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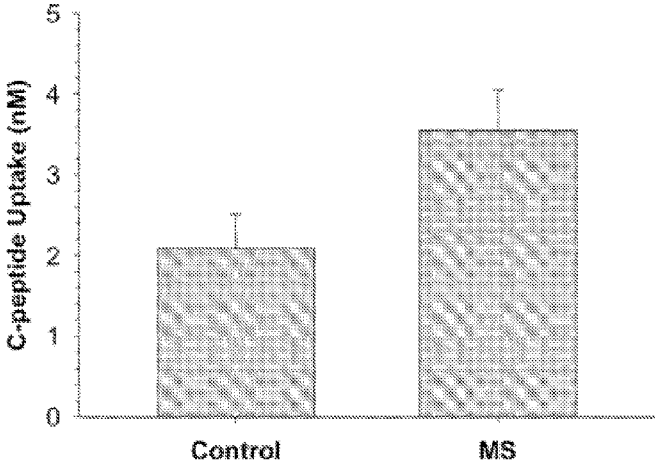


Fig. 1

(57) Abstract: Compositions and methods for measuring C-peptide binding by cells, including cells expressing Glut1, using a C-peptide binding facilitator, such as an albumin. Such methods include incubating the cell with a known amount of C-peptide and a C-peptide binding facilitator, and determining the amount of C-peptide bound to the incubated cells. Also provided are methods for detecting, monitoring, and/or treating immune-mediated diseases, such as multiple sclerosis (MS), using method of the present technology for measuring C-peptide binding by cells obtained from a human or other animal subject.

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COMPOSITIONS AND METHODS FOR MEASURING C-PEPTIDE BINDING AND DIAGNOSING IMMUNE-MEDIATED DISEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/132,213, filed on March 12, 2015. The entire disclosure of the above application is incorporated herein by reference.

BACKGROUND

[0002] The present technology relates to compositions and *in vitro* methods for measuring C-peptide binding by cells. Such compositions and methods may be used in methods of diagnosing, monitoring, and treating multiple sclerosis (MS) and other immune-mediated or autoimmune diseases.

[0003] MS is a demyelinating disease that is associated with the immune-mediated destruction of myelin that insulates and protects axons in the central nervous system. MS is one of a group of disorders that can be characterized as autoimmune diseases.

[0004] About 1 in 1,000 people in the United States has MS, while about 1-2 million people worldwide are thought to have MS. The number of people tested for MS is significantly higher. This is largely because not all symptoms are present in all MS patients, and MS can be characterized, in some cases, as episodic, with short to long periods of clinical remission. Common symptoms of MS include, *e.g.*, fatigue, weakness, spasticity, balance problems, bladder and bowel problems, numbness, vision loss, tremor and vertigo. Due to the broad range of symptoms and the manner in which they present (which may be very subtle), the diagnosis of MS may take months to years. For example, physicians often must combine detailed patient histories and perform both neurological and physiological examinations of people presenting with symptoms. A definitive diagnosis of MS requires time (*i.e.*, at least two separate symptomatic events or changes on Magnetic Resonance Imaging, MRI, over time) and measurable dissemination of the disease (*i.e.*, at least two separate locations within the central nervous system, which can be demonstrated by MRI or neurological exam). Thus, proper and effective treatment of patients may be delayed during this diagnostic phase. Furthermore, current tests for MS can be costly and invasive - *e.g.*, MRI scans, electrophysiological tests, and analysis of

cerebrospinal fluid. In short, there is a need for new methods and compositions for diagnosing MS and other immune-mediated diseases.

SUMMARY

[0005] The present technology is based, at least in part, on the discovery that albumin facilitates the uptake of C-peptide and Zn^{2+}, Fe^{2+}, and/or Cr^{3+} by red blood cells (RBCs), *i.e.*, erythrocytes, *in vitro*. In various embodiments, the present technology exemplifies the use of a C-peptide binding facilitator, such as an albumin, in methods for detecting or otherwise measuring C-peptide binding by RBCs. As discussed below, without limiting the scope or function of the present technology, it has been found that RBCs from patients with MS exhibit increased C-peptide uptake and Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake, with concomitant adenosine triphosphate (ATP) release, relative to RBCs from healthy humans without MS. In some embodiments, the present technology provides a variety of diagnostic and therapeutic applications (*e.g.*, methods for diagnosing and/or treating an immune-mediated/autoimmune disease, such as MS), which involve the methods of measuring C-peptide binding to cells, or information derived therefrom.

[0006] Accordingly, in one aspect, the present technology provides *in vitro* methods for measuring C-peptide binding to cells, such as RBCs or other cells expressing the glucose 1 transporter protein (Glut1). Such methods comprise incubating the cells with a C-peptide mixture comprising an amount, which may be a known amount, of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture, and determining the amount of C-peptide that bound to the incubated cells.

[0007] In some embodiments, C-peptide binding facilitators comprise albumins, leptin, collagen, and mixtures thereof. In various preferred embodiments, the C-peptide binding facilitator is an albumin, is human albumin or a non-human albumin (*e.g.*, bovine). In some embodiments, the albumin is obtained from plasma, serum, or whole blood of a mammal. In some embodiments, the albumin is recombinantly produced albumin. In some embodiments, the albumin is a variant form of a naturally-occurring albumin protein, *e.g.*, one that shares at least 80 (*e.g.*, at least 85, 90, 95, or 99) % identity with a naturally-occurring albumin protein (*e.g.*, human albumin).

[0008] In various embodiments, the C-peptide may be a polypeptide comprising the amino acid sequence EGSLQ (SEQ ID NO:17). For example, the C-peptide is

selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the C-peptide binding facilitator.

[0009] For example, such methods may comprise contacting, in the presence of albumin, a RBC (or a plurality of RBCs) with C-peptide to form a mixture; incubating the mixture under conditions that allow for binding of C-peptide by the RBC or cells; and determining the binding (*e.g.*, determining the uptake) of C-peptide by the RBCs. The binding can be determined by measuring the amount of C-peptide not bound by the cell or cells; the amount of C-peptide bound by the cell or cells; the amount of C-peptide not bound by the cell or cells; and/or the resultant ATP release from the cells contacted with C-peptide (*e.g.*, C-peptide and Zn^{2+}).

[0010] In some embodiments of any of the methods described herein, the amount of C-peptide present in the RBCs after the incubation is measured as the measure of binding or uptake. In some embodiments, the amount of free C-peptide in the mixture remaining after the incubation is measured as the measure of binding or uptake. In some embodiments, binding or uptake of C-peptide by the RBCs is measured as a function of the amount of ATP released from the RBCs after the incubation.

[0011] Thus, in various embodiments, determining the binding of C-peptide may be assessed by direct measurement of binding of C-peptide by cells, or indirectly such as through measuring the amount of C-peptide that is not bound by the cells. For example, the determining of an amount of C-peptide bound to the incubated cells may comprise:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) a combination of (i) and (ii).

In some embodiments, the amount of C-peptide bound by cells is determined by incubating cells with a known amount of C-peptide, separating the incubated cells to form a supernatant, and measuring the amount of C-peptide in the supernatant. The amount is

then determined by subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide.

[0012] Measuring C-peptide may be performed by methods among those known in the art. For example, the measuring may comprise directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

[0013] The present technology also provides *in vitro* methods for assessing the status of an immune-mediated disease in a mammalian subject, for example assessing the probability that a mammalian subject has an immune-mediated disease, the method comprising:

incubating cells that express Glut1 obtained from the subject with a C-peptide composition comprising an amount, *e.g.*, a known amount, of C-peptide and an albumin under conditions that allow for the cells to bind the C-peptide to form incubated cells in an incubated cellular mixture;

separating the incubated cells from the incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

[0014] In yet another aspect, the present technology features an *in vitro* method for assessing the probability that a mammalian subject has an immune-mediated disease. The method includes: contacting cells that express Glut1 obtained from the subject with an amount, *e.g.*, a known amount, of C-peptide and an albumin to form a C-peptide cellular mixture; incubating the C-peptide cellular mixture under conditions that allow for the cells to bind the C-peptide, to form incubated cells in an incubated cellular mixture; separating the incubated cells from the incubated cellular mixture; and determining the amount of C-peptide bound to the incubated cells.

[0015] The present technology also provides *in vitro* methods for diagnosing a mammalian subject as having an immune-mediated disease, such methods comprising:

incubating, in the presence of albumin, RBCs from the subject with C- peptide under conditions that allow for binding of C-peptide by RBCs; and

determining the amount of C-peptide bound by the RBCs, wherein increased binding of C-peptide by the RBCs, relative to a control C-peptide binding level, indicates that the subject has the immune-mediated disease. Associated methods include those for managing MS in a subject in need thereof, the method comprising:

(a) effecting an assay for measuring C-peptide binding on cells obtained from the subject, the assay comprising

(i) incubating the cells with a C-peptide composition comprising an amount, *e.g.*, a known amount, of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

(ii) determining the amount of C-peptide bound to the incubated cells.

(b) comparing the amount of C-peptide bound by the cells to a reference amount determined by performing the assay on cells obtained from a second subject that does not have the immune-mediated disease;

(c) administering a first treatment for MS; and

(d) assessing the efficacy of the administering by repeating the effecting and comparing.

[0016] The present technology also provides kits for use measuring C-peptide binding by cells, the kit comprising a C-peptide binding facilitator, C-peptide, and a container. The container may be operable for mixing a solvent with one or both of the C-peptide binding facilitator and the C-peptide. In various embodiments, the container is a first container containing the C-peptide binding facilitator, and the kit further comprises a second container containing C-peptide. The C-peptide binding facilitator may be albumin in powdered form, preferably wherein the albumin is lyophilized. In various embodiments, the kit further comprises a third container containing a solvent for one or both of the C-peptide binding facilitator and the C-peptide. For example, the solvent may comprise phosphate buffered saline, physiological saline solution, tris(hydroxymethyl)aminomethane (Tris) buffer, or phosphate buffer.

[0017] The present technology also provides reagents for use in measuring C-peptide binding to cells that express Glut1, the reagent consisting essentially of albumin dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of C-peptide, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof. Also provided are reagents for use in measuring C-peptide binding to cells that express Glut1, the reagent consisting essentially of C-peptide dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of albumin, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof. For example, the buffering agent may be selected from the group consisting of phosphate salts, Tris, and mixtures thereof.

DRAWINGS

[0018] The drawings described herein are for illustrative purposes only of selected embodiments and not all possible implementations, and are not intended to limit the scope of the present technology.

[0019] Fig. 1 is a bar graph depicting the level of C-peptide uptake by RBCs from patients with multiple sclerosis (MS) (n=25) and healthy human volunteers (control or C) (n=17). The Y axis represents C-peptide binding in picomoles.

[0020] Fig. 2 is a diagram of an exemplary kit composition. The kit includes 3 vials and an ELISA well. In (A) buffer is moved from the first vial to a second vial that contains solid albumin. In (B), the content of the third vial, C-peptide in water, is moved to the vial now containing the albumin in buffer. In (C) red blood cells (RBCs) from the donor are added to the vial containing C-peptide and albumin in buffer. This solution is centrifuged and analyzed by ELISA for remaining C-peptide in the supernatant. This value is subtracted from the moles of C- peptide originally in the vial #3 (typically 20 picomoles), resulting in the amount of C-peptide bound to the RBCs.

[0021] Fig. 3 is a bar graph depicting the results from the adenosine triphosphate (ATP) release studies, showing that the ATP release from the RBCs of MS patients was found to be an average of 344.7 ± 46.8 nM where the average release from healthy controls was 132.1 ± 14.1 nM. When the RBCs of the MS patients were incubated with a CFTR inhibitor, glybenclamide, the ATP release is decreased below the amount of the healthy controls, to a level of 65.3 ± 11.6 nM, suggesting that the increase in ATP release of the flowing RBCs of MS patients is not the result of RBC lysis. The error is reported as standard error of the mean, for N = 19 MS patients, 10 healthy controls and 12 glybenclamide inhibitions.

[0022] Fig. 4 is a bar graph depicting that the amount of $^{65}Zn^{2+}$ able to bind with the RBCs of MS patients is significantly higher, at a value of 3.61 ± 0.22 picomoles, than that of healthy controls, at a value of 2.26 ± 0.24 picomoles. The amount of C-peptide binding to the RBC correlates to this very well, as shown in Fig. 1. The error is reported as standard error of the mean for N = 22 MS patients and 11 healthy controls.

[0023] Fig. 5 is a bar graph showing C-peptide binding to blood from control patients, patients with MS, and patients with a non-MS neurological disease (OND).

[0024] Fig. 6 is receiver operating characteristic (ROC) curve generated by performing a method according to the current technology.

[0025] Fig. 7 is a diagnostic odds ratio (DOR) plot showing the sensitivity and specificity of a method according to the current technology.

[0026] Fig. 8 is a bar graph showing relative amounts of the glucose 1 transporter protein (Glut1) in lysates from healthy control patients, lysates from MS patients, and lysates from patients with a non-MS neurological disease (OND).

[0027] Fig. 9 is a bar graph showing relative amount of glucose associated with healthy control cells and cells form MS patients; with and without Zn^{2+} and C-peptide.

[0028] Corresponding reference numerals indicate corresponding parts throughout the several views of the drawings.

DETAILED DESCRIPTION

[0029] The following description of technology is merely exemplary in nature of the subject matter, manufacture and use of one or more inventions, and is not intended to limit the scope, application, or uses of any specific invention claimed in this application or in such other applications as may be filed claiming priority to this application, or patents issuing therefrom. In particular, the following description sets forth example embodiments and otherwise provides information that enables a person skilled in the art to make and use the subject matter set forth in the appended embodiments, but may omit certain details already well-known in the art. The following description is, therefore, to be taken as illustrative and not limiting. A non-limiting discussion of terms and phrases intended to aid understanding of the present technology is provided at the end of this Detailed Description.

[0030] The present technology provides, among other things, methods, compositions, reagents and kits that are useful for *in vitro* methods for measuring the binding of C-peptide to cells. Also provided are associated diagnostic methods, as well as therapeutic applications, which are useful for detecting, monitoring, and/or treating immune-mediated diseases, which affect such *in vitro* methods or involve use of information obtained from such *in vitro* methods.

[0031] In general, the present technology includes *in vitro* methods (also referred to herein as "assays") comprising:

incubating cells with C-peptide mixture comprising a known or unknown amount of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

[0032] Determining the amount of C-peptide bound to the incubated cells generally includes separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and measuring:

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from a known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

[0033] Embodiments described herein can involve, *e.g.*, contacting, in the presence of a C-peptide binding facilitator, such as albumin, RBCs from a subject of interest with C-peptide to form a mixture.

Materials

C-peptide

[0034] C-peptide, also known as the connecting peptide, is an approximately 30 amino acid polypeptide that connects the A and B chain of mature insulin within the proinsulin molecule. Proteolytic processing of proinsulin to mature insulin liberates the C-peptide. In addition to facilitating the proper folding of insulin, C-peptide possesses characteristics of a hormone, stimulating intracellular signaling mechanisms and even inducing beneficial physiological effects in renal and vascular tissues from diabetic subjects. Nordquist et al. (2008) *Biologics* @:441-452. C-peptide has been shown to facilitate Zn^{2+}, Fe^{2+}, and/or Cr^{3+} delivery to RBCs, and to stimulate the release of ATP from RBCs in a Zn^{2+}-, Fe^{2+}-, and/or Cr^{3+}-dependent manner. Medawala et al. (2009) *Rev Diabetic Studies* @:148. The cellular effects of this Zn^{2+}, Fe^{2+}, and/or Cr^{3+} delivery are manifold; glucose uptake is significantly enhanced into the cells, and ATP release from the cells is also increased. That latter feature is important for maintenance of proper blood flow, as ATP is a known stimulus of nitric oxide (NO) production in endothelial cells and is also an established vessel dilator and platelet inhibitor.

[0035] The term "C-peptide" as used herein includes all forms of C-peptide (also known as proinsulin C-peptide), including naturally derived, native, synthetic peptides or semi-synthetic peptides. Such C-peptides may be may be isolated from any species, including the human peptide, peptides from other animal species and genera, preferably

mammals. In various embodiments, the C-peptide comprises carboxyl C-peptide pentapeptide having the sequence EGSLQ (SEQ ID NO:17) In various embodiments, the C-peptide is a fraction of a corresponding full length C-peptide having at least 5 amino acids.

[0036] As referred to herein, the terms "polypeptide," "peptide," and "protein" are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification. As noted below, the polypeptides described herein can be, *e.g.*, wild-type proteins, functional fragments of the wild-type proteins, or variants of the wild-type proteins or fragments. Variants, in accordance with the present technology, can contain amino acid substitutions, deletions, or insertions. The substitutions can be conservative or non-conservative. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine.

[0037] In some embodiments, the C-peptide is a human C-peptide. An exemplary amino acid sequence for the human C-peptide is as follows: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ (SEQ ID N0:1). In some embodiments, the C-peptide is a porcine C-peptide. An exemplary sequence for porcine C-peptide is as follows: ELEDPQVEQTELGMGLGAGGLQPLALEMALQ (SEQ ID N0:2). In some embodiments, the C-peptide is a bovine C-peptide, *e.g.*, one having the following amino acid sequence: EVEGPQVGALELAGGPGAGGLEGPPQ (SEQ ID N0:3).

[0038] In some embodiments, the C-peptide is a chicken C-peptide, *e.g.*, comprising or consisting of the following amino acid sequence: DVEQPLVSSPLRGEAGVLPFQQEEYEKV (SEQ ID N0:4). In some embodiments, the C-peptide is from a zebrafish, *e.g.*, having or consisting of the following sequence: DVEPLLGFLPPKSAQETEV ADFAFKDHAELI (SEQ ID N0:5).

[0039] In some embodiments, the C-peptide is a variant of a naturally-occurring C-peptide. For example, the C-peptide may, in some embodiments, consist of or comprise an amino acid sequence that is at least 70 (*e.g.*, at least 75, 80, 85, 90, 95, or 99) % identical to the amino acid sequence depicted in any one of SEQ ID NOs:1-5. As used herein, percent (%) amino acid sequence identity is defined as the percentage of amino acids in a candidate sequence that are identical to the amino acids in a reference sequence,

after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software, such as BLAST software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

[0040] In some embodiments, the C-peptide comprises or consists of an amino acid sequence that has no more than 15 (*e.g.*, no more than 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1) amino acid substitutions, deletions, insertions, or a mixture of the above, relative to any one of SEQ ID NOs:1-5. The substitutions can be conservative, non-conservative, or a mixture of both. In some embodiments, the C-peptide is a fraction of a corresponding full length C-peptide having at least 5 amino acids of the sequence EGSLQ (SEQ ID NO:17).

[0041] As used herein, the term "conservative substitution" refers to the replacement of an amino acid present in the native sequence in a given polypeptide with a naturally or non-naturally occurring amino acid having similar steric properties. Where the side-chain of the native amino acid to be replaced is either polar or hydrophobic, the conservative substitution should be with a naturally occurring amino acid, a non-naturally occurring amino acid that is also polar or hydrophobic, and, optionally, with the same or similar steric properties as the side-chain of the replaced amino acid. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine. One letter amino acid abbreviations are as follows: alanine (A); arginine (R); asparagine (N); aspartic acid (D); cysteine (C); glycine (G); glutamine (Q); glutamic acid (E); histidine (H); isoleucine (I); leucine (L); lysine (K); methionine (M); phenylalanine (F); proline (P); serine (S); threonine (T); tryptophan (W), tyrosine (Y); and valine (V).

[0042] The phrase "non-conservative substitutions" as used herein refers to replacement of the amino acid as present in the parent sequence by another naturally or non-naturally occurring amino acid, having different electrochemical and/or steric properties. Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the native amino acid being substituted and/or

can have functional groups with significantly different electronic properties than the amino acid being substituted.

[0043] Variant forms of C-peptide retain at least 50 (*e.g.*, at least 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 65, 70, 75, 80, 85, 90, 95, 99, or even 100) % of the activity (*e.g.*, uptake by RBCs, delivery of Zn^{2+} to RBCs, and/or stimulation of ATP release from RBCs) of the wild-type C-peptide from which they were derived. For example, a variant form of human C-peptide having three amino acid substitutions relative to SEQ ID NO: 1 would retain at least 50% of the ability of wild-type human C-peptide of SEQ ID NO: 1 to bind RBCs, delivery of Zn^{2+} to RBCs, and/or stimulate ATP release from RBCs. Methods for measuring C-peptide binding to RBCs, uptake of C-peptide by RBCs, and/or ATP release by RBCs are known in the art and described and exemplified herein.

[0044] Methods for making C-peptide, including recombinant methods, are well known in the art. See, *e.g.*, U.S. Patent Nos. 6,558,924 and 8,691,755. C-peptide is also available from commercial sources, *e.g.*, Sigma-Aldrich® (St. Louis, MO; product number C5051).

[0045] In some embodiments, a C-peptide can be modified, *e.g.*, with a heterologous moiety. The heterologous moiety can be, *e.g.*, a heterologous polypeptide, a therapeutic agent (*e.g.*, a toxin or a drug), or a detectable label such as, but not limited to, a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a luminescent label, or an affinity tag such as biotin or streptavidin. Suitable heterologous polypeptides include, *e.g.*, an antigenic tag, *e.g.*, FLAG (DYKDDDDK; SEQ ID N0:8), polyhistidine (6-His; HHHHHH; SEQ ID N0:6), hemagglutinin (HA; YPYDVPDY A; SEQ ID N0:7), glutathione-S-transferase (GST), or maltose-binding protein (MBP)) for use in purifying the antibodies or fragments. Heterologous polypeptides also include polypeptides (*e.g.*, enzymes) that are useful as diagnostic or detectable markers, for example, luciferase, a fluorescent protein (*e.g.*, green fluorescent protein (GFP)), or chloramphenicol acetyl transferase (CAT). Suitable radioactive labels include, *e.g.*, 32P, 33P, 14C, 1251, 1311, 35 S, and 3H. Suitable fluorescent labels include, without limitation, fluorescein, fluorescein isothiocyanate (FITC), green fluorescent protein (GFP), DyLight™ 488, phycoerythrin (PE), propidium iodide (PI), PerCP, PE-Alexa Fluor® 700, Cy5, allophycocyanin, and Cy7. Luminescent labels include, *e.g.*, any of a variety of luminescent lanthanide (*e.g.*, europium or terbium) chelates. For example, suitable europium chelates include the europium chelate of diethylene triamine

pentaacetic acid (DTPA) or tetraazacyclododecane-1 ,4,7,10- tetraacetic acid (DOTA). Enzymatic labels include, *e.g.*, alkaline phosphatase, CAT, luciferase, and horseradish peroxidase.

[0046] Two molecules can be cross-linked using any of a number of known chemical cross linkers. Examples of such cross linkers are those that link two amino acid residues via a linkage that includes a "hindered" disulfide bond. In these linkages, a disulfide bond within the cross-linking unit is protected (by hindering groups on either side of the disulfide bond) from reduction by the action, for example, of reduced glutathione or the enzyme disulfide reductase. One suitable reagent, 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio) toluene (SMPT), forms such a linkage between two proteins utilizing a terminal lysine on one of the proteins and a terminal cysteine on the other. Heterobifunctional reagents that cross-link by a different coupling moiety on each protein can also be used. Other useful cross-linkers include, without limitation, reagents that link two amino groups (*e.g.*, N-5-azido-2-nitrobenzoyloxysuccinimide), two sulfhydryl groups (*e.g.*, 1,4-bis-maleimidobutane), an amino group and a sulfhydryl group (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester), an amino group and a carboxyl group (*e.g.*, 4-[p-azidosalicylamido]butylamine), and an amino group and a guanidinium group that is present in the side chain of arginine (*e.g.*, p-azidophenyl glyoxal monohydrate).

[0047] In some embodiments, a radioactive label can be directly conjugated to the amino acid backbone of a protein agent. Alternatively, the radioactive label can be included as part of a larger molecule (*e.g.*, ^{125}I in meta-[^{125}I]iodophenyl-N-hydroxysuccinimide ([^{125}I]miPNHS)) that binds to free amino groups to form meta-iodophenyl (miP) derivatives of relevant proteins (see, *e.g.*, Rogers et al. (1997) *J Nucl Med* 38:1221-1229) or chelate (*e.g.*, to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or diethylene triamine pentaacetic acid (DTPA)) which is in turn bound to the protein backbone. Methods of conjugating the radioactive labels or larger molecules/chelates containing them to the antibodies or antigen-binding fragments described herein are known in the art. Such methods involve incubating the proteins with the radioactive label under conditions (*e.g.*, pH, salt concentration, and/or temperature) that facilitate binding of the radioactive label or chelate to the protein (see, *e.g.*, U.S. Patent No. 6,001,329).

[0048] Methods for conjugating a fluorescent label (sometimes referred to as a "fluorophore") to a protein (*e.g.*, an antibody) are known in the art of protein chemistry. For example, fluorophores can be conjugated to free amino groups (*e.g.*, of lysines) or sulfhydryl groups (*e.g.*, cysteines) of proteins using succinimidyl (NHS) ester or tetrafluorophenyl (TFP) ester moieties attached to the fluorophores. In some embodiments, the fluorophores can be conjugated to a heterobifunctional cross-linker moiety such as sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). Suitable conjugation methods involve incubating an antibody protein, or fragment thereof, with the fluorophore under conditions that facilitate binding of the fluorophore to the protein. See, *e.g.*, Welch and Redvanly (2003) "Handbook of Radiopharmaceuticals: Radiochemistry and Applications," John Wiley and Sons (ISBN 0471495603).

[0049] In some embodiments, the heterologous moiety is joined to the C-peptide as a fusion protein (*e.g.*, an antigenic tag useful for detection of uptake of tag-bearing C-peptide).

[0050] The present technology provides the results of experiments showing that albumin (from any one of several species) facilitates binding of C-peptide by RBCs and thus enhances the sensitivity of C-peptide binding-related assays. The improved methods are useful for, among other things, detecting increased binding of C-peptide by RBCs from MS patients as, *e.g.*, a diagnostic or prognostic indicator.

C-peptide Binding Facilitators

[0051] As discussed above, the methods and compositions of the present technology employ a C-peptide binding facilitator. As used herein, the term "C-peptide binding facilitator" refers to a component that facilitates binding of C-peptide on a cell, such "binding" being the transfer, uptake, or other association of C-peptide in (*e.g.*, within the cell) or on (*e.g.*, on the outer surface or in the cell membrane) a cell. Thus, "binding" may include association of C-peptide to an outer surface of a cell membrane, binding to a receptor expressed on a cell membrane, or uptake of into a cell. Accordingly, in various embodiments, binding effects an association of C-peptide with a cell such that if the cell is removed from a solution, the molecule or atom remains associated with the cell. As noted above, without limiting the function or scope of the present technology, it has been found that the binding of C-peptide is enhanced in the presence of a C-peptide binding facilitator

and, in some situations, C-peptide does not bind to cells without use of a C-peptide binding facilitator. Moreover, it has been discovered that, in some embodiments, the binding of C-peptide to the cells may be affected by the presence of a disease state or disorder in the subject from whom the cells are obtained.

[0052] A person of ordinary skill in the art can easily determine whether a component of interest is a "C-peptide binding facilitator" by combining the component of interest, C-peptide, and cells, such as cells that express Glut1, together to form a mixture, incubating the mixture for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C, and determining whether a portion of the C-peptide binds to the cells. C-peptide binding to the cells can be performed by directly measuring C-peptide bound to the cells or C-peptide remaining in solution or indirectly measuring C-peptide bound to the cells or C-peptide remaining in solution, such as, for example, by measuring Zn^{2+}, Fe^{2+}, and/or Cr^{3+} associated with the C-peptide.

[0053] C-peptide binding facilitators useful herein include albumins, leptin, collagen, and mixtures thereof. As used herein, the term "albumin" or "serum albumin" includes all forms of the globular proteins known as albumins, as well as albuminoids, and variants thereof. Albumins among those useful herein may be native, naturally derived, synthetic or semi-synthetic, including albumins isolated from any species (and amino acid sequences for albumin proteins from a wide variety of species are known in the art and publicly accessible). Such albumin polypeptides may be derived from the tissues of humans or other animals, such as from whole blood or blood fractions obtained from human, cow, chicken or pig blood, or from the eggs of chickens or other fowl. In some embodiments, the human albumin has the amino acid sequence provided by SEQ ID NO:9. In some embodiments, the human albumin protein has the sequence provided by SEQ ID NO:10, which is a human albumin protein without its amino-terminal leader sequence.

[0054] In some embodiments, the albumin protein is a bovine albumin protein. In some embodiments, the bovine albumin protein comprises the amino acid sequence provided by SEQ ID NO:11. In some embodiments, the bovine albumin protein comprises or consists of the amino acid sequence of SEQ ID NO:12, which lacks a propeptide/leader sequence.

[0055] In some embodiments, the albumin protein is a chicken albumin protein. In some embodiments, the chicken albumin protein comprises or consists of the amino acid

sequence provided by SEQ ID NO:13. In some embodiments, the chicken albumin protein comprises or consists of the amino acid sequence provided by SEQ ID NO:14, which lacks the amino-termiano propeptide/leader sequence.

[0056] In some embodiments, the albumin protein is a porcine albumin protein. In some embodiments, the porcine albumin protein comprises or consists of the amino acid sequence provided by SEQ ID NO:15. In some embodiments, the porcine albumin protein comprises or consists of the amino acid sequence provided by SEQ ID NO:16, which lacks the amino-terminal propeptide/leader sequence.

[0057] In some embodiments, the albumin protein is a variant of a naturally-occurring albumin protein. For example, the albumin protein may, in some embodiments, consist of or comprise an amino acid sequence that is at least 70 (*e.g.*, at least 75, 80, 85, 90, 95, or 99) % identical to the amino acid sequence depicted in any one of SEQ ID NOs:9-16. In some embodiments, the C- peptide comprises or consists of an amino acid sequence that has no more than 40 (*e.g.*, no more than 39, 38, 37,36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16,15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1) amino acid substitutions, deletions, insertions, or a mixture of the above, relative to any one of SEQ ID NOs:9-16. The substitutions can be conservative, non-conservative, or a mixture of both. It is understood that albumin proteins described herein can be from a natural source or be produced recombinantly. The albumin proteins can comprise, in some embodiments, a heterologous moiety (*e.g.*, a detectable label), *e.g.*, via fusion protein or chemical conjugation (see above).

[0058] A variant form of wild-type albumin proteins retain at least 50 (*e.g.*, at least 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 65, 70, 75, 80, 85, 90, 95, 99, or even 100) % of the activity of the wild-type albumin protein from which it was derived. For example, a variant form of human albumin having 10 amino acid substitutions relative to SEQ ID NO:10 would retain at least 50% of the ability of wild-type human albumin of SEQ ID NO:10 to facilitate delivery of C-peptide to RBCs. Methods for measuring C-peptide binding to RBCs, binding of C- peptide by RBCs, and/or ATP release from RBCs are known in the art and described and exemplified herein.

[0059] Methods for making albumin protein, including recombinant methods, are well known in the art. See, *e.g.*, U.S. Patent Application Publication No. 20120165509, European Patent No. 0319067, and International Patent Application Publication No. WO

2014/094406. Albumin from many species is also available from commercial sources, *e.g.*, Sigma-Aldrich® (St. Louis, MO; product numbers A4503, A9511, and A1830).

Methods for Measuring C-peptide Binding by Cells

[0060] As discussed above, the present technology provides *in vitro* methods for measuring the binding of C-peptide by cells, comprising incubating the cells with C-peptide and an albumin or other C-peptide binding facilitator. As referred to herein, "measuring" (or "determining") refers to a qualitative or quantitative process to detect or measure (*e.g.*, quantitatively measure), directly or indirectly, the existence or degree of binding (as defined above) of C-peptide by a cell of interest. Such cells include cells obtained from any animal, preferably humans or another mammalian subject. In particular, as discussed below, such cells may be obtained from a human or other animal subject having, or suspected of having, disease disorder (*e.g.*, MS or other immune-mediated disease) associated with C-peptide. The cells are preferably cells that express Glut1, such as RBCs, macrophages, neutrophils, or endothelial cells.

[0061] As referred to herein, "incubating" refers to the contacting of cells with the C-peptide and C-peptide binding facilitator, preferably under conditions that allow for binding (as defined above) C-peptide by the cells. In various embodiments, incubating comprises forming a C-peptide cellular mixture comprising the cells with one or both of the C-peptide and the C-peptide binding facilitator. The mixture may be an intimate admixture of cells, C-peptide and C-peptide binding facilitator, or other formulation by which the cells are in sufficient contact with the C-peptide and C-peptide binding facilitator so as to allow binding of the C-peptide by the cells.

[0062] In various embodiments, a C-peptide cellular mixture is incubated under conditions (from about 5°C to about 50°C, *e.g.*, room temperature, and/or for at least 1, 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes, or from about 1 minute to about 24 hours) sufficient to allow binding and/or uptake of the C-peptide by the RBC forming an aqueous mixture. Following incubation, the RBCs (containing bound or internalized C-peptide) can be separated from the mixture (containing free C-peptide). Separation can be accomplished, *e.g.*, by way of centrifugation. In some embodiments, the RBCs are diluted to a 7% hematocrit, which is approximately 780 million cells per milliliter, for incubation with the C-peptide. However, it is understood that other dilutions are acceptable, such as hematocrits of from about 2% to about 20%.

[0063] As noted above and further discussed below, the cells may be contacted with a known amount of C-peptide, so as to facilitate indirect determination of C-peptide binding by the cells through direct measurement of C-peptide remaining in solution after incubation (*i.e.*, in the supernatant after the incubated cells are removed from the incubated C-peptide cellular mixture). Thus, a "known" amount is an amount of C-peptide that is quantified prior to formation of the C-peptide cellular mixture, such as by mixing a pre-determined amount of C-peptide with cells (and the C-peptide binding facilitator) to form the C-peptide cellular mixture.

[0064] In various embodiments, the method includes mixing from about 0.05 mL to about 20 mL, or from about 1 mL to about 5 mL of a cell suspension from a test subject (such as a red blood cell suspension having a hematocrit of from about 2% to about 20% or purified RBCs having a hematorcrit of from about 20% to about 90%) with from about 1 pmole to about 500 pmoles or from about 10 pmoles to about 50 pmoles of C-peptide, and from about 0.05% (w/v) to about 25% (w/v), or from about 1% (w/v) to about 10% (w/v) of a C-peptide binding facilitator. In another embodiment, cells, C-peptide, and a C-peptide binding facilitator are combined to generate an isotonic solution including the cells at a hematocrit of from about 2% to about 20%, from about 100 pM to about 1 mM or from about 500 pM to about 1000 nM C-peptide, and from about 0.05% (w/v) to about 25% (w/v), or from about 1% (w/v) to about 10% (w/v) of the C-peptide binding facilitator. The methods also includes incubating the isotonic solution at from about 5°C to about 50°C for from about 1 minute to about 24 hours and determining the amount of C-peptide bound to the cells. Obtaining RBCs from a subject (*e.g.*, a human) is a routine procedure and well- known to those of skill in the art (*e.g.*, using phlebotomy). For example, blood can be obtained from subjects by venipuncture using a syringe containing, *e.g.*, citrate or heparin. Horn et al. (1995) *J Physiol* 489(1):73-80. Isolated blood is then centrifuged at approximately 500g at room temperature (about 20°C) for 10 minutes, and the plasma, buffy coat, and uppermost layer of RBCs are removed by aspiration. The remaining RBCs can then be resuspended and, optionally, washed in a physiologic buffer, *e.g.*, containing (in mM) 21.0 Tris, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgS0_4$, 5.5 glucose, and 0.5% bovine serum albumin (BSA) with the pH adjusted to 7.4 (or other suitable buffer or wash solution). After the final wash, the hematocrit of the isolated RBCs can be measured. RBCs can be prepared on the day of use in the assay or stored until the day of use in the assay. See, *e.g.*, Richards et al. (2013) *Am J Physiol Regul Integr Camp*

Physiol 305(11):R862- 868. In some embodiments, an assay is performed up to a step of separating cells from a supernatant and the supernatant is stored for further processing at a later time.

[0065] As noted above, determining the binding of C-peptide may be assessed by direct measurement of binding of C-peptide by cells, or indirectly such as through measuring the amount of C-peptide that is not bound by the cells relative to a known amount of C-peptide with which the cells are mixed to form the incubated cellular mixture. For example, measuring uptake and/or binding of C-peptide to RBCs can be accomplished, *e.g.*, by detecting or otherwise measuring the amount of C-peptide bound to the RBCs, measuring or detecting the amount of free C-peptide remaining in the mixture, or, as described below, measuring the ATP release from the RBCs. In some embodiments, the amount of free C-peptide is measured against the starting amount of C-peptide added to the mixture. In some embodiments, the amount of C-peptide bound to, or internalized by, the RBCs can be detected or measured (*e.g.*, by fluorescence-assisted cell sorting (FACS)/flow cytometry, or by lysis of the RBCs and detection or measurement of C-peptide associated with the lysate).

[0066] Methods for detecting or quantifying C-peptide (*e.g.*, labeled or unlabeled C-peptide) are known in the art. For example, free C-peptide can be detected and/or quantified using a variety of techniques such as, but not limited to, BioLayer Interferometry (BLI), Western blot, dot blot, surface plasmon resonance method (SPR), enzyme-linked immunosorbent assay (ELISA), AlphaScreen® or AlphaLISA® assays, or mass spectrometry based methods. The capture or detection reagent can be, *e.g.*, an antibody that binds to C-peptide (*e.g.*, an epitope of C-peptide that is conserved across several species), an antibody that binds to an epitope tag (*e.g.*, a flag, his, or HA tag, in embodiments in which a C-peptide comprising such a tag is used). Antibodies that bind to C-peptide are known in the art and described in, *e.g.*, EP1191337 and U.S. Patent Application Publication No. 20050037448. Commercially available anti-C-peptide antibodies include, *e.g.*, 5B8 (AbD Serotec®). Methods for making antibodies that bind to C-peptide are also well known in the art.

[0067] A variety of immunoassay techniques, including competitive and non-competitive immunoassays, can be used. The term "immunoassay" encompasses techniques including, without limitation, flow cytometry, FACS, enzyme immunoassays (EIA), such as enzyme multiplied immunoassay technique (EMIT), enzyme-linked

immunosorbent assay (ELISA), IgM antibody capture ELISA (MAC ELISA) and microparticle enzyme immunoassay (MEIA), furthermore capillary electrophoresis immunoassays (CEIA), radio-immunoassays (RIA), immunoradiometric assays (IRMA), fluorescence polarization immunoassays (FPIA) and chemiluminescence assays (CL). If desired, such immunoassays can be automated.

[0068] Immunoassays can also be used in conjunction with laser induced fluorescence. Liposome immunoassays, such as flow-injection liposome immunoassays and liposome immunosensors, are also suitable for use in the present invention. In addition, nephelometry assays, in which, for example, the formation of protein/antibody complexes results in increased light scatter that is converted to a peak rate signal as a function of the marker concentration, are suitable for use in the methods of the present invention. In a preferred embodiment of the present invention, the incubation products are detected by ELISA, RIA, fluoro immunoassay (FIA) or soluble particle immune assay (SPIA).

[0069] In some embodiments, detecting or measuring the amount of C-peptide uptake can be accomplished by measuring ATP release by the RBCs contacted with C-peptide (and, optionally, Zn^{2+}, Fe^{2+}, and/or Cr^{3+}). Methods for measuring ATP release by RBCs are known in the art and described in, *e.g.*, Faris and Spence (2008) *Analyst* 133(5):678-682; Fischer et al. (2003) *Analyst* 128(9):1163-1168; Petruzzi et al. *(1994)Metabalism* 43(4):435-440; Richards et al. (2013) *Am J Physiol Regul Integr Comp* Physiol 305(11):R1331-1336; and Richards et al. (2014) *Am J Physiol Regul Integr Comp* Physiol 307(7):R862-868. For example, ATP can be measured using a luciferin-luciferase assay. RBCs contacted with C-peptide, albumin and Zn^{2+}, Fe^{2+}, and/or Cr^{3+} can be added to a cuvette containing firefly lantern extract (*e.g.*, 10 mg/ml distilled water, FLE 250; Sigma-Aldrich®, St. Louis, MO) and d-luciferin solution (*e.g.*, 10 mg/20 ml distilled water; Research Products International, Mount Prospect, IL). The light emitted from the reaction with ATP can be quantified using a luminometer. A standard curve can be generated for each experiment and ATP values can be normalized to the amount released from a given number of cells. Richards et al. (2013) *Am J Physiol Regul Integr Comp* Physiol 305(11):R1331-1336. Fig. 3 shows a bar graph depicting results from an ATP release study.

[0070] In some embodiments, *e.g.*, those in which RBCs are contacted with C-peptide and Zn^{2+}, Fe^{2+}, and/or Cr^{3+}, in the presence of albumin or other C-peptide binding

facilitator, Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake by RBCs can be measured (*e.g.*, as a proxy for C-peptide uptake). Methods for detecting or measuring Zn^{2+} uptake by RBCs are known in the art and described in, *e.g.*, Horn et al. (1995) *J Physiol* 489(1):73-80 (describing the use of radiolabeled Zn^{2+}). Fig. 4 shows a bar graph that shows a difference in Zn^{2+} binding between control cells and cells from MS patients.

[0071] In various embodiments, the present technology provides methods for measuring C-peptide binding by cells. The method includes: contacting cells with a known amount of C-peptide and a C-peptide binding facilitator to form a C-peptide cellular mixture; incubating the C-peptide cellular mixture to produce incubated cells in an incubated cellular mixture; and determining the amount of C-peptide bound to the incubated cells. As discussed below, such methods can be used to analyze the risk of developing a disorder in a mammalian subject or to determine the likelihood that a mammalian subject has a disorder. Determining an amount of C-peptide bound to the incubated cells includes separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and measuring: (i) the amount of C-peptide in the incubated cellular fraction; or (ii) the amount of C-peptide in the supernatant; or (iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or (iv) any combination of (i), (ii), and (iii).

[0072] All of the above embodiments are suitable for development into high-throughput platforms.

Diagnostic Methods

[0073] The present technology also provides methods for managing, or providing information for use in managing, the health care of human or other animal subjects. As discussed above, it has been found that binding of C-peptide by the cells of a human or other animal subject may be altered (*e.g.*, increased) in some disease states or disorders. For example, it has been found that the binding of C-peptide by cells (*e.g.*, RBCs) in subjects having MS is increased relative to the binding of C-peptide by cells in normal subjects (*i.e.*, subjects not having MS). Accordingly, the present technology provides compositions and methods for use in detecting or diagnosing such diseases or disorders, and for use in the course of treatment of such diseases and disorders. Such methods

include any of the methods for measuring C-peptide binding by cells described above and elsewhere in this disclosure.

[0074] Diseases and disorders among those for which the methods of this technology may be used include, in various embodiments, immune-mediated diseases and/or autoimmune diseases. As used herein, the term "immune-mediated disease" includes diseases and other disorders that are associated with an abnormal immunological response, including autoimmune diseases. As used herein, the term "autoimmune disease" means a disease resulting from an immune response against a self-tissue or tissue component, including both self-antibody responses and cell-mediated responses. Such diseases include organ-specific autoimmune diseases, in which an autoimmune response is directed against a single tissue, such as Crohn's disease, ulcerative colitis, myasthenia gravis, vitiligo, Graves' disease, Hashimoto's disease, Addison's disease and autoimmune gastritis and autoimmune hepatitis, and inflammatory demyelinating disorders. Autoimmune diseases also include non-organ specific autoimmune diseases, in which an autoimmune response is directed against a component present in several or many organs throughout the body. Such autoimmune diseases include, for example, rheumatoid disease, systemic lupus erythematosus, progressive systemic sclerosis and variants, polymyositis and dermatomyositis. Additional autoimmune diseases include pernicious anemia including some of autoimmune gastritis, primary biliary cirrhosis, autoimmune thrombocytopenia, Sjogren's syndrome, MS, and psoriasis. One skilled in the art understands that the methods of the invention can be applied to these or other autoimmune diseases, as desired. In various embodiments, the methods of the present technology are used in assessing an inflammatory demyelinating disorder, such as MS. In various embodiments, the MS is relapsing remitting MS or chronic progressive MS, *e.g.*, primary-progressive, secondary progressive, or progressive relapsing MS.

[0075] For example, the present technology provides methods for assessing the status of an immune-mediated disease (*e.g.*, MS) in a human or other animal subject. Such "assessing the status" includes methods to assess the risk of, diagnose, or monitor an immune-mediated disease associated with C-peptide in subjects having an immune-mediated disease or at risk of having an immune-mediated disease, as well as to provide information useful in such assessment, diagnosis or monitoring.

[0076] In some embodiments of any of the methods described herein, the subject is one suspected of having an immune-mediated disease or at risk for developing an

immune-mediated disease. In some embodiments, the subject is suspected of having, or is at risk for developing, MS. In some embodiments, any of the methods described herein can be used to assess the status (*e.g.*, diagnose) of an immune-mediated disease in a subject as having an immune-mediated disease (*e.g.*, MS), even when the subject is in clinical remission (*e.g.*, smoldering disease).

[0077] Accordingly, the present technology provides methods for assessing the probability that a mammalian subject has an immune-mediated disease. The methods includes contacting cells (*e.g.*, RBCs) from a subject with a detection reagent in a constricted environment, such that the RBCs release ATP and the detection reagent facilitates detection of the ATP. RBCs from mammalian subjects that have an immune-mediated disease or are at risk of developing an immune-mediated disease, such as MS, release at least 2 fold more ATP relative to RBCs from normal mammalian subjects that do not have an immune-mediated disease or are not at risk of developing an immune-mediated disease. No C-peptide is required for this particular method. Accordingly, blood from a mammalian subject that generates greater than or equal to 2 fold more ATP relative to ATP released from normal blood obtained from a normal subject that does not have and is not at risk of developing an immune-mediated disease indicates a high probability that the mammalian subject has an immune-mediated disease, such as MS.

[0078] In other aspects, the present technology provides methods for diagnosing a disorder, such as MS, in a mammalian subject, comprising obtaining cells from the subject and performing an *in vitro* method for measuring the binding of C-peptide by cells according to the present technology. Also provided are methods for analyzing the risk of developing a disorder, such as MS, in a mammalian subject, comprising obtaining cells from the subject and performing an *in vitro* method for measuring the binding of C-peptide by cells according to the present technology. Also provided are methods, for determining the likelihood that a mammalian subject has a disorder, such as MS, comprising obtaining cells from the subject and performing an *in vitro* method for measuring the binding of C-peptide by cells according to the present technology.

[0079] In various embodiments, the present technology provides *in vitro* methods for assessing the probability that a mammalian subject has an immune-mediated disease, the method comprising:

incubating cells that express Glut1 obtained from the subject with a C-peptide composition comprising an amount, *e.g.*, a known amount, of C-peptide and an albumin

under conditions that allow for the cells to bind the C-peptide to form incubated cells in an incubated cellular mixture;

separating the incubated cells from the incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells. As discussed above, in various embodiments, determining the binding of C-peptide may be assessed by direct measurement of binding of C-peptide by cells, or indirectly such as through measuring the amount of C-peptide that is not bound by the cells.

[0080] In various embodiments, a C-peptide binding of greater than or equal to about 2000, greater than or equal to about 2100, greater than or equal to about 2200, greater than or equal to about 2300, greater than or equal to about 2400, greater than or equal to about 2500, greater than or equal to about 2600, greater than or equal to about 2700, greater than or equal to about 2800, greater than or equal to about 2900, or greater than or equal to about 3000 C-peptide molecules per cell indicates a high probability that the test subject has an immune-mediated disease, such as MS. In other embodiments, a C-peptide binding of from about 2300 to about 2800 C-peptide molecules per cell indicates a high probability that the test subject has an immune-mediated disease, such as MS. C-peptide binding in healthy individuals that do not have MS is about 1500 C-peptide molecules per cell.

[0081] As referred to herein, such methods of "assessing the probability" provide information that may be useful by a health care provider (*e.g.*, physician) in diagnosing, monitoring or treating a subject as currently having, or being at risk of having, an immune-mediated disease. In some embodiments, a method for assessing the probability is performed by a technician or other medical practitioner (*e.g.*, in a diagnostic laboratory), providing information regarding the amount of C-peptide bound to incubated cells which is then used by a physician or other health care provider to diagnose, monitor or treat a subject having, or at risk of having, an immune-mediated disease. Such information may be used, together with other diagnostic information, to determine whether a subject actually has the immune-mediated disease or whether the subject is at risk of developing the immune-mediated disease. Thus, in some embodiments, the methods of the present technology further comprise receiving a request, *e.g.*, by a diagnostic technician from a physician or other health care provider, to determine whether a subject has an immune-mediated disease.

[0082] Thus, in some embodiments, the present technology provides methods for determining whether a subject has an immune-mediated disease, such as MS. An exemplary method for detecting the amount involves, optionally, obtaining RBCs from a subject and contacting the red bloods, in the presence of albumin or other C-peptide binding facilitator (and optionally Zn^{2+}, Fe^{2+}, and/or Cr^{3+}), with a C-peptide, such as a human C-peptide, and: (1) detecting or measuring the amount of C-peptide bound to the RBCs, (2) measuring or detecting the amount of free C-peptide remaining in the mixture; (3) detecting or measuring the ATP release from the RBCs; and/or (4) detecting or measuring Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake by the RBCs. An increased amount of C-peptide uptake by the RBCs, a reduced amount of free C-peptide, an increase in ATP release by RBCs, and/or an increase in Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake by the RBCs, indicates that the subject has an immune-mediated disease.

[0083] In some embodiments, the amount of C-peptide bound to the incubated cells is compared to a reference amount determined by performing the incubating, separating and determining using cells obtained from a second subject that does not have the immune-mediated disease. In some embodiments, an amount of C-peptide bound to the incubated cells greater than or equal to about a factor of from about 1.2 to about 5 fold (*e.g.*, about 1.5 fold) relative to the reference amount is indicative of a high probability of the subject currently having the immune-mediated disease

[0084] In some embodiments, an increase in C-peptide uptake of at least 10 (*e.g.*, at least 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more than 100)% over a normal control level (*e.g.*, normal control C-peptide uptake level) indicates that the subject has an immune-mediated disease. In some embodiments, an increase in C-peptide uptake of at least 1.5 (*e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40) fold over a normal control level (*e.g.*, normal control C-peptide uptake level) indicates that the subject has an immune-mediated disease.

[0085] The term "control" refers to any reference standard suitable to provide a comparison to the test sample. As described above, the methods described herein can involve comparing the C-peptide binding, remaining free C-peptide, Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake, or ATP release to a control amount. In some embodiments, the control is a control sample obtained from a normal, healthy subject of the same species who does not have, is not suspected of having, and/or is not at risk for developing an immune-mediated disease, such as, for example, MS. In some embodiments, the control can be (or can be

based on), *e.g.*, a collection of samples obtained from two or more (*e.g.*, two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) healthy individuals (*e.g.*, a mean or median level). In some embodiments, the control can be (or can be based on), *e.g.*, one sample or a collection of samples obtained from two or more (*e.g.*, two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) individuals (*e.g.*, a mean or median level) determined to be in clinical remission of an immune-mediated disease (*e.g.*, MS). In some embodiments, the control amount is detected or measured concurrently with the test sample. In some embodiments, the control level or amount is a pre-determined range or threshold based on, *e.g.*, average levels from a control group (*e.g.*, normal healthy volunteer subjects). Thus, a normal control C-peptide uptake level can be the C-peptide uptake level determined from a RBCs obtained from a healthy subject of the same species. A normal control C-peptide uptake level can be the mean, or a range of values around the mean, of obtained from C-peptide uptake measurements from two or more normal healthy subjects of the same species as the subject of interest. In some embodiments, the normal control C-peptide uptake level is a threshold value (*e.g.*, determined based on the average C-peptide uptake levels from subjects with a particular immune-mediated disease, *e.g.*, MS) or a particular form of an immune-mediated disease (secondary progressive MS), above which indicates that a subject has an immune-mediated disease.

[0086] In some embodiments, the control is a control sample obtained from a subject of the same species who has, is suspected of having, and/or is at risk for developing an immune-mediated disease (*e.g.*, immune-mediated disease, such as MS). In some embodiments, the control can be (or can be based on), *e.g.*, a collection of samples obtained from two or more (*e.g.*, two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) individuals of the same species (*e.g.*, a mean or median level) who have an immune-mediated disease. Thus, an MS control C-peptide uptake level can be the C-peptide uptake level determined from RBCs obtained from a subject of the same species who has MS. An MS control C-peptide uptake level can be the mean, or a range of values around the mean, of obtained from C-peptide uptake measurements from two or more immune-mediated disease-afflicted subjects of the same species as the subject of interest.

[0087] The methods of the present invention are not limited to use of a specific cut-point in comparing a level (*e.g.*, C-peptide uptake level) in the test sample to the

control. Without limiting the scope or function of the present technology, this may be seen, for example, from the data presented below.

[0088] In some embodiments, the methods further involve obtaining a control biological sample (*e.g.*, biological sample from a subject who does not have an immune-mediated disease) or a biological sample from the subject during treatment to monitor the regression or further progression of the immune-mediated disease.

[0089] In some embodiments, the methods described herein can be used to determine whether a subject with an immune-mediated disease is responsive to treatment, as further described below. For example, C-peptide can be incubated, in the presence of a C-peptide binding facilitator (*e.g.*, albumin), with cells (*e.g.*, RBCs) from a subject under conditions that allow for binding of C-peptide by RBCs, wherein the subject has an immune-mediated disease, such as MS, and is being treated with an immunosuppressant or anti-inflammatory agent; and determining the amount of binding of C-peptide by the RBCs, wherein decreased binding of C-peptide by the RBCs, relative to a control C-peptide uptake level (*e.g.*, an MS control C-peptide uptake level), indicates that the subject is responsive to treatment with the immunosuppressant or the anti-inflammatory agent. (One of skill in the art would appreciate based on the present technology that variations of such a method involving Zn^{2+}, Fe^{2+}, and/or Cr^{3+} uptake and/or ATP release would also be useful for monitoring responsiveness.)

[0090] In addition to monitoring disease progression using the methods described herein, an MS therapy, *e.g.*, can be deemed to be effective if there is a statistically significant difference in the rate or proportion of exacerbation-free or relapse-free patients between the treated group and the placebo group for either of these measurements. In addition, time to first exacerbation and exacerbation duration and severity may also be measured. A measure of effectiveness as therapy in this regard is a statistically significant difference in the time to first exacerbation or duration and severity in the treated group compared to control group. An exacerbation-free or relapse- free period of greater than one year, 18 months, or 20 months is particularly noteworthy.

[0091] Clinical measurements include the relapse rate in one and two-year intervals, and a change in EDSS (expanded disability status scale), including time to progression from baseline of 1.0 unit on the EDSS that persists for six months. On a Kaplan-Meier curve, a delay in sustained progression of disability shows efficacy. Other criteria include a change in area and volume of T2 images on MRI, and the number and

volume of lesions determined by gadolinium enhanced images. MRI can be used to measure active lesions using gadolinium-diethylenetriamine pentaacetic acid (DTPA)-enhanced imaging (McDonald et al., Ann. Neurol. 36:14, 1994) or the location and extent of lesions using T2 -weighted techniques. Briefly, baseline MRIs are obtained. The same imaging plane and patient position are used for each subsequent study. Positioning and imaging sequences can be chosen to maximize lesion detection and facilitate lesion tracing. The same positioning and imaging sequences can be used on subsequent studies. The presence, location and extent of MS lesions can be determined by radiologists. Areas of lesions can be outlined and summed slice by slice for total lesion area. Three analyses may be done: evidence of new lesions, rate of appearance of active lesions, percentage change in lesion area (Paty et al. (1993) Neurology 43:665). Improvement due to therapy can be established by a statistically significant improvement in an individual patient compared to baseline or in a treated group versus a placebo group.

[0092] The present technology also provides *in vitro* methods for diagnosing a mammalian subject as having an immune-mediated disease, the method comprising:

incubating, in the presence of albumin, RBCs from the subject with C- peptide under conditions that allow for binding of C-peptide by RBCs; and

determining the amount of C-peptide bound by the RBCs, wherein increased binding of C-peptide by the RBCs, relative to a control C-peptide binding level, indicates that the subject has the immune-mediated disease.

In various embodiments, the immune-mediated disease is an inflammatory demyelinating disorder, such as MS. In some aspects, such *in vitro* methods for diagnosing a subject as having an immune-mediated disease. Such method comprise: contacting, in the presence of albumin, RBCs from a subject of interest with C-peptide to form a mixture; incubating the mixture under conditions that allow for binding of C-peptide by RBCs; and determining the amount of binding of C-peptide by the RBCs. An increased binding of C-peptide by the RBCs, relative to a control C-peptide uptake level (*e.g.*, a normal control C-peptide uptake level), indicates that the subject has an immune-mediated disease.

[0093] In some embodiments, *in vitro* methods for diagnosing a subject as having an immune-mediated disease, comprise: contacting, in the presence of albumin, RBCs from a subject of interest with C-peptide to form a mixture; incubating, in the presence of albumin, RBCs from a subject of interest with C-peptide under conditions that allow for binding of C-peptide by RBCs; contacting free C-peptide with an agent that specifically

binds to the free C-peptide to form an agent-C-peptide complex; and applying a detection reagent that detects the agent-C-peptide complex to thereby determine the amount of free C-peptide, wherein a decreased amount of free C-peptide, relative to a control free C-peptide level (*e.g.*, a normal control free C-peptide level), indicates that the subject has an immune-mediated disease.

[0094] In some embodiments, *in vitro* methods for diagnosing a subject as having MS comprise: incubating, in the presence of albumin, RBCs from a subject of interest with C-peptide under conditions that allow for binding of C-peptide by RBCs; and determining the amount of uptake of C-peptide by the RBCs, wherein increased binding of C-peptide by the RBCs, relative to a normal control C-peptide uptake level, indicates that the subject has MS.

[0095] The present technology also provides methods for effecting an assessment of the status of an immune-mediated disease (*e.g.*, MS). Such effected methods include those of the present technology associated with the management of an immune-mediated disease, *e.g.*, methods for analyzing the risk of developing a disorder, methods for diagnosing a disorder, and methods for determining the likelihood that a subject has a disorder, as described above. As referred to herein, such "effecting" includes performing such methods, or causing such methods to be performed. Thus, in some embodiments, effecting such methods may be performed by a physician or other health care professional directly or indirectly, wherein (for example) the *in vitro* a method for measuring the binding of C-peptide by cells according to the present technology is performed by another health care provider such as a diagnostic lab.

Methods of Treatment

[0096] Also provided herein are methods for treating a subject having an immune-mediated disease comprising performing or effecting an *in vitro* method of measuring the C-peptide binding of cells according to the present technology. Such methods comprise may one or more methods for management of an immune-mediated disease, *e.g.*, methods for analyzing the risk of developing a disorder, methods for diagnosing a disorder, and methods for determining the likelihood that a subject has a disorder, as described above.

[0097] In various embodiments, such methods for treating an immune-mediated disease comprise methods of treating such disorders among those known in the art. For example, a medical professional can administer to a subject an anti-inflammatory

compound or an immunosuppressant in an amount effective to treat an immune-mediated disease, wherein the subject has been diagnosed as having the immune-mediated disease by any of the *in vitro* methods described herein. In some embodiments, the professional can request the results of a test used to determine whether the subject has an immune-mediated disease, which test is, or includes, one of the *in vitro* methods for measuring C-peptide binding described herein. In some embodiments, the subject has been diagnosed as having MS. In some embodiments, the subject has been diagnosed as having relapsing-remitting MS, secondary progressive MS, primary progressive MS, or progressive relapsing MS. As used herein, a subject can be a human, a non-human primate (*e.g.*, monkey, baboon, or chimpanzee), a horse, a cow, a pig, a sheep, a goat, a dog, a cat, a rabbit, a guinea pig, a gerbil, a hamster, a rat, or a mouse. In some embodiments, the mammal is an infant (*e.g.*, a human infant).

[0098] As used herein, a subject "in need of prevention," "in need of treatment," or "in need thereof," refers to one, who by the judgment of an appropriate medical practitioner (*e.g.*, a doctor, a nurse, or a nurse practitioner in the case of humans; a veterinarian in the case of non-human mammals), would reasonably benefit from a given treatment.

[0099] The term "preventing" is art-recognized, and when used in relation to a condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject mammal relative to a subject which does not receive the composition.

[0100] As used herein, a subject "at risk for developing" an immune-mediated disease is a subject having one or more (*e.g.*, two, three, four, five, six, seven, or eight or more) risk factors for developing an immune-mediated disease. A subject "suspected of having" an immune-mediated disease is one having one or more symptoms of the immune-mediated disease. It should be understood that mammal at risk for developing, or suspected of having, an immune-mediated disease does not include all mammals within the species of interest.

[0101] Exemplary symptoms associated with MS, which can be treated with the methods described herein, include: optic neuritis, diplopia, nystagmus, ocular dysmetria, internuclear ophthalmoplegia, movement and sound phosphenes, afferent pupillary defect, paresis, monoparesis, paraparesis, hemiparesis, quadraparesis, plegia, paraplegia,

hemiplegia, tetraplegia, quadraplegia, spasticity, dysarthria, muscle atrophy, spasms, cramps, hypotonia, clonus, myoclonus, myokymia, restless leg syndrome, footdrop, dysfunctional reflexes, paraesthesia, anaesthesia, neuralgia, neuropathic and neurogenic pain, proprioceptive dysfunction, trigeminal neuralgia, ataxia, intention tremor, dysmetria, vestibular ataxia, vertigo, speech ataxia, dystonia, dysdiadochokinesia, bladder spasticity, flaccid bladder, detrusor- sphincter dyssynergia, erectile dysfunction, frigidity, constipation, fecal urgency, fecal incontinence, depression, cognitive dysfunction, dementia, mood swings, emotional lability, euphoria, bipolar syndrome, anxiety, aphasia, dysphasia, fatigue, uhthoff s symptom, gastroesophageal reflux, and sleeping disorders. See, *e.g.*, International Patent Application Publication No. 2006/055871.

[0102] Suitable therapeutics for treating immune-mediated diseases include those known in the art. For example, treatments for MS include an anti-alpha 4 integrin antibody, interferon-beta-la (*e.g.*, Avonex®, Rebif®), Cinno Vex), or a derivative ofinterferon-beta-1a. Suitable therapies also include, but are not limited to, natalizumab (Tysabri®), peginterferon (Plegridy™), glatiramer acetate (Copaxone®), fingolimod (Gilenya®), or alemtuzumab (Campath®). Any of the methods described herein can also include treatments, such as interferon beta-1b (Betaseron®), mitoxantrone (Novantrone®), teriflunomide (Aubagio®), and dimethyl fumarate (BG 12, Tecfidera®).

[0103] The compositions described herein can be administered to a subject, *e.g.*, a human subject, using a variety of methods that depend, in part, on the route of administration. The route can be, *e.g.*, intravenous injection or infusion (IV), subcutaneous injection (SC), intraperitoneal (IP) injection, or intramuscular injection (IM).

[0104] Administration can be achieved by, *e.g.*, local infusion, injection, or by means of an implant. The implant can be of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. The implant can be configured for sustained or periodic release of the composition to the subject. See, *e.g.*, U.S. Patent Application Publication No. 20080241223; U.S. Patent Nos. 5,501,856; 4,863,457; and 3,710,795; EP488401; and EP 430539, the disclosures of each of which are incorporated herein by reference in their entirety. The composition can be delivered to the subject by way of an implantable device based on, *e.g.*, diffusive, erodible, or convective systems, *e.g.*, osmotic pumps, biodegradable implants, electrodiffusion systems, electroosmosis systems, vapor pressure pumps, electrolytic pumps, effervescent pumps, piezoelectric pumps, erosion-based systems, or electromechanical systems.

[0105] As used herein the term "effective amount" or "therapeutically effective amount", in an in vivo setting, means a dosage sufficient to treat, inhibit, or alleviate one or more symptoms of the disorder being treated or to otherwise provide a desired pharmacologic and/or physiologic effect (*e.g.*, modulate (*e.g.*, enhance) an immune response to an antigen. The precise dosage will vary according to a variety of factors such as subject-dependent variables (*e.g.*, age, immune system health, etc.), the disease, and the treatment being effected. Therapeutically effective amounts of the agents disclosed herein enhance an immune response by a mammal to a target antigen. Suitable human doses of any of the antibodies or fragments thereof described herein can further be evaluated in, *e.g.*, Phase I dose escalation studies. See, *e.g.*, van Gurp et al. (2008) *Am J Transplantation* 8(8):1711-1718; Hanouska et al. (2007) *Clin Cancer Res* 13(2, part 1):523-531; and Hetherington et al. (2006) *Antimicrobial Agents and Chemotherapy* 50(10):3499-3500.

[0106] Toxicity and therapeutic efficacy of such compositions can be determined by known pharmaceutical procedures in cell cultures or experimental animals (*e.g.*, animal models of cancer, vaccination, or infection). These procedures can be used, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Agents that exhibits a high therapeutic index is preferred. While compositions that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue and to minimize potential damage to normal cells and, thereby, reduce side effects.

[0107] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such antibodies or antigen- binding fragments thereof lies generally within a range of circulating concentrations of the antibodies or fragments that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. A therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the antibody which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine

useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. In some embodiments, *e.g.*, where local administration is desired, cell culture or animal modeling can be used to determine a dose required to achieve a therapeutically effective concentration within the local site.

[0108] Accordingly, in various embodiments, the present technology provides methods for treating a subject having an immune-mediated disease, comprising administering to the subject an anti-inflammatory compound or an immunosuppressant in an amount effective to treat the immune-mediated disease, wherein the subject has been diagnosed as having the immune-mediated disease by any of the *in vitro* methods described herein. In another aspect, the present technology features a method for treating a subject having an immune-mediated disease. The method comprises: (i) requesting the results of a test to determine whether the subject has an immune-mediated disease, which test utilizes any of the *in vitro* methods described herein; and (ii) administering to the subject an anti-inflammatory compound or an immunosuppressant in an amount effective to treat the immune-mediated disease, if the results of the test indicate that the subject has an immune-mediated disease, such as MS.

[0109] In some embodiments, the present technology provides methods for determining whether a subject with MS is responsive to a treatment for MS, comprising: incubating, in the presence of albumin, RBCs from a subject with C-peptide under conditions that allow for binding of C-peptide by RBCs, wherein the subject has MS and is being treated with an immunosuppressant or anti-inflammatory agent; and determining the amount of binding of C-peptide by the RBCs, wherein decreased binding of C-peptide by the RBCs, relative to a control C-peptide uptake level (*e.g.*, an MS control C-peptide uptake level), indicates that the subject is responsive to treatment with the immunosuppressant or the anti-inflammatory agent.

[0110] In various embodiments, the present technology provides methods of managing MS in a subject in need thereof, the method comprising:

(a) effecting an assay for measuring C-peptide binding on cells obtained from the subject, the assay comprising

(i) incubating the cells with a C-peptide composition comprising a known amount of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

(ii) determining the amount of C-peptide bound to the incubated cells.

(b) comparing the amount of C-peptide bound by the cells to a reference amount determined by performing the assay on cells obtained from a second subject that does not have the immune-mediated disease;

(c) administering a first treatment for MS; and

(d) assessing the efficacy of the administering by repeating the effecting and comparing.

In some embodiments, such methods further comprise administering a second treatment of MS after the assessing, wherein the second treatment is altered from the first treatment based on the results of the assessing. The present technology also provides methods of assessing the responsiveness of a mammalian subject having MS to a treatment for the MS.

Kits

[0111] The present technology also provides kits for use in the methods of the present technology, as well as methods for using such kits. In various embodiments, such a "kit" is any manufacture (*e.g.*, a package or container) comprising at least one reagent, *e.g.*, an albumin protein, an C-peptide (*e.g.*, detectably-labeled C-peptide), Zn^{2+}, Fe^{2+}, and/or Cr^{3+} (*e.g.*, solid or an aqueous solution of $ZnCl_2$), or an antibody that binds to C-peptide, for detecting C-peptide binding, free C-peptide, Zn^{2+}, Fe^{2+}, and/or Cr^{3+} binding, or ATP release, *e.g.*, for the diagnosis of an immune-mediated disease. In some embodiments, the present technology provides kits for use measuring C-peptide binding by cells, the kit comprising a C-peptide binding facilitator, C-peptide, and a container. In various embodiments, a kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. Fig. 2 shows an exemplary kit composition according to the present technology.

[0112] In some embodiments, the container is operable for mixing a solvent with one or both of the C-peptide binding facilitator and the C-peptide, and may be operable for mixing one or both of the C-peptide binding facilitator and the C-peptide with a suspension of cells.

[0113] In some embodiments, the container is a first container containing the C-peptide binding facilitator, and the kit further comprises a second container containing C-peptide. The kit may further comprise a third container containing a solvent for one or both of the C-peptide binding facilitator and the C-peptide. In some embodiments, one or

both of the C-peptide and the C-peptide binding facilitator are in powder form, preferably as lyophilized powders. The solvent may comprise phosphate buffered saline, physiological saline solution, Tris buffer, or phosphate buffer. In some embodiments, a kit comprises a fourth container, wherein components of the first, second and third containers are mixed, optionally with a suspension of cells. In some aspects, the kits are used in methods comprising transferring a predetermined volume of the solvent in the third container to one of the first container or the second container and dissolving the contents of the first container or the second container in the solution to form a first working solution;

transferring the partial working solution to the other of the first container or the second container and dissolving the contents of the other of the first container or the second container to form a second working solution;

incubating a suspension of the cells with the second working solution to generate a C-peptide cellular mixture under conditions that allow for the cells to bind the C-peptide.

[0114] In certain embodiments, the kit may further comprise a reference standard (normal RBCs) and/or one or more suitable buffers (*e.g.*, phosphate buffered saline or other physiological buffers). Reagents in the kit may be provided in individual containers or as mixtures of two or more reagents in a single container. In some embodiments, the kits include at least two containers, wherein one container comprises C-peptide (*e.g.*, detectably-labeled C-peptide) and at least one container comprises albumin. In some embodiments, the kit comprises a container comprising Zn^{2+}, Fe^{2+}, and/or Cr^{3+}. Albumin or C-peptide can be provided in aqueous form or lyophilized. In some embodiments, the kits comprise one or more buffers for use in rehydrating a lyophilized product. In addition, instructional materials which describe the use of the compositions within the kit can be included.

[0115] In some embodiments, the kit comprises a device that is operable for obtaining RBCs from a subject (*e.g.*, a syringe or a lancet). In some embodiments, the kit may comprise an analytical device or reagent for detecting or measuring C-peptide, such as an anti-C-peptide antibody or an antibody that binds to a specific epitope tag, where the C-peptide is epitope-tagged, such as discussed above.

Reagents

[0116] The present technology also provides reagents for use in measuring C-peptide binding to cells, such as cells that express Glut1. In some embodiments, a reagent consists essentially of peptide binding facilitator (*e.g.*, albumin), dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of C-peptide, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof. In some embodiments, a reagent consists essentially of C-peptide dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of a C-peptide binding facilitator (*e.g.*, albumin), a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof.

[0117] Reagents may include a sufficient amount of C-peptide, such that from about 1 pmole to about 50 pmoles of the C-peptide in the reagent contact cells when the reagent is combined with the cells. Reagents may also include, or alternatively include, a sufficient amount of C-peptide binding facilitator, such that from about 0.05% (w/v) to about 10% (w/v) of the C-peptide binding facilitator in the reagent contact cells when the reagent is combined with the cells. In various embodiments, a reagent includes from about 100 pM to about 1 mM C-peptide and from about 0.05% (w/v) to about 25% (w/v) C-peptide binding facilitator, as described above. The reagent may also include a buffering agent, such as piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), (3-(*N*-morpholino)propanesulfonic acid) (MOPS), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES), tris(hydroxymethyl)aminomethane (TRIS), tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCL), *N*-[Tris(hydroxymethyl)methyl]glycine (TRICINE), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS), *N,N*-Bis(2-hydroxyethyl)glycine (BICINE), N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS), or phosphate as non-limiting examples. Such buffering agents, and others, are known in the art.

Examples

[0118] The following examples are meant to illustrate, not to limit, the present technology.

Example 1.

[0119] The amount of C-peptide binding to RBCs obtained from MS patients was compared to controls run on the same day. All samples were handled in an identical manner. Briefly, about 10 mL of blood were drawn from each donor; the RBCs were purified by centrifugation. Next, 20 picomoles of human C-peptide were added to a buffer containing 75 micromolar albumin. RBCs were added to this C-peptide and albumin-containing buffer and allowed to incubate for 2 hours. Next, the samples were centrifuged and the moles of C-peptide remaining in the supernatant are subtracted from the original amount added. This provided a value corresponding to the moles of C-peptide bound to the RBCs. Fig. 1 shows the average amount of C-peptide bound to the RBCs obtained from patients with MS and healthy controls. Importantly, of the 25 MS patient samples analyzed, the values ranged from 2.7 to 4.8 picomoles of C-peptide bound to the RBCs, with 18 of the 25 above 3.2 picomoles. In comparison, one control was 3.1, another was 2.8; all others were below 2.45. Thus, separation exists between groups -- approximately 2.7 picomoles of bound C-peptide difference.

[0120] Additional samples from healthy controls and MS patients were analyzed for C-peptide binding, ATP release (10 healthy control patients, 10 MS patients, and 12 MS patients with glybenclamide), and Zn^{2+} binding (11 healthy control patients and 22 MS patients). The ATP release from the RBCs of MS patients was found to be an average of 344.7 ± 46.8 nM where the average release from healthy controls was 132.1 ±14.1 nM. As shown in Fig. 4, the amount of $^{65}Zn^{2+}$ that is able to interact with the RBCs of MS patients is significantly higher, at a value of 3.61 ± 0.22 picomoles, than that of healthy controls, at a value of 2.26 ± 0.24 picomoles. Here, 20 nM $^{65}Zn^{2+}$ was incubated with the RBCs and 20 nM C-peptide. It is noteworthy that in 32 of these 34 samples, MS ATP release, C-peptide and/or Zn^{2+} binding, was higher than the control samples analyzed simultaneously with the MS samples.

[0121] Similar studies were performed using serum albumin from other species (human, porcine, chicken); all of these forms deliver C-peptide to RBCs and are useful in the methods described herein.

Example 2.

[0122] The amount of C-peptide binding to RBCs obtained from 86 MS patients and 75 patients diagnosed with a non-MS neurological disease (OND) was compared to

controls blood from 39 patients without MS or OND in a binding experiment (to demonstrate a diagnostic test).

[0123] The MS patients were 75% female and ages ranged between all the MS patients from 21 – 81 years (52 years average). The MS patients had the disease for an average of 13.9 years. 24% of the patients were not on any disease-modifying therapy.

[0124] The OND patients were 46% female and ages ranged between all the OND patients from 21 – 88 years (63 years average). OND diseases include myasthenia gravis, brain tumor, optic neuropathy, sixth nerve palsy, vestibular neuronitis, Guillain-Barre syndrome, neuromyelitis optica, vertigo, seizures, neurofibromatosis type 1, mitochondria disease (POLG mutation), mylopathy, Parkinson's disease, Parkinson's disease/MS, stroke, central retinal artery occlusion, neuromyelitis optic, neuromyelitis optic/MS, diabetes type 2/MS, unknown/toxic optic neuropathy, migraines, facial spasms, Horner's syndrome, chronic inflammatory demyelinating neuropathy, diplopia, Meniere's disease, rheumatoid arthritis, and epilepsy.

[0125] All samples were handled in an identical manner. Briefly, about 10 mL of blood was drawn from each donor; the RBCs were purified by centrifugation; and the RBCs were suspended in physiological salt solution (PSS). Then RBCs were combined with PSS (containing bovine serum albumin (BSA) as a C-peptide binding facilitator) and C-peptide to generate a mixture having a 7% hematocrit, 20 nM C-peptide, and 75 μM BSA (0.5%). The mixture was allowed to incubate for 2 hours at 37°C. Next, the samples were centrifuged to separate the cells from solution, the solution was diluted 1:50 with double distilled water, and the amount of C-peptide remaining in the solution was determined by ELISA. This provided a value corresponding to the moles of C-peptide bound to the RBCs. Fig. 5 shows the average amount of C-peptide bound to the RBCs obtained from patients with MS, OND, and healthy controls. Whereas blood from the healthy controls bound an average of 1.99 ± 0.32 pmoles C-peptide, blood from MS patients and OND patients bound averages of 3.51 ± 0.59 pmoles of C-peptide and 2.23 ± 0.51 pmoles of C-peptide, respectively. As shown by p values of less than 0.001, the increase in C-peptide binding relative to healthy controls and OND patients is significant.

[0126] A Receiver Operating Characteristic (ROC) Curve was generated to demonstrate the tradeoff between sensitivity and specificity, where any increase in sensitivity is accompanied by a decrease in specificity. The ROC Curve plots the true positive rate (TPR) against a false positive rate (FPR) for different possible cutpoints (C-

peptide binding to RBCs) of a diagnostic test. An area under a curve (AUC) of 1 is representative of a perfect diagnostic and AUCs greater than 0.9 are generally considered excellent. Fig. 6 shows a ROC curve generated for the binding experiment including 200 patients; 86 MS patients, and 114 controls (39 patients without MS or OND and 75 patients with OND). The area under the curve is 0.97, which confirms that the diagnostic test is excellent. Moreover, when the healthy controls were not considered, the resulting ROC curve still demonstrated an excellent AUC of 0.96 (data not shown).

[0127] To further demonstrate the sensitivity and specificity of the binding experiment, a diagnostic odds ratio (DOR) was determined. The DOR compares the odds of positivity in diseased relative to the odds of positivity in the non-diseased. DOR values range from 0 to infinity, with higher values indicating better discriminatory test performance. With the same sensitivity to the test, DOR increases with an increase of a test specificity. Fig. 7 shows a DOR plot generated for the binding experiment including the 200 patients. The plot shows a DOR of greater than 315 across cutpoints between 2.99 and 3.08 pmoles of C-peptide binding. The plot shows a maximum DOR of 481 at 3.04 pmoles of C-peptide binding. Therefore, the DOR further demonstrates excellent sensitivity and specificity of the binding experiment. When healthy controls are not included in the control data set, the DOR was determined to be greater than 200 across cutpoints between 2.99 and 3.08 picomoles of C-peptide binding with a maximum DOR of 312 at 3.04 picomoles of C-peptide binding (data not shown).

Example 3

[0128] Experiments were performed to determine the effect of hematocrit on C-peptide binding to RBCs. The experiment was conducted as described above in Experiment 2, except various hematocrits were tested. The results are provided in Table 1. Table 1 shows that there is less C-peptide binding to RBCs when a lower hematocrit is used. Conversely, the table shows that there is greater C-peptide binding to RBCs when a higher hematocrit is used. In general, increase the hematocrit results in a substantially linear increase in C-peptide binding when the RBCs are saturated with C-peptide.

Table 1. Effect of hematocrit on C-peptide binding by RBCs

Hematocrit	Control (pmoles)	MS (pmoles)
3.50%	1.18	1.63
7%	2.18	3.38
14%	4.78	6.25

Example 4

[0129] Experiments were performed to determine the stability of the whole blood. The experiment was conducted as described above in Experiment 2, except the blood was stored in citrate tubes at 4°C over a period of 4 days during the experiment. Only blood from healthy patients was tested. The results are provided in Table 2. Table 2 shows that C-peptide binding slightly decreases from day-to-day over the 4-day period.

Table 2. Effect of storing blood on C-peptide binding by RBCs

Day	C-peptide binding (pmoles)
1	2.38
2	2.23
3	2.14
4	1.83

Example 5

[0130] Experiments were performed to determine the stability of supernatant generated after separating cells from solution as described in Experiment 2. The protocol of Experiment 2 was repeated on blood from healthy patients, but the supernatant was stored at 4°C for from 1 to 3 days prior to determining C-peptide binding by the RBCs. The results are provided in Table 3. Table 3 shows that storing the supernatant at 4°C for from 1 to 3 days has little effect on the amount of C-peptide determined to be in the supernatant.

Table 3. Effect of short-term storage of supernatant on C-peptide binding calculations

Day	C-peptide binding (pmoles)
1	2.23
2	2.17
3	2.20

Example 6

[0131] Experiments were performed to determine the stability of supernatant generated after separating cells from solution as described in Experiment 2. The protocol of Experiment 2 was repeated on blood from healthy patients, but the supernatant was stored at -20°C from 0 (day of draw) to 6 weeks prior to determining C-peptide binding by the RBCs. The results are provided in Table 4. Table 4 shows that freezing the supernatant for from 0 to 6 weeks resulted in only a slight decrease in C-peptide binding over the 6 week period.

Table 4. Effect of long-term storage of supernatant on C-peptide binding calculations

Week	C-peptide binding (pmoles)
0	2.45
1	2.48
3	2.30
6	2.34

Example 7

[0132] Experiments were performed to determine the effect of C-peptide binding facilitator concentration on C-peptide binding by RBCs. The protocol of Experiment 2 was repeated, but with increasing BSA concentrations. The results are shown in Table 5. Table 5 shows that increasing the BSA concentration from 0.5% to 2.0% has little effect on C-peptide binding. Only a slight increase in C-peptide binding is realized.

Table 5. Effect of BSA concentration of C-peptide binding to RBCs

[BSA]	C-peptide binding (pmoles)
0.5%	2.14 ± 0.22
1.0%	2.19 ± 0.16
2.0%	2.26 ± 0.13

Example 8

[0133] Experiments were performed to test various potential C-peptide binding facilitators. The experiments were performed as described in example 2, but the C-peptide binding facilitator, BSA, was replaced with other albumins (porcine serum albumin and ovalbumin) or other proteins (leptin, casein, transferrin, and collagen). The results are shown in Tables 6 and 7.

Table 6. Effect of other albumins on C-peptide binding to RBCs

Albumin	Control (pmoles; n=4)	MS (pmoles; n=1)
porcine serum albumin	4.01 ± 1.01	6.33
ovalbumin	0.39 ± 0.22	0.67

Table 7. Effect of other proteins on C-peptide binding to RBCs

Protein	Control (pmoles; n=3)	MS (pmoles; n=1)
leptin	0.74 ± 0.11	0.97
casein	No binding detected	No binding detected
transferrin	No binding detected	No binding detected
collagen	0.21 ± 0.14	0.56

Example 9

[0134] Experiments were performed to determine if RBCs from MS patients (n = 19) release more ATP than blood from healthy control patients (n = 10) or blood from MS patents combined with the ATP release inhibitor, glybenclamide (Gly; n = 12) when subjected to a constricted environment. RBCs from the patients were washed in PSS and suspended to a 7% hematocrit. A dual syringe pump provided two syringes; a first syringe containing a blood sample and a second syringe containing a detection facilitator (a luciferin/luciferase mixture). Collectively the blood sample and detection facilitator are referred to as "the solutions." The solutions were individually pumped at 6.7 μL/min through 50 μm internal diameter microbore tubes having a polyimide coating. The solutions met at a mixing T-junction, where they were combined and pumped collectively through 75 μm internal diameter microbore tubing having a portion of the polyimide coating removed. A constricted environment was provided as the solutions combined because the total volume was doubling, but the inner diameter of the tubing was increased by only 50%. As the RBCs are deformed in the constricted environment, they released ATP. The portion of tubing having the polyimide coating removed was positioned over a light-excluding box containing a photomultiplier tube (PMT), such that chemiluminescence generated from the ATP contacting the detection facilitator was detected. The concentrations of ATP were compared to a standard curve prepared by pumping standard concentrations of ATP (instead of blood samples) and detection facilitator (a luciferin/luciferase mixture) through the tubing and over the PMT in the same manner. The cells combined with Gly act as a control to demonstrate that ATP was not

released due to cell lysis. The results are shown in Fig. 3. Whereas the blood from the healthy control patients released 132.1 ± 14.1 nM ATP, the blood from the MS patients released 344.7 ± 46.8 nM of ATP; a significantly higher amount (over 2.5 fold higher). However, when blood from MS patients was combined with Gly, the amount of ATP release dropped to 65.3 ± 11.6 nM, which is lower than the blood from the healthy control. Therefore, measuring ATP release from blood can be used to indicate a high probably of having MS.

Example 10

[0135] Additional experiments were performed to determine whether a C-peptide facilitator can facilitate binding of C-peptide to cells expressing Glut1 other than RBCs. Endothelial cells (bovine pulmonary artery endothelial cells) were grown to confluency on 12-well tissue culture plates. When the cell reached confluency, they were incubated in Dulbecco's Modified Eagle Medium (DMEM) containing serum for 24 hours. Following incubation, the cells were washed and incubated for 1 hour at 37°C in the presence of 10 nM Zn^{2+} and C-peptide in DMEM containing serum. The endothelial cells were determined to have bound 2.0 ± 0.1 attomoles of C-peptide per endothelial cell.

[0136] Neutrophils from healthy control patients were also tested. The neutrophils were isolated using techniques known in the art. Briefly, blood was drawn into a heparin-coated vacutainer. An equal volume of 3% Dextran was added to drawn whole blood in sterile 50 mL tubes. After inverting, the tubes were set upright for 30 minutes at room temperature to generate a plurality of layers. The top layer was aspirated and transferred to a new 50 mL tube and centrifuged at 500g for 10 minutes at 4°C. The resulting top layer was discarded. The resulting pellet was resuspended in 10 mL 0.9% saline. After pooling suspensions together in a single tube, it was underplayed with 10 mL of hypaque-ficoll and centrifuged at 800g for 15 minutes at room temperature. The resulting liquid band was removed and the remainder was resuspended in 20 mL 0.2% saline and mixed. 20 mL of 1.6% saline was then added to the suspension and it was centrifuged at 250g for 6 minutes at 4°C. The supernatant was removed and the pellet was suspended in PBS for use in experiments. The cells were counted and then incubated with 20 nM C-peptide for 1 hour at 37°C to generate an incubated sample. The incubated sample was centrifuged and the amount of C-peptide in the supernatant was determined by ELISA. The neutrophils were determined to have bound 2.68 attomoles of C-peptide per neutrophil.

Example 11

[0137] Experiments were performed to determine the relative expression level of Glut1 in RBCs from healthy controls (n = 44), MS patients (n = 66), and patients with OND (n = 57). RBCs were isolated from the controls and patients as described above. The cells were lysed with a lysis buffer and centrifuged at 23,000 x g for 15 minutes at 4°C. A resulting supernatant was discarded and the pellet was subjected to a lysis buffer and then centrifuged again at 23,000 x g for 15 minutes at 4°C. A resulting supernatant was discarded and the remaining pellet included cell lysates. Equal amounts of each lysate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were then prepared using anti-Glut1 primary antibodies. Bands corresponding to Glut1 in RBC lysate from the healthy controls, MS patients, and OND patients were scanned and quantified (normalized to the control).

[0138] The results of the experiment are shown in Fig. 8. Fig. 8 is a graph showing the control as having 1.03 ± 0.026 normalize Glut1, MS as having 1.34 ± 0.045 normalized Glut1, and OND (Non-MS Neuro) as having 1.08 ± 0.047. Whereas the OND lysates contained substantially the same amount of Glut1 as the control lysates, the MS lysates contained about 1.3 fold more Glut1 relative to the control lysates ($p < 0.005$).

Example 12

[0139] Experiments were performed to determine how much glucose RBCs from MS patients (n = 22) bind relative to RBCs from healthy controls (n = 11). 7% solutions of purified RBCs from healthy control and MS patients were incubated with ^{14}C-glucose alone or with ^{14}C-glucose with 20 nM Zn^{2+} and C-peptide. The solutions were incubated for 4 hours at 37°C and then centrifuged at 500g for 4 minutes. A resulting supernatant was discarded and the cells were resuspended in physiological salt solution (PSS). Centrifuging and washing with PSS was repeated twice. The RBCs were then lysed with bleach and incubated for 30 min. to generate lysates. 200 µL of lysate was combined with scintillation cocktail and read, along with standards, on a liquid scintillation counter. Values were normalized to lysates form the healthy control without Zn^{2+} and C-peptide.

[0140] The results of the experiment are shown in Fig. 9. Fig. 9 is a graph showing that, in both RBCs form healthy controls and MS patients, the addition of Zn2+ and C-peptide greatly increases glucose uptake relative to RBCs not contacted with Zn2+

and C-peptide. Moreover, the graph shows that RBCs from MS patients uptake significantly more glucose relative to corresponding controls, with or without Zn^{2+} and C-peptide.

Sequence Listing

[0141] Various amino acid sequences are described herein with sequence identifiers (*e.g.*, SEQ ID NO:_) that correspond to sequences in a sequence listing. The sequence listing constitutes a part of this disclosure.

Recitation of Exemplary Embodiments

[0142] The following is a recitation of embodiments exemplifying the materials, methods, kits and reagents of the present technology.

1. A method for measuring C-peptide binding by cells, the method comprising:

incubating the cells with C-peptide mixture comprising an amount, *e.g.*, known amount, of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

2. The method according to Embodiment 1, wherein the C-peptide binding facilitator is selected from the group consisting of albumins, leptin, collagen, and mixtures thereof.

3. The method according to Embodiment 2, wherein the C-peptide binding facilitator is an albumin.

4. The method according to Embodiment 3, wherein the albumin is obtained from whole blood or a blood fraction obtained from a human, cow, chicken or pig blood.

5. The method according to Embodiment 3, wherein the albumin is recombinant albumin.

6. The method according to any one of the preceding Embodiments, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

7. The method according to Embodiment 6, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

8. The method according to any one of Embodiments 1 – 5, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the C-peptide binding facilitator.

9. The method according to Embodiment 8, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

10. The method according to Embodiment 8, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

11. The method according to Embodiment 10, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

12. The method according to Embodiment 6, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

13. The method according to any one of the preceding Embodiments, wherein the C-peptide is modified with a heterologous moiety.

14. The method according to Embodiment 13, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

15. The method according to any one of the preceding Embodiments, wherein the C-peptide binding facilitator comprises a tag for quantifying the C-peptide, preferably

wherein the tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

16. The method according to any one of the preceding Embodiments, wherein the cells express glucose 1 transporter protein (Glut1).

17. The method according to Embodiment 16, wherein the cells are red blood cells (RBCs), macrophages, neutrophils, or endothelial cells.

18. The method according to any one of the preceding Embodiments, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

19. The method according to any one of the preceding Embodiments, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

20. The method according to Embodiment 19, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

21. The method according to any one of the preceding Embodiments, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of the C-peptide, and (iii) the c-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

22. The method according to Embodiment 21, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

23. The method according to Embodiment 20, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

24. The method according to any one of Embodiments 21 - 23, wherein the C-peptide and c-peptide binding facilitator are components of an isotonic solution.

25. The method according to Embodiment 24, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 25% (w/v).

26 The method according to Embodiment 24 or Embodiment 25, wherein the solution comprises a buffer selected from the group consisting of phosphate buffered saline, physiological saline solution, Tris buffer, and phosphate buffer.

27. The method according to any one of Embodiments 24 – 26, wherein the isotonic solution is admixed with the suspension of the cells.

28. A method of assessing the metabolic activity of cells, comprising performing a method according to any of the preceding Embodiments.

29. A method of detecting a metabolic disorder in a cell, comprising performing a method according to any one of Embodiments 1 – 27.

30. The method according to Embodiment 29, wherein a determination of the amount of C-peptide bound to the cells of greater than or equal to about 2000 C-peptide molecules per cell is indicative of the metabolic disorder.

31. A method for diagnosing a disorder in a mammalian subject, comprising obtaining cells from the subject and performing a method according to any one of Embodiments 1 – 27.

32. A method for analyzing the risk of developing a disorder in a mammalian subject, comprising obtaining cells from the subject and performing a method according to any one of Embodiments 1 – 27.

33. A method for determining the likelihood that a mammalian subject has a disorder, comprising obtaining cells from the subject and performing a method according to any one of Embodiments 1 – 27.

34. The method according to any one of Embodiment 31 – 33, wherein the disorder is an immune-mediated disease.

35. The method according to Embodiment 34, wherein the disorder is multiple sclerosis (MS).

36. A method for diagnosing a disorder in a mammalian subject, comprising effecting a method for measuring C-peptide in cells obtained from the subject, the method comprising a method according to any one of Embodiments 1 - 27.

37. A method for analyzing the risk of developing a disorder in a mammalian subject, comprising effecting a method for measuring C-peptide binding in cells obtained from the subject, the assay comprising a method according to any one of Embodiments 1 - 27.

38. A method for determining the likelihood that a mammalian subject has a disorder, comprising effecting an assay for measuring C-peptide in cells obtained from the subject, the assay comprising a method according to any one of Embodiments 1 - 27.

39. The method according to any one of Embodiments 36 – 38, wherein the disorder is an immune-mediated disease.

40. The method according to Embodiment 35, wherein the disorder is MS.

41. An *in vitro* method for assessing the probability that a mammalian subject has an immune-mediated disease, the method comprising:

incubating cells that express glucose 1 transporter protein (Glut1) obtained from the subject with a C-peptide composition comprising an amount, *e.g.*, known amount, of C-peptide and an albumin under conditions that allow for the cells to bind the C-peptide to form incubated cells in an incubated cellular mixture;

separating the incubated cells from the incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

42. The method according to Embodiment 41, further comprising comparing the amount of C-peptide bound to the incubated cells to a reference amount determined by performing the incubating, separating and determining using cells obtained from a second subject that does not have the immune-mediated disease.

43. The method according to Embodiment 42, wherein an amount of C-peptide bound to the incubated cells greater than or equal to about 1.5 fold relative to the reference amount is indicative of a high probability of the subject having the immune-mediated disease.

44. The method according to Embodiment 41, wherein a level of C-peptide bound to the incubated cells of greater than or equal to about 2000 molecules/cell is indicative of the immune-mediated disease in the subject.

45. The method according to Embodiment 41, wherein the albumin is obtained from whole blood or a blood fraction obtained from human, cow, chicken or pig blood.

46. The method according to Embodiment 45, wherein the albumin is recombinant albumin.

47. The method according to any one of Embodiments 41 – 46, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

48. The method according to Embodiment 47, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

49. The method according to any one of Embodiments 41 – 46, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the C-peptide binding facilitator.

50. The method according to Embodiment 49, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

51. The method according to Embodiment 49, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

52. The method according to Embodiment 51, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

53. The method according to Embodiment 47, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

54. The method according to any one of Embodiments 41 – 53, wherein the C-peptide is modified with a heterologous moiety.

55. The method according to Embodiment 54, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

56. The method according to any one of Embodiments 41 – 55, wherein the C-peptide binding facilitator comprises a tag for quantifying the C-peptide, preferably wherein the

tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

57. The method according to any one of Embodiments 41 – 56, wherein the cells express Glut1.

58. The method according to Embodiment 57, wherein the cells are red blood cells (RBCs), macrophages, neutrophils, or endothelial cells.

59. The method according to any one of the preceding Embodiments, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

60. The method according to any one of Embodiments 41 – 59, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

61. The method according to Embodiment 60, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

62. The method according to any one of Embodiments 41 – 61, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of C-peptide, and (iii) C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

63. The method according to Embodiment 62, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

64. The method according to Embodiment 61, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

65. The method according to any one of Embodiments 62 - 64, wherein the C-peptide and C-peptide binding facilitator are components of an isotonic solution.

66. The method according to Embodiment 65, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 25% (w/v).

67. The method according to any one of Embodiments 41 – 66, wherein the immune-mediated disease is MS.

68. A method of analyzing the risk of an immune-mediated disease in a mammalian subject, comprising effecting an assay on cells obtained from the subject, the assay comprising a method according to any one of Embodiments 41-67.

69. A kit for use measuring C-peptide binding by cells, the kit comprising a C-peptide binding facilitator, C-peptide, and a container.

70. A kit according to Embodiment 69, wherein the container is operable for mixing a solvent with one or both of the C-peptide binding facilitator and the C-peptide.

71. A kit according to Embodiment 70, wherein the container is operable for mixing one or both of the C-peptide binding facilitator and the C-peptide with a suspension of the cells.

72. A kit according to Embodiment 69, wherein the container is a first container containing the C-peptide binding facilitator, and the kit further comprises a second container containing C-peptide.

73. The kit according to any one of Embodiments 69 – 72, wherein the C-peptide binding facilitator is an albumin.

74. The kit according to Embodiment 73, wherein the albumin is in powdered form, preferably wherein the albumin is lyophilized.

75. The kit according to any one of Embodiments 69 – 74, wherein the C-peptide is in powdered form, preferably wherein the C-peptide is lyophilized.

76. The kit according to Embodiment 72, further comprising a third container containing a solvent for one or both of the C-peptide binding facilitator and the C-peptide.

77. The kit according to Embodiment 76, wherein the solvent comprises phosphate buffered saline, physiological saline solution, Tris buffer, or phosphate buffer.

78. The kit according to Embodiment 76 or Embodiment 77, further comprising a fourth container operable for receiving the cells.

79. The kit according to any one of Embodiments 69 – 78, further comprising a reagent for determining an amount of C-peptide bounded by the cells.

80. A method for measuring C-peptide binding by cells using the kit according to any one of Embodiments 69 – 79.

81. The method according to Embodiment 80, the method comprising:

mixing the C-peptide binding facilitator and the C-peptide to form a C-peptide/facilitator mixture;

incubating the cells with the C-peptide/facilitator mixture to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

82. A method for measuring C-peptide binding by cells using the kit according to Embodiment 76 or Embodiment 77, the method comprising:

mixing the C-peptide binding facilitator and the C-peptide to form a C-peptide/facilitator mixture;

incubating the cells with the C-peptide/facilitator mixture to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

83. The method according to Embodiment 82, wherein C-peptide/facilitator mixture is an isotonic solution comprising the C-peptide and C-peptide binding facilitator.

84. The method according to Embodiment 82, wherein the isotonic solution comprises the solvent in the third container.

85. The method according to Embodiment 81, wherein the mixing and contacting are performed simultaneously or sequentially in the first container or second container.

86. The method according to Embodiment 82, wherein the mixing and contacting are performed simultaneously or sequentially in the third container.

87. The method according to Embodiment 82, wherein the kit comprises a fourth container and the mixing and contacting are performed simultaneously or sequentially in the fourth container.

88. The method according to Embodiment 82 comprising transferring a predetermined volume of the solvent in the third container to one of the first container or the second container and dissolving the contents of the first container or the second container in the solution to form a first working solution;

transferring the partial working solution to the other of the first container or the second container and dissolving the contents of the other of the first container or the second container to form a second working solution;

incubating a suspension of the cells with the second working solution to generate a C-peptide cellular mixture under conditions that allow for the cells to bind the C-peptide.

89. The method according to Embodiment 88, further comprising:
contacting a detection reagent with the second working solution; and
measuring the amount of C-peptide in the second working solution.

90. The method according to any one of Embodiments 81 – 89, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of the C-peptide, and (iii) the C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

91. The method according to Embodiment 90, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

92. The method according to Embodiment 90, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

93. The method according to any one of Embodiments 81 –92, wherein the concentration of C-peptide is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 25% (w/v).

94. A reagent for use in measuring C-peptide binding to cells that express glucose 1 transporter protein (Glut1), the reagent consisting essentially of albumin dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of C-peptide, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof.

95. The reagent according to Embodiment 94, wherein the buffering agent is selected from the group consisting of phosphate salts, tris(hydroxymethyl)aminomethane, and mixtures thereof.

96. The reagent according to Embodiment 94, containing C-peptide.

97. A method for measuring C-peptide binding by cells using the reagent of any of Embodiments 94 – 96.

98. A method for detecting a metabolic disorder in a cell using the reagent of any of Embodiments 94 –96.

99. The method according to Embodiment 97 or Embodiment 98, comprising incubating from about 0.05 mL to about 5 mL of a the suspension of the cells with the reagent, wherein the reagent comprises from about 1 to about 50 pmoles of the C-peptide, and a C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 10% (w/v).

100. The method according to Embodiment 99, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

101. The method according to Embodiment 100, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

102. The method according to any one of Embodiments 97 – 101, wherein the concentration of C-peptide in the reagent is from about 100 pM to about e and the concentration of the C-peptide binding facilitator in the reagent is from about 0.05% (w/v) to about 10% (w/v).

103. A reagent for use in measuring C-peptide binding to cells that express glucose 1 transporter protein (Glut1), the reagent consisting essentially of C-peptide dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of albumin, a buffering agent, Zn, Fe, Cr, and mixtures thereof.

104. The reagent according to Embodiment 103, containing a buffering agent is selected from the group consisting of phosphate salts, tris(hydroxymethyl)aminomethane, and mixtures thereof.

105. The reagent according to Embodiment 103, containing albumin.

106. A method for measuring C-peptide binding to cells using the reagent of any of Embodiments 103 – 105.

107. A method for detecting a metabolic disorder in a cell using the reagent of any of Embodiments 103 – 105.

108. The method according to Embodiment 106 or Embodiment 107, comprising contacting from about 0.05 mL to about 20 mL of a suspension of the cells with the reagent, wherein the reagent comprises from about 1 to about 500 pmoles of the C-peptide, and C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

109. The method according to Embodiment 108, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

110. The method according to Embodiment 109, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

111. The method according to any one of Embodiments 106 – 110, wherein the concentration of C-peptide in the reagent is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator in the reagent is from about 0.05% (w/v) to about 25% (w/v).

112. An *in vitro* method for diagnosing a mammalian subject as having an immune-mediated disease, the method comprising:

incubating, in the presence of albumin, red blood cells (RBCs) from the subject with C-peptide under conditions that allow for binding of C-peptide by red blood cells; and

determining the amount of C-peptide bound by the RBCs, wherein increased binding of C-peptide by the RBCs, relative to a control C-peptide binding level, indicates that the subject has the immune-mediated disease.

113. The method according to Embodiment 112, wherein the immune-mediated disease is an inflammatory demyelinating disorder.

114. The method according to Embodiment 113, wherein the inflammatory demyelinating disorder is MS.

115. The method according to Embodiment 112, wherein the albumin is obtained from whole blood or a blood fraction obtained from human, cow, chicken or pig blood.

116. The method according to Embodiment 112, wherein the albumin is recombinant albumin.

117. The method according to any one of Embodiments 112 – 116, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

118. The method according to Embodiment 117, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

119. The method according to Embodiment 112, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the albumin.

120. The method according to Embodiment 119, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

121. The method according to Embodiment 119, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

122. The method according to Embodiment 121, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

123. The method according to Embodiment 117, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

124. The method according to any one of Embodiments 112 – 123, wherein the C-peptide is modified with a heterologous moiety.

125. The method according to Embodiment 124, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

126. The method according to any one of Embodiments 112 – 125, wherein the albumin comprises a tag for quantifying the C-peptide, preferably wherein the tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

127. The method according to any one of Embodiments 112 – 126, wherein the cells express glucose 1 transporter protein (Glut1).

128. The method according to Embodiment 127, wherein the cells are RBCs, macrophages, neutrophils, or endothelial cells.

129. The method according to any one of Embodiments 112 – 128, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

130. The method according to any one of Embodiments 112 – 129, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

131. The method according to Embodiment 130, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

132. The method according to any one of Embodiments 112 – 131, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of the C-peptide, and (iii) the C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

133. The method according to Embodiment 132, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

134. The method according to Embodiment 131, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

135. The method according to any one of Embodiments 132 – 134, wherein the C-peptide and C-peptide binding facilitator are components of an isotonic solution.

136. The method according to Embodiment 135, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1 mM and the concentration of the albumin is from about 0.05% (w/v) to about 25% (w/v).

137. A method of managing MS in a subject in need thereof, the method comprising:

(a) effecting an assay for measuring C-peptide binding on cells obtained from the subject, the assay comprising

(i) incubating the cells with a C-peptide composition comprising an amount, *e.g.*, known amount, of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

(ii) determining the amount of C-peptide bound to the incubated cells.

(b) comparing the amount of C-peptide bound by the cells to a reference amount determined by performing the assay on cells obtained from a second subject that does not have the immune-mediated disease;

(c) administering a first treatment for MS; and

(d) assessing the efficacy of the administering by repeating the effecting and comparing.

138. The method of Embodiment 137, further comprising administering a second treatment of MS after the assessing, wherein the second treatment is altered from the first treatment based on the results of the assessing.

139. A method of assessing the responsiveness of a mammalian subject having MS to a treatment for the MS, comprising a method according to Embodiment 137.

Non-limiting Discussion of Terminology

[0143] The headings (such as "Introduction" and "Summary") and sub-headings used herein are intended only for general organization of topics within the present technology, and are not intended to limit the present technology or any aspect thereof. In particular, subject matter disclosed in the "Introduction" may include novel technology

and may not constitute a recitation of prior art. Subject matter disclosed in the "Summary" is not an exhaustive or complete disclosure of the entire scope of the technology or any embodiments thereof. Classification or discussion of a material within a section of this specification as having a particular utility is made for convenience, and no inference should be drawn that the material must necessarily or solely function in accordance with its classification herein when it is used in any given composition or method.

[0144] The description and specific examples, while indicating embodiments of the technology, are intended for purposes of illustration only and are not intended to limit the scope of the technology. Moreover, recitation of multiple embodiments having stated features is not intended to exclude other embodiments having additional features, or other embodiments incorporating different combinations of the stated features. Specific examples are provided for illustrative purposes of how to make and use the compositions and methods of this technology and, unless explicitly stated otherwise, are not intended to be a representation that given embodiments of this technology have, or have not, been made or tested. Equivalent changes, modifications and variations of some embodiments, materials, compositions and methods can be made within the scope of the present technology, with substantially similar results.

[0145] As used herein, the words "preferred" or "preferable" or "preferably" refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be desirable, under the same or other circumstances. Furthermore, the recitation of one or more desired embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the technology.

[0146] Although the open-ended term "comprising," as a synonym of non-restrictive terms such as including, containing, or having, is used herein to describe an embodiment of the present technology, embodiments may alternatively be described using more limiting terms such as "consisting of" or "consisting essentially of." Thus, for any given embodiment reciting materials, components or process steps, the present technology also specifically includes embodiments consisting of, or consisting essentially of, such materials, components or processes excluding additional materials, components or processes (for consisting of) and excluding additional materials, components or processes affecting the significant properties of the embodiment (for consisting essentially of), even

though such additional materials, components or processes are not explicitly recited in this application. For example, recitation of a composition or process reciting elements A, B and C specifically envisions embodiments consisting of, and consisting essentially of, A, B and C, excluding an element D that may be recited in the art, even though element D is not explicitly described as being excluded herein.

[0147] As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features.

[0148] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present technology pertains. In case of conflict, this disclosure, including definitions, will control.

[0149] Throughout this disclosure, the numerical values represent approximate measures or limits to ranges to encompass minor deviations from the given values and embodiments having about the value mentioned as well as those having exactly the value mentioned. All numerical values of parameters (*e.g.*, of quantities or conditions) in this specification, including the appended embodiments, are to be understood as being modified in all instances by the term "about" whether or not "about" actually appears before the numerical value. "About" indicates that the stated numerical value allows some slight imprecision (with some approach to exactness in the value; approximately or reasonably close to the value; nearly). If the imprecision provided by "about" is not otherwise understood in the art with this ordinary meaning, then "about" as used herein indicates at least variations that may arise from ordinary methods of measuring and using such parameters.

[0150] Disclosure of values and ranges of values for specific parameters (such as temperatures, molecular weights, weight percentages, etc.) are not exclusive of other values and ranges of values useful herein. It is envisioned that two or more specific exemplified values for a given parameter may define endpoints for a range of values that may be claimed for the parameter. For example, if Parameter X is exemplified herein to

have value A and also exemplified to have value Z, it is envisioned that parameter X may have a range of values from about A to about Z. Similarly, it is envisioned that disclosure of two or more ranges of values for a parameter (whether such ranges are nested, overlapping or distinct) subsume all possible combination of ranges for the value that might be claimed using endpoints of the disclosed ranges. For example, if parameter X is exemplified herein to have values in the range of 1 - 10, or 2 - 9, or 3 - 8, it is also envisioned that Parameter X may have other ranges of values including 1 - 9, 1 - 8, 1 - 3, 1 - 2, 2 - 10, 2 - 8, 2 - 3, 3 - 10, and 3 - 9. As referred to herein, ranges are, unless specified otherwise, inclusive of endpoints and include disclosure of all distinct values and further divided ranges within the entire range. Thus, for example, a range of “from A to B” or “from about A to about B” is inclusive of A and of B.

[0151] As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items.

[0152] Although the terms first, second, third, etc. may be used herein to describe various elements, components, regions, layers and/or sections, these elements, components, regions, layers and/or sections should not be limited by these terms. These terms may be only used to distinguish one element, component, region, layer or section from another region, layer or section. Terms such as “first,” “second,” and other numerical terms when used herein do not imply a sequence or order unless clearly indicated by the context. Thus, a first element, component, region, layer or section discussed below could be termed a second element, component, region, layer or section without departing from the teachings of the example embodiments

[0153] While the present technology has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the technology. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present technology. All such modifications are intended to be within the scope of the technology.

CLAIMS

What is claimed is:

1. A method for measuring C-peptide binding by cells, the method comprising:

incubating the cells with a C-peptide mixture comprising a known or unknown amount of C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

2. The method according to Claim 1, wherein the C-peptide binding facilitator is selected from the group consisting of albumins, leptin, collagen, and mixtures thereof.

3. The method according to Claim 2, wherein the C-peptide binding facilitator is an albumin.

4. The method according to Claim 3, wherein the albumin is obtained from whole blood or a blood fraction obtained from a human, cow, chicken or pig blood.

5. The method according to Claim 3, wherein the albumin is recombinant albumin.

6. The method according to any one of the preceding Claims, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

7. The method according to Claim 6, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

8. The method according to any one of Claims 1 – 5, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the C-peptide binding facilitator.

9. The method according to Claim 8, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

10. The method according to Claim 8, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

11. The method according to Claim 10, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

12. The method according to Claim 6, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

13. The method according to any one of the preceding Claims, wherein the C-peptide is modified with a heterologous moiety.

14. The method according to Claim 13, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

15. The method according to any one of the preceding Claims, wherein the C-peptide binding facilitator comprises a tag for quantifying the C-peptide, preferably wherein the tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

16. The method according to any one of the preceding Claims, wherein the cells express Glut1.

17. The method according to Claim 16, wherein the cells are red blood cells (RBCs), macrophages, neutrophils, or endothelial cells.

18. The method according to any one of the preceding Claims, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

19. The method according to any one of the preceding Claims, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

20. The method according to Claim 19, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

21. The method according to any one of the preceding Claims, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of the C-peptide, and (iii) the c-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

22. The method according to Claim 21, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

23. The method according to Claim 20, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

24. The method according to any one of Claims 21 - 23, wherein the C-peptide and c-peptide binding facilitator are components of an isotonic solution.

25. The method according to Claim 24, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 25% (w/v).

26 The method according to Claim 24 or Claim 25, wherein the solution comprises a buffer selected from the group consisting of phosphate buffered saline, physiological saline solution, tris(hydroxymethyl)aminomethane buffer, and phosphate buffer.

27. The method according to any one of Claims 24 – 26, wherein the isotonic solution is admixed with the suspension of the cells.

28. A method of assessing the metabolic activity of cells, comprising performing a method according to any of the preceding Claims.

29. A method of detecting a metabolic disorder in a cell, comprising performing a method according to any one of Claims 1 – 27.

30. The method according to Claim 29, wherein a determination of the amount of C-peptide bound to the cells of greater than or equal to about 2000 C-peptide molecules per cell is indicative of the metabolic disorder.

31. A method for diagnosing a disorder in a mammalian subject, comprising obtaining cells from the subject and performing a method according to any one of Claims 1 – 27.

32. A method for analyzing the risk of developing a disorder in a mammalian subject, comprising obtaining cells from the subject and performing a method according to any one of Claims 1 – 27.

33. A method for determining the likelihood that a mammalian subject has a disorder, comprising obtaining cells from the subject and performing a method according to any one of Claims 1 – 27.

34. The method according to any one of Claim 31 – 33, wherein the disorder is an immune-mediated disease.

35. The method according to Claim 34, wherein the disorder is multiple sclerosis (MS).

36. A method for diagnosing a disorder in a mammalian subject, comprising effecting a method for measuring C-peptide in cells obtained from the subject, the method comprising a method according to any one of Claims 1 - 27.

37. A method for analyzing the risk of developing a disorder in a mammalian subject, comprising effecting a method for measuring C-peptide binding in cells obtained from the subject, the assay comprising a method according to any one of Claims 1 - 27.

38. A method for determining the likelihood that a mammalian subject has a disorder, comprising effecting an assay for measuring C-peptide in cells obtained from the subject, the assay comprising a method according to any one of Claims 1 - 27.

39. The method according to any one of Claims 36 – 38, wherein the disorder is an immune-mediated disease.

40. The method according to Claim 35, wherein the disorder is MS.

41. An *in vitro* method for assessing the probability that a mammalian subject has an immune-mediated disease, the method comprising:

incubating cells that express glucose 1 transporter protein (Glut1) obtained from the subject with a C-peptide composition comprising a known or unknown amount of C-peptide and an albumin under conditions that allow for the cells to bind the C-peptide to form incubated cells in an incubated cellular mixture;

separating the incubated cells from the incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

42. The method according to Claim 41, further comprising comparing the amount of C-peptide bound to the incubated cells to a reference amount determined by performing the incubating, separating and determining using cells obtained from a second subject that does not have the immune-mediated disease.

43. The method according to Claim 42, wherein an amount of C-peptide bound to the incubated cells greater than or equal to about 1.5 fold relative to the reference amount is indicative of a high probability of the subject having the immune-mediated disease.

44. The method according to Claim 41, wherein a level of C-peptide bound to the incubated cells of greater than or equal to about 2000 molecules/cell is indicative of the immune-mediated disease in the subject.

45. The method according to Claim 41, wherein the albumin is obtained from whole blood or a blood fraction obtained from human, cow, chicken or pig blood.

46. The method according to Claim 45, wherein the albumin is recombinant albumin.

47. The method according to any one of Claims 41 – 46, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

48. The method according to Claim 47, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

49. The method according to any one of Claims 41 – 46, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the C-peptide binding facilitator.

50. The method according to Claim 49, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

51. The method according to Claim 49, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

52. The method according to Claim 51, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

53. The method according to Claim 47, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

54. The method according to any one of Claims 41 – 53, wherein the C-peptide is modified with a heterologous moiety.

55. The method according to Claim 54, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

56. The method according to any one of Claims 41 – 55, wherein the C-peptide binding facilitator comprises a tag for quantifying the C-peptide, preferably wherein the tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

57. The method according to any one of Claims 41 – 56, wherein the cells express Glut1.

58. The method according to Claim 57, wherein the cells are red blood cells (RBCs), macrophages, neutrophils, or endothelial cells.

59. The method according to any one of the preceding Claims, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

60. The method according to any one of Claims 41 – 59, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from the known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

61. The method according to Claim 60, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

62. The method according to any one of Claims 41 – 61, wherein the incubating comprises admixing (i) from about 0.05 mL to about 5 mL of a suspension of the cells, (ii) about 1 to about 50 pmoles of C-peptide, and (iii) C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 10% (w/v).

63. The method according to Claim 62, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

64. The method according to Claim 61, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

65. The method according to any one of Claims 62 - 64, wherein the C-peptide and C-peptide binding facilitator are components of an isotonic solution.

66. The method according to Claim 65, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1000 nM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 10% (w/v).

67. The method according to any one of Claims 41 – 66, wherein the immune-mediated disease is MS.

68. A method of analyzing the risk of an immune-mediated disease in a mammalian subject, comprising effecting an assay on cells obtained from the subject, the assay comprising a method according to any one of Claims 41-67.

69. A kit for use measuring C-peptide binding by cells, the kit comprising a C-peptide binding facilitator, C-peptide, and a container.

70. A kit according to Claim 69, wherein the container is operable for mixing a solvent with one or both of the C-peptide binding facilitator and the C-peptide.

71. A kit according to Claim 70, wherein the container is operable for mixing one or both of the C-peptide binding facilitator and the C-peptide with a suspension of the cells.

72. A kit according to Claim 69, wherein the container is a first container containing the C-peptide binding facilitator, and the kit further comprises a second container containing C-peptide.

73. The kit according to any one of Claims 69 – 72, wherein the C-peptide binding facilitator is an albumin.

74. The kit according to Claim 73, wherein the albumin is in powdered form, preferably wherein the albumin is lyophilized.

75. The kit according to any one of Claims 69 – 74, wherein the C-peptide is in powdered form, preferably wherein the C-peptide is lyophilized.

76. The kit according to Claim 72, further comprising a third container containing a solvent for one or both of the C-peptide binding facilitator and the C-peptide.

77. The kit according to Claim 76, wherein the solvent comprises phosphate buffered saline, physiological saline solution, tris(hydroxymethyl)aminomethane buffer, or phosphate buffer.

78. The kit according to Claim 76 or Claim 77, further comprising a fourth container operable for receiving the cells.

79. The kit according to any one of Claims 69 – 78, further comprising a reagent for determining an amount of C-peptide bounded by the cells.

80. A method for measuring C-peptide binding by cells using the kit according to any one of Claims 69 – 79.

81. The method according to Claim 80, the method comprising:

mixing the C-peptide binding facilitator and the C-peptide to form a C-peptide/facilitator mixture;

incubating the cells with the C-peptide/facilitator mixture to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

82. A method for measuring C-peptide binding by cells using the kit according to Claim 76 or Claim 77, the method comprising:

mixing the C-peptide binding facilitator and the C-peptide to form a C-peptide/facilitator mixture;

incubating the cells with the C-peptide/facilitator mixture to produce incubated cells in an incubated cellular mixture; and

determining the amount of C-peptide bound to the incubated cells.

83. The method according to Claim 82, wherein C-peptide/facilitator mixture is an isotonic solution comprising the C-peptide and C-peptide binding facilitator.

84. The method according to Claim 82, wherein the isotonic solution comprises the solvent in the third container.

85. The method according to Claim 81, wherein the mixing and contacting are performed simultaneously or sequentially in the first container or second container.

86. The method according to Claim 82, wherein the mixing and contacting are performed simultaneously or sequentially in the third container.

87. The method according to Claim 82, wherein the kit comprises a fourth container and the mixing and contacting are performed simultaneously or sequentially in the fourth container.

88. The method according to Claim 82 comprising transferring a predetermined volume of the solvent in the third container to one of the first container or the second container and dissolving the contents of the first container or the second container in the solution to form a first working solution;

transferring the partial working solution to the other of the first container or the second container and dissolving the contents of the other of the first container or the second container to form a second working solution;

incubating a suspension of the cells with the second working solution to generate a C-peptide cellular mixture under conditions that allow for the cells to bind the C-peptide.

89. The method according to Claim 88, further comprising:
contacting a detection reagent with the second working solution; and
measuring the amount of C-peptide in the second working solution.

90. The method according to any one of Claims 81 – 89, wherein the incubating comprises admixing (i) from about 0.05 mL to about 5 mL of a suspension of the cells, (ii) about 1 to about 50 pmoles of the C-peptide, and (iii) the C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 10% (w/v).

91. The method according to Claim 90, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

92. The method according to Claim 90, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

93. The method according to any one of Claims 81 –92, wherein the concentration of C-peptide is from about 100 pM to about 1000 nM and the concentration of the C-peptide binding facilitator is from about 0.05% (w/v) to about 10% (w/v).

94. A reagent for use in measuring C-peptide binding to cells that express glucose 1 transporter protein (Glut1), the reagent consisting essentially of albumin dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of C-peptide, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof.

95. The reagent according to Claim 94, wherein the buffering agent is selected from the group consisting of phosphate salts, tris(hydroxymethyl)aminomethane, and mixtures thereof.

96. The reagent according to Claim 94, containing C-peptide.

97. A method for measuring C-peptide binding by cells using the reagent of any of Claims 94 – 96.

98. A method for detecting a metabolic disorder in a cell using the reagent of any of Claims 94 –96.

99. The method according to Claim 97 or Claim 98, comprising incubating from about 0.05 mL to about 5 mL of a the suspension of the cells with the reagent, wherein the reagent comprises from about 1 to about 50 pmoles of the C-peptide, and a C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 10% (w/v).

100. The method according to Claim 99, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

101. The method according to Claim 100, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

102. The method according to any one of Claims 97 – 101, wherein the concentration of C-peptide in the reagent is from about 100 pM to about 1mM and the concentration of the C-peptide binding facilitator in the reagent is from about 0.05% (w/v) to about 10% (w/v).

103. A reagent for use in measuring C-peptide binding to cells that express glucose 1 transporter protein (Glut1), the reagent consisting essentially of C-peptide dissolved in an isotonic solution and, optionally, further consisting of an optional component selected from the group consisting of albumin, a buffering agent, Zn^{2+}, Fe^{2+}, Cr^{3+}, and mixtures thereof.

104. The reagent according to Claim 103, containing a buffering agent is selected from the group consisting of phosphate salts, tris(hydroxymethyl)aminomethane, and mixtures thereof.

105. The reagent according to Claim 103, containing albumin.

106. A method for measuring C-peptide binding to cells using the reagent of any of Claims 103 – 105.

107. A method for detecting a metabolic disorder in a cell using the reagent of any of Claims 103 – 105.

108. The method according to Claim 106 or Claim 107, comprising contacting from about 0.05 mL to about 20 mL of a suspension of the cells with the reagent, wherein the reagent comprises from about 1 to about 500 pmoles of the C-peptide, and C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

109. The method according to Claim 108, wherein the suspension of the cells comprises red blood cells (RBCs) and has a hematocrit of from about 2% to about 20%.

110. The method according to Claim 109, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

111. The method according to any one of Claims 106 – 110, wherein the concentration of C-peptide in the reagent is from about 100 pM to about 1 mM and the concentration of the C-peptide binding facilitator in the reagent is from about 0.05% (w/v) to about 25% (w/v).

112. An *in vitro* method for diagnosing a mammalian subject as having an immune-mediated disease, the method comprising:

incubating, in the presence of albumin, red blood cells (RBCs) from the subject with C- peptide under conditions that allow for binding of C-peptide by RBCs; and

determining the amount of C-peptide bound by the red blood cells, wherein increased binding of C-peptide by the red blood cells, relative to a control C-peptide binding level, indicates that the subject has the immune-mediated disease.

113. The method according to Claim 112, wherein the immune-mediated disease is an inflammatory demyelinating disorder.

114. The method according to Claim 113, wherein the inflammatory demyelinating disorder is MS.

115. The method according to Claim 112, wherein the albumin is obtained from whole blood or a blood fraction obtained from human, cow, chicken or pig blood.

116. The method according to Claim 112, wherein the albumin is recombinant albumin.

117. The method according to any one of Claims 112 – 116, wherein the C-peptide is a polypeptide comprising the amino acid sequence EGSLQ.

118. The method according to Claim 117, wherein the C-peptide is a mammalian C-peptide, preferably human C-peptide.

119. The method according to Claim 112, wherein the C-peptide is selected from the group consisting of a full length wild type C-peptide, a C-peptide molecule having an amino acid sequence at least 75% identical to the amino acid sequence of wild type C-peptide, fractions thereof comprising at least 5 amino acids, and combinations thereof, wherein the C-peptide has the ability to bind to cells in the presence of the albumin.

120. The method according to Claim 119, wherein the C-peptide is a fraction thereof comprising the five terminal amino acids of a wild type C-peptide, optionally consisting of the amino acid sequence EGSLQ.

121. The method according to Claim 119, wherein the C-peptide has an amino acid sequence at least 75% identical to the amino acid sequence of a full length C-peptide.

122. The method according to Claim 121, wherein the full length C-peptide is isolated from a human, pig, cow, chicken, or zebrafish.

123. The method according to Claim 117, wherein the C-peptide is recombinant C-peptide or C-peptide synthesized *in vitro*.

124. The method according to any one of Claims 112 – 123, wherein the C-peptide is modified with a heterologous moiety.

125. The method according to Claim 124, wherein the heterologous moiety is a radioactive label, an enzymatic label, a fluorescent label, a heavy metal label, a fluorescent label, a luminescent label, or an affinity tag.

126. The method according to any one of Claims 112 – 125, wherein the albumin comprises a tag for quantifying the C-peptide, preferably wherein the tag comprises FLAG, polyhistidine, hemagglutinin, glutathione-S-transferase, or maltose-binding protein.

127. The method according to any one of Claims 112 – 126, wherein the cells express glucose 1 transporter protein (Glut1).

128. The method according to Claim 127, wherein the cells are RBCs, macrophages, neutrophils, or endothelial cells.

129. The method according to any one of Claims 112 – 128, wherein the incubating is performed for from about 1 minute to about 24 hours at a temperature of from about 5° C to about 50° C.

130. The method according to any one of Claims 112 – 129, wherein the determining an amount of C-peptide bound to the incubated cells comprises:

separating the incubated cells from the incubated cellular mixture, forming an incubated cell fraction and a supernatant; and

measuring

(i) the amount of C-peptide in the incubated cellular fraction; or

(ii) the amount of C-peptide in the supernatant; or

(iii) the amount of C-peptide in the supernatant, and subtracting the amount of C-peptide in the supernatant from a known amount of C-peptide; or

(iv) any combination of (i), (ii), and (iii).

131. The method according to Claim 130, wherein the measuring comprises directly detecting C-peptide by detecting a signal provided by a tag coupled to the C-peptide or indirectly detecting C-peptide by detecting an antibody or antibody fraction that binds to the C-peptide.

132. The method according to any one of Claims 112 – 131, wherein the incubating comprises admixing (i) from about 0.05 mL to about 20 mL of a suspension of the cells, (ii) about 1 to about 500 pmoles of the C-peptide, and (iii) the C-peptide binding facilitator to a final concentration of from about 0.05% (w/v) to about 25% (w/v).

133. The method according to Claim 132, wherein the suspension of the cells comprises RBCs and has a hematocrit of from about 2% to about 20%.

134. The method according to Claim 131, wherein the suspension of the cells is whole blood, or a blood fraction comprising RBCs, membranes of RBCs, neutrophils, macrophages, or leukocytes.

135. The method according to any one of Claims 132 – 134, wherein the C-peptide and C-peptide binding facilitator are components of an isotonic solution.

136. The method according to Claim 135, wherein the concentration of C-peptide in the isotonic solution is from about 100 pM to about 1 mM and the concentration of the albumin is from about 0.05% (w/v) to about 25% (w/v).

137. A method of managing MS in a subject in need thereof, the method comprising:

(a) effecting an assay for measuring C-peptide binding on cells obtained from the subject, the assay comprising

(i) incubating the cells with a C-peptide composition comprising C-peptide and a C-peptide binding facilitator to produce incubated cells in an incubated cellular mixture; and

(ii) determining the amount of C-peptide bound to the incubated cells.

(b) comparing the amount of C-peptide bound by the cells to a reference amount determined by performing the assay on cells obtained from a second subject that does not have the immune-mediated disease;

(c) administering a first treatment for MS; and

(d) assessing the efficacy of the administering by repeating the effecting and comparing.

138. The method of Claim 137, further comprising administering a second treatment of MS after the assessing, wherein the second treatment is altered from the first treatment based on the results of the assessing.

139. A method of assessing the responsiveness of a mammalian subject having MS to a treatment for the MS, comprising a method according to Claim 137.

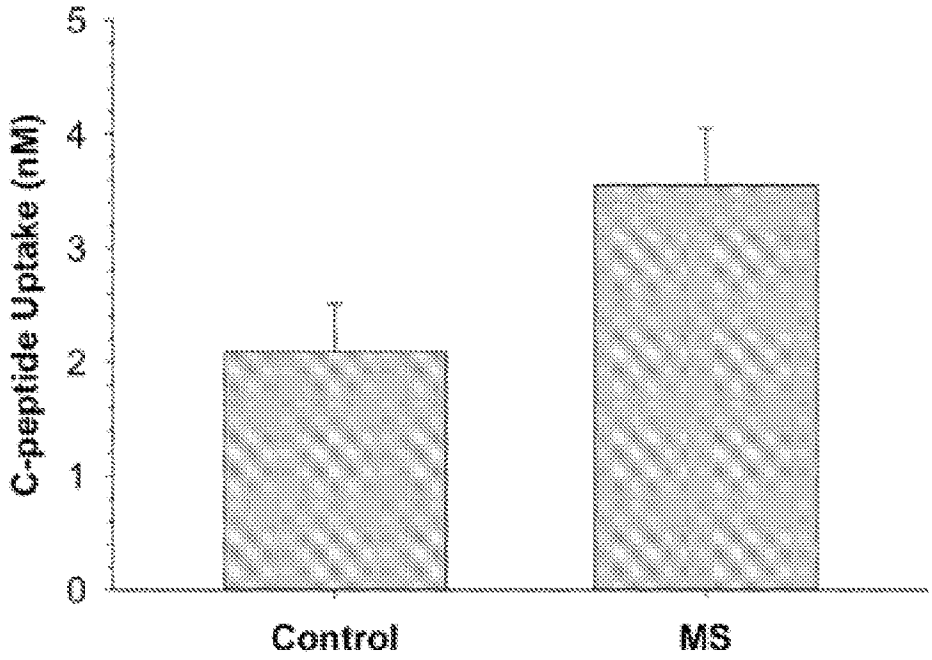


Fig. 1

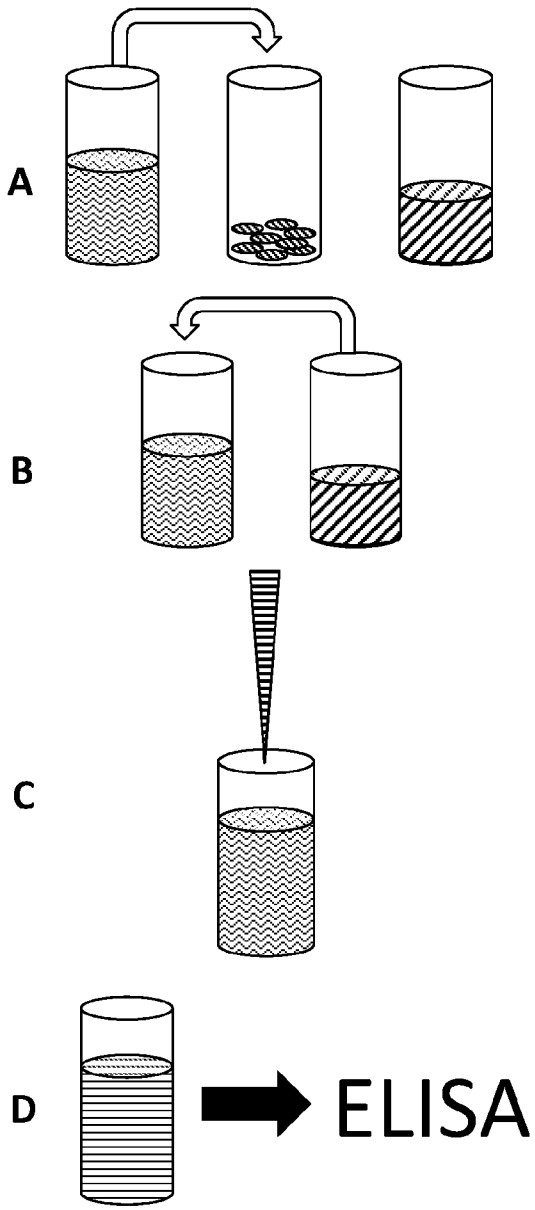


Fig. 2

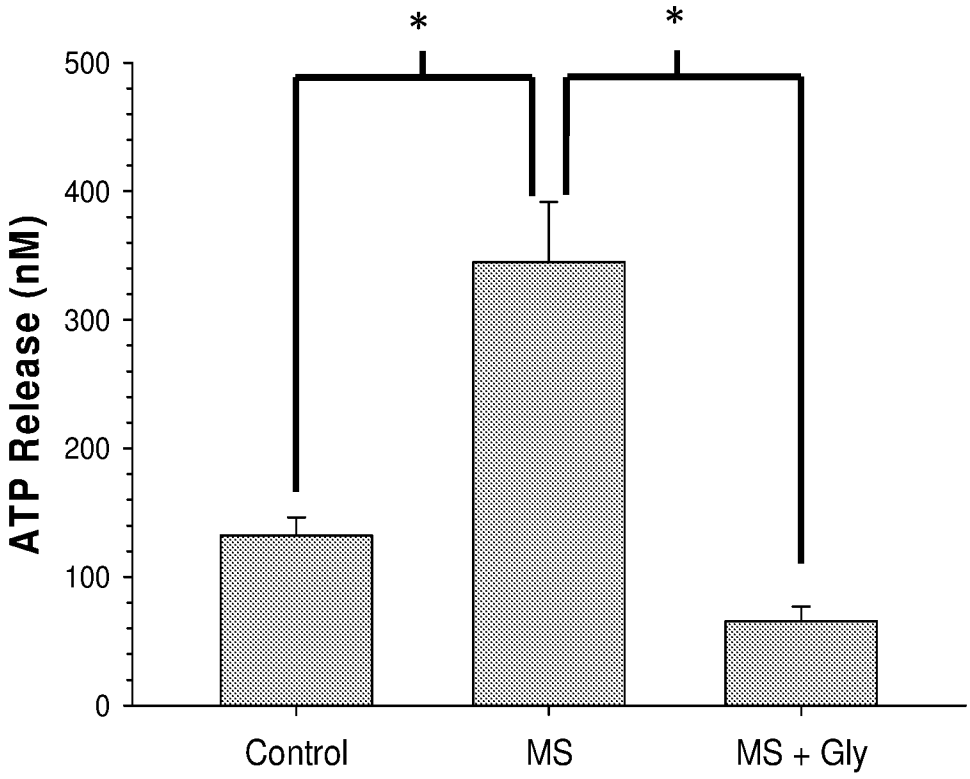


Fig. 3

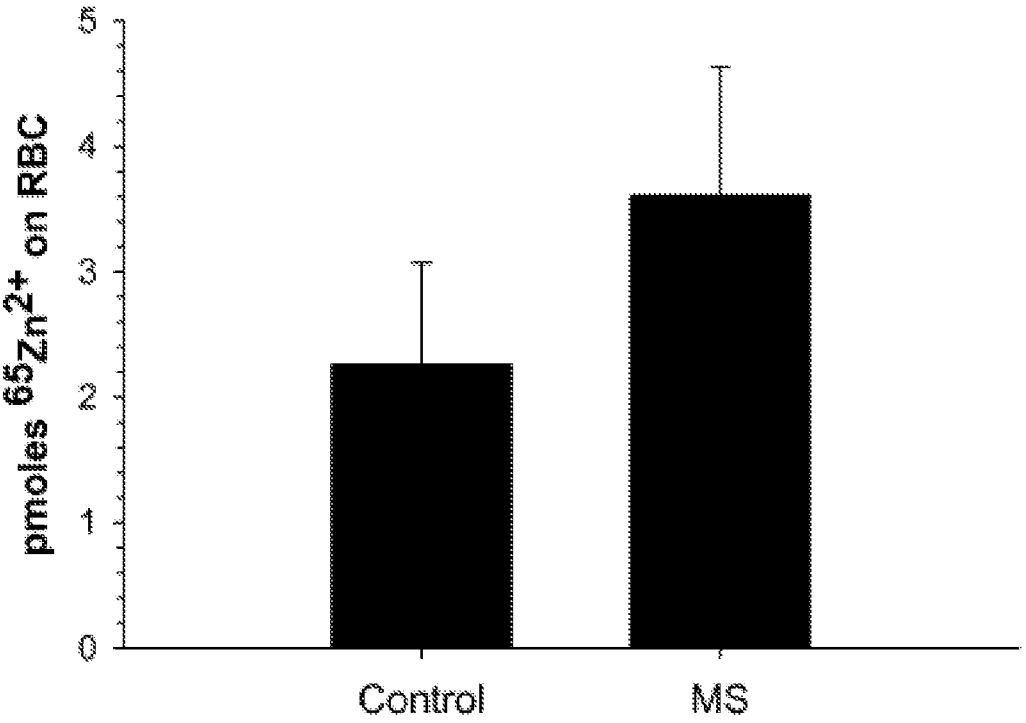


Fig. 4

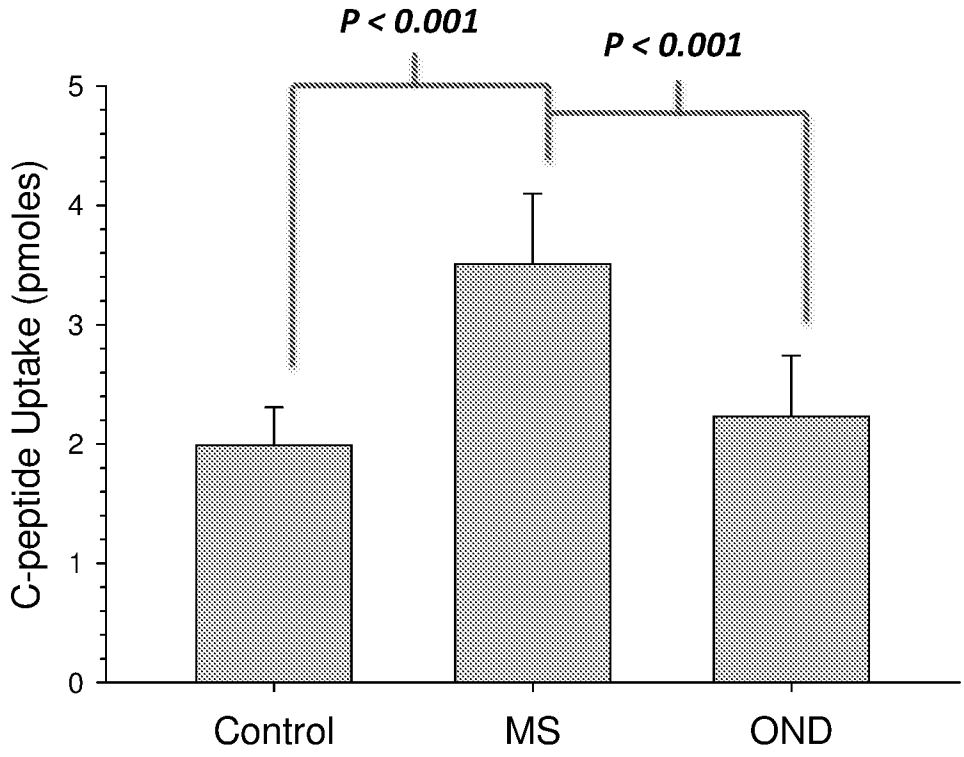


Fig. 5

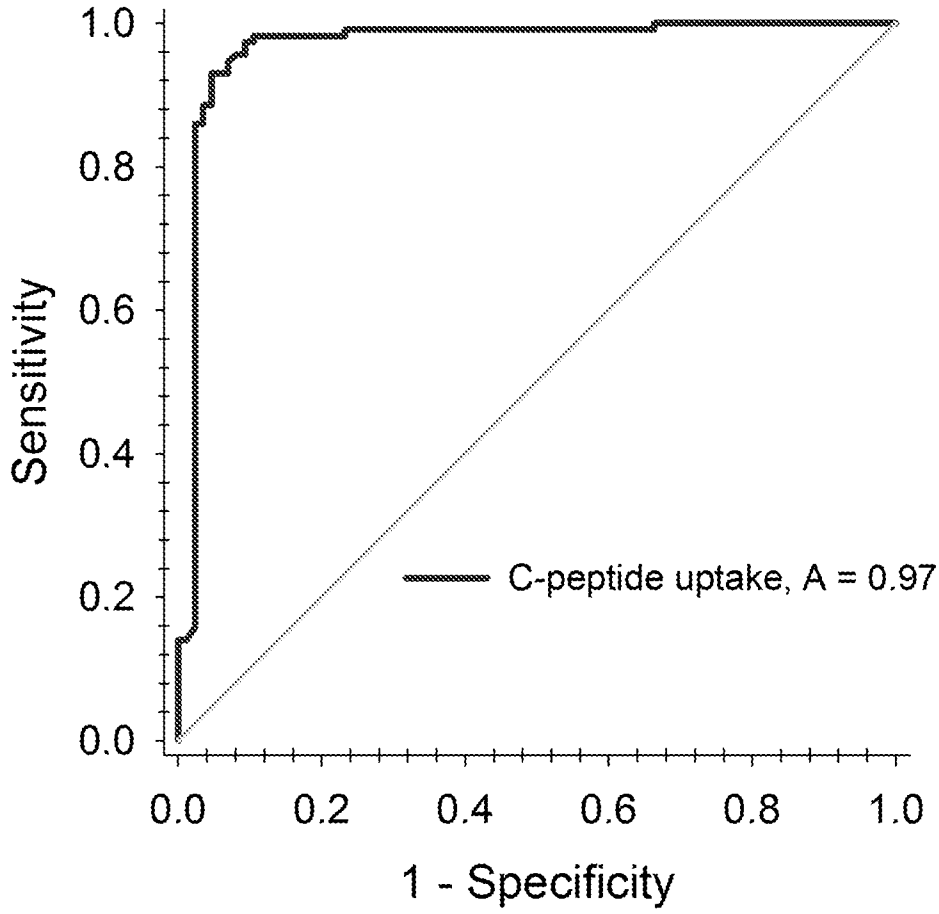

1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
C-peptide uptake, A = 0.97
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Fig. 6

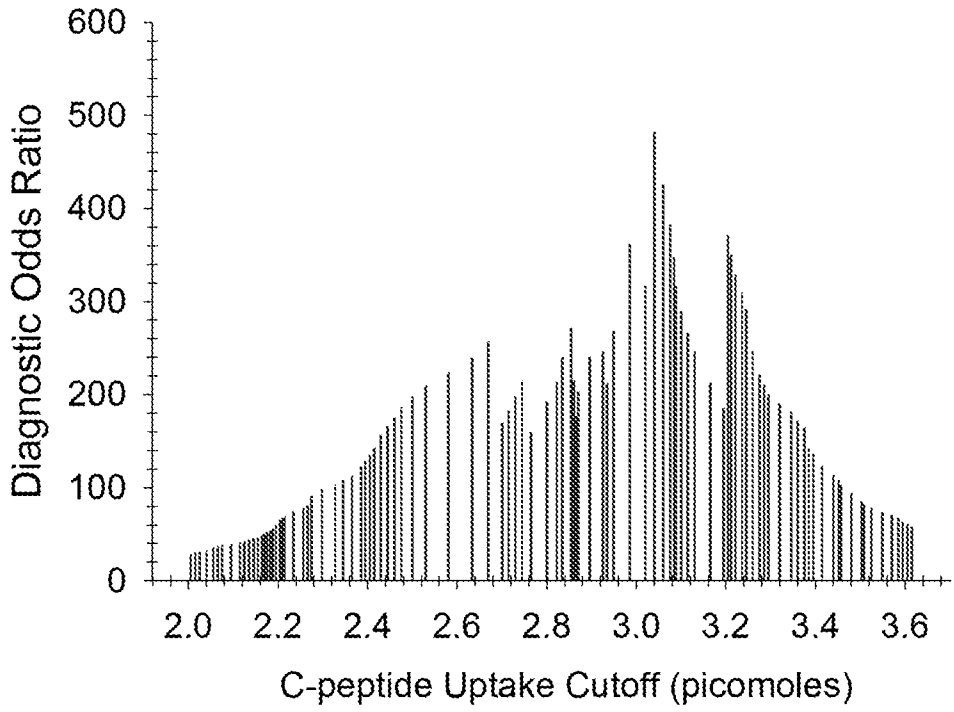

600
500
400
300
200
100
0
Diagnostic Odds Ratio
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
C-peptide Uptake Cutoff (picomoles)

Fig. 7

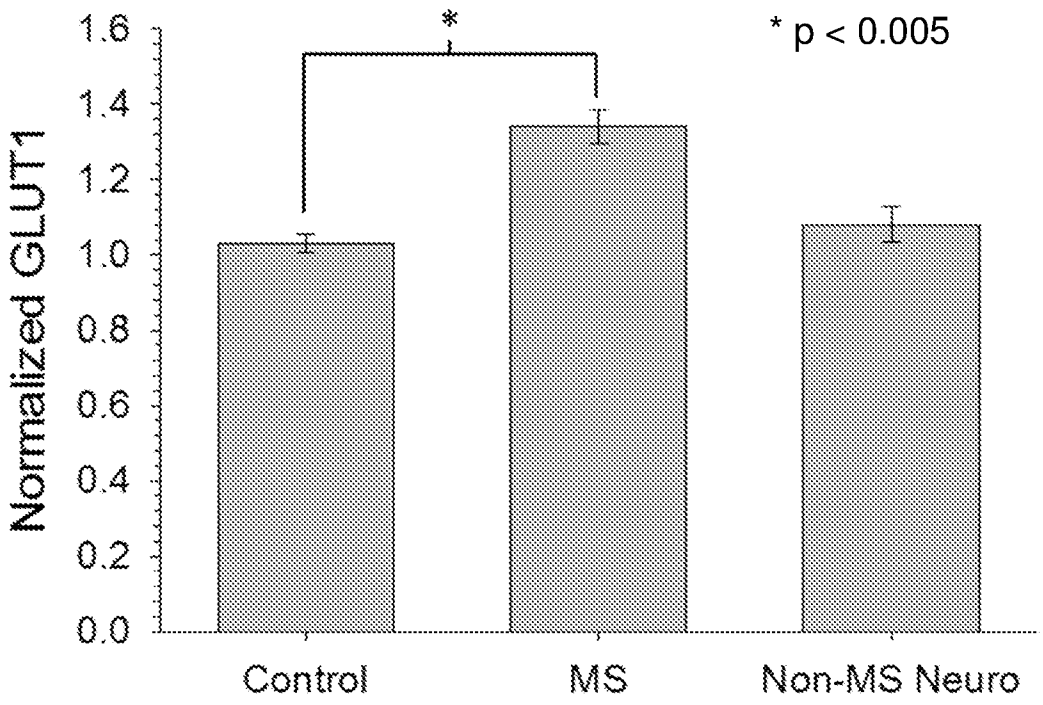

Normalized GLUT1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
* p < 0.005
Control
MS
Non-MS Neuro

Fig. 8

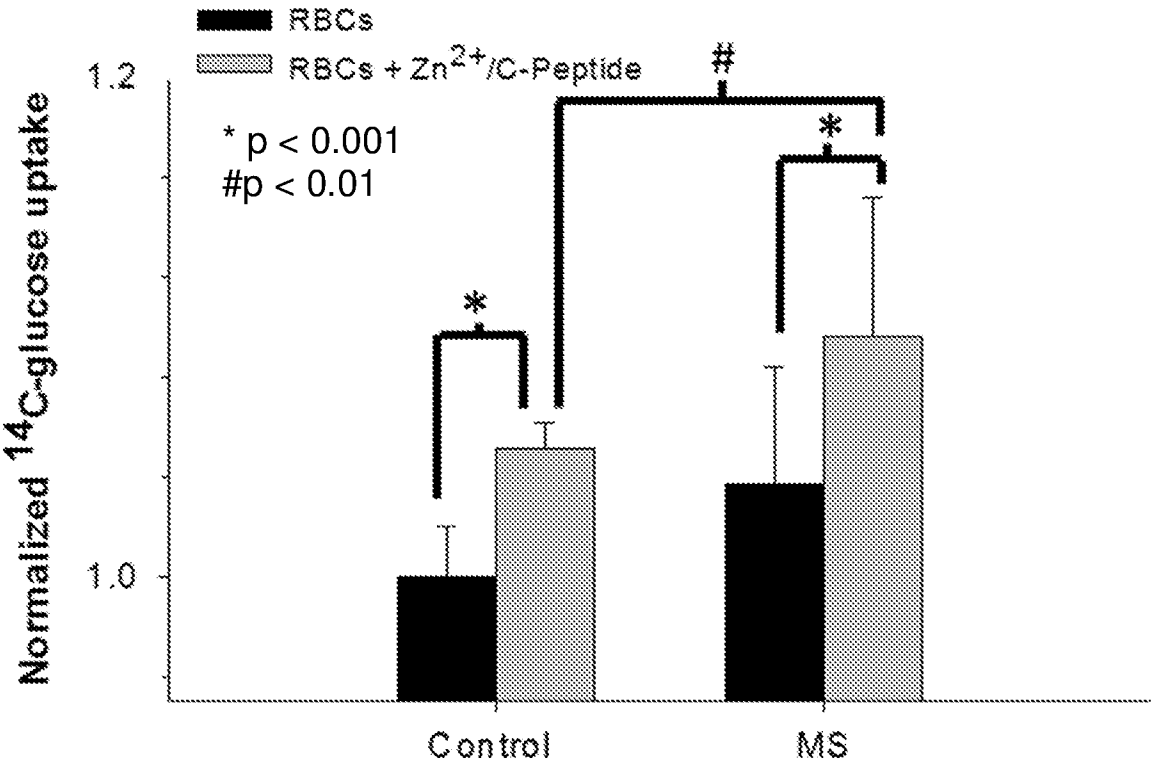

RBCs
RBCs + Zn2+/C-Peptide
* p < 0.001
#p < 0.01
Normalized 14C-glucose uptake
1.2
1.0
*
*
#
Control
MS

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/022148

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 33/30; A61K 38/17; A61K 38/28; A61K 38/38; A61P 3/00 (2016.01)
CPC - A61K 33/24; A61K 38/28; A61K 45/06; G01N 2333/62 (2016.05)
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)
IPC(8) - A61K 33/30; A61K 38/17; A61K 38/28; A61K 38/38; A61P 3/00 (2016.01)
CPC - A61K 33/24; A61K 38/28; A61K 45/06; G01N 2333/62 (2016.05)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/288.1; 514/263.31; 530/324 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, Google, PubMed.
Search terms used: C-peptide, connecting peptide, insulin; binding; immune-mediated, autoimmune; multiple sclerosis; red blood cells, RBC, erythrocytes; GLUT1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	RIGLER et al. "Specific binding of proinsulin C-peptide to human cell membranes". Proc Natl Acad Sci U S A, 09 November 1999 (09.11.1999), Vol. 96, Pgs. 13318-23, entire document	1-4, 6-12 ---------- 5
X -- Y	FLATT et al. "Specific Binding of the C-Peptide of Proinsulin to Cultured B-Cells from a Transplantable Rat Islet Cell Tumor". Biosdience Reports, 01 February 1986 (01.02.1986), Vol. 6, Pgs. 193-199, entire document	94-97, 103-106 ---------- 82-84, 86-89, 98, 107
X --- Y	CN 103852584 A (CHONGQING ELEMENT IN BIOLOGICAL TECHNOLOGY CO LTD) 11 June 2014 (11.06.2014) entire document; see machine translation	69 ---------- 70-74, 76-78, 82-84, 86-89
Y	US 2009/0171070 A1 (VAN URK et al) 02 July 2009 (02.07.2009) entire document	5
Y	US 2002/0160435 A1 (KITAJIMA et al) 31 October 2002 (31.10.2002) entire document	70-72, 74, 76-78, 82-84, 86-89
Y	US 2014/0141509 A1 (THE CHILDREN'S HOSPITAL OF PHILADELPHIA) 22 May 2014 (22.05.2014) entire document	71, 78
Y	KLEIN et al. "Albumin-bound basal insulin analogues (insulin detemir and NN344): comparable time-action profiles but less variability than insulin glargine in type 2 diabetes," Diabetes Obes Metab., 01 May 2007 (01.05.2007), Vol. 9, Pgs. 290-299. entire document	73, 74

☒ 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	Date of mailing of the international search report
06 May 2016	21 JUN 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/022148

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 13-25, 27-40, 54-68, 75, 79-81, 85, 90-93, 99-102, 108-111, 126-136
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/022148

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KUNT et al. "The effect of human proinsulin C-peptide on erythrocyte deformability in patients with Type I diabetes mellitus," Diabetologia, 01 April 1999 (01.04.1999), Vol. 42, Pgs. 465-471. entire document	98, 107
A	US 5,491,216 A (HOFFMANN et al) 13 February 1996 (13.02.1996) entire document	1-12, 41-53, 69-74, 76-78, 82-84, 86-89, 94-98, 103-107, 112-125, 137-139
A	US 2011/0020471 A1 (SPENCE et al) 27 January 2011 (27.01.2011) entire document	1-12, 41-53, 69-74, 76-78, 82-84, 86-89, 94-98, 103-107, 112-125, 137-139
A	DOUGHTY et al. "Antigen receptor–mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth," BLOOD, 01 June 2006 (0 1.06.2006), Vol. 107, Pgs. 4458-4465. entire document	1-12, 41-53, 69-74, 76-78, 82-84, 86-89, 94-98, 103-107, 112-125, 137-139
A	MEYER et al. "Metal-activated C-peptide Facilitates Glucose Clearance and the Release of a Nitric Oxide Stimulus via the GLUT1 Transporter," Diabetologia. 27 October 2007 (27.10.2007), Vol. 51, Pgs. 175–182. entire document	1-12, 41-53, 69-74, 76-78, 82-84, 86-89, 94-98, 103-107, 112-125, 137-139