



(43) International Publication Date
18 June 2015 (18.06.2015)

(10) International Publication Number
WO 2015/085368 A1

- (51) **International Patent Classification:**
G01N 33/50 (2006.01) *C12Q 1/68* (2006.01)
- (21) **International Application Number:**
PCT/AU2014/050415
- (22) **International Filing Date:**
10 December 2014 (10.12.2014)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
2013904799 10 December 2013 (10.12.2013) AU
- (71) **Applicant:** THE UNIVERSITY OF QUEENSLAND
[AU/AU]; The University of Queensland, St Lucia,
Queensland 4072 (AU).
- (72) **Inventor:** THOMAS, Ranjeny; 172 Barton Road,
Hawthorne, Queensland 4171 (AU).
- (74) **Agent:** DAVIES COLLISON CAVE; Level 10/301
Coronation Drive, Milton, Queensland 4064 (AU).
- (81) **Designated States** (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) **Title:** KITS AND METHODS FOR THE DIAGNOSIS, TREATMENT, PREVENTION AND MONITORING OF DIABETES

(57) **Abstract:** The present invention discloses a method and kit for making clinical assessments, such as early diagnostic, diagnostic, disease stage, disease severity, disease subtype, disease susceptibility, response to therapy or prognostic assessments. More particularly, the present invention discloses methods and kits for identifying a subject with or at risk of developing, Type 1 diabetes (T1D), or stratifying a subject with risk of development of T1D to a treatment regimen based on a biomarker profile.



WO 2015/085368 A1

TITLE OF THE INVENTION

"KITS AND METHODS FOR THE DIAGNOSIS, TREATMENT, PREVENTION AND MONITORING OF DIABETES"

FIELD OF THE INVENTION

[0001] The present invention relates to a method and kit for making clinical assessments, such as early diagnostic, diagnostic, disease stage, disease severity, disease subtype, disease susceptibility, response to therapy or prognostic assessments. More particularly, the present invention relates to methods and kits for identifying a subject with or at risk of developing, Type 1 diabetes (T1D), or stratifying a subject with risk of development of T1D to a treatment regimen based on a biomarker profile.

[0002] Bibliographic details of certain publications numerically referred to in this specification are collected at the end of the description.

BACKGROUND OF THE INVENTION

[0003] In Type 1 diabetes (T1D), autoimmune-mediated destruction or dysfunction of insulin-producing β -cells of the pancreatic islets results in diabetes onset during childhood or young adulthood, requiring life-long insulin replacement to maintain glucose control. Before the onset of clinical diabetes, it is thought that a progressive decline in β -cell reserve occurs. The incidence of T1D has been increasing by about 3% per year since the mid 1950s. Genetic and environmental factors each contribute to the development of the disease. The strongest genetic associations with T1D susceptibility lie in the HLA locus. High risk HLA alleles include DRB1*0301-DQB1*0201 and DRB1*04-DQB1*0302 in Caucasians¹. Autoimmunity is marked by the appearance of autoantibodies (AB) directed towards islet antigens, including glutamic acid decarboxylase (GAD), insulin/pro-insulin and insulinoma-associated protein (IA-2), which can precede expression of clinical diabetes by up to 8 years. AB status is an important indicator of T1D risk. The presence of multiple islet AB is generally established by the age of 14 and prospectively identifies individuals who go on to develop T1D². Among T1D first-degree relatives (T1D FDR) recruited to the Diabetes Prevention Trial-Type 1 (DPTT1), the risk of developing T1D within 5 years was 25% for individuals with 1 AB, 50-60% for 2 AB and 70% for 3 AB³. Glucose intolerance and insulin resistance are additional predictors of the progression to T1D in AB⁺ individuals^{4,5}. The expansion in new cases is preferentially occurring in younger children, and in children carrying lower risk T1D HLA haplotypes^{1,6,7}, suggesting that environmental

factors are promoting the expression of T1D in children who would otherwise have been at lower genetic risk of disease⁸.

[0004] In the general community, the low predictive value of HLA genotyping for T1D development and the low frequency of positive AB tests precludes screening to identify at-risk subjects, particularly children, without a family history. However, the majority of individuals who develop T1D have no family history, and there are no biomarkers for population screening to identify at-risk individuals. Thus, identification of at-risk individuals without a family history from the general population, and the discrimination of high risk AB⁻ individuals in whom preventive strategies might be employed remains a significant challenge. Furthermore, while several immunotherapies, including anti-CD3, anti-CD20 and CTLA4Ig have appeared promising in small trials of T1D patients, results have been disappointing in later phase trials. Hence, there is a clear need for the development of new methods for identifying biomarkers additional to the currently-used islet AB, HLA typing, glucose intolerance and insulin resistance that are capable of identifying subjects that are at risk of developing T1D and of stratifying at-risk AB⁻ and AB⁺ FDR, especially biomarkers that would elucidate disease immunopathogenesis and identify possible novel therapeutic targets.

SUMMARY OF THE INVENTION

[0005] The present invention is predicated, in part, on the surprising finding that subjects at risk of developing T1D, such as first degree relatives (*i.e.*, siblings) of individuals with T1D, have a biomarker profile that distinguishes them from individuals who are not considered at risk of developing T1D, including healthy individuals. In this regard, the present inventors have found that several biomarkers, including inflammatory biomarkers, are differentially expressed in subjects who are at risk of developing T1D as compared to healthy controls. Surprisingly, the differential expression of these biomarkers in at-risk individuals was apparent in islet autoantibody negative and islet autoantibody positive subjects, indicating that their differential expression is independent of islet autoantibody status. Based on this finding, a subject's biomarker profile can be used as a diagnostic tool to determine the subject's risk of developing T1D.

[0006] Accordingly, in one aspect, the present invention provides a method for determining whether a subject is at risk of developing Type 1 diabetes (T1D), the method comprising: (1) correlating a reference biomarker profile with the risk of development of T1D, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of interleukin 12B (IL12B), platelet-

derived growth factor BB (PDGFBB), adiponectin, neutrophil-activating protein-2 (NAP2) and Angiopoietin-like 4 (ANGPTL4), Monocyte chemotactic protein 2 (MCP-2); Chemokine (C-C motif) ligand 2 (CCL2)), fractalkine, vascular endothelial cell growth factor receptor 1 (VEGFR1) and serum amyloid P (SAP); (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) determining whether the subject has an increased risk of developing T1D based on the sample biomarker profile and the reference biomarker profile.

[0007] The inventors' findings enable treatment regimens, which can be adopted or prescribed, particularly at an earlier stage in the progression towards T1D, with a view to preventing or delaying the onset of T1D in a subject. Thus, in another aspect, the present invention provides a method for preventing or delaying the onset of T1D or a symptom thereof in a subject, the method comprising:

- (a) determining whether a subject is at risk of developing T1D according to the method broadly described above and elsewhere herein; and
- (b) exposing the subject, on the basis that the subject has an increased risk or likelihood of developing T1D, to a treatment regimen for preventing or delaying the onset of T1D or a symptom thereof.

[0008] The inventors' findings also enable methods of monitoring the efficacy of a treatment regimen for preventing or delaying the onset of T1D and determining a subject's response to such treatment (*e.g.*, whether it is a positive or negative response to such treatment).

[0009] Thus, in another aspect, a method is provided for monitoring the efficacy of a treatment regimen in a subject at risk of developing T1D, the method comprising: (1) providing a correlation of a reference biomarker profile with a likelihood of having a healthy condition, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; (2) obtaining a corresponding biomarker profile of a subject at risk of developing T1D after commencement of a treatment regimen, wherein a similarity of the subject's biomarker profile after commencement of the treatment regimen to the reference biomarker profile indicates the likelihood that the treatment regimen is effective for changing (*e.g.*, improving) the health status of the subject.

[0010] In another aspect, the present invention provides a method of correlating a reference biomarker profile with an effective treatment regimen for

preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine, the method comprising: (1) determining a sample biomarker profile from a subject at risk of developing T1D prior to commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (2) correlating the sample biomarker profile with a treatment regimen that is effective for preventing or delaying the onset of T1D, or a symptom thereof.

[0011] In another aspect, the present invention provides a method of determining whether a treatment regimen is effective for preventing or delaying the onset of T1D or a symptom thereof in a subject at risk of developing T1D, the method comprising: (1) correlating a reference biomarker profile prior to treatment with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) obtaining a sample biomarker profile from the subject after commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker, and wherein the sample biomarker profile after commencement of treatment indicates whether the treatment regimen is effective for preventing or delaying the onset of T1D, or a symptom thereof, in the subject.

[0012] In another aspect, the present invention provides a method of correlating a biomarker profile with a positive or negative response to a treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, the method comprising: (1) obtaining a sample biomarker profile from a subject at risk of developing T1D following commencement of the treatment regimen, wherein the biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) correlating the sample biomarker profile from the subject with a positive or negative response to the treatment regimen.

[0013] In another aspect, the present invention provides a method of determining a positive or negative response to a treatment regimen by a subject at risk of developing T1D, the method comprising: (a) correlating a reference biomarker profile with a positive or negative response to the treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the

reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; (b) determining a sample biomarker profile from the subject following commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (c) determining a positive or negative response to the treatment regimen based on a comparison of the sample biomarker profile and the reference biomarker profile.

[0014] The present inventors have also found that the biomarker profile of a subject at risk of developing T1D, independent of the subject's islet autoantibody status, stratifies the subject into an inflammatory and/or metabolic phenotype, allowing the subject determined to be at risk of developing T1D to be segregated to a targeted therapeutic regimen specific for an inflammatory and/or metabolic phenotype.

[0015] Thus, in another aspect, the present invention provides a method of stratifying a subject at risk of developing T1D to an anti-inflammatory treatment regimen, the method comprising (1) correlating a reference biomarker profile with an inflammatory phenotype, wherein the reference biomarker profile evaluates at least one inflammatory biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having an inflammatory phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to an anti-inflammatory treatment regimen.

[0016] In another aspect, the present invention provides a method of stratifying a subject at risk of developing T1D to a metabolic phenotype-targeted treatment regimen, the method comprising (1) correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having a metabolic phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to a metabolic phenotype-targeted treatment regimen.

[0017] In a related aspect, the present invention provides a kit comprising one or more reagents and/or devices for use in performing any one of the methods of the present invention as broadly described above and elsewhere herein.

[0018] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgement or admission or any form of suggestion that the prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1 shows constitutive inflammatory activity in peripheral blood of healthy children at familial risk of T1D. RELB (a) and RELA (b) DNA binding activity in whole PBMC was quantified by chemiluminescent ELISA. IL12A (c) and TNF (d) mRNA expression in whole PBMC was quantified by real time PCR. Serum sVEGFR (e) and serum amyloid P (SAP) (f) were assayed by the Luminex multiplex platform respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (g): Hierarchical clustering of subjects and markers that were significantly correlated with variables discriminating AB- FDR from healthy (Spearman's rank correlation, average linkage). The bar at the bottom of Figure 1g indicates the AB- FDR (dark/red shading) and healthy controls (HC) (lighter/yellow shading). The expression of each marker is expressed relative to the median value for that marker across all subjects, and is coloured according to the scale shown. Grey blocks represent missing data.

[0020] **Figure 2** shows clinical parameters associated with presence of multiple autoantibodies in FDR. Blood glucose levels at fasting (dark circles) and 120 minutes (light circles) after oral glucose in an oral glucose tolerance test (a), BMI age percentile (b) HbA1c (c) and HOMA-IR (d) are plotted in AB⁺ FDR. HOMA-IR cut-off at 2.6 is shown (dotted line). 120 min blood glucose levels were compared by one-way ANOVA with post-hoc test for trend and other measures were compared by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$.

[0021] **Figure 3** shows correlates of biomarkers that predict FDR with multiple AB. Hierarchical clustering of subjects and serum markers that were significantly correlated with variables discriminating AB⁺ from AB_{2/3}⁺ FDR (Spearman's rank correlation, average linkage). The bar at the bottom of Figure 3 is colour-coded to indicate the AB number, as shown. The expression of each marker is expressed relative to the median value for that marker across all subjects, and is coloured according to the scale shown on the left. Grey blocks represent missing data.

[0022] **Figure 4** shows hierarchical clustering of subjects and serum markers that were significantly correlated with variables discriminating HC from AB- FDR, and AB⁺ from AB2/3⁺ FDR (Spearman's rank correlation, average linkage). Branches are coloured according to AB number, as indicated. The expression of each marker is expressed relative to the median value for that marker across all subjects, and is coloured according to the scale shown on the left. Grey blocks represent missing data.

TABLE 1**BRIEF DESCRIPTION OF THE SEQUENCES**

Gene	Nucleotide Sequence
IL12A	Forward 5'-GCTCCAGAAGGCCAGACAAA-3' (SEQ ID NO:1)
	Reverse 5'-GCCTCCACTGTGCTGGTTTT-3' (SEQ ID NO:2)
GRP78	Forward 5'-AACACAGTGGTGCCTACCAAGAA-3' (SEQ ID NO:3)
	Reverse 5'-GTT TTTGTCAGGGGTCTTTCACCTT-3' (SEQ ID NO:4)
TNF	Forward 5'-CCTGTAGCCCATGTTGTAGCAAAC-3' (SEQ ID NO:5)
	Reverse 5'-GGG TCTCTCAGCTCCACGCCATT-3' (SEQ ID NO:6)

DETAILED DESCRIPTION OF THE INVENTION

[0023] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[0024] The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of

example, "a biomarker" means one biomarker or more than one biomarker, unless otherwise indicated.

[0025] Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. Thus, use of the term "*comprising*" and the like indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0026] The present invention is predicated, in part, on the inventors' surprising finding that subjects at risk of developing T1D, such as first degree relatives (*i.e.*, siblings) of individuals with T1D, have a biomarker profile that distinguishes them from individuals who are not considered at risk of developing T1D, including healthy individuals. In this regard, the present inventors have found that several biomarkers, including inflammatory biomarkers, are differentially expressed in subjects who are at risk of developing T1D as compared to healthy controls. Surprisingly, the differential expression of these biomarkers in at-risk individuals was apparent in islet autoantibody negative and autoantibody positive subjects, indicating that their differential expression is independent of islet autoantibody status. Based on this finding, a subject's biomarker profile can be used as a diagnostic tool to determine the subject's risk of developing T1D.

[0027] Thus, disclosed herein are methods for determining whether a subject is at risk of developing Type 1 diabetes (T1D), which comprise: (1) correlating a reference biomarker profile with the risk of development of T1D, wherein the reference biomarker profile evaluates at least one biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) determining whether the subject is at risk of developing T1D based on the sample biomarker profile and the reference biomarker profile. In some embodiments, the methods further comprise determining the islet autoantibody status of the subject. In illustrative examples of this type, the subject is islet autoantibody negative.

Biomarkers

[0028] The term "biomarker" typically refers to a measurable characteristic that reflects the presence or nature (*e.g.*, severity) of a physiological and/or pathophysiological state, including an indicator of risk of developing a particular physiological or pathophysiological state. For example, a biomarker may be present

in a sample obtained from a subject before the onset of a physiological or pathophysiological state, including a symptom, thereof. Thus, the presence of the biomarker in a sample obtained from the subject is likely to be indicative of an increased risk that the subject will develop the physiological or pathophysiological state or symptom thereof. Alternatively, or in addition, the biomarker may be normally expressed in an individual, but its expression may change (*i.e.*, it is increased (upregulated; over-expressed) or decreased (downregulated; under-expressed) before the onset of a physiological or pathophysiological state, including a symptom thereof. Thus, a change in the level of expression of the biomarker is likely to be indicative of an increased risk that the subject will develop the physiological or pathophysiological state or symptom thereof. Alternatively, or in addition, a change in the expression of a biomarker may reflect a change in a particular physiological or pathophysiological state, or symptom thereof, in a subject, thereby allowing the nature (*e.g.*, severity) of the physiological or pathophysiological state, or symptom thereof, to be tracked over a period of time. This approach may be useful in, for example, monitoring a treatment regimen for the purpose of assessing its effectiveness (or otherwise) in a subject. As herein described, reference to the expression of a biomarker includes the concentration of the biomarker, or a gene expression product thereof (*e.g.*, peptide, pro-peptide, metabolite thereof), as will be described in more detail below. Reference to the expression of a biomarker also includes the activity of a biomarker. For example, where the biomarker is an enzyme, its expression may be determined or measured by the level of activity of the enzyme on a known substrate.

[0029] The term "reference biomarker" is used herein to denote a biomarker that has been identified as being associated with a risk of developing T1D; particularly an increased risk of developing T1D. For example, a reference biomarker can be differentially expressed for a sample population of reference individuals at risk of developing T1D as compared to healthy controls. Reference individuals include, but are not limited to, first degree relatives (*i.e.*, siblings) of individuals who have T1D, also referred to herein as "T1D FDR". A reference biomarker profile provides a compositional analysis (*e.g.*, concentration, number ratio or mole percentage (%)) of the biomarker) in which one or more, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twelve or more, fifteen or more, twenty or more, fifty or more, one-hundred or more or a greater number of biomarkers are evaluated.

[0030] A suitable biomarker is typically a biological characteristic that can be detected and measured in a subject *in situ* or in a biological sample obtained from an subject (e.g., *ex vivo* or *in vitro*). Examples of suitable biomarkers include specific cells (e.g., CD14CD16 cells), molecules, or genes, gene products, enzymes, or hormones. Complex organ functions or general characteristic changes in biological structures can also serve as biomarkers. For example, body temperature is a well-known biomarker for fever and blood pressure can be used to determine the risk of stroke.

[0031] Not limiting examples of suitable biomarkers include at least one biomarker (e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more or 10) selected from the group consisting of interleukin 12B (IL12B), platelet-derived growth factor BB (PDGFBB), adiponectin, neutrophil-activating protein-2 (NAP2) and Angiopoietin-like 4 (ANGPTL4), Monocyte chemotactic protein 2 (MCP-2; Chemokine (C-C motif) ligand 2 (CCL2)), fractalkine, vascular endothelial cell growth factor receptor 1 (VEGFR1), serum amyloid P (SAP), and their corresponding transcripts.

[0032] In some embodiments, the biomarker is selected from the group consisting of VEGFR1 and SAP. As shown in Figures 1e and f, herein, the level of expression of VEGFR1 and SAP has been shown by the inventors to be significantly altered in subjects at risk of developing T1D as compared to healthy controls. For instance, the level of expression of VEGFR1 in subjects at risk of developing T1D (e.g., AB- T1D FDR) has been shown by the inventors to be significantly lower than the level of expression found in healthy individuals (see Figure 1e). Conversely, the level of expression of SAP in subjects at risk of developing T1D has been shown by the inventors to be significantly higher than levels found in healthy individuals (see Figure 1f).

[0033] The present inventors have found that subjects at risk of developing T1D can also be distinguished from healthy controls on the basis of differential expression of RELB (a marker of NF κ B DNA binding), tumor necrosis factor (TNF), 78kDa glucose-related protein (GRP78), CD14^{LOW}CD16⁻ cells and CD14^{HIGH}CD16⁺ cells and serum IL-12p40. Thus, in some embodiments, the reference biomarker profile further evaluates at least one (e.g., 1, at least 2, at least 3, at least 4, at least 5, at least 6 or 7) other biomarker selected from the group consisting of RELB, tumor necrosis factor (TNF), 78kDa glucose-related protein (GRP78), CD14^{LOW}CD16⁻ cells and CD14^{HIGH}CD16⁺ cells and serum IL-12p40 and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

[0034] The level of NF- κ B activation has been found to be significantly greater in blood cells of T1D FDR individuals who are islet autoantibody (AB) negative as compared to the level of NF- κ B activation in blood cells of healthy controls. NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA. NF- κ B is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF- κ B plays a key role in regulating the immune response to infection (κ light chains are critical components of immunoglobulins). Incorrect regulation of NF- κ B has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF- κ B activation can be determined by any means known to persons skilled in the art. In an illustrative example, NF- κ B activation (NF- κ B DNA binding) is assessed by measuring the level of expression of RELA (sc-372) and/or RELB (sc-226), which are components of the NF- κ B DNA binding complex. In some embodiments, the at least one biomarker is RELB, also referred to herein as RELB DNA binding. Methods of measuring NF- κ B activation in a sample are also known to persons skilled in the art, with some illustrative examples disclosed herein.

[0035] CD14^{LOW}CD16⁻ cell numbers and a CD14^{HIGH}CD16⁺ cell numbers need not be evaluated as absolute values. In some embodiments, cell numbers may be expressed as a percentage or ratio of the total number of cells in the blood (e.g., cells per mL blood) or of the total number of a subset of cells, such as peripheral blood mononuclear cells (PBMC). Methods by which the number of CD14^{LOW}CD16⁻ cells and a CD14^{HIGH}CD16⁺ cells can be determined will be known to persons skilled in the art. As an illustrative example, cell numbers may be measured by fluorescence-activated cell sorting (FACS) using detectable binding agents (e.g., fluorescein labelled antibodies) that selectively bind to CD14 and CD16 on the surface of the cells. FACS can then be used to determine whether the cells are CD14^{LOW}CD16⁻ cells and/or CD14^{HIGH}CD16⁺ cells by measuring the presence and intensity of staining of the anti-CD14 and anti-CD16 antibodies.

[0036] In some embodiments, it may be desirable to measure the biomarker at the protein level. As an illustrative example, the biomarker is soluble VEGFR1 (sVEGFR1) protein, as measured, for example, in blood. However, it will be understood that, in some instances, the biomarker can be a gene expression product such as transcript (e.g., mRNA) levels. Methods of measuring expression products such as proteins and transcripts are known to persons skilled in the art, with some illustrative examples described below.

[0037] Thus, a biomarker can be a gene expression product, including a polynucleotide or polypeptide. The term "gene" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. The term "gene" is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression. In this regard, the gene may further comprise control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals. The DNA sequences may be cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

[0038] The term "nucleic acid" or "polynucleotide" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA or RNA. "Protein," "polypeptide" and "peptide" are also used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same.

[0039] In some embodiments, evaluation of the biomarker comprises determining the level of the at least one biomarker. As used herein the terms "level" and "amount" are used interchangeably herein to refer to a quantitative amount (e.g., weight or moles or number), a semi-quantitative amount, a relative amount (e.g., weight % or mole % within class or a ratio), a concentration, and the like. Thus, these terms encompasses absolute or relative amounts or concentrations of biomarkers in a sample, including ratios of levels of biomarkers, and odds ratios of levels or ratios of odds ratios. biomarker levels in cohorts of subjects may be represented as mean levels and standard deviations as shown in some of the Tables and Figures herein.

[0040] Biomarkers may be quantified or detected using any suitable technique, including, but not limited to, nucleic acid- and protein-based assays.

[0041] In illustrative nucleic acid-based assays, nucleic acid is isolated from cells contained in a biological sample according to standard methodologies (Sambrook, *et al.*, 1989, *supra*; and Ausubel *et al.*, 1994, *supra*). The nucleic acid is typically fractionated (e.g., poly A⁺ RNA) or whole cell RNA. Where RNA is used as the subject of detection, it may be desired to convert the RNA to a complementary

DNA. In some embodiments, the nucleic acid is amplified by a template-dependent nucleic acid amplification technique. A number of template dependent processes are available to amplify the biomarker sequences present in a given template sample. An exemplary nucleic acid amplification technique is the polymerase chain reaction (referred to as PCR), which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, Ausubel *et al.* (*supra*), and in Innis *et al.*, ("PCR Protocols", Academic Press, Inc., San Diego Calif., 1990). Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the biomarker sequence. An excess of deoxynucleotide triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If a cognate biomarker sequence is present in a sample, the primers will bind to the biomarker and the polymerase will cause the primers to be extended along the biomarker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the biomarker to form reaction products, excess primers will bind to the biomarker and to the reaction products and the process is repeated. A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989, *supra*. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art.

[0042] In certain embodiments, the template-dependent amplification involves quantification of transcripts in real-time. For example, RNA or DNA may be quantified using the Real-Time PCR technique (Higuchi, 1992, *et al.*, *Biotechnology* **10**: 413-417). By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundance of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundance is only true in the linear range of the PCR reaction. The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. In specific embodiments, multiplexed, tandem PCR (MT-PCR) is employed, which

uses a two-step process for gene expression profiling from small quantities of RNA or DNA, as described for example in US Pat. Appl. Pub. No. 20070190540. In the first step, RNA is converted into cDNA and amplified using multiplexed gene specific primers. In the second step each individual gene is quantitated by real time PCR.

[0043] In certain embodiments, target nucleic acids are quantified using blotting techniques, which are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provides different types of information, although cDNA blotting is analogous, in many aspects, to blotting of RNA species. Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter. Subsequently, the blotted target is incubated with a probe (usually labelled) under conditions that promote denaturation and rehybridisation. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above. Following detection/quantification, one may compare the results seen in a given subject with a control reaction or a statistically significant reference group or population of control subjects as defined herein. In this way, it is possible to correlate the amount of a biomarker nucleic acid detected with the likelihood that a subject is at risk of developing T1D.

[0044] Also contemplated are biochip-based technologies such as those described by Hacia *et al.* (1996, *Nature Genetics* 14: 441-447) and Shoemaker *et al.* (1996, *Nature Genetics* 14: 450-456). Briefly, these techniques involve quantitative methods for analysing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ biochip technology to segregate target molecules as high-density arrays and screen these molecules on the basis of hybridization. See also Pease *et al.* (1994, *Proc. Natl. Acad. Sci. U.S.A.* 91: 5022-5026); Fodor *et al.* (1991, *Science* 251: 767-773). Briefly, nucleic acid probes to biomarker polynucleotides are made and attached to biochips to be used in screening and diagnostic methods, as outlined herein. The nucleic acid probes attached to the biochip are designed to be substantially complementary to specific expressed biomarker nucleic acids, *i.e.*, the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occur. This complementarity need not be

perfect; there may be any number of base pair mismatches, which will interfere with hybridization between the target sequence and the nucleic acid probes of the present invention. However, if the number of mismatches is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. In certain embodiments, more than one probe per sequence is used, with either overlapping probes or probes to different sections of the target being used. That is, two, three, four or more probes, with three being desirable, are used to build in a redundancy for a particular target. The probes can be overlapping (*i.e.* have some sequence in common), or separate.

[0045] In an illustrative biochip analysis, oligonucleotide probes on the biochip are exposed to or contacted with a nucleic acid sample suspected of containing one or more biomarker polynucleotides under conditions favouring specific hybridization. Sample extracts of DNA or RNA, either single or double-stranded, may be prepared from fluid suspensions of biological materials, or by grinding biological materials, or following a cell lysis step which includes, but is not limited to, lysis effected by treatment with SDS (or other detergents), osmotic shock, guanidinium isothiocyanate and lysozyme. Suitable DNA, which may be used in the method of the invention, includes cDNA. Such DNA may be prepared by any one of a number of commonly used protocols as for example described in Ausubel, *et al.*, 1994, *supra*, and Sambrook, *et al.*, *et al.*, 1989, *supra*.

[0046] Suitable RNA, which may be used in the method of the invention, includes messenger RNA, complementary RNA transcribed from DNA (cRNA) or genomic or subgenomic RNA. Such RNA may be prepared using standard protocols as for example described in the relevant sections of Ausubel, *et al.* 1994, *supra* and Sambrook, *et al.* 1989, *supra*).

[0047] cDNA may be fragmented, for example, by sonication or by treatment with restriction endonucleases. Suitably, cDNA is fragmented such that resultant DNA fragments are of a length greater than the length of the immobilized oligonucleotide probe(s) but small enough to allow rapid access thereto under suitable hybridization conditions. Alternatively, fragments of cDNA may be selected and amplified using a suitable nucleotide amplification technique, as described for example above, involving appropriate random or specific primers.

[0048] Usually the target biomarker polynucleotides are detectably labelled so that their hybridization to individual probes can be determined. The target polynucleotides are typically detectably labelled with a reporter molecule illustrative

examples of which include chromogens, catalysts, enzymes, fluorochromes, chemiluminescent molecules, bioluminescent molecules, lanthanide ions (e.g., Eu^{3+}), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like. Illustrative labels of this type include large colloids, for example, metal colloids such as those from gold, selenium, silver, tin and titanium oxide. In some embodiments, in which an enzyme is used as a direct visual label, biotinylated bases are incorporated into a target polynucleotide.

[0049] The hybrid-forming step can be performed under suitable conditions for hybridizing oligonucleotide probes to test nucleic acid including DNA or RNA. In this regard, reference may be made, for example, to NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH (Homes and Higgins, eds.) (IRL press, Washington D.C., 1985). In general, whether hybridization takes place is influenced by the length of the oligonucleotide probe and the polynucleotide sequence under test, the pH, the temperature, the concentration of mono- and divalent cations, the proportion of G and C nucleotides in the hybrid-forming region, the viscosity of the medium and the possible presence of denaturants. Such variables also influence the time required for hybridization. The preferred conditions will therefore depend upon the particular application. Such empirical conditions, however, can be routinely determined without undue experimentation.

[0050] After the hybrid-forming step, the probes are washed to remove any unbound nucleic acid with a hybridization buffer. This washing step leaves only bound target polynucleotides. The probes are then examined to identify which probes have hybridized to a target polynucleotide.

[0051] The hybridization reactions are then detected to determine which of the probes has hybridized to a corresponding target sequence. Depending on the nature of the reporter molecule associated with a target polynucleotide, a signal may be instrumentally detected by irradiating a fluorescent label with light and detecting fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be detected using a spectrophotometer; or detection of a dye particle or a coloured colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label or chemiluminescent molecule employing a radiation counter or autoradiography. Accordingly, a detection means may be adapted to detect or scan light associated with the label which light may include fluorescent, luminescent, focussed beam or laser light. In such a case, a

charge couple device (CCD) or a photocell can be used to scan for emission of light from a probe:target polynucleotide hybrid from each location in the micro-array and record the data directly in a digital computer. In some cases, electronic detection of the signal may not be necessary. For example, with enzymatically generated colour spots associated with nucleic acid array format, visual examination of the array will allow interpretation of the pattern on the array. In the case of a nucleic acid array, the detection means is suitably interfaced with pattern recognition software to convert the pattern of signals from the array into a plain language genetic profile. In certain embodiments, oligonucleotide probes specific for different biomarker polynucleotides are in the form of a nucleic acid array and detection of a signal generated from a reporter molecule on the array is performed using a 'chip reader'. A detection system that can be used by a 'chip reader' is described for example by Pirrung *et al* (U.S. Patent No. 5,143,854). The chip reader will typically also incorporate some signal processing to determine whether the signal at a particular array position or feature is a true positive or maybe a spurious signal. Exemplary chip readers are described for example by Fodor *et al* (U.S. Patent No., 5,925,525). Alternatively, when the array is made using a mixture of individually addressable kinds of labelled microbeads, the reaction may be detected using flow cytometry.

[0052] In other embodiments, biomarker protein levels are assayed using protein-based assays known in the art. For example, when a biomarker protein is an enzyme, the protein can be quantified based upon its catalytic activity or based upon the number of molecules of the protein contained in a sample.

[0053] Antibody-based techniques may also be employed to determine the level of a biomarker in a sample, non-limiting examples of which include immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

[0054] In specific embodiments, protein-capture arrays that permit simultaneous detection and/or quantification of a large number of proteins are employed. For example, low-density protein arrays on filter membranes, such as the universal protein array system (Ge, 2000 *Nucleic Acids Res.* 28(2):e3) allow imaging of arrayed antigens using standard ELISA techniques and a scanning charge-coupled device (CCD) detector. Immuno-sensor arrays have also been developed that enable the simultaneous detection of clinical analytes. It is now possible using protein arrays, to profile protein expression in bodily fluids, such as in sera of healthy or diseased subjects, as well as in subjects pre- and post-drug treatment.

[0055] Exemplary protein capture arrays include arrays comprising spatially addressed antigen-binding molecules, commonly referred to as antibody arrays, which can facilitate extensive parallel analysis of numerous proteins defining a proteome or subproteome. Antibody arrays have been shown to have the required properties of specificity and acceptable background, and some are available commercially (e.g., BD Biosciences, Clontech, BioRad and Sigma). Various methods for the preparation of antibody arrays have been reported (see, e.g., Lopez *et al.*, 2003 *J. Chromatogr. B* 787:19-27; Cahill, 2000 *Trends in Biotechnology* 7:47-51; U.S. Pat. App. Pub. 2002/0055186; U.S. Pat. App. Pub. 2003/0003599; PCT publication WO 03/062444; PCT publication WO 03/077851; PCT publication WO 02/59601; PCT publication WO 02/39120; PCT publication WO 01/79849; PCT publication WO 99/39210). The antigen-binding molecules of such arrays may recognise at least a subset of proteins expressed by a cell or population of cells, illustrative examples of which include growth factor receptors, hormone receptors, neurotransmitter receptors, catecholamine receptors, amino acid derivative receptors, cytokine receptors, extracellular matrix receptors, antibodies, lectins, cytokines, serpins, proteases, kinases, phosphatases, ras-like GTPases, hydrolases, steroid hormone receptors, transcription factors, heat-shock transcription factors, DNA-binding proteins, zinc-finger proteins, leucine-zipper proteins, homeodomain proteins, intracellular signal transduction modulators and effectors, apoptosis-related factors, DNA synthesis factors, DNA repair factors, DNA recombination factors and cell-surface antigens.

[0056] Individual spatially distinct protein-capture agents are typically attached to a support surface, which is generally planar or contoured. Common physical supports include glass slides, silicon, microwells, nitrocellulose or PVDF membranes, and magnetic and other microbeads.

[0057] Particles in suspension can also be used as the basis of arrays, providing they are coded for identification; systems include colour coding for microbeads (e.g., available from Luminex, Bio-Rad and Nanomics Biosystems) and semiconductor nanocrystals (e.g., QDots™, available from Quantum Dots), and barcoding for beads (UltraPlex™, available from Smartbeads) and multimetal microrods (Nanobarcodes™ particles, available from Surromed). Beads can also be assembled into planar arrays on semiconductor chips (e.g., available from LEAPS technology and BioArray Solutions). Where particles are used, individual protein-capture agents are typically attached to an individual particle to provide the spatial definition or separation of the array. The particles may then be assayed separately,

but in parallel, in a compartmentalized way, for example in the wells of a microtitre plate or in separate test tubes.

[0058] In an illustrative example, a protein sample, which is optionally fragmented to form peptide fragments (see, *e.g.*, U.S. Pat. App. Pub. 2002/0055186), is delivered to a protein-capture array under conditions suitable for protein or peptide binding, and the array is washed to remove unbound or non-specifically bound components of the sample from the array. Next, the presence or amount of protein or peptide bound to each feature of the array is detected using a suitable detection system. The amount of protein bound to a feature of the array may be determined relative to the amount of a second protein bound to a second feature of the array. In certain embodiments, the amount of the second protein in the sample is already known or known to be invariant.

[0059] In another illustrative example of a protein-capture array is Luminex-based multiplex assay, which is a bead-based multiplexing assay, where beads are internally dyed with fluorescent dyes to produce a specific spectral address. Biomolecules (such as an oligo or antibody) can be conjugated to the surface of beads to capture analytes of interest. Flow cytometric or other suitable imaging technologies known to persons skilled in the art can then be used for characterization of the beads, as well as for detection of analyte presence. The Luminex technology enables are large number of proteins, genes or other gene expression products (*e.g.*, 100 or more, 200 or more, 300 or more, 400 or more) to be detected using very small sample volume (*e.g.*, in a 96 or 384-well plate). In some embodiments, the protein-capture array is Bio-Plex Luminex-100 Station (Bio-Rad) as described previously.

[0060] For analysing differential expression of proteins between two cells or cell populations, a protein sample of a first cell or population of cells is delivered to the array under conditions suitable for protein binding. In an analogous manner, a protein sample of a second cell or population of cells to a second array is delivered to a second array that is identical to the first array. Both arrays are then washed to remove unbound or non-specifically bound components of the sample from the arrays. In a final step, the amounts of protein remaining bound to the features of the first array are compared to the amounts of protein remaining bound to the corresponding features of the second array. To determine the differential protein expression pattern of the two cells or populations of cells, the amount of protein bound to individual features of the first array is subtracted from the amount of protein bound to the corresponding features of the second array.

[0061] In some embodiments, the level of a biomarker is normalized against a housekeeping biomarker. The term "housekeeping biomarker" refers to a biomarker or group of biomarkers (e.g., polynucleotides and/or polypeptides), which are typically found at a constant level in the cell type(s) being analysed and across the conditions being assessed. In some embodiments, the housekeeping biomarker is a "housekeeping gene." A "housekeeping gene" refers herein to a gene or group of genes which encode proteins whose activities are essential for the maintenance of cell function and which are typically found at a constant level in the cell type(s) being analysed and across the conditions being assessed.

[0062] In some embodiments, the determination is carried out in the absence of comparing the level of the at least one biomarker in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile. For example, in some embodiments, a ratio among two, three, four or more biomarkers can be determined. Changes or perturbations in biomarker ratios can be advantageous in indicating where there are blocks (or releases of such blocks) or other alterations in cellular pathways associated with a risk of developing T1D, response to treatment, development of side effects, and the like. Thus, in some embodiments, the method of the present invention comprises comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and determining whether a subject is at risk of developing T1D based on that ratio.

Biomarker Profiles

[0063] As used herein, the terms "profile" and "biomarker profile" are used interchangeably herein to denote any set of data that represents the distinctive features or characteristics associated with a condition of interest, such as with a particular prediction, diagnosis and/or prognosis of a specified condition as taught herein. The term generally encompasses quantification of one or more biomarkers, *inter alia*, nucleic acid profiles, such as, for example, gene expression profiles (e.g., sets of gene expression data that represents mRNA levels of one or more genes associated with a condition of interest), as well as protein, polypeptide or peptide profiles, such as, for example, protein expression profiles (e.g., sets of protein expression data that represents the levels of one or more proteins associated with a condition of interest), the number of cell types associated with the condition of interest (e.g., peripheral blood mononuclear cells or subsets thereof), and any combinations thereof.

[0064] The term "reference biomarker profile" is used herein to denote a pattern of expression of at least one biomarker for a sample population of reference individuals at risk of developing T1D (e.g., T1D AB- FDR or T1D FDR with single or multiple AB). A reference biomarker profile may be identified based on reference data measured for individuals in the sample population (e.g., T1D FDR). Reference data typically include the measurement of at least one biomarker. The measurement may include information regarding the activity, such as its level or abundance, of any expression product or measurable molecule, as will be described in more detail herein. The reference data may also include other additional relevant information, such as clinical data, including, but not limited to, information regarding age-adjusted body-mass index (BMI) percentile, BMI standard deviation score (BMI-SDS), waist circumference, fasting lipid profile and homeostatic model assessment of insulin resistance (HOMA-IR), the presence, absence, degree, severity or progression of a symptom associated with T1D, phenotypic information, such as details of phenotypic traits, genetic or genetically regulated information associated with T1D, amino acid or nucleotide related genomics information associated with T1D and the like and this is not intended to be limiting, as will be apparent from the description below.

[0065] The reference data may be acquired in any appropriate manner, such as obtaining gene expression product data from a plurality of subjects, selected to include individuals at risk of developing T1D (e.g., T1D FDR). The terms "expression" or "gene expression" refer to production of RNA message or translation of RNA message into proteins or polypeptides, or both. Detection of either types of gene expression in use of any of the methods described herein is encompassed by the present invention. Gene expression product data are collected, for example, by obtaining a biological sample, such as a blood sample from the subject, and performing a quantification, semi-quantification or qualification process, such as sequence-specific nucleic acid amplification, including PCR (Polymerase Chain Reaction) or the like, in order to assess the expression, and in particular, the level or abundance of one or more reference biomarker. Quantified values indicative of the relative activity can then be stored as part of the reference data.

[0066] Biomarker profiles may be created in a number of ways and may be the combination of measurable biomarkers or aspects of biomarkers using methods such as ratios, or other more complex association methods or algorithms (e.g., rule-based methods), as discussed for example in more detail below. A biomarker profile comprises at least one measurement. However, in some embodiments, the

biomarker profile evaluates at least 2 biomarkers (e.g., 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 25 or more, 30 or more, 40 or more, 50 or more, 60 or more, 70 or more, 80 or more, 90 or more, 100 or more, 200 or more). Where the biomarker profile comprises two or more measurements, the measurements can correspond to the same or different biomarkers. For example, distinct reference profiles may represent the degree of risk (e.g., an abnormally elevated risk) of having or developing a specified condition, as compared no or normal risk of having or developing the specified condition. In another example, distinct reference profiles may represent predictions of differing degrees of risk of having or developing a specified condition.

[0067] A reference biomarker profile or a sample biomarker profile can be quantitative, semi-quantitative and/or qualitative. For example, the biomarker profile can evaluate the presence or absence of at least one biomarker, can evaluate the presence of at least one biomarker above or below a particular threshold, and/or can evaluate the relative or absolute amount of at least one biomarker.

[0068] In some embodiments, the subject's risk of developing T1D is determined by comparing the biomarker profile in a sample obtained from the subject (*i.e.*, the sample biomarker profile) with a reference biomarker profile in a healthy control population. An illustrative example is provided in Figures 1c-e, which show a comparison of the level of expression of biomarkers in T1D FDR and healthy controls. Alternatively, the subject's risk of developing T1D is determined by comparing the biomarker profile in a sample obtained from the subject (*i.e.*, the sample biomarker profile) with a reference biomarker profile from an at-risk AB-negative FDR population. For example, a subject's risk of developing T1D is determined by comparing the level of expression of a biomarker in a sample obtained from the subject with a level that is representative of a mean or median level of the expression in population of at-risk individuals, non-limiting examples of which include AB- and/or AB+ T1D FDR.

[0069] In some embodiments, the expression of the at least one biomarker in a sample population of reference individuals, as broadly defined herein, is used to generate a biomarker profile; namely, of subjects at risk of developing T1D (the reference group) and healthy controls (the control group). For instance, a particular biomarker may be more abundant or less abundant in the reference group as compared to the control group. The data may be represented as an overall

signature score or the profile may be represented as a barcode, heat-map, z-score, receiver-operator characteristics (ROC) curve or other graphical representation known to persons skilled in the art to facilitate the determination of a test subject's risk of developing T1D. The expression of the corresponding biomarker in a test subject may be represented in the same way, thereby providing a sample biomarker profile, such that a comparison of the sample profile with the reference profile may be undertaken to determine the test subject's risk of developing T1D.

[0070] The number of biomarkers measured for use as a reference biomarker profile may vary depending upon the preferred implementation or degree of sensitivity and selectivity for determining whether a subject is at risk of developing T1D. Persons skilled in the art would appreciate that the greater the number of reference biomarkers measured, the greater the power of predicting a subject's risk of developing T1D. For example, the number of reference biomarkers in a reference biomarker profile for use in accordance with the present invention may include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 5000, 10000 or above, although this is not intended to be limiting.

[0071] Additionally, the reference data may include details of one or more phenotypic traits of the individuals and/or their relatives. Phenotypic traits can include information such as the gender, ethnicity, age, and the like. Additionally, in the case of the technology being applied to individuals other than humans, this can also include information such as designation of a species, breed or the like. Accordingly, in one example, the reference data can include for each of the reference individuals an indication of the activity of a plurality of reference biomarkers, a presence, absence degree or progression of a condition, phenotypic information such as phenotypic traits, genetic information and a physiological score such as a SOFA score.

[0072] It will be appreciated that once collected, the reference data can be stored in a database allowing them to be subsequently retrieved, for example, by a processing system for subsequent use in accordance with the present invention. The processing system may also store an indication of the identity of each of the reference biomarkers as a reference biomarker collection or panel where there are two or more reference biomarkers.

[0073] In illustrative examples, the reference biomarker profile evaluates at least one biomarker (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60 or more) as listed in Figure 1g, Figure 3 or Figure 4.0, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 5000, 10000

Corresponding biomarker

[0074] By "corresponding biomarker" or "corresponding biomarker" is meant a biomarker that is structurally and/or functionally similar to a reference biomarker. Representative corresponding biomarkers include expression products of allelic variants (same locus), homologs (different locus), and orthologs (different organism) of reference biomarker genes. Nucleic acid variants of reference biomarker genes and encoded biomarker polynucleotide expression products can contain nucleotide substitutions, deletions, inversions and/or insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product). For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of a reference T1D polypeptide.

[0075] Generally, variants of a particular biomarker gene or polynucleotide will have at least about 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs known in the art using default parameters.

[0076] Corresponding biomarkers also include amino acid sequences that display substantial sequence similarity or identity to the amino acid sequence of a reference biomarker polypeptide. In general, an amino acid sequence that corresponds to a reference amino acid sequence will display at least about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% or even up to 100% sequence similarity or identity to a reference amino acid as determined by sequence alignment programs known in the art using default parameters.

[0077] In some embodiments, calculations of sequence similarity or sequence identity between sequences can be performed. For example, to determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the

sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In some embodiments, the length of a reference sequence aligned for comparison purposes is at least 30%, usually at least 40%, more usually at least 50%, 60%, and even more usually at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide at the corresponding position in the second sequence, then the molecules are identical at that position. For amino acid sequence comparison, when a position in the first sequence is occupied by the same or similar amino acid residue (*i.e.*, conservative substitution) at the corresponding position in the second sequence, then the molecules are similar at that position.

[0078] The percent identity between the two sequences is a function of the number of identical amino acid residues or nucleotides shared by the sequences at individual positions, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. By contrast, the percent similarity between two amino acid sequences is a function of the number of identical and similar amino acid residues shared by the sequences at individual positions, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0079] The comparison of sequences and determination of percent identity or percent similarity between sequences can be accomplished using a mathematical algorithm. In certain embodiments, the percent identity or similarity between amino acid sequences is determined using the Needleman and Wunsch, (1970, *J. Mol. Biol.* 48: 444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In specific embodiments, the percent identity between nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A non-limiting set of parameters (and the one that should be used unless otherwise specified) includes a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0080] In some embodiments, the percent identity or similarity between amino acid or nucleotide sequences can be determined using the algorithm of E. Meyers and W. Miller (1989, *Cabios*, 4: 11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[0081] The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.*, (1990, *J. Mol. Biol.*, 215: 403-10). BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to 53010 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to 53010 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997, *Nucleic Acids Res.*, 25: 3389-3402). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

[0082] Corresponding biomarker polynucleotides also include nucleic acid sequences that hybridize to reference biomarker polynucleotides, or to their complements, under stringency conditions described below. As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and optionally washing. "Hybridization" is used herein to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA, U pairs with A and C pairs with G. In this regard, the terms "match" and "mismatch" as used herein refer to the hybridization potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridize efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides that do not hybridize efficiently.

[0083] Guidance for performing hybridization reactions can be found in Ausubel *et al.*, (1998, *supra*), Sections 6.3.1-6.3.6. Aqueous and non-aqueous methods are described in that reference and either can be used. Reference herein to low stringency conditions include and encompass from at least about 1% v/v to at least

about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization at 42° C, and at least about 1 M to at least about 2 M salt for washing at 42° C. Low stringency conditions also may include 1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS for hybridization at 65° C, and (i) 2 × SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM NaHPO₄ (pH 7.2), 5% SDS for washing at room temperature. One embodiment of low stringency conditions includes hybridization in 6 × sodium chloride/sodium citrate (SSC) at about 45° C, followed by two washes in 0.2 × SSC, 0.1% SDS at least at 50° C (the temperature of the washes can be increased to 55° C for low stringency conditions). Medium stringency conditions include and encompass from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization at 42° C, and at least about 0.1 M to at least about 0.2 M salt for washing at 55° C. Medium stringency conditions also may include 1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS for hybridization at 65° C, and (i) 2 × SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM NaHPO₄ (pH 7.2), 5% SDS for washing at 60-65° C. One embodiment of medium stringency conditions includes hybridizing in 6 × SSC at about 45°C, followed by one or more washes in 0.2 × SSC, 0.1% SDS at 60° C. High stringency conditions include and encompass from at least about 31% v/v to at least about 50% v/v formamide and from about 0.01 M to about 0.15 M salt for hybridization at 42° C, and about 0.01 M to about 0.02 M salt for washing at 55° C. High stringency conditions also may include 1% BSA, 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS for hybridization at 65° C, and (i) 0.2 × SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM NaHPO₄ (pH 7.2), 1% SDS for washing at a temperature in excess of 65° C. One embodiment of high stringency conditions includes hybridizing in 6 × SSC at about 45°C, followed by one or more washes in 0.2 × SSC, 0.1% SDS at 65° C.

[0084] In certain embodiments, a corresponding biomarker polynucleotide is one that hybridizes to a disclosed nucleotide sequence under very high stringency conditions. One embodiment of very high stringency conditions includes hybridizing 0.5 M sodium phosphate, 7% SDS at 65° C, followed by one or more washes at 0.2 × SSC, 1% SDS at 65° C.

[0085] Other stringency conditions are well known in the art and a skilled addressee will recognize that various factors can be manipulated to optimize the specificity of the hybridization. Optimization of the stringency of the final washes can serve to ensure a high degree of hybridization. For detailed examples, see

Ausubel *et al.*, *supra* at pages 2.10.1 to 2.10.16 and Sambrook *et al.* (1989, *supra*) at sections 1.101 to 1.104.

[0086] In some embodiments, the individual level of a biomarker in the reference group is at least 101%, 102%, 103%, 104%, 105%, 106%, 107%, 108%, 109%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% (i.e. an increased or higher level), of the level of a corresponding biomarker in the control group.

[0087] In some embodiments, the individual level of a biomarker in the reference group is at least 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% or at least 10%, (i.e. a decreased or lower level), of the level of a corresponding biomarker in the control group.

Risk or likelihood of developing T1D

[0088] The term "risk" is used to denote a subject's likelihood, based on the sample biomarker profile as determined for that subject, of developing T1D (or not) on the basis of the reference biomarker profile, as herein described. Accordingly, the terms "risk" and "likelihood" are used interchangeably herein, unless otherwise stated.

[0089] It would be apparent to persons skilled in the art that the risk that a subject will develop T1D will vary, for example, from being at *low* or decreased risk of developing T1D to being at *high* or increased risk of developing T1D. By "low or decreased risk" is meant that the subject is less likely to develop T1D as compared to a subject determined to be a "high or increased risk" subject. Conversely, a "high or increased risk" subject is a subject who is more likely to develop T1D as compared to a subject who is not at risk or a "low risk" subject. For example, a healthy subject may be regarded as being at low risk of developing T1D.

[0090] Likelihood is suitably based on mathematical modeling. An increased likelihood, for example, may be relative or absolute and may be expressed qualitatively or quantitatively. For instance, an increased risk may be expressed as simply determining the subject's level of a given biomarker and placing the test subject in an "increased risk" category, based upon the corresponding reference biomarker profile as determined, for example, from previous population studies. Alternatively, a numerical expression of the test subject's increased risk may be determined based upon biomarker level analysis.

[0091] As used herein, the term "probability" refers to the probability of class membership for a sample as determined by a given mathematical model and is construed to be equivalent likelihood in this context.

[0092] In some embodiments, likelihood is assessed by comparing the level or abundance of at least one biomarker to one or more preselected level, also referred to herein as a threshold or reference levels. Thresholds may be selected that provide an acceptable ability to predict risk, treatment success, etc. In illustrative examples, receiver operating characteristic (ROC) curves are calculated by plotting the value of a variable versus its relative frequency in two populations in which a first population is considered at risk of developing T1D (e.g., T1D FDR) and a second population that is not considered to be at risk, or have a low risk, of developing T1D (called arbitrarily, for example, "healthy controls").

[0093] In some embodiments, the subject is considered at risk of developing T1D where the at least one biomarker in the sample biomarker profile for the subject is upregulated or downregulated as compared to the corresponding biomarker in a healthy subject.

[0094] For any particular biomarker, a distribution of biomarker levels for subjects who are at risk or not at risk of developing T1D may overlap. Under such conditions, a test may not absolutely distinguish a subject who is at risk of developing T1D from a subject who is not at risk of developing T1D with absolute (i.e., 100%) accuracy, and the area of overlap indicates where the test cannot distinguish the two subjects. A threshold can be selected, above which (or below which, depending on how a biomarker changes with risk) the test is considered to be "positive" and below which the test is considered to be "negative." The area under the ROC curve (AUC) provides the C-statistic, which is a measure of the probability that the perceived measurement will allow correct identification of a condition (see, e.g., Hanley et al., Radiology 143: 29-36 (1982)).

[0095] Alternatively, or in addition, thresholds may be established by obtaining a biomarker profile from the same patient, to which later results may be compared. In these embodiments, the individual in effect acts as their own "control group." In biomarkers that increase with, for example, prognostic risk, an increase over time in the same patient can indicate a failure of a treatment regimen, while a decrease over time can indicate success of a treatment regimen.

[0096] In some embodiments, a positive likelihood ratio, negative likelihood ratio, odds ratio, and/or AUC or receiver operating characteristic (ROC) values are used as a measure of a method's ability to predict risk of developing T1D. As used

herein, the term "likelihood ratio" is the probability that a given test result would be observed in a subject with a likelihood of such risk, divided by the probability that that same result would be observed in a subject without a likelihood of such risk. Thus, a positive likelihood ratio is the probability of a positive result observed in subjects with the specified risk divided by the probability of a positive results in subjects without the specified risk. A negative likelihood ratio is the probability of a negative result in subjects without the specified risk divided by the probability of a negative result in subjects with specified risk. The term "odds ratio," as used herein, refers to the ratio of the odds of an event occurring in one group (e.g., a healthy control group) to the odds of it occurring in another group (e.g., a T1D FDR group), or to a data-based estimate of that ratio. The term "area under the curve" or "AUC" refers to the area under the curve of a receiver operating characteristic (ROC) curve, both of which are well known in the art. AUC measures are useful for comparing the accuracy of a classifier across the complete data range. Classifiers with a greater AUC have a greater capacity to classify unknowns correctly between two groups of interest (e.g., a healthy control group and a T1D risk group). ROC curves are useful for plotting the performance of a particular feature (e.g., any of the biomarkers described herein and/or any item of additional biomedical information) in distinguishing or discriminating between two populations (e.g., cases having a condition and controls without the condition). Typically, the feature data across the entire population (e.g., the cases and controls) are sorted in ascending order based on the value of a single feature. Then, for each value for that feature, the true positive and false positive rates for the data are calculated. The sensitivity is determined by counting the number of cases above the value for that feature and then dividing by the total number of cases. The specificity is determined by counting the number of controls below the value for that feature and then dividing by the total number of controls. Although this definition refers to scenarios in which a feature is elevated in cases compared to controls, this definition also applies to scenarios in which a feature is lower in cases compared to the controls (in such a scenario, samples below the value for that feature would be counted). ROC curves can be generated for a single feature as well as for other single outputs, for example, a combination of two or more features can be mathematically combined (e.g., added, subtracted, multiplied, etc.) to produce a single value, and this single value can be plotted in a ROC curve. Additionally, any combination of multiple features, in which the combination derives a single output value, can be plotted in a ROC curve. These combinations of features may comprise a test. The ROC curve is the plot of the sensitivity of a test against the specificity of the test, where sensitivity is traditionally presented on the vertical axis and

specificity is traditionally presented on the horizontal axis. Thus, "AUC ROC values" are equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. An AUC ROC value may be thought of as equivalent to the Mann-Whitney U test, which tests for the median difference between scores obtained in the two groups considered if the groups are of continuous data, or to the Wilcoxon test of ranks.

[0097] In some embodiments, at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) biomarker or a panel of biomarkers is selected to discriminate between subjects with or without risk of developing T1D with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% accuracy or having a C-statistic of at least about 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95.

[0098] In the case of a positive likelihood ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "T1D risk" and "healthy control" groups; a value greater than 1 indicates that a positive result is more likely in the T1D risk group; and a value less than 1 indicates that a positive result is more likely in the healthy control group. In this context, "T1D risk group" is meant to refer to a population of reference individuals considered to be at risk of developing T1D (e.g., T1D FDR) and a "control group" is meant to refer to a group of subjects considered not to be at risk of developing T1D (e.g., healthy controls). In the case of a negative likelihood ratio, a value of 1 indicates that a negative result is equally likely among subjects in both the "T1D risk" and "control" groups; a value greater than 1 indicates that a negative result is more likely in the "T1D risk" group; and a value less than 1 indicates that a negative result is more likely in the "control" group. In the case of an odds ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "T1D risk" and "control" groups; a value greater than 1 indicates that a positive result is more likely in the "T1D risk" group; and a value less than 1 indicates that a positive result is more likely in the "control" group. In the case of an AUC ROC value, this is computed by numerical integration of the ROC curve. The range of this value can be 0.5 to 1.0. A value of 0.5 indicates that a classifier (e.g., a biomarker profile) is no better than a 50% chance to classify unknowns correctly between two groups of interest, while 1.0 indicates the relatively best diagnostic accuracy. In certain embodiments, biomarkers and/or biomarker panels are selected to exhibit a positive or negative likelihood ratio of at least about 1.5 or more or about 0.67 or less, at least about 2 or more or about 0.5 or less, at least about 5 or more or about 0.2 or less, at least about 10 or more or about 0.1 or less, or at least about 20 or more or about 0.05 or less.

[0099] In certain embodiments, the at least one biomarker is selected to exhibit an odds ratio of at least about 2 or more or about 0.5 or less, at least about 3 or more or about 0.33 or less, at least about 4 or more or about 0.25 or less, at least about 5 or more or about 0.2 or less, or at least about 10 or more or about 0.1 or less.

[0100] In certain embodiments, the at least one biomarker is selected to exhibit an AUC ROC value of greater than 0.5, preferably at least 0.6, more preferably 0.7, still more preferably at least 0.8, even more preferably at least 0.9, and most preferably at least 0.95.

[0101] In some cases, multiple thresholds may be determined in so-called "tertile," "quartile," or "quintile" analyses. In these methods, the "T1D risk" and "control" groups are considered together as a single population, and are divided into 3, 4, or 5 (or more) "bins" having equal numbers of individuals. The boundary between two of these "bins" may be considered "thresholds." The degree of risk can then be assigned based on which "bin" a test subject falls into.

[0102] In other embodiments, particular thresholds for the reference biomarker(s) measured are not relied upon to determine if the biomarker level(s) obtained from a subject are correlated to risk of developing T1D. For example, a temporal change in the biomarker(s) can be used to rule in or out such risk. Alternatively, biomarker(s) are correlated to such risk by the presence or absence of one or more biomarkers in a particular assay format. In the case of biomarker profiles, the present invention may utilize an evaluation of the entire profile of biomarkers to provide a single result value (e.g., a "panel response" value expressed either as a numeric score or as a percentage risk). In such embodiments, an increase, decrease, or other change (e.g., slope over time) in a certain subset of biomarkers may be sufficient to indicate risk of developing T1D in a subject, while an increase, decrease, or other change in a different subset of biomarkers may be sufficient to indicate the same risk in another subject.

[0103] In certain embodiments, a panel of biomarkers is selected to assist in distinguishing between "T1D risk" and "control" groups with at least about 70%, 80%, 85%, 90% or 95% sensitivity, suitably in combination with at least about 70%, 80%, 85%, 90% or 95% specificity. In some embodiments, both the sensitivity and specificity are at least about 75%, 80%, 85%, 90% or 95%.

[0104] The phrases "assessing the likelihood" and "determining the likelihood," as used herein, refer to methods by which the skilled artisan can predict a subject's risk of developing T1D. The skilled artisan will understand that this phrase includes

within its scope an increased probability that the subject will develop T1D; that is, such risk is more likely to be present or absent in a subject. For example, the probability that an individual identified as being at risk of developing T1D may be expressed as a "positive predictive value" or "PPV." Positive predictive value can be calculated as the number of true positives divided by the sum of the true positives and false positives. PPV is determined by the characteristics of the predictive methods of the present invention as well as the prevalence of the condition in the population analysed. The statistical algorithms can be selected such that the positive predictive value in a population considered to be at risk of developing T1D is in the range of 70% to 99% and can be, for example, at least 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0105] In other examples, the probability that a subject is identified as not being at risk of developing T1D may be expressed as a "negative predictive value" or "NPV." Negative predictive value can be calculated as the number of true negatives divided by the sum of the true negatives and false negatives. Negative predictive value is determined by the characteristics of the diagnostic or prognostic method, system, or code as well as the prevalence of risk in the population analysed. The statistical methods and models can be selected such that the negative predictive value in a population considered at risk of developing T1D is in the range of about 70% to about 99% and can be, for example, at least about 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0106] In some embodiments, a subject is determined as being at significant risk of developing T1D. By "significant risk" is meant that the subject has a reasonable probability (e.g., 0.6, 0.7, 0.8, 0.9 or more) of developing T1D.

[0107] The methods of the present invention, as broadly described herein, also permit the generation of high-density data sets that can be evaluated using informatics approaches. High data density informatics analytical methods are known and software is available to those in the art, e.g., cluster analysis (Pirouette, Informetrix), class prediction (SIMCA-P, Umetrics), principal components analysis of a computationally modeled dataset (SIMCA-P, Umetrics), 2D cluster analysis (GeneLinker Platinum, Improved Outcomes Software), and metabolic pathway analysis (biotech.icmb.utexas.edu). The choice of software packages offers specific tools for questions of interest (Kennedy et al., Solving Data Mining Problems Through Pattern Recognition. Indianapolis: Prentice Hall PTR, 1997; Golub et al., (2999) Science 286:531-7; Eriksson et al., Multi and Megavariate Analysis

Principles and Applications: Umetrics, Umea, 2001). In general, any suitable mathematic analyses can be used to evaluate at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, et.) biomarker in a biomarker profile with respect to determining the likelihood that the subject is at risk of developing T1D. For example, methods such as multivariate analysis of variance, multivariate regression, and/or multiple regression can be used to determine relationships between dependent variables (e.g., clinical measures) and independent variables (e.g., levels of biomarkers). Clustering, including both hierarchical and non-hierarchical methods, as well as nonmetric Dimensional Scaling can be used to determine associations or relationships among variables and among changes in those variables.

[0108] In some embodiments, a biomarker profile is used to assign a risk score which describes a mathematical equation for evaluation or prediction of risk. The evaluation of risk may also take into account genotype (including described HLA genes), islet autoantibodies species (e.g., the number of autoantibody target antigens) and other clinical features into account, including age-adjusted BMI, fasting and 2h glucose measurements on an oral glucose tolerance test, age and first-phase insulin response to a glucose load.

[0109] In addition, principal component analysis is a common way of reducing the dimension of studies, and can be used to interpret the variance-covariance structure of a data set. Principal components may be used in such applications as multiple regression and cluster analysis. Factor analysis is used to describe the covariance by constructing "hidden" variables from the observed variables. Factor analysis may be considered an extension of principal component analysis, where principal component analysis is used as parameter estimation along with the maximum likelihood method. Furthermore, simple hypothesis such as equality of two vectors of means can be tested using Hotelling's T squared statistic.

[0110] In some embodiments, the data sets corresponding to biomarker profiles are used to create a diagnostic or predictive rule or model based on the application of a statistical and machine learning algorithm. Such an algorithm uses relationships between a biomarker profile and risk of developing T1D observed in control subjects or typically cohorts of control subjects (sometimes referred to as training data), which provides combined control or reference biomarker profiles for comparison with biomarker profiles of a subject. The data are used to infer relationships that are then used to predict the status of a subject and the presence or absence of risk of developing T1D.

[0111] Persons skilled in the art of data analysis will recognize that many different forms of inferring relationships in the training data may be used without materially changing the present invention. The data presented in the Tables and Examples herein has been used to generate illustrative minimal combinations of biomarkers (models) that differentiate between T1D risk and control using feature selection based on AUC maximisation in combination with support vector machine classification.

Subject

[0112] The terms "*subject*," "*individual*" and "*patient*" are used interchangeably herein to refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, any member of the subphylum Chordata including primates, rodents (*e.g.*, mice rats, guinea pigs), lagomorphs (*e.g.*, rabbits, hares), bovines (*e.g.*, cattle), ovines (*e.g.*, sheep), caprines (*e.g.*, goats), porcines (*e.g.*, pigs), equines (*e.g.*, horses), canines (*e.g.*, dogs), felines (*e.g.*, cats), avians (*e.g.*, chickens, turkeys, ducks, geese, companion birds such as canaries, budgerigars *etc.*), marine mammals (*e.g.*, dolphins, whales), reptiles (snakes, frogs, lizards, *etc.*), and fish. A preferred subject is a primate (*e.g.*, a human, ape, monkey, chimpanzee).

Sample

[0113] In some embodiments, individual biomarkers are detected or measured in a biological sample. A biological sample may include a sample that may be extracted, untreated, treated, diluted or concentrated from a subject. In some embodiments, the biological sample has not been extracted from the subject, particularly where the determination steps in accordance with the present invention (*e.g.*, measurement of the at least one biomarker) can be performed *in situ*. In preferred embodiments, the biological sample is a sample obtained from the subject that is reasonably expected to comprise the at least one biomarker of interest. Non-limiting examples of biological samples include, but are not limited to, tissue, bodily fluid (for example, blood, serum, plasma, saliva, urine, tears, peritoneal fluid, ascitic fluid, vaginal secretion, breast fluid, breast milk, lymph fluid, cerebrospinal fluid or mucosa secretion), umbilical cord blood, chorionic villi, amniotic fluid, an embryo, embryonic tissues, lymph fluid, cerebrospinal fluid, mucosa secretion, or other body exudate, fecal matter, an individual cell or extract of the such sources that contain the nucleic acid of the same, and subcellular structures such as mitochondria, obtained using protocols well established within the art. In certain embodiments, the biological sample contains blood, especially

peripheral blood, or a fraction or extract thereof. Typically, the biological sample comprises blood cells such as mature, immature or developing leukocytes, including lymphocytes, polymorphonuclear leukocytes, neutrophils, monocytes, reticulocytes, basophils, coelomocytes, hemocytes, eosinophils, megakaryocytes, macrophages, dendritic cells, natural killer cells, or fraction of such cells (e.g., a nucleic acid or protein fraction). In specific embodiments, the biological sample comprises leukocytes including peripheral blood mononuclear cells (PBMC).

[0114] In some embodiments disclosed herein, the biological sample is a whole blood sample. In some embodiments, the biological sample is a serum sample.

[0115] The biological sample may be processed and analyzed for the purpose of determining the sample biomarker profile, in accordance with the present invention, almost immediately following collection (i.e., as a fresh sample), or it may be stored for subsequent analysis. If storage of the biological sample is desired or required, it would be understood by persons skilled in the art that it should ideally be stored under conditions that preserve the integrity of the biomarker of interest within the sample (e.g., at -80°C).

[0116] By "obtained" is meant to come into possession. Biological or reference samples so obtained include, for example, nucleic acid extracts or polypeptide extracts isolated or derived from a particular source. For instance, the extract may be isolated directly from a biological fluid or tissue of a subject.

Monitoring a response to treatment

[0117] The methods of the present invention, as broadly described herein, can also be used to monitor the efficacy of treatment regimen for preventing or delaying the onset of T1D. Therefore, the present invention further contemplates methods for (i) determining whether a treatment regimen is effective for preventing or delaying the onset of T1D or a symptom thereof in a subject, (ii) monitoring the efficacy of a treatment regimen in a subject at risk of developing T1D; (iii) correlating a reference biomarker profile with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, (iv) determining whether a treatment regimen is effective for preventing or delaying the onset of T1D or a symptom thereof in a subject at risk of developing T1D, (v) correlating a biomarker profile with a positive or negative response to a treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, and (vi) determining a positive or negative response to a treatment regimen by a subject at risk of developing T1D.

[0118] For example, in a method of monitoring the efficacy of a treatment regimen in a subject at risk of developing T1D, the method may comprise: (1) providing a correlation of a reference biomarker profile with a likelihood of having a healthy condition, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) obtaining a corresponding biomarker profile of a subject at risk of developing T1D after commencement of a treatment regimen, wherein a similarity of the subject's biomarker profile after commencement of the treatment regimen to the reference biomarker profile indicates a likelihood that the treatment regimen is effective for changing the health status of the subject.

[0119] With respect to correlating a reference biomarker profile with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, the method may comprise: (1) determining a sample biomarker profile from a subject at risk of developing T1D prior to commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (2) correlating the sample biomarker profile with a treatment regimen that is effective for preventing or delaying the onset of T1D, or a symptom thereof. The reference biomarker profile will evaluate, for example, SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine.

[0120] For determining whether a treatment regimen is effective for preventing or delaying the onset of T1D or a symptom thereof in a subject at risk of developing T1D, the method may comprise: (1) correlating a reference biomarker profile prior to treatment with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) obtaining a sample biomarker profile from the subject after commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker, and wherein the sample biomarker profile after commencement of treatment, when compared to the reference biomarker profile, indicates whether the treatment regimen is effective for preventing or delaying the onset of T1D, or a symptom thereof, in the subject.

[0121] For correlating a biomarker profile with a positive or negative response to a treatment regimen for preventing or delaying the onset of T1D, or a symptom

thereof, the method may comprise: (1) obtaining a sample biomarker profile from a subject at risk of developing T1D following commencement of the treatment regimen, wherein the biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) correlating the sample biomarker profile from the subject with a positive or negative response to the treatment regimen. This enables an evaluation as to whether an at-risk subject is responding (*i.e.*, a positive response) or not responding (*i.e.*, a negative response) to a treatment regimen.

[0122] The invention also provides methods of determining a positive and/or negative response to a treatment regimen by a subject. This aspect of the invention can be practiced to identify responders or non-responders relatively early in the treatment process, *i.e.*, before clinical manifestations of efficacy. In this way, the treatment regimen can optionally be discontinued, a different treatment protocol can be implemented and/or supplemental therapy can be administered. The method may comprise: (a) correlating a reference biomarker profile with a positive or negative response to the treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; (b) determining a sample biomarker profile from the subject following commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (c) determining a positive or negative response to the treatment regimen based on a comparison of the sample biomarker profile and the reference biomarker profile.

[0123] In some embodiments, the reference biomarker profile further evaluates at least one other biomarker selected from the group consisting of RELB, tumor necrosis factor (TNF), 78kDa glucose-related protein (GRP78), CD14^{LOW}CD16⁻ cells and CD14^{HIGH}CD16⁺ cells and serum IL-12p40 and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

[0124] In some embodiments, the methods comprise the analysis of a series of biological samples obtained over a period of time from the subject during treatment. Without being bound by theory or a particular mode of practice, it is expected that a change in the sample biomarker profile over the period of time will be indicative of treatment efficacy and a change in the subject's risk of developing

T1D. Conversely, it would be understood that no change in the sample biomarker profile over the period of time is indicative of lack of an effective treatment regimen, where that treatment regimen was prescribed for reducing the subjects risk of developing T1D.

[0125] Where there has been no change or an increase in the likelihood that a subject will develop T1D, based on the sample biomarker profile and reference biomarker profile in accordance with the present invention, the method may further comprises exposing the subject to a treatment regimen for preventing or delaying the onset of T1D. This may comprise administering to the subject additional doses of the same agent with which they are being treated or changing the dose and/or type of medication. Illustrative examples of suitable treatment regimens will be discussed in more detail herein below.

[0126] The diagnostic method of the present invention, as disclosed herein, further enables determination of endpoints in pharmacotranslational studies. For example, clinical trials can take many months or even years to establish the pharmacological parameters for a medicament to be used in preventing or delaying the onset of T1D, particularly in subjects at risk of developing T1D. However, these parameters may be associated with the biomarker profiles as herein described. Hence, the clinical trial can be expedited by selecting a treatment regimen (e.g., medicament and pharmaceutical parameters), which results in a biomarker profile associated with low or lower risk of developing T1D, including a healthy state (e.g., healthy condition). This may be determined for example by (1) providing a correlation of a reference biomarker profile with the likelihood of having the healthy condition; (2) obtaining a sample biomarker profile from a subject suspected of being at risk of developing T1D, wherein a similarity of the subject's biomarker profile after treatment to the reference biomarker profile indicates the likelihood that the treatment regimen is effective for changing the health status of the subject to the desired health state (e.g., healthy condition). This aspect of the present invention advantageously provides methods of monitoring the efficacy of a particular treatment regimen in a subject (for example, in the context of a clinical trial) already diagnosed as being at risk of developing T1D. Thus, in another aspect, the present invention provides a method of correlating a reference biomarker profile with an effective treatment regimen for T1D, wherein the reference biomarker profile evaluates at least one inflammatory biomarker, the method comprising: (1) determining a sample biomarker profile from a subject prior to commencement of the treatment regimen, wherein the sample biomarker profile evaluates for an individual biomarker in the reference biomarker profile a

corresponding biomarker; and (2) correlating the sample biomarker profile with a treatment regimen that is effective for preventing or delaying the onset of T1D, or a symptom thereof.

[0127] The term "correlating" generally refers to determining a relationship between one type of data with another or with a state (physiological and/or pathophysiological). In various embodiments, correlating a biomarker profile with the presence or absence of risk of developing T1D comprises determining the presence, absence or amount of at least one biomarker in a subject that suffers from that condition; or in persons known to be free of that condition. In specific embodiments, a profile of biomarker levels, absences or presences is correlated to a global probability or a particular outcome, using receiver operating characteristic (ROC) curves.

[0128] Thus, in some embodiments, evaluation of biomarkers includes determining the levels of individual biomarkers, which correlate with the presence, absence or degree of risk of developing T1D, as herein described. In certain embodiments, the techniques used for detection of biomarkers will include internal or external standards to permit quantitative or semi-quantitative determination of those biomarkers, to thereby enable a valid comparison of the level of the biomarkers in a biological sample with the corresponding biomarkers in a reference sample or samples. Such standards can be determined by the skilled practitioner using standard protocols, illustrative examples of which are disclosed herein.

[0129] In some embodiments, the methods comprise comparing the expression of at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc.) biomarker in the subject's sample biomarker profile to the expression of a corresponding biomarker in a reference biomarker profile from at least one control subject or population of subjects selected from a healthy control subject or group (i.e., "reference biomarker profile"), wherein a similarity between the expression of the at least one biomarker in the sample biomarker profile and the expression of the corresponding biomarker in the reference biomarker profile identifies that the subject has a biomarker profile that correlates with the presence of a healthy condition, or alternatively the absence of risk (or low risk) of developing T1D and/or wherein a similarity between the expression of the at least one biomarker in the sample biomarker profile and the expression of the corresponding biomarker in the reference biomarker profile identifies that the subject has a biomarker profile that correlates with an increased risk of developing T1D or, alternatively, the absence of a healthy condition.

Stratifying a subject to an anti-inflammatory treatment regimen

[0130] As outlined above, the present inventors have found that subjects at risk of developing T1D can be stratified into an inflammatory phenotype and a metabolic phenotype based on the sample biomarker profile for that subject. As used herein, the term "inflammatory phenotype" is characterised by at least one inflammatory biomarker in the sample biomarker profile being expressed at a level that is higher than a level that is representative of a mean or median level of expression of a corresponding biomarker in a population of subjects considered at risk of developing T1D or healthy controls (which is to be understood as including subjects who are not considered at risk of developing T1D). Thus, an at-risk subject will typically have an inflammatory phenotype where the sample biomarker profile for that subject identifies at least one inflammatory biomarker whose expression is equal to or greater than a level of expression that is representative of a mean or median level of expression of the inflammatory biomarker in a sample population, such as a sample population of subjects considered at risk of developing T1D or healthy controls.

[0131] Similarly, a subject will typically have a "metabolic phenotype" where the sample biomarker profile for that subject identifies at least one metabolic biomarker whose expression is equal to or greater than a level of expression that is representative of a median level of expression of the at least one metabolic biomarker in a sample population, such as a sample population of subjects considered at risk of developing T1D or healthy controls. Alternatively, a subject may have a metabolic phenotype where the sample biomarker profile for that subject identifies at least one inflammatory biomarker whose expression is less than a level of expression that is representative of a mean or median level of expression of the at least one inflammatory biomarker in a sample population, such as a sample population of subjects considered at risk of developing T1D or healthy controls, even where the subject's sample biomarker profile does not identify a metabolic biomarker whose expression is equal to or greater than a level of expression that is representative of a median level of expression of the at least one metabolic biomarker in a sample population

[0132] The term "inflammatory phenotype", as used herein, also includes an at-risk subject whose sample biomarker profile identifies at least one inflammatory biomarker whose expression is equal to or greater than a level of expression that is representative of a mean or median level of expression of the inflammatory biomarker in a sample population, such as a sample population of subjects considered at risk of developing T1D or healthy controls, even though the sample

biomarker profile for that subject also identifies at least one metabolic biomarker whose expression is equal to or greater than a level of expression that is representative of a median level of expression of the at least one metabolic biomarker in a sample population. That is, an at-risk subject is regarded as having an inflammatory phenotype even in the presence of an underlying sample biomarker profile that suggests a metabolic phenotype.

[0133] This is the first time that subjects at risk of developing T1D, independent of their islet autoantibody status, have been identified as having an inflammatory or metabolic phenotype. This surprising finding suggests that the progression to T1D in subjects identified as being at risk of developing T1D and having an inflammatory phenotype may be prevented or delayed by stratifying these subjects to treatment regimens that are designed to ameliorate inflammation (*i.e.*, anti-inflammatory treatment regimens). Similarly, the progression to T1D in subjects identified as being at risk of developing T1D and having a metabolic phenotype may be prevented or delayed by stratifying these subjects to treatment regimens that are designed to ameliorate the metabolic phenotype (also referred to herein as a metabolic phenotype-targeted treatment regimens).

[0134] Therefore, disclosed herein is a method of stratifying a subject at risk of developing T1D to an anti-inflammatory treatment regimen, the method comprising (1) correlating a reference biomarker profile with an inflammatory phenotype, wherein the reference biomarker profile evaluates at least one inflammatory biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having an inflammatory phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to an anti-inflammatory treatment regimen.

[0135] The present inventors have shown that the stratification of an at-risk subject into an inflammatory or metabolic phenotype is independent of islet autoantibody status. Thus, for the purpose of stratifying an at-risk subject to an inflammatory or metabolic phenotype in accordance with the present invention, the subject can be an islet autoantibody positive subject or an islet autoantibody negative subject.

[0136] Persons skilled in the art will know that there are a number of different autoantibodies that are used to characterise an at-risk subject with respect to. Non-limiting examples include autoantibodies that specifically bind to at least one islet

antigen selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2). For the purpose of stratifying an at-risk subject to an inflammatory or metabolic phenotype in accordance with the present invention, the subject will have circulating islet autoantibodies that specifically bind to at least one islet antigen (e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more). In some embodiments, the methods further comprise determining the islet autoantibody status of the subject. In illustrative examples of this type, the subject is islet autoantibody positive.

[0137] In some embodiments, the reference biomarker profile evaluates at least one inflammatory biomarker as broadly described above and elsewhere herein. In illustrative examples, the inflammatory biomarker is selected from the group listed in Figure 3. The reference biomarker profile is typically correlated with an inflammatory phenotype where the at least one inflammatory biomarker is higher than a level that is representative of a mean or median level a corresponding biomarker in a population of subjects at risk of developing T1D. This is illustrated, for example, in the biomarker profiles that are diagrammatically represented in Figures 3.

[0138] For an at-risk subject that is islet autoantibody positive, the inventors have shown that the reference biomarker profile is correlated with an inflammatory phenotype where the reference biomarker profile evaluates at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) inflammatory biomarker selected from the group consisting of IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, thymic stromal lymphopoietin (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF-2), GLP-1, parathyroid hormone (PTH), glucagon, adiponectin, Adrenocorticotrophic hormone (ACTH), amylin (islet amyloid polypeptide precursor; IAPP) and Chemokine (C motif) ligand (XCL1), soluble CD30 (sCD30), soluble interleukin 6 receptor (sIL-6R), stem cell factor (SCF), eotaxin-2, macrophage inflammatory protein 1d (MIP1d), Apolipoprotein A-1 (APOA1), peptide tyrosine tyrosine (peptide YY; PYY), and osteopontin (OPN).

[0139] In another embodiment, for an at-risk subject that is islet autoantibody positive, the inflammatory biomarker is selected from the group consisting of IL-23, IL-11, and leukemia inhibitory factor (LIF).

[0140] In some embodiments, a metabolic phenotype in an islet autoantibody positive subject is characterised by at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) metabolic biomarker selected from

increased expression of insulin resistance (such as HOMA-IR, or waist circumference and serum triglycerides), a measure of age-adjusted BMI, such as BMI-SDS or BMI percentile, a measure of glucose tolerance (such as fasting and 2 hour glucose after oral glucose tolerance test), a measure of insulin secretion, (such as insulin, proinsulin, proinsulin/insulin ratio or c-peptide), serum amyloid P (SAP), leptin, CFH, anti-thrombin III, sIL-1RII, PDFBB, TGFB1, ENA78, SAA, GCP2, VEGF, GIP and low levels of the inflammatory biomarkers described above (*e.g.*, XCL1, TSLP, ACTH, IL-33, IL-23, IL-28A, IL-6).

[0141] In some embodiments, the at least one metabolic biomarker is selected from the group listed in Figure 3. In specific embodiments, the at least one metabolic biomarker is selected from the group consisting of insulin resistance, age-adjusted BMI, a measure of glucose tolerance, insulin, proinsulin, proinsulin/insulin ratio, c-peptide, serum amyloid P (SAP), leptin, complement factor H (CFH), anti-thrombin III, sIL-1RII, PDFBB, transforming growth factor beta 1 (TGF β 1), chemokine (C motif) ligands (*e.g.*, XCL1 and ENA78), serum amyloid A (SAA), Granulocyte Chemotactic Protein 2 (GCP2), vascular endothelial growth factor (VEGF) and Gastric inhibitory polypeptide (GIP). In some embodiments, the reference biomarker profile is correlated with a metabolic phenotype where the level of the at least one metabolic biomarker is higher than a level that is representative of a mean or median level of the same biomarker in a population of subjects at risk of developing T1D.

[0142] In some embodiments, the metabolic biomarker is SAP.

[0143] Alternatively, or in addition, the reference biomarker profile is correlated with a metabolic phenotype where the level of the at least one inflammatory biomarker is lower than a level that is representative of a mean or median level of the same biomarker in a population of subjects considered at risk of developing T1D. In an illustrative example, the inflammatory biomarker whose level of expression is lower than a level that is representative of a mean or median level of the same biomarker in a population of subjects considered at risk of developing T1D is selected from the group consisting of XCL1, TSLP, ACTH, IL-33, IL-23, IL-28A and IL-6. In another illustrative example, the inflammatory biomarker whose level of expression is lower than a level that is representative of a mean or median level of the same biomarker in a population of subjects considered at risk of developing T1D is selected from the group consisting of IL-11, IL-23, and LIF.

[0144] In some embodiments, the reference biomarker profile further evaluates at least one other biomarker selected from the group consisting of IL20, PYY, RELB

DNA binding, IL-12A, TNF, GRP78, VEGFR1, CD14^{LOW}CD16⁻ cells, CD14^{HIGH}CD16⁺ cells and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

[0145] For a subject that is islet autoantibody negative, the reference biomarker profile may be correlated with an inflammatory phenotype where the reference biomarker profile evaluates at least one inflammatory biomarker that is different from the inflammatory biomarker(s) that correlate with an inflammatory phenotype in an islet autoantibody positive subject. Alternatively, the reference biomarker profile may be correlated with an inflammatory phenotype where the reference biomarker profile evaluates fewer inflammatory biomarkers than as compared to the reference biomarker profile that correlate with an inflammatory phenotype in an islet autoantibody positive subject. In some embodiments, the inflammatory biomarker is selected from the group consisting of IL-12B, *TNF*, *IL12A*, PDGFB.

[0146] In an illustrative example, a metabolic phenotype of an islet autoantibody negative subject is characterised by low levels of adiponectin and high PDGFB, *IL12A*, *TNF* or their encoding transcripts.

[0147] In some embodiments, the method comprises comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and stratifying the subject to the anti-inflammatory treatment regimen based on the ratio. In non-limiting examples, the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

[0148] Without being bound by theory or mode of operation, it is postulated that an inflammatory phenotype precedes a metabolic phenotype after development of islet antibodies in the pathogenesis of T1D (*i.e.*, in the progression towards the onset of T1D). It is therefore proposed that treating an at-risk subject who is identified as having an inflammatory phenotype with an anti-inflammatory treatment regimen may assist to prevent or delay the onset of T1D in that subject. Therefore, also disclosed herein is a method that further comprises correlating a reference biomarker profile with an inflammatory phenotype, wherein the reference biomarker profile evaluates at least one inflammatory biomarker and wherein the sample biomarker profile further evaluates, for the at least one inflammatory biomarker in the reference biomarker profile, a corresponding biomarker. Subjects who are determined to be at risk of developing T1D and are further identified as

having an inflammatory phenotype (but, in some embodiments, not a metabolic phenotype), can be stratified to an anti-inflammatory treatment regimen, as disclosed herein. In doing so, it is postulated that the progression of the subject to a metabolic phenotype, which is postulated to follow the inflammatory phenotype and to precede onset of diabetes, will be delayed or prevented.

[0149] Also disclosed herein is a method of stratifying a subject at risk of developing T1D to a metabolic phenotype-targeted treatment regimen, the method comprising (1) correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having a metabolic phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to a metabolic phenotype-targeted treatment regimen.

[0150] In some embodiments, where the at-risk subject is an islet autoantibody positive subject, the metabolic phenotype is characterised by increased or decreased expression of at least one of the metabolic biomarkers listed in Figure 3. In non-limiting examples, the at least one metabolic biomarker is selected from the group consisting of insulin resistance, age-adjusted BMI, a measure of glucose tolerance, insulin, proinsulin, proinsulin/insulin ratio, c-peptide, SAP, leptin, complement factor H (CFH), anti-thrombin III, sIL-1RII, PDFBB, transforming growth factor beta 1 (TGF β 1), chemokine (C motif) ligands (e.g., XCL1 and ENA78), serum amyloid A (SAA), Granulocyte Chemotactic Protein 2 (GCP2), vascular endothelial growth factor (VEGF) and Gastric inhibitory polypeptide (GIP). In other non-limiting examples, the at least one metabolic biomarker is SAP. Increased expression includes, but is not limited to, the expression of the at least one metabolic biomarker being higher than a level that is representative of a mean or median level a corresponding biomarker in a population of subjects at risk of developing T1D or healthy controls.

[0151] In some embodiments, the method further comprises correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker and wherein the sample biomarker profile further evaluates, for the at least one metabolic biomarker in the reference biomarker profile, a corresponding biomarker. In some embodiments, the at least one metabolic biomarker is selected from the group consisting of insulin resistance (such as HOMA-IR, or waist circumference and

serum triglycerides), age-adjusted BMI, such as BMI-SDS or BMI percentile, a measure of glucose tolerance (such as fasting and 2 hour glucose after oral glucose tolerance test), a measure of insulin secretion, (such as insulin proinsulin, proinsulin/insulin ratio or c-peptide), serum amyloid P (SAP), leptin, complement factor H (CFH), anti-thrombin III, sIL-1RII, PDFBB, transforming growth factor beta 1 (TGF β 1), chemokine (C motif) ligands (e.g., XCL1 and ENA78), serum amyloid A (SAA), Granulocyte Chemotactic Protein 2 (GCP2), vascular endothelial growth factor (VEGF) and Gastric inhibitory polypeptide (GIP).

[0152] In some embodiments, the at least one metabolic biomarker is SAP.

[0153] In specific embodiments, the term "at-risk subject" and the like are taken to mean a subject that is determined to be at risk of developing T1D in accordance with any one of the methods broadly described and disclosed herein. In some embodiments, the term "at-risk subject" and the like are taken to mean a subject who has been determined to be at risk of developing T1D independent of the diagnostic methods of the present invention, as broadly described and disclosed herein. In a non-limiting example, the subject is considered to be at-risk of developing T1D by virtue of an identified or suspected genetic predisposition to T1D, such as being a first degree relative of an individual with T1D.

Treatment regimen

[0154] The present invention also extends to the management of risk of developing T1D in a subject. The management of said risk can include identification and amelioration of the underlying cause and use of therapeutic agents or treatment regimens for preventing or delaying the onset of T1D, or a symptom thereof. Treatment regimens may include dietary restrictions (e.g., limiting caloric intake) and exercise. In some embodiments, a treatment regimen will be administered in pharmaceutical (or veterinary) compositions together with a pharmaceutically acceptable carrier and in an effective amount to achieve their intended purpose. The dose of active compounds administered to a subject should be sufficient to achieve a beneficial response in the subject. The quantity of the pharmaceutically active compound(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the active compound(s) for administration will depend on the judgment of the practitioner. In determining the effective amount of the active compound(s) to be administered for preventing or delaying the onset of T1D, the medical practitioner or veterinarian may evaluate severity of any symptom associated with the presence of T1D including abnormal

blood pressure and vascular disease (e.g., atherosclerosis). In any event, those of skill in the art may readily determine suitable dosages of the therapeutic agents and suitable treatment regimens without undue experimentation.

[0155] Thus, also disclosed herein is a method for preventing or delaying the onset of T1D or a symptom thereof in a subject, the method comprising:

- (a) determining whether a subject is at risk of developing T1D in accordance with the method of the present invention, as broadly described above and elsewhere herein; and
- (b) exposing the subject, on the basis that the subject has an increased likelihood of developing T1D, to a treatment regimen for preventing or delaying the onset of T1D or a symptom thereof.

[0156] As used herein, the term "treatment regimen" typically refers to a prophylactic regimen (*i.e.*, before the onset of T1D), unless the context specifically indicates otherwise. The term "treatment regimen" encompasses natural substances and pharmaceutical agents (*i.e.*, "drugs") as well as any other treatment regimen including but not limited to dietary treatments, physical therapy or exercise regimens, surgical interventions, and combinations thereof.

[0157] The term "treating" as used herein, unless otherwise indicated, means alleviating, inhibiting the progress of, or preventing, either partially or completely, the onset of T1D, or a symptom thereof. The term "treatment" as used herein, unless otherwise indicated, refers to the act of treating.

[0158] Following diagnosis, the treatment regimen to be adopted or prescribed may depend on several factors, including the age, weight and general health of the subject. Another determinative factor may be the degree of risk of developing T1D determined by the sample biomarker profile in accordance with the present invention, as herein described. For instance, where the subject is determined to be at high risk of developing T1D, a more aggressive treatment regimen may be prescribed as compared to a subject who is determined to be at low risk of developing T1D. The treatment regimen may also depend on existing clinical parameters relevant to T1D, including body mass index, weight, glucose intolerance and homeostatic insulin resistance.

[0159] Thus, the present invention contemplates exposing the subject to a treatment regimen if the subject is determined to be at risk of developing T1D in accordance with the methods of the present invention. Non-limiting examples of such treatment regimens include exposing the at-risk subject to metformin,

glucagon-like peptide (GLP)-1, diet (e.g., caloric intake restrictions), exercise, anti-CD3 monoclonal antibodies (mAb), rituximab, abatacept, IL-1-receptor antagonist, TNF-inhibitors, other anti-cytokine mAb or soluble receptors, strategies to induce antigen-specific tolerance (including curcusesomes encapsulating islet antigenic peptides, DNA vaccines encoding islet antigenic peptides, islet antigenic peptide immunotherapy, dendritic cell targeting strategies using monoclonal antibodies fused to islet antigens).

[0160] In some embodiments, the treatment regimen is an anti-inflammatory treatment regimen. Illustrative examples include *ex vivo* and *in vivo* approaches to reduce the expression of inflammatory biomarkers, whether said biomarkers are the same as those of the reference biomarker profile, or others. It would be recognised by persons skilled in the art that an effective anti-inflammatory response may still be achieved where the treatment regimen targets other inflammatory factors or pathways in the subject. Illustrative examples include, but are not limited to, antagonists of pro-inflammatory agents (including neutralising antibodies, or neutralising antigen-binding fragments thereof), anti-inflammatory compounds that directly or indirectly activate anti-inflammatory pathways in the subject to an extent that the pro-inflammatory state identified in the subject is ameliorated, inhibited, reversed or neutralised. These approaches may rely on the use of molecules to target inflammatory pathways. The effector molecule may be, for example, an antibody specific for some marker on the surface of an immune cell, such as a T cell, a B cell, a macrophage, a monocyte and a natural killer cell, or subsets thereof, so as to lyse or otherwise neutralise the immune cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually facilitate cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent.

[0161] Suitable anti-inflammatory treatment regimens may include antagonists of any one or more of the biomarkers identified as being differentially expressed in subjects at risk of developing T1D, as herein described. Illustrative examples are the biomarkers listed in Figures 3, particularly the biomarkers whose expression has been shown to be upregulated (i.e., IL-1R, IL-20, IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, TSLP, GM-CSF, XCL1 or LIF) as compared to a median level of expression of the corresponding biomarker in a population of subjects considered at risk of developing T1D.

[0162] In some embodiments, it may be appropriate to target a metabolic phenotype. In a non-limiting example, the metabolic phenotype-targeted treatment

regimen comprises a strategy for inducing antigen-specific tolerance in the subject, which applies to at-risk subjects that are either islet autoantibody negative or islet autoantibody positive. For an at-risk subject that is islet autoantibody negative, non-limiting examples of suitable metabolic phenotype-targeted treatment regimens include exercise, caloric intake restriction and the administration of a therapeutically effective amount of metformin.

[0163] In another non-limiting example, the metabolic phenotype-targeted treatment regimen or anti-inflammatory treatment regimen comprises a strategy for inducing or increasing the expression of fractalkine (Chemokine (C-X3-C motif) ligand 1 (CX3CL1)) in the at-risk subject. This may include, for example, administering to the at-risk subject fractalkine, a non-limiting example of which is human recombinant fractalkine.

[0164] In some embodiments, the subject is exposed to a combination of two or more additional treatment regimens (*e.g.*, 2, 3 or more, 4 or more, 5 or more, 6 or more), including, but not limited to, the administration of agents that induce antigen-specific tolerance in the subject (*i.e.*, metabolic phenotype-targeted treatment regimens), optionally in combination with an anti-inflammatory regimen. Illustrative examples for the anti-inflammatory regimens are antagonists of the biomarkers listed in Figures 3 shown to be upregulated (*i.e.*, increased IL-20, IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, TSLP, GM-CSF, XCL1 or LIF) as compared to a mean or median level of expression of the corresponding biomarker in a population of subjects considered at risk of developing T1D. Illustrative examples of agents or regimens for inducing antigen-specific tolerance include curcusesomes encapsulating islet antigenic peptides, DNA vaccines encoding islet antigenic peptides, islet antigenic peptide immunotherapy, tolerogenic dendritic cell and islet antigen therapy, and dendritic cell targeting strategies using mAb fused to islet antigens.

[0165] The term "upregulated," "overexpressed" and the like, as used herein, refer to an upward deviation in the level of expression of a biomarker as compared to a baseline expression level of a corresponding biomarker in a control sample. Conversely, the term "downregulated," "underexpressed" and the like refer to a downward deviation in the level of expression of a biomarker as compared to a baseline expression level of a corresponding biomarker in a control sample.

[0166] One of skill in the art would know that the list of possible target inflammatory biomarkers, as show, for example, in Figures 3 and 4, is not

exhaustive of the types of targets to which the proposed treatment regimens may be designed.

[0167] In some embodiments, the treatment regimen includes exposing the subject to a therapeutically effective amount of an anti-TSLP antibody, a therapeutically effective amount of an anti-IL33 antibody, TLR7 antagonists (e.g., imiquimod), a therapeutically effective amount of an anti-OX40L antibody, JAK inhibitors, or any combination thereof (see, e.g., Ito *et al*, 2012, *Allergy International*, 61:35-43).

[0168] Without being bound by theory or a particular mode of operation, the present inventors have shown that the inflammatory phenotype in islet autoantibody positive at-risk subjects included cytokines, chemokines and alarmins, as well as type 1 interferon-mediated viral response proteins typically produced by epithelial and infiltrating inflammatory cells in atopic skin and lung disease and skin psoriasis (including without limitation IL-33, TSLP, eotaxin, IL-23, IL-15, IL-6, IL-28A and IL-29), suggesting an infectious, potentially viral, trigger to the pathogenesis and/or progression towards T1D in this cohort. Furthermore, the inflammatory phenotype also included hormones, including the appetite suppressing hormones PYY and amylin, the calcium regulator PTH, and ACTH, an activator of the hypothalamic-pituitary-adrenal (HPA) axis. Conversely, AB+ FDR with the insulin resistant "metabolic" biomarker phenotype had lower levels of these inflammatory cytokines and hormones but higher levels of c-peptide, proinsulin, insulin, leptin and acute-phase proteins serum amyloid A and P (Figure 4). AB+ FDR with the insulin resistant "metabolic" biomarker phenotype also had higher levels of SAP. Thus, strategies that seek to restore the balance of these biomarkers in at-risk subjects towards levels seen in healthy individuals may represent, or form the basis of, suitable treatment regimens.

Kits

[0169] In another aspect there is provided a kit comprising one or more reagents and/or devices for use in performing the method of the present invention, as herein described. The kits may contain reagents for obtaining a sample biomarker profile in accordance with the methods as herein described. Kits for carrying out the methods of the present invention typically include, in suitable container means, (i) a reagent for detecting the at least one biomarker, (ii) a probe that comprises an antibody or nucleic acid sequence that specifically binds to the at least one biomarker, (iii) a label for detecting the presence of the probe and (iv) instructions for how to measure the level of expression of the at least one

biomarker. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe and/or other container into which a first antibody specific for the at least one biomarker or a first nucleic acid specific for the at least one biomarker may be placed and/or suitably aliquoted. Where a second and/or third and/or additional component is provided, the kit will also generally contain a second, third and/or other additional container into which this component may be placed. Alternatively, a container may contain a mixture of more than one reagent, each reagent specifically binding a different biomarker in accordance with the present invention, when required. The kits of the present invention will also typically include means for containing the reagents (*e.g.*, nucleic acids, polypeptides *etc.*) in close confinement for commercial sale. Such containers may include injection and/or blow-moulded plastic containers into which the desired vials are retained.

[0170] The kits may further comprise positive and negative controls, including a reference biomarker profile, as well as instructions for the use of kit components contained therein, in accordance with the methods of the present invention.

[0171] In some embodiments, the kit comprises a set of nucleic acid primers listed herein in Table 1.

[0172] All the essential materials and reagents required for detecting and quantifying biomarker expression products may be assembled together in a kit, which is encompassed by the present invention. The kits may also optionally include appropriate reagents for detection of labels, positive and negative controls, washing solutions, blotting membranes, microtiter plates dilution buffers and the like. For example, a nucleic acid-based detection kit may include (i) a biomarker polynucleotide (which may be used as a positive control), (ii) a primer or probe that specifically hybridizes to a biomarker polynucleotide. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (Reverse Transcriptase, *Taq*, Sequenase™, DNA ligase *etc.* depending on the nucleic acid amplification technique employed), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits also generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each primer or probe. Alternatively, a protein-based detection kit may include (i) a biomarker polypeptide (which may be used as a positive control), (ii) an antibody that binds specifically to a biomarker polypeptide. The kit can also feature various devices (*e.g.*, one or more) and reagents (*e.g.*, one or more) for performing one of the assays described herein; and/or printed instructions for using the kit to quantify the expression of a biomarker gene.

[0173] It will be appreciated that the above described terms and associated definitions are used for the purpose of explanation only and are not intended to be limiting.

[0174] In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

INFLAMMATORY AND METABOLIC PATHWAYS IMPLICATED IN THE PRECLINICAL DEVELOPMENT OF TYPE I DIABETES

EXAMPLE 1

CONSTITUTIVE INFLAMMATORY ACTIVITY IN PB OF HEALTHY CHILDREN AT FAMILIAL RISK OF T1D

[0175] An overview of constitutive innate and adaptive immune activation amongst 119 healthy children, of whom 93 were FDR of patients with T1D and 26 were unrelated, was obtained by measuring DNA binding activity of NF- κ B RELA and RELB by chemiluminescent ELISA in whole cell extracts generated from PBMC. Constitutive RELB DNA binding was significantly higher in PBMC of AB- FDR than healthy controls (Figure 1a, $p < 0.0001$), and RELA DNA binding was increased in a subset of these FDR (Figure 1b $p = 0.018$). RELB and RELA DNA binding activity were correlated ($\rho = 0.53$, $p = 0.003$).

[0176] Quantitative polymerase chain reaction (qPCR) was used to survey mRNA expression of RELA- and RELB-regulated genes in PBMC, focussing on mRNAs that were expressed in freshly-isolated PBMC, including IL12A (IL12p35) and TNF. IL12A (Figure 1c, $p < 0.0001$) and TNF (Figure 1d, $p < 0.001$) expression was increased in AB- FDR relative to HC PBMC, and the expression of each gene correlated with RELB DNA binding ($\rho = 0.50$, $p = 0.004$ and $\rho = 0.62$, $p < 0.001$, respectively).

[0177] 133 serum analytes were then screened using ELISA assays and a Luminex multiplex platform (see Table 5) in the cohort of subjects, together with integrated previously-collected flow cytometric data from the same PB samples¹⁶. Extensive data mining was conducted using Feature Selection to evaluate possible individual serum analytes, myeloid subsets, PBMC NF- κ B binding and inflammatory and endoplasmic reticulum (ER) stress gene expression that predict AB- T1D FDR amongst healthy children. Compared to healthy controls, AB- FDR were significantly more likely to have higher levels of RELB DNA binding, TNF expression, SAP and

%CD14^{lo}CD16⁻ cells, and significantly lower levels of the ER stress gene GRP78, serum soluble VEGFR1 (sVEGFR1) and %CD14^{hi}CD16⁺ cells (Figures 1a-f and Table 2).

[0178] Since healthy controls were fasting and AB- FDR were non-fasting for these analyses, healthy control data were compared to data derived from two further cohorts of non-fasted healthy subjects. No significant differences were found in sVEGFR1 (median and IQR for fasting HC = 397 (73-596), for non-fasting HC = 541 (297-499), $p=0.9$) in fasting and non-fasting subjects, using Mann-Whitney tests.

Table 2: Variables identified by the Feature Selection process differentiating AB- FDR and Healthy Controls and significance obtained from univariate logistic regression

Marker	Median (IQR)		Feature Selection		Logistic regression	
	HC	FDR	Chi-square	p value	p value	
RelB DNA binding	0.94 (0.8, 1.2)	1.57 (1.2, 2.1)	37.76	<0.001	0.001	
Serum sVEGFR1	514 (103, 597)	162 (103, 283)	24.43	<0.001	0.012	
% PB CD14 ^{hi} CD16 ⁺ cells	5.9 (4.8, 9)	3.5 (2.4, 4.3)	23.97	0.002	<0.001	
% PB CD14 ^{lo} CD16 ⁻ cells	10.6 (10, 17.6)	16 (13, 21.8)	22.08	0.005	0.025	
GRP78 mRNA	0.46 (0.3, 0.9)	0.14 (0.1, 0.6)	16.29	0.012	0.001	
TNF mRNA	0.34 (0.8, 1.1)	5.11 (1.4, 30.9)	15.45	0.004	<0.001	

[0179] NF- κ B, flow cytometric, mRNA, Luminex and ELISA variables were analysed using Feature Selection and univariate logistic regression.

[0180] To further investigate the relationships between these candidate risk markers and associated biological pathways, a set of PB markers was identified that

significantly correlated with variables differentiating AB- FDR and healthy controls; that is, RELB, sVEGFR1, PB CD14hiCD16+ and CD14loCD16- cells, TNF and GRP78 ($p < 0.05$, Spearman's rank correlation, $\rho > 0.2$). Data were normalised for each variable to the median value for that variable across all subjects so that clinical, mRNA and serum data could be visualised on the same scale, and clustered according to the degree of Spearman's rank correlation between variables (Figure 1g). Visual inspection of the tree hierarchy indicated that most AB- FDR were separated from HC by their differential expression of TNF, IL12A, RelB, CD14HIGHCD16+ cells, serum IL-12p40 and sVEGFR1. Surprisingly, AB- FDR were heterogeneous, and clustered into two major phenotypic groups characterised by differential expression of inflammatory, metabolic and vascular biomarkers, including adiponectin, platelet-derived growth factor BB (PDGFB), angiopoietin-ligand 4 (ANGPTL4), IL12A and TNF. These data identify biomarkers that distinguish AB- FDR from HC and identify heterogeneity among AB- FDR.

EXAMPLE 2

Insulin resistance, glucose intolerance and serum biomarkers associated with increasing numbers of islet AB in FDR

[0181] To stratify at-risk AB+ FDR, and to discover markers elucidating disease immunopathogenesis, serum analytes were screened and PCR carried out for inflammatory and ER stress genes. Clinical (age, BMI, fasting and 120 min blood glucose measurements during an oral glucose tolerance test, OGTT) but not HLA typing data were available for AB+ FDR. Insufficient numbers of AB+ FDR were assessed for NF- κ B DNA binding ($n=5$) or flow cytometric assays ($n=3$) to meaningfully assess their relationship to AB status. Compared to FDR with a single AB, those with 2 or 3 AB were likely to have significantly higher blood glucose 120 min after an oral glucose load in the OGTT, higher BMI percentile, higher HbA1c, higher levels of insulin resistance as determined by HOMA-IR, and significantly lower levels of serum IL-20, peptide YY (PYY) and XCL1 (Table 3, Figures 2a-d). Seven of 19 subjects with multiple AB (38%) were insulin-resistant, using a HOMA-IR cut-off of 2.624 (Figure 2d). These data confirm previously identified clinical features of increased T1D risk among AB+ subjects, and identify further serum biomarkers associated with presence of multiple AB and thus higher risk.

Table 3. Variables identified by the Feature Selection process differentiating AB1⁺ FDR and AB2/3⁺ FDR and significance obtained from univariate logistic regression

Marker	Median (IQR)		Feature Selection		Logistic regression
	AB1 ⁺	AB2/3 ⁺	Chi-square	P value	P value
BMI percentile	70 (50, 77)	88 (70, 100)	13.01	0.011	0.003
120 min glucose	5.7 (4.8, 6)	7.1 (6, 8.6)	12.83	0.005	0.021
HOMA-IR	0.16 (0.04, 1.57)	1.91 (1.23, 5.06)	10.20	0.017	0.053
Serum peptide YY	138 (68, 185)	96 (89, 108)	9.55	0.022	0.052
Serum XCL1	101 (87, 144)	89.40 (72, 118)	9.29	0.026	0.004
HbA1c	4.9 (4.7, 5.1)	5.4 (5.1, 5.6)	8.64	0.013	0.004
Serum IL-20	214 (129, 246)	105 (37, 187)	8.09	0.018	0.023

[0182] Clinical NF- κ B, flow cytometric, mRNA, Luminex and ELISA variables were analysed using Feature Selection and univariate logistic regression.

[0183] To further investigate the biological pathways associated with candidate risk markers emerging from analysis of AB⁺ FDR, a set of markers that was significantly correlated with variables differentiating single from multiple AB⁺ FDR (*i.e.* BMI percentile, 120 min stimulated glucose, HOMA-IR, HbA1c, PYY, XCL1 and IL-20 ($p < 0.05$, Spearman's rank correlation, $\rho > 0.25$)) were identified and clustered as described above (Figure 3). Visual inspection of the tree hierarchy again indicated heterogeneity among AB⁺ FDR. Similar to AB⁻ FDR, the groups differed in their expression of a distinctive set of serum pro-inflammatory cytokines and chemokines (*e.g.* IL-28A, IL-33, IL-23, TSLP), hormones and adipokines (PYY, fibroblast growth factor-21 (FGF-21), glucagon, GLP1, parathyroid hormone (PTH), ACTH, amylin (IAPP) and adiponectin). Of interest, insulin resistant individuals (high HOMA-IR) were identifiable amongst the AB2/3⁺ FDR, and were also characterised by high serum levels of insulin, proinsulin, 120 minute glucose and acute phase proteins previously associated with steatohepatitis (*e.g.* serum amyloid P and A, leptin). On the other hand, serum levels of the above-mentioned pro-inflammatory cytokine/chemokine and hormonal signature were generally lower. These data suggest that clinical and serum markers separate "inflammatory" and "metabolic" phenotypic subgroups of AB⁺ FDR, as shown in Figure 3.

[0184] Given that the cohort of at-risk FDR was heterogeneous, regardless of the presence of islet AB, PB markers that were significantly correlating with variables differentiating single from multiple AB+ FDR, and AB- FDR from HC, were combined and clustered in the entire cohort of FDR (Figure 4). Clinical variables were not included as they were not measured in AB- FDR. Visual inspection of the tree demonstrates a characteristic signature driven by a set of inflammatory and hormonal biomarkers. As expected, AB+ individuals did not cluster together. These PB biomarkers provide additional immunophenotypic information to that provided by clinical data and AB status in FDR at risk of T1D.

Discussion

[0185] While islet AB and analysis of glucose tolerance are the main screening tools to determine risk of progression to T1D, additional biomarkers are needed to stratify risk, to elucidate disease immunopathogenesis and to identify novel therapeutic and screening targets. In particular, identification of novel targets and disease pathways may afford new treatment strategies and stratify patients appropriately for existing immunotherapies. In this cross-sectional study, biomarkers of dendritic cell activation, inflammation and angiogenesis, including RelB DNA binding, TNF and GRP78 expression, sVEGFR1, and circulating antigen-presenting cell subsets, were identified that predicted AB- FDR of children with T1D from healthy controls. Clinical, hormone and inflammatory biomarkers, including BMI percentile, 120 minute blood glucose during OGTT, HbA1c, IL-20, XCL1 and PYY predicted multiple AB+ FDR from amongst those with islet AB. However, non-hierarchical clustering of a broader set of biomarker correlates of these variables identified an immunophenotypic signature of inflammatory cytokines, chemokines and hormones which was differentially expressed among the AB- FDR. Clinical, hormone and inflammatory biomarkers, including BMI percentile, 120 minute blood glucose during OGTT, HbA1c, IL-20, XCL1 and PYY predicted multiple AB+ FDR from amongst those with islet AB. Consistent with previous reports, more than one third of FDR with multiple AB within the cohort were insulin resistant. Interestingly, non-hierarchical clustering of correlates of these variables demonstrated heterogeneity within the AB+ FDR, with emergence of apparently "inflammatory" and "metabolic" immunophenotypic patterns or profiles. The "metabolic" biomarker pattern of the insulin resistant group was characterised by high levels of acute phase proteins, associated with adiposity/metabolic syndrome²¹.

[0186] It is intriguing that the "inflammatory" immunophenotypic signature in AB+ FDR included cytokines, chemokines and alarmins, as well as type 1 interferon-mediated viral response proteins typically produced by epithelial and

infiltrating inflammatory cells in atopic skin and lung disease and skin psoriasis (including non-limiting IL-33, TSLP, eotaxin, IL-23, IL-15, IL-6, IL-28A and IL-29), suggesting an infectious, potentially viral, trigger. Furthermore, the signature also included hormones, including the appetite suppressing hormones PYY and amylin, the calcium regulator PTH, and ACTH, an activator of the hypothalamic-pituitary-adrenal (HPA) axis. It is of note that the immune system is a major consumer of energy-rich fuel, particularly glucose, at night when feeding, brain and muscle are at rest and HPA axis activity and insulin levels are low²⁵. Inflammatory cytokines have been shown to activate ACTH and the HPA axis, to promote anorexia through appetite-suppressing hormones, and to mobilise calcium through PTH-mediated bone resorption^{25,26}. Conversely, AB+ FDR with the insulin resistant "metabolic" biomarker phenotype had lower levels of these inflammatory cytokines and hormones but higher levels of c-peptide, proinsulin, insulin, leptin and acute-phase proteins serum amyloid A and P (Figure 4). Insulin resistance of liver, adipose tissue and muscle would tend to conserve glucose for the immune system, which does not become insulin resistant²⁵. Thus, both chronic inflammation and insulin resistance have major consequences for energy regulation and pancreatic demand, which would impact on T1D pathogenesis. The identified immunophenotypic diversity, even amongst AB- FDR subjects in this study, suggests that heterogeneity is present very early in T1D immunopathogenesis. Without being bound by theory, this might reflect underlying differences in T1D-associated genes, and/or epigenetic responses to pregnancy, birthweight, growth and environmental inflammatory triggers.

[0187] The current studies support the concept that immune activation in individuals at familial risk of T1D is abnormal relative to healthy controls, and that there are distinct patterns of abnormality during the pre-clinical period associated with high or lower levels of innate and adaptive immune activation, adipokines and acute phase proteins, and glucose-regulating hormones. The current studies also suggest that pre-clinical immune interventions may be designed appropriate to the immune and inflammatory background of the patient, given that subjects at risk of developing T1D can be stratified into an "inflammatory" or "metabolic" phenotype . Furthermore, demonstration of efficacy of immunotherapy in subjects with or at-risk of T1D may become more feasible when accounting for this inflammatory heterogeneity.

Materials and Methods

Study Design:

[0188] In a cross-sectional design, children presenting to a public teaching hospital with new-onset T1D between 2010 and 2012, and their first degree relatives (FDR; siblings), were recruited for these studies. Blood was collected non-fasting from 93 islet autoantibody-negative (AB⁻) FDR of new-onset T1D patients recruited at a routine new-onset diabetes clinic visit. Fasting blood was collected from 34 islet autoantibody-negative (AB⁺) FDR on the day of oral glucose tolerance test screening. FDR from 70% of all families presenting to the clinic participated in the study. Non-participants included very young children and those with behavioural disorders where venesection was impractical. Demographic details of participants are shown in Table 4, below.

Table 4. Demographic details of subjects studied.

Group	n	Male n (%)	Age (months) Mean (SD)	Autoantibodies		
				GAD n (%)	IA-2 n (%)	Insulin n (%)
Healthy Controls	28	7 (26)	131 (55)	N.A.	N.A.	N.A.
AB ⁻ FDR	93	36 (49)	131 (60)	0 (0)	0 (0)	0 (0)
AB ⁺ FDR	13	6 (46)	115 (54)	10 (77)	1 (8)	2 (15)
AB2 ⁺ FDR	16	5 (31)	172 (57)	16 (100)	11 (69)	5 (31)
AB3 ⁺ FDR	5	3 (60)	112 (79)	5 (100)	5 (100)	5 (100)

N.A. = not available

[0189] There was no significant difference in age between groups (Kruskal-Wallis test with Dunn's multiple comparisons test). FDR testing positive for one or more islet AB to glutamic acid decarboxylase (GADA), insulinoma-associated (IA-2) antigen and insulin by radioimmunoassay (Mater Pathology, Mater Health Services) were classified as islet AB⁺ and those testing negative for islet AB were classified as islet AB⁻. AB⁺ children were assessed clinically for height, weight, HbA1c and oral glucose tolerance test, from which fasting and 120 min blood glucose measurements were recorded. Age-adjusted BMI percentile, BMI-SDS and HOMA-IR were calculated as previously described. Non-fasting blood was collected from 93 AB⁻ FDR of new-onset T1D patients recruited at a routine new-onset diabetes clinic visit.

[0190] Children with newly-diagnosed T1D were insulin-dependent and did not have overt concomitant infection at the time of venesection. The diagnosis of T1D was based on clinical and biochemical parameters, including age, weight loss, episodes of keto-acidosis, islet auto-antibody status (GAD, IA-2, insulin) and autoimmune diathesis at presentation. None of the T1D patients received immunosuppressive medication; however they were treated with other necessary medications, such as anti-hypertensives, diuretics and lipid-lowering agents. Healthy control children without a family history of autoimmune disease or other chronic illness undergoing elective surgery were recruited at their pre-operative assessment and PB was obtained just before or immediately after induction of anaesthesia, in the fasting state. These healthy controls were included as a group of unrelated children, for comparison to FDR, including AB- otherwise-healthy subjects. The study was approved by the human ethics committees of the Mater Health Services and The University of Queensland.

[0191] Two additional datasets were interrogated to determine whether fasting status impacted the levels of various serum analytes: (1) plasma from 47 non-fasted Chinese subjects collected from students at the National University of Singapore, and (2) serum from 23 non-fasted healthy control children collected at Children's Hospital of Pittsburgh and University of Florida.

Sample processing

[0192] All blood samples were processed to PBMC and serum within 16 hours of collection. Total PBMC RNA was extracted using the RNeasy Mini RNA purification kit with on-column DNase digestion (Qiagen). Total PBMC protein extracts were prepared as previously described. Sera, RNA and cell lysates were stored at -80°C.

RELB and RELA DNA binding assays

[0193] Protein concentration was measured using the Bradford assay (Biorad Laboratories). NF-κB DNA binding was measured using a chemiluminescent ELISA-based 96 well plate⁴⁶, adding 10 µg whole cell extract per well. NF-κB family members were detected with rabbit antibodies against RELA (sc-372) and RELB (sc-226) (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit HRP (1:30,000, Pierce). To standardize readings between runs, measurements were normalized to the average value (in photon units) of healthy control samples included in each run (n=7-12).

Serum assays

[0194] Sera were further tested in a high-throughput multiplex assay for a select panel of 130 analytes (see Table 5, below) detectable post-thaw in human serum samples stored at -80°C with Beadlyte technology (MPXHCTYO-60K; Millipore) using the Bio-Plex Luminex-100 Station (Bio-Rad) as described previously. Human bead mates determined the levels of several cytokines with the Human Multi-Cytokine Flex Kit using detection protocol B (Upstate) and duplicate standards. All markers were analyzed in triplicate from a $600\mu\text{l}$ sample. Bio-Plex Manager 4.1 software with a five-parameter logistic curve-fitting algorithm applied for standard curve calculations, determined cytokine concentrations.

Table 5. Serum analytes measured in this study

Luminex Panels	Analytes tested
Human Panel I	EGF, CCL11, FGF2, FLT3L, CX3CL1, GCSF, GMCSF, CXCL1, IL12B, IL15, IL6, IL7, IL8, CXCL10, CCL3, CCL4, VEGF, sCD40L, PDGFA, PDGFB
Human Cytokine Panel II	CCL21, CXCL13, CCL27, CXCL5, CCL24, CCL26, CCL1, IL16, IL20, IL21, IL23, IL28A, IL33, LIF, CCL8, CCL13, CCL15, SCF, CXCL12, CCL17, TPO, TRAIL, TSLP
Human Cytokine Panel III	GCP2, HCC1, CXCL11, IL11, IL29, XCL1, MCSF, CXCL9, CCL20, CCL19, NAP2
Human cytokine receptor	sCD30, sEGFR, sIL1RI, sIL1RII, sIL2Ra, sIL4R, sIL6R, sRAGE, sTNFR1, sTNFR2, sVEGFR1, sVEGFR2, sVEGFR3, sGP130
Human Cardiovascular Panel 2	CRP, SAP, SAA
Human Apolipoproteins	APOAI, APOAII, APOB, APOCII, APOCIII, APOE
Human Circulating Cancer Biomarker Panel 2	SERPINC1, F13A1, CFH, (ECM1), GC (VITDBP), VTN
Human Liver Protein	AFP, ANGPTL3, ANGPTL4, ANGPTL6, FABP1, FGF19, FGF21, FGF23, HGF
Human Neurodegenerative Disease Panel 4	S100B, A β 1-40, A β 1-42, GDVF, sRAGE
TGF β 3-Plex	TGF β 1, TGF β 2, TGF β 3

Quantitative PCR assays

[0195] RNA concentration and purity were measured using a Nanodrop (Thermo Scientific). Oligo-dT-primed cDNA was synthesised from 1 µg total PBMC RNA (Quantace). Quantitative RT-PCR was performed using Sensimix SYBR mastermix (Quantace) on an ABI HT7900 cyclor in 384 well plates. Primer sequences used in this study were as follows: IL12A (forward primer): GCTCCAGAAGGCCAGACAAA (SEQ ID NO:1), IL12A (reverse primer): GCCTCCACTGTGCTGGTTTT (SEQ ID NO:2), GRP78 (forward primer): AACACAGTGGTGCCTACCAAGAA (SEQ ID NO:3), GRP78 (reverse primer): TTTGTCAGGGGTCTTTCACCTT (SEQ ID NO:4), TNF (forward primer): CCTGTAGCCCATGTTGTAGCAAAC (SEQ ID NO:5), TNF (reverse primer): TCTCTCAGCTCCACGCCATT (SEQ ID NO:6). Expression of each transcript was determined relative to the HPRT housekeeping gene using the ddct method, with normalisation to a calibrator sample run on each plate.

Statistical and cluster analysis

[0196] Basic statistics for the study parameters are presented as number (percentage) or mean (SD) or median (IQR). The non-parametric Feature Selection procedure, followed by logistic regression modelling, was used to identify biomarkers differentially expressed between 1) HC and AB- FDR, and 2) FDR with single or multiple AB. The degree of relationship between variables was evaluated using the non-parametric (Spearman's) rank correlation in R and the variables that were significantly correlated with biomarkers of interest ($p < 0.05$, Spearman's $\rho > 0.2$) were selected. Data for each variable were normalised to the median value for that variable across all samples so that all variables could be visualised on the same scale. Variables with data missing from more than 50% of subjects were omitted. Hierarchical clustering of subjects and variables was performed using Genespring (Agilent Technologies) using Spearman's rank correlation to assign inter-variable relationships and the average distance between pairwise clusters to build the final hierarchical cluster (average linkage). Significance is indicated as $*P < 0.05$, $**P < 0.005$ and $***P < 0.001$. All error bars represent median and inter-quartile range.

[0197] This application claims priority to Australian Provisional Application No. 2013904799 entitled "Kits and methods for the diagnosis, treatment, prevention and monitoring of diabetes" filed 10 December 2013, the contents of which are incorporated herein by reference in their entirety.

[0198] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[0199] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

[0200] Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appended claims.

BIBLIOGRAPHY

1. Aly, T.A. et al. Extreme genetic risk for type 1A diabetes. *Proc Natl Acad Sci U S A* **103**, 14074-9 (2006).
2. LaGasse, J.M. et al. Successful prospective prediction of type 1 diabetes in schoolchildren through multiple defined autoantibodies: an 8-year follow-up of the Washington State Diabetes Prediction Study. *Diabetes Care* **25**, 505-11 (2002).
3. Orban, T. et al. Pancreatic islet autoantibodies as predictors of type 1 diabetes in the Diabetes Prevention Trial-Type 1. *Diabetes Care* **32**, 2269-74 (2009).
4. Xu, P. et al. Role of insulin resistance in predicting progression to type 1 diabetes. *Diabetes Care* **30**, 2314-20 (2007).
5. Furlanos, S., Narendran, P., Byrnes, G.B., Colman, P.G. & Harrison, L.C. Insulin resistance is a risk factor for progression to type 1 diabetes. *Diabetologia* **47**, 1661-7 (2004).
6. Hamalainen, A.M. and Knip, M. Autoimmunity and familial risk of type 1 diabetes. *Curr Diab Rep* **2**, 347-53 (2002).
7. Atkinson, M.A. The pathogenesis and natural history of type 1 diabetes. *Cold Spring Harb Perspect Med* **2** (2012).
8. Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet* **355**, 873-6 (2000).
9. Furlanos, S. et al. The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes. *Diabetes Care* **31**, 1546-9 (2008).
10. Gillespie, K.M. et al. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. *Lancet* **364**, 1699-700 (2004).
11. Oresic, M. et al. Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. *J Exp Med* **205**, 2975-84 (2008).
12. Devaraj, S. et al. Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. *Diabetes* **55**, 774-9 (2006).
13. Galgani, M. et al. Meta-Immunological Profiling of Children With Type 1 Diabetes Identifies New biomarkers to Monitor Disease Progression. *Diabetes* **62**, 2481-2491 (2013).

14. Shih, V.F. et al. Control of RelB during dendritic cell activation integrates canonical and noncanonical NF-kappaB pathways. *Nat Immunol* **13**, 1162-70 (2012).
15. O'Sullivan, B.J. & Thomas, R. CD40 Ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-kappaB. *J Immunol* **168**, 5491-5498 (2002).
16. Hoffmann, A. & Baltimore, D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev* **210**, 171-86 (2006).
17. Mollah, Z.U.A. et al. Abnormal NF-kappa B function characterizes human type 1 diabetes dendritic cells and monocytes. *J Immunol* **180**, 3166-3175 (2008).
18. Irvine, K.M. et al. Peripheral Blood Monocyte Gene Expression Profile Clinically Stratifies Patients With Recent-Onset Type 1 Diabetes. *Diabetes* **61**, 1281-1290 (2012).
19. Unek, I.T. et al. Enhanced levels of soluble CD40 ligand and C-reactive protein in a total of 312 patients with metabolic syndrome. *Metabolism* **59**, 305-13 (2010).
20. Scheid, M. and Sweeney, G. The role of adiponectin signaling in metabolic syndrome and cancer. *Reviews in Endocrine and Metabolic Disorders*, 1-11 (2013).
21. Ascaso, J.F. et al. Diagnosing insulin resistance by simple quantitative methods in subjects with normal glucose metabolism. *Diabetes Care* **26**, 3320-5 (2003).
22. Festa, A., D'Agostino, R., Tracy, R.P. & Haffner, S.M. Elevated Levels of Acute-Phase Proteins and Plasminogen Activator Inhibitor-1 Predict the Development of Type 2 Diabetes: The Insulin Resistance Atherosclerosis Study. *Diabetes* **51**, 1131-1137 (2002).
23. Heinig, M. et al. A trans-acting locus regulates an anti-viral expression network and type 1 diabetes risk. *Nature* **467**, 460-4 (2010).
24. Yeung, W.C., Rawlinson, W.D. and Craig, M.E. Enterovirus infection and type 1 diabetes mellitus: systemic review and meta-analysis of observational molecular studies. *BMJ* **342**, d35 (2011).
25. Straub, R.H., Cutolo, M., Buttgereit, F. and Pongratz, G. Energy regulation and neuroendocrine-immune control in chronic inflammatory diseases. *J Intern Med* **267**, 543-60 (2010).
26. Gisslinger, H. et al. Interferon-alpha stimulates the hypothalamic-pituitary-adrenal axis in vivo and in vitro. *Neuroendocrinology* **57**, 489-95 (1993).

27. Kugler, D.G., Mittelstadt, P.R., Ashwell, J.D., Sher, A. and Jankovic, D. CD4+ T cells are trigger and target of the glucocorticoid response that prevents lethal immunopathology in toxoplasma infection. *J Exp Med* **210**, 1919-27 (2013).
28. Turcot, V. et al. Thymic stromal lymphopoietin: an immune cytokine gene associated with the metabolic syndrome and blood pressure in severe obesity. *Clin Sci (Lond)* **123**, 99-109 (2012).
29. Miller, A.M. and Liew, FLY. The IL-33/ST2 pathway – A new therapeutic target in cardiovascular disease. *Pharmacol Ther* **131**, 179-86 (2011).
30. Quinn, L.S. and Anderson, B.G. Interleukin-15, IL-15 Receptor-Alpha, and Obesity: Concordance of Laboratory Animal and Human Genetic Studies. *J Obes* **2011**, 456347 (2011).
31. Mallone, R. et al. Functional avidity directs T-cell fate in autoreactive CD4+ T cells. *Blood* **106**, 2798-805 (2005).
32. Standifer, N.E., Burwell, E.A., Gersuk, V.H., Greenbaum, C.J. & Nepom, G.T. Changes in autoreactive T cell avidity during type 1 diabetes development. *Clin Immunol* **132**, 312-20 (2009).
33. Pettit, A.R. et al. Nuclear localization of RelB is associated with effective antigen-presenting cell function. *J Immunol* **159**, 3681-3691 (1997).
34. Platzner, B., Jorgl, A., Taschner, S., Hocher, B. & Strobl, H. RelB regulates human dendritic cell subset development by promoting monocyte intermediates. *Blood* **104**, 3655-63 (2004).
35. Gringhuis, S.I. et al. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat Immunol* **10**, 203-13 (2009).
36. Villalta, S.A. et al. Inhibition of VEGFR-2 reverses type 1 diabetes in NOD mice by abrogating insulinitis and restoring islet function. *Diabetes* **62**, 2870-8 (2013).
37. Louvet, C. et al. Tyrosine kinase inhibitors reverse type 1 diabetes in nonobese diabetic mice. *Proc Natl Acad Sci USA* **105**, 18895-900 (2008).
38. Poligone, B., Weaver, D.J., Jr., Sen, P., Baldwin, A.S., Jr. & Tisch, R. Elevated NF-kappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* **168**, 188-96. (2002).
39. Dahlen, E., Dawe, K., Ohlsson, L. & Hedlund, G. Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. *J Immunol* **160**, 3585-93 (1998).

40. Uno, S. et al. Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes. *Diabetologia* **50**, 596-601 (2007).
41. Chen, X. et al. RelB sustains IkappaBalpha expression during endotoxin tolerance. *Clin Vaccine Immunol* **16**, 104-10 (2009).
42. Martin, E., O'Sullivan, B., Low, P. & Thomas, R. Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* **18**, 155-67 (2003).
43. Liu, T.F., Yoza, B.K., El Gazzar, M., Vachharajani, V.T. & McCall, C.E. NAD⁺-dependent SIRT1 deacetylase participates in epigenetic reprogramming during endotoxin tolerance. *J Biol Chem* **286**, 9856-64 (2011).
44. Vuckovic, S. et al. Decreased blood dendritic cell counts in type 1 diabetic children. *Clin Immunol* **123**, 281-8 (2007).
45. Segura, E. et al. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* **38**, 336-48 (2013).
46. Scholin, A. et al. Proinsulin/C-peptide ratio, glucagon and remission in new-onset Type 1 diabetes mellitus in young adults. *Diabet Med* **28**, 156-61 (2011).
47. Kaas, A. et al. Proinsulin, GLP-1, and glucagon are associated with partial remission in children and adolescents with newly diagnosed type 1 diabetes. *Pediatr Diabetes* **13**, 51-8 (2012).
48. Kaufmann, J., Kielstein, V., Kilian, S., Stein, G. and Hein, G. Relation between body mass index and radiological progression in patients with rheumatoid arthritis. *J Rheumatol* **30**, 2350-5 (2003).
48. Kaufmann, J., Kielstein, V., Kilian, S., Stein, G. and Hein, G. Relation between body mass index and radiological progression in patients with rheumatoid arthritis. *J Rheumatol* **30**, 2350-5 (2003).
49. Scheuing, N. et al. Is there a benefit to use calculated percent body fat or age- and gender-adjusted BMI-SDS(LMS) to predict risk factors for cardiovascular disease? A German/Austrian multicenter DPV-Wiss analysis on 42 048 type 2 diabetic patients. *Exp Clin Endocrinol Diabetes* **121**, 67-74 (2013).
50. Schaan, B.D., Portal, V.L., de Ugarte, M.T., Dias, A.A. & Hatem, D.M. Emerging risk factors and early atherosclerosis indices in subjects with impaired glucose tolerance. *Diabetes Metab* **31**, 581-7 (2005).
51. Bhattacharya, N. et al. High-throughput detection of nuclear factor-kappaB activity using a sensitive oligo-based chemiluminescent enzyme-linked immunosorbent assay. *Int J Cancer* **127**, 404-11 (2010).

52. Edupuganti, S. et al. A randomized, double-blind, controlled trial of the 17D yellow fever virus vaccine given in combination with immune globulin or placebo: comparative viremia and immunogenicity. *Am J Trop Med Hyg* **88**, 172-7 (2013).
53. Saeys, Y., Inza, I. & Larranaga, P. A review of feature selection techniques in bioinformatics. *Bioinformatics* **23**, 2507-17 (2007).

WHAT IS CLAIMED IS:

1. A method for determining whether a subject is at risk of developing Type 1 diabetes (T1D), the method comprising: (1) correlating a reference biomarker profile with the risk of development of T1D, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of interleukin 12B (IL12B), platelet-derived growth factor BB (PDGFBB), adiponectin, neutrophil-activating protein-2 (NAP2) and Angiopoietin-like 4 (ANGPTL4), Monocyte chemotactic protein 2 (MCP-2; Chemokine (C-C motif) ligand 2 (CCL2)), fractalkine, vascular endothelial cell growth factor receptor 1 (VEGFR1) and serum amyloid P (SAP); (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) determining whether the subject is at risk of developing T1D based on the sample biomarker profile and the reference biomarker profile.
2. The method of Claim 1, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.
3. The method of Claim 2 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and determining whether the subject is at risk of developing T1D based on that ratio.
4. The method of Claim 3, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.
5. The method of any one of Claims 1 to 4, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting VEGFR1 and SAP.
6. The method of Claim 5, wherein the reference biomarker profile evaluates VEGFR1.
7. The method of Claim 6, wherein the VEGFR1 is soluble VEGFR1 (sVEGFR1).

8. The method of Claim 5, wherein the reference biomarker profile evaluates SAP.
9. The method of Claim 6 or Claim 7, wherein the subject is determined to be at risk of developing T1D where the at least one biomarker in the sample biomarker profile for the subject is downregulated or under-expressed as compared to a corresponding biomarker in a healthy subject.
10. The method of Claim 8, wherein the subject is determined to be at risk of developing T1D where the at least one biomarker in the sample biomarker profile for the subject is upregulated or over-expressed as compared to a corresponding biomarker(s) in a healthy subject.
11. A method of monitoring the efficacy of a treatment regimen in a subject at risk of developing T1D, the method comprising: (1) providing a correlation of a reference biomarker profile with a likelihood of having a healthy condition, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; (2) obtaining a corresponding biomarker profile of a subject at risk of developing T1D after commencement of a treatment regimen, wherein a similarity of the subject's biomarker profile after commencement of the treatment regimen to the reference biomarker profile indicates the likelihood that the treatment regimen is effective for changing the health status of the subject.
12. The method of Claim 11, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.
13. The method of Claim 12 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and determining the likelihood that the treatment regimen is effective for changing the health status of the subject based on that ratio.
14. The method of Claim 13, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

15. A method of correlating a reference biomarker profile with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine, the method comprising: (1) determining a sample biomarker profile from a subject at risk of developing T1D prior to commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (2) correlating the sample biomarker profile with a treatment regimen that is effective for preventing or delaying the onset of T1D, or a symptom thereof.

16. The method of Claim 15, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.

17. The method of Claim 16 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and correlating the sample biomarker profile with a treatment regimen that is effective for preventing or delaying the onset of T1D, or a symptom thereof, based on that ratio.

18. The method of Claim 17, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

19. A method of determining whether a treatment regimen is effective for preventing or delaying the onset of T1D or a symptom thereof in a subject at risk of developing T1D, the method comprising: (1) correlating a reference biomarker profile prior to treatment with an effective treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) obtaining a sample biomarker profile from the subject after commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker,

and wherein the sample biomarker profile after commencement of treatment, when compared to the reference biomarker profile, indicates whether the treatment regimen is effective for preventing or delaying the onset of T1D, or a symptom thereof, in the subject.

20. The method of Claim 19, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.

21. The method of Claim 20 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and determining whether the treatment regimen is effective for preventing or delaying the onset of T1D, or a symptom thereof, in the subject based on that ratio.

22. The method of Claim 21, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

23. A method of correlating a biomarker profile with a positive or negative response to a treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, the method comprising: (1) obtaining a sample biomarker profile from a subject at risk of developing T1D following commencement of the treatment regimen, wherein the biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; and (2) correlating the sample biomarker profile from the subject with a positive or negative response to the treatment regimen.

24. The method of Claim 23, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.

25. The method of Claim 24 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and correlating the sample biomarker profile from the subject with a positive or negative response to the treatment regimen based on that ratio.

26. The method of Claim 25, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

27. A method of determining a positive or negative response to a treatment regimen by a subject at risk of developing T1D, the method comprising: (a) correlating a reference biomarker profile with a positive or negative response to the treatment regimen for preventing or delaying the onset of T1D, or a symptom thereof, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting of SAP, VEGFR1, IL12B, PDGFBB, adiponectin, NAP2, ANGPTL4, MCP-2 and fractalkine; (b) determining a sample biomarker profile from the subject following commencement of the treatment regimen, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (c) determining a positive or negative response to the treatment regimen based on a comparison of the sample biomarker profile and the reference biomarker profile.

28. The method of Claim 27 further comprising: determining a first sample biomarker profile from the subject prior to commencing the treatment regimen, wherein the first sample biomarker profile evaluates the at least one biomarker; and comparing the first sample biomarker profile with a second sample biomarker profile from the subject determined after commencement of the treatment regimen, wherein the second sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker.

29. The method of Claim 27 or Claim 28, wherein evaluation of the at least one biomarker comprises determining the level of the at least one biomarker.

30. The method of Claim 29 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and determining a positive or negative response to the treatment regimen based on that ratio.

31. The method of Claim 30, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample

biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

32. The method of any one of Claims 11 to 31, wherein the reference biomarker profile evaluates at least one biomarker selected from the group consisting VEGFR1 and SAP.

33. The method of Claim 32, wherein the reference biomarker profile evaluates VEGFR1.

34. The method of Claim 33, wherein the VEGFR1 is soluble VEGFR1 (sVEGFR1).

35. The method of Claim 32, wherein the reference biomarker profile evaluates SAP.

36. The method of any one of Claims 1 to 33 wherein the reference biomarker profile further evaluates at least one other biomarker selected from the group consisting of RELB DNA binding, tumor necrosis factor (TNF), interleukin 12A (IL-12A), 78kDa glucose-related protein (GRP78), CD14^{LOW}CD16⁻ cells and CD14^{HIGH}CD16⁺ cells and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

37. The method of any one of Claims 1 to 32, wherein the subject is considered at risk of developing T1D where the at least one biomarker in the sample biomarker profile for the subject is upregulated as compared to the same biomarker(s) in a healthy subject.

38. The method of any one of Claims 1 to 37, wherein the subject is an islet antibody negative subject.

39. A method for preventing or delaying the onset of T1D or a symptom thereof in a subject, the method comprising:

- (a) determining whether a subject is at risk of developing T1D in accordance with the method of any one of Claims 1 to 10; and

(b) exposing the subject determined as being at risk of developing T1D, to a treatment regimen for preventing or delaying the onset of T1D or a symptom thereof.

40. A method of stratifying a subject at risk of developing T1D to an anti-inflammatory treatment regimen, the method comprising (1) correlating a reference biomarker profile with an inflammatory phenotype, wherein the reference biomarker profile evaluates at least one inflammatory biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having an inflammatory phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to an anti-inflammatory treatment regimen.

41. The method of Claim 40, wherein the subject is an islet autoantibody positive subject.

42. The method of Claim 41, wherein the islet autoantibody positive subject has circulating islet autoantibodies that specifically bind to at least one islet antigen selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

43. The method of Claim 41, wherein the islet autoantibody positive subject has circulating islet autoantibodies that specifically bind to one islet antigen selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

44. The method of Claim 41, wherein the subject has circulating islet autoantibodies that specifically bind to two islet antigens selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

45. The method of Claim 41, wherein the subject has circulating islet autoantibodies that specifically bind to glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

46. The method of any one of Claims 41 to 45, wherein the at least one inflammatory biomarker is selected from the group consisting listed in Figure 3 or Figure 4.

47. The method of any one of Claims 41 to 45, wherein the at least one inflammatory biomarker is selected from the group consisting of IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, thymic stromal lymphopoietin (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF-2), fibroblast growth factor 21 (FGF-21), GLP-1, parathyroid hormone (PTH), glucagon, adiponectin, Adrenocorticotrophic hormone (ACTH), amylin (islet amyloid polypeptide precursor; IAPP) and Chemokine (C motif) ligand (XCL1), soluble CD30 (sCD30), soluble interleukin 6 receptor (sIL-6R), stem cell factor (SCF), eotaxin-2, macrophage inflammatory protein 1d (MIP1d), Apolipoprotein A-1 (APOA1), peptide tyrosine tyrosine (peptide YY; PYY), and osteopontin (OPN).

48. The method of Claim 47, wherein the reference biomarker profile is correlated with an inflammatory phenotype where the at least one inflammatory biomarker is higher than a level that is representative of a mean or median level a corresponding biomarker in a population of subjects at risk of developing T1D.

49. The method of any one of Claims 41 to 48, further comprising correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker and wherein the sample biomarker profile further evaluates, for the at least one metabolic biomarker in the reference biomarker profile, a corresponding biomarker.

50. The method of Claim 49, wherein the at least one metabolic biomarker is selected from the group listed in Figure 3 or Figure 4.

51. The method of Claim 49 or Claim 50, wherein the at least one metabolic biomarker is selected from the group consisting of insulin resistance, age-adjusted BMI, a measure of glucose tolerance, insulin, proinsulin, proinsulin/insulin ratio, c-peptide, serum amyloid P (SAP), leptin, complement factor H (CFH), anti-thrombin III, sIL-1RII, PDFBB, transforming growth factor beta 1 (TGF β 1), chemokine (C motif) ligands, serum amyloid A (SAA), Granulocyte Chemotactic Protein 2 (GCP2), vascular endothelial growth factor (VEGF) and Gastric inhibitory polypeptide (GIP).

52. The method of any one of Claims 49 to 51, wherein the reference biomarker profile is correlated with a metabolic phenotype where the level of the at least one metabolic biomarker is higher than a level that is representative of a mean or median level of the same biomarker in a population of subjects at risk of developing T1D.

53. The method of any one of Claims 40 to 52, wherein the reference biomarker profile is correlated with a metabolic phenotype where the level of the at least one inflammatory biomarker is lower than a level that is representative of a mean or median level of the same biomarker in a population of subjects considered at risk of developing T1D.

54. The method of Claim 53, wherein the at least one inflammatory biomarker is selected from the group consisting of IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, thymic stromal lymphopoietin (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF-2), GLP-1, parathyroid hormone (PTH), glucagon, adiponectin, Adrenocorticotrophic hormone (ACTH), amylin (islet amyloid polypeptide precursor; IAPP) and Chemokine (C motif) ligand (XCL1), soluble CD30 (sCD30), soluble interleukin 6 receptor (sIL-6R), stem cell factor (SCF), eotaxin-2, macrophage inflammatory protein 1d (MIP1d), Apolipoprotein A-1 (APOA1), peptide tyrosine tyrosine (peptide YY; PYY), and osteopontin (OPN).

55. The method of Claim 53, wherein the at least one inflammatory biomarker is selected from the group consisting of XCL1, TSLP, ACTH, IL-33, IL-23, IL-28A and IL-6.

56. The method of any one of Claims 41 to 55, wherein the reference biomarker profile further evaluates at least one other biomarker selected from the group consisting of IL20, PYY, RELB DNA binding, IL-12A, TNF, GRP78, VEGFR1, CD14^{LOW}CD16⁻ cells, CD14^{HIGH}CD16⁺ cells and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

57. The method of Claim 56, wherein evaluation of the at least one biomarker and/or the at least one other biomarker comprises determining a level of the at least one biomarker and/or a level of the at least one other biomarker.

58. The method of Claim 57 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and stratifying the subject to the anti-inflammatory treatment regimen based on the ratio.

59. The method of Claim 58, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

60. The method of Claim 40, wherein the subject is a islet autoantibody negative subject.

61. The method of Claim 60, wherein the at least one inflammatory biomarker is selected from the group consisting of IL-12B, *TNF*, *IL12A*, PDGFB.

62. The method of Claim 60 or Claim 61, further comprising correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker and wherein the sample biomarker profile further evaluates, for the at least one metabolic biomarker in the reference biomarker profile, a corresponding biomarker.

63. The method of Claims 62, wherein the at least one metabolic biomarker is adiponectin.

64. The method of any one of Claims 60 to 63, wherein evaluation of the at least one biomarker comprises determining a level of the at least one biomarker.

65. The method of any one of Claims 60 to 64, wherein the subject is determined as having an inflammatory phenotype where the least one inflammatory biomarker in the sample biomarker profile is expressed at a level that is higher than a level that is representative of a mean or median level of expression of a corresponding biomarker in a population of subjects considered at risk of developing T1D.

66. The method of any one of Claims 60 to 65 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second

biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and stratifying the subject to the anti-inflammatory treatment regimen based on the ratio.

67. The method of Claim 66, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

68. The method of any one of Claims 11 to 67, wherein the subject is determined to be at risk of developing T1D in accordance with the method of any one of Claims 1 to 10.

69. A method of stratifying a subject at risk of developing T1D to a metabolic phenotype-targeted treatment regimen, the method comprising (1) correlating a reference biomarker profile with a metabolic phenotype, wherein the reference biomarker profile evaluates at least one metabolic biomarker; (2) obtaining a sample biomarker profile from the subject, wherein the sample biomarker profile evaluates, for an individual biomarker in the reference biomarker profile, a corresponding biomarker; and (3) stratifying the subject determined as having a metabolic phenotype based on the sample biomarker profile and the reference biomarker profile, to thereby stratify the subject to a metabolic phenotype-targeted treatment regimen.

70. The method of Claim 69, wherein the subject is an islet autoantibody positive subject.

71. The method of Claim 70, wherein the islet autoantibody positive subject has circulating islet autoantibodies that specifically bind to at least one islet antigen selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

72. The method of Claim 70, wherein the islet autoantibody positive subject has circulating islet autoantibodies that specifically bind to one islet antigen selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

73. The method of Claim 70, wherein the subject has circulating islet autoantibodies that specifically bind to two islet antigens selected from the group consisting of glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

74. The method of Claim 70, wherein the subject has circulating islet autoantibodies that specifically bind to glutamic acid decarboxylase (GAD), insulin/pro-insulin and islet cell antigen 512 (ICA512/IA-2).

75. The method of any one of Claims 69 to 74, wherein the at least one metabolic biomarker is selected from the group consisting listed in Figure 3 or Figure 4.

76. The method of any one of Claims 69 to 75, wherein the at least one metabolic biomarker is selected from the group consisting of insulin resistance, age-adjusted BMI, a measure of glucose tolerance, insulin, proinsulin, proinsulin/insulin ratio, c-peptide, SAP, leptin, complement factor H (CFH), anti-thrombin III, sIL-1RII, PDFBB, transforming growth factor beta 1 (TGF β 1), chemokine (C motif) ligands, serum amyloid A (SAA), Granulocyte Chemotactic Protein 2 (GCP2), vascular endothelial growth factor (VEGF) and Gastric inhibitory polypeptide (GIP).

77. The method of Claim 76, wherein the reference biomarker profile is correlated with a metabolic phenotype where the at least one metabolic biomarker is higher than a level that is representative of a mean or median level a corresponding biomarker in a population of subjects at risk of developing T1D.

78. The method of any one of Claims 69 to 77, further comprising correlating a reference biomarker profile with an inflammatory phenotype, wherein the reference biomarker profile evaluates at least one inflammatory biomarker and wherein the sample biomarker profile further evaluates, for the at least one inflammatory biomarker in the reference biomarker profile, a corresponding biomarker.

79. The method of Claim 78, wherein the at least one metabolic biomarker is selected from the group listed in Figure 3 or Figure 4.

80. The method of Claim 78, wherein the at least one inflammatory biomarker is selected from the group consisting of IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-

15, eotaxin, thymic stromal lymphopoietin (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF-2), fibroblast growth factor 21 (FGF-21), GLP-1, parathyroid hormone (PTH), glucagon, adiponectin, Adrenocorticotrophic hormone (ACTH), amylin (islet amyloid polypeptide precursor; IAPP) and Chemokine (C motif) ligand (XCL1), soluble CD30 (sCD30), soluble interleukin 6 receptor (sIL-6R), stem cell factor (SCF), eotaxin-2, macrophage inflammatory protein 1d (MIP1d), Apolipoprotein A-1 (APOA1), peptide tyrosine tyrosine (peptide YY; PYY), and osteopontin (OPN).

81. The method of any one of Claims 78 to 80, wherein the reference biomarker profile is correlated with a metabolic phenotype where the level of the at least one inflammatory biomarker is lower than a level that is representative of a mean or median level of the same biomarker in a population of subjects considered at risk of developing T1D.

82. The method of Claim 81, wherein the at least one inflammatory biomarker is selected from the group consisting of IL-28A, IL-33, IL-23, IL-6, IL-11, IL-29, IL-15, eotaxin, thymic stromal lymphopoietin (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF-2), GLP-1, parathyroid hormone (PTH), glucagon, adiponectin, Adrenocorticotrophic hormone (ACTH), amylin (islet amyloid polypeptide precursor; IAPP) and Chemokine (C motif) ligand (XCL1), soluble CD30 (sCD30), soluble interleukin 6 receptor (sIL-6R), stem cell factor (SCF), eotaxin-2, macrophage inflammatory protein 1d (MIP1d), Apolipoprotein A-1 (APOA1), peptide tyrosine tyrosine (peptide YY; PYY), and osteopontin (OPN).

83. The method of Claim 81, wherein the at least one inflammatory biomarker is selected from the group consisting of XCL1, TSLP, ACTH, IL-33, IL-23, IL-28A and IL-6.

84. The method of any one of Claims 78 to 83, wherein the subject is determined as having an inflammatory phenotype where the least one inflammatory biomarker in the sample biomarker profile is expressed at a level that is higher than a level that is representative of a mean or median level of expression of a corresponding biomarker in a population of subjects considered at risk of developing T1D.

85. The method of any one of Claims 69 to 84, wherein the reference biomarker profile further evaluates at least one other biomarker selected from the group consisting of IL20, PYY, RELB DNA binding, IL-12A, TNF, GRP78, VEGFR1, CD14^{LOW}CD16⁻ cells, CD14^{HIGH}CD16⁺ cells and wherein the sample biomarker profile further evaluates, for the at least one other biomarker in the reference biomarker profile, a corresponding biomarker.

86. The method of any one of Claims 69 to 85, wherein evaluation of the at least one biomarker and/or the at least one other biomarker comprises determining a level of the at least one biomarker and/or a level of the at least one other biomarker.

87. The method of any one of Claims 69 to 86 comprising comparing the level of a first biomarker in the sample biomarker profile with the level of a second biomarker in the sample biomarker profile to provide a ratio between the first and second biomarkers and stratifying the subject to the treatment regimen based on the ratio.

88. The method of Claim 87, wherein the determination is carried out in the absence of comparing the level of the first or second biomarkers in the sample biomarker profile to the level of a corresponding biomarker in the reference biomarker profile.

89. The method of any one of Claims 70 to 88, wherein the metabolic phenotype-targeted treatment comprises a strategy for inducing antigen-specific tolerance in the subject.

90. The method of Claim 69, wherein the subject is a islet autoantibody negative subject.

91. The method of Claim 90, wherein the metabolic phenotype-targeted treatment comprises a treatment regimen selected from the group consisting of exercise, caloric intake restriction and metformin.

92. The method of any one of Claims 11 to 91, wherein the subject is determined to be at risk of developing T1D in accordance with the method of any one of Claims 1 to 10.

93. A kit comprising one or more reagents and/or devices for use in performing the method of any one of Claims 1 to 92.

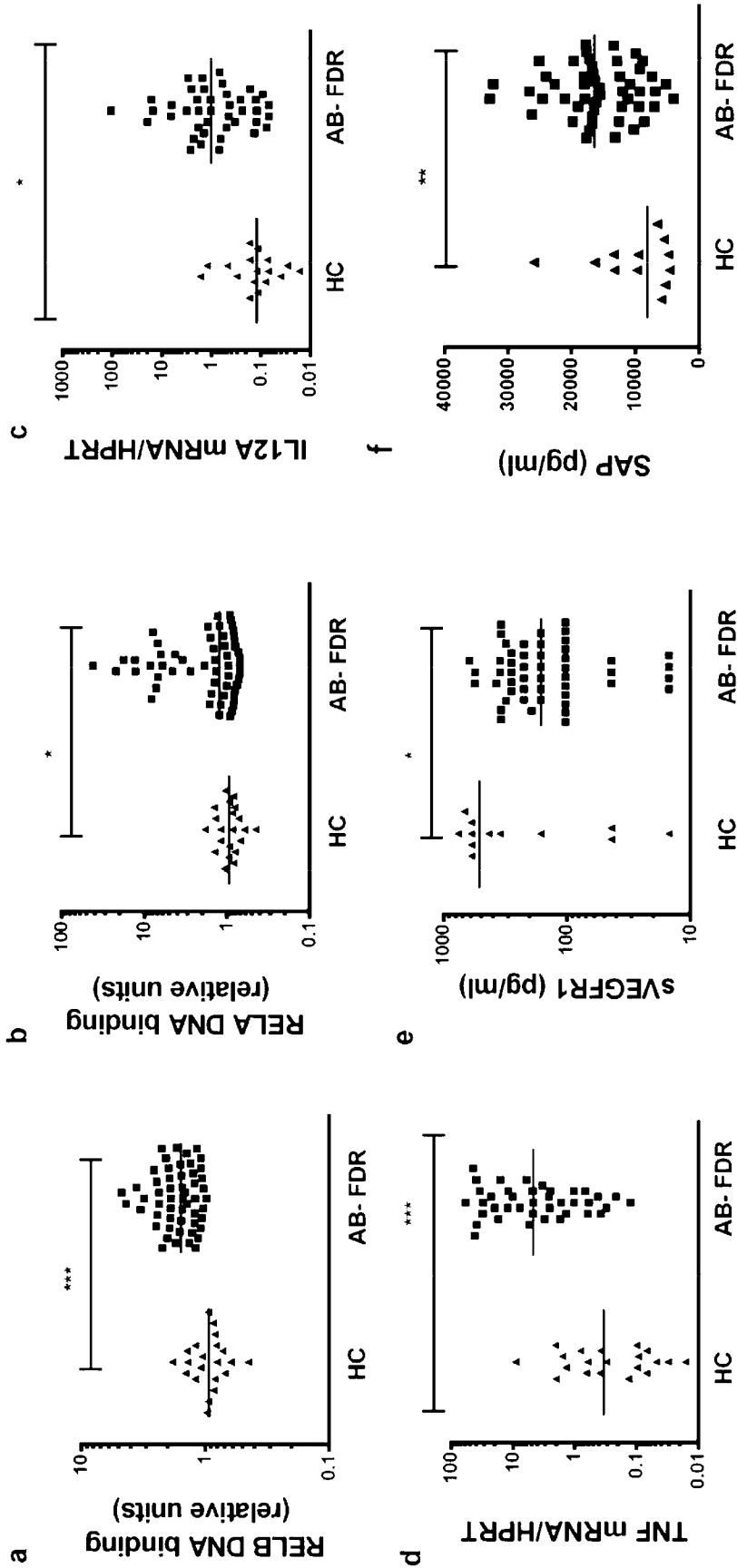


FIGURE 1
Substitute Sheet
(Rule 26) RO/AU

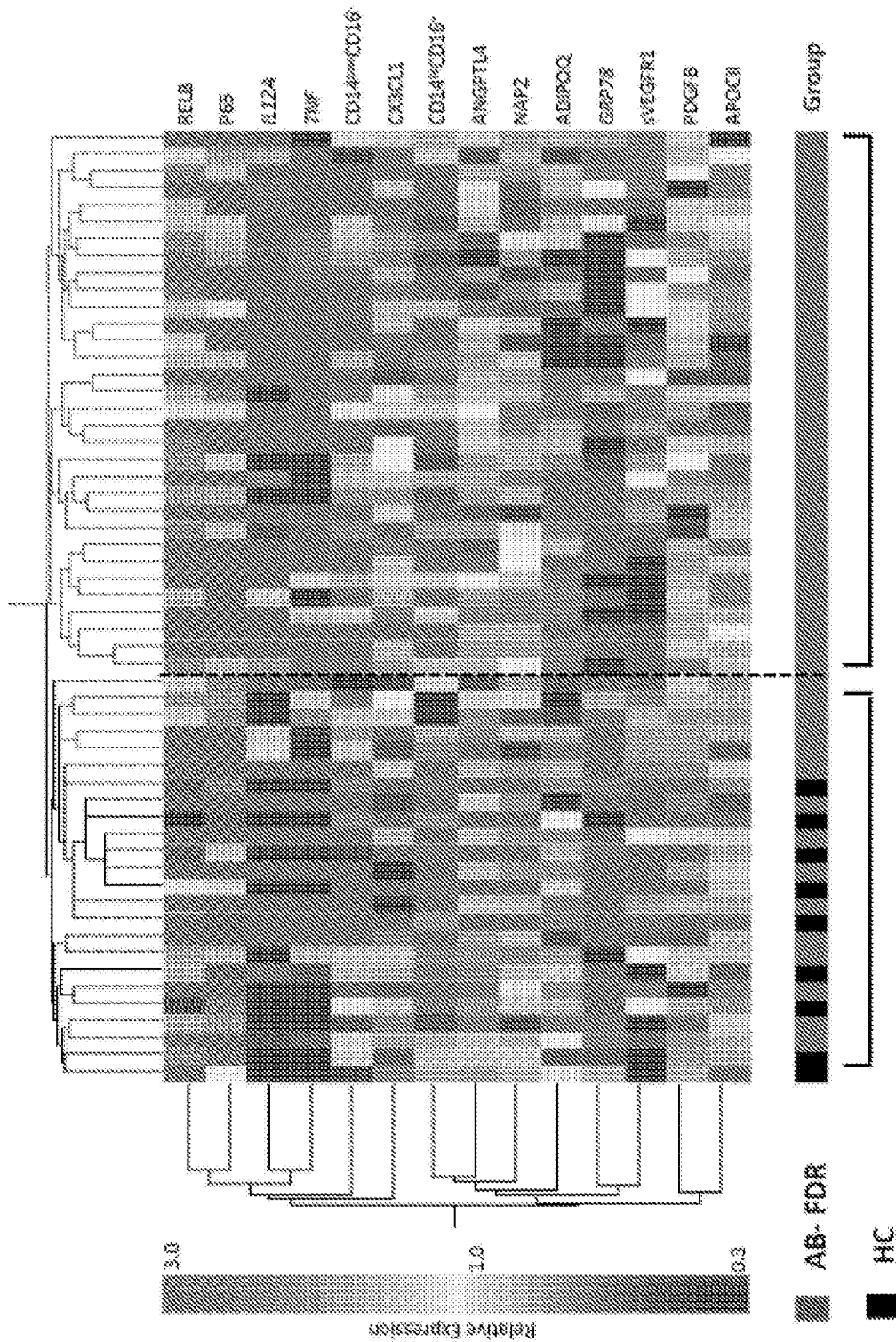


FIGURE 2

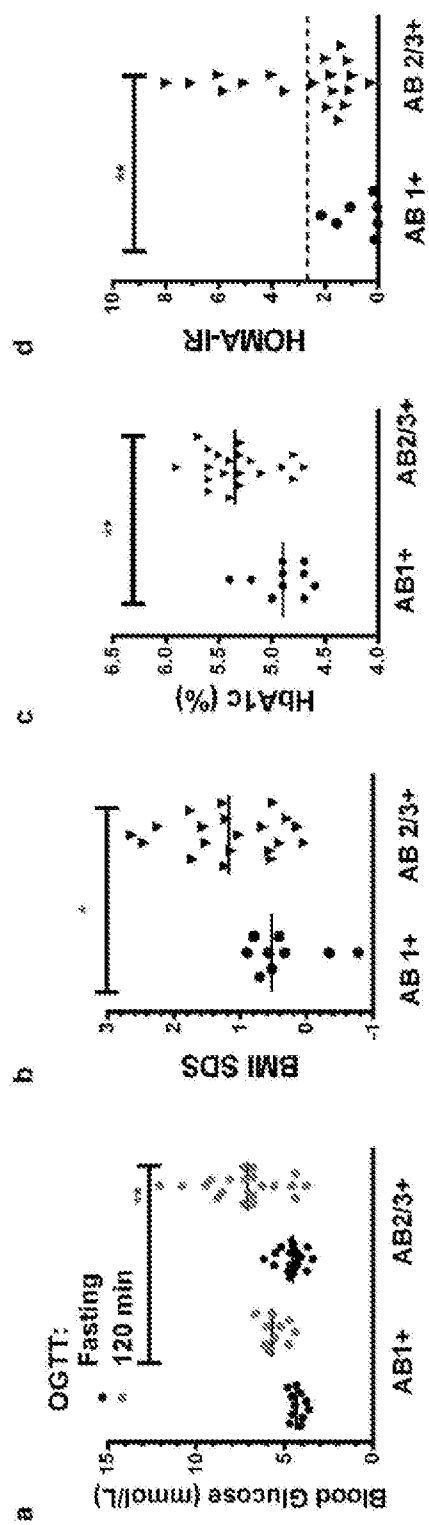


FIGURE 3

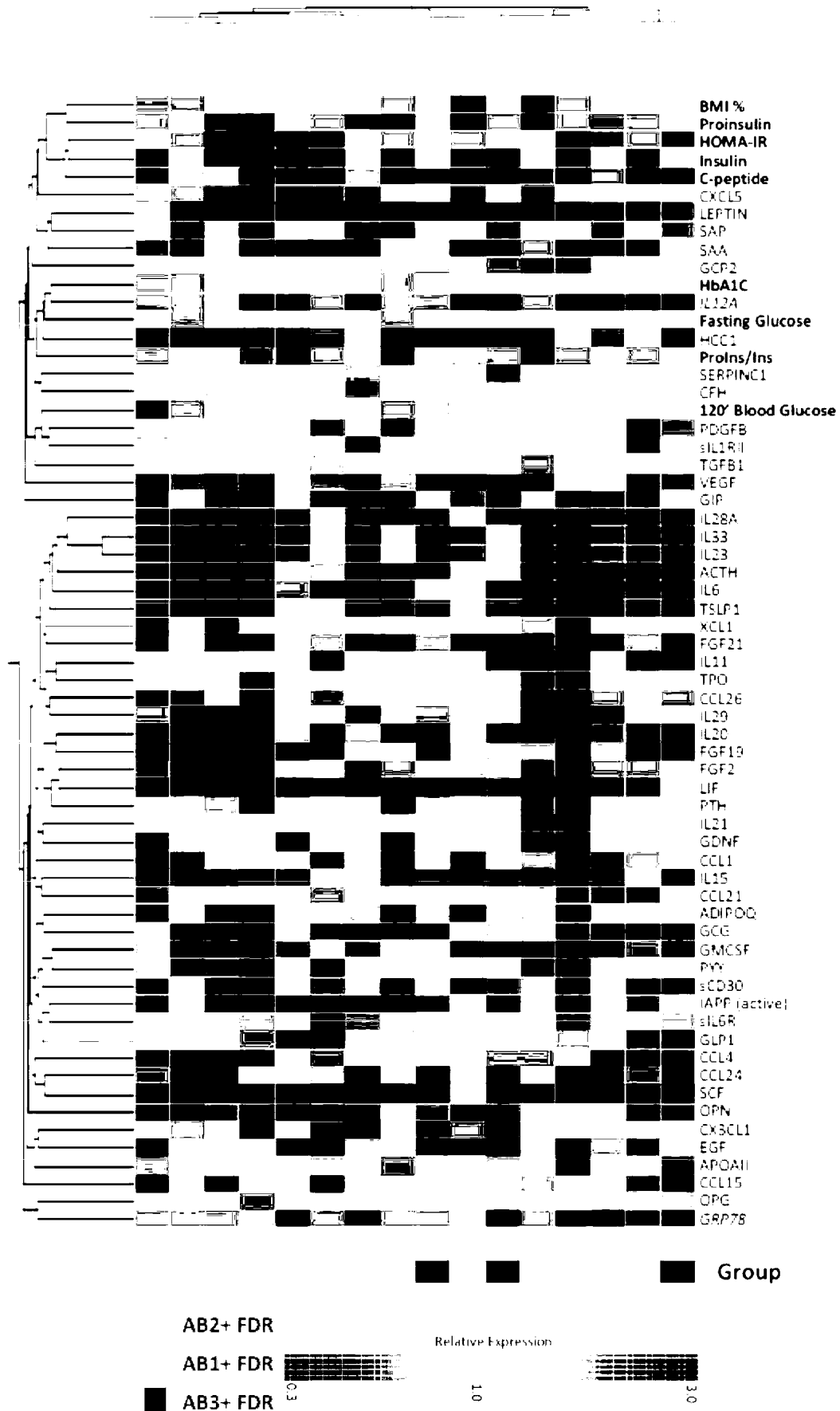
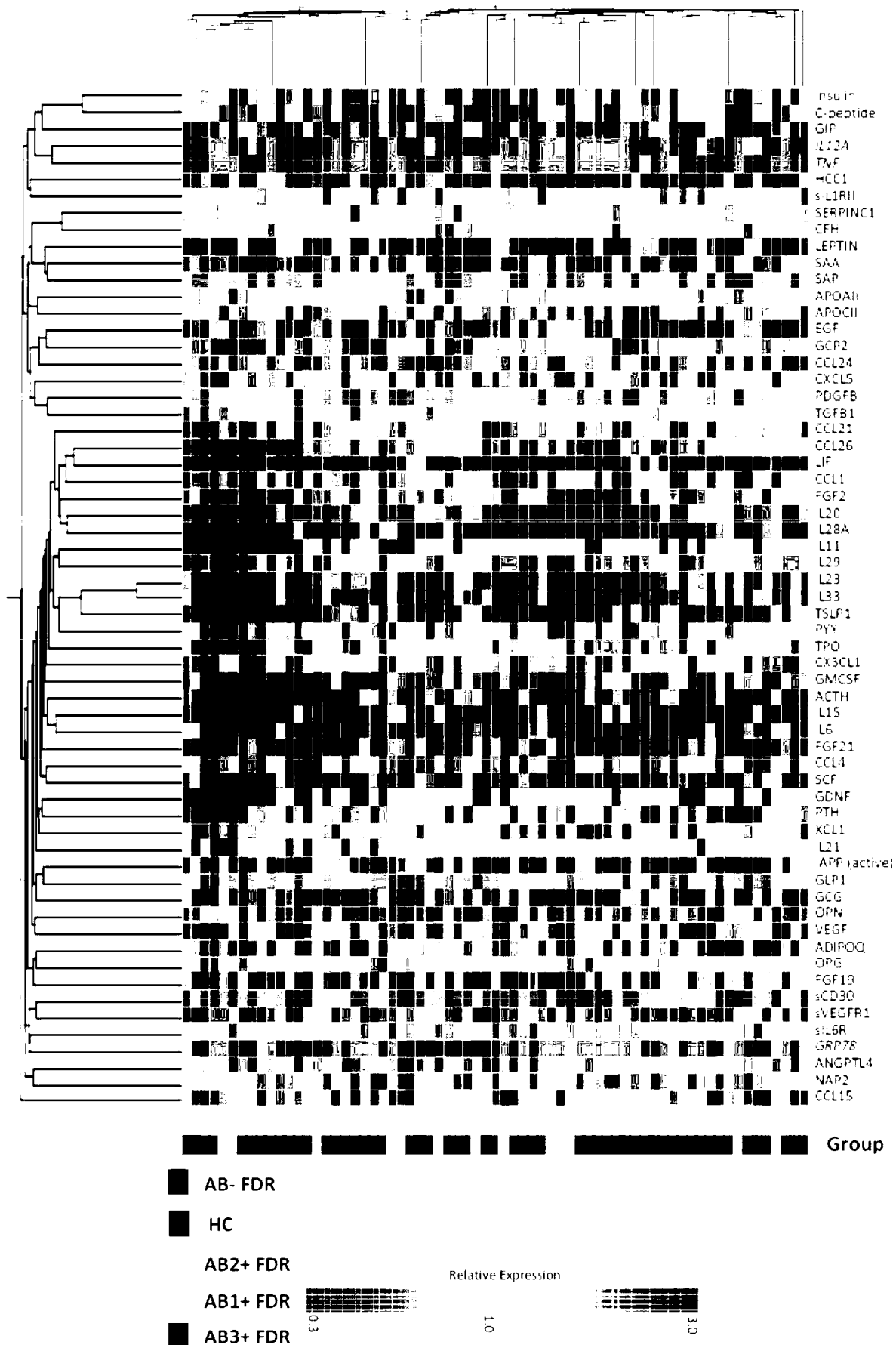


FIGURE 4
Substitute Sheet
(Rule 26) RO/AU

**FIGURE 5**

Substitute Sheet
(Rule 26) RO/AU

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050415

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/50 (2006.01) C12Q 1/68 (2006.01)

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)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: MEDLINE, HCAPLUS, WPIAP, EPODOC; Keywords: type 1 diabetes, risk, first degree relative, predisposed, develop, subset, phenotype, profile, signature, classify, stratify, inflammatory, cytokine, biomarker, metabolic, insulin sensitivity, prevent, prophylactic, treatment and similar terms. Databases: Google Scholar, Patentscope; Keywords: Ranjeny, diabetes

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 23 March 2015		Date of mailing of the international search report 23 March 2015	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Daniel Sheahan AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6283 7969	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2014/050415
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FOURLANOS, S. et al., "Insulin resistance is a risk factor for progression to type 1 diabetes", Diabetologia, 2004, Vol. 47, pages 1661-1667 abstract; figure 1; page 1662, paragraph 6; page 1664, paragraph 2; page 1666, paragraphs 2 and 4; Tables 1 and 2	69-77, 86-88, 90, 91
X	US 2012/0177660 A1 (SHE et al.) 12 July 2012 abstract; [0010]-[0018], [0051], [0057], [0060], [0090], [0094], claims 5, 8, 11-20	40-48, 60, 64, 93
X A	YEUNG, W-C.G. et al., "Children with islet autoimmunity and enterovirus infection demonstrate a distinct cytokine profile", Diabetes, 2012, Vol. 61, pages 1500-1508 page 1501, paragraph 5 page 1507, paragraph 5; Table 2	93 40-67
A	XU, P. et al., "Prognostic performance of metabolic indexes in predicting onset of type 1 diabetes", Diabetes Care, Vol. 33, No. 12, pages 2508-2513 abstract; page 2512, paragraphs 2-5; Table 2	69-91
A	STECHOVA, K. et al., "Healthy first-degree relatives of patients with type 1 diabetes exhibit significant differences in basal gene expression pattern of immunocompetent cells compared to controls: expression pattern as predeterminant of autoimmune diabetes", Scandinavian Journal of Immunology, 2011, Vol. 75, pages 210-219 abstract; page 215, paragraph 2 to page 216, paragraph 8	40-67
A	IRVINE, K.M. et al., "Peripheral blood monocyte gene expression profile clinically stratifies patients with recent-onset type 1 diabetes", Diabetes, 2012, Vol. 61, pages 1281-1290 abstract; page 1282, paragraph 6 to page 1286, paragraph 1; page 1286, paragraph 3; Figures 2, 7	40-67, 69-91
A	VON HERRATH, M. et al., "Progress in immune-based therapies for type 1 diabetes", 2013, Clinical and Experimental Immunology, Vol. 172, pages 186-202 Tables 1 and 2	40-67, 69-91
Form PCT/ISA/210 (fifth sheet) (July 2009)		

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:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
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.:
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:

See Supplemental Box for Details

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.:
40-67, 69-91 (wholly) and 93 (in part)

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/AU2014/050415
Supplemental Box	
Continuation of: Box III This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups: • <u>Invention 1</u>: Claims 1-39, 68, 92 and 93 (all in part) relate to methods for determining whether a subject is at risk of developing Type 1 diabetes (T1D) and methods for monitoring a preventative treatment for T1D wherein the level of interleukin 12B (IL-12B) is evaluated. The feature of IL-12B is specific to this invention. • <u>Inventions 2-9</u>: Claims 1-39, 68, 92 and 93 (all in part) relate to methods for determining whether a subject is at risk of developing T1D and methods for monitoring a preventative treatment for T1D wherein the level of at least one marker selected from platelet-derived growth factor BB (PDGFBB), adiponectin, neutrophil-activating protein-2 (NAP2), angiopoietin-like 4 (ANGPTL4), monocyte chemotactic protein 2 (MCP-2), fractalkine, vascular endothelial cell growth factor receptor 1 (VEGFR1) and serum amyloid P (SAP) is evaluated. Each different marker is specific to a separate invention. • <u>Invention 10</u>: Claims 40-67, 69-91 (wholly) and claims 68, 92 and 93 (in part) relate to methods of stratifying a subject at risk of developing T1D to either an anti-inflammatory treatment regimen based on the evaluation of an inflammatory biomarker or to a metabolic treatment regimen based on the evaluation of a metabolic biomarker. PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art. When there is no special technical feature common to all the claimed inventions there is no unity of invention. In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is that they relate to the prediction and monitoring of T1D development. The only feature common to inventions 1-9 is that they all relate to biomarkers for determining the risk of developing T1D. However neither of these features make a contribution over the prior art because they are disclosed in numerous documents, for example US 2012/0177660 A1. Therefore in light of this document these common features cannot be special technical features. Therefore there is no special technical feature common to all of the claimed inventions and the requirements for unity of invention are consequently not satisfied <i>a posteriori</i>. No additional fees were paid by the applicant, however it was agreed to search the subject matter corresponding to Invention 10 instead of the first invention mentioned in the claims. It is noted that the search and opinion has also been reserved for claims 68 and 92 as they also include subject matter corresponding to inventions 1-9.	
Form PCT/ISA/210 (Supplemental Box) (July 2009)	