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(54) **Title**: DIAGNOSTIC FOR DETERMINING SKELETAL HEALTH

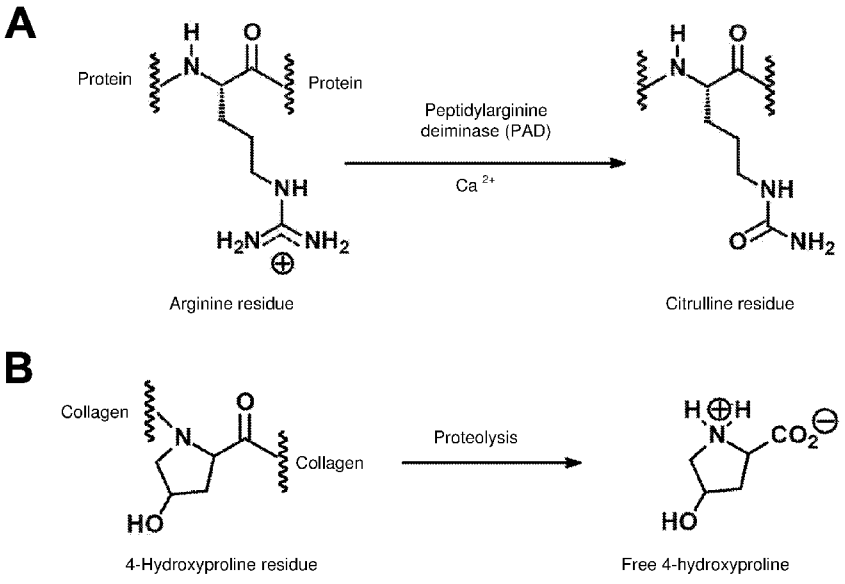

Figure 1

(57) **Abstract**: The invention concerns the use of Citrullinated proteins (CP) in a diagnostic method for determining skeletal health before or during a treatment regimen and particularly for determining whether an individual, human or animal, has a skeletal disorder such as, but not limited to osteoarthritis and/or is responding to treatment for said condition.

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DIAGNOSTIC FOR DETERMINING SKELETAL HEALTH

The invention relates to a diagnostic method for determining skeletal health before or during a treatment regimen and particularly, but not exclusively, for determining whether an individual, human or animal, has arthritis such as, but not limited to rheumatoid arthritis or osteoarthritis and/or is responding to treatment for said condition. The invention has application in the medical and veterinary fields.

INTRODUCTION

The formation of citrulline residues in proteins is a post-translational modification of arginine residues catalysed by members of the peptidylarginine deiminase (PAD) family of enzymes (1) – as illustrated in part A of Figure 1. Citrullinated proteins (CP) are immunogenic *in vivo* and may be involved in autophagic presentation of antigens in antigen presenting cells. Anti–cyclic citrullinated peptide (anti-CCP) antibodies are found in rheumatoid arthritis (RA). They are considered to be highly specific for this disease and can be detected several years before the onset of clinical symptoms and be involved in disease pathogenesis. There is the expectation, therefore, that CP is present in synovial fluid and plasma of RA patients and may, similarly, be a specific marker for the early development of RA.

Osteoarthritis (OA) is a degenerative joint disease involving mechanical abnormalities such as joint degradation, both of the articular cartilage and bone, particularly subchondral bone. Bone changes often precede loss of cartilage. It can be caused by a variety of factors including hereditary, developmental, metabolic, and mechanical. Symptoms may include joint pain causing loss of movement, tenderness, stiffness, locking, and sometimes an effusion. Patients may also experience muscle spasm and contractions in the tendons. Occasionally, the joints may also be filled with fluid. As a result of decreased movement, adjacent muscles may atrophy, and ligaments may become more lax.

OA commonly affects the hands, feet, spine, and the large weight bearing joints, such as the hips and knees, although in theory, any joint in the body can be affected. As OA progresses, the affected joints change their shape, are stiff and painful, and usually feel better with gentle use but worse with excessive or prolonged use.

In smaller joints, such as at the fingers, hard bony enlargements, called Heberden's nodes (on the distal interphalangeal joints) and/or Bouchard's nodes (on the proximal interphalangeal joints), may form, and though they are not necessarily painful, they do limit the movement of the fingers significantly. OA at the toes leads to the formation of bunions, rendering them red or swollen. Some people notice these physical changes before they experience any pain.

OA is the most common form of arthritis, and the leading cause of chronic disability in the United States. It affects nearly 27 million people in the United States and about 8 million people in the United Kingdom.

Citrullinated protein as a marker of inflammation, when combined with increased free hydroxyproline (hyp), may find application in the diagnosis of early stage decline of skeletal health in osteoporosis (2) and Crohn's disease (3), risk of fractures in type 2 diabetes, metabolic syndrome and related disorders (4), loosening of hip arthroplasty (5) and other inflammatory disorders with complications of declining skeletal health. Citrullinated protein measurement may be a diagnostic factor, alone or combined with other diagnostic factors, for improved diagnosis of type 1 diabetes mellitus (6), vascular disease (7) and autoimmune disease (8).

The presence of anti-CCP antibodies in OA is sporadic and evidence of increased PAD activity and CP uncertain. Anti-CCP antibodies have been detected in synovial fluid and serum of OA and psoriatic arthritis (PsA) but at lower levels than those detected in RA (9;10). These studies indicate that synovial fluid and serum levels of anti-CCP antibodies (and IgA rheumatoid factor IgA-RF) are significantly increased in patients with RA in comparison with patients with PsA or OA.

The direct detection and quantitation of CP in plasma, synovial fluid and elsewhere poses a significant analytical challenge. The citrulline residue epitope in proteins is present in trace amounts (0.1 – 0.01% arginine residues), there is a 10 – 50 fold higher concentration of free, dialyzable citrulline - a metabolite of the urea cycle and co-product of nitric oxide synthases (11), and there are multiple trace arginine residues-derived adducts that may degrade to citrulline during pre-analytic processing (12).

Hitherto, immunochemical techniques have been employed to indirectly detect CP and they have involved putative citrulline residue detection by prior derivatisation with diacetylmonoxime/antipyrine under strong acid conditions (13) or the use of commercial

anti-citrulline antibodies (14). These techniques have been used mainly for qualitative analysis without investigation of analytical recovery and interferences. A robust technique for quantitation of CP is currently lacking.

In this application we describe a novel method for the direct quantitation of CP in proteins of body fluids such as, but not limited to, plasma or synovial fluid, and the application of this procedure for determining skeletal health such as, but without limitation, the presence of osteoarthritis or other arthritic disease, including a determination of disease progression and/or the success of on-going therapy or treatment regimen.

We also describe a novel method for the quantification of dialyzable or free hydroxyproline (hyp) as a marker of bone remodelling (turnover and/or resorption) (15).

STATEMENTS OF INVENTION

According to a first aspect of the invention there is provided a diagnostic method for quantifying citrullinated proteins (CP) as a marker for determining skeletal health comprising:

(a) undertaking an assay for direct detection of CP in a body fluid sample obtained from a test individual;

(b) quantifying the amount of CP in said sample; and

(c) where said amount of CP is higher than normal levels, concluding said individual has a skeletal disorder.

In one embodiment, part c) involves determining whether said amount of CP is statistically significantly higher than normal levels. In accordance with this embodiment, if the amount of CP is statistically significantly higher than normal levels, it can be concluded that the individual has a skeletal disorder (e.g. OA). By way of example, part c) may involve determining whether the amount of CP in the test sample is more than 1.5 fold above normal levels, such as more than two fold above normal levels, or more than three- or four- or five- fold above normal levels and, if they are, concluding said individual has said skeletal disorder (e.g. OA).

Reference herein to the term skeletal disorder includes reference to a 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 OA (eOA) or advanced OA (aOA). Inflammatory arthritic conditions include without limitation rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis. Joint or bone disorders also include arthropathy, non-specific synovitis (non-RA), loosening of hip arthroplasty, osteoporosis, and bone resorption disease. Examples of conditions that give rise to a lack of skeletal health or integrity include without limitation Crohn's disease or other inflammatory bowel disorders, inflammatory vascular disease, autoimmune disease disorders with complications of declining skeletal health, type 1 or 2 diabetes, metabolic syndrome and related disorders. In one embodiment, the method is for diagnosis of a non-inflammatory arthritic condition. In one embodiment, the method is for diagnosis of osteoarthritis, such as in particular eOA or aOA.

The invention herein described is for use on a body fluid sample obtained from a mammal, more particularly a human, but the invention also has veterinary application and so can be used on a body fluid sample from other mammals including, but not limited to, equine, porcine, canine, feline, ungulate, primate animals or, indeed, in relation to any load-bearing limbed animal that is known to be susceptible to, or suffer from, a skeletal health disorder.

In a preferred embodiment of the invention said sample is any body fluid, such as but not limited to, eye fluid, urine, whole blood, blood serum, blood plasma, lymphatic fluid, saliva, synovial fluid, seminal fluid, cerebrospinal fluid, sebaceous secretions, or sputum. Most ideally said sample is taken from eye fluid, blood plasma, synovial fluid, or urine. In one embodiment, said sample is a blood plasma sample. In one embodiment, said sample is a synovial fluid sample. In one embodiment, said sample is a urine sample. As will be appreciated by those skilled in the art, said sample may be pre-treated for analysis, typically, by using conventional techniques as described herein and known by those skilled in the art.

Most ideally, said body fluid sample is from an individual who is not presenting with symptoms characteristic of a skeletal disease such as arthritis, in particular RA or OA. However, in an alternative embodiment, said body fluid sample is from an individual who is known to have a skeletal disorder (e.g. OA), or is presenting with symptoms

characteristic of a skeletal disorder (e.g. OA). In one embodiment, said individual has received or is receiving treatment for said skeletal disorder (e.g. RA).

Reference herein to normal levels includes reference to the quantity of the marker (i.e. CP, and optionally or hyp, and/or arg) that is observed in a healthy individual that exhibits no signs of a skeletal disorder and so it is reasonable for those skilled in the art to conclude the individual is free from such a disorder. A sample obtained from such a healthy individual can be used as a normal control sample. Alternatively, a reference sample may be available as a normal control sample. Thus, step c) of the diagnostic method comprises comparing the amount of CP determined in the sample from the test individual to the amount of CP in a normal, control sample. The diagnostic method may optionally comprise the step of quantifying the amount of CP in the normal, control sample (typically, the same direct detection assay will be used to detect CP in the test sample and in the normal, control sample). The method of the invention involves the direct detection of CP in the sample. Any direct detection assay can be used in the invention. Particularly suitable direct detection assays include immunoassays (such as ELISAs) for CP. The sensitivity and specificity of an immunoassay for CP can be demonstrated by corroboration with a best available reference technique, such as stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS).

In this regard, a particularly suitable embodiment of stable isotopic dilution analysis LC-MS/MS direct detection assay reference method involves enzymatic hydrolysis of proteins to component amino acids and direct quantitation by liquid chromatography-mass spectrometry.

In one embodiment, the assay for direct detection of CP comprises:

(i) exposing protein extracted from a body fluid sample from a test individual to enzymatic hydrolysis; and

(ii)undertaking LC-MS/MS analysis on the hydrolysed protein obtained in part (i) to detect CP.

The method may additionally comprise the initial step of extracting protein from the body fluid sample (or the protein may have been pre-extracted).

In the above method, protein hydrolysis by enzymatic digestion has been developed to avoid the severe conditions of acid hydrolysis which may compromise the analyte, or

CP, content of the sample during pre-analytic processing. Thus, the method for quantitation of trace amino acid residues involves hydrolysis of proteins to component amino acids and quantitation by stable isotopic dilution analysis LC-MS/MS.

Ideally, said enzymatic digestion involves 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 is present in the extracellular matrix.

Analysis of CP by LC-MS/MS combines the high specificity of detection response – based on combination of molecular mass, molecular fragment mass and chromatographic retention time – with the high sensitivity and dynamic range of electrospray ionisation mass spectrometry and quantitation by stable isotopic dilution analysis.

In a preferred method of the invention we employ washing of proteins by ultrafiltration to eliminate the interference of free citrulline (where absence of interference is verifiable by assay of citrulline prior to protein hydrolysis). Preferably, we also use automated exhaustive enzymatic hydrolysis which avoids harsh, pre-analytic processing. Ideally we also use normalisation of CP to total arginine residue content.

The method of the invention may further comprise detection (and optionally quantification) of one or more additional skeletal health markers in the body fluid sample. By way of example, the sample may additionally be assayed for the presence of a marker of bone turnover/ resorption, such as free (dialyzable) hydroxyproline (hyp).

When hyp is measured, a sample, such as a plasma protein sample or urine sample, is assayed for CP and another, or the same, sample is also assayed for hyp and, where the CP is increased as described above, one concludes said individual has a skeletal disorder. By way of example, in one embodiment of the method of the invention, the sample is a plasma sample and it is further assayed for hyp; and where both CP and hyp are more than 2 fold above normal levels one concludes that said individual has a skeletal disorder. Specifically, where both plasma CP and hyp are increased one concludes the individual from whom the sample has been taken has early stage OA; where only the plasma CP is increased one concludes the individual from whom the sample has been taken has early stage RA.

Additionally, or alternatively, a urine sample may be used in the above method to assay for hyp. An elevated level of hyp in the test sample (as compared to normal levels) may indicate that the test individual has a skeletal disorder such as OA. In one embodiment, a statistically significantly elevated level of hyp in the test sample (e.g. a urine sample) as compared to normal levels (in a normal control sample) may indicate that the test individual has a skeletal disorder such as OA. In one embodiment, where hyp in a urine sample is more than 50% above normal levels of hyp in the urine of a healthy individual (normal, control sample), one concludes that the test individual has a skeletal disorder. In an alternative, or additional embodiment, said urine sample is further assayed for creatinine, and the amount of hyp in the test sample (and in said healthy control sample) is normalised, having regard to the amount of creatinine present in said sample.

Additionally, and advantageously, the assay for free (dialyzable) 4-hydroxyproline (hyp) is performed using said LC-MS/MS analysis. Preferably, a filtered amount of said sample, prepared by ultrafiltration, is used for this purpose.

The method of the invention may further comprise assaying the sample for anti-CCP and/or RF; and where anti-CCP and/or RF as well as CP are higher than normal levels, thereby concluding said individual has a skeletal disorder (e.g. OA). In one embodiment, where the amount of anti-CCP and/or RF as well as CP in the test sample is statistically significantly higher than normal levels (in a normal, control sample), one can conclude that the test individual has a skeletal disorder (e.g. OA). For example, the amount of anti-CCP and/ or RF as well as CP in the test sample may be 1.5-fold, 2-fold, 3-, 4- or 5-fold higher than normal levels. This can improve sensitivity and specificity of the conclusion.

The method of our invention involves the direct detection of citrulline (optionally and arg, optionally and hyp) and so avoids analyte degradation or formation during derivatisation used in other methods. Although there is some minor thermal degradation of arginine to citrulline in the electrospray source of the mass spectrometer, interference from this is preferably avoided by chromatographic separation of arginine and citrulline. Thus, in a further preferred method of the invention, said sample is assayed for arginine and the quantification of CP in part b) of the method involves normalisation of the data having regard to the amount of arginine present. In a specific embodiment, this typically involves concurrent quantitation of citrulline and arginine contents by stable isotopic

dilution analysis and then dividing the amount of citrulline detected (pmol) by the amount of arginine (nmol) detected.

For example, a measurement in pmol per mg protein involves correction for the specific content of arginine residues in the test fluid protein – although these were similar herein (*ca.* 320 plasma and 312 synovial nmol arg/mg protein, respectively). For a comparison of CP concentration in plasma and synovial fluid (μM; μmol citrulline residues per litre), correction for the protein content of plasma and synovial fluid protein is also required. The protein content of synovial fluid is *ca.* 30 – 40% lower than that of plasma in OA and RA (16). Hence, herein the median concentration of CP in plasma and synovial fluid of patients with eRA was similar at *ca.* 4.8 μM whereas in patients with early OA CP concentrations were 6.3 μM in plasma and 2.6 μM in synovial fluid. This indicates that there is no gradient of CP from synovial fluid to plasma in early RA and there is a negative gradient from plasma to synovial fluid in early OA.

We also developed a stable isotopic dilution analysis LC-MS/MS method for the quantitation of free hyp in plasma and synovial fluid. Other methods for hyp measurement in human serum of healthy people reported hyp estimates of 11.1 ± 3.5 μM (17) and 8.8 ± 6.2 (18), and estimate of hyp in synovial fluid of patients with RA of 71 ± 11 μM (19). The lower median estimates obtained herein, plasma hyp of healthy people 1.3 μM and synovial fluid of patients with RA hyp *ca.* 2.4 μM, are likely due to the higher specificity of detection by LC-MS/MS compared to previously employed techniques. Free hyp is a marker of bone turnover and resorption with contributions from skin collagen turnover and dietary collagen and gelatine – although recent quantitative clinical studies suggest 62% of variation in plasma hyp relates to bone turnover and resorption.

In many cases, it may also be useful to obtain information regarding the progression of a skeletal health disorder in an individual. Accordingly, by monitoring progression of the disease, this will permit clinical decisions to be tailored specifically to the individual's needs.

Therefore, in yet a further preferred embodiment of the invention, the method further comprises repeating steps a)-b) above to quantify the amount of CP after a set time-interval, and comparing the quantity of CP after a set time interval to the quantity of CP determined at an earlier time point, and where:

i. a decrease in CP quantity is observed concluding that the individual has improved skeletal health;
ii. an increase in CP quantity is observed concluding that the individual has worsening skeletal health; or
iii. zero, or marginal difference is observed in CP quantity concluding that the individual has stable skeletal health.

This aspect of the diagnostic method of the invention is useful for determining or monitoring disease progression; a treatment regimen; general skeletal health; or the effectiveness of exercise, physiotherapy, foods or supplements on skeletal health.

By way of example, in the above method of the invention the CP marker can be used to assess how a treatment regimen is working, for example, by assaying plasma CP and, optionally plasma hyp, during the course of a given therapy to determine if elevated CP, and optionally elevated hyp, has declined in response to said treatment.

Additionally, in healthy people plasma CP may be a marker of general skeletal health. We were able to detect CP and hyp in all samples tested. In healthy people plasma CP correlated positively with plasma hyp. Thus, as discussed herein, elevated CP is a marker of inflammation linked bone desorption and so CP can be used to assess skeletal health in normal individuals or individuals not diagnosed as having a skeletal disorder, and may advantageously be used as a marker for assessing the effectiveness of exercise, physiotherapy, foods or supplements to promote skeletal health.

In embodiments of the above method in which an amount of CP is detected that is more than twice the normal median plasma value, we have achieved 89% sensitivity and 81% specificity for diagnosing early RA and early OA. This sensitivity can be increased to 96% by increasing the level of detection of CP, in part c) above, to three or four or five times that found in a normal sample i.e. more than three or four times the normal median plasma value. This means our test out-performs or is as good as the anti-CCP antibody test for eRA and, moreover, is also applicable to eOA – see below.

The assay has good day-to-day reproducibility and sample stability during batch analysis.

Our findings indicate that increased plasma CP is a characteristic of both early-stage RA and OA. Accordingly, elevated plasma CP is a diagnostic indicator, or biomarker, for both diseases.

Surprisingly CP was detected in plasma and synovial fluid of patients with OA. Plasma CP was increased 5-fold in patients with early-stage OA, compared to healthy individuals, moreover, plasma CP was also higher than CP of synovial fluid in early-stage OA. However, as OA progresses this relationship changes and plasma CP is lower than synovial CP in severe OA. Notably, plasma CP correlated positively with synovial fluid hyp in early-stage and advanced OA. For eOA the anti-CCP antibody test had a poor response with sensitivity of 6% whereas our test had a sensitivity of 100% and a specificity of 80% for plasma CP more than twice the normal median value.

Anti-CCP antibodies are often present in the serum of patients who have early stage RA. The anti-CCP antibodies assay measures the affinity of host antibodies (which may be present in a plasma or serum sample from a test individual) to a synthetic citrullinated peptide. It is not known if immunoaffinity for synthetic citrullinated peptide in this assay is linked to the presence of CP in physiological samples. Hitherto there has not been a robust method for measurement of CP. We found a 4-fold increase in plasma CP in early RA i.e. less than that for early OA. Moreover, this value was linked to anti-CCP antibodies. Further, synovial fluid CP was increased in patients positive for rheumatoid factor (RF). Thus, plasma CP is linked to rheumatoid factor autoimmunity and anti-CCP antibody positivity in RA.

Our findings also indicate that the gradient of CP from plasma to synovial fluid changes with progression of OA, being negative in early OA and positive in advanced OA whilst no noticeable gradient in early RA but positive in advanced RA. This has implications for diagnosis and etiological relationships in RA and OA, specifically it implies CP formation occurs outside of the joint in early OA and it may suggest vascular or other non-joint factors are causative factors for OA. This is counterintuitive to current understanding.

Accordingly, in a preferred method of the invention parts a)-b) above involve determining both plasma and synovial protein CP values to distinguish early RA and OA, wherein:

i. if CP content is greater in plasma protein than synovial protein, i.e. the gradient is negative, concluding under part c) that the individual has early OA; or

ii. if CP content in plasma protein and synovial protein is the same, or similar, concluding under part c) that the individual has early RA.

Furthermore, in this regard it is possible to determine the gradient of CP from plasma to synovial fluid in addition to the hyp values.

Consequently, unexpectedly in this instance, it has been found that in early stage detection, OA and RA are distinguishable by relative levels of plasma CP and hyp, specifically, with an increase in plasma CP and hyp observed in OA, compared to an increase in plasma CP without increase in plasma hyp in RA. Notably, plasma CP correlated positively with synovial fluid hyp in early-stage and advanced OA. Thus plasma CP and/or hyp are markers for OA and, when plasma CP and plasma hyp markers are both present, they represent a combined, strong diagnostic indicator of the disease.

Therefore, in a further preferred embodiment of the invention the diagnostic method may further comprise determining both plasma and synovial protein CP and hyp values to distinguish between OA and RA, wherein:

i) if elevated quantities of CP and hyp are observed (in either or both of said samples), concluding that the individual has OA; or

ii) if elevated quantities of CP is observed and not hyp (in either of both of said samples), concluding that the individual has RA.

Additionally, or alternatively, a urine sample may be used in the above method to assay for hyp and in this instance the amount of hyp is normalised, having regard to the amount of creatinine present in said sample.

In this instance, it is therefore also possible to distinguish between advanced OA and advanced RA. Accordingly, in a yet a further preferred method of the invention parts a)-b) above involve determining both plasma and synovial protein CP and hyp values to distinguish between advanced OA and advanced RA, wherein:

i. if CP content is greater in plasma protein than synovial protein, i.e. the gradient is negative, and hyp level is elevated, concluding under part c) that the individual has advanced OA; or

ii. if CP content is greater in plasma protein than synovial protein, i.e. the gradient is negative, and hyp level is the same, or similar, concluding under part c) that the individual has advanced RA.

Additionally, or alternatively, a urine sample may be used in the above method to assay for hyp and in this instance the amount of hyp is normalised, having regard to the amount of creatinine present in said sample.

According to a yet further aspect of the invention there is provided the use of CP as a marker for skeletal health.

In accordance with this aspect of the invention, the CP may advantageously be used alone as a marker for skeletal health, or may be used in combination with one or more additional markers. In one embodiment, the CP is used in combination with a marker of bone turnover/ resorption, such as hyp. In a further embodiment, the CP is used in combination with hyp and additionally one or both of creatinine and arginine.

In one embodiment, the CP (alone or in combination with hyp, and further optionally in combination with creatinine and/or arginine) is used as a marker for a joint disorder or a bone disorder as defined herein, such as a non-inflammatory arthritic condition - e.g. as a marker for OA, such as early OA or advanced OA.

According to a yet further aspect of the invention there is provided a diagnostic method of determining skeletal health comprising:

(a) quantifying citrullinated proteins (CP) as a first marker by undertaking an assay for direct detection of CP in a body fluid sample obtained from a test individual and by quantifying the amount of CP in said sample;

(b) quantifying at least one further marker by undertaking an assay for detection of that further marker; and

(c) determining the skeletal health of the test individual in dependence on the markers as inputs.

By determining the skeletal health of the test individual in dependence on the markers (both the first marker and the at least one further marker) as inputs, the reliability of the determination can be enhanced, and in particular both the specificity and the sensitivity of the determination can be optimised. For example, if the CP quantity is elevated, but not high enough to determine the test individual's skeletal health with adequate confidence, then the combination with the further (secondary) marker(s) can provide better confidence.

In an example, the test individual's plasma CP quantity may be less than twice the normal median plasma value, but above the normal median plasma value. In this case if further markers are also slightly higher than their normal values, then it can be determined that the test individual has a skeletal disorder, even though the plasma CP quantity on its own might not be a sufficiently strong indication.

In another example the test individual's plasma CP quantity may be twice the normal median plasma value, and, as mentioned above, it can be determined that the test individual has a skeletal disorder with a sensitivity at 89%. If the further (secondary) markers are also considered (e.g. if they are also slightly higher than their normal values), then it can be determined that the test individual has a skeletal disorder with a sensitivity greater than 89%.

Preferably the assay for detection of the further marker is undertaken on said body fluid sample and/or on a further body fluid sample obtained from the test individual. This can enable the use of for example plasma CP and synovial CP as markers, which can enable particularly reliable determination of (or classification between) early RA and early OA, as set out above.

Preferably said at least one further marker includes at least one, and preferably all, of: hydroxyproline (hyp), anti–cyclic citrullinated peptide antibodies (anti-CCP antibody), rheumatoid factor (RF), CP, creatinine, arginine, age and gender of the test individual. These further markers in combination can be particularly informative for the determination of skeletal health (or classification). 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 further marker may be CP if taken from a further body fluid sample, or if quantified by undertaking an assay for indirect detection, for example. Preferably the at least one further marker includes: hydroxyproline (hyp), anti–cyclic citrullinated peptide antibodies (anti-CCP antibody), age and gender of the test individual. This combination is particularly informative.

Preferably determining skeletal health comprises classifying the skeletal health. Preferably classifying is into a class of at least one of: healthy; osteoarthritic; early-stage osteoarthritic; advanced-stage osteoarthritic; rheumatoid arthritic; early-stage rheumatoid arthritic; advanced-stage rheumatoid arthritic; non-rheumatoid arthritic; early-stage non-rheumatoid arthritic; advanced-stage non-rheumatoid arthritic; having a joint disorder; having a bone disorder; having arthropathy; having non-specific synovitis;

loosening of hip arthroplasty; having a bone resorption disease; having osteoporosis; having Crohn's disease; having inflammatory bowel disorder; having inflammatory vascular disease; having autoimmune disease disorder with complications of declining skeletal health; having type 1 or 2 diabetes; and having metabolic syndrome.

Preferably classification is by a classification algorithm.

Preferably the classification algorithm is trained on a set of markers from a population of individuals with known skeletal health. This can enable reliable classification of the test individual.

Preferably the classification algorithm is an ensemble algorithm comprising different types of classification algorithms.

Preferably the classification algorithm comprises a decision tree based algorithm. Other types of algorithms, such as regression algorithms and neural networks, may also be used.

Preferably the classification algorithm comprises a random forest algorithm. This can provide particularly reliable classification.

Preferably the classification algorithm is an ensemble algorithm comprising a random forest algorithm and a generalized linear model regression algorithm, preferably with elastic net.

According to a further aspect of the invention there is provided apparatus for determining skeletal health comprising:

means for receiving a quantification of citrullinated proteins (CP) as a first marker, obtained by direct detection of CP in a body fluid sample obtained from a test individual;

means for receiving a quantification of at least one further marker, obtained by an assay for detection of that further marker; and

means for determining the skeletal health of the test individual in dependence on the markers as inputs.

According to a further aspect of the invention there is provided a diagnostic method for quantifying citrullinated proteins (CP) as a marker for determining skeletal health comprising:

a) exposing protein extracted from a body fluid sample from a test individual to enzymatic hydrolysis;

b) undertaking LC-MS/MS analysis on the hydrolysed protein of part a) to detect and quantify CP; and

c) where said amount of CP is increased as compared to normal levels, concluding said individual has a skeletal disorder.

In one embodiment, part c) of said method involves determining whether the amount of CP is statistically significantly increased as compared to normal levels (i.e. the level in a normal, control sample), such as whether the amount of CP is increased by 1.5-fold, 2-fold, or 3-, 4- or 5-fold as compared to normal levels.

In one embodiment, said method comprises the initial step of extracting protein from the body fluid sample (alternatively, the protein may be pre-extracted). Enzymatic hydrolysis and LC-MS/MS analysis may be performed as outlined above.

Throughout the present description, reference to terms such as “higher than”, “increased”, “elevated” (and derivations thereof), and terms such as “lower than”, “decreased”, “reduced” (and derivations thereof) include reference to statistically significant differences between values, such as 1.5-fold or 2-fold differences, or 3-, 4- or 5-fold differences.

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

The invention also provides a signal embodying a computer program for carrying out any of the methods described herein and/or for embodying any of the apparatus features 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 described herein and/or for embodying any of the apparatus features described herein.

Any apparatus feature as described herein may also be provided as a method feature, and vice versa. As used herein, means plus function features may be expressed

alternatively in terms of their corresponding structure, such as a suitably programmed processor and associated memory.

It should also be appreciated that particular combinations of the various features described and defined in any aspects of the invention can be implemented and/or supplied and/or used independently.

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.

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.

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 invention will now be described, by way of example only, with reference to the following figures and tables wherein:-

Figure 1 **A** illustrates formation of citrullinated protein. **B** illustrates formation of free 4-hydroxyproline;

Figure 2 shows detection of citrulline by stable isotopic dilution analysis LC-MS/MS. MRM chromatograms for: **A** and **B** Detection of citrulline and arg residues, respectively, in early OA plasma protein; **C** and **D** [5-^{13}C-4,4,5,5-$^{2}H_4$]citrulline (5 pmol) and [guanidino-$^{15}N_2$]arg (10 nmol), respectively; **E** and **F** Detection of hydroxyproline and 4,5-[$^{13}C_2$]hydroxyproline (25 pmol), respectively, in early OA plasma;

Figure 3 shows citrullinated protein in plasma and synovial fluid of healthy human subjects and patients with arthritic disease. **A** Plasma and **B** Synovial fluid. Significance: *, ** and ***, P<0.05, P<0.01 and P<0.001 with respect to control (healthy subjects); † and †††, P<0.05 and P<0.001 for aRA with respect to eRA and for aOA with respect to eOA . Data are median (lower – upper quartile);

Figure 4 shows free hydroxyproline in plasma and synovial fluid of healthy human subjects and patients with arthritic disease. **A** Plasma and **B** Synovial fluid. Significance: *, ** and ***, P<0.05, P<0.01 and P<0.001 with respect to control (healthy people). Data are median (lower – upper quartile);

Figure 5 shows algorithm classifiers for **A** healthy controls, **B** subjects with early-stage osteoarthritis, **C** subjects with early-stage rheumatoid arthritis and **D** subjects with non-rheumatoid arthritis; and

Figure 6 shows receiver operating characteristic plots for **A** healthy controls, **B** subjects with early-stage osteoarthritis, **C** subjects with early-stage rheumatoid arthritis and **D** subjects with non-rheumatoid arthritis.

Table 1. shows clinical characteristics of healthy people and patients with arthritis. Significance: *, ** and ***, *P*>0.05, *P*<0.01 and *P*<0.001 with respect to control (healthy people); ^{OO} and ^{OOO}, *P*<0.01 and *P*<0.001 with respect to non-RA; and †††, P<0.001 for aRA with respect to eRA and for aOA with respect to eOA. For Medications, numbers in

parentheses are number of patients receiving treatment with the drugs indicated. Data are mean ± SD (parametric) and median [lower – upper quartile] (non-parametric).

Table 2. Shows analytical variables and assay characteristics for quantitation of citrulline, arginine and 4-hydroxyproline by stable isotopic dilution analysis tandem mass spectrometry.

PATIENTS & METHODS

Patients and samples – Patients with longstanding history or established severe, advanced RA (aRA) and advanced OA (aOA) were recruited at the Department of Rheumatology, Ipswich Hospital, U.K. and Peninsula Medical School, Exeter, UK. OA patients were undergoing therapeutic knee aspiration and corticosteroid instillation or total knee replacement (TKR) due to longstanding symptoms of osteoarthritis with corresponding radiographic changes. Patients with non-rheumatoid arthritis, non-specific synovitis (non-RA), and early rheumatoid arthritis (eRA) were recruited from those attending the Rapid Access Rheumatology Clinic, City Hospital, Birmingham, U.K. Synovial fluid and peripheral venous blood samples were collected at initial presentation. In the non-RA group, all symptoms were self-limiting and eventually resolved: 6 patients had a reactive arthritis, 1 patient had pseudogout, and 3 patients were unclassified. Patients consented for the study prior to an ultrasound-guided diagnostic joint aspiration. Patients with early OA (eOA) and advanced OA (aOA) were recruited from Orthopaedic Clinics, University Hospital Coventry & Warwickshire, Coventry, U.K., presenting with new onset knee pain. All patients in this group had normal radiographs of the symptomatic knee and were undergoing routine arthroscopy. Macroscopic findings on arthroscopy were classified according to the Outerbridge classification. Patients found to have changes (Outerbridge grade I/II) during routine arthroscopy were recruited for eOA. Normal healthy control subjects were recruited from friends of the patients and investigators with no history of joint symptoms. 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 samples from healthy people were collected after overnight fasting. Blood cells were separated by centrifugation (2000g, 10 min) and the resulting plasma was stored at – 80 ^{o}C until analysis. Synovial fluid was aspirated (1 – 5 ml) from the knee joints of patients and stored at – 80 ^{o}C until analysis. The collection of samples from patients and healthy subjects was achieved with informed consent and use of them was approved by the local medical ethics committee and was conducted in accordance with the Declaration of Helsinki. Subject's characteristics, including medications, are given in Table 1.

The content of citrulline residues in plasma/serum and synovial proteins was quantified in exhaustive enzymatic digests by stable isotopic dilution analysis LC-MS/MS by methods described below.

Plasma, serum or synovial fluid (100 µl) was diluted 5-fold with water 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 de-lipidified by extraction 3-times with an equal volume of water-saturated ether. Residual ether was removed in a centrifugal evaporation and protein concentration determined by Bradford method. For enzymatic hydrolysis, an aliquot of protein (100 µg in 20 µl water, free citrulline < 62 fmol or <0.002 mmol/mol arg) was mixed with 100 mM HCl (10 µl), pepsin (2 mg/ml in 20 mM HCl; 5 µl) and thymol (2 mg/ml in 20 mM HCl; 5 µl) in HPLC vials with fused glass inserts. The samples were gassed with argon by 3 cycles of placing under vacuum (20 mmHg, 2 min) and re-filling with argon in a centrifugal evaporator. Samples were incubated at 37 °C for 24 h. The samples were then neutralized and buffered at pH 7.4 by the addition of firstly 12.5 µl 100 mM potassium phosphate buffer, pH 7.4, and then 5 µl 260 mM KOH. Pronase E (2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 5 µl) and penicillin-streptomycin solution (1000 units/ml and 1 mg/ml respectively; 5 µl) was added and the samples were incubated at 37 °C for a further 24 h. Finally aminopeptidase (2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 5 µl) and prolidase solution (2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4; 5 µl) were added and the samples incubated at 37 °C for a further 48 h.

All reagents were sterile-filtered, gassed with argon and added automatically by a PAL HTS sample autoprocessor (CTC-PAL Analytics, Zwingen, Switzerland). Protein hydrolysate (25 µl) was spiked with isotopic standards ([*guanidino*-$^{15}N_2$]arg, 5 nmol and [5-^{13}C-4,4,5,5-$^{2}H_4$]citrulline, 25 pmol; 25 µl) 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 column was 150 mm x 2.1 mm Hypercarb™ (3 µm particle size; Thermo, Runcorn, U.K.) at 30°C. The mobile phase was 0.1% trifluoroacetic acid (TFA) from 0 - 5 min and a linear gradient of 0 – 2.5% acetonitrile from 5 - 20 min; the flow rate was 0.2 ml/min. Eluate was directed to the mass spectrometer from 4 – 20 min. Analytes were detected by electrospray positive ionization, multiple reaction monitoring (MRM). The ionization source and desolvation gas temperatures were 120 °C and 350 °C, respectively. The cone gas and desolvation gas flow rates were 100 and 900 l/h, respectively. The capillary voltage was 3.55 kV. Argon gas (2.7×10-3 mbar) was in the collision cell. Programmed molecular ion and fragment ion masses optimized to ± 0.1 Da and collision energies were ± 1 eV for MRM detection. CP contents of plasma and

synovial fluid protein are normalised to arginine content and given as mmol/mol arg. For normalisation the amount of citrulline (pmol) in the sample is divided by the amount of arg (nmol) in the same sample.

Free 4-hydroxyproline (hyp) was analysed in plasma and synovial fluid by similar method except 25 µl ultrafiltrate, prepared by 3 kDa cut-off microspin ultrafilter with 25 pmol 4,5-[$^{13}C_2$]hyp, was analysed. 4,5-[$^{13}C_2$]Hyp synthesised as described (20) from [$^{13}C_2$]glyoxal (21). Hyp concentrations are given in µM. Analytical characteristics of the assays are given in Table 2. For 4,5-[$^{13}C_2$]Hydroxyproline, [$^{13}C_2$]ethylene glycol (100 µmol) was oxidized by alcohol oxidase (41 U/ml) in the presence of (2360 U/ml) to form [$^{13}C_2$]glyoxal in 80 mM phosphate buffer with 240 mM oxaloacetic acid at pH 7.4 with stirring at 37°C for 24 h, then at 10°C for 48 h. Enzymes were removed by using 12 kDa microspin filters. The concentrated solution was diluted to 5 ml with water and 500µl of concentrated NH_4OH was added and stirred for 10 min. Sodium borohydride (50 mg) was then added and stirred at room temperature for 10 min. The reaction mixture was acidified to pH 1 with 6 M HCl, lyophilised and purified on a preparative HPLC system with isocratic elution in 0.1% TFA. Structure was confirmed by mass spectrometry and ^{13}C NMR (270 MHz, D_2O) where chemical shift δ_C (ppm) values for the two [^{13}C] labelled carbons were: 71 (pyrrolidineCOH, 1C), and 59 (pyrrolidine-CH_2, 1C).

Statistics. Significance of 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. Data were analysed using SPSS, version 16.0. Limit of detection (LOD) was defined as analyte equivalent to 3 SD of the zero analyte control deduced from response calibration curves.

RESULTS

Quantification of citrulline residues in the protein of plasma and synovial fluid of patients with rheumatoid arthritis.

Citrulline and arginine were detected and quantified in exhaustive enzymatic hydrolysates of protein of plasma and synovial fluid – Figure 2. The stable isotopic dilution analysis-LC-MS/MS method for detection of citrulline had high specificity, good

linearity of response from 62-50,000 fmol and high sensitivity. The LOD was 62 fmol – equivalent to 0.006 mmol of CP/mol arg in plasma protein under assay conditions (Table 2).

CP was detected in plasma protein of healthy people. CP content of plasma protein was 0.053 (0.043 – 0.091) mmol/mol arg (n = 16). This was increased 4-fold in early RA but not in patients with non-RA or advanced RA. CP was detected in synovial fluid in all patients and was similar in all study groups.

Comparing CP content of plasma and synovial fluid within patient groups, CP content of synovial fluid was higher than that of plasma in patients with non-RA (0.235 versus 0.126 mmol/mol arg, $P<0.05$) and aRA (0.432 versus 0.089 mmol/mol arg, $P<0.001$) but not in early RA where CP contents of plasma and synovial fluid were similar. CP content of synovial fluid was higher in patients with non-RA and RA positive for rheumatoid factor (RF) than in patients negative for RF (1.08 versus 0.23, $P<0.001$), whereas plasma CP was not. Plasma CP was increased in patients with eRA and positive for anti-CCP antibodies with respect to those negative for anti-CCP antibodies (0.259 versus 0.133 mmol/mol arg, $P<0.02$) – Figure 3.

Quantification of citrulline residues in the protein of plasma and synovial fluid of patients with osteoarthritis.

CP was detected and quantified in protein of plasma and synovial fluid of patients with OA. Plasma CP was increased 5-fold in patients with early OA with respect to healthy people and also increased in patients with advanced OA but only by 37%. Plasma CP of patients with early OA was 4-fold higher than in patients with advanced OA whereas CP of synovial fluid was increased 44% in patients with advanced OA with respect to patients with early OA. Comparing CP content of plasma and synovial fluid protein within patient groups, plasma CP was higher than in synovial fluid CP in patients with early OA (0.290 versus 0.170 mmol/mol arg, $P<0.05$) whereas in patients with advanced OA this was reversed where synovial fluid CP was higher than in plasma CP (0.245 versus 0.079 mmol/mol arg, $P<0.001$). Although increased plasma and synovial fluid CP has been previously associated only with RA, herein we found protein citrullination was also associated with OA. Plasma CP of patients with early OA was similar to that of patients with early RA and 2-fold higher than in patients with non-RA ($P<0.05$) and 3-fold higher

than patients with RA (*P*<0.001). Synovial fluid CP of patients with advanced OA was similar to that of patients with non-RA, eRA and aRA – Figure 3.

Free hydroxyproline concentration in plasma and synovial fluid of patients with rheumatoid arthritis and osteoarthritis.

The stable isotopic dilution analysis-LC-MS/MS method for detection of hyp had high specificity, good linearity of response from 0.1 – 50 pmol and high sensitivity. The LOD was 102 fmol (Table 2). The median concentration of hyp in plasma of healthy people was 1.26 (0.86 – 1.86) μM. Plasma hyp was increased 58% in non-RA, 44% in eOA and 102% in aOA but not increased significantly in eRA or aRA. Synovial fluid hyp was 2.90 (2.34 – 4.43) μM in patients with non-RA and not significantly different in synovial fluid of patients with RA or OA (Figure 4). Synovial fluid hyp was higher than plasma hyp in patients with non-RA (2.90 versus 1.99 μM, *P*<0.01), early RA (2.49 versus 1.71 μM, *P*<0.01), eOA (4.24 versus 1.81 μM, *P*<0.001) and advanced OA (4.06 versus 2.54 μM, *P*<0.01) – Figure 4.

Correlation analysis.

In healthy people plasma CP correlated positively with plasma hyp (r = 0.63, P<0.05). Plasma CP correlated positively with synovial fluid CP in patients with early RA (r = 0.81, *P*<0.05) but not in advanced RA (aRA). In non-RA and early RA there were strong positive correlations of hyp in plasma with hyp in synovial fluid (r = 0.95 and r = 0.88, respectively; *P*<0.001). In early OA and advanced OA (aOA) there were also positive correlations of hyp in plasma and with hyp in synovial fluid (r = 0.70, P<0.01 and r = 0.56, P<0.05, respectively).

Effect of drug therapy in rheumatoid arthritis

The effect of drug treatment of patients with aRA on CP and hyp was examined. Patients receiving anti-tumour necrosis factor-α (anti-TNFα) therapy had lower plasma hyp with respect to those not receiving anti-TNFα therapy (0.96 versus 3.37 μM, P<0.01). Patients receiving treatment with non-steroidal anti-inflammatory drugs (NSAIDs) had lower synovial fluid CP (0.15 versus 1.02 mmol/mol arg, P<0.05) and plasma hyp (0.99 versus 3.16 μM, P<0.01) with respect to those not receiving NSAIDs. However, patients receiving treatment with prednisolone had higher synovial fluid CP (1.72 versus 0.24 mmol/mol arg, P<0.01) and plasma hyp (2.87 versus 0.94 μM, P<0.05) with respect to those not receiving prednisolone. Treatment with or without methotrexate (MTX) and

opiate analgesics was not associated with differences in these variables. These differences may be due to patient treatment linked to severity of symptoms. For NSAIDs and prednisolone, patients received treatment with one of these agents but not both. NSAID treatment was applied for mild and moderate severity cases and hence this treatment appears linked to low synovial fluid CP and plasma hyp whereas prednisolone was applied for severe cases and hence this treatment appears linked to high synovial fluid CP and plasma hyp.

DISCUSSION

Development of a specific, sensitive and quantitative method of CP measurement has revealed that CP are present in both health and disease and show distinctive links to the plasma and synovial compartments and the bone turnover and resorption biomarker free hyp.

The presence of CP in plasma of healthy people implicates PADs in pre-symptomatic low grade inflammation which may be activated and increase in susceptible individuals and environments. The correlation of CP with hyp in plasma of healthy subjects found herein reveals, for the first time, a link of protein citrullination with bone resorption of pre-symptomatic arthropathy. Thus, CP analysis in healthy people now represents a marker of change in skeletal health.

The autoimmune response to CP may be particularly marked in RA. This likely underlies the diagnostic utility of anti-CCP antibody measurement for eRA. We found high levels of plasma CP in patients with eRA and association of this with anti-CCP antibodies, consistent with formation and immunogenicity of CP in this response. At this early stage of RA, CP concentrations of plasma and synovial fluid compartments were similar, consistent with CP being formed in the synovium and leaking freely into plasma and also possibly formation of CP beyond the synovium. The positive correlation of plasma and synovial fluid CP in eRA is consistent with inter-compartment flow and related factors influencing CP formation in both compartments.

An outstanding finding of this study is high levels of plasma CP in early OA – particularly impressive as the levels of plasma CP in early OA were similar to those of early RA although declining in advanced OA. Also remarkable was higher CP concentration in plasma than in synovial fluid in early OA and reversal of this in advanced OA. Plasma

CP in early and advanced OA correlated positively with synovial hyp. This may suggest a vascular inflammatory component of OA in early stage development leading to joint degradation which becomes focussed in the joint and dominant in advanced disease.

In conclusion, the aim of this study was to develop a sensitive method for the quantitation of total CP with a view to determining differences in the CP content of plasma and synovial fluid and links to symptoms and treatment in skeletal disorders such as RA and OA. We found the first evidence of CP in healthy subjects and an increase in CP in plasma of patients with early OA similar to those of patients with early RA. We also found a link of plasma CP in healthy people and patients with arthritis, such as OA, with a marker of bone resorption, hyp. Quantitation of plasma CP may have an important diagnostic role in the detection of early OA and other arthritic diseases with a link to the bone turnover/resorption marker hyp. Non-RA, eRA and eOA may be distinguished by differential changes in plasma CP and hyp, with respect to healthy subjects. Additionally, quantitation of plasma CP, and possibly also hyp, in healthy people may provide an objective biochemical assessment of early-stage decline in skeletal health.

MACHINE LEARNING

Clinical and biochemical variables are combined in a machine learning approach to develop an algorithm using the optimum combination of reporter variables (features) to detect and determine skeletal health by classification, and distinguish between eOA, eRA, non-RA and healthy controls. The machine learning algorithm is trained on a data set. To validate the machine learning algorithm and analyse the predictive performance of the machine learning algorithm, leave-one-out cross-validation analyses are performed, comparing in turn each of the classes with the other three. This allows estimation of the performance of the machine learning algorithms.

A variety of different machine learning algorithms may be used for the classification. Four different machine learning algorithms were evaluated in more detail: stepwise generalized linear model (GLM); GLM with elastic net (GLMNET); a random forest algorithm (a nonlinear, classification tree-based method); and an ensemble algorithm that uses the mean of predictions from the random forest method and the GLMNET method.

The stepwise GLM algorithm combines logistic regression with stepwise feature selection. The stepwise GLM algorithm can be implemented using the R programming language function 'stepAIC'. The GLMNET algorithm (with elastic net) uses a sparse form of logistic regression, where it is assumed that parameters for some input variables will be exactly zero (and hence they do not contribute to the analysis). For the GLMNET algorithm the R package 'glmnet' can be used.

The random forest algorithm is a well-known and very powerful non-linear machine learning method that forms ensembles of decision trees. This allows it to capture in particular complex structure in the data that linear methods cannot. The R package 'randomForest' can be used.

The cross-validation analysis shows that two algorithms give particularly good performance: the random forest algorithm, and an ensemble model consisting of the mean of predictions from the random forest algorithm and GLMNET algorithms.

The cross-validation analysis also allows robust identification of the variables that are selected consistently by both the random forest algorithm and the ensemble model as being informative.

The random forest algorithm gives the best outcome with the following variables as input:

- age;
- gender;
- plasma CP (citrullinated protein);
- hyp (hydroxyproline);
- anti-CCP antibody (anti–cyclic citrullinated peptide antibodies); and
- RF (rheumatoid factor).

Figure 5 shows algorithm classifiers for the random forest algorithm. This illustrates which features are particularly informative for the four classes of healthy, early OA, early RA, and non-RA. The table below provides per cent proportion of times that each feature is selected in the leave-one-out cross-validation (with 100% indicating that the feature is always selected, and highly informative).

Feature / Class	Age	Gender	Plasma CP	RF	Plasma hyp	Anti-CCP
Healthy controls (Figure 5A)	100 %	6 %	100 %	100 %	100 %	100 %
Early OA (Figure 5B)	100 %	11 %	100 %	100 %	100 %	100 %
Early RA (Figure 5C)	100 %	4 %	92 %	100 %	4 %	100 %
Non-RA (Figure 5D)	100 %	58 %	98 %	100 %	6 %	100 %

There was redundancy with both anti-CCP antibody and RF in the diagnostic algorithm, and only one or the other is required for diagnostic power. Therefore, algorithms were evaluated with exclusion of RF and retaining features of subject age, gender, plasma CP, plasma hyp and anti-CCP antibody.

To quantify the performance of the classification, receiver operating characteristic plots are produced as shown in Figure 6 for the random forest algorithm. The areas under the curves provide a classification performance metric and are:

- healthy controls (Figure 6 A): 0.80 (95% confidence interval: 0.68 – 0.93)
- early OA (Figure 6 B): 0.92 (95% confidence interval: 0.83 – 1.00)
- early RA (Figure 6 C): 1.00 (95% confidence interval: 1.00 – 1.00)
- non-RA (Figure 6 D): 0.75 (95% confidence interval: 0.55 – 0.96)

For the ensemble algorithm the receiver operating characteristic plots have the following areas under the curves:

- healthy controls: 0.80 (95% confidence interval: 0.64 – 0.96)
- early OA: 0.93 (95% confidence interval: 0.85 – 1.00)
- early RA: 1.00 (95% confidence interval: 1.00 – 1.00)
- non-RA: 0.70 (95% confidence interval: 0.50 – 0.91)

Sensitivity and specificity results are obtained by combining the four leave-one-out cross-validation analyses. These give the probability of each sample belonging to each of the four disease groups. Each sample is predicted to belong to the group for which it

has the highest probability. These predictions are then compared to the ground truth and sensitivity and specificity values computed.

The sensitivity and specificity of the random forest algorithm are:

- Healthy controls – sensitivity 0.88, specificity 0.78
- Early OA – sensitivity 0.94, specificity 0.94
- Early RA – sensitivity 1.00, specificity 1.00
- Non-RA – sensitivity 0.20, specificity 0.98

The sensitivity and specificity of the ensemble algorithm are:

- Healthy controls – sensitivity 0.75, specificity 0.81
- Early OA – sensitivity 0.94, specificity 0.86
- Early RA – sensitivity 0.9, specificity 1.00
- Non-RA – sensitivity 0.3, specificity 0.98

Using the features of age, gender, plasma CP, hyp, and anti-CCP antibody as markers in combination can provide particular powerful classification. Especially the classification by means of the random forests algorithm, or by the ensemble algorithm (which takes the random forest method into account), can provide a diagnostic for biochemical diagnosis of eOA and improve detection of the development of eRA.

It is surprising and remarkable that high levels of plasma CP can provide a marker for eOA, and also that high levels of plasma CP in combination with plasma free hydroxyproline, anti-CCP antibodies and RF can provide the basis for sensitive and specific detection of eOA, improved detection of eRA and also non-RA. A combination of plasma CP and hydroxyproline, anti-CCP antibodies and RF measurement can thus provide for early and accurate diagnosis between arthritis phenotypes. Detection and quantification can provide an objective biochemical assessment of early-stage decline in musculoskeletal health. The observed reversal of CP gradient from eOA to aOA can also provide diagnostic utility.

It will be understood that the present invention has been described above purely by way of example, and modifications of detail can be made within the scope of the invention.

Each feature disclosed in the description, and (where appropriate) the claims and drawings may be provided independently or in any appropriate combination.

Reference numerals appearing in the claims are by way of illustration only and shall have no limiting effect on the scope of the claims.

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Table 1. Clinical characteristics of healthy people and patients with arthritis.

Subject group	N	Age (yr)	Gender M/F	Duration of disease (yr)	Medications	Co-morbidities
Control	16	50 ± 8	9/7	----------	None	None
non-RA	10	37 ± 12**	8/2	0.1 (0.1 - 0.2)	NSAID (9); opiate (4); antibiotic (2); anti-hypertensive (1)	Hypertension (1)
eRA	10	65 ± 11***,OOO	4/6	0.2 (0.1 - 0.3)	NSAID (10); statin (3); anti-hypertensive (2); hypoglycemic (1)	Dyslipidaemia (3); hypertension (2); type 2 diabetes (1)
aRA	22	60 ± 15 *,OOO	8/14	7 (3 – 20) OOO, †††	Anti-TNF therapy (15); NSAID (11); methotrexate (10); prednisone (9), opiate (9); sulfasalazine, statin (3); anti-hypertensive (2); bronchodilator, muscle relaxant, antithrombotic, anti-ulcerogenic, antibiotic (1)	Asthma, hypertension (2); uveitis (1)
eOA	16	48 ± 8 OO	9/7	0.3 (0.1 – 1.5)	Anti-ulcerogenic (3); NSAID, anti-hypertensive (2); anti-epileptic, opiate, antithrombotic, tamsulosin, glucosamine, thyroxine (1).	Asthma, hypertension, deep vein thrombosis (2); developmental hip dysplasia, pancreatitis, obesity, epilepsy, bilateral gastro-oesophageal

						reflux disease (1)
aOA	17	69 ± 9***,OOO, †††	7/11	2 (2 – 7) OOO, †††	NSAID (11), anti-hypertensive (5), anti-depressant (3), bronchodilator (2), statin (2), opiate, anxiolytic, antipsychotic, antithrombotic, gabapentin, Ca^{2+} channel blocker, antibiotic, allopurinol (1)	Hypertension (5); dyslipidemia (2); type 2 diabetes, asthma, obesity, pneumococcal meningitis (1).

Significance: *, ** and ***, *P*>0.05, *P*<0.01 and *P*<0.001 with respect to control (healthy people); OO and OOO, *P*<0.01 and *P*<0.001 with respect to non-RA; and †††, P<0.001 for aRA with respect to eRA and for aOA with respect to eOA. For Medications, numbers in parentheses are number of patients receiving treatment with the drugs indicated. Data are mean ± SD (parametric) and median [lower – upper quartile] (non-parametric).

Table 2. Analytical variables and assay characteristics for quantitation of citrulline, arginine and 4-hydroxyproline by stable isotopic dilution analysis tandem mass spectrometry.

Analyte	Citrulline	Arginine	4-Hydroxyproline
Retention time R_t (min)	13.5	16.5	5.6
Molecular ion M+1 (Da)	176.1	175.1	132.0
Fragment ion (Da)	70.1	70.1	86.1
Cone voltage (V)	20	30	26
Collision energy (eV)	21.0	24.0	12.0
Neutral fragment losses	H_2CO_2, $NH_2C(=O)NH_2$	H_2CO_2, $NH_2C(=NH)NH_2$	H_2CO_2
Internal standard	[5-^{13}C,4,4,5,5-$^{2}H_4$]citrulline	[*guanidino*-$^{15}N_2$]arg	4,5-[$^{13}C_2$]Hyp
LOD (fmol)	62	520	102
Intra- and interbatch CV (%; n = 6)	1.3 and 6.0	1.0 and 1.8	1.0 and 1.5
Recovery (%)	88 (protein digest)	94 (protein digest)	100 (ultrafiltrate)

CLAIMS

1. A diagnostic method for quantifying citrullinated proteins (CP) as a marker for determining skeletal health comprising:

(a) undertaking an assay for direct detection of CP in a body fluid sample obtained from a test individual;

(b) quantifying the amount of CP in said sample; and

(c) where said amount of CP is increased above normal levels, concluding said individual has a skeletal disorder.

2. The diagnostic method according to Claim 1 wherein part c) involves determining whether the level of CP is more than 1.5-fold or more than 2-fold above the normal level and, if it is, concluding said individual has said skeletal disorder.

3. The diagnostic method according to Claim 1 wherein part c) involves determining whether the level of CP is more than three or four or five fold above the normal level and, if it is, concluding said individual has said skeletal disorder.

4. The diagnostic method according to any one of Claims 1 to 3, wherein said skeletal disorder is a joint or bone disorder selected from the group comprising: OA, such as early OA or advanced OA; an arthritic condition including arthropathy, RA, such as early RA or advanced RA, non-specific synovitis (non-RA), loosening of hip arthroplasty; bone resorption disease or osteoporosis;

or wherein said skeletal disorder is a condition that gives rise to a lack of skeletal health or integrity, such as Crohn's disease or other inflammatory bowel disorders; inflammatory vascular disease; autoimmune disease disorders with complications of declining skeletal health; type 1 or 2 diabetes, metabolic syndrome and related disorders.

5. The diagnostic method according to Claim 4, wherein said skeletal disorder is OA, such as early OA or advanced OA.

6. The diagnostic method according to any one of the preceding claims, wherein said body fluid sample is selected from the group consisting of synovial fluid, whole blood, blood serum, blood plasma, urine, lymphatic fluid, saliva, eye fluid, seminal fluid, cerebrospinal fluid, sebaceous secretions, or sputum.

7. The diagnostic method according to any one of the preceding claims, wherein said sample is a plasma sample and it is further assayed for hyp; and where both CP and hyp are higher than normal levels concluding said individual has a skeletal disorder.

8. The diagnostic method according to any one of Claims 1-6, further comprising assaying a urine sample from the test individual for hyp.

9. The diagnostic method according to Claim 8, wherein said urine sample is further assayed for creatinine; and where the quantification of hyp in the test sample and said healthy sample involves normalisation of the data having regard to the amount of creatinine present.

10. The diagnostic method according to any one of the preceding claims, wherein said sample is further assayed for arginine; and where the quantification of CP in part b) above involves normalisation of the data having regard to the amount of arginine present.

11. The diagnostic method according to any one of the preceding claims, wherein parts a)-b) above are repeated after a selected time interval and the quantity of CP after said time interval is compared to the quantity of CP determined at an earlier time point, and where:

(i) a decrease in CP quantity after said selected time interval is observed, concluding that the individual has improved skeletal health;

(ii) an increase in CP quantity after said selected time interval is observed, concluding that the individual has worsening skeletal health; or

(iii) zero, or marginal difference is observed in CP quantity after said selected time interval, concluding that the individual has stable skeletal health.

12. The diagnostic method according to Claim 11 for use in determining or monitoring disease progression, a treatment regimen, general skeletal health, or the effectiveness of exercise, physiotherapy, foods or supplements on skeletal health.

13. The diagnostic method according to any one of Claims 1-6, wherein parts a)-b) are undertaken on both a plasma and synovial fluid sample and said plasma and synovial fluid samples are further assayed for hyp, and where:

i) if elevated quantities of CP and hyp are observed in either or both said plasma or synovial sample, concluding under part c) that the individual has OA; or

ii) if elevated quantities of CP are observed, but not hyp, in either or both said plasma or synovial sample, concluding under part c) that the individual has RA.

14. The diagnostic method according to any one of Claims 1-6, wherein parts a)-b) are undertaken on both a plasma and synovial fluid sample, and where:

i) the CP content is greater in the plasma fluid sample than the synovial fluid sample (i.e. the gradient is negative), concluding under part c) that the individual has early OA; or

ii) the CP content in the plasma fluid sample and the synovial fluid sample is the same, or similar, concluding under part c) that the individual has early RA.

15. The diagnostic method according to any one of Claims 1-6, wherein parts a)-b) are undertaken on both a plasma and synovial fluid sample and said plasma and synovial fluid samples are further assayed for hyp, and where:

i) the CP content is greater in the plasma fluid sample than the synovial fluid sample (i.e. the gradient is negative), and hyp is elevated, concluding under part c) that the individual has advanced OA; or

ii) the CP content is greater in the plasma fluid sample than the synovial fluid sample (i.e. the gradient is negative), and hyp is the same or similar in both samples, concluding under part c) that the individual has advanced RA.

16. The diagnostic method according to any one of the preceding claims, wherein said assay for direct detection of CP is an immunoassay, such as an ELISA assay.

17. The diagnostic method according to any one of Claims 1-15, wherein said assay for direct detection of CP comprises:

(i) exposing protein extracted from a body fluid sample taken from a test individual to enzymatic hydrolysis; and

(ii) undertaking LC-MS/MS analysis on the hydrolysed protein from the sample of part (i) to detect CP.

18. The diagnostic method according to any one of Claims 1-17, wherein said sample is further assayed for anti-CCP and/or RF; and where anti-CCP and/or RF as well as CP are higher than normal levels, such as 1.5-fold or 2-fold higher, or 3-fold, 4-fold or 5-fold higher, thereby concluding said individual has a skeletal disorder.

19. The use of CP, alone or in combination with hyp, as a marker for skeletal health.

20. The use according to Claim 19, wherein said CP is used in combination with (i) hyp and (ii) creatinine and/or arginine.

21. The use according to Claim 19 or 20, as a marker for OA, such as early OA or advanced OA.

22. A diagnostic method of determining skeletal health comprising:

(a) quantifying citrullinated proteins (CP) as a first marker by undertaking an assay for direct detection of CP in a body fluid sample obtained from a test individual and quantifying the amount of CP in said sample;

(b) quantifying at least one further marker by undertaking an assay for detection of that further marker; and

(c) determining the skeletal health of the test individual in dependence on the markers as inputs.

23. The diagnostic method according to Claim 22, wherein the assay for detection of the further marker is undertaken on said body fluid sample and/or on a further body fluid sample obtained from the test individual.

24. The diagnostic method according to Claim 22 or 23, wherein said at least one further marker includes at least one, and preferably all, of: hydroxyproline (hyp), anti–cyclic citrullinated peptide antibodies (anti-CCP antibody), rheumatoid factor (RF), CP, creatinine, arginine, age and gender of the test individual.

25. The diagnostic method according to any of Claims 22 to 24, wherein determining skeletal health comprises classifying the skeletal health.

26. The diagnostic method according to Claim 25, wherein classifying is by a classification algorithm.

27. The diagnostic method according to Claim 26, wherein the classification algorithm is trained on a set of markers from a population of individuals with known skeletal health.

28. The diagnostic method according to Claim 26 or 27, wherein the classification algorithm is an ensemble algorithm comprising different types of classification algorithms.

29. The diagnostic method according to any of Claims 26 to 28, wherein the classification algorithm comprises a decision tree based algorithm.

30. The diagnostic method according to Claim 29, wherein the classification algorithm comprises a random forest algorithm.

31. Apparatus for determining skeletal health comprising:

means for receiving a quantification of citrullinated proteins (CP) as a first marker, obtained by direct detection of CP in a body fluid sample obtained from a test individual;

means for receiving a quantification of at least one further marker, obtained by an assay for detection of that further marker; and

means for determining the skeletal health of the test individual in dependence on the markers as inputs.

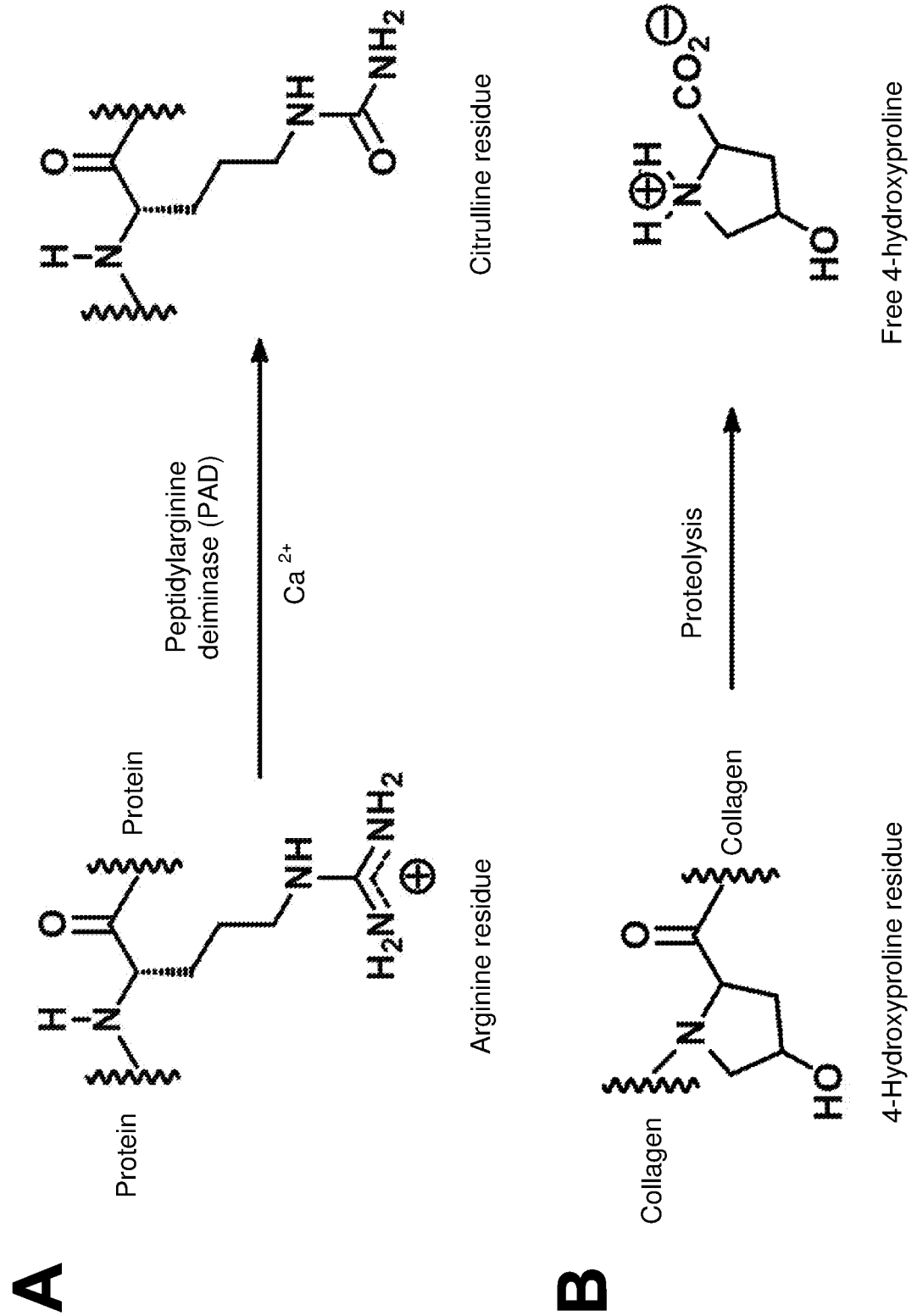
A
Protein
Protein
Arginine residue
Peptidylarginine deiminase (PAD)
Ca 2+
Citrulline residue
B
Collagen
Collagen
4-Hydroxyproline residue
Proteolysis
Free 4-hydroxyproline

Figure 1

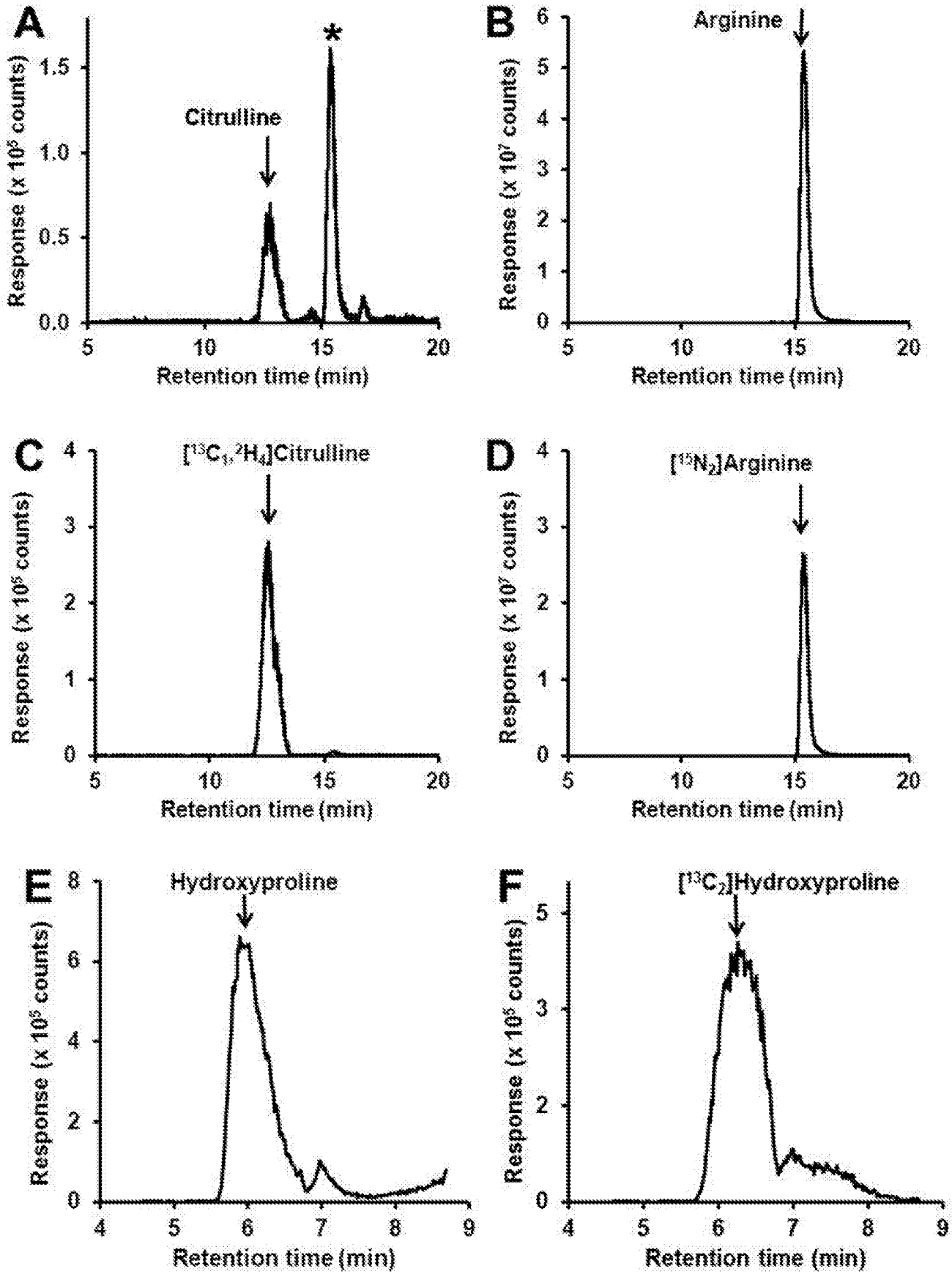
A
Citrulline
*
Response (x 10⁵ counts)
Retention time (min)
B
Arginine
Response (x 10⁷ counts)
Retention time (min)
C
[¹³C₁,²H₄]Citrulline
Response (x 10⁵ counts)
Retention time (min)
D
[¹⁵N₂]Arginine
Response (x 10⁷ counts)
Retention time (min)
E
Hydroxyproline
Response (x 10⁵ counts)
Retention time (min)
F
[¹³C₂]Hydroxyproline
Response (x 10⁵ counts)
Retention time (min)

Figure 2

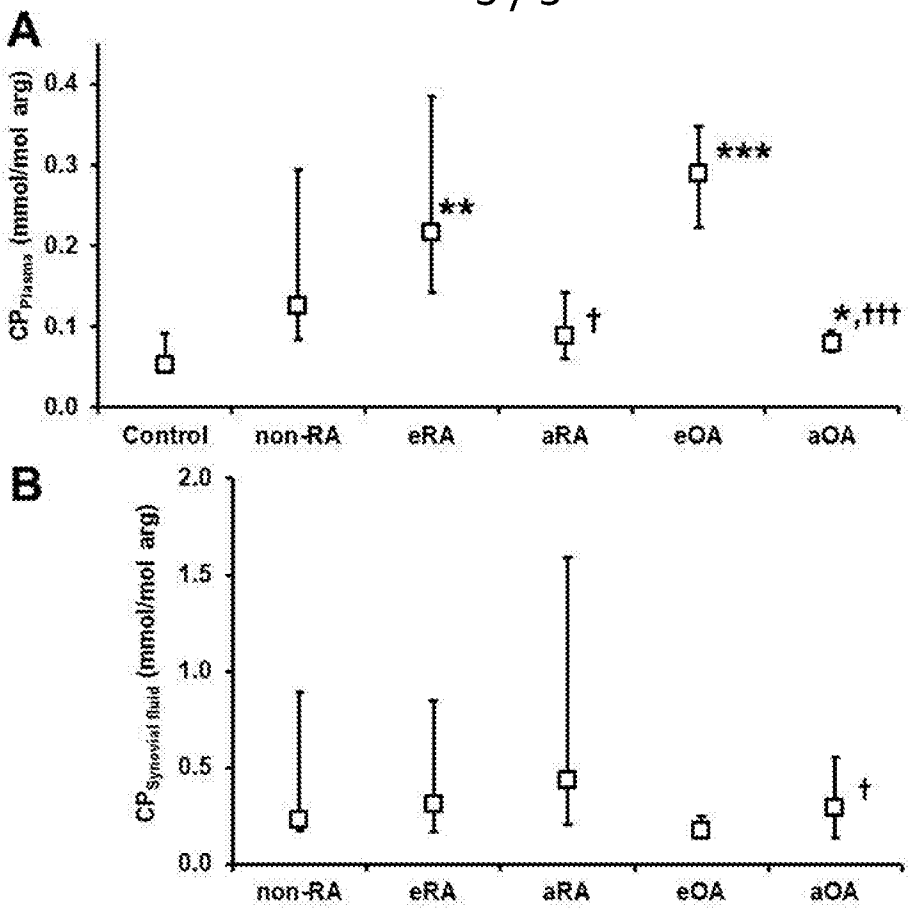

Figure 3

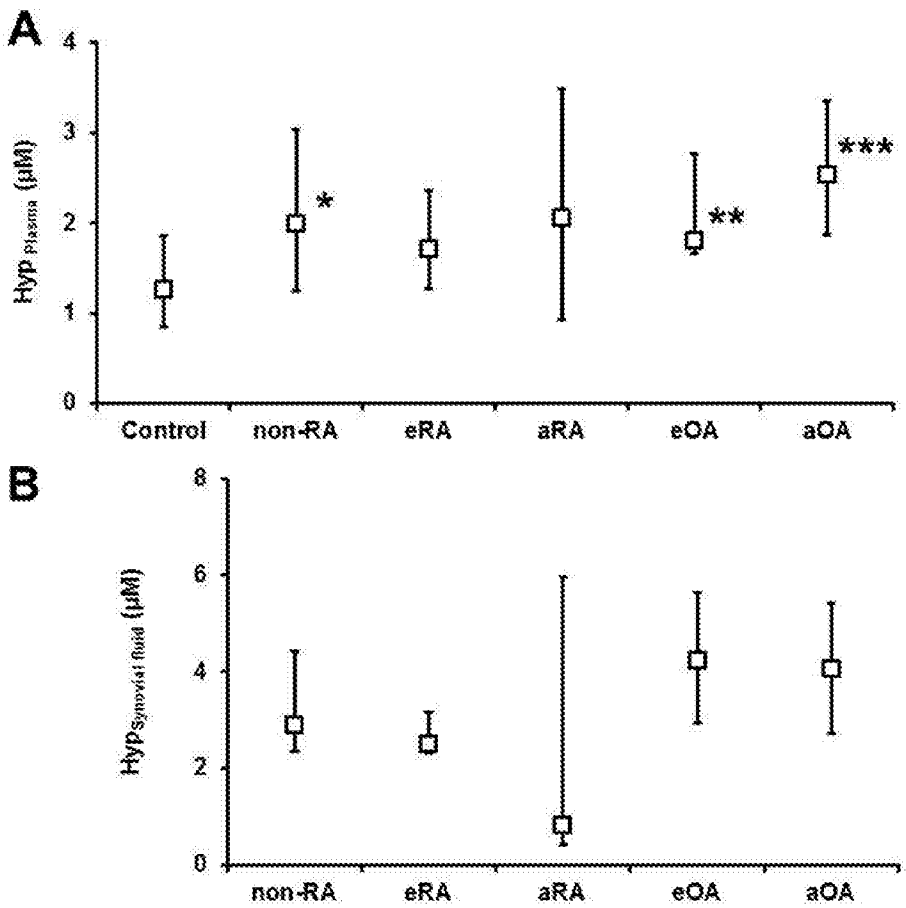

Figure 4

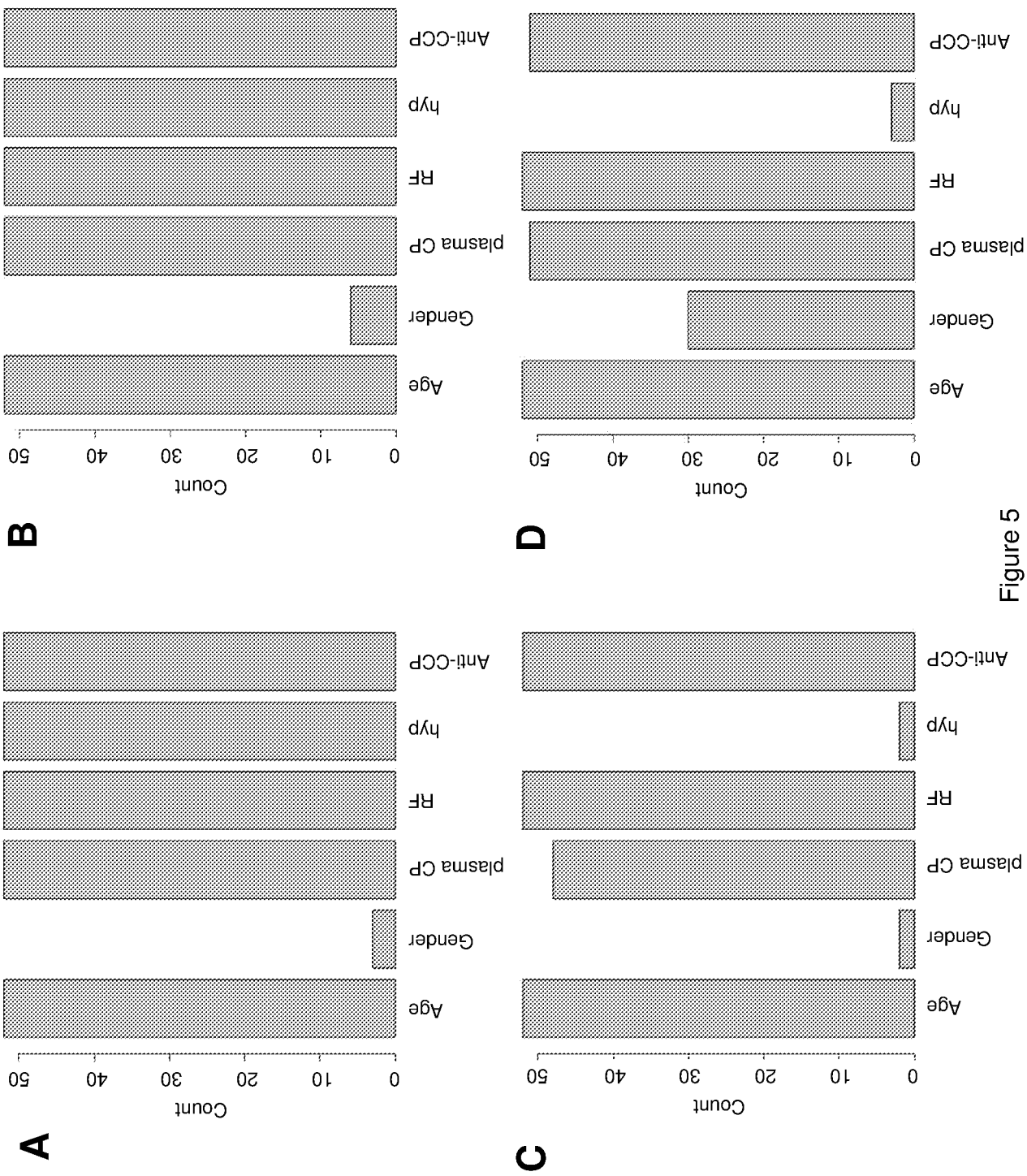

Figure 5

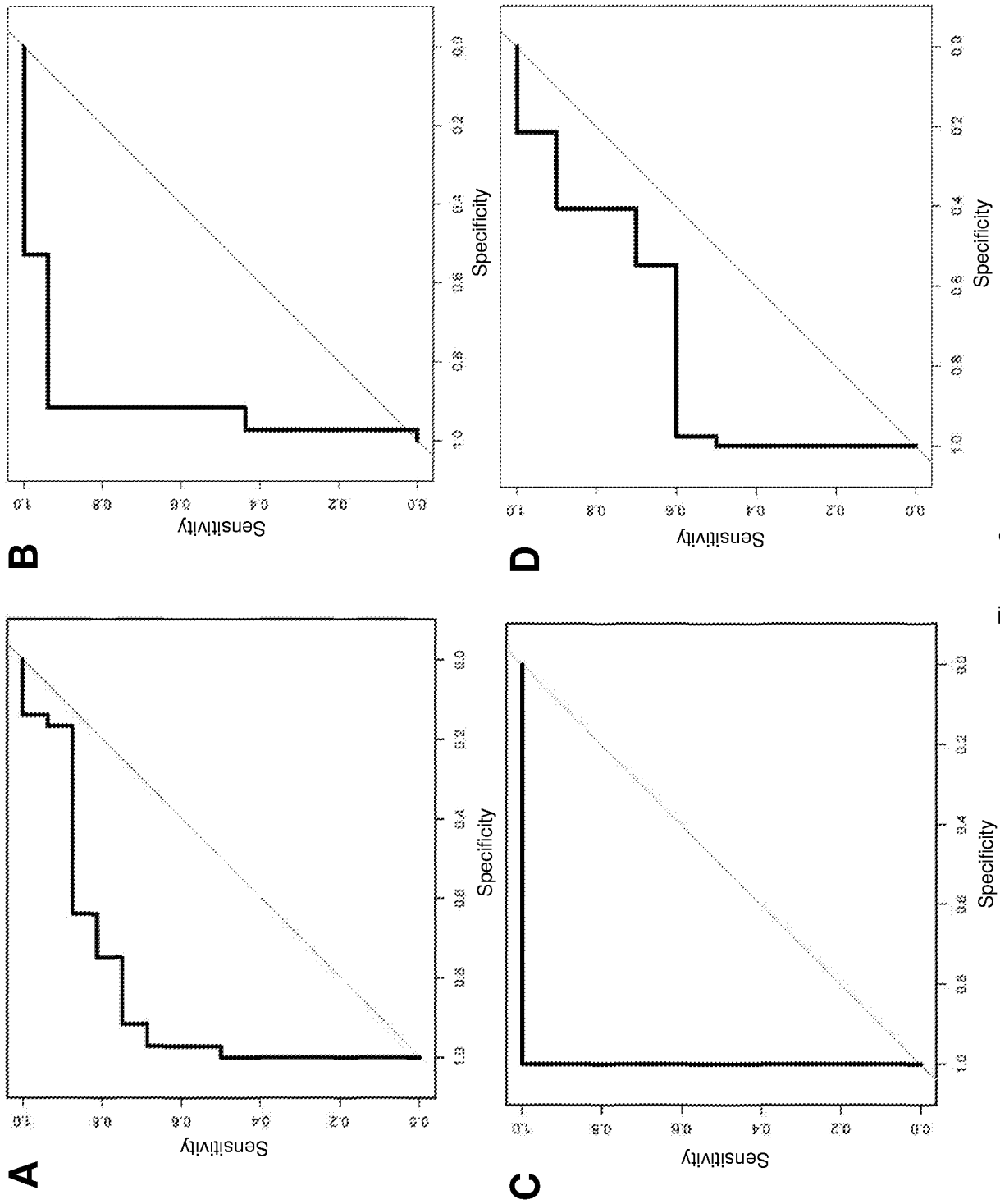

Figure 6