stage skeletal disorder, such as those for treating early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving.

In one embodiment, the treatment administered to the individual improves the skeletal health of the individual.

The method for determining the skeletal health of an individual (and for treating a skeletal disorder in an individual), and the method for determining whether an individual has an early-stage skeletal disorder (and for treating an early-stage skeletal disorder in an individual), may further comprise repeating the ‘quantification’ and ‘comparison’ steps of the method after a selected time interval and comparing the amount of each marker quantified after said time interval to the amount quantified for each marker at an earlier time point, to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

The method for determining the skeletal health of an individual (and for treating a skeletal disorder in an individual), and the method for determining whether an individual has an early-stage skeletal disorder (and for treating an early-stage skeletal disorder in an individual), may further comprise repeating the ‘quantification’ and ‘classification’ steps of the method after a selected time interval and comparing the classification of skeletal health obtained after said time interval to the classification of skeletal health obtained at an earlier time point, to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

The method for determining the skeletal health of an individual (and for treating a skeletal disorder in an individual), and the method for determining whether an individual has an early-stage skeletal disorder (and for treating an early-stage skeletal disorder in an individual), may further comprise repeating the ‘quantification’ and ‘comparison’ steps of the method using a body fluid sample obtained from the test individual at one or more later time points and comparing the amount of each marker quantified at the one or more later time points to the amount quantified for each marker at a first (or earlier) time point, to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

The method for determining the skeletal health of an individual (and for treating a skeletal disorder in an individual), and the method for determining whether an individual has an early-stage skeletal disorder (and for treating an early-stage skeletal disorder in an individual), may further comprise repeating the ‘quantification’ and ‘classification’ steps of the method using a body fluid sample obtained from the test individual at one or more later time points and comparing the classification of skeletal health determined for the one or more later time points to the classification of skeletal health determined for a first (or earlier) time point, to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

This may be useful for monitoring the skeletal health of a test individual. In one embodiment, the method may be useful for monitoring the severity of a skeletal disorder; and/or the effectiveness of a treatment regimen on skeletal health.

By repeating the diagnostic methods of the invention at one or more later time point, the disease status of the patient can be re-classified to determine whether there has been a change or no change in the disease status of the patient.

For example, when the levels of the markers return towards (or becomes increasingly statistically similar to) the levels typically observed for the reference value representative of a healthy individual, and/or increasingly statistically deviates from the level typically observed for the reference value representative of a skeletal disorder (such as a particular type of early stage or advanced stage skeletal disorder), this indicates that there has been an improvement or regression of the skeletal disease in the test individual. Likewise, when the levels of the markers increasingly statistically deviates from the levels typically observed for the reference value representative of a healthy individual, and/or remains statistically similar to (or becomes increasingly statistically similar to) the level typically observed for the reference value representative of a skeletal disorder (such as a particular type of early stage or advanced stage skeletal disorder), this indicates that there has been a worsening or progression of the skeletal disorder in the test individual.

Monitoring of the severity of skeletal disorder in a patient may comprise monitoring of the progression, regression, aggravation, alleviation or recurrence of the disorder. Monitoring of the severity of skeletal disorder in a patient may comprise determining whether the skeletal disorder is progressing towards a more advanced form of the disorder, or regressing towards normalcy. Monitoring may also comprise determining whether the skeletal disorder has remained stable.

As used herein, the term "progression" refers to an increase or worsening in the symptoms of a disease or disorder, and the term "regression" refers to a decrease or improvement in the symptoms of disease or disorder.

Monitoring of the skeletal health in a patient may be used to determine the effectiveness of a treatment regimen on skeletal health.

The treatment regimen may include all known treatments (e.g. pharmacological treatments) for early-stage and advantage-stage skeletal disorder. The skilled person will be familiar with treatments for early-stage skeletal disorder, such as those for treating early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or other inflammatory joint disease that may be self-resolving; and for advanced-stage skeletal disorder, such as those for treating advanced-stage osteoarthritis (aOA), advanced -stage rheumatoid arthritis (eaRA).

The treatment regimen may include a program of exercise and/or physiotherapy; and/or administration of foods and/or supplements that improve skeletal health.

In one embodiment, the method for monitoring the skeletal health of a test individual may comprise:

- (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual at a first time point, and classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm;
  - (ii) quantifying markers of skeletal health in a body fluid sample obtained from a test individual at one or more later time points, and classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm; and
  - (iii) comparing the classification determined in step (i) to the classification determined in step (ii) to determine whether the skeletal health of the test individual has improved, worsened, or remained stable;
- wherein the skeletal markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp), and wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

All embodiments described above for the method for determining the skeletal health of a test individual apply equally to the method for monitoring skeletal health of the test individual.

In one embodiment, the method for monitoring the skeletal health of a test individual may comprise:

- (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual at a first time point, and classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm;
  - (ii) quantifying markers of skeletal health in a body fluid sample obtained from a test individual at one or more later time points, and classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm; and
  - (iii) comparing the classification determined in step (i) to the classification determined in step (ii) to determine whether the skeletal health of the test individual has improved, worsened, or remained stable;
- wherein the skeletal markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody, and wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder.

All embodiments described above for the method for determining whether an individual has an early-stage skeletal disorder apply equally to the method for monitoring skeletal health of the test individual.

The diagnostic algorithm used in the monitoring method may comprise a two-stage classification with a first stage for classification as having or not having a skeletal disorder, and a second stage for classification as having an early-stage skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or self-resolving inflammatory joint disease. The diagnostic algorithm used in the first stage classification may be trained on corresponding values for each marker obtained from a population of individuals having known skeletal health. The diagnostic algorithm used in the second stage classification may be trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder.

The skeletal markers used in the first stage classification may comprise the markers described above for the method for determining the skeletal health of a test individual. For example, the skeletal markers used in the first stage classification may comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

The skeletal markers used in the second stage classification may comprise the markers described above for the method for determining whether an individual has an early-stage skeletal disorder. For example, the skeletal markers used in the second stage classification may comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

The "time interval" of the method may comprise a time period of at least 24 hours (e.g. at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 8 weeks, at least 12 weeks, at least 16 weeks, at least 20 weeks, at least 30 weeks, at least 40 weeks, at least 52 weeks, at least 2 years, at least 3 years, or at least 4 years).

The sample obtained from the test individual at the "one or more later time points" may be obtained at least 24 hours (e.g. at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 8 weeks, at least 12 weeks, at least 16 weeks, at least 20 weeks, at least 30 weeks, at least 40 weeks, at least 52 weeks, at least 2 years, at least 3 years, or at least 4 years) after the sample was obtained from the test individual at a first (or earlier) time point.

In one embodiment, when the method is for monitoring the effectiveness of a treatment regimen for a skeletal disorder in a test individual, the sample obtained from the test individual at a first (or earlier) time point is obtained from the test individual before or during the course of treatment. For example, the sample may be obtained from the test individual at least 1 hour (e.g. at least 2 hours, at least 4 hours, at least 8 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 1 week, at least 2 weeks, at least 3 weeks, or at least 4 weeks) before treatment. The sample obtained from the test individual at one or more later time points is obtained during or after a course of treatment. For example, the sample may be obtained from the test individual at least 24 hours (e.g. at least 48 hours, at least 96 hours, at least 120 hours, at least 1 week, at least 2 weeks, at least 4 weeks, at least 8 weeks, at least 12 weeks, at least 16 weeks, at least 20 weeks, at least 30 weeks, at least 40 weeks, at least 52 weeks, at least 2 years, at least 3 years, or at least 4 years) after a treatment regimen has begun or has been completed.

The data presented in Figures 3-8 and 13 demonstrates that markers of skeletal health differ in abundance in skeletal disorders of varying severity. Table 2 of Example 4 summarises the key markers that were observed to significantly differ in abundance in advanced-stage skeletal disorders versus early-stage skeletal disorders. These differences in marker abundance provide a useful way of distinguishing between advanced-stage skeletal disorder and early-stage skeletal disorder. These include methods for distinguishing between advanced-stage osteoarthritis and early-stage osteoarthritis; and between advanced-stage rheumatoid arthritis and early-stage rheumatoid arthritis.

The invention therefore provides a method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, comprising:

(a) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL);

(b) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health;

and thereby determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis.

All embodiments of the 'quantifying' step described above for the 'method of determining the skeletal health of an individual' and the 'method of determining whether an individual has an early-stage skeletal disorder' apply equally to the 'method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis'.

In one embodiment, the method comprises quantifying at least one (such as at least 2, 3, 4, 5 or 6) marker selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL).

If 2 or more markers are selected, the method may comprise classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

- 5 The data presented in Figures 3-8 and 13 demonstrates that markers of skeletal health differ in abundance in advanced-stage and early-stage osteoarthritis. When testing blood plasma or blood serum, an increase in the abundance of MetSO, NFK, CEL and CMA was observed in advanced-stage osteoarthritis as compared to early-stage osteoarthritis, and a decrease in the abundance of DT and 3-NT was observed in advanced-stage osteoarthritis as compared to early-stage osteoarthritis. When testing  
10 synovial fluid, an increase was observed in the abundance of MetSO, and NFK in advanced-stage osteoarthritis as compared to early-stage osteoarthritis, and a decrease was observed in the abundance of FL in advanced-stage osteoarthritis as compared to early-stage osteoarthritis.

- In one embodiment, the method is performed using blood plasma or blood serum as the body fluid sample, and comprises quantifying at least one (such as at least 2, 3, 4, or 5) marker selected from the  
15 oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and N<sub>ω</sub>-carboxymethylarginine (CMA). In one embodiment, the method is performed using blood plasma or blood serum as the body fluid sample, and comprises quantifying the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-  
20 NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and N<sub>ω</sub>-carboxymethylarginine (CMA).

- In one embodiment, the method is performed using synovial fluid as the body fluid sample, and comprises quantifying at least one (such as at least 2) marker selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), and N<sub>ε</sub>-fructosyl-lysine (FL). In one  
25 embodiment, the method is performed using synovial fluid as the body fluid sample, and comprises quantifying the combination of the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), and N<sub>ε</sub>-fructosyl-lysine (FL).

- The method involves a step of comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals  
30 having known skeletal health. Alternatively the method involves a step of classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm. Such a diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

- All embodiments of the “comparison step” and/or the “classification step” described above for the ‘method  
35 of determining the skeletal health of an individual’ and the ‘method of determining whether an individual has an early-stage skeletal disorder’ apply equally to the ‘method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis’.

The “reference value” and/or the “diagnostic algorithm” used in the method of determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis is as defined above.

By comparing the amount of markers quantified in a sample obtained from a test individual to the amount of markers quantified for a reference value obtained from a population of individuals having known skeletal health, it is possible to determine whether the individual has early-stage or advanced-stage OA. By classifying the skeletal health based on the amount of markers with a diagnostic algorithm trained on corresponding values obtained from a population of individuals having known skeletal health, it is possible to determine whether the individual has early-stage or advanced-stage OA. The method permits classification of the individual as belonging to or not belonging to the reference population (ie. by determining whether the amounts of marker quantified in the individual are statistically similar to the reference population or statistically deviate from the reference population). Hence, classification of the individual's marker profile (ie. the overall pattern of change observed for the markers quantified) as corresponding to the profile derived from a particular reference population is predictive that the patient falls (or does not fall) within the reference population.

In one embodiment, an individual may be diagnosed as having early-stage OA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having early-stage OA. In one embodiment, an individual may be diagnosed as having advanced-stage OA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having advanced-stage OA.

All embodiments of the “population of individuals having known skeletal health” described above for the method of determining the skeletal health of an individual apply equally to the method of determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis.

In one embodiment, when performing the method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, the population of individuals used to obtain reference values for the diagnostic algorithm and/or used to train the diagnostic algorithm may comprise: at least one healthy individual having no skeletal disorder; at least one individual having early-stage osteoarthritis; and/or at least one individual having advanced-stage osteoarthritis. In one embodiment, the population of individuals may comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder; multiple (eg. at least 10) individuals having early-stage osteoarthritis; and/or multiple (eg. at least 10) individuals having advanced-stage osteoarthritis.

In one embodiment, when performing the method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, the population of individuals used to obtain reference values for the diagnostic algorithm and/or used to train the diagnostic algorithm may comprise: at least one healthy individual having no skeletal disorder; and at least one individual having advanced-stage osteoarthritis. In one embodiment, the population of individuals may comprise: multiple (eg. at least 10)

healthy individuals having no skeletal disorder; and multiple (eg. at least 10) individuals having advanced-stage osteoarthritis.

The step of comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health may be performed using a diagnostic algorithm. The step of comparing may be part of a classification algorithm. All embodiments of the diagnostic algorithm described above apply equally to the diagnostic algorithm used in this method.

The method of the invention for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis provides a useful way for assessing the effectiveness of treatment for advanced-stage osteoarthritis. By looking at the abundance of individual markers or a combination of markers in a test individual undergoing treatment, and comparing the abundance of those markers to values obtained from a population of individuals having known skeletal health (eg. individuals having advanced-stage osteoarthritis, early-stage osteoarthritis and/or no skeletal disorder), it is possible to classify the skeletal health of the test individual to determine whether treatment has improved the skeletal health of the individual.

In one embodiment, the method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis may be performed to determine whether treatment for advanced-stage osteoarthritis improves the skeletal health of a test individual diagnosed with advanced-stage osteoarthritis.

As used herein, the phrase “determining whether the treatment for advanced-stage osteoarthritis improves the skeletal health of a test individual diagnosed with advanced-stage osteoarthritis” means determining whether the test individual diagnosed with advanced-stage osteoarthritis shows an improvement in their skeletal health as a result of treatment, on the basis of the abundance of markers quantified for the test individual. This means determining whether the abundance of skeletal markers quantified for the test individual is characteristic of an individual having advanced-stage osteoarthritis, or is characteristic of an individual having early-stage osteoarthritis or no skeletal disorder (thereby demonstrating that the test individual has improved skeletal health).

In one embodiment, the body fluid sample may be obtained from a test individual undergoing treatment for advanced-stage osteoarthritis. The body fluid sample may be obtained from the test individual at any stage of the treatment process. In one embodiment, the body fluid sample is obtained following at least 1 week (such as at least 2, 4, 8, 12, 20, 30, 40, or 52 weeks, or 2, 3, or 4 years) of treatment for advanced-stage osteoarthritis. In one embodiment, the body fluid sample is obtained from the test individual once treatment has been completed.

In order to provide further insight into the effect of treatment on the skeletal health of the test individual, the method may be performed using multiple body fluid samples obtained from the test individual at different time points during the treatment process. In one embodiment, the method is performed using a body fluid sample obtained from the test individual prior to commencement of treatment for advanced-

stage osteoarthritis, and a body fluid sample obtained from the test individual during treatment (eg. following at least 1, 2, 4, 8, 12, 20, 40, or 52 weeks, or 2, 3, or 4 years of treatment).

The invention further provides the use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL) as markers for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis. In one embodiment, the use is for determining whether treatment for advanced-stage osteoarthritis improves the skeletal health of a test individual diagnosed with advanced-stage osteoarthritis.

10 The invention further provides a method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, comprising:

(a) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine;

15 (b) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health;

and thereby determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis.

20 All embodiments of the 'quantifying' step described above for the 'method of determining the skeletal health of an individual' and the 'method of determining whether an individual has an early-stage skeletal disorder' apply equally to the 'method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis'.

25 In one embodiment, the method comprises quantifying at least one (such as at least 2) marker selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine. In one embodiment, the method comprises quantifying the combination of the oxidised, and glycated free: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine.

30 If 2 or more markers are selected, the method may comprise classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

35 The data presented in Figures 3-8 and 13 demonstrate that markers of skeletal health differ in abundance in advanced-stage and early-stage rheumatoid arthritis. When testing blood plasma or blood serum, an increase was observed in the abundance of MetSO, DT and pentosidine in advanced-stage rheumatoid arthritis as compared to early-stage rheumatoid arthritis. When testing synovial fluid, an increase was observed in the abundance of DT and pentosidine in advanced-stage rheumatoid arthritis as compared to early-stage rheumatoid arthritis.

In one embodiment, the method is performed using blood plasma or blood serum as the body fluid sample, and comprises quantifying at least one (such as at least 2) marker selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine. In one embodiment, the method is performed using blood plasma or blood serum as the body fluid sample, and comprises quantifying the combination of the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine.

In one embodiment, the method is performed using synovial fluid as the body fluid sample, and comprises quantifying at least one marker selected from the oxidised free adduct dityrosine (DT), and the glycated free adduct pentosidine. In one embodiment, the method is performed using synovial fluid as the body fluid sample, and comprises quantifying the combination of the oxidised free adduct dityrosine (DT), and the glycated free adduct pentosidine.

The method involves a step of comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health. Alternatively the method involves a step of classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm. Such a diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health.

All embodiments of the “comparison step” and/or the “classification step” described above for the ‘method of determining the skeletal health of an individual’ and the ‘method of determining whether an individual has an early-stage skeletal disorder’ apply equally to the ‘method for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis’.

The “reference value” and/or the “diagnostic algorithm” used in the method of determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis is as defined above.

By comparing the amount of markers quantified in a sample obtained from a test individual to the amount of markers quantified for a reference value obtained from a population of individuals having known skeletal health, it is possible to determine whether the individual has early-stage or advanced-stage RA. By classifying the skeletal health based on the amount of markers with a diagnostic algorithm trained on corresponding values obtained from a population of individuals having known skeletal health, it is possible to determine whether the individual has early-stage or advanced-stage RA. The method permits classification of the individual as belonging to or not belonging to the reference population (ie. by determining whether the amounts of marker quantified in the individual are statistically similar to the reference population or statistically deviate from the reference population). Hence, classification of the individual's marker profile (ie. the overall pattern of change observed for the markers quantified) as corresponding to the profile derived from a particular reference population is predictive that the patient falls (or does not fall) within the reference population.

In one embodiment, an individual may be diagnosed as having early-stage RA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained

from a population of individuals having early-stage RA. In one embodiment, an individual may be diagnosed as having advanced-stage RA when the amount of markers quantified is statistically similar to the amount determined for the corresponding values obtained from a population of individuals having advanced-stage RA.

5 All embodiments of the "population of individuals having known skeletal health" described above for the method of determining the skeletal health of an individual apply equally to the method of determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis.

10 In one embodiment, when performing the method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, the population of individuals used to obtain reference values for the diagnostic algorithm may comprise: at least one healthy individual having no skeletal disorder; at least one individual having early-stage rheumatoid arthritis; and/or at least one individual having advanced-stage rheumatoid arthritis. In one embodiment, the population of individuals  
15 may comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder; multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis; and/or multiple (eg. at least 10) individuals having advanced-stage rheumatoid arthritis.

In one embodiment, when performing the method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, the population of individuals used to obtain  
20 reference values for the diagnostic algorithm and/or used to train the diagnostic algorithm may comprise: at least one individual having early-stage rheumatoid arthritis; and at least one individual having advanced-stage rheumatoid arthritis. In one embodiment, the population of individuals may comprise: multiple (eg. at least 10) individuals having early-stage rheumatoid arthritis; and multiple (eg. at least 10)  
25 individuals having advanced-stage rheumatoid arthritis.

In one embodiment, the population of individuals having known skeletal health may comprise: at least one healthy individual having no skeletal disorder; and at least one individual having advanced-stage rheumatoid arthritis. In one embodiment, the population of individuals having known skeletal health may  
30 comprise: multiple (eg. at least 10) healthy individuals having no skeletal disorder; and multiple (eg. at least 10) individuals having advanced-stage rheumatoid arthritis.

The step of comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health is performed using a diagnostic algorithm. The step of comparing may be part of a  
35 classification algorithm. All embodiments of the diagnostic algorithm described above apply equally to the diagnostic algorithm used in this method.

The method of the invention for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis provides a useful way for assessing the effectiveness of treatment for advanced-stage rheumatoid arthritis. By looking at the abundance of individual markers or a  
40 combination of markers in a test individual undergoing treatment, and comparing the abundance of those markers to values obtained from a population of individuals having known skeletal health (eg. individuals

having advanced-stage rheumatoid arthritis, early-stage rheumatoid arthritis and/or no skeletal disorder), it is possible to classify the skeletal health of the test individual to determine whether treatment has improved the skeletal health of the individual.

In one embodiment, the method for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis may be performed to determine whether treatment for advanced-stage rheumatoid arthritis improves the skeletal health of a test individual diagnosed with advanced-stage rheumatoid arthritis.

As used herein, the phrase “to determine whether the treatment for advanced-stage rheumatoid arthritis improves the skeletal health of a test individual diagnosed with advanced-stage rheumatoid arthritis” means determining whether the test individual diagnosed with advanced-stage rheumatoid arthritis shows an improvement in their skeletal health as a result of their treatment, on the basis of the abundance of markers quantified for the test individual. This means determining whether the abundance of skeletal markers quantified for the test individual is characteristic of an individual having advanced-stage rheumatoid arthritis, or is characteristic of an individual having early-stage rheumatoid arthritis or no skeletal disorder (thereby demonstrating that the test individual has improved skeletal health).

In one embodiment, the body fluid sample may be obtained from a test individual undergoing treatment for advanced-stage rheumatoid arthritis. The body fluid sample may be obtained from the test individual during any stage of the treatment process. In one embodiment, the body fluid sample is obtained following at least 1 week (such as at least 2, 4, 8, 12, 20, 30, 40, or 52 weeks, or 2, 3, or 4 years) of treatment for advanced-stage rheumatoid arthritis. In one embodiment, the body fluid sample is obtained from the test individual once treatment has been completed.

In order to provide further insight into the effect of treatment on the skeletal health of the test individual, the method may be performed using multiple body fluid samples obtained from the test individual at different time points during the treatment process. In one embodiment, the method is performed using a body fluid sample obtained from the test individual prior to commencement of treatment for advanced-stage rheumatoid arthritis, and a body fluid sample obtained from the test individual during treatment (eg. following at least 1, 2, 4, 8, 12, 20, 30, 40, or 52 weeks, or 2, 3, or 4 years of treatment).

The invention further provides the use of the combination of the oxidised free adducts: methionine sulfoxide (MetSO), and dityrosine (DT), and the glycated free adduct pentosidine as markers for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis. In one embodiment, the use is for determining whether treatment for advanced-stage rheumatoid arthritis improves the skeletal health of a test individual diagnosed with advanced-stage rheumatoid arthritis.

The invention also provides a kit comprising reagents for quantification of markers of skeletal health, wherein said markers comprise at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least 11 or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-

derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT); 3-nitrotyrosine (3-NT); N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT); 3-nitrotyrosine (3-NT); N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT); 3-nitrotyrosine (3-NT); N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N<sub>ω</sub>-carboxymethylarginine (CMA); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: N-formylkynurenine (NFK), dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine and N-formylkynurenine (NFK); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: N<sub>ω</sub>-carboxymethylarginine (CMA), dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts:

methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA) and N-formylkynurenine (NFK); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone (MG-H1); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), and dityrosine (DT); and optionally further comprise one or more markers selected from: the glycated free adduct glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and glyoxal-derived hydroimidazolone (G-H1); and optionally further comprise N<sub>ε</sub>-fructosyl-lysine (FL) and/or hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine

(CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and N<sub>ε</sub>-fructosyl-lysine (FL); and optionally further comprise glyoxal-derived hydroimidazolone (G-H1) and/or hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), pentosidine, N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK) N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and hydroimidazolone (G-H1); and optionally further comprises the marker hydroxyproline (Hyp).

In one embodiment, the kit may further comprise reagents for quantification of anti-cyclic citrullinated peptide antibody.

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), and glucosepane (GSP); and may further comprise one or more markers selected from: the oxidised and glycated free adducts: N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), and N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone (MG-H1); and optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), and dityrosine (DT); and

optionally further comprise one or more markers selected from: the oxidised and glycated free adducts: glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and N<sub>ε</sub>-fructosyl-lysine (FL); and optionally further comprise glyoxal-derived hydroimidazolone (G-H1) and/or hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), and glyoxal-derived hydroimidazolone (G-H1); and optionally further comprise N<sub>ε</sub>-fructosyl-lysine (FL) and/or hydroxyproline (Hyp).

In one embodiment, the kit may comprise reagents for quantification of markers of skeletal health, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), 3-nitrotyrosine (3-NT), N<sub>ω</sub>-carboxymethylarginine (CMA), N-formylkynurenine (NFK), glucosepane (GSP), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), dityrosine (DT), glyoxal-derived hydroimidazolone (G-H1), and N<sub>ε</sub>-fructosyl-lysine (FL); and optionally further comprises hydroxyproline (Hyp).

In one embodiment, the kit may additionally comprise reagents for quantification of the oxidised free adducts or adduct residues: AASA and/ or GSA.

The invention also provides a kit comprising reagents for quantification of markers of early-stage skeletal disorder. The kit may comprise reagents for quantifying the above combinations of markers.

The kit may comprise reagents for quantification of markers of early-stage skeletal disorder, wherein said markers comprise at least 6 (eg. at least 7, at least 8, at least 9, at least 10, at least 11, or all 12) markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

In one embodiment, the kit may comprise reagents for quantification of markers of early-stage skeletal disorder, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free

adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and anti-cyclic citrullinated peptide antibody.

- 5 In one embodiment, the kit may comprise reagents for quantification of markers of early-stage skeletal disorder, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody.
- 10 In one embodiment, the kit may comprise reagents for quantification of markers of early-stage skeletal disorder, wherein said markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H),
- 15 pentosidine and glucosepane; and anti-cyclic citrullinated peptide antibody.

In one embodiment, the kit may further comprise reagents for quantification of hydroxyproline (hyp).

In one embodiment, the kit may further comprise reagents for quantification of the oxidised free adducts or adduct residues: AASA and/ or GSA.

- 20 The invention also provides a kit comprising reagents for quantification of markers for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis, wherein said markers comprise: the combination of the oxidised, nitrated and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL). In one
- 25 embodiment, the kit comprises reagents for quantification of markers for determining whether treatment for advanced-stage osteoarthritis improves the skeletal health of a test individual diagnosed with advanced-stage osteoarthritis

- 30 The invention also provides a kit comprising reagents for quantification of markers for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis, wherein said markers comprise the combination of the oxidised free adducts: methionine sulfoxide (MetSO), and dityrosine (DT), and the glycated free adduct pentosidine. In one embodiment, the kit comprises reagents for quantification of markers for determining whether treatment for advanced-stage
- 35 rheumatoid arthritis improves the skeletal health of a test individual diagnosed with advanced-stage rheumatoid arthritis.

In all embodiments of the kits of the invention, the reference to reagents for quantification of oxidised, nitrated, and glycated free adducts (or adduct residues) and the reference to reagents for quantification of

hydroxyproline include a reference to reagents for quantification of their related stable isotope substituted compounds (isotopomers).

In one embodiment, the reagents for quantification of markers are for quantification of the markers in a body fluid sample obtained from a test individual.

As used herein, the "reagents for quantification of markers" may comprise any reagent that allows the amount of the markers described herein to be determined. In one embodiment, the reagents are for quantification of the oxidised, nitrated, and glycated free adducts by isotopic dilution analysis.

In one embodiment, the reagent for quantifying MetSO is *methyl*-[<sup>2</sup>H<sub>3</sub>]MetSO. In one embodiment, the reagent for quantifying Hyp is [<sup>13</sup>C<sub>2</sub>]Hyp. In one embodiment, the reagent for quantifying NFK is [<sup>15</sup>N<sub>2</sub>]NFK. In one embodiment, the reagent for quantifying DT is *ring*-[<sup>2</sup>H<sub>6</sub>]DT. In one embodiment, the reagent for quantifying 3-NT is *ring*-[<sup>2</sup>H<sub>3</sub>]3-NT. In one embodiment, the reagent for quantifying CEL is *lysyl*-[<sup>13</sup>C<sub>6</sub>]CEL. In one embodiment, the reagent for quantifying CML is *lysyl*-[<sup>13</sup>C<sub>6</sub>]CML. In one embodiment, the reagent for quantifying FL is *lysyl*-[<sup>13</sup>C<sub>6</sub>]FL. In one embodiment, the reagent for quantifying CMA is *carboxymethyl*-[<sup>13</sup>C<sub>2</sub>]CMA. In one embodiment, the reagent for quantifying G-H1 is *guanidino* [<sup>15</sup>N<sub>2</sub>]G-H1. In one embodiment, the reagent for quantifying MG-H1 is *guanidino*-[<sup>15</sup>N<sub>2</sub>]MG-H1. In one embodiment, the reagent for quantifying 3DG-H is *guanidino*-[<sup>15</sup>N<sub>2</sub>]3DG-H. In one embodiment, the reagent for quantifying AASA is [<sup>2</sup>H<sub>3</sub>]α-Aminoadipic acid. In one embodiment, the reagent for quantifying GSA is [<sup>2</sup>H<sub>3</sub>]α-Aminoadipic acid. In one embodiment, the reagent for quantifying GSP is [<sup>13</sup>C<sub>6</sub>]Glucosepane. In one embodiment, the reagent for quantifying Pyrraline is [<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]Pyrraline. In one embodiment, the reagent for quantifying pentosidine is [<sup>13</sup>C<sub>6</sub>]pentosidine. Alternative stable isotopic substitution may be used in these compounds, as may be selected by those skilled in the art of stable isotopic dilution analysis.

In one embodiment, the reagents for isotopic dilution analysis may comprise at least one (eg. at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or all 16) reagents selected from the group consisting of: [<sup>13</sup>C<sub>2</sub>]Hyp, *methyl*-[<sup>2</sup>H<sub>3</sub>]MetSO, [<sup>15</sup>N<sub>2</sub>]NFK, *ring*-[<sup>2</sup>H<sub>6</sub>]DT; *ring*-[<sup>2</sup>H<sub>3</sub>]3-NT; *lysyl*-[<sup>13</sup>C<sub>6</sub>]CEL, *lysyl*-[<sup>13</sup>C<sub>6</sub>]CML, *lysyl*-[<sup>13</sup>C<sub>6</sub>]FL, *carboxymethyl*-[<sup>13</sup>C<sub>2</sub>]CMA, *guanidino* [<sup>15</sup>N<sub>2</sub>]G-H1, *guanidino*-[<sup>15</sup>N<sub>2</sub>]MG-H1, *guanidino*-[<sup>15</sup>N<sub>2</sub>]3DG-H, [<sup>2</sup>H<sub>3</sub>]α-Aminoadipic acid, [<sup>13</sup>C<sub>6</sub>]Glucosepane, [<sup>13</sup>C<sub>6</sub>]pentosidine, and [<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]Pyrraline.

In one embodiment, the reagents may comprise the combination of: [<sup>13</sup>C<sub>2</sub>]Hyp, *methyl*-[<sup>2</sup>H<sub>3</sub>]MetSO, [<sup>15</sup>N<sub>2</sub>]NFK, *ring*-[<sup>2</sup>H<sub>6</sub>]DT; *ring*-[<sup>2</sup>H<sub>3</sub>]3-NT; *lysyl*-[<sup>13</sup>C<sub>6</sub>]CEL, *lysyl*-[<sup>13</sup>C<sub>6</sub>]CML, *lysyl*-[<sup>13</sup>C<sub>6</sub>]FL, *carboxymethyl*-[<sup>13</sup>C<sub>2</sub>]CMA, *guanidino* [<sup>15</sup>N<sub>2</sub>]G-H1, *guanidino*-[<sup>15</sup>N<sub>2</sub>]MG-H1, *guanidino*-[<sup>15</sup>N<sub>2</sub>]3DG-H, [<sup>2</sup>H<sub>3</sub>]α-Aminoadipic acid, [<sup>13</sup>C<sub>6</sub>]Glucosepane, [<sup>13</sup>C<sub>6</sub>]pentosidine and [<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]Pyrraline.

The kits of the invention may further comprise a known quantity or concentration of the markers described herein (or their related stable isotope substituted compounds (isotopomers)) for use as a standard.

The kit of the invention may further comprise instructions for carrying out the methods and uses of the invention as described herein.

The invention also provides a method

- for determining the skeletal health of an individual and/or
- of treating a skeletal disorder in an individual and/or
- of determining whether an individual has an early-stage skeletal disorder and/or
- of treating an early-stage skeletal disorder in an individual and/or
- 5     • for determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis and/or
- for determining whether a test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis;

the method comprising:

- 10     (a) optionally determining the skeletal health of an individual by performing a method comprising the steps of:

(i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 15     3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine and glucosepane; and hydroxyproline (Hyp);

(ii) comparing the amount of each marker quantified in the test sample to corresponding 20     reference values for each marker in a diagnostic algorithm, wherein the reference values are obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual;

- (b) optionally determining whether the individual has an early-stage skeletal disorder by performing a method comprising the steps of:

25     (i) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycosylated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 30     3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;

(ii) comparing the amount of each marker quantified in the test sample to corresponding 35     reference values for each marker in a second diagnostic algorithm, wherein the reference values are obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder;

- (c) optionally determining whether the individual has advanced-stage osteoarthritis or early-stage osteoarthritis by performing a method comprising the steps of:

(i) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from: the oxidised, nitrated, and glycosylated 40     free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), and N<sub>ε</sub>-fructosyl-lysine (FL);

(ii) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health, and thereby determining whether a test individual has advanced-stage osteoarthritis or early-stage osteoarthritis;

5 (d) optionally determining whether the individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis by performing a method comprising the steps of:

(i) quantifying at least one marker of skeletal health in a body fluid sample obtained from a test individual, wherein the at least one marker is selected from the oxidised, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), and pentosidine;

10 (ii) comparing the amount of the at least one marker quantified in the test sample to a corresponding reference value for the marker obtained from a population of individuals having known skeletal health, and thereby determining whether the test individual has advanced-stage rheumatoid arthritis or early-stage rheumatoid arthritis;

15 (d) optionally selecting an individual identified as having a skeletal disorder, optionally an early-stage skeletal disorder; and

(e) optionally administering a treatment for the skeletal disorder, optionally the early-stage skeletal disorder, to the individual.

20 The invention also provides a computer program and a computer program product for carrying out any of the methods and uses described herein, and a computer readable medium having stored thereon a program for carrying out any of the methods/ uses described herein.

25 The invention also provides a signal embodying a computer program for carrying out any of the methods and uses described herein, a method of transmitting such a signal, and a computer product having an operating system which supports a computer program for carrying out any of the methods and uses described herein.

Furthermore, features implemented in hardware may generally be implemented in software, and vice versa. Any reference to software and hardware features herein should be construed accordingly.

30 Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", mean "including but not limited to" and do not exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

35 All references, including any patent or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art. Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith. Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

The present invention is discussed in more detail by means of the Examples described below, and by the Figures.

Figure 1 illustrates compound data bar charts of changes in the levels of oxidised, nitrated, and glycated free adducts and adduct residues of protein in plasma and synovial fluid of patients with early and advanced arthritis. The values used to generate the bar chart are shown in Figures 2-8. Bar chart values are normalised to levels in plasma of healthy control subjects, converted to  $\log_2$  scale where zero change is the bar midpoint and horizontal scale range is - 6 - +6.

Figure 2 illustrates the training and validation of the diagnostic algorithms for detection of impaired skeletal health and discrimination of early-stage osteoarthritis, rheumatoid arthritis and other inflammatory joint disorders (using the data for "marker group 1"). (a) Training set and test set study groups for detection of impaired skeletal health. A ROC curve is given for the training set. Area under the curve (AUC) was: 0.99 (95% confidence interval 0.97 – 1.00). A random outcome is 0.5. (b) Training set and test set study groups for discrimination of eOA, eRA and non-RA. ROC curves are given for the training set with AUC and confidence intervals: eOA, 0.98 (0.96 – 1.00); eRA, 0.91 (0.81 – 1.00); and non-RA, 0.68 (0.50 – 0.86). A random outcome is 0.33.

Figure 3 provides a summary of the data obtained for the oxidation and nitration adduct residues of protein in patient plasma and synovial fluid.

Figure 4 provides a summary of the data obtained for the oxidation and nitration free adducts in patient plasma and synovial fluid.

Figure 5 provides a summary of the data obtained for lysine-derived glycation adduct residues in patient plasma and synovial fluid.

Figure 6 provides a summary of the data obtained for arginine-derived glycation adduct residues in patient plasma and synovial fluid.

Figure 7 provides a summary of the data obtained from lysine-derived glycation free adducts in patient plasma and synovial fluid.

Figure 8 provides a summary of the data obtained from arginine derived glycation free adducts in patient plasma and synovial fluid.

Figure 9 provides a summary of the homotypic correlation of protein oxidation, nitration and glycation adducts in patient synovial fluid and plasma compartments.

Figure 10 provides a summary of the predictive algorithm outcomes for training set cross-validation using Random Forest (based on "marker group 1").

Figure 11 provides a summary of the predictive algorithm outcomes for test set cross-validation using Random Forest (based on "marker group 1").

Figure 12 provides a summary of the predictive algorithm outcomes for test set validation using Random Forest (based on "marker group 1").

Figure 13 provides a summary of the data obtained for the glycation free adduct glucosepane and the oxidation free adducts AASA and GSA in patient serum/plasma.

Figure 14 provides a summary of the predictive algorithm outcomes for 2-fold cross validation using Support Vector Machines (based on "marker group 2").

Figure 15 illustrates (A) MACH-1 mechanical testing system (Biomomentum, Canada); and (B) a view of guinea pig femoral condyle with a position grid superimposed.

Figure 16 provides representative pictures of medial compartment of right guinea pig knees of each group over time. Safranin-O/fast green/hematoxylin staining, 4x magnification.

Figure 17 provides a summary of the total OA score in each guinea pig group. Mean with SEM, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\*\* =  $p < 0.0001$ , One-way ANOVA with TUKEY post-test.

Figure 18 provides a summary of global synovial histological score in each guinea pig group. Mean with SEM, \*\* =  $p < 0.01$ , One-way ANOVA with TUKEY post-test.

Figure 19 illustrates the distribution of thickness (mm) and Young modulus (MPa) in each guinea pig group (femoral condyles and tibial plateaus). Mean with SEM, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\*\* =  $p < 0.0001$ ; one-way ANOVA with TUKEY post-test;  $n = 12$  per group.

Figure 20 illustrates the correlation between the global histological score and OA cartilage thickness or Young modulus.

Figure 21 provides a summary of serum concentrations of glycated amino acids in the guinea pig model of osteoarthritis. (A) FL, (B) MG-H1, (C) G-H1, (D) CMA, (E) 3DG-H, and (F) glucosepane. Data are

mean with SEM (nM). Significance: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ ; one-way ANOVA with TUKEY post-test.

Figure 22 provides a summary of serum concentrations of oxidised and nitrated amino acids in the guinea pig model of osteoarthritis. (A) AASA, (B) GSA, (C) Dityrosine, (D) NFK and (E) 3-NT. Data are mean with SEM (nM). Significance: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ ; one-way ANOVA with TUKEY post-test

Figure 23 provides a summary of serum concentrations of hydroxyproline and citrullinated protein (mmol/mol arg) in the guinea pig model of osteoarthritis. Data are mean with SEM. Significance: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ ; one-way ANOVA with TUKEY post-test.

Figure 24 illustrates the correlation between glycation, oxidation and nitration free adducts and hydroxyproline measured in the guinea pig model of osteoarthritis. Correlations statically significance ( $P < 0.05$ ) after a Bonferroni correction of 15 was applied.

Figure 25 provides a summary of the levels of plasma glycated, oxidised, nitrated and citrullinated protein quantified in the guinea pig model of osteoarthritis over time.

Figure 26 illustrates the correlation between glycated, oxidised, nitrated protein and citrullinated protein in the guinea pig model of osteoarthritis. Correlations statically significance ( $P < 0.05$ ) after a Bonferroni correction of 15 was applied.

Figure 27 illustrates the correlation of glycation, oxidation and nitration free adducts and citrullinated protein and global histological score in the guinea pig model of osteoarthritis. Correlation coefficients in bold are statistically significant. \*Correlation coefficient significant after Bonferroni correction of 16 applied.

Figure 28 illustrates the correlation of markers with OA score in the guinea pig model of osteoarthritis.

Figure 29 summarises the correlation analysis of glycation, oxidation and nitration free adducts with joint biomechanical properties measured by Mach-1 parameters. \*Correlation coefficient significant after Bonferroni correction of 16 applied.

## **EXAMPLES**

**EXAMPLE 1: Quantifying levels of oxidised, nitrated and glycated free adducts and adduct residues in samples obtained from healthy individuals and individuals having skeletal disorders**

### **1) Patients, healthy subjects and sampling**

Patients with longstanding history or established severe, advanced OA (aOA), early-stage OA (eOA), advanced RA (aRA), early-stage RA (eRA) and self-resolving inflammatory joint disease (non-RA) were recruited at the Department of Rheumatology, Ipswich Hospital NHS Trust, U.K., Orthopaedic Clinics,

University Hospital Coventry & Warwickshire, Coventry, U.K., Department of Rheumatology, Ipswich Hospital NHS Trust, U.K, Department of Rheumatology, Royal Devon and Exeter NHS Foundation Trust, Exeter, U.K., and Rapid Access Rheumatology Clinic, City Hospital, Birmingham, U.K..

5 Criteria for eOA were: subjects presenting with new onset knee pain, normal radiographs of the symptomatic knee and routine exploratory arthroscopy with macroscopic findings classified as grade I/II on the Outerbridge scale.

10 Criteria for eRA were: A diagnosis of early rheumatoid arthritis (eRA) was made according to the 1987 American Rheumatoid Association criteria (Arnett, F. C. *et al.* American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis & Rheumatism* **31**, 315-324 (1988)).

15 Criteria for nonRA were: A diagnosis of non-RA was made where alternative rheumatological diagnoses explained the inflammatory arthritis (Raza, K. *et al.*, Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis Research & Therapy* **7**, 1-12 (2005)). For example, training-set non-RA subjects had reactive arthritis (n = 6), pseudogout (n = 1), and unclassified (n = 3).

20 Patients with eRA and non-RA were recruited within 5 months of the onset of symptoms of inflammatory arthritis with synovial fluid and peripheral venous blood samples collected at initial presentation. Diagnostic outcomes were determined at follow-up.

25 Criteria for aOA were: longstanding or established severe symptoms of OA ( $\geq 2$  years duration of disease) with corresponding radiographic changes (Kellgren-Lawrence grade IV changes on plain x-rays) undergoing therapeutic knee aspiration and corticosteroid instillation or total knee replacement.

30 Criteria for aRA were: joint stiffness in the mornings of at least one hour duration; symmetrical swelling in three or more joints; radiographic evidence of bone erosions; rheumatoid nodules with increased serum rheumatoid factor (RF); and symptoms of  $\geq 2$  years duration.

35 Normal healthy control subjects were recruited according to the following criteria: Inclusion criteria – no history of joint symptoms with no arthritic disorder or other morbidity; and Exclusion criteria – a history of knee injury or knee pain in either knee, taking medication (excepting oral contraceptives and vitamins), and any abnormality at physical examination of the knee.

Control subjects and patients with early-stage disease were recruited as two independent cohorts for training set and independent test set for data analysis in machine learning methods.

40 Peripheral venous blood samples were collected with EDTA anti-coagulant from patients pre-operatively and synovial fluid obtained intraoperatively from patients, as appropriate. Peripheral venous blood and synovial fluid were collected and stored and subject characteristics are as previously described (Ahmed,

U. *et al.* Biomarkers of early-stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. *Sci. Rep.* **5**, 9259 (9251-9257) (2015).

Peripheral venous blood samples from healthy subjects and patients with eOA were collected after overnight fasting with EDTA anti-coagulant. Venous blood samples for eRA, non-RA and aOA study groups were collected in the non-fasted state. For analytes studied herein, diurnal variation in serum Hyp from healthy subjects was 20 % and for other amino acids up to 13–25 %, depending on the analyte (Gasser AB, Biological variation in free serum hydroxyproline concentration. *Clin Chim Acta.* 1980;106(1):39–43; Thompson DK, Daily variation of serum acylcarnitines and amino acids. *Metabolomics.* 2012;8(4):556–65). Diurnal variation in plasma protein glycation and oxidative stress in healthy subjects was <15 %, as judged by plasma fructosamine and plasma cysteine, respectively (Brunnbauer M, Diurnal variations of fructosamine in patients with type II diabetes mellitus. *Wien Klin Wochenschr.* 1990;180:72–3; Blanco RA, Diurnal variation in glutathione and cysteine redox states in human plasma. *Am J Clin Nutr.* 2007;86(4):1016–23).

Synovial fluid was collected from patients with aOA and aRA and eOA, eRA and with non-RA recruited for the training set cohort. Synovial fluid was not collected from patients with eOA, eRA or non-RA in the test set cohort in which analytes were determined only in plasma/serum.

Blood samples were centrifuged (2000g, 10 min) and the plasma and synovial fluid supernatant removed and stored at – 80 °C until analysis. Samples were centrifuged within one hour of collection.

## **2) Quantifying oxidised, nitrated, and glycated free adducts and adduct residues in patient plasma/serum and synovial fluid samples.**

The content of oxidation, nitration, and glycation adduct residues in plasma/serum and synovial proteins was quantified in exhaustive enzymatic digests by stable isotopic dilution analysis LC-MS/MS. Oxidation, nitration, and glycation free adducts were similarly determined in the ultrafiltrates of the same samples. Serum was available for eRA and non-RA study groups, and plasma for all others; there was no significance difference in oxidation, nitration and glycation markers of plasma and serum.

### Sample processing:

Ultrafiltrate (50-100 µl) of plasma/serum or synovial fluid was collected by microspin ultrafiltration (10 kDa cut-off) at 4°C. Retained protein was diluted with water to 500 µl and washed by 4 cycles of concentration to 50 µl and dilution to 500 µl with water over a microspin ultrafilter (10 kDa cut-off) at 4°C. The final washed protein (100 µl) was delipidated and hydrolysed enzymatically as described, designed and validated to maintain protein oxidation, nitration and glycation adduct content during processing (Rabbani, N., Shaheen, F., Anwar, A., Masania, J. & Thomalley, P. J. Assay of methylglyoxal-derived protein and nucleotide AGEs. *Biochem.Soc.Trans.* **42**, 511-517 (2014); Ahmed, U. *et al.* Biomarkers of early-stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. *Sci. Rep.* **5**, 9259 (9251-9257) (2015)).

Quantification of adduct residues and free adducts:

Protein hydrolysate (25 µl, 32 µg equivalent) or ultrafiltrate (5 µl) was spiked with stable isotopic standard analytes, and analysed by LC-MS/MS using an Acquity™ UPLC system with a Quattro Premier tandem mass spectrometer (Waters, Manchester, U.K.). Samples were maintained at 4°C in the autosampler during batch analysis. The columns were 2.1 x 50 mm and 2.1 mm x 250 mm, 5 µm particle size Hypercarb™ (Thermo Scientific), in series with programmed switching, at 30 °C.

Chromatographic retention is necessary to resolve oxidised analytes from their amino acid precursors to avoid interference from partial oxidation of the latter in the electrospray ionization source of the mass spectrometric detector. Analytes were detected by electrospray positive ionization, multiple reaction monitoring (MRM) mode where analyte detection response is specific for mass/charge ratio of the analyte molecular ion and major fragment ion generated by collision-induced dissociation in the mass spectrometer collision cell. The ionization source and desolvation gas temperatures were 120°C and 350°C, respectively. The cone gas and desolvation gas flow rates were 99 and 900 l/h, respectively, and the capillary voltage was 0.60 kV. Argon gas ( $5.0 \times 10^{-3}$  mbar) was in the collision cell. Programmed molecular ion and fragment ion masses optimized to  $\pm 0.1$  Da and collision energies were  $\pm 1$  eV for MRM detection. Correction for autohydrolysis of hydrolytic enzymes was made as described (Rabbani, N., Shaheen, F., Anwar, A., Masania, J. & Thornalley, P. J. Assay of methylglyoxal-derived protein and nucleotide AGEs. *Biochem. Soc. Trans.* **42**, 511-517 (2014)).

Analytes determined were:

**Oxidation adducts:** MetSO, dityrosine (DT), N-formylkynurenine (NFK),  $\alpha$ -amino adipic semialdehyde (AASA), and glutamic semialdehyde (GSA);

**Nitration adduct:** 3-NT;

**Glycation adducts:** FL, N $\epsilon$ -carboxymethyl-lysine (CML), N $\epsilon$ -(1-carboxyethyl)lysine (CEL), N $\omega$ -carboxymethylarginine (CMA), hydroimidazolones derived from glyoxal, methylglyoxal and 3-deoxyglucosone (G-H1, MG-H1 and 3DG-H), respectively), pentosidine, glucosepane, methylglyoxal-derived lysine dimer (MOLD); and

**Others:** Hyp and amino acids – arg, lys, tyr, trp, met and val.

Valine is determined in protein hydrolysates for the protease autohydrolysis correction. Oxidation, nitration and glycation adduct residues are normalised to their amino acid residue precursors and given as mmol/mol amino acid modified; and related free adducts are given in nM. Anti-CCP antibody positivity was assessed by automated enzymatic immunoassay (EliA CCP; Phadia, Uppsala, Sweden).

Results:

Changes in protein oxidation, nitration, and glycation status in plasma and synovial fluid in patients with eOA, aOA, eRA, aRA, and non-RA with respect to plasma levels in healthy controls are summarised in a heatmap – Figure 1. The heatmap shows there are changes in protein oxidation, nitration and glycation adduct status in both protein and free adducts, in plasma and synovial fluid compartments.

A summary of the data is given in Figures 3-8 and 13. Data are presented as mean  $\pm$  SD for parametric distributions and median (lower – upper quartile) for non-parametric distributions. Significance of the difference between means of parametric data was analysed by Student's *t*-test and between medians of non-parametric data were analysed by Mann-Whitney U test for independent samples and Wilcoxon's signed ranks test for paired samples (analytes of plasma and synovial fluid of the same donor). Correlation analysis was performed by the Spearman method.

#### Discussion of results presented in Figures 3-8

In the results presented in Figures 3-8, there were 8 marked changes (>50% decrease or 2-fold increase) in oxidation, nitration, and glycation free adduct concentrations and only one marked change of protein adduct residue content, indicating that plasma free adducts are more responsive to change in early-stage disorder than protein adduct residue contents. Similar analysis for early-stage and advanced disorder indicates that plasma free adducts are also changed in eOA to aOA, and eRA to aRA progressions.

Surprisingly there were few significant increases of protein oxidation, nitration, and glycation adduct residue contents of synovial protein, compared to plasma protein of the same subject group. Indeed, in eRA, MetSO and NFK residue contents of synovial protein were lower than in plasma, although 3-NT residue content was higher. In aOA synovial protein had higher 3-NT, CEL, pentosidine and G-H1 residue contents than plasma protein. Similarly oxidation, nitration, and glycation free adducts were rarely increased in the synovial fluid compared to plasma: exceptions being DT, FL and CEL in aOA and CML in eRA. Nevertheless there were many increases of protein oxidation, nitration, and glycation adduct contents of protein and free adduct concentrations in both plasma and synovial fluid compartments with respect to levels in plasma of healthy controls, suggesting that protein oxidation, nitration and glycation is often increased in early and advanced arthritis but it tends to equilibrate between the plasma and synovial fluid compartments.

The methods and uses of the invention therefore rely on the patterns of increase and decrease observed in the oxidised, nitrated and glycated free adducts and adduct residues (as discussed in detail below) as indicators of skeletal health in a test individual, so as to determine the skeletal health of the individual, or determine whether the test individual has an early-stage or an advanced-stage skeletal disorder (such as early-stage or advanced-state osteoarthritis or rheumatoid arthritis).

When performing the 'comparison' step or 'classification' step of the method for determining the skeletal health of an individual, the abundance of markers quantified for a test individual is compared to reference values used to train the diagnostic algorithm (such as those obtained for a healthy individual and/ or individuals having known skeletal disorders). Because of the patterns of increase and decrease of markers that are associated with different skeletal conditions (described below), it is possible to determine/classify the skeletal health of the individual. Similarly, when performing the 'comparison' step or 'classification' step of the method for determining whether the test individual has an early-stage skeletal disorder (or for determining whether an individual has early-stage or advanced-state osteoarthritis or rheumatoid arthritis), the abundance of markers quantified for the test individual is compared to reference values used to train the diagnostic algorithm (such as those obtained for a healthy

individual and/ or individuals having known skeletal disorders). Because of the patterns of increase and decrease observed in markers in early-stage and advanced-stage skeletal disorders (as discussed below), it is possible to determine whether an individual has an early-stage or advanced-stage skeletal disorder.

5

Oxidation adduct residues of protein in plasma and synovial fluid (Figure 3)

MetSO residue content of plasma protein was increased *ca.* 2-fold in patients with eOA, eRA, aRA and non-RA and increased *ca.* 3-fold in patients with aOA, with respect to healthy controls. MetSO residue content of synovial fluid protein was increased 2 to 3 fold in eOA and aOA whereas it was decreased 53%  
10 in eRA, with respect to plasma protein content of healthy controls. MetSO residue content of synovial fluid protein of patients with aRA was increased *ca.* 3-fold with respect to patients with eRA.

The increased abundance of MetSO adduct residue observed in the plasma of individuals having early-stage and advanced-stage skeletal disorders as compared to healthy individuals can therefore be used in  
15 the methods and uses of the invention as an indicator of skeletal health, in particular in determining whether an individual has a skeletal disorder. The increased abundance of MetSO adduct residue observed in the synovial fluid of individuals having early-stage OA and advanced-stage OA as compared to healthy individuals, and the increased abundance of MetSO observed in the synovial fluid of individuals having advanced-stage RA as compared to individuals having early-stage RA can be used in the  
20 methods and uses of the invention as an indicator of skeletal health, e.g. to distinguish between early-stage and advanced-stage RA.

NFK residue content of plasma protein was little changed in the study groups except for *ca.* 3-fold increase in patients with aOA with respect to patient with eOA. The NFK residue content of synovial fluid  
25 protein was decreased 85% and 83% in patients with non-RA and eRA, with respect to NFK residue content of plasma protein of healthy controls. There was also a *ca.* 5-fold increase in NFK residue content of synovial fluid protein in aOA, with respect to NFK residue content of plasma protein of healthy controls.

The increase observed in the abundance of NFK adduct residue in the plasma of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the  
30 methods and uses of the invention to distinguish between early-stage and advanced-stage OA. The increase observed in the abundance of NFK adduct residue in the synovial fluid of individuals having advanced-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular as an indicator of advanced-stage OA. The  
35 decrease observed in the abundance of NKF adduct residues in the synovial fluid of individuals having early-stage RA and non-RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of early-stage skeletal disorder.

40 DT residue content of plasma protein was increased *ca.* 55-fold and 56-fold in patients with aRA and aOA whereas it was decreased 68% and 74% in patients with eRA and non-RA, respectively, with respect to healthy controls. DT residue content of plasma protein was markedly increased in advanced versus early-

stage disorder. DT residue content of plasma protein was increased ca. 30 fold in aOA with respect to eOA and increased ca. 14-fold in aRA with respect eRA. Similar effects were found for DT residue content of synovial fluid protein: DT residue content of synovial fluid protein was increased ca. 88 fold in aOA with respect to eOA and increased ca. 29-fold in aRA with respect to eRA.

5 The increased abundance of DT adduct residue in the plasma and synovial fluid of individuals having advanced-stage skeletal disorder as compared to individuals having early-stage skeletal disorder can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. to distinguish between early-stage and advanced-stage skeletal disorders.

10 Surprisingly, 3-NT residue content of plasma protein decreased in patients with eOA (-85%), eRA (- 83%) and non-RA (- 80%) but remained unchanged in patients with aOA and aRA, with respect to healthy controls. In synovial fluid, the 3-NT residue content of protein was decreased 83% in patients with eOA and increased ca. 4-fold in patients with aOA. 3-NT residue content was increased ca. 6-fold in synovial  
15 fluid protein, compared to plasma protein, of patients with aOA and eRA – Figure 3.

The decrease in the abundance of 3-NT observed in the plasma of individuals having early-stage skeletal disorders as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular as an indicator of early-stage skeletal disorders.  
20 The increase in the abundance of 3-NT observed in the synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the methods and uses of the invention to distinguish between early-stage and advanced-stage OA.

#### Oxidation and nitration free adducts of plasma and synovial fluid (Figure 4)

25 MetSO free adduct concentration of plasma was increased ca. 4-fold in patients with eOA, ca. 6-fold in patients with aOA, ca. 3-fold in patients with eRA, ca. 6-fold in patients with aRA and ca. 4-fold in patients with non-RA, with respect to healthy controls. It was increased ca. 2-fold in advanced disorder, comparing aOA versus eOA and aRA versus eRA. In synovial fluid of patients, MetSO free adduct concentration of plasma was increased ca. 5-fold in eOA, ca. 15-fold in aOA, ca. 5-fold in eRA, ca. 10-fold in aRA and ca.  
30 4-fold in non-RA, with respect to plasma of healthy controls.

The increased abundance of MetSO free adducts observed in plasma and synovial fluid of individuals having early-stage and advanced-stage skeletal disorders as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular  
35 in determining whether an individual has a skeletal disorder. The increased abundance of MetSO free adduct observed in the plasma and synovial fluid of individuals having advanced-stage skeletal disorders as compared to individuals having early-stage skeletal disorders can also be used in the methods and uses of the invention to distinguish between early-stage and advanced-stage skeletal disorders.

40 NFK free adduct concentration of plasma was increased ca. 4-fold in patients with aOA, eRA and non-RA, and ca. 5 in patients with aRA, with respect to healthy controls. NFK free adduct concentration of plasma was increased ca. 3-fold in aOA with respect to eOA. In synovial fluid the concentration of NFK

free adduct was increased *ca.* 2-fold in eOA, *ca.* 6-fold in aOA, *ca.* 16-fold in eRA, *ca.* 5-fold in aRA and *ca.* 17-fold in non-RA, with respect to plasma of healthy controls.

The increased abundance of NFK free adducts observed in plasma and synovial fluid of individuals having early-stage and advanced-stage skeletal disorders as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular in determining whether an individual has a skeletal disorder. The increased abundance of NFK free adduct observed in the plasma and synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA can also be used in the methods and uses of the invention to distinguish between early-stage and advanced-stage OA.

DT free adduct concentration of plasma was decreased 60% in patients with aOA, 70% in patients with eRA and 75% in patients with non-RA, with respect to healthy controls. It was decreased 80% in aOA versus eOA whereas it was increased 4-fold in aRA versus eRA. DT free adduct concentration of synovial fluid was increased *ca.* 2-fold in eOA and aOA and unchanged in other patient study groups, compared to plasma of healthy controls.

The decrease in the abundance of DT free adduct in the plasma of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular for distinguishing early-stage and advanced-stage OA. The increase in the abundance of DT free adduct in the plasma of individuals having advanced-stage RA as compared to individuals having early-stage RA can therefore be used in the methods and uses of the invention as an indicator of skeletal health, in particular for distinguishing early-stage and advanced-stage RA. The increase in the abundance of DT free adduct in the synovial fluid of individuals having early-stage and advanced-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health.

Plasma 3-NT free adduct concentration was decreased 70% and 64% in plasma and synovial fluid of aOA with respect to healthy controls and unchanged in all other patient groups – Figure 4. The decrease in the abundance of 3-NT free adduct observed in the plasma and synovial fluid of individuals having advanced-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of advanced-stage OA.

#### Glycation adducts residues of plasma and synovial protein (Figures 5 and 6)

For lysine-derived adducts (Figure 5), FL residue content of plasma protein was increased *ca.* 2-fold in patients with eRA and non-RA, with respect to healthy controls. FL residue content of synovial fluid protein was increased *ca.* 2-fold with respect to plasma protein of patients with eOA.

The increase in the abundance of FL adduct residue observed in the plasma of individuals having early-stage RA and non-RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of early-stage skeletal disorders.

CML residue content of plasma protein was increased *ca.* 5-fold in patients with aOA and aRA, with respect to healthy controls. It was increased markedly in advanced disorder: *ca.* 9-fold increase in aOA versus eOA and aRA versus eRA. A similar effect was found in synovial fluid protein where CML residue content was increased *ca.* 11-fold in aOA versus eOA and *ca.* 7-fold in aRA versus eRA.

5 The increase in the abundance of CML adduct residue in the plasma and synovial fluid of individuals having advanced-stage skeletal disorders as compared to individuals having early-stage skeletal disorders can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. for distinguishing early-stage and advanced-stage disorders.

10 CEL residue content of plasma protein was increased *ca.* 2-fold in patients with eOA and aRA only, with respect to healthy controls. CEL residue content of synovial fluid protein was increased 2-fold with respect to plasma protein in patients with aOA. The increase in the abundance of CEL adduct residue in the plasma of individuals having early-stage OA and advanced-stage RA as compared to healthy  
15 individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health.

MOLD residue content of plasma protein and synovial fluid protein was unchanged in all study groups.

20 Pentosidine residue content of plasma protein was increased *ca.* 23-fold in patients with eOA, *ca.* 9-fold in patients with aOA and *ca.* 7-fold in patients with aRA, with respect to healthy controls. Pentosidine residue content of synovial fluid protein was unchanged. In patients with aOA, pentosidine residue content of synovial fluid protein was *ca.* 7-fold higher than in plasma protein – Figure 5. The increase in the abundance of pentosidine adduct residue observed in the plasma of individuals having early-stage  
25 OA, early-stage RA and advanced-stage RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health.

For arginine-derived adducts (Figure 6), G-H1 residue content of plasma protein was increased *ca.* 3-fold in patients with aRA, with respect to healthy controls. G-H1 residue content of synovial fluid protein was  
30 unchanged, with respect to plasma protein healthy controls. There were *ca.* 4-fold, 2-fold and 3-fold increases in G-H1 residue content of synovial protein compared to plasma protein in patients with aOA, aRA and non-RA, respectively. The increase in the abundance of G-H1 adduct residue observed in the plasma of individuals having advanced-stage RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of  
35 advanced-stage RA.

MG-H1 residue content of plasma protein was decreased 64% in patients with eRA but increased 2 - 3-fold in patients with aRA; hence it increased *ca.* 7-fold in aRA versus eRA. MG-H1 residue content of synovial fluid protein was unchanged, with respect to plasma protein healthy controls. It increased *ca.* 3-  
40 fold, however, in aOA versus eOA. There were *ca.* 4-fold and 2-fold increases in MG-H1 residue content of synovial protein compared to plasma protein in patients with eRA and non-RA, respectively. The increase in the abundance of MG-H1 adduct residue observed in the plasma and synovial fluid of

individuals having advanced-stage skeletal disorders as compared to individuals having early-stage skeletal disorders can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. for distinguishing early-stage and advanced-stage disorders.

5 3DG-H residue content of plasma protein and synovial fluid protein was unchanged, with respect to plasma protein of healthy controls. It was increased, however, ca. 9-fold in plasma protein and ca. 4-fold in synovial protein in aOA with respect to eOA. The increase in the abundance of 3DG-H adduct residue in the plasma and synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. for distinguishing early-stage and advanced-stage OA.

CMA residue content of plasma protein was increased ca. 6-fold in aRA, ca. 2-fold in non-RA and was unchanged in synovial fluid protein, with respect to plasma protein of healthy controls. It was increased ca. 4-fold in plasma protein of aOA versus eOA – Figure 6. The increase in the abundance of CMA adduct residue in the plasma of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. for distinguishing early-stage and advanced-stage OA.

#### Glycation free adducts of plasma and synovial protein (Figures 7 and 8)

20 For lysine derived adducts (Figure 7), FL free adduct concentration in plasma and synovial fluid was little changed in the study groups except it was increased 2 to 3 fold in synovial fluid of patients with eOA.

CML free adduct concentration in plasma and synovial fluid was not changed in the study groups.

25 CEL free adduct concentration in plasma was increased ca. 4-fold in patients with aOA and 2-fold in patients with aRA, with respect to healthy controls. CEL free adduct concentration of synovial fluid of patients with aOA was 61% lower than in plasma. The increase in the abundance of CEL free adduct observed in the plasma and synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA, and in the plasma and synovial fluid of individuals having advanced-stage RA as compared to individuals having early-stage RA, can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. to distinguish between early-stage and advanced-stage skeletal disorders.

35 MOLD free adduct concentration in plasma was decreased 82% in patients with eOA with respect to healthy controls. MOLD free adduct concentration in synovial fluid was decreased 77% and 68% and in eOA and aOA whereas it was increased ca. 6-fold and 4-fold in eRA and non-RA, with respect to plasma of healthy controls.

40 Pentosidine free adduct concentration in plasma was increased ca. 5-fold in patients with aRA, with respect to healthy controls. In synovial fluid it was increased ca. 2-fold in eOA, 3-fold in aOA and 4-fold in aRA, with respect to plasma of healthy controls. It was increased ca. 3-fold in aRA versus eRA in both plasma and synovial fluid – Figure 7. The increase in the abundance of pentosidine free adduct in the

plasma and synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA, and in the plasma and synovial fluid of individuals having advanced-stage RA as compared to individuals having early-stage RA can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. for distinguishing early-stage and advanced-stage skeletal disorders.

For arginine-derived adducts (Figure 8), G-H1, MG-H1 and 3DG-H free adduct concentrations in plasma were little changed in the study groups except for a 2-fold increase in MG-H1 free adduct concentration in plasma of patients with aOA and 49% decrease in 3DG-H free adduct concentration in patients with eOA.

There was ca. 2-fold increases of G-H1 and MG-H1 free adducts in synovial fluid of patients with eRA and a 57% decrease in 3DG-H free adduct concentration in patients with eOA, with respect to plasma of healthy controls. The increase in the abundance of MG-H1 free adduct in the plasma of individuals having advanced-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of advanced-stage OA. The decrease in the abundance of 3DG-H free adduct in the plasma of individuals having early-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of early-stage OA. The increase in the abundance of G-H1 and MG-H1 free adducts in synovial fluid of individuals having early-stage RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of early-stage RA. The decrease in abundance of 3DG-H free adduct observed in the synovial fluid of individuals having early-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health, e.g. as an indicator of early-stage OA.

CMA free adduct concentration in plasma was far more responsive: it increased ca. 2-fold in patients with eOA, 4-fold in patients with aOA, and 3-fold in patients with eRA and non-RA, with respect to healthy controls. CMA free adduct concentration increased similarly in synovial fluid - Figure 8. The increase in the abundance of CMA free adduct in the plasma and synovial fluid of individuals having early-stage OA, early-stage RA, non-RA and advanced-stage OA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health. The increase in the abundance of CMA free adduct in the plasma and synovial fluid of individuals having advanced-stage OA as compared to individuals having early-stage OA can therefore be used in the methods and uses of the invention to distinguish between early-stage and advanced-stage OA.

#### Homotypic correlation of markers between plasma and synovial fluid (Figure 9)

As free adducts are relatively rapidly exchanged between plasma and synovial fluid compartments, analytes in synovial fluid may be used in algorithms in place of those in plasma where there is evidence of positive association. This is indicated by the many homotypic correlations (correlations of free adducts of the same type in plasma and synovial fluid compartments) of glycated, oxidised and nitrated amino acids. Figure 9 provides data relating to the homotypic correlation of free adducts and adduct residues observed in synovial fluid and plasma compartments. There were more homotypic correlations for free adducts than protein adducts in aOA, eRA and non-RA. The reverse was found for eOA and aRA.

Discussion of data presented in Figure 13

The concentrations of AASA, GSA and glucosepane are presented in Figure 13. In summary, plasma GSP was observed to increase in early and advanced OA – and also eRA and non-RA, whereas AASA and GSA were unchanged in early-stage OA and decreased in advanced OA.

The increase in the abundance of glucosepane in the serum/plasma of individuals having all forms of OA and non-RA as compared to healthy individuals can therefore be used in the methods and uses of the invention as an indicator of skeletal health. The increase in the abundance of glucosepane in the serum/plasma of individuals having early-stage RA as compared to individuals having early-stage OA and non-RA can therefore be used in the methods and uses of the invention to distinguish between different types of early-stage skeletal disease.

**EXAMPLE 2: Generating a predictive diagnostic algorithm for diagnosing early-stage skeletal disorder**

Two diagnostic algorithms were developed, the first to discriminate between individuals having a skeletal disorder and individuals having no skeletal disorder (e.g. Figure 2(a)), and the second to discriminate between individuals having early-stage OA, early-stage RA and non-RA (e.g. Figure 2(b)). This pair of algorithms could be used separately or in combination to form a two-stage diagnostic testing regime.

The data obtained in Example 1 was used to generate diagnostic algorithms for diagnosing skeletal disorder. Various subsets of markers were identified for use in a diagnostic algorithm enabling highly sensitive and specific determination of skeletal health.

**Marker group 1:**

To generate the diagnostic algorithms, the data obtained in Example 1 for the 12 oxidised, nitrated and glycated free adducts (MetSO, DT, NFK, 3-NT, FL, CML, CEL, C-HI, MG-HI, 3DG-H, CMA and Pentosidine) was used together with data obtained for plasma hydroxyproline, anti-CCP antibodies, and rheumatoid factor (RF).

The subject groups used to train the diagnostic algorithms were the healthy control, eOA, eRA and non-RA subject groups. Data were analysed using SPSS, version 22.0, with R version 3.1.3 used for the diagnostic algorithm analysis.

**Training the algorithm:**

The two predictive algorithms were trained on a training data set, before being used to predict the disorder class for each sample in a test data set. The clinical characteristics of training and test set study groups are as given in Ahmed, U. *et al.* Biomarkers of early-stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. *Sci. Rep.* **5**, 9259 (9251-9257) (2015).

Machine learning analysis on subject groups with and without early-stage arthritis was performed to assess the predictive power of the measured biomarkers in early-stage skeletal disorder. The training data set used to generate the first diagnostic algorithm for predicting impaired skeletal health was obtained from 52 individuals (including healthy controls, and individuals with impaired skeletal health).

The training data set used to generate the second diagnostic algorithm for distinguishing early-stage skeletal disorders (eOA, eRA and non-RA) was obtained from 36 individuals (including individuals with eOA, eRA, and non-RA).

5 Various machine learning algorithms were tested for performance:

- (i) Random Forests – a nonlinear, tree-based method (Breiman, L. Random Forests. **45**, 5-32 (2001)); multi-class logistic regression (GLM));
- (ii) multi-class sparse logistic regression (GLMNET) (Friedman, J., Hastie, T. & Tibshirani, R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *J. Statistical Software* **33**, 1-22 (2010)); and
- (iii) Support Vector Machines (SVM).

ROC curve AUC statistic was used as a measure of performance, with 95% CI determined via bootstrap analysis, using the R package “pROC” (Robin, X. *et al.* pROC: an open-source package for R and S plus to analyze and compare ROC curves. *BMC Bioinformatics* **12**, 77-85 (2011)). Example sensitivity/specificity values were produced from the ROC curves via an automated procedure that finds the point on the ROC curve where the sensitivity/specificity values are most similar. The sensitivity and specificity values can however be tuned in other ways.

Five-fold cross-validation was carried out on the training set data. This was used to give an initial estimate of predictive performance, and to identify the best-performing machine learning method. Random Forest was consistently the best-performing method for the training set cross-validation. In the training set cross-validations, a panel of 15 plasma biomarkers was used consisting of: RF, anti-CCP antibody positivity, Hyp, and free adducts of MetSO, DT, NFK, 3-NT, FL, CML, CEL, CMA, G-H1, MG-H1, 3DG-H and pentosidine.

A Random Forest algorithm was trained on the entire training set, before making predictions for the held-out test data set. The predictive algorithm outcomes for training set cross-validation using the Random Forest algorithm are shown in Figure 10.

Training set cross-validation was also used to perform a stepwise removal of features that were not improving the mean AUC score. These markers can be omitted without reduction in the training set cross-validation AUC. This reduced feature set was used in the test set validation, but not the training set cross-validation (where it would upwardly bias the result). It was observed that for the disorder-versus-control algorithm, the RF, anti-CCP antibody positivity, CML, and FL markers could be removed without affecting sensitivity and specificity of the algorithm. For the early-disorder-type algorithm, it was observed that the RF, NFK, G-H1, and Hyp markers could be removed without affecting sensitivity and specificity of the algorithm.

The outcome of each analysis was to assign, for each test set sample, a set of probabilities corresponding to each of the disorder/control groups. The predicted group is then the one for which the probability is highest.

Testing the algorithm

The two predictive algorithms were tested using test data that were held separate from the algorithm training, and no algorithm settings were adjusted after generating the test set results – providing for a rigorous estimate of predictive performance on previously unseen cases.

The test data set used to test the first diagnostic algorithm for predicting impaired skeletal health was obtained from 129 individuals (including healthy controls, and individuals with impaired skeletal health). The test data set used to test the second diagnostic algorithm for distinguishing early-stage skeletal disorders (eOA, eRA and non-RA) was obtained from 97 individuals (including individuals with eOA, eRA, and non-RA).

The set of markers used in the disorder-versus-control algorithm included a total of 11 markers consisting of: hydroxyproline (Hyp), and the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT); 3-nitrotyrosine (3-NT); N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine.

The set of markers used in the early-disease-type algorithm included a total of 11 markers consisting of: anti-cyclic citrullinated peptide antibody, and the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine.

Anti-CCP antibody positivity was most important in arthritis type discrimination rather than skeletal health detection. Conversely, RF was not a key marker and its value compared to the anti-CCP antibody test has been questioned (Symmons, D. P. M. Classification criteria for rheumatoid arthritis—time to abandon rheumatoid factor? *Rheumatology* **46**, 725-726, doi:10.1093/rheumatology/ke1418 (2007)).

To give further insight into the results, 5-fold cross-validation was carried out on the test set data. The results of the cross-validations and test set hold-out validation are given in Figures 11 and 12. The first diagnostic algorithm was able to detect and discriminate early-stage skeletal disorder with test set cross-validation sensitivity/ specificity of 0.89/0.90, and with test set validation sensitivity/ specificity of 0.73/0.73. Area under the curve (AUC) for receiver operating characteristic (ROC) curve was: 0.99; and a random outcome is 0.5. Positive likelihood ratio and positive predictive value for the disease-versus-control algorithm indicate that this test gives definitive evidence for skeletal health impairment with a very low, 4% false positive rate. This is highly preferred for a health screening test. The second diagnostic algorithm was able to discriminate between the three early-stage disease types with test set validation sensitivities/specificities of 0.81/0.80 (non-RA), 0.57/0.56 (early RA), 0.83/0.84 (early OA). Mean AUC of ROC curves: 0.86; and a random outcome is 0.33.

Testing the algorithm with a reduced set of skeletal markers

Algorithms combine those features which improve the diagnostic performance of the test based on increase in area under the curve on the receiver operating characteristic (ROC) curve which relates to the probability of assigning a random sample to the correct clinical group (Hanley JA, McNeil BJ: A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology 1983;148:839-843). It was therefore investigated whether fewer skeletal markers can be used in the predictive algorithm of the invention. By varying the skeletal markers used in the algorithm, it was possible to characterise the loss of diagnostic performance in the predictive algorithm for determining skeletal health.

Below in the table are computed changes for AUC on the ROC plot when a feature is removed for the healthy versus arthritic disease comparison. A negative value indicates an important contribution to the test comparison.

Table 1. Comparison of importance of features in the algorithm for the Control vs Arthritic disease (any).

| Feature     | $\Delta$ AUC of ROC curve on feature removal Control vs arthritic disease (any) comparison |
|-------------|--------------------------------------------------------------------------------------------|
| RF          | 0.0000                                                                                     |
| CCPAb       | 0.0023                                                                                     |
| Hyp         | 0.0010                                                                                     |
| FL          | - 0.0023                                                                                   |
| G-H1        | 0.0023                                                                                     |
| MG-H1       | - 0.0047                                                                                   |
| 3DG-H       | - 0.0070                                                                                   |
| CML         | 0.0023                                                                                     |
| CEL         | - 0.0058                                                                                   |
| CMA         | - 0.0062                                                                                   |
| MetSO       | - 0.0104                                                                                   |
| 3-NT        | - 0.0070                                                                                   |
| NFK         | - 0.0062                                                                                   |
| DT          | - 0.0004                                                                                   |
| Pentosidine | - 0.0070                                                                                   |

Some of the skeletal markers were observed to be more useful than others for diagnostic performance of the algorithm. The order of utility of the skeletal markers observed was as follows:

MetSO > 3DG-H  $\approx$  3-NT  $\approx$  pentosidine > CMA  $\approx$  NFK > CEL > MG-H1 > DT > FL  $\approx$  G-H1

**Marker group 2**

In another example, the diagnostic algorithms were trained using a different subset of the markers measured in Example 1. This subset of markers included: MetSO, DT, NFK, 3-NT, FL, CML, CEL, CMA,

G-H1, MG-H1, 3DG-H, pentosidine, glucosepane (GSP), glutamic semialdehyde (GSA) and  $\alpha$ -amino adipic semialdehyde (AASA), as well as RF, anti-CCP antibody and Hyp.

Support Vector Machines were used to train the algorithm (Sajda P: Machine learning for detection and diagnosis of disease. *Annual Review of Biomedical Engineering* 2006, **8**(1):537-565). For training 50% of the data was used and the remaining 50% data was used as test set for cross validation. The algorithm was validated by 2-fold cross-validation, using 10 randomized repeat trials for improved robustness. A two-stage approach was taken: classification stage (i) to distinguish between disease and healthy control; and classification stage (ii) to distinguish between eOA, eRA and non-RA.

The training set cross-validations used a panel of biomarkers consisting of: RF, anti-CCP antibody positivity, Hyp, and free adducts of MetSO, DT, NFK, 3-NT, FL, CML, CEL, CMA, G-H1, MG-H1, 3DG-H, pentosidine, glucosepane (GSP), glutamic semialdehyde (GSA) and  $\alpha$ -amino adipic semialdehyde (AASA).

The predictive algorithm outcomes for training set cross-validation using the Support Vector Machines algorithm are shown in Figure 14. Diagnostic characteristics, including area under-the-curve of the receiver operating characteristic (ROC) plot (AUROC), are given 95% CI determined via bootstrap analysis using the R package "pROC". Algorithm feature selection was optimized for maximum classification accuracy.

Again it was investigated whether fewer skeletal markers can be used in the predictive algorithm of the invention. By varying the skeletal markers used in the algorithm, it was possible to characterise the loss of diagnostic performance in the predictive algorithm for determining skeletal health. The preferred minimum sub-set of markers observed for the diagnostic algorithm was as follows:

Optimum features for classification stage (i), the distinction between disease and healthy control, were: Hyp, G-H1, MG-H1, 3DG-H, CEL, CMA, MetSO, 3-NT, NFK, DT, and glucosepane.

Optimum features for classification stage (ii), the distinction between eOA, eRA and non-RA, were: CCP-Ab, FL, 3DG-H, CML, CEL, MetSO, 3-NT, and glucosepane.

There was high accuracy for classification stage (i), 88.4%; and particularly high accuracy for distinguishing eOA from eRA and non-RA, 95.5%. High positive predictive values of 93.6% and 92.8%, respectively, also indicate very low false positive rates for the test (see Figure 14).

### **EXAMPLE 3: Effect of drug therapy on hydroxyproline and protein oxidation, nitration and glycation adducts in plasma and synovial fluid of patients with aRA.**

Patients receiving anti-TNF $\alpha$  therapy, compared to those not receiving anti-TNF $\alpha$  therapy, had lower plasma Hyp (0.96 versus 3.37  $\mu$ M,  $P < 0.01$ ) and MOLD free adduct (43.5 versus 0.5 nM,  $P < 0.05$ ) and

lower synovial fluid NFK adduct free (43.2 versus 5.6 nM,  $P<0.05$ ), but increased plasma DT free adduct (6.29 versus 4.75 nM,  $P<0.05$ ).

Patients receiving treatment with non-steroidal anti-inflammatory drugs (NSAIDs), compared to those not receiving NSAIDs, had: in plasma – lower Hyp (0.99 versus 3.16  $\mu$ M,  $P<0.01$ ), 3DG-H free adduct (181 versus 19 nM,  $P<0.01$ ), MOLD free adduct (27.2 versus 0.5 nM,  $P<0.01$ ), 3-NT free adduct (3.7 versus 0.7 nM,  $P<0.01$ ), 3DG-H protein adduct (0.245 versus 0.014 mmol/mol arg,  $P<0.05$ ) and MetSO protein adduct (30 versus 14 mmol/mol met,  $P<0.05$ ), but higher DT free adduct (5.1 versus 7.1 nM) and CEL protein adduct (0.030 versus 0.099 mmol/mol arg,  $P<0.05$ ); and in synovial fluid – lower G-H1 free adduct (60 versus 19 nM,  $P<0.01$ ), but higher CEL protein adduct (0.028 versus 0.094 mmol/mol arg,  $P<0.05$ ).

Patients receiving treatment with prednisolone, compared to those not receiving prednisolone, had: in plasma – higher Hyp (2.87 versus 0.94  $\mu$ M,  $P<0.05$ ), CML free adduct (230 versus 99 nM,  $P<0.05$ ), 3DG-H, and MetSO protein adduct (0.283 versus 0.016 mmol/mol arg and 47 versus 16 mmol/mol met, respectively,  $P<0.05$ ); and in synovial fluid – lower CEL free adduct (69 versus 453 nM,  $P<0.05$ ), but high 3DG-H free adduct (253 versus 26 nM,  $P<0.05$ )

Treatment with or without methotrexate was associated with increased MG-H1 free adduct in plasma and synovial fluid (plasma: 307 versus 665 nM,  $P<0.05$ ; synovial fluid, 308 versus 829 nM,  $P<0.01$ ) and decreased synovial fluid 3-NT residue content (0.0053 versus 0.0022 mmol/mol tyr,  $P<0.05$ ).

#### EXAMPLE 4: MONITORING TREATMENT OF ADVANCED-STAGE SKELETAL DISORDERS

The data presented in Figures 3-8 demonstrate that a number of oxidised, nitrated and glycated free adducts change in abundance in advanced-stage skeletal disorders as compared to early-stage skeletal disorders. A summary of the skeletal markers showing the most significant changes is shown below in Table 2.

**Table 2. Protein oxidation, nitration and glycation free adducts in plasma and synovial fluid compartments changed in advanced versus early-stage disease.**

| Compartment    | Study group | Free adduct                    |
|----------------|-------------|--------------------------------|
| Plasma         | aOA         | MetSO, NFK, DT, 3-NT, CEL, CMA |
|                | aRA         | MetSO, DT, pentosidine         |
| Synovial fluid | aOA         | MetSO, NFK, FL                 |
|                | aRA         | DT, pentosidine                |

The skeletal markers shown in Table 2 can be used individually or in combination to distinguish between early-stage and advanced-stage skeletal disorders. This can be particularly helpful for determining the effectiveness of treatment for advanced-stage skeletal disorders.

**EXAMPLE 5: GUINEA PIG MODEL OF OSTEOARTHRITIS**

To further investigate markers of skeletal disease, an experiment was performed to quantify glycated, nitrated and oxidised free adducts in serum obtained from a guinea pig model of osteoarthritis. The progression of histological and mechanical properties (stiffness measurement or young modulus and the thickness of the articular cartilage) in the guinea pigs spontaneously developing OA was also measured to correlate biomarker levels with the severity of the histological lesions and cartilage biomechanical properties assessed by Mach-1<sup>®</sup> micro-mechanical tester.

Sixty, male, 3-week-old Dunkin-Hartley guinea pigs were included in this study. At 4-weeks-old and every 8 weeks until week 36, twelve Hartley guinea pigs were sacrificed. Blood sampling was performed every 8 weeks and glycated, oxidised and nitrated amino acids were analysed in plasma. Histological severity of the lesion was evaluated using OARSI score and rheological properties of cartilage were assessed by the Mach-1<sup>®</sup> micro-mechanical tester. In the guinea pig model of OA, severity of OA was observed to increase progressively with age. Plasma levels of glycated and oxidised amino acids showed different age-related changes: progressive increase with age (glucosepane, dityrosine and NFK), increased only at 36 weeks (FL, G-H1, GSA), initially decreased and then increased at 36 weeks (MG-H1, CMA, 3DG-H and AASA) and decreased with age (3-NT and Hyp). Some glycated and oxidised amino acids increased and correlated with the global OARSI histological score, cartilage thickness and Young's modulus. Plasma levels of glucosepane correlated positively with the severity of histological lesions ( $r=0.617$ ,  $p<0.0001$ ) and with Young's modulus ( $r=0.561$ ;  $p < 0.0001$ ).

**Materials and methods****Guinea pig OA model**

The Dunkin-Hartley guinea pig was used for the experiment. This model is well-characterized for spontaneous knee OA which resembles the development of clinical disease. The attraction of the guinea pig as an OA model system is its histopathological similarity to human disease. This guinea pig OA model is characterized by an early collagen fibril disruption occurring in articular cartilage (2 months). This is followed by the formation of bone cysts (2-3 months), subchondral bone thickening and osteophytes (3-12 months), proteoglycan loss (4-6 months) and fibrillations (8-12 months).

The appearance of joint pathology in the guinea pig and human is both age-related and subject to a variety of well-known risk factors, including the weight and mechanical stress. Spontaneous lesions in the knee are balanced and are more pronounced in the medial compartment in the area not covered by the meniscus. This corresponds to the location of lesions in the primary idiopathic OA, in human subjects.

**Animals**

All experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal care and Use Ethics Committee of the University of Liège (Belgium), reference 1648.

Sixty, male, 3-week-old Dunkin-Hartley guinea pigs were provided by from Charles River Laboratories (Paris, France). The identification of animals was made by microchip. The guinea pigs were bred under specific pathogen-free conditions and received free access to water. Animals were housed 3 per cage in

solid bottom cages and fed with a standard guinea-pig chow provided by SDS (Special Diets Service, Essex, England) containing Vitamin C (393.53 mg/kg) and Vitamin D3 (1972.54 IU/kg). PVC pipes were added to the cages to improve housing conditions and minimize stress. All animals allowed 2 weeks for acclimatization to housing conditions prior to start experimentation. Twelve young, 4-weeks old guinea pigs were studied as a reference group. The 48 remaining guinea pigs were randomized into 4 groups for sacrifice at 12, 20, 28 and 36 weeks. The number of animals per group was chosen according to the Osteoarthritis Research Society International (OARSI) recommendation (Kraus VB et al.; The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2010;18 Suppl 3:S35-52.).

Animal body weight was recorded weekly.

#### Blood sampling

Peripheral venous blood samples were collected in the morning at weeks 4, 12, 20 and 28 at the superficial veins of the ears under ketamine (32 mg/kg)/xylazine (3mg/kg) subcutaneous anesthesia. Additional blood samples were collected by intracardiac puncture, under general anesthesia (sodium pentobarbital 200 mg/kg, intraperitoneally) immediately before euthanasia. Blood samples were centrifuged at 2000 g for 5 min, and serum stored at -80°C until analysis.

#### Histology

At euthanasia of animal study groups, cartilage samples were processed for the histological evaluation. The right knee joint (femoral condyles and tibial plateaus) from each animal was fixed for 24 h in 4 % paraformaldehyde, followed by decalcification in hydrochloric acid (DC2 medium, Labonord, Templemars, France) for 4 h at 4°C before embedding in paraffin. The right kidney and a piece of liver were fixed in 4% paraformaldehyde and included in paraffin. Sections (6 µm) of the femoral condyles and tibial plateaus were cut with a microtome in the central area not covered by meniscus following the Cushin plane, as recommended by OARSI (Kraus VB, The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2010;18 Suppl 3:S35-52). Three sections at 200 µm of intervals were stained with hematoxylin, fast green and safranin-O, and one supplementary central section was stained with toluidine blue. Each compartment of the section (tibial median, tibial lateral, femoral median and femoral lateral) was scored by 2 trained experts blinded from sample identity following OARSI recommendations for the guinea pig model. Briefly, the evaluation considered the cartilage surface integrity (0-8), the proteoglycan content (0-6), the cellularity (0-3), the tidemark integrity (0-1) and the osteophyte (0-3), with a maximum of 21 per compartment. The mean of three sections score were calculated for each knee compartment. To assess the global OA score, scores of each compartment were added, giving a maximal score of 84.

Lateral and medial synovial membranes were also scored (synovial lining cells hyperplasia 0-2, villous hyperplasia 0-3, degree of cellular infiltration by perivascular lymphocytes and mononuclear cells 0-5) and the mean of lateral and median membrane was calculated to assess the global synovial score (maximum score of 10) (Kraus VB, The OARSI histopathology initiative - recommendations for histological

assessments of osteoarthritis in the guinea pig. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2010;18 Suppl 3:S35-52).

#### Biomechanical testing by Mach-1<sup>®</sup> micro-mechanical tester

The left knee joint (femoral condyles and tibial plateaus) of each animal was used for testing the biomechanical properties of articular cartilage assessed using a Mach-1<sup>®</sup> micromechanical tester (Mach-1, Biomomentum Inc., Canada) (Figure 15). Prior to testing, samples were thawed at room temperature in phosphate buffered saline (PBS) for 30 min to equilibrate before starting the experiment. Subsequently, femoral condyle or tibial plateau was fixed with Loctite<sup>®</sup> 4013 glue (Henkel, USA) in a small plastic container. Throughout the testing, each sample was kept moist with PBS. Using top view picture of each sample, at least 50 positions per articular surface, were tested using the automated indentation and thickness mapping protocol. The Young's modulus – a measure of cartilage stiffness, and cartilage thickness were calculated using the Mach-1 Analysis software.

#### Biomarker quantification

Glycated, oxidised, and nitrated adduct residues and free adducts were measured in the plasma/serum of guinea pigs using stable isotopic dilution analysis LC-MS/MS described above. Plasma Hyp and citrullinated protein in guinea pigs were also measured by stable isotopic dilution analysis LC-MS/MS (Ahmed, U *et al.*. Biomarkers of early stage osteoarthritis, rheumatoid arthritis and musculoskeletal health. Sci. Rep. 5, 9259 (9251-9257) (2015)).

#### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Following a normality test, a parametric one-way analysis of variance (one-way ANOVA) with Tukey's post-test was performed for histology, MACH-1 and biomarkers (Graphpad Prism 6.0). Statistical significance was represented as  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) or  $p < 0.0001$  (\*\*\*\*). Pearson correlations were performed (GraphPad Prism 6.0) between global OA score, parameters of MACH-1 and biomarkers. For glycated, oxidized and nitrated amino acids, hydroxyproline and serum citrullinated protein analytes analyzed without pre-conceived hypothesis, a Bonferroni correction of 15 is applied.

### **Results**

#### Housing and weight evolution

All animals were subjected to a daily examination during the study. Two guinea pigs of the last group died during the study (weeks 30 and 31). At baseline, guinea pigs had the same weight ( $282 \pm 3$  g). During the study, the 5 groups gained weight in the same way. No difference of weight between groups was observed. At the end of the study (week 36), the weight of the guinea pigs was  $1016 \pm 25$  g.

#### Toxicity and stress

The liver and kidney were examined during euthanasia. No sign of toxicity were observed. The liver and adrenal glands were weighed and no significant differences between the guinea pigs of the same group were observed.

Histology

Histological assessment of cartilage lesions as recommended by the OARSI shows that guinea pigs spontaneously develop severe knee osteoarthritis (Figure 16). In all animals, the global histological score increased significantly with age until week 28 ( $p < 0.0001$  between week 4 and 28) and then stabilized (between weeks 28 and 36) (Figure 17). A significant and progressive increase of synovial score between week 4 and 36 was observed ( $p < 0.01$ , Figure 18). The global histological score is well correlated with the global synovial histological score ( $r = 0.55$ ,  $p < 0.0001$ ).

Biomechanical testing by Mach-1<sup>®</sup> micro-mechanical tester

Significant differences in thickness and young modulus between groups over time were observed (Figure 19). The cartilage thickness gradually decreased to week 20 and then remained stable between weeks 28 and 36. A significantly positive correlation was observed between the global OARSI histological score and the Young's modulus (condyle:  $r = 0.566$ ,  $p < 0.0001$ ; tibial plateau:  $r = 0.442$ ,  $p < 0.0012$ ). When histological items were analyzed individually, it appears that the structure of the cartilage and the proteoglycan content were better correlated with the instantaneous modulus of the femoral condyle ( $r = 0.58X$ ,  $p < 0.0001$ ;  $r = 0.517$ ,  $p < 0.0001$ ) than with other items. At the tibial plateaus, the strongest associations were found between the items cartilage structure and integrity of tidemark and the young modulus ( $r = 0.435$ ,  $p = 0.0014$ ;  $r = 0.433$ ,  $p = 0.0015$ ). Conversely, a significantly negative correlation was also observed between the global histological score and OA cartilage thickness (condyle:  $r = -0.346$ ,  $p = 0.009$ ; plateau:  $r = -0.273$ ,  $p = 0.045$ ) (Figure 20).

Analysis of glycated, oxidised and nitrated free adducts and glycated, oxidised and nitrated protein in plasma/serum in the guinea pig model of osteoarthritis

For glycated amino acids, serum concentrations of the early glycation adduct FL and AGE free adducts, G-H1 and CEL were unchanged from 4 – 28 weeks and then increased 2 – 3 fold at 36 weeks. Pyrraline free adduct was decreased at 20 and 28 with respect to 4, 12 and 36 weeks. MG-H1, 3DG-H and CML free adducts initially decreases at 12 and 20 weeks, compared to baseline levels, returned to baseline levels at 28 weeks and then increased 2 - 3 fold at 36 weeks. CMA free adduct showed a similar trend except decreasing at 20 and 28 weeks. In contrast, glucosepane free adduct was unchanged at 12 weeks and then increased progressively at 20, 28 and 36 weeks to 3-fold higher than baseline levels. For oxidised amino acids, MetSO, AASA and GSA free adducts were generally increased only at week 36 and by 2 – fold, compared to baseline. Dityrosine and NFK free adducts were increased progressively at weeks 28 and 36 up to 2-fold, compared to baseline. Serum 3-NT concentration was decreased by 29 – 32% at 12 – 36 weeks, compared to baseline. Serum Hyp, a bone resorption marker was decreased at weeks 12, 20 and 28, with respect to baseline level ( $P < 0.001$ ). See Figures 21-23 and 25.

In correlation analysis (Figure 24), CMA and 3-NT free adducts correlated with pyrraline ( $r = 0.73$  and  $r = 0.52$ ,  $P < 0.0001$ ), suggesting dietary absorption was only a major source for CMA, 3-NT (and pyrraline). Surprisingly, NFK free adduct correlated negatively with pyrraline which may indicate that dietary antioxidants decrease NFK formation. FL, AGEs except CMA and oxidation markers except NFK and 3-NT correlated positively together ( $r = 0.48 - 0.92$ ,  $P < 0.001$ ). Plasma Hyp correlated positively with CML

(0.59,  $P < 0.001$ ) whereas there were no correlations of glycated, oxidised and nitrated free adducts with citrullinated proteins (CPs).

For plasma glycated, oxidised, nitrated and citrullinated protein (Figures 25 and 26), FL and pentosidine residue content of plasma protein tended to increase with age whereas other AGE content and oxidation adduct residues except for dityrosine tended to decrease with increased age. 3-NT residue content of plasma protein was unchanged and CP tended to decrease with age.

Considering association of protein glycation, oxidation and nitration with the global histological score (Figure 27), CMA free adduct correlated negatively with global histological score whereas other glycation adducts correlated positively: FL ( $r = 0.33$ ), G-H1 ( $r = 0.26$ ) and 3DG-H ( $r = 0.30$ , all  $P < 0.05$ ), and glucosepane ( $r = 0.62$ ,  $P < 0.0001$ ). Similarly, oxidation free adducts correlated positively with global histological score: AASA ( $r = 0.47$ ,  $P < 0.001$ ), GSA ( $r = 0.41$ ,  $P < 0.01$ ), dityrosine ( $r = 0.46$ ,  $P < 0.001$ ) and NFK ( $r = 0.42$ ,  $P < 0.001$ ). In contrast, 3-NT free adduct correlated negatively with global histological score ( $r = -0.47$ ,  $P < 0.001$ ). Plasma CP correlated negatively with the global histological score ( $r = -0.52$ ,  $P < 0.001$ ). Homocitrulline levels decreased significantly between week 4 and week 12 (1.4-fold decrease,  $p < 0.05$ ) and increased between week 12 and week 20 (1.5-fold increase,  $p < 0.01$ ).

The raw data relating to correlation of OA score and biomarker levels is presented in Figure 28.

The serum levels of biomarkers were also correlated with the Mach-1 parameters – Figure 29. Cartilage thickness correlated negatively with GSA free adduct (condyle,  $r = -0.28$  and plateau  $r = -0.33$ ,  $P < 0.05$ ) and positively with 3-NT free adduct (condyle,  $r = 0.33$  and plateau,  $r = 0.29$ ;  $P < 0.05$ ) and Hyp (condyle  $r = 0.47$ ,  $P < 0.001$  and plateau  $r = 0.39$ ,  $P < 0.01$ ). Young's modulus correlated positively with: glucosepane free adduct (condyle  $r = 0.52$  and plateau  $r = 0.56$ ,  $P < 0.0001$ ), AASA free adduct (condyle:  $r = 0.27$ ,  $P < 0.05$  and plateau  $r = 0.40$ ,  $P < 0.01$ ), dityrosine free adduct (condyle  $r = 0.34$ ,  $P < 0.01$  and plateau  $r = 0.35$ ,  $P < 0.05$ ) and NFK free adduct (condyle  $r = 0.36$ ,  $P < 0.01$  and plateau  $r = 0.33$ ,  $P < 0.05$ ). In contrast, there was a negative correlation of Young's modulus with: 3-NT free adduct (condyle  $r = -0.46$ ,  $P < 0.001$  and plateau  $r = -0.41$ ,  $P < 0.01$ ) and CP (femoral condyle  $r = -0.53$ ,  $P < 0.0001$ ) and tibial plateau ( $r = -0.33$ ,  $P < 0.05$ ).

**CLAIMS**

1. A method for determining the skeletal health of an individual comprising:

(a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp);

(b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known skeletal health, and thereby determining the skeletal health of the test individual.

2. A method according to claim 1, wherein the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp).

3. A method according to claim 1, wherein the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp).

4. A method according to any of claims 1-3, wherein the markers further comprise anti-cyclic citrullinated peptide antibody (anti-CCP antibody).

5. A method according to any of claims 1-4, wherein the individual is diagnosed as having no skeletal disorder.

6. A method according to any of claims 1-5, wherein the individual is diagnosed as having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or self-resolving inflammatory joint disease.

7. A method according to any of claims 1-6, wherein the population of individuals having known skeletal health comprises at least one healthy individual having no skeletal disorder, and/or at least one individual having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or self-resolving inflammatory joint disease.

8. A method according to any of claims 1-7, wherein the body fluid sample is selected from at least one of: synovial fluid, blood serum, blood plasma, urine and sputum.

9. A method according to claim 8, wherein the body fluid sample is blood plasma or serum.

10. A method according to any of claims 1-9, wherein the method further comprises an initial step of isolating the hydroxyproline, and/or the oxidised, nitrated, and glycated free adducts from the body fluid sample by ultrafiltration.

11. A method according to any of claims 1-10, wherein the hydroxyproline, and/ or the oxidised, nitrated, and glycated free adducts are quantified using isotope dilution mass spectroscopy.

12. A method according to any of claims 1-11, wherein the diagnostic algorithm comprises a random forest algorithm and/or a support vector machine algorithm.

13. A method according to any of claims 1-12, wherein the diagnostic algorithm comprises a two-stage classification with a first stage for classification as having or not having a skeletal disorder, and a second stage for classification as having a skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or self-resolving inflammatory joint disease.

14. A method of determining whether an individual has an early-stage skeletal disorder comprising:  
 (a) quantifying markers of skeletal health in a body fluid sample obtained from a test individual, wherein said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody;  
 (b) classifying the skeletal health based on the amount of each marker quantified in the test sample with a diagnostic algorithm, wherein the diagnostic algorithm is trained on corresponding values for each marker obtained from a population of individuals having known early-stage skeletal disorder, and thereby determining whether the test individual has an early-stage skeletal disorder.

15. A method according to claim 14, wherein the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and anti-cyclic citrullinated peptide antibody.

16. A method according to claim 14, wherein the markers comprise the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody.

17. A method according to any of claims 14-16, wherein the method is for determining whether an individual has an early-stage skeletal disorder selected from: early-stage osteoarthritis, early-stage rheumatoid arthritis and self-resolving inflammatory joint disease.
- 5 18. A method according to any of claims 14-17, wherein the population of individuals having known early-stage skeletal disorder comprises: at least one individual having early-stage osteoarthritis, at least one individual having early-stage rheumatoid arthritis, and/ or at least one individual having self-resolving inflammatory joint disease.
- 10 19. A method according to any of claims 14-18, wherein the body fluid sample is selected from at least one of: synovial fluid, blood serum, blood plasma, urine and sputum.
20. A method according to claim 19, wherein the body fluid sample is blood plasma or serum.
- 15 21. A method according to any of claims 14-20, wherein the method further comprises an initial step of isolating the oxidised, nitrated, and glycated free adducts from the body fluid sample by ultrafiltration.
22. A method according to any of claims 14-21, wherein the oxidised, nitrated, and glycated free adducts are quantified using isotope dilution mass spectroscopy.
- 20 23. A method according to any of claims 14-22, wherein the skeletal health of the test individual has been previously determined using the method according to any of claims 1-13, and the test individual has been diagnosed as having a skeletal disorder.
- 25 24. A method according to claim 15, wherein the skeletal health of the test individual has been previously determined using the method according to claim 2, and the test individual has been diagnosed as having a skeletal disorder.
- 30 25. A method according to claim 16, wherein the skeletal health of the test individual has been previously determined using the method according to claim 3, and the test individual has been diagnosed as having a skeletal disorder.
26. A method according to any of claims 14-25, wherein the diagnostic algorithm comprises a random forest algorithm and/or a support vector machine algorithm.
- 35 27. A method according to any of claims 14-26, wherein the diagnostic algorithm comprises a two-stage classification with a first stage for classification as having or not having a skeletal disorder, and a second stage for classification as having an early-stage skeletal disorder selected from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), or self-resolving inflammatory joint disease.
- 40

28. A method for monitoring the skeletal health of a test individual, comprising:

(i) classifying the skeletal health of a test individual using a method according to any of claims 1-13 and/or any of claims 14-27, wherein the body fluid sample is obtained from the test individual at a first time point;

(ii) classifying the skeletal health of a test individual using a method according to any of claims 1-13 and/or any of claims 14-27, wherein the body fluid sample is obtained from the test individual at one or more later time points;

(iii) comparing the classification determined in step (i) to the classification determined in step (ii) to determine whether the skeletal health of the test individual has improved, worsened, or remained stable.

29. A method according to claim 28 for monitoring the severity of a skeletal disorder and/or the effectiveness of a treatment regimen on skeletal health.

30. The use of at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine and glucosepane; and hydroxyproline (Hyp) for determining skeletal health.

31. The use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and hydroxyproline (Hyp) as markers for determining skeletal health.

32. The use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and hydroxyproline (Hyp) as markers for determining skeletal health.

33. The use of at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) for determining whether a test individual has an early-stage skeletal disorder.

34. The use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and pentosidine; and  
5 anti-cyclic citrullinated peptide antibody (anti-CCP antibody) as markers for determining whether a test individual has an early-stage skeletal disorder.

35. The use of the combination of the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-  
10 carboxyethyl)lysine (CEL), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), and glucosepane; and anti-cyclic citrullinated peptide antibody (anti-CCP antibody) as markers for determining whether a test individual has an early-stage skeletal disorder.

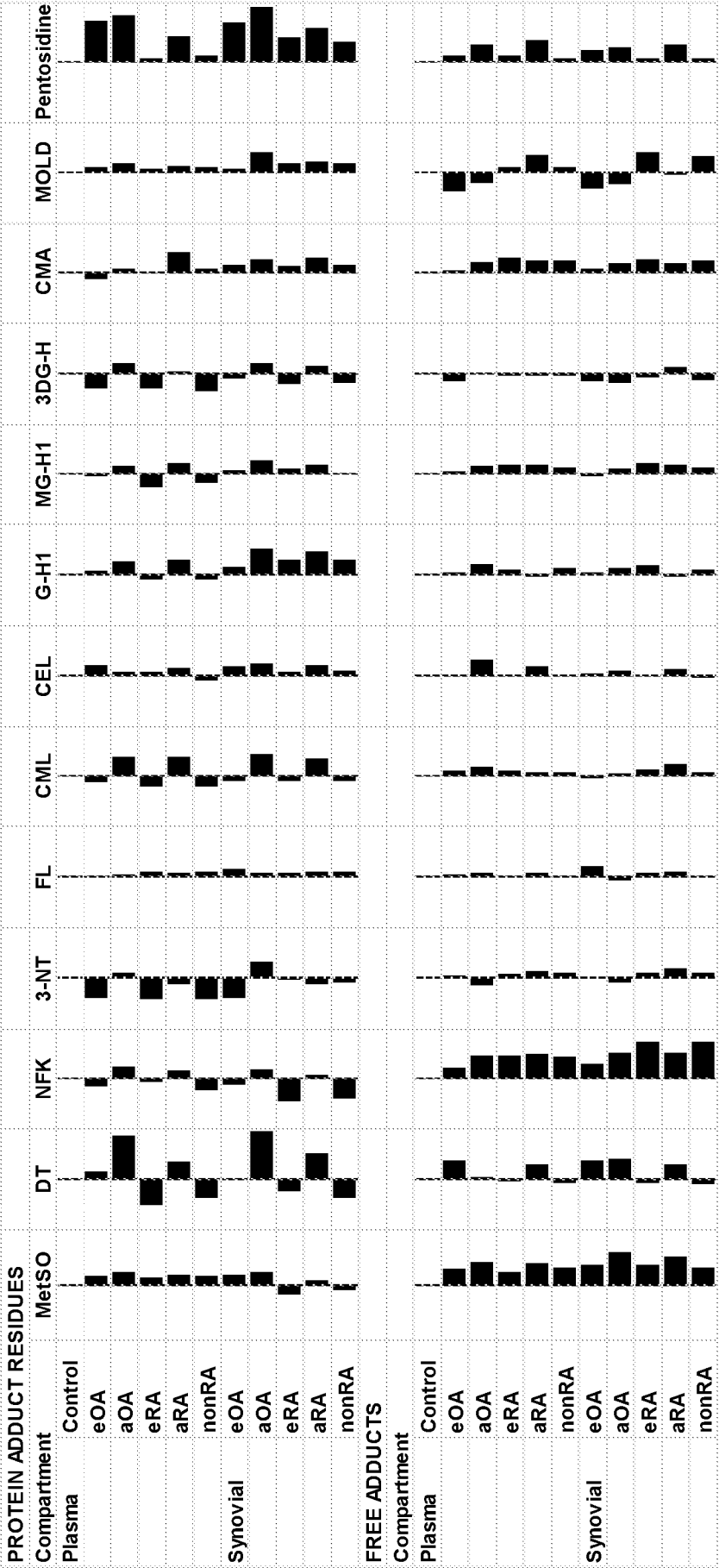
36. The use according to any of claims 33-35, wherein the early-stage skeletal disorder is selected  
15 from at least one of: early-stage osteoarthritis (eOA), early-stage rheumatoid arthritis (eRA), and self-resolving inflammatory joint disease.

37. A kit comprising reagents for quantification of markers of skeletal health, wherein said markers  
20 comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), N-formylkynurenine (NFK), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), glyoxal-derived hydroimidazolone (G-H1), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H), pentosidine, and glucosepane; and hydroxyproline (Hyp).

38. A kit comprising reagents for quantification of markers of early-stage skeletal disorder, wherein  
25 said markers comprise at least 6 markers selected from: the oxidised, nitrated, and glycated free adducts: methionine sulfoxide (MetSO), dityrosine (DT), 3-nitrotyrosine (3-NT), N<sub>ε</sub>-fructosyl-lysine (FL), N<sub>ε</sub>-carboxymethyl-lysine (CML), N<sub>ε</sub>-(1-carboxyethyl)lysine (CEL), N<sub>ω</sub>-carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolone (MG-H1), 3-deoxyglucosone-derived hydroimidazolone (3DG-H),  
30 pentosidine, and glucosepane; and anti-cyclic citrullinated peptide antibody.

39. A kit according to claim 37 or 38, wherein the reagents for quantification of the oxidised, nitrated,  
and glycated free adducts are for quantification of the markers by isotope dilution mass spectroscopy.

Figure 1



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Figure 2

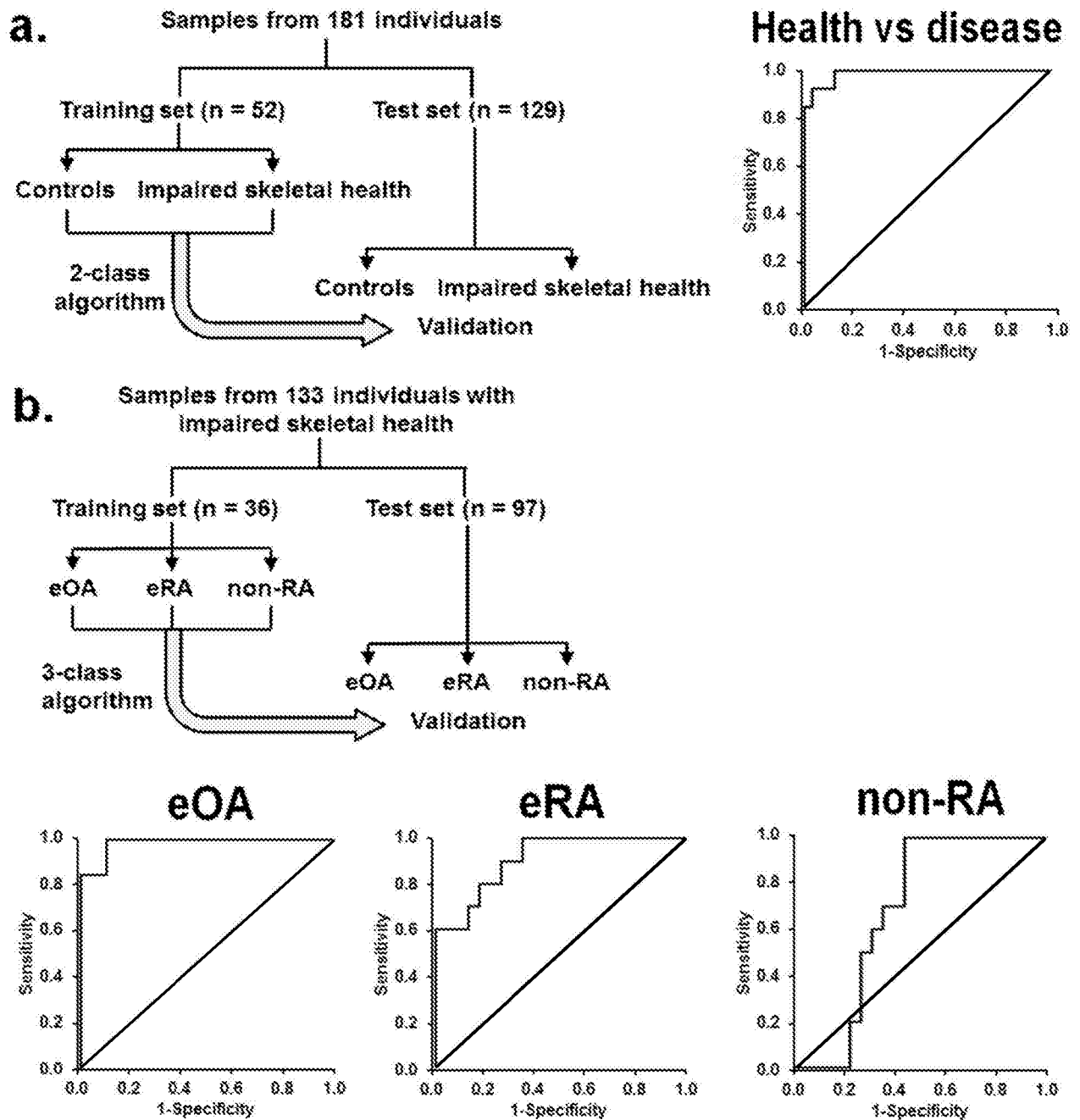


Figure 3. Oxidation and nitration adduct residues of protein in patient plasma and synovial fluid.

| Compartment    | Group   | MetSO (mmol/mol met)             | NFK (mmol/mol trp)                   | DT (mmol/mol tyr)                          | 3-NT (mmol/mol tyr)                          |
|----------------|---------|----------------------------------|--------------------------------------|--------------------------------------------|----------------------------------------------|
| Plasma         | Control | 7.6 (5.3 – 10.1)                 | 0.205 (0.076 – 0.593)                | 0.0038 (0.0024 – 0.0082)                   | 0.0054 (0.0030 – 0.0086)                     |
|                | eOA     | 15.3 (11.8 – 18.5)***            | 0.107 (0.074 – 0.148)                | 0.0069 (0.0028 – 0.0137)                   | 0.0008 (0.0002 – 0.0019)**                   |
|                | aOA     | 21.3 (13.7 – 28.6)***            | 0.551 (0.442 – 0.763) <sup>ooo</sup> | 0.2083 (0.1038 – 0.2553)*** <sup>ooo</sup> | 0.0077 (0.0029 – 0.0089) <sup>ooo</sup>      |
|                | eRA     | 15.7 (14.3 – 17.7)**             | 0.294 (0.167 – 0.360)                | 0.0012 (0.0004 – 0.0021)*                  | 0.0009 (0.0007 – 0.0011)**                   |
|                | aRA     | 17.4 (13.1 – 41.4)***            | 0.390 (0.199 – 0.720)                | 0.0171 (0.0081 – 0.0570)** <sup>ooo</sup>  | 0.0035 (0.0017 – 0.0054)                     |
|                | Non-RA  | 18.2 (15.3 – 19.2)***            | 0.097 (0.068 – 0.128)                | 0.0010 (0.0007 – 0.0018)**                 | 0.0011 (0.0008 – 0.0016)*                    |
| Synovial fluid | eOA     | 18.3 (14.9 – 24.4)***            | 0.125 (0.048 – 0.405)                | 0.0040 (0.0011 – 0.0101)                   | 0.0009 (0.0006 – 0.0016)***                  |
|                | aOA     | 22.9 (17.5 – 26.8)***            | 0.440 (0.258 – 0.915) <sup>oo</sup>  | 0.3522 (0.2070 – 0.5433)*** <sup>ooo</sup> | 0.0220 (0.0050 – 0.0300)* <sup>ooo</sup> , † |
|                | eRA     | 3.6 (3.3 – 4.8)*, †††            | 0.026 (0.015 – 0.055)***, ††         | 0.0013 (0.0009 – 0.0018)*                  | 0.0046 (0.0038 – 0.0059)††                   |
|                | aRA     | 10.4 (8.2 – 32.4) <sup>ooo</sup> | 0.274 (0.153 – 0.455)                | 0.0374 (0.0112 – 0.1258)* <sup>ooo</sup>   | 0.0032 (0.0018 – 0.0067)                     |
|                | Non-RA  | 5.1 (3.6 – 9.4)†††               | 0.034 (0.012 – 0.047)**              | 0.0007 (0.0005 – 0.0009)***                | 0.0037 (0.0027 – 0.0066)                     |

Data are median (lower – upper quartile). Significance: \*, \*\* and \*\*\* with respect to plasma levels of healthy controls; oo and ooo, P<0.01 and P<0.001 for aRA and aOA with respect to eRA and eOA, respectively; and †, †† and †††, P<0.05, P<0.01 and P<0.001 for synovial fluid with respect to plasma of the same study group; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

Figure 4. Oxidation and nitration free adducts in patient plasma and synovial fluid.

| Compartment    | Study group | MetSO (nM)                  | NFK (nM)                     | Dityrosine (nM)            | 3-NT (nM)                 |
|----------------|-------------|-----------------------------|------------------------------|----------------------------|---------------------------|
| Plasma         | Control     | 22.4 (15.4 – 37.5)          | 3.46 (1.84 – 4.85)           | 4.56 (4.04 – 7.78)         | 1.85 (1.02 – 2.76)        |
|                | eOA         | 84.1 (74.8 – 133.6)***      | 4.93 (2.81 – 8.97)           | 8.95 (4.44 – 9.89)         | 1.12 (1.05 – 1.19)        |
|                | aOA         | 140.4 (100.0 – 300.2)***,°° | 14.34 (10.58 – 28.75)***,°°° | 1.82 (1.29 – 3.24),***,°°° | 0.55 (0.43 – 0.89)***,°°° |
|                | eRA         | 67.0 (54.8 – 81.9)**        | 13.97 (10.74 – 23.89)**      | 1.36 (1.15 – 1.63)***      | 1.26 (1.14 – 1.43)        |
|                | aRA         | 124.0 (99.9 – 202.4)***,°°° | 16.24 (10.21 – 20.45)***     | 5.55 (4.49 – 6.64)°°°      | 1.79 (0.78 – 4.15)        |
|                | Non-RA      | 94.5 (61.5 – 118.6)**       | 13.62 (8.52 – 15.11)**       | 1.15 (0.87 – 1.36)***      | 1.49 (1.26 – 2.23)        |
| Synovial fluid | eOA         | 120 (69 – 182)***           | 6.72 (4.96 – 8.69)***        | 7.95 (5.79 – 9.85)**       | 0.97 (0.83 – 1.11)        |
|                | aOA         | 335 (233 – 469)***,°°       | 19.6 (12.2 – 28.1)***,°°     | 9.20 (7.80 – 12.40)***,†   | 0.66 (0.50 – 0.84)***     |
|                | eRA         | 120 (80 – 157)***           | 55.1 (42.6 – 62.4)***        | 1.25 (0.92 – 1.59)         | 1.46 (1.29 – 1.59)        |
|                | aRA         | 225 (134 – 283)***          | 18.1 (14.1 – 41.7)***        | 6.03 (5.10 – 7.63)°°°      | 2.25 (0.63 – 5.25)        |
|                | Non-RA      | 88 (71 – 99)***             | 60.0 (42.3 – 81.3)***        | 1.04 (0.90 – 1.39)         | 1.52 (1.37 – 1.93)        |

Data are median (lower – upper quartile). Significance: \*\* and \*\*\*, P<0.01 and P<0.001 with respect to plasma levels of healthy controls; °° and °°°, P<0.01 and P<0.001 for aRA and aOA with respect to eRA and eOA, respectively; †, P<0.05 for synovial fluid with respect to plasma of the same study group; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

Figure 5. Lysine-derived glycation adduct residues in patient plasma and synovial protein.

| Compartment    | Group   | FL                                | CML                                     | CEL                   | MOLD                                  | Pentosidine                  |
|----------------|---------|-----------------------------------|-----------------------------------------|-----------------------|---------------------------------------|------------------------------|
| Plasma         | Control | 4.63 (3.51 – 6.01)                | 0.075 (0.043 – 0.135)                   | 0.019 (0.016 – 0.025) | 0.0014 (0.0006 – 0.0103)              | 0.0005 (0.0002 – 0.0007)     |
|                | eOA     | 4.72 (4.56 – 4.96)                | 0.044 (0.033 – 0.051)                   | 0.046 (0.014 – 0.064) | 0.0030 (0.0017 – 0.0033)              | 0.0117 (0.0009 – 0.0209)***  |
|                |         |                                   |                                         | *                     |                                       |                              |
|                | aOA     | 5.12 (4.63 – 5.97)                | 0.401 (0.165 – 0.674)*** <sup>ooo</sup> | 0.023 (0.016 – 0.030) | 0.0047 (0.0011 – 0.0103)              | 0.0047 (0.0011 – 0.0103)**   |
|                | eRA     | 7.59 (6.99 – 8.10)***             | 0.045 (0.029 – 0.048)                   | 0.033 (0.023 – 0.051) | 0.0033 (0.0025 – 0.0050)              | 0.0024 (0.0006 – 0.0051)     |
|                | aRA     | 5.73 (5.02 – 6.35) <sup>ooo</sup> | 0.389 (0.146 – 0.474)*** <sup>ooo</sup> | 0.035 (0.020 – 0.103) | 0.0037 (0.0020 – 0.0080)              | 0.0037 (0.0027 – 0.0094) *** |
| Synovial fluid | Non-RA  | 8.13 (7.18 – 8.68)***             | 0.033 (0.023 – 0.037)                   | 0.028 (0.013 – 0.036) | 0.0035 (0.0029 – 0.0051)              | 0.0014 (0.0008 – 0.0027)     |
|                | eOA     | 8.82 (7.16 – 10.58) †             | 0.050 (0.042 – 0.081)                   | 0.042 (0.013 – 0.075) | 0.0028 (0.0021 – 0.0060)              | 0.0110 (0.0033 – 0.0129)     |
|                | aOA     | 6.00 (5.13 – 6.75) <sup>ooo</sup> | 0.537 (0.301 – 0.650) <sup>ooo</sup>    | 0.056 (0.029 – 0.103) | 0.0130 (0.080 – 0.017) <sup>ooo</sup> | 0.0341 (0.0117 – 0.0633) ††† |
|                | eRA     | 6.19 (5.27 – 6.75)                | 0.050 (0.042 – 0.060)                   | 0.024 (0.021 – 0.033) | 0.0046 (0.0018 – 0.0063)              | 0.0032 (0.0019 – 0.0041)     |
|                | aRA     | 6.53 (5.29 – 7.82)                | 0.349 (0.156 – 0.479) <sup>ooo</sup>    | 0.045 (0.022 – 0.082) | 0.0054 (0.0027 – 0.0081)              | 0.0069 (0.0040 – 0.0095)     |
|                | Non-RA  | 6.41 (5.29 – 7.04)                | 0.049 (0.034 – 0.071)                   | 0.026 (0.021 – 0.033) | 0.0044 (0.0023 – 0.0062)              | 0.0023 (0.0017 – 0.0030)     |

Data are median (lower – upper quartile); mmol/mol lys. Significance: \*, \*\* and \*\*\*, P<0.05, P<0.01 and P<0.001 for plasma levels with respect to Control; ooo, P P<0.001 for aRA and aOA with respect to eRA and eOA, respectively; † and †††, P<0.05 and P<0.001 for synovial fluid with respect to plasma of the same study group; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

Figure 6. Arginine-derived glycation adduct residues of protein in patient plasma and synovial fluid.

| Compartment    | Study group | G-HI                        | MG-HI                      | 3DG-H                    | CMA                          |
|----------------|-------------|-----------------------------|----------------------------|--------------------------|------------------------------|
| Plasma         | Control     | 0.030 ± 0.019               | 0.288 (0.126 – 0.374)      | 0.085 (0.022 – 0.309)    | 0.024 ± 0.007                |
|                | eOA         | 0.031 (0.024 – 0.036)       | 0.257 (0.242 – 0.339)      | 0.022 (0.016 – 0.046)    | 0.015 (0.008 – 0.026)        |
|                | aOA         | 0.070 (0.008 – 0.139)       | 0.574 (0.174 – 1.175)      | 0.207 (0.043 – 0.524)    | 0.033 (0.019 – 0.051)        |
|                | eRA         | 0.021 (0.015 – 0.040)       | 0.104 (0.091 – 0.128)*     | 0.031 (0.023 – 0.035)    | 0.034 (0.025 – 0.044)        |
|                | aRA         | 0.081 (0.045 – 0.182)***.00 | 0.720 (0.386 – 1.208)*.000 | 0.096 (0.013 – 0.283)    | 0.144 (0.105 – 0.268)***.000 |
| Synovial fluid | Non-RA      | 0.028 (0.016 – 0.047)       | 0.138 (0.129 – 0.160)      | 0.021 (0.018 – 0.024)    | 0.045 (0.034 – 0.061)*       |
|                | eOA         | 0.046 (0.035 – 0.061)       | 0.353 (0.276 – 0.421)      | 0.059 (0.041 – 0.067)    | 0.050 (0.033 – 0.059)        |
|                | aOA         | 0.253 (0.204 – 0.408)***.†  | 0.900 (0.449 – 1.300)      | 0.200 (0.157 – 0.260)*** | 0.078 (0.038 – 0.104)        |
|                | eRA         | 0.090 (0.034 – 0.128)       | 0.429 (0.262 – 0.563)†     | 0.036 (0.029 – 0.057)    | 0.043 (0.034 – 0.055)        |
|                | aRA         | 0.195 (0.078 – 0.456)†      | 0.653 (0.332 – 1.119)      | 0.168 (0.018 – 0.415)    | 0.088 (0.036 – 0.189)        |
| Synovial fluid | Non-RA      | 0.083 (0.066 – 0.131)†      | 0.288 (0.252 – 0.371)††    | 0.039 (0.030 – 0.043)    | 0.045 (0.031 – 0.049)        |

Data are median (lower – upper quartile); mmol/mol arg. Significance: \* and \*\*\*, P<0.05 and P<0.001 for plasma levels with respect to Control; oo and ooo, P<0.01 and P<0.001 for aRA and aOA for eRA and eOA, respectively; and † and ††, P<0.05 and P<0.01 for synovial fluid with respect to plasma of the same study group; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

**Figure 7. Lysine-derived glycation free adducts in patient plasma and synovial fluid.**

| Compartment    | Study group | FL (nM)                             | CMIL (nM)             | CEL (nM)                               | MOLD (nM)                        | Pentosidine (nM)                       |
|----------------|-------------|-------------------------------------|-----------------------|----------------------------------------|----------------------------------|----------------------------------------|
| Plasma         | Control     | 72.4 (64.4 – 104.7)                 | 89.6 (18.6 – 196.1)   | 89.6 (33.5 – 135.6)                    | 1.54 (1.04 – 2.82)               | 0.67 (0.37 – 1.24)                     |
|                | eOA         | 84.1 (74.8 – 133.6)                 | 124.4 (87.2 – 164.7)  | 88.8 (79.9 – 122.3)                    | 0.27 (0.17 – 0.35)***            | 1.01 (0.67 – 1.51)                     |
|                | aOA         | 99.1 (53.6 – 172.5)                 | 127.4 (191.8 – 215.7) | 353.7 (134.7 – 715.2)*** <sup>oo</sup> | 0.60 (0.21 – 1.26)               | 2.57 (1.51 – 3.59)                     |
|                | eRA         | 71.6 (61.5 – 79.7)                  | 133.5 (72.7 – 165.0)  | 89.7 (67.6 – 104.6)                    | 2.16 (1.82 – 2.70)               | 1.02 (0.83 – 1.36)                     |
|                | aRA         | 88.8 (69.7 – 164.0)                 | 121 (90 – 224)        | 190.6 (115.4 – 400.5)*                 | 7.01 (0.47 – 35.13)              | 3.23 (2.45 – 3.81)*** <sup>oo</sup>    |
| Synovial fluid | Non-RA      | 71.9 (47.2 – 83.6)                  | 118.5 (86.9 – 176.5)  | 94.4 (72.1 – 113.7)                    | 2.09 (1.71 – 2.63)               | 0.88 (0.64 – 1.13)                     |
|                | eOA         | 185.4 (108.3 – 202.6)*              | 81.9 (51.7 – 132.7)   | 99.5 (79.1 – 123.1)                    | 0.36 (0.24 – 0.57)***            | 1.54 (1.36 – 1.71)***                  |
|                | aOA         | 59.4 (41.6 – 72.8) <sup>oo, †</sup> | 106.3 (71.7 – 140.5)  | 138.4 (97.3 – 332.5) ††                | 0.50 (0.01 – 0.80)***            | 1.89 (1.51 – 3.38)***                  |
|                | eRA         | 92.5 (76.9 – 120.9)                 | 153 (99 – 206) †      | 91.9 (73.9 – 111.2)                    | 8.55 (7.16 – 8.78)***            | 0.80 (0.71 – 1.07)                     |
|                | aRA         | 101.8 (59.8 – 232.6)                | 244 (138 – 325)       | 153.4 (75.2 – 463.5)                   | 1.29 (0.57 – 1.74) <sup>oo</sup> | 2.47 (2.19 – 5.84) <sup>ooo, ***</sup> |
|                | Non-RA      | 68.4 (61.5 – 88.4)                  | 118 (94 – 162)        | 82.6 (70.5 – 91.1)                     | 6.41 (3.38 – 11.78)***           | 0.85 (0.69 – 1.38)                     |

Data are median (lower – upper quartile). Significance: \* and \*\*\*, P<0.05 and P<0.001 with respect to plasma levels of healthy controls; oo and ooo, P<0.01 and P<0.001 for aRA and aOA with respect to eRA and eOA, respectively; † and ††, P<0.05 and P<0.01 for synovial fluid with respect to plasma of the same study group; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

**Figure 8. Arginine-derived glycation free adducts in patient plasma and synovial fluid.**

| Compartment    | Group   | G-HI (nM)               | MG-HI (nM)        | 3DG-H (nM)            | CMA (nM)                  |
|----------------|---------|-------------------------|-------------------|-----------------------|---------------------------|
| Plasma         | Control | 49.1 (36.8 – 71.1)      | 260 (131 – 447)   | 66.7 (41.5 – 112.0)   | 5.8 (3.4 – 6.7)           |
|                | eOA     | 55.3 (41.7 – 76.6)      | 278 (226 – 496)   | 34.2 (25.6 – 47.6)*   | 6.17 (4.68 – 9.76)        |
|                | aOA     | 114.5 (34.8 – 256.1)    | 511 (306 – 766)*  | 69.5 (21.1 – 99.6)    | 14.2 (12.3 – 20.1)***.000 |
|                | eRA     | 70.4 (60.8 – 103.7)     | 551 (303 – 1160)  | 57.3 (43.7 – 63.3)    | 21.1 (14.4 – 27.8)***     |
|                | aRA     | 45.8 (35.8 – 94.6)      | 597 (314 – 742)   | 58.0 (18.5 – 193.4)   | 16.1 (10.7 – 22.9)***     |
|                | Non-RA  | 78.4 (56.1 – 90.4)      | 438 (383 – 906)   | 60.8 (47.6 – 76.6)    | 16.9 (14.9 – 25.3)***     |
| Synovial fluid | eOA     | 56.3 (48.6 – 72.9)      | 223 (198 – 325)   | 34.2 (30.9 – 50.3)    | 7.2 (6.2 – 10.1)          |
|                | aOA     | 83.9 (39.6 – 154.6)     | 360 (275 – 656)   | 29.0 (23.0 – 46.3)*** | 12.9 (7.3 – 17.6)***      |
|                | eRA     | 101.0 (84.2 – 126.3)*** | 615 (367 – 1343)* | 50.5 (41.0 – 72.7)    | 17.5 (11.7 – 26.6)***     |
|                | aRA     | 44.0 (19.7 – 77.8)      | 562 (289 – 838)   | 107.3 (23.9 – 251.9)  | 13.4 (9.9 – 19.5)***      |
|                | Non-RA  | 76.7 (67.1 – 117.4)     | 462 (318 – 672)   | 39.9 (30.7 – 57.6)    | 16.0 (13.3 – 22.5)***     |

Data are median (lower – upper quartile). Significance: \* and \*\*\*,  $P < 0.05$  and  $P < 0.001$  with respect to plasma levels of healthy controls; 000,

$P < 0.001$  for aRA and aOA with respect to eRA and eOA, respectively; *Mann-Whitney U*. A Bonferroni correction of 12 was applied.

Figure 9. Homotypic correlation of protein oxidation, nitration and glycation adducts in synovial fluid and plasma compartments.

| Study group | Protein adduct residue                                                                                                                                                                                                           | Free adduct                                                                                                                                                                    |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| eOA         | CML (r = 0.60, P<0.05), CEL (r = 0.82, P<0.001), MetSO (r = 0.61, P<0.05), DT (r = 0.53, P<0.05)                                                                                                                                 | CEL (r = 0.59, P<0.05), 3DG-H (r = 0.62, P<0.05), pentosidine (r = 0.71)                                                                                                       |
| aOA         | FL (r = 0.58, P<0.05), MG-H1 (r = 0.80, P<0.001)                                                                                                                                                                                 | FL (r = 0.75, P<0.01), CEL (r = 0.63, P<0.05), G-H1 (r = 0.71, P<0.01), MG-H1 (r = 0.91, P<0.001), NFK (r = 0.73, P<0.05)                                                      |
| eRA         | 3DG-H (r = 0.72, P<0.01), MetSO (r = 0.63, P<0.05)                                                                                                                                                                               |                                                                                                                                                                                |
|             | Pentosidine (r = 0.82, P<0.01)                                                                                                                                                                                                   | FL (r = 0.87, P<0.01), CML (r = 0.67, P<0.05), CEL (r = 0.81, P<0.01), MG-H1 (r = 0.92, P<0.001), 3DG-H (r = 0.83, P<0.01), CMA (0.72, P<0.05), pentosidine (r = 0.78, P<0.01) |
| aRA         | CML (r = 0.56, P<0.05), CEL (r = 0.70, P<0.01), G-H1 (r = 0.52, P<0.05), MG-H1 (r = 0.86, P<0.001), 3DG-H (r = 0.86, P<0.001), CMA (0.50, P<0.05), pentosidine (r = 0.83, P<0.01), NFK (r = 0.56, P<0.05), DT (r = 0.64, P<0.01) | G-H1 (r = 0.53, P<0.05), MG-H1 (r = 0.87, P<0.001), 3DG-H (r = 0.73, P<0.01), 3-NT (0.67, P<0.01)                                                                              |
| Non-RA      | Pentosidine (r = 0.76, P<0.05)                                                                                                                                                                                                   | CEL (r = 0.73, P<0.05), MG-H1 (r = 0.76, P<0.05), MOLD (r = 0.81, P<0.01)                                                                                                      |

Figure 10. Predictive algorithm outcomes for training set cross-validation using Random Forest (95% CI for sensitivity, specificity given in brackets).

A. Algorithm diagnostic characteristics.

|                           | Algorithm 1            |                    | Algorithm 2        |                    |
|---------------------------|------------------------|--------------------|--------------------|--------------------|
|                           | Disease-versus-control | eOA                | eRA                | non-RA             |
| nCorrect                  | 44/46                  | 13/13              | 6/8                | 8/12               |
| Sensitivity               | 0.92 (0.64 - 1)        | 0.92 (0.64 - 1)    | 0.80 (0.44 - 0.97) | 0.70 (0.35 - 0.93) |
| Specificity               | 0.91 (0.76 - 0.98)     | 0.90 (0.68 - 0.99) | 0.78 (0.56 - 0.93) | 0.65 (0.43 - 0.84) |
| AUC                       | 0.99 (0.97 - 1)        | 0.98 (0.96 - 1)    | 0.91 (0.82 - 1)    | 0.68 (0.49 - 0.86) |
| Positive predictive value | 1.0                    | 1.0                | 0.60               | 0.80               |
| Negative predictive value | 0.85                   | 1.0                | 0.84               | 0.83               |
| Positive likelihood ratio | 10.2                   | 9.2                | 3.6                | 2.0                |
| Negative likelihood ratio | 0.09                   | 0.09               | 0.26               | 0.46               |

B. Confusion matrix for algorithm 1 (training set cross-validation)

|                |         | Predicted class |         |
|----------------|---------|-----------------|---------|
|                |         | Control         | Disease |
| Clinical class | Control | 11              | 0       |
|                | Disease | 2               | 33      |

C. Confusion matrix for algorithm 2 (training set cross-validation)

|                |        | Predicted class |     |        |
|----------------|--------|-----------------|-----|--------|
|                |        | eOA             | eRA | Non-RA |
| Clinical class | eOA    | 13              | 0   | 0      |
|                | eRA    | 0               | 6   | 2      |
|                | Non-RA | 0               | 4   | 8      |

Figure 11. Predictive algorithm outcomes for test set cross-validation using Random Forest (95% CI for sensitivity, specificity given in brackets).

A. Algorithm diagnostic characteristics.

|                           | Algorithm 1        |                    | Algorithm 2        |                    |
|---------------------------|--------------------|--------------------|--------------------|--------------------|
|                           | Disease-vs-control | eOA                | eRA                | non-RA             |
| nCorrect                  | 119/134            | 30/31              | 22/27              | 27/39              |
| Sensitivity               | 0.89 (0.75 - 0.97) | 0.83 (0.65 - 0.94) | 0.77 (0.6 - 0.9)   | 0.72 (0.53 - 0.86) |
| Specificity               | 0.90 (0.82 - 0.95) | 0.84 (0.73 - 0.92) | 0.76 (0.63 - 0.86) | 0.71 (0.58 - 0.81) |
| AUC                       | 0.96 (0.93 - 0.99) | 0.93 (0.88 - 0.98) | 0.87 (0.8 - 0.94)  | 0.77 (0.68 - 0.86) |
| Positive predictive value | 0.96               | 0.97               | 0.81               | 0.69               |
| Negative predictive value | 0.75               | 1.0                | 0.81               | 0.91               |
| Positive likelihood ratio | 8.9                | 5.2                | 3.2                | 2.5                |
| Negative likelihood ratio | 0.12               | 0.20               | 0.30               | 0.39               |

B. Confusion matrix for algorithm 1 (training set cross-validation)

|                |         | Predicted class |         |
|----------------|---------|-----------------|---------|
|                |         | Control         | Disease |
| Clinical class | Control | 33              | 4       |
|                | Disease | 11              | 86      |

C. Confusion matrix for algorithm 2 (training set cross-validation)

|                |        | Predicted class |     |        |
|----------------|--------|-----------------|-----|--------|
|                |        | eOA             | eRA | Non-RA |
| Clinical class | eOA    | 30              | 0   | 0      |
|                | eRA    | 1               | 22  | 12     |
|                | Non-RA | 0               | 5   | 27     |

Figure 12. Predictive algorithm outcomes for test set validation using Random Forest (95% CI for sensitivity, specificity given in brackets).

A. Algorithm diagnostic characteristics.

| Algorithm 1               |                    | Algorithm 2        |                    |                    |
|---------------------------|--------------------|--------------------|--------------------|--------------------|
|                           | Disease-vs-control | eOA                | eRA                | non-RA             |
| nCorrect                  | 63/134             | 18/19              | 8/35               | 31/32              |
| Sensitivity               | 0.73 (0.56 - 0.86) | 0.83 (0.65 - 0.94) | 0.60 (0.42 - 0.76) | 0.81 (0.64 - 0.93) |
| Specificity               | 0.72 (0.62 - 0.81) | 0.84 (0.73 - 0.92) | 0.60 (0.46 - 0.72) | 0.80 (0.68 - 0.89) |
| AUC                       | 0.77 (0.69 - 0.85) | 0.91 (0.84 - 0.99) | 0.62 (0.5 - 0.75)  | 0.84 (0.77 - 0.92) |
| Positive predictive value | 0.62               | 0.60               | 0.23               | 0.97               |
| Negative predictive value | 0.05               | 0.99               | 0.97               | 0.43               |
| Positive likelihood ratio | 2.6                | 5.2                | 1.5                | 4.1                |
| Negative likelihood ratio | 0.38               | 0.20               | 0.67               | 0.24               |

B. Confusion matrix for algorithm 1 (training set cross-validation)

|                |         | Predicted class |         |
|----------------|---------|-----------------|---------|
|                |         | Control         | Disease |
| Clinical class | Control | 2               | 36      |
|                | Disease | 35              | 61      |

C. Confusion matrix for algorithm 2 (training set cross-validation)

|                |        | Predicted class |     |        |
|----------------|--------|-----------------|-----|--------|
|                |        | eOA             | eRA | Non-RA |
| Clinical class | eOA    | 18              | 0   | 1      |
|                | eRA    | 2               | 8   | 0      |
|                | Non-RA | 10              | 27  | 31     |

Figure 13. Selected plasma oxidation and glycation free adducts in patients with early and advanced osteoarthritis and other early-stage arthritic disease

| Study group      | Glucosepane (nM)     | AASA (nM)             | GSA (nM)           |
|------------------|----------------------|-----------------------|--------------------|
| Control (n = 29) | 20.8 (15.6 – 29.9)   | 127 (84 - 158)        | 253 (208 – 299)    |
| eOA (n = 28)     | 30.2 (18.6 – 53.5)*  | 120 (66 - 139)        | 231 (127 - 371)    |
| aOA (n = 18)     | 24.9 (18.5– 75.3)*   | 3.31 (2.4 - 25)***oo  | 51.9 (33.2 - 447)o |
| eRA (n = 35)     | 46.1(31.1 – 77.8)*** | 77.1 (17.3 - 121)*    | 141 (115 - 248)**  |
| Non-RA (n = 32)  | 31.2 (20.3– 45.2)**  | 78.9 (62.6 - 109.2) * | 138 (113 - 165)    |
| Significance     | P<0.001              | P>0.05                | P<0.001            |

Data are median (lower – upper quartile). Significance: P-values for 5 study groups are given above; *Kruskal-Wallis*.

For binary comparisons: \*, \*\* and \*\*\*, P<0.05, P<0.01 and P<0.001 with respect to plasma levels of healthy controls; o and oo, P<0.05 and P<0.01 with respect to plasma levels

**Figure 14. Predictive algorithm outcomes for 2-fold cross-validation using SVM**

| <b>Algorithm diagnostic characteristics.</b> | <b>Algorithm 1<br/>(features:<br/>Hyp, G-H1, MG-H1, 3DG-H, CEL, CMA, MetSO, 3-NT, NFK, DT, GSP)</b> | <b>Algorithm 2:<br/>(features: CCP-Ab, FL, 3DG-H, CML, CEL, MetSO, 3-NT, GSP)</b> |                                    |                                    |
|----------------------------------------------|-----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------|------------------------------------|
|                                              |                                                                                                     | <b>eOA versus non-RA &amp; eRA</b>                                                | <b>eRA versus non-RA &amp; eOA</b> | <b>Non-RA versus eRA &amp; eOA</b> |
| <b>Classification</b>                        | <b>Disease-versus-control</b>                                                                       |                                                                                   |                                    |                                    |
| <b>Accuracy</b>                              | 88.4%<br>(86.9 – 90.0)                                                                              | 95.5%<br>(93.7 – 97.3)                                                            | 78.1%<br>(74.3 – 82.0)             | 78.9%<br>(74.7 – 83.2)             |
| <b>Sensitivity</b>                           | 90.4%<br>(88.7 – 92.1)                                                                              | 94.0%<br>(88.8 – 99.3)                                                            | 69.1%<br>(54.4 – 83.7)             | 67.5%<br>(46.2 – 88.8)             |
| <b>Specificity</b>                           | 83.2%<br>(77.4 – 89.0)                                                                              | 96.1%<br>(92.6 – 99.6)                                                            | 83.2%<br>(71.8 – 94.6)             | 84.6%<br>(77.1 – 92.1)             |
| <b>AUROC</b>                                 | 0.93<br>(0.92 – 0.94)                                                                               | 0.98<br>(0.97 – 0.99)                                                             | 0.86<br>(0.81 – 0.90)              | 0.88<br>(0.86 – 0.90)              |
| <b>Positive likelihood ratio</b>             | 8.26<br>(5.77 – 10.75)                                                                              | 16.11<br>(9.56 – 22.66)                                                           | 7.66<br>(2.94 – 12.37)             | 4.96<br>(3.31 – 6.60)              |
| <b>Negative likelihood ratio</b>             | 0.11<br>(0.10 – 0.13)                                                                               | 0.06<br>(0.01 – 0.11)                                                             | 0.34<br>(0.20 – 0.48)              | 0.36<br>(0.14 – 0.57)              |
| <b>Positive predictive value</b>             | 93.6%<br>(91.7 – 95.6)                                                                              | 92.8%<br>(86.5 – 99.0)                                                            | 76.3%<br>(65.1 – 87.4)             | 71.8%<br>(62.7 – 80.8)             |
| <b>Negative predictive value</b>             | 77.3%<br>(74.4 – 80.2)                                                                              | 97.5%<br>(95.3 – 99.6)                                                            | 84.4%<br>(79.0 – 89.8)             | 86.2%<br>(78.9 – 93.4)             |
| <b>F-score</b>                               | 0.92<br>(0.91 – 0.93)                                                                               | 0.93<br>(0.90 – 0.96)                                                             | 0.69<br>(0.62 – 0.75)              | 0.64<br>(0.49 – 0.79)              |

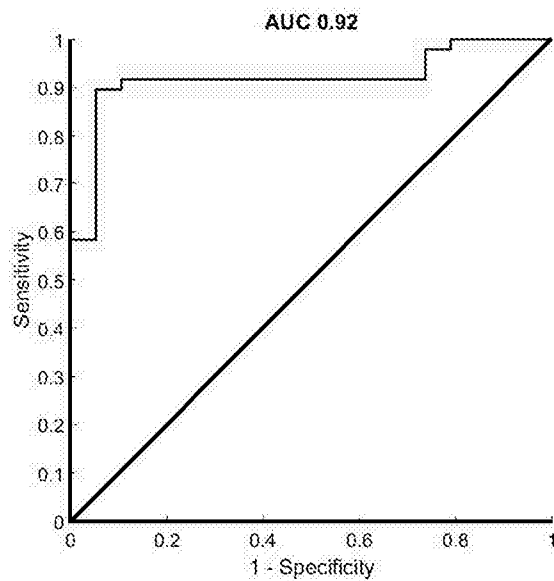
(Mean estimates 95% are given)

Confusion matrix and nCorrect

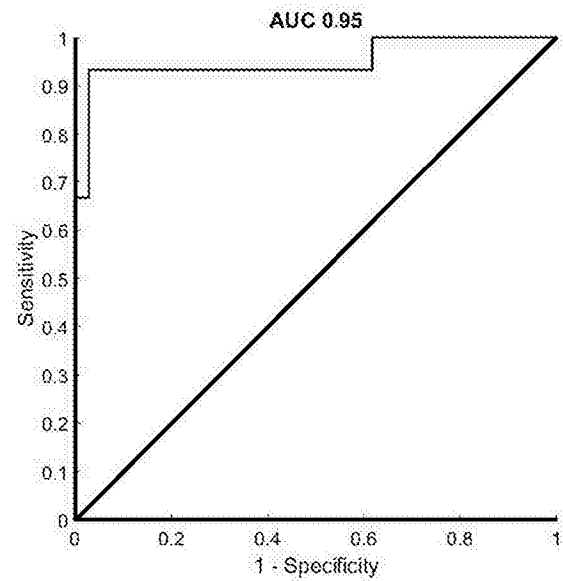
|                |         | Algorithm 1     | Algorithm 2 |                 |        |       |
|----------------|---------|-----------------|-------------|-----------------|--------|-------|
|                |         | Diseased        |             | Non-RA          | eRA    | eOA   |
|                |         |                 |             |                 |        |       |
| nCorrect       |         | 42/48           |             | 11/17           | 13/18  | 14/14 |
|                |         | Predicted class |             |                 |        |       |
|                |         | Predicted class |             | Predicted class |        |       |
|                |         | Disease         | Control     |                 | Non-RA | eRA   |
| Clinical class | Disease | 42              | 6           | Non-RA          | 11     | 5     |
|                | Control | 1               | 18          | eRA             | 5      | 13    |
|                |         |                 |             | oRA             | 0      | 0     |
|                |         |                 |             |                 |        | 14    |

From 1 trial of 2-fold cross-validation. The values are representative of classification on 50% data as test set cross validation while the training was performed on the remaining 50% of the data.

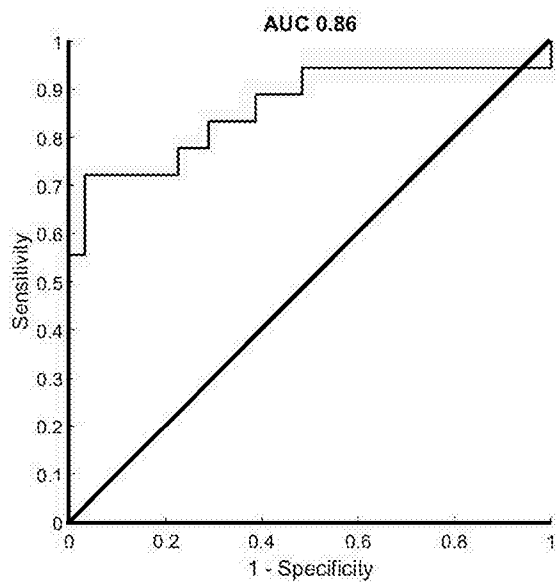
Healthy vs disease



Early OA vs Non-RA/ early RA



Early RA vs non-RA/ early OA



non-RA vs early RA/ early OA

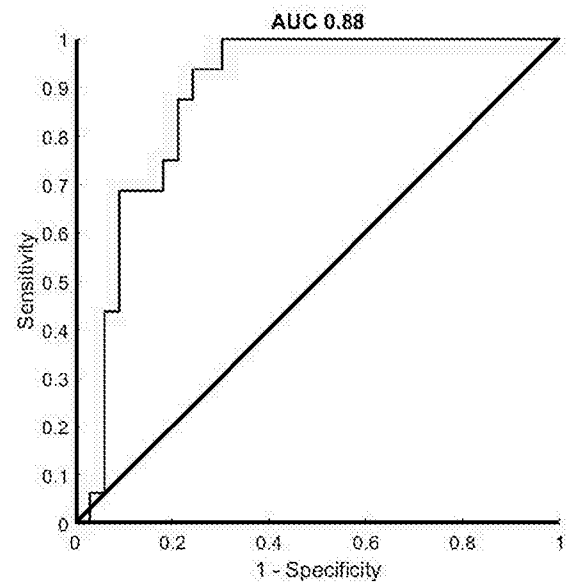
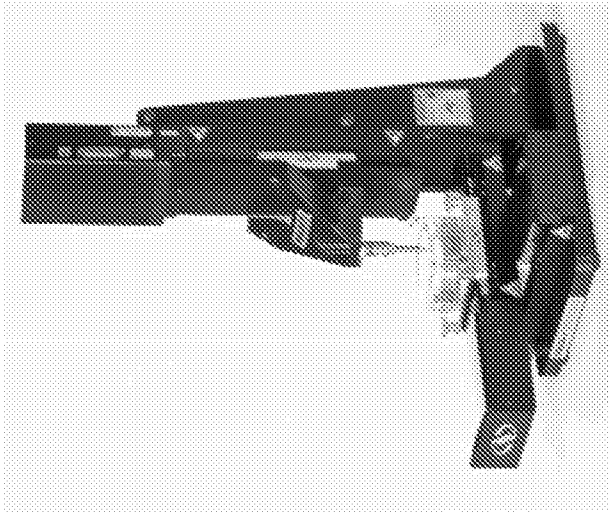


Figure 15.

(A) MACH-1 mechanical testing system (Biomomentum, Canada)

(B) View of a guinea pig femoral condyle with a position grid superimposed.

A



B

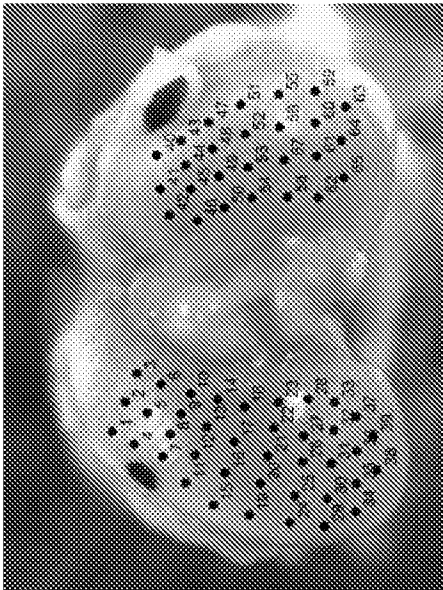
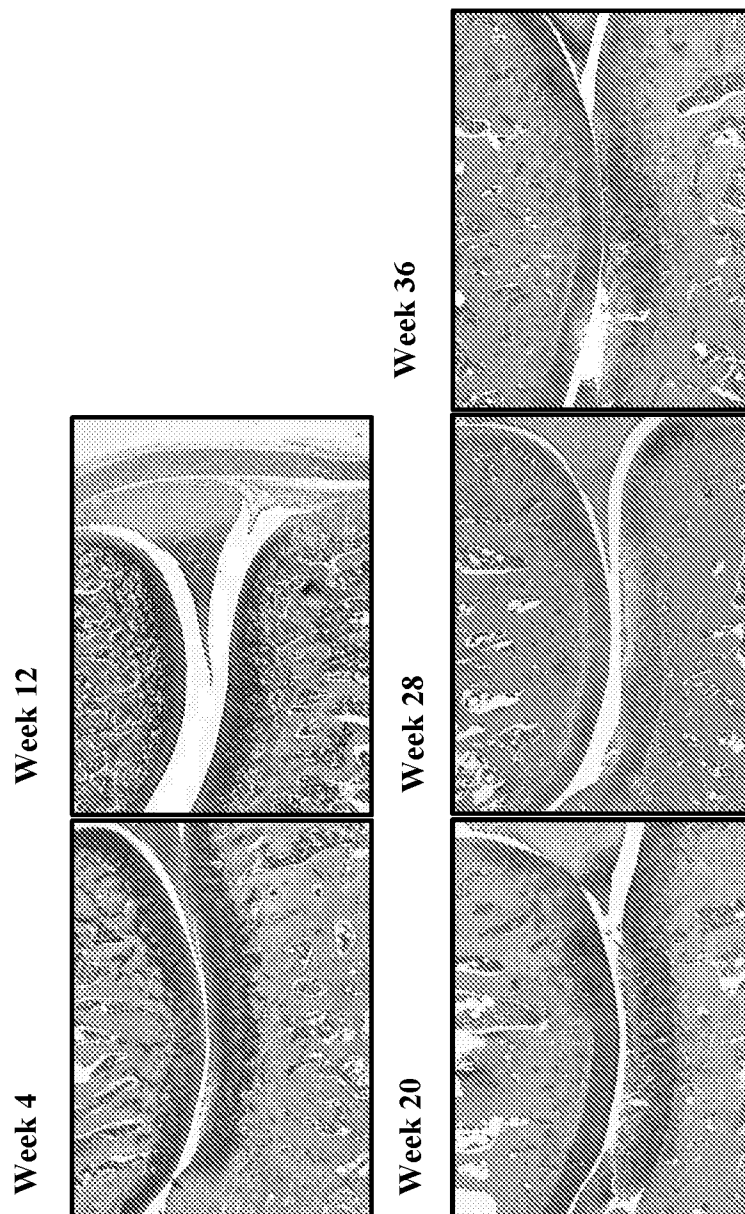
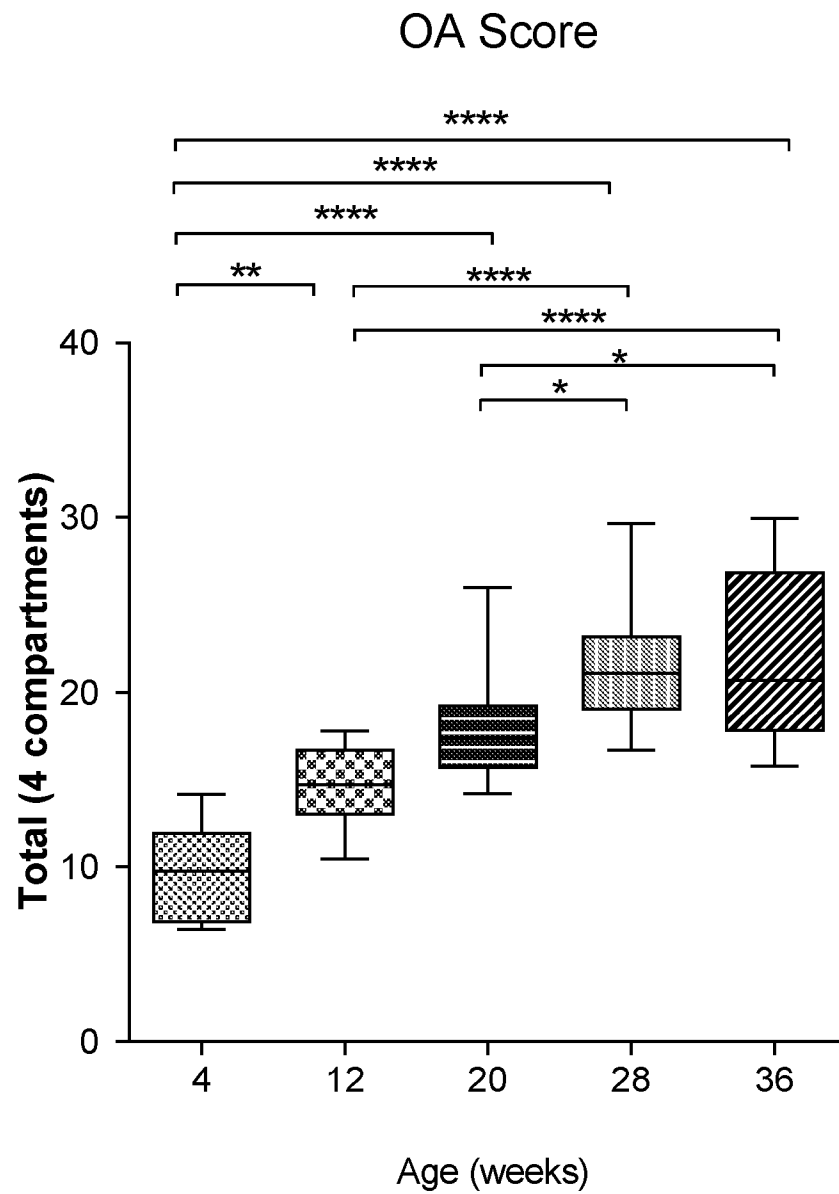


Figure 16. Representative pictures of medial compartment of right guinea pig knees over time



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**Figure 17. Total OA score in the guinea pig model of OA over time**

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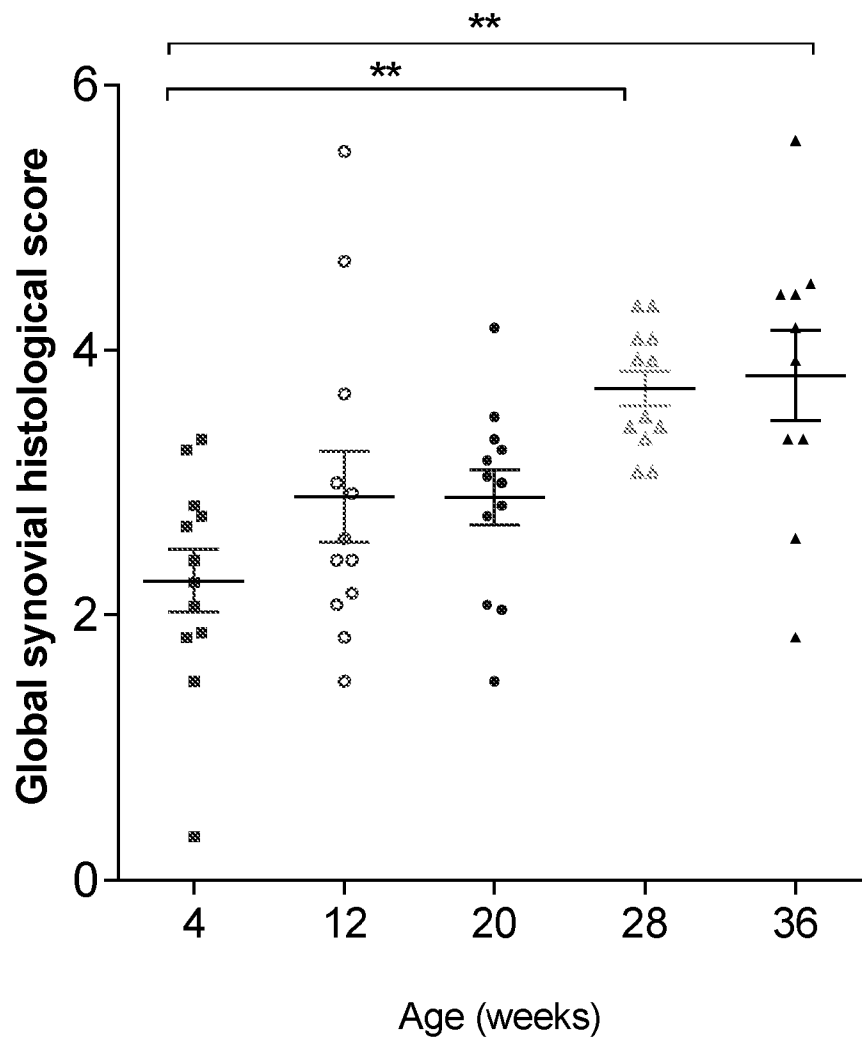
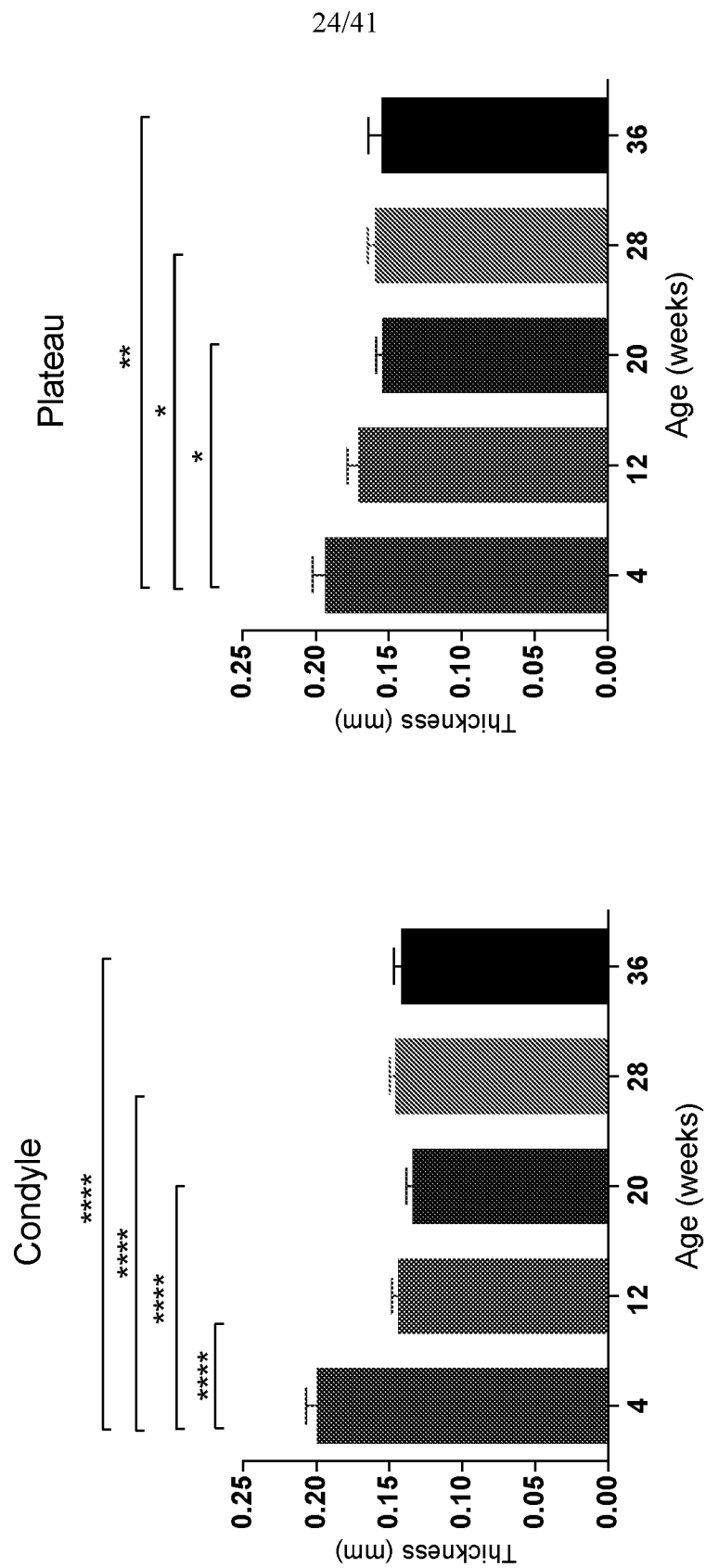
**Figure 18. Global synovial histological score in the guinea pig model of OA over time**

**Figure 19. Distribution of thickness (mm) and Young modulus (MPa) in the guinea pig model of OA over time**



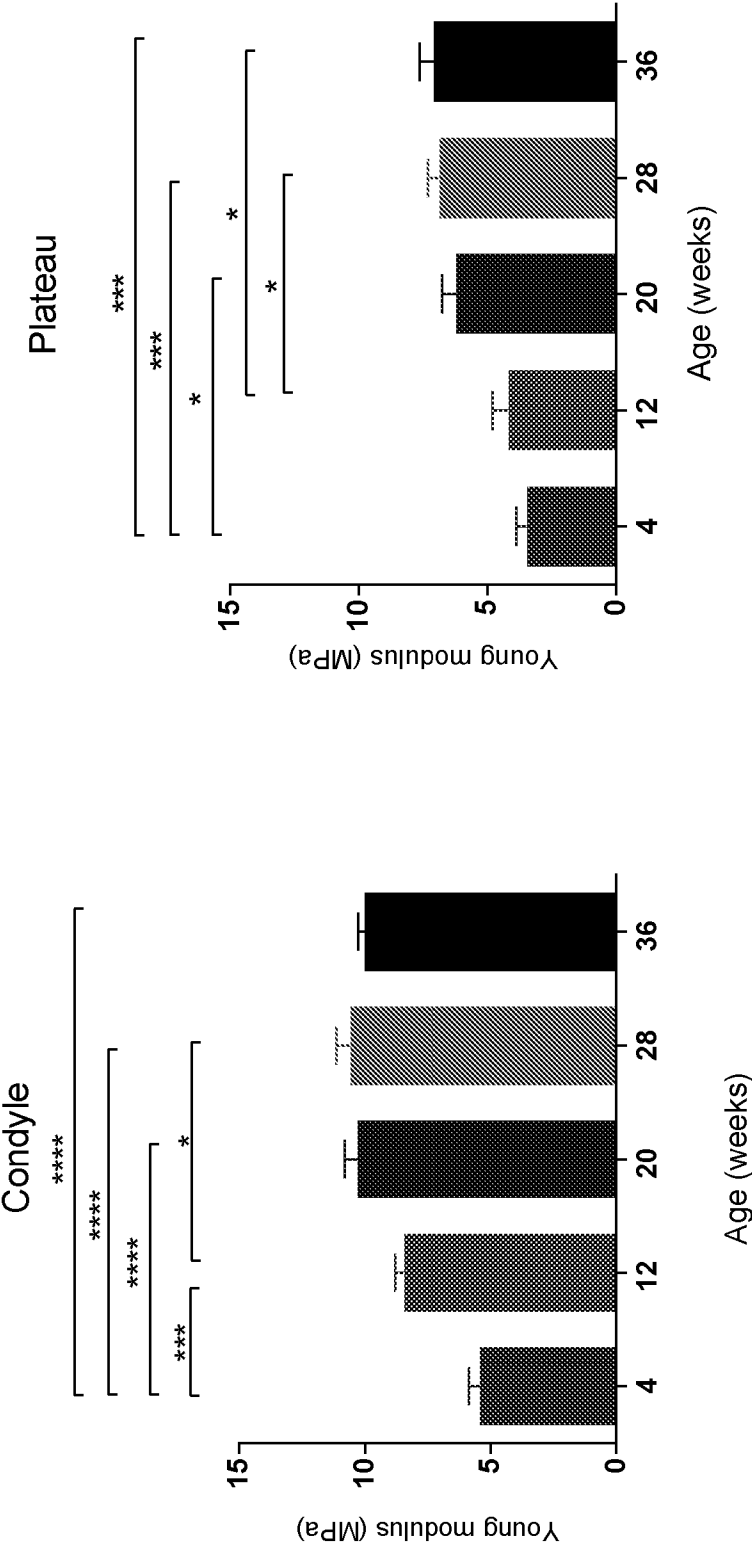
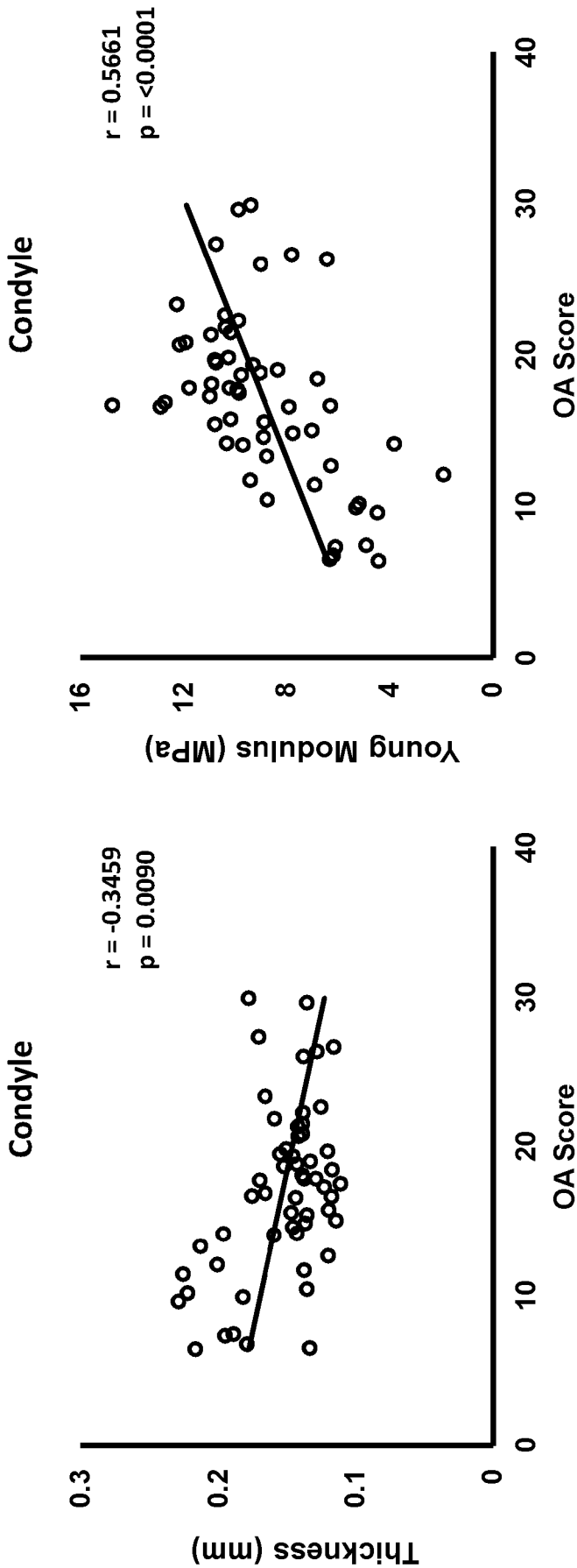


Figure 20. Correlations between the global histological score and OA cartilage thickness or Young modulus.



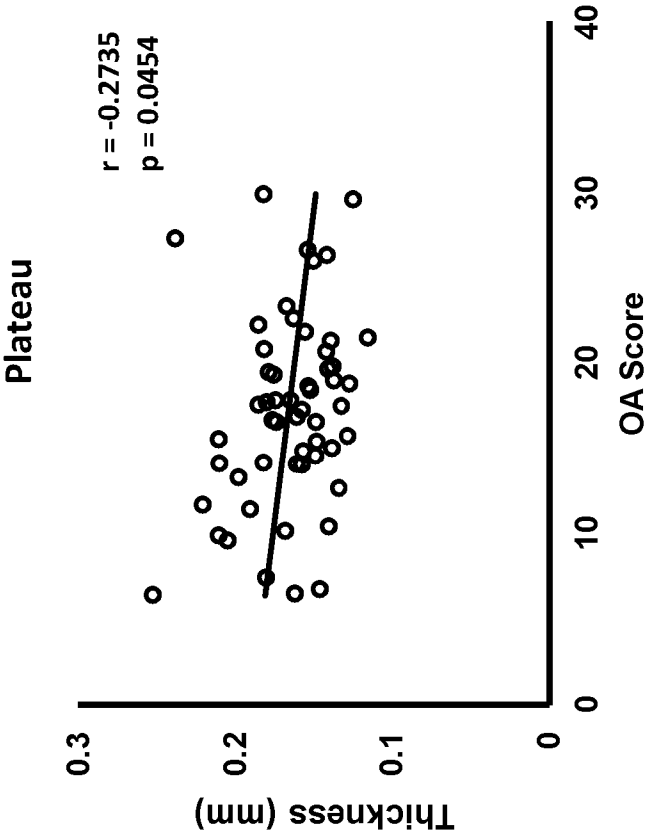
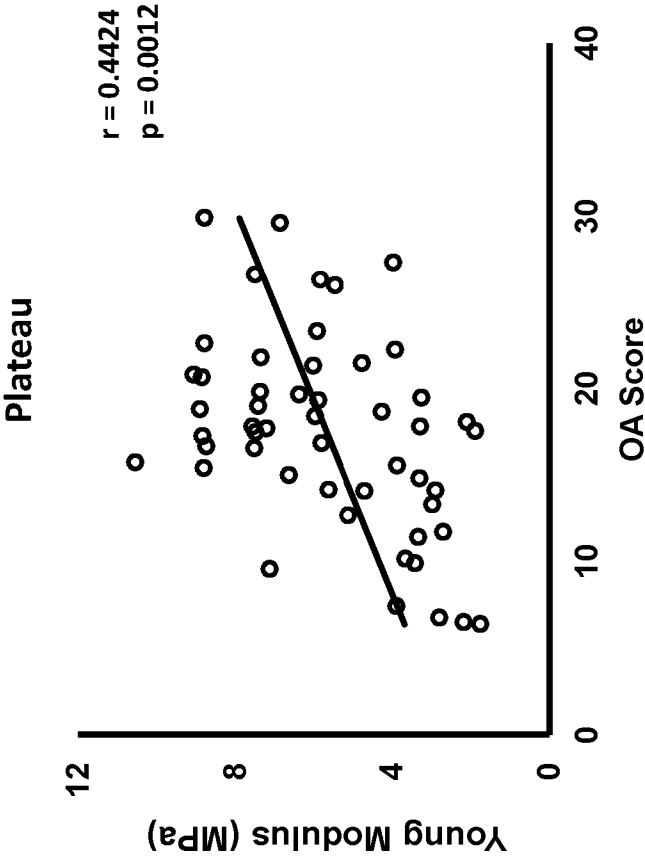
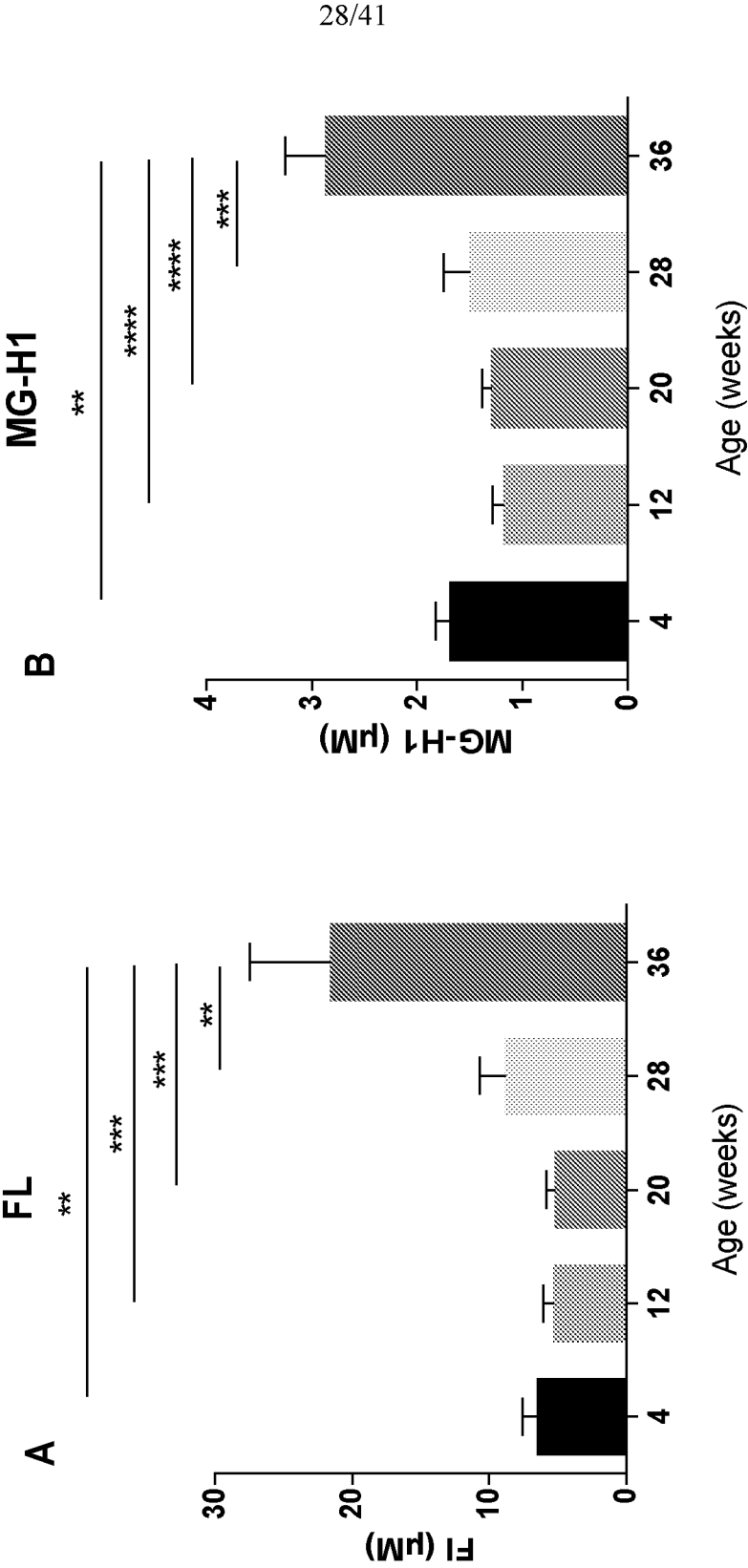
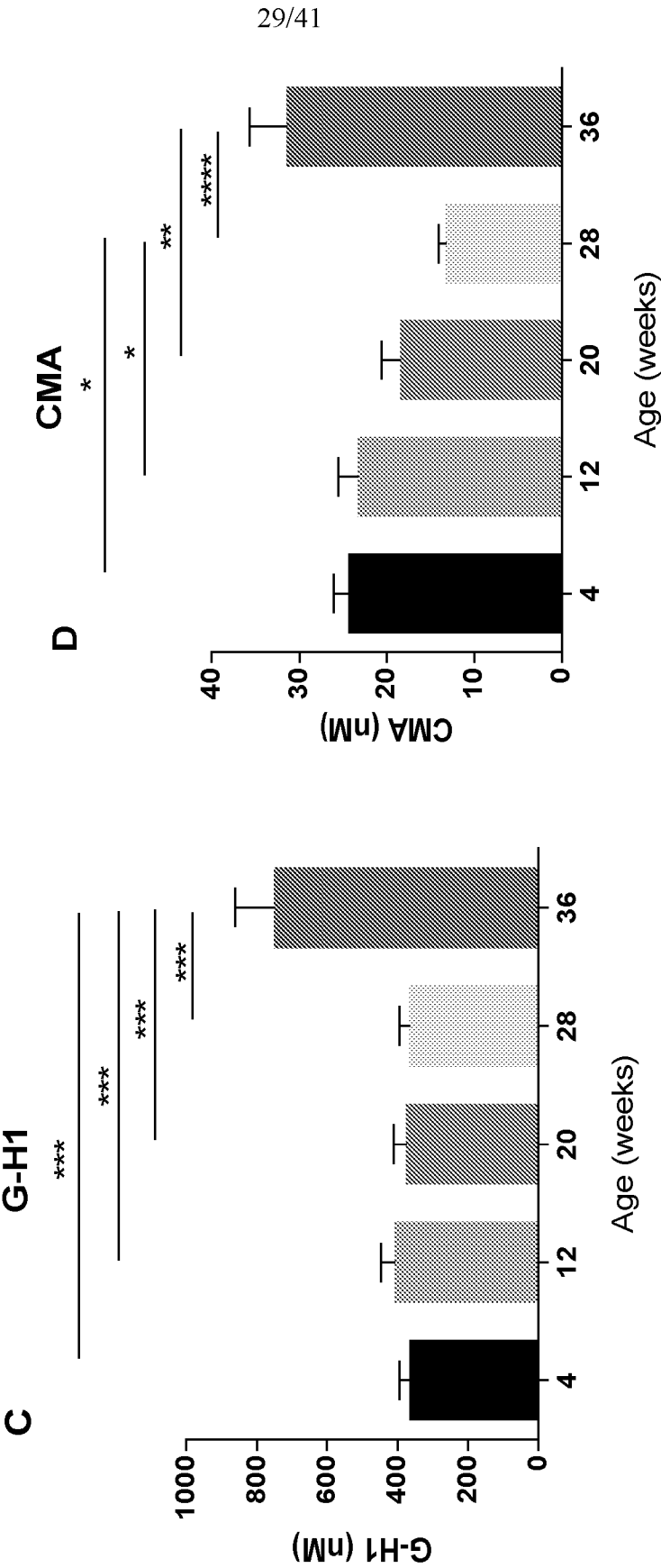


Figure 21. Serum concentrations of glycated amino acids in the guinea pig model of osteoarthritis.





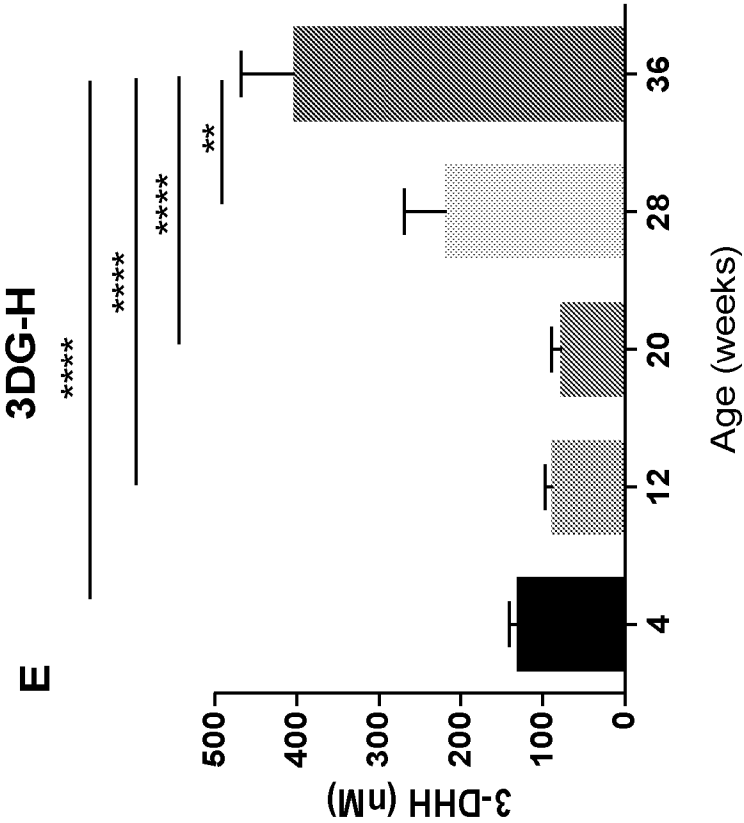
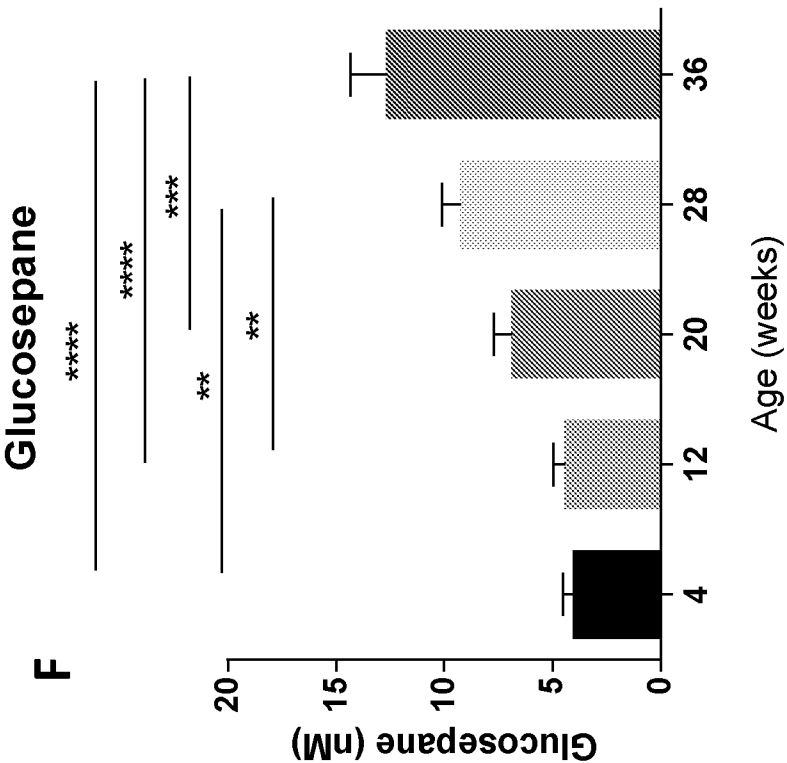
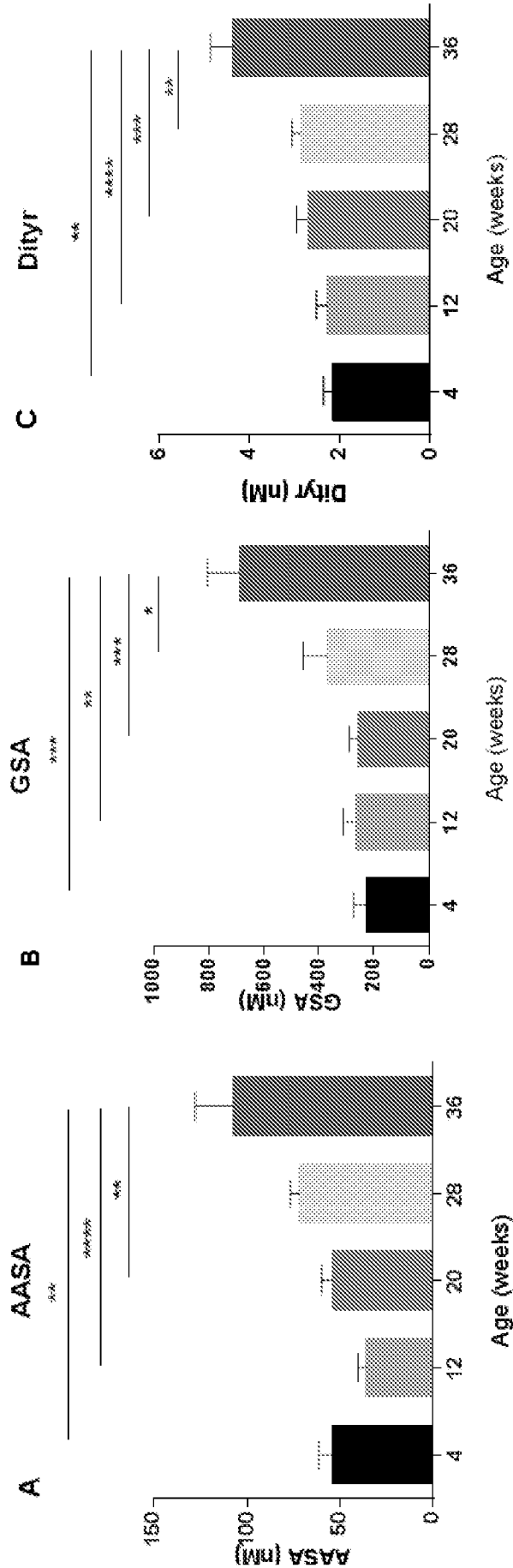


Figure 22. Serum concentrations of oxidized and nitrated amino acids in the guinea pig model of osteoarthritis.



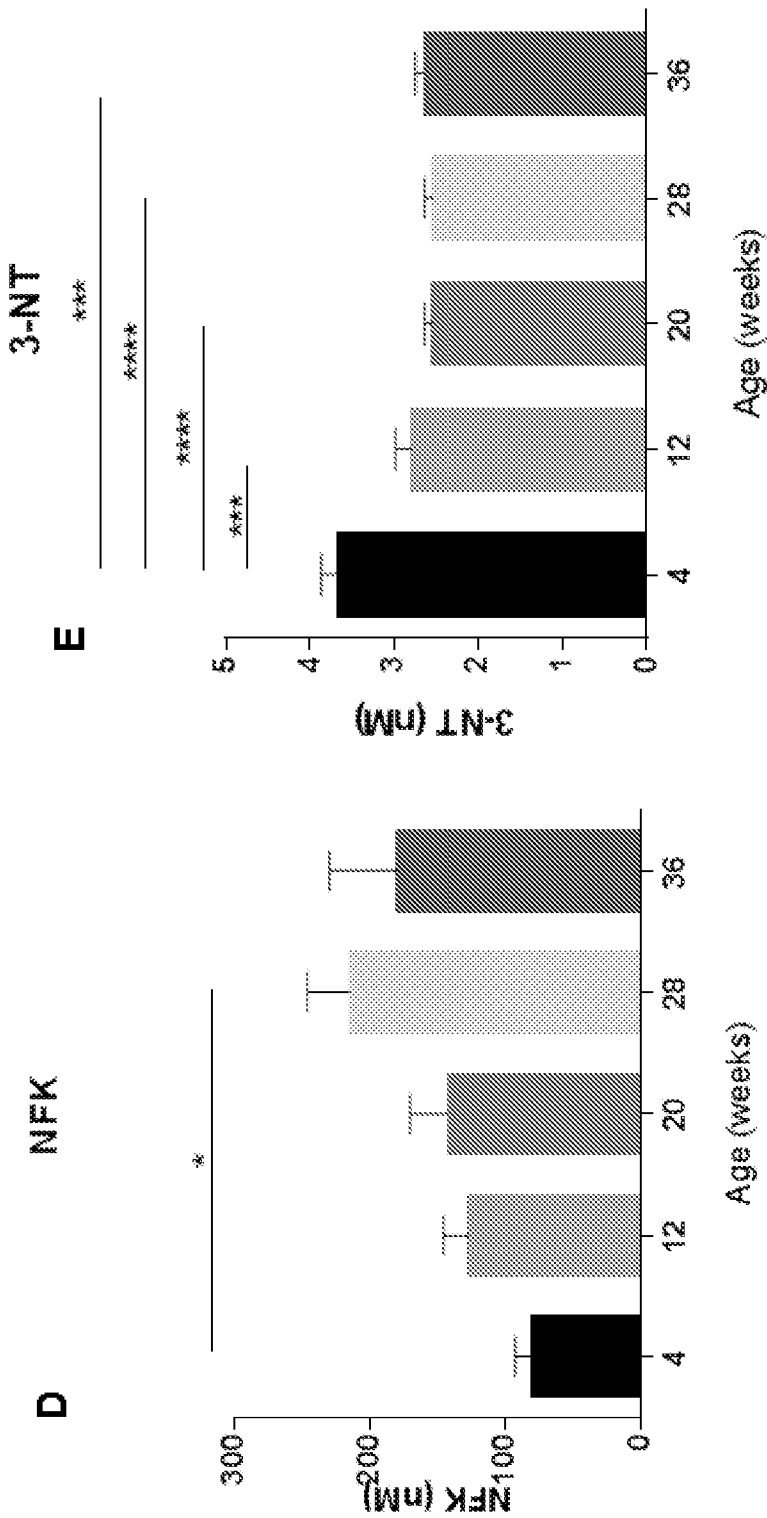


Figure 23. Serum concentrations of hydroxyproline and citrullinated protein in the guinea pig model of osteoarthritis.

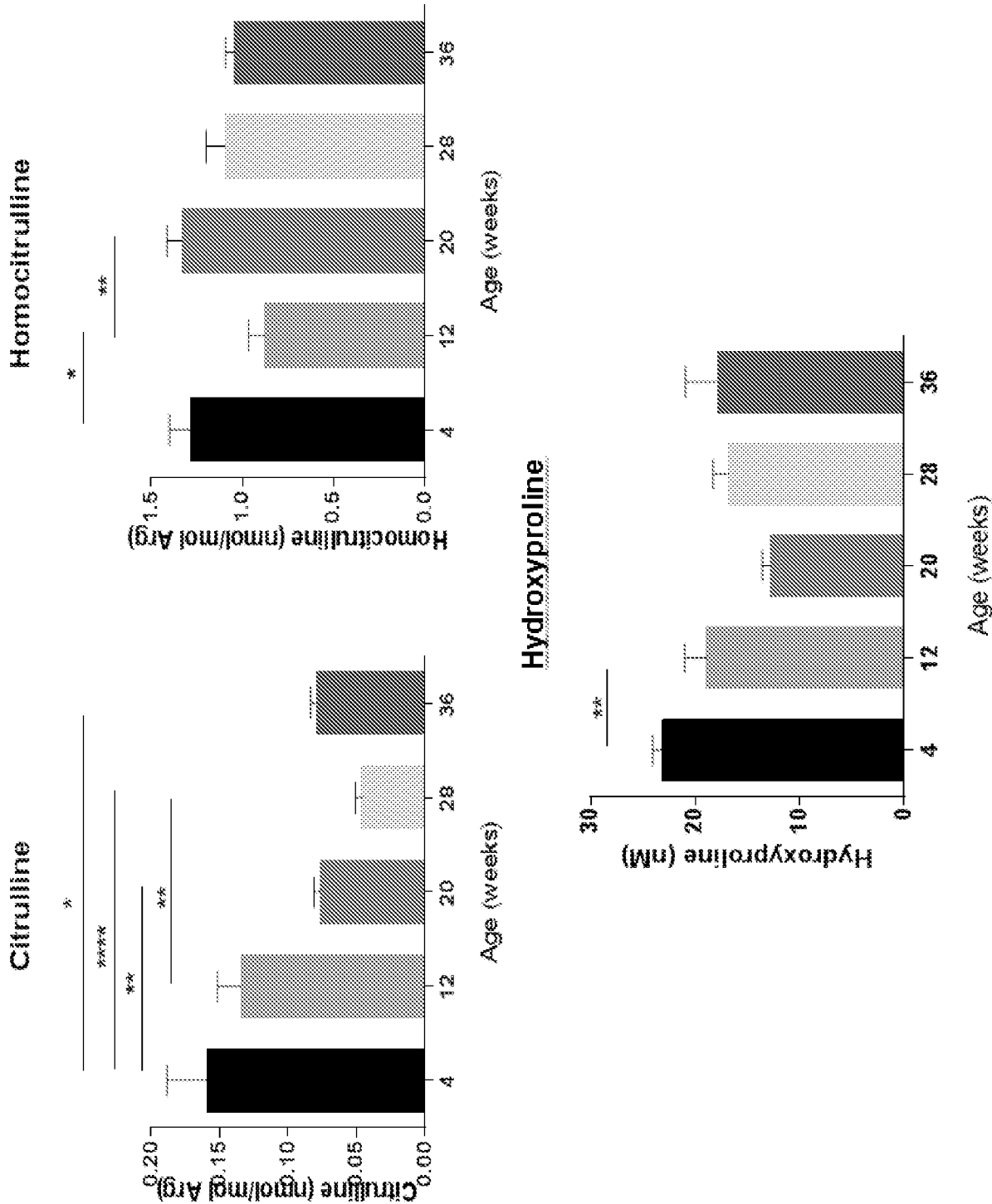




Figure 25. Plasma glycated, oxidized, nitrated and citrullinated protein in the guinea pig model of osteoarthritis.

| Analyte                    | Age (weeks)     |                 |                   |                    |                  |
|----------------------------|-----------------|-----------------|-------------------|--------------------|------------------|
|                            | 4               | 12              | 20                | 28                 | 36               |
| FL (mmol/mol lys)          | 2.82 ± 0.78     | 3.70 ± 1.51     | 5.16 ± 0.84***    | 4.52 ± 0.99***     | 4.34 ± 0.90***   |
| CML (mmol/mol lys)         | 0.564 ± 0.187   | 0.481 ± 0.211   | 0.206 ± 0.048***  | 0.183 ± 0.026***   | 0.218 ± 0.030*** |
| CEL (mmol/mol lys)         | 0.090 ± 0.068   | 0.090 ± 0.061   | 0.093 ± 0.038     | 0.066 ± 0.030      | 0.095 ± 0.034    |
| G-HI (mmol/mol arg)        | 0.321 ± 0.285   | 0.333 ± 0.279   | 0.017 ± 0.006**   | 0.018 ± 0.009**    | 0.018 ± 0.007**  |
| MG-HI (mmol/mol arg)       | 0.246 ± 0.199   | 0.270 ± 0.144   | 0.202 ± 0.042     | 0.203 ± 0.089      | 0.221 ± 0.029    |
| 3DG-H (mmol/mol arg)       | 0.263 ± 0.117   | 0.296 ± 0.198   | 0.099 ± 0.057***  | 0.061 ± 0.023***   | 0.054 ± 0.016*** |
| CMA (mmol/mol arg)         | 0.524 ± 0.255   | 0.416 ± 0.314   | 0.034 ± 0.019***  | 0.050 ± 0.015***   | 0.076 ± 0.016*** |
| Pentosidine (mmol/mol lys) | 0.0014 ± 0.0004 | 0.0015 ± 0.0008 | 0.0021 ± 0.0005** | 0.0020 ± 0.0002*** | 0.0018 ± 0.0005* |
| GSP (mmol/mol lys)         | 0.175 ± 0.255   | 0.163 ± 0.126   | 0.093 ± 0.026     | 0.078 ± 0.026      | 0.095 ± 0.036    |
| AASA (mmol/mol lys)        | 1.24 ± 0.35     | 0.79 ± 0.30**   | 0.50 ± 0.16***    | 0.48 ± 0.15***     | 0.55 ± 0.24***   |
| GSA (mmol/mol arg)         | 0.283 ± 0.223   | 0.236 ± 0.103   | 0.108 ± 0.030*    | 0.115 ± 0.018*     | 0.141 ± 0.029    |
| DT (mmol/mol tyr)          | 0.034 ± 0.044   | 0.077 ± 0.074   | 0.174 ± 0.069***  | 0.190 ± 0.060***   | 0.185 ± 0.111*** |
| NFK (mmol/mol trp)         | 0.251 ± 0.158   | 0.265 ± 0.137   | 0.163 ± 0.035     | 0.207 ± 0.031      | 0.168 ± 0.048    |
| 3-NT (mmol/mol tyr)        | 0.0104 ± 0.006  | 0.0097 ± 0.0023 | 0.0099 ± 0.0027   | 0.0095 ± 0.0039    | 0.0117 ± 0.0038  |
| CP (mmol/mol arg)          | 0.159 ± 0.102   | 0.134 ± 0.059   | 0.077 ± 0.015*    | 0.046 ± 0.016*     | 0.079 ± 0.014*   |



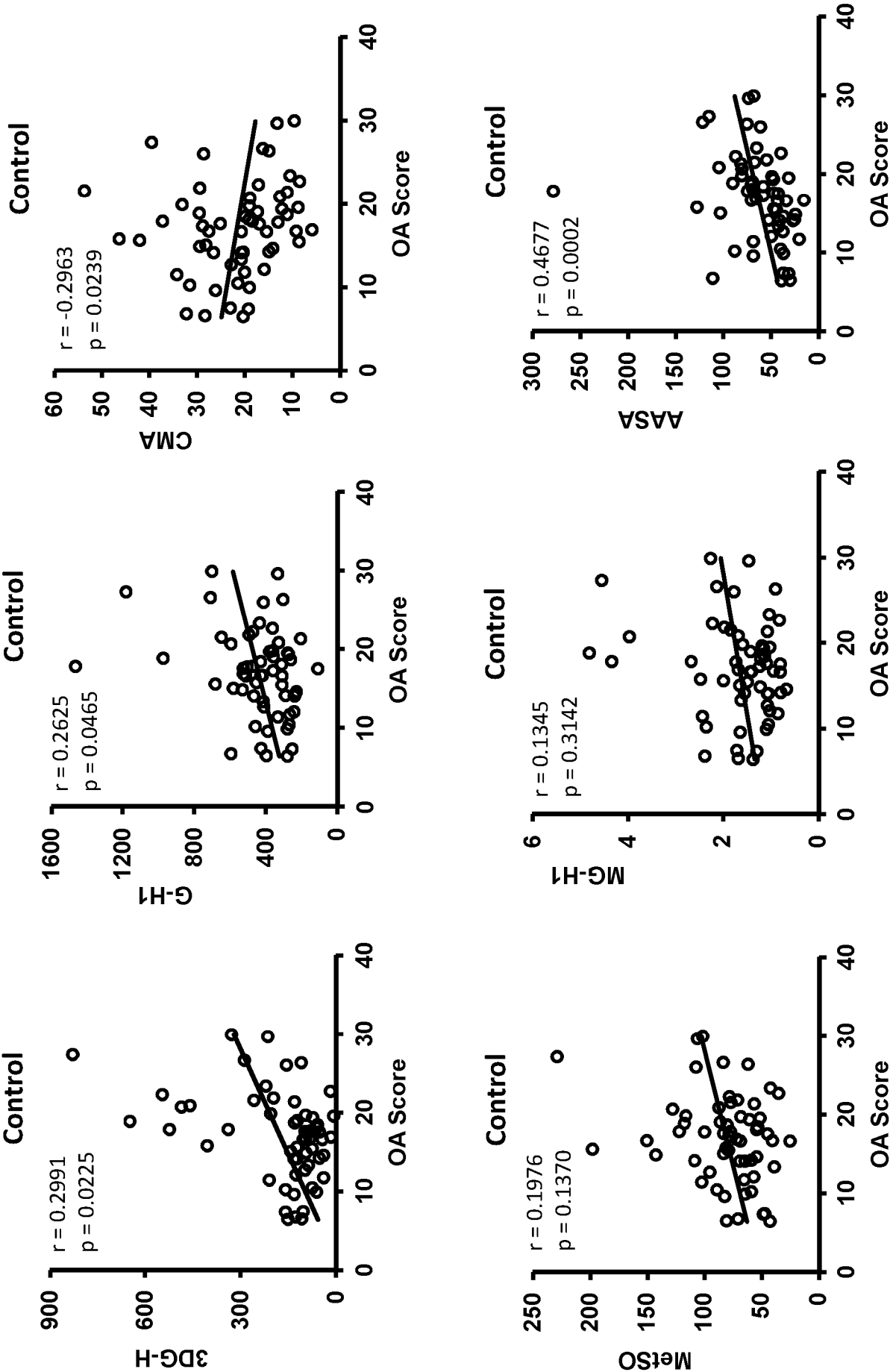
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**Figure 27. Correlation of glycation, oxidation and nitration free adducts and citrullinated protein and global histological score.**

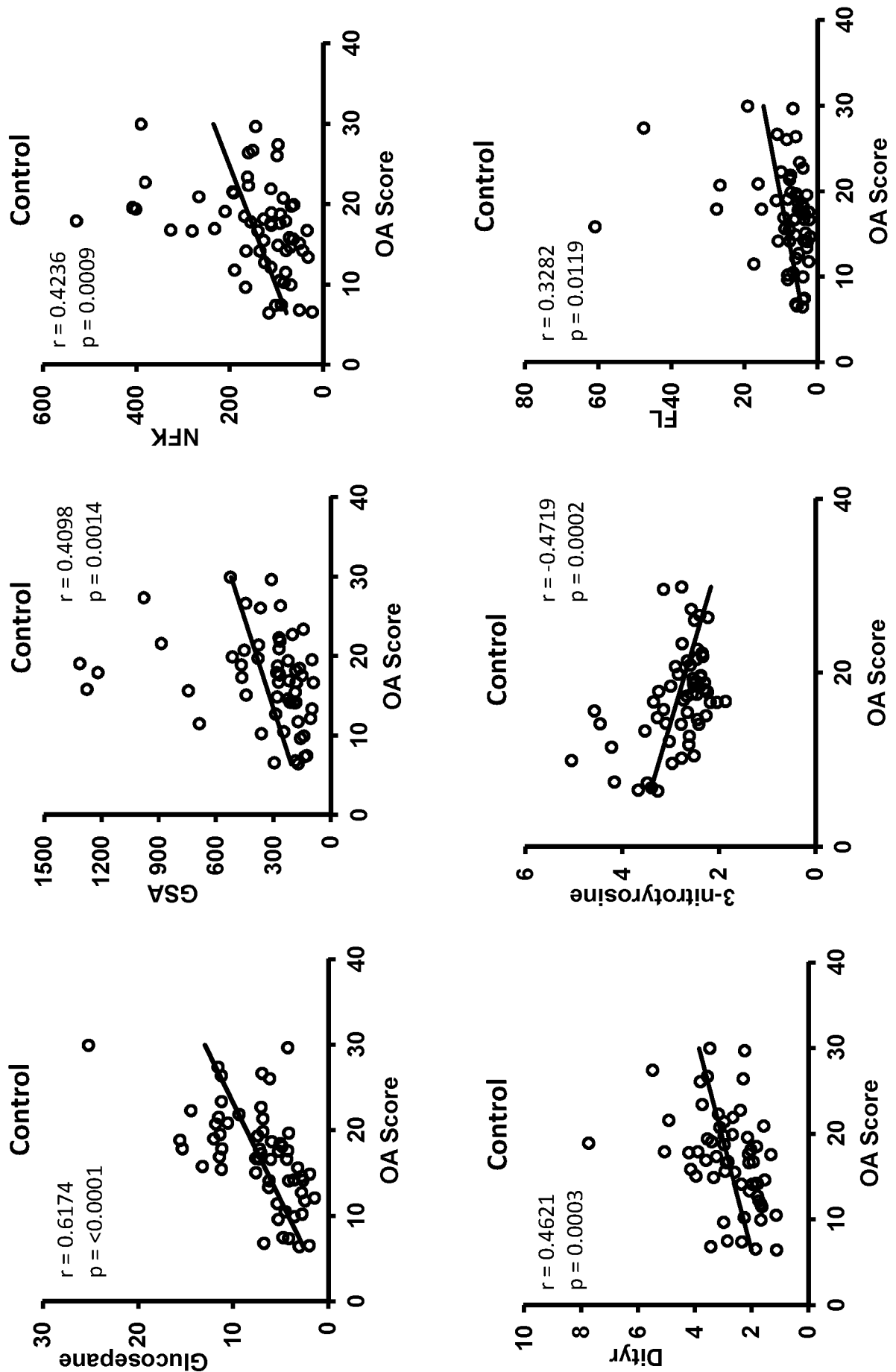
| <b>Global histological score</b> |                                  |                    |
|----------------------------------|----------------------------------|--------------------|
|                                  | <b>Correlation coefficient r</b> | <b>P-value</b>     |
| <b>Biomarkers</b>                |                                  |                    |
| <i>Glycation free adduct</i>     |                                  |                    |
| FL                               | <b>0.33</b>                      | <b>0.012</b>       |
| G-H1                             | <b>0.26</b>                      | <b>0.047</b>       |
| CMA                              | <b>-0.30</b>                     | <b>0.024</b>       |
| 3DG-H                            | <b>0.30</b>                      | <b>0.023</b>       |
| Glucosepane                      | <b>0.62</b>                      | <b>&lt;0.0001*</b> |
| <i>Oxidative free adduct</i>     |                                  |                    |
| AASA                             | <b>0.47</b>                      | <b>0.0002*</b>     |
| GSA                              | <b>0.41</b>                      | <b>0.0014*</b>     |
| Dityrosine                       | <b>0.46</b>                      | <b>0.0003*</b>     |
| NFK                              | <b>0.42</b>                      | <b>0.0009*</b>     |
| <i>Nitration free adduct</i>     |                                  |                    |
| 3-NT                             | <b>-0.47</b>                     | <b>0.0002*</b>     |
| <i>Other</i>                     |                                  |                    |
| <i>Citrullinated protein</i>     |                                  |                    |
| Citrulline                       | <b>-0.52</b>                     | <b>&lt;0.0001*</b> |

Correlation coefficients in bold are statistically significant. \*Correlation coefficient significant after Bonferroni correction of 16 applied.

Figure 28. Correlation of markers with OA score in the guinea pig model of OA



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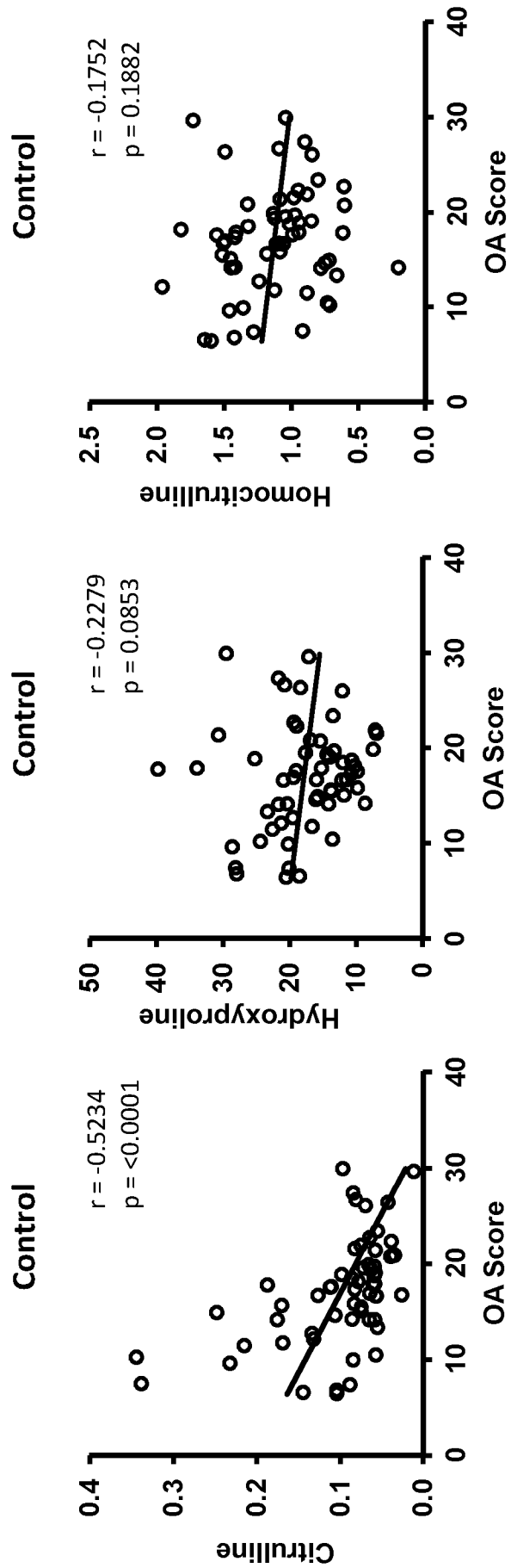


Figure 29.

Correlation analysis of glycation, oxidation and nitration free adducts with joint biomechanical properties measured by Mach-1 parameters.

| Biomarkers               | Thickness               |         |                         |         | Condyle                 |          | Plateau                 |         | Condyle                 |         | Plateau                 |         | Young Modulus           |          |
|--------------------------|-------------------------|---------|-------------------------|---------|-------------------------|----------|-------------------------|---------|-------------------------|---------|-------------------------|---------|-------------------------|----------|
|                          | Pearson's correlation r | p-value | Pearson's correlation r | p-value | Pearson's correlation r | p-value  | Pearson's correlation r | p-value | Pearson's correlation r | p-value | Pearson's correlation r | p-value | Pearson's correlation r | p-value  |
| <i>Glycation adducts</i> |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| FL                       |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         | 0.30                    | 0.031    |
| MG-H1                    |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| G-H1                     |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| CMA                      |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| 3DG-H                    |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| Glucosepane              |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| <i>Oxidation adduct</i>  |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| AASA                     |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         | 0.56                    | <0.0001* |
| GSA                      | -0.29                   | 0.033   | -0.33                   | 0.015   | 0.27                    | 0.043    |                         |         | 0.40                    | 0.004   |                         |         | 0.35                    | 0.013    |
| Dityrosine               |                         |         |                         |         | 0.34                    | 0.010    |                         |         | 0.36                    | 0.010   |                         |         | 0.36                    | 0.010    |
| NFK                      |                         |         |                         |         | 0.37                    | 0.006    |                         |         | 0.33                    | 0.018   |                         |         | 0.33                    | 0.018    |
| <i>Nitration adduct</i>  |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| 3-NT                     | 0.33                    | 0.013   | 0.29                    | 0.034   | -0.46                   | 0.0004*  |                         |         | -0.41                   | 0.003*  |                         |         |                         |          |
| <i>Other</i>             |                         |         |                         |         |                         |          |                         |         |                         |         |                         |         |                         |          |
| Hyp                      | 0.47                    | 0.0003  | 0.39                    | 0.003   | -0.38                   | 0.0037   |                         |         |                         |         |                         |         |                         |          |
| CP                       |                         |         |                         |         | -0.53                   | <0.0001* |                         |         | -0.33                   | 0.018   |                         |         |                         |          |

\*Correlation coefficient significant after Bonferroni correction of 16 applied.

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB2017/050546

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/564 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, COMPENDEX, EMBASE, INSPEC, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y         | U. AHMED ET AL: "Oxidative biomarkers increases in patients with increasing severity of osteoarthritis", FREE RADICAL BIOLOGY AND MEDICINE, vol. 53, 1 September 2012 (2012-09-01), pages S168-S169, XP055362058, US | 1,4-14,<br>17-30,<br>33,36    |
| A         | ISSN: 0891-5849, DOI: 10.1016/j.freeradbiomed.2012.08.354 the whole document                                                                                                                                         | 2,3,15,<br>16,31,<br>32,34,35 |
|           | -----<br>-/--                                                                                                                                                                                                        |                               |



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

6 April 2017

Date of mailing of the international search report

18/04/2017

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## INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2017/050546

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                     | Relevant to claim No.         |
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| A         | the whole document<br>in particular page 21, lines 11-13; page 23, lines 1-5                                                                                                                                                                                                                           | 2,3,15,<br>16,31,<br>32,34,35 |
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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2017/050546

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                                                                                                                                                                                   | Publication<br>date                                                                                                                                    |
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