(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 November 2016 (10.11.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/176726 Al

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/AU2016/050313

(22) International Filing Date:
29 April 2016 (29.04.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

2015901567	1 May 2015 (01.05.2015)	AU
2015904991	2 December 2015 (02.12.2015)	AU
2016901468	20 April 2016 (20.04.2016)	AU

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(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))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: DIAGNOSTIC METHODS

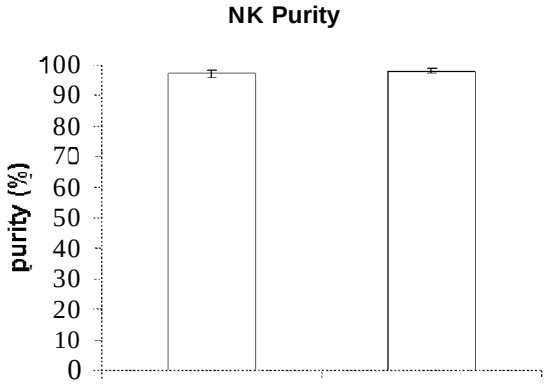

Figure 1

(57) Abstract: In one aspect the invention relates to the use of single nucleotide polymorphisms (SNPs) in transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) genes as probes, tools or reagents for identifying, screening, diagnosing, monitoring or managing/treating subjects with, or predisposed to, medical conditions (or symptoms thereof), such as chronic fatigue syndrome (CFS), myalgic encephalomyelitis (ME), Gulf war syndrome (GWS), irritable bowel syndrome (IBS), multiple chemical sensitivity (MCS), fibromyalgia, and migraine, as well as some medical conditions caused by dysregulation in calcium, acetylcholine, TRP and ADR, and dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems. In another aspect the invention relates to methods, kits and assays for identifying, screening, diagnosing, monitoring or managing/treating subjects with one or more of those medical conditions or symptoms.

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TITLE

Diagnostic Methods

TECHNICAL FIELD

[0001] In some aspects the present invention broadly relates to the use of single nucleotide polymorphisms (SNPs) in transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) genes as probes, tools or reagents for identifying, screening, diagnosing, monitoring or managing/treating subjects with, or predisposed to, medical conditions (or symptoms thereof), such as chronic fatigue syndrome (CFS), myalgic encephalomyelitis (ME), Gulf war syndrome (GWS), irritable bowel syndrome (IBS), multiple chemical sensitivity (MCS), fibromyalgia, and migraine, as well as some medical conditions caused by dysregulation in calcium, acetylcholine, TRP and ADR, and dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems.

[0002] In other aspects the present invention relates to the use of calcium metabolism testing for identifying, screening, diagnosing, monitoring or managing/treating a subject having, or at risk of developing, a medical condition or symptom thereof. This aspect may involve testing any suitable calcium-dependent biochemical process.

[0003] In other aspects the present invention relates to identifying or diagnosing a subject having a medical condition or symptom thereof, by testing cells obtained from the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[0004] In other aspects the present invention relates to the use of one or more differentially regulated calcium-dependent kinase genes for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof.

[0005] Other aspects concern probes, tools or reagents based on, or developed from, the various aspects of the invention described above.

[0006] Yet other aspects relate to methods, kits and assays for identifying, screening, diagnosing, monitoring or managing/treating subjects with one or more of those medical conditions or symptoms.

BACKGROUND ART

[0007] It will be clearly understood that, if a prior art publication is referred to herein, this reference does not constitute an admission that the publication forms part of the common general knowledge in the art in Australia or in any other country.

[0008] Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is known to affect about 1-4% of individuals worldwide [la, 2a]. CFS/ME has an unknown aetiology and there is no specific diagnostic test. Chronic fatigue syndrome (CFS) is an unexplained disorder with multiple physiological impairments. The illness is characterised by significant impairment in physical activity and debilitating fatigue accompanied by impairment in memory, cognition and concentration, enhanced experience of pain as well as dysregulation of the gastrointestinal, cardiovascular and immune systems [14a-31a]. Research to date suggests significant immune impairment. However, the mechanism of this disorder remains to be determined. CFS patients may have reactions to a number of environmental and biological factors [11a-13a]. Moreover, there is evidence to suggest that CFS may have an allergic component [14a-16a].

[0009] Gulf war syndrome (GWS) is a serious condition that affects at least a quarter of the 697,000 US veterans who served in the 1900-1991 Gulf war [le]. GWS comprises a complex of multiple concurrent symptoms, being typified by persistent memory and concentration problems, chronic headaches, wide-spread pain, gastrointestinal problems and other chronic abnormalities, not explained but well established by diagnoses. No effective treatments have been identified for GWS and studies indicate that few veterans recover over time.

[0010] Irritable bowel syndrome (IBS) is characterised by abnormally-increased motility of the small and large intestines of unknown origins. Most patients are young adults who complain of diarrhoea and occasionally pain in the lower abdomen. No organic disease has been identified in IBS to date.

[0011] Multiple chemical sensitivity (MCS) is the most common term used to describe a condition presenting as a complex array of symptoms linked to low level chemical exposures [2e]. The underlying mode(s) of action of MCS, i.e. the biological mechanisms by which the chemical sensitivity occurs, remain uncertain. In terms of sensitivities involving chemicals, the terms "MCS" and "chemical sensitivity" (sometimes known as "chemical intolerance") are often used interchangeably. However, "chemical sensitivity" in its wider context can describe several distinct types of reactions encompassing classical adverse toxicological reactions, immunological "allergic" sensitivities, individual chemical idiosyncrasies and intolerances

through to aversions to particular odours. Broadly, on the basis of Consensus Criteria, MCS is distinguished from other types of chemical sensitivities or intolerances predominantly on the basis of reactions to multiple, diverse chemical substances, the wide spectrum of non-specific symptoms reported in multiple organ systems and the extremely low levels of environmental exposures linked to responses. Symptoms include headache, fatigue, confusion, depression, shortness of breath, arthralgia, myalgia, nausea, dizziness, memory problems, gastrointestinal symptoms and respiratory symptoms. Medical conditions caused by dysregulation in calcium, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia and migraine), are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; respiratory symptoms and immunological "allergic" sensitivities; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; and gastrointestinal symptoms.

[0012] Medical conditions caused by dysregulation in acetylcholine are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems.

[0013] Medical conditions caused by dysregulation in TRP are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems.

[0014] Medication conditions caused by dysregulation in ADR are typified by specific symptoms such as respiratory difficulties including shortness or breath, air hunger, colds and nasalpharynx congestion, cardiovascular conditions such as as hypertension, and palpitations, gastrointestinal illness, kidney disease, diabetes, and autonomic function including sweating episodes.

[0015] Medical conditions caused by dysregulation of the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; urinary frequency or discomfort and respiratory symptoms.

[0016] Transient receptor potential (TRP) ion channels are expressed on almost all cells and have a significant effect on physiological functions [3b]. A number of channelopathies have been associated with TRP genes as these have consequences for cellular function [4b, 18b, 19b]. Dysregulation in TRPs has been associated with pathological conditions and diseases including chronic pain, overactive bladder, diabetes, chronic obstructive pulmonary disease, cardiac hypertrophy, familial Alzheimer's disease, skin diseases, skeletal dysplasias, motor neuropathies, neuro-sensory neuropathies (including Charcot-Marie-Tooth disease (type 2C) and cancer [4b-8b]. TRP ion channels have an important role in Ca^{2+} signalling. TRP ion channels are activated following fluctuations or deviations in the cellular environment. Factors that may influence these changes are stressors including pathogens, temperature, pressure, chemicals, oxidation/reduction, toxins, osmolality and pH [9b, 10b]. TRP ion channels are activated in the presence of irritants, inflammatory products, and xenobiotic toxins.

[0017] Mammalian TRPs are comprised of six main groups including the TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) [lb, 2b]. Generally, the TRPC channels are nonselective cation channels, only two are highly permeable Ca^{2+} channels and two are impermeable for Ca^{24}. Several TRPs are permeable for Mg^{2+} andZn^{2+} [3b].

[0018] Acetylcholine is principally a neurotransmitter. The physiological functions of acetylcholine (ACh) are mediated by two membrane proteins, namely the muscarinic (mAChR) and nicotinic receptors (nAChR). Both receptor types have numerous subtypes and are located in the central and peripheral nervous system including the autonomic system. Furthermore, ACh performs non-neuronal functions, termed the non-neuronal cholinergic system (NNCS), where ACh performs endocrine functions of tissue located on smooth muscle, β pancreatic cells, glial cells, lymphocytes, ocular lens cells and brain vascular endothelium [lc] as well as in the CNS [2c-6c]. The degradation of ACh into choline and acetate is catalysed by the enzymes acetylcholinesterase (AChE) [7c, 8c].

[0019] There are five main mAChR subtypes - Ml, M2, M3, M4 and M5, where M2 and M4 are inhibitory receptors, and Ml, M2 and M3 are excitatory receptors [7c, 8c]. mAChRs are G protein coupled receptors that regulate intracellular signalling second messengers as well as ion channel activities. Once activated each subtype has distinctive functions - Ml, M3 and M5 receptors form inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG), resulting in increased intracellular calcium. Activated M2 and M4 receptors inhibit adenylate cyclase activity as well as mediating function of non-selective cation channels, transient receptor potential channels and potassium channels [7c-10c].

[0020] nAChRs are fast ionotropic cationic nicotinic receptor channels which allow for the influx of cations such as potassium, calcium and sodium ions into the cell. nAChRs are comprised of different subunits: a subunits (αi-α_{10}), β subunits (βι—β4), one δ subunits, one γ subunit and one ε subunit [11c]. Depending upon combinational subunit binding AChRs can form either heteromers or homomers [11c].

[0021] Previous research has reported anomalies in acetylcholine signalling in CFS/ME patients. Peripheral cholinergic function is noted to be abnormal in CFS/ME patients exposed to ACh challenge whereby blood flow peaks take a longer time to return to normal. Increased sensitivity to ACh is noted in peripheral vascular endothelium [30c, 31c]. Moreover it is documented that ACh influences immune cell function [32c] and is manufactured and secreted by a wide range of immune cells including lymphocytes [33c, 34c, 32c, 35c]. The present inventors, along with others, have previously reported profound changes in immune cell and function as well as noting cardiac and neurological effects in CFS/ME patients [14c-16c, 18c-20c, 22c, 24c, 26c, 27c, 29c].

[0022] Single nucleotide polymorphisms (SNPs) occur in coding sequences of genes, non-coding regions of genes, or in the intergenic regions of genes. SNPs located within a coding sequence may or may not necessarily change the amino acid sequence of the protein that is produced. As such SNPs that do not alter the polypeptide sequence are termed synonymous (sometimes called silent variants) while SNPs that result in different polypeptide sequences are referred to as non-synonymous. Non-synonymous single nucleotide polymorphisms (nsSNPs) result in changes to protein expression that may result in aberrant signalling, such as loss or gain of function in their effect. Importantly, silent variants have been reported to affect splicing and may lead to human disease [lOd, lid]. Splicing affecting gene variants can induce exon skipping and activate alternate splice isoforms of the gene transcript, potentially resulting in altered gene transcripts and disease phenotypes.

[0023] Despite intensive research, to date, the pathophysiology of CFS/ME is not yet fully understood and clear diagnostic tools remain elusive. Therefore, there remains a need for rapid, cost-effective and reliable means for identifying, screening, diagnosing, monitoring and/or managing/treating individuals having, or at risk of developing, a medical condition such as CFS/ME.

SUMMARY OF INVENTION

[0024] The present invention, in a first aspect, broadly concerns the use of one or more single nucleotide polymorphisms (SNPs) in one or more transient receptor potential (TRP) ion channel, acetylcholine receptor (AChR) or adrenergic receptor (ADR) genes as probes, tools or reagents for identifying, screening, diagnosing, monitoring or managing/treating subjects with, or predisposed to, medical conditions or specific symptoms thereof, such as chronic fatigue syndrome (CFS), myalgic encephalomyelitis (ME), Gulf war syndrome (GWS), irritable bowel syndrome (IBS), multiple chemical sensitivity (MCS), fibromyalgia, or migraine, as well as some medical conditions caused by dysregulation in calcium, acetylcholine, TRP or ADR, and dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary or immune systems.

[0025] In a second aspect, the present invention broadly relates to the use of calcium metabolism testing for identifying, screening, diagnosing, monitoring or managing/treating a subject having, or at risk of developing, a medical condition or symptom thereof.

[0026] In a third aspect, the present invention broadly relates to identifying, screening, diagnosing, monitoring or managing/treating a subject having a medical condition or symptom thereof, by testing cells obtained from the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[0027] In a fourth aspect, the present invention broadly relates to the use of at least one differentially regulated calcium-dependent kinase gene for identifying, screening, diagnosing, monitoring or managing/treating a subject having, or at risk of developing, a medical condition or symptom thereof.

[0028] In a fifth aspect, the invention broadly concerns at least one probe, tool or reagent based on or developed from any one of the first to fourth aspects, for identifying, screening, diagnosing, monitoring or managing/treating the medical condition or symptom thereof.

[0029] In a sixth aspect, the present invention broadly concerns methods, kits or assays based on or developed from any one of the first to fifth aspects, for identifying, screening, diagnosing, monitoring or managing/treating subjects with one or more of the medical conditions or symptom thereof.

[0030] Preferred features, embodiments and variations of the invention may be discerned from the following Detailed Description which provides sufficient information for those skilled in the art to perform the invention. The Detailed Description is not to be regarded as limiting the scope of the preceding Summary of Invention in any way. The Detailed Description will make reference to a number of drawings as follows.

[0031] **BRIEF DESCRIPTION OF THE DRAWINGS**

[0032] Figure 1: Natural Killer Cell Purity. The purity of NK cells represents minimal contamination from other cells types. Data shown for ME/CFS (n=39), and non-fatigued controls (n = 30), and presented as mean ± SEM.

[0033] Figure 2: Reduced NK cytotoxic activity in CFS/ME. In vivo assessment of NK cytotoxic activity of tumour cell lines K562 in CFS/ME (n=39) and unfatigued controls (n=30). Lytic activity represented by percentage lysis of target cells on the y-axis. Data presented as mean ± SE *P<0.05.

[0034] Figure 3. TRPM3 expression (%) on B lymphocytes and NK cells gated from HC (n=19) and CFS/ME (n=18) peripheral mononuclear cells. (A) NK cells subsets were characterized as $CD56^{Bright}$ NK cells and $CD56^{Dim}$ NK cells. Identification of TRPM3 surface expression on the NK cell subsets was analyzed using indirect flow cytometry. (B) B cells were characterized as total B cells ($CD3^{-}CD19^{+}$) and indirect flow cytometry was employed to identify TRPM3 surface expression on B cells. Histograms report the means ±SEM. ^Denotes p<0.05. HC: healthy controls; CFS: Chronic Fatigue Syndrome; ME: myalgic encephalomyelitis.

[0035] Figure 4: Fura-AM cytoplasmic calcium influx in $CD19^{+}$B cells and $CD56^{Bright}$ NK cells. (A). $CD19^{+}$ B cells calcium influx response curve reported as area under the curve was measured during Anti-IgM and anti-CD21 conjugated biotins were cross-linked with streptavidin or in the presence of ionomycin, 2-APB or Thapsigargin using flow cytometry. (B). Fura-AM cytoplasmic calcium influx response during $CD56^{Bright}$ NK cell receptors, Anti-CD314 and anti-CD335 conjugated biotins were cross-linked with streptavidin or in the presence of ionomycin, 2-APB or Thapsigargin using flow cytometry. Histograms report the means ±SEM. ^Denotes statistically significance at p<0.05.

[0036] Figure 5: Representative flow cytometric plot of $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$ NK cell phenotypes (A). Comparisons of $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$ NK cell phenotypes between CFS/ME and NFC revealed no significant differences (B). Data are presented as median percentage with interquartile range.

[0037] Figure 6: $CD56^{bright}CD16^{dim/-}$ NK cell ERK1/2 flow cytometric plot for a representative individual (A). ERK1/2 in $CD56^{bright}CD16^{dim/-}$ NK cells were compared between CFS/ME and NFC groups and no significant differences were observed. PMA/I stimulation caused a significant increase in ERK1/2 phosphorylation compared to US (***p<0.001) and K562 cells (****p <0.0001) in both CFS/ME and NFC. Data are presented as MFI with interquartile range.

[0038] Figure 7: Representative flow cytometric plot for MEKl/2 in $CD56^{dim}CD16^{+}$ NK cells (A). No significant differences were observed when MEKl/2 was compared between CFS/ME and NFC (B). In both CFS/ME and NFC, PMA/I stimulation resulted in a significant increase in phosphorylated MEKl/2 compared to US (****p<0.0001) and K562 stimulation (**** p<0.0001). Data are presented as MFI with interquartile range.

[0039] Figure 8: p38 representative flow cytometric plot in $CD56^{dim}CD16^{+}$ NK cells (A). p38 was compared between CFS/ME and NFC and no significant differences were observed (B). Stimulation with PMA/I caused a significant increase in phosphorylated p38 when compared to US and K562 incubated cells (*p<0.05). Data are presented as MFI with interquartile range.

[0040] Figure 9: Representative Stat-3 flow cytometric plots in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. Comparison of Stat-3 in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells between CFS/ME and NFC revealed no significant differences. In $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells, stimulation with PMA/I caused a significant increase in Stat-3 when compared to US (****p<0.0001) and K562 (****p<0.0001) in both CFS/ME and NFC.

[0041] Figure 10: Representative flow cytometric analysis of NF-κβ in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. No significant differences were observed when NF-κβ was compared between CFS/ME and NFC in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells. Phosphorylated NF-κβ significantly increased after PMA/I stimulation in both $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells compared to US (****p<0.0001) and K562 (^^O.OOOl) in CFS/ME and NFC. Data are presented as MFI with interquartile range.

[0042] Figure 11: Iκβ representative flow cytometric plots in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cdls . Iκβ was compared in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells from CFS/ME and NFC and no significant differences were observed. Stimulation with PMA/I caused a *significant* reduction in Iκβ in both $CD56^{dim}CD16^{+}$ (*p<0.05) and $CD56^{bright}CD16^{dim/-}$ (***p<0.001) NK cells from CFS/ME and NFC. Incubation with PMA/I also caused a significant reduction (*p<0.05) in Iκβ in $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients.

[0043] Figure 12: Representative flow cytometric plots for the analysis of PKC-a in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. PKC-a was compared in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells from CFS/ME and NFC and no significant differences were observed. In $CD56^{dim}CD16^{+}$ NK cells from NFC, stimulation with PMA/I caused a significant increase (**p<0.01) in PKC-a phosphorylation compared to K562 cells. PKC-a was significantly increased in $CD56^{bright}CD16^{dim/-}$ NK cells after PMA/I stimulation when compared to US (*p<0.05) and K562 (***p<0.001) in NFC.

[0044] Figure 13: Flow cytometric analysis of JNK in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. No significant differences were observed when JNK was compared in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells from CFS/ME and NFC. Significant increases in phosphorylated JNK were observed in both $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells after pMA/I stimulation when compared to US (**p<0.01) and K562 (***p<0.001) in CFS/ME and NFC.

[0045] Figure 14: NK cell cytotoxic activity in CFS/ME and NFC at three E:T ratios.

[0046] Figure 15: Representative flow cytometry plots for CD107a in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. CD107a was measured in US cells and after stimulation with either K562 cells or PMA/I. Comparison of CD107a on $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells between CFS/ME and NFC revealed no significant differences. CD107a expression significantly increased after K562 and PMA/I (****ρ<0.0001) stimulation in $CD56^{dim}CD16^{+}$ NK cells from both CFS/ME and NFC. In $CD56^{bright}CD16^{dim/-}$ NK cells, PMA/I stimulation significantly increased expression of CD107a when compared to K562 and US cells (**«p<0.0001) from CFS/ME and NFC.

[0047] Figure 16: Flow cytometric analysis of CD107b on $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. No significant differences were observed when CD107b expression was compared between CFS/ME and NFC on $CD56^{dim}CD16^{+}$ (C) and

$CD56^{bright}CD16^{dim/-}$ (D) NK cells. In $CD56^{dim}CD16^{+}$ NK cells, stimulation with K562 cells (*p<0.05) and PMA/I (^^p-cO.OOOl) caused a significant increase in CD107b expression in both CFS/ME and NFC compared to US. PMA/I stimulation significantly increased CD107b expression on $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME and NFC when compared to K562 and US (****p<0.0001).

[0048] Figure 17: Perforin, Granzymes A and B and CD57 from $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients.

[0049] Figure 18: Perforin, Granzymes A and B and CD57 from $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients.

[0050] Figure 19: Representative flow cytometric plots for $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cell production of IFN-γ. Comparison of IFN-γ production in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells between CFS/ME and NFC revealed no significant differences. IFN-γ production significantly increased after PMA/I stimulation in both $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells when compared to US and K562 (****p<0.0001).

[0051] Figure 20: Flow cytometric plots for TNF-a in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cellg. Between CFS/ME and NFC cohorts, TNF-a production in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells were not significantly different. In $CD56^{dim}CD16^{+}$ NK cells, PMA/I stimulation significantly increased TNF-a production when compared to US and K562 incubated cells (****p<0.0001) in both CFS/ME and NFC.

[0052] Figure 21: Flow cytometric analysis of GM-CSF production in $CD56^{dim}CD16^{+}$ (A) and $CD56^{bright}CD16^{dim/-}$ (B) NK cells. Production of GM-CSF in $CD56^{dim}CD16^{+}$ (C) and $CD56^{bright}CD16^{dim/-}$ (D) NK cells were not significantly different when compared between CFS/ME and NFC cohorts. Stimulation with PMA/I caused a significant increase in $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ GM-CSF production in both CFS/ME and NFC compared to US and K562 incubated cells (****p<0.0001, ***p<0.001).

[0053] Figure 22. Natural Killer cell purity. NK cell purity measurements are represented as total % of $CD3^{-}CD56^{+}$ cells. Data are presented as mean ± SD for CFS/ME group (n= 24) and control group (n=11).

[0054] Figure 23. Heat map of kinase gene expression showing (A) significantly upregulated and (B) significantly downregulated genes from severe CFS/ME patients compared

with non-fatigued controls.

[0055] Figure 24: Frequency of SNPs per chromosome.

[0056] Figure 25: Manhattan plot of Fisher's exact test on 950 SNPs.

[0057] Figure 26: Frequency of top 10 SNPs from Fisher's exact test. Cases: CFS/ME group; Controls: Healthy control group; MAF: Minor allele.

[0058] Figure 27: Proportion of CFS/ME patients ("Cases") and healthy control group ("Controls") being homozygous major (GG), heterozygous (AG) or homozygous minor (AA) for adrenergic a1A (ADRA1A) SNP rs2322333.

DETAILED DESCRIPTION

[0059] Chronic fatigue syndrome (CFS) and myalgic encephalomyelitis (ME) are significantly debilitating medical conditions characterised by persistent fatigue and other specific symptoms that last for a minimum of six months. CFS and ME are often used interchangeably to describe the same illness, although this need not be the case. The fatigue experienced by human subjects suffering from CFS is not due to exertion or caused by other medical condition, and is not significantly relieved by rest. It is a complex disease involving dysregulation of immune and central nervous systems, dysfunction of cellular energy metabolism and ion transport, and cardiovascular abnormalities.

[0060] CFS/ME patients may further be categorised into mild, moderate, severe or very severely affected by their illness. Mild CFS/ME patients are mobile and often still employed, moderate CFS/ME patients have reduced mobility and are restricted in daily tasks, such as household chores, severe CFS/ME patients are only able to perform minimal necessary hygiene-related tasks and are wheelchair dependent while those with very severe CFS/ME are unable to carry out any daily task for themselves and are essentially bedridden [3e]. The ICC is the most recent and accurate set of criteria used for CFS/ME diagnosis and contains reference to these severity subgroups of CFS/ME patients, although it is not a necessary component of the guidelines [4e].

[0061] A number of healthcare initiatives have been undertaken to advance research into the likely cause(s), mechanism, preventive measures and potential therapeutic strategies for CFS/ME. Presently, none of these initiatives has been successful and the medical community remains baffled by the illness.

[0062] Currently there are no commercially available diagnostic tests or definitive methods for screening of CFS/ME.

[0063] The most puzzling aspect of CFS/ME is its multifactorial, multi-symptom nature and resulting difficulty in the diagnosis of CFS/ME. The current method of diagnosis is to rule out other potential causes of the symptoms presented by the patients. When symptoms are attributable to certain other conditions, the diagnosis of CFS/ME is excluded. As a result, there is a prolonged 'elimination' process often including several attempted unsuccessful treatment strategies. This process can often take from 6 to 18 months. Accordingly, it is a serious financial burden to the subject and to the healthcare system and economy.

[0064] Although there is no specific treatment for CFS/ME, it can be appropriately managed once a patient is diagnosed as suffering from CFS. Additionally, there is some evidence to suggest that earlier a management regime is adopted the greater the chance of improvement, although no cure exists and improvements are largely empirically based. A diagnostic/screening test would significantly help in diagnosis/screening of CFS/ME, thereby reducing the patient suffering and healthcare costs associated with waiting for many months before being diagnosed with CFS/ME.

[0065] The present invention is described in more detail below.

[0066] The present inventors have, for the first time, identified SNPs of TRP ion channel, ACh receptor and ADR genes that correlate with CFS and ME or specific symptoms thereof. The inventors believe that the identified SNPs of TRP ion channel, ACh receptor and ADR genes also correlate with other medical conditions or symptoms thereof such as IBS, MCS, fibromyalgia, and migraine, as well as some medical conditions caused by dysregulation in calcium, acetylcholine, TRP and ADR, and dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems.

[0067] "Medical condition" as used hereon in the specification can include (but is not limited to): CFS or specific symptoms thereof; ME or specific symptoms thereof; GWS, IBS; MCS; non-allergic rhinitis; fibromyalgia; migraine; or rheumatoid arthritis. "Medical condition" as used hereon in the specification can also include (but is not limited to) conditions or symptoms: caused by dysregulation in calcium (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in acetylcholine (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in TRP (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in ADR; caused by dysregulation of the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems (especially in respect of CFS, ME, GWS, IBS, MCS, non-allergic rhinitis, fibromyalgia or migraine).

[0068] Preferably, the medical condition is CFS or ME.

[0069] Specific symptoms of CFS or ME include: neuromuscular fatigue, particularly

fatigue upon exertion; memory and concentration difficulties; muscle and joint pain; altered blood pressure, particularly postural orthostatic tachycardia syndrome; headache; immunological dysregulation; sore throat; swollen lymph nodes/glands; gastrointestinal symptoms including IB, diarrhoea, constipation and abdominal pain; chemical sensitives; and intolerances to drugs and chemicals.

[0070] MCS conditions/symptoms are characterised by reactions to multiple diverse chemical substances, the wide spectrum of non-specific symptoms reported in multiple organ systems, and the extremely low levels of environmental exposures linked to responses. Symptoms include: headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; or respiratory symptoms.

[0071] Medical conditions caused by dysregulation in calcium, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation such as: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and immunological "allergic" sensitivities.

[0072] Medical conditions caused by dysregulation in acetylcholine, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation such as: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems (immunological "allergic" sensitivities).

[0073] Medical conditions caused by dysregulation in TRP are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems (immunological "allergic" sensitivities).

[0074] Medication conditions caused by dysregulation in ADR are typified by specific symptoms such as respiratory difficulties including shortness or breath, air hunger, colds and nasalpharynx congestion, cardiovascular conditions such as as hypertension, and palpitations, gastrointestinal illness, kidney disease, diabetes, and autonomic function including sweating episodes.

[0075] Medical conditions caused by dysregulation of the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; urinary frequency or discomfort; respiratory symptoms; and immunological "allergic" sensitivities.

[0076] Therefore, one or more of those SNPs can be used for identifying, screening, diagnosing or monitoring subjects with, or predisposed to, those medical conditions or symptoms thereof.

[0077] Moreover, yet one or more other TRP ion channel, ACh receptor or ADR gene/allele-based or gene product-based probes, tools, reagents, methods and assays can be used for identifying, screening, diagnosing, monitoring or managing/treating subjects with, or predisposed to, those medical conditions or symptoms thereof.

[0078] The TRP ion channel can be selected from one or more of the following: TRPC4, TRPA1 (ankyrin), TRPM3 (melastatin) and TRPM4. The TRP ion channel gene can be selected from one or more of the following genes: Gene ID 80036, 7223, 101927086 and 54795. (Searchable at the ncbi.nlm.nih.gov website.)

[0079] The at least one SNP of a TRP ion channel gene can be selected from a SNP listed in one or more of the Tables, such as Tables 1, 3, 4, 7, 9, 10, 12, 13, 15, 16, 17, 26, 27, 34a and 34b.

[0080] The at least one SNP of a TRP ion channel gene can be one or more (eg. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13) of the following SNPs: rsl2682832, rslll42508, rsll60742, rs4454352, rsl328153, rs3763619, rs7865858, rsl504401 or rslOl 15622 of *TRPM3;* rs2383844 or rs4738202 of *TRPA1;* or rs6650469 or rs655207 of *TRPC4.*

[0081] The ACh receptor can be selected from one or more of the following: muscarinic acetylcholine receptor, especially mAChRM3; and nicotinic acetylcholine alpha receptors, especially nAChRa2, nAChRa5 or nAChRalO. The AChR gene can be selected from one or

more of the following genes: Gene ID 1131, 417, 4928, 57053, 100873984, 1138 and 1142.

[0082] The at least one SNP of an ACh receptor gene can be selected from a SNP listed in one or more of the Tables, such as Tables 2, 5, 6, 7, 9, 10, 12, 13, 14, 16, 17, 26, 28. 34a and 34b.

[0083] The at least one SNP of an ACh receptor gene can be one or more (eg. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) of the following SNPs: rs4463655, rs589962, rsl072320, rs7543259, rs6661621, rs7520974, rs726169, rsrs6669810 or rsrs6429157 of mAChRM3 ; rs2672211, rs2672214, rs2741868, rs2741870 or rs2741862 of nACh alpha 10; rs951266 or rs7180002 of nACh alpha 5; or rs2565048 of nACh alpha 2.

[0084] The ADR may be any suitable member of the adrenergic receptor family, such as a or β, or any suitable subtype thereof. (See Protein Sci. 1993 Aug; 2(8): 1198-1209, for example.)

[0085] The ADR can be adrenergic receptor a l (ADRA1A), Gene ID. 148. (Searchable at the ncbi.nlm.nih.gov website.)

[0086] The at least one SNP of the ADRA1 A gene can be rs2322333.

[0087] The at least one SNP of an ADR gene can be selected from a SNP listed in a Table, such as Table 34a or 34b.

[0088] The at least one SNP can be one or more non-synonymous SNPs. The non-synonymous SNP can be located in an intron, exon or regulatory region.

[0089] More information about the aforementioned SNPs/polymorphisms as well as other polymorphisms in the TRP ion channel, ACh receptor and ADR genes can be found in the NCBI SNP database, searchable at the ncbi.nlm.nih.gov website.

[0090] The at least one probe, tool or reagent based on or developed from a TRP ion channel, ACh receptor or ADR gene or gene product can, for example, specifically bind, detect, identify, characterise or quantify the gene or part of the gene, the RNA gene product or part of the RNA gene product (RNA transcript), the polypeptide gene product or part of the polypeptide gene product (protein).

[0091] Of course, in an embodiment, the at least one probe, tool or reagent can identify a TRP ion channel, ACh receptor or ADR gene SNP of interest.

[0092] The probe, tool or reagent can be, but is not limited to, an oligonucleotide, primer, nucleic acid, polynucleotide, DNA, cDNA, RNA, peptide or polypeptide. These can be, for example, single stranded or double stranded, naturally occurring, isolated, purified, chemically modified, recombinant or synthetic.

[0093] The probe, tool or reagent can be, but is not limited to, an antibody or other type of molecule or chemical entity capable of detecting the gene or gene product (RNA or polypeptide).

[0094] The at least one probe, tool or reagent can be any number or combination of the

above, and the number and combination will depend on the desired result to be achieved - eg. detection of a polymorphism at the genomic level (genotyping), at the RNA transcription level or translation polypeptide level, or quantitative or qualitative measurement of RNA transcription or translation.

[0095] In one preferred embodiment, the at least one probe, tool or reagent is for detection of a polymorphism at the genomic level, at the transcription level or polypeptide level.

[0096] In another preferred embodiment, the at least one probe, tool or reagent is for quantitative or qualitative measurement of RNA transcription or translation.

[0097] In yet another preferred embodiment, the at least one probe, tool or reagent is for assaying TRP ion channel, or ACh receptor protein/polypeptide expression on the surface of cells, preferably blood cells such as NK, T and/or B cells.

[0098] In yet another preferred embodiment, the at least one probe, tool or reagent is for assaying ADR protein/polypeptide expression in or on cells.

[0099] In a preferred embodiment, the probe, tool or reagent is for detecting at least one polymorphism as listed in a Table, such as any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

[00100] The probe, tool or reagent can be derived from or based on one or more SNPs recited in a Table, such as any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

[00101] The probe, tool or reagent can be derived from or based on any relevant region or regions of the TRP ion channel, ACh receptor or ADR genes. This includes the promoter region, 5' UTR, coding region (exon), intronic region or 3' UTR.

[00102] The probe, tool or reagent (1) can have a sequence as listed in Table 35 or Table 36, or (2) can have a sequence substantially identical to that shown in Table 35 or Table 36, or (3) can have a reverse complementary sequence to (1) or (2). The at least one probe, tool or reagent can, for example, be used to specifically bind, detect, identify, amplify, characterise or quantify the gene or part of the gene, the RNA gene product or part of the RNA gene product, or any synthetic or recombinant nucleic acid based on these.

[00103] With the foregoing in view, the present invention, in a preferred first form, resides broadly in at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene for use as an indicator of a medical condition or symptom thereof.

[00104] For clarity, the term "indicator" signifies that the SNP positively correlates with the medical condition or symptom thereof.

[00105] For clarity, the expression "TRP ion channel, ACh receptor and/or ADR" and like

expressions as used herein mean any individual gene/protein or any combination of 2 genes/proteins, or the combination of 3 genes/proteins.

[00106] In a second form, the present invention resides broadly in at least one probe, tool or reagent based on or developed from a TRP ion channel, ACh receptor and/or ADR gene or gene product for use as an indicator of a medical condition or symptom thereof.

[00107] For clarity, the term "indicator" signifies that a result produced by the probe, tool or reagent positively correlates with the medical condition or symptom thereof.

[00108] In a third form, the present invention resides in the use of at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof.

[00109] In a first preferred form, the present invention resides in a method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

[001 10] (a) genotyping said subject for at least one polymorphism in a TRP ion channel, ACh receptor and/or ADR gene to obtain a result; and

[00111] (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

[00112] In another preferred form, the present invention resides in a method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

[00113] (a) testing said subject for a TRP ion channel, ACh receptor and/or ADR gene product to obtain a result; and

[00114] (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

[001 15] The TRP ion channel, ACh receptor or ADR gene product may be transcribed RNA, nascent RNA, mRNA or polypeptide. Testing may involve, for example, detecting aberrant mRNA or a difference in the level of gene expression (ie. dysregulation).

[00116] Testing may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells, preferably blood cells such as NK, T and/or B cells, whereby altered or reduced expression of TRP ion channel and/or ACh receptor is indicative of the subject having the medical condition or symptom thereof or a predisposition to the medical condition or symptom thereof.

[00117] Testing may involve, for example, assaying ADR expression in or on cells whereby

altered or reduced expression of ADR is indicative of the subject having the medical condition or symptom thereof or a predisposition to the medical condition or symptom thereof.

[00118] In a fourth form, the present invention resides broadly in the use of at least one probe, tool or reagent based on or developed from a TRP ion channel, ACh receptor and/or ADR gene or gene product for identifying, screening, diagnosing, monitoring or managing/treating a subject having, or at risk of developing, a medical condition or symptom thereof.

[00119] In a fifth form, the present invention resides in at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

[00120] In a sixth form, the present invention resides in at least one probe, tool or reagent based on or developed from a TRP ion channel, ACh receptor and/or ADR gene or gene product when used as an indicator of a medical condition or symptom thereof, when used in identifying, screening, diagnosing, monitoring or managing/treating a subject having a medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

[00121] In a seventh form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

[00122] Preferably, this method comprises the step of testing a biological sample obtained from the subject for the at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

[00123] In an eighth form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of assaying the subject for a property of a TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

[00124] Preferably, this method comprises the step of testing a biological sample obtained from the subject for the property.

[00125] The property may be a polymorphism at the genomic level, at the transcription level or polypeptide level. That is, the property may relate to a polymorphism at the genomic level, or altered RNA, altered mRNA or altered polypeptide/protein expression.

[00126] The method may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells (such as blood cells), whereby altered or reduced expression of TRP ion channel and/or ACh receptor is indicative of the subject having the medical condition or symptom thereof.

[00127] The method may involve, for example, assaying ADR expression in or on cells whereby altered or reduced expression of ADR is indicative of the subject having the medical condition or symptom thereof.

[00128] In a ninth form, the present invention resides in a method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of testing the subjects for at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

[00129] Preferably, this method comprises the step of testing a biological sample obtained from each of the subjects for the at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene.

[00130] In a tenth form, the present invention resides in a method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a property of a TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

[00131] Preferably, this method comprises the step of testing a biological sample obtained from each of the subjects for the property.

[00132] Again, as for other forms of the invention, the property may be a polymorphism at the genomic level, at the transcription level or polypeptide level. That is, the property may relate to a polymorphism at the genomic level, or altered RNA or mRNA, or altered polypeptide/protein expression. The method may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells (such as blood cells), whereby altered or reduced expression of TRP ion channel and/or ACh receptor is indicative of the subject having the medical condition or symptom thereof. The method may involve, for example, assaying ADR expression in or on cells, whereby altered or reduced ADR is indicative of the subject having the medical condition or symptom thereof.

[00133] In view of the fact that SNPs/genes for the medical condition or symptom thereof

have been discovered and characterised, this enables management/treatment of a subject that has been identified as having the medical condition or symptom thereof, and identifying whether a subject having the medical condition or symptom thereof is likely to respond to, or is responding to, management/treatment of that illness.

[00134] In an eleventh form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

[00135] (1) testing the subject for at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof; and

[00136] (2) managing the subject if the subject has been found to have the at least one SNP of a TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

[00137] Preferably, this method comprises the step of testing a biological sample obtained from the subject for the at least one SNP.

[00138] In a twelfth form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

[00139] (1) assaying the subject for a property of a TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof; and

[00140] (2) managing the subject if the subject has been found to have the property of the TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

[00141] Preferably, this method comprises the step of assaying a biological sample obtained from the subject for the property.

[00142] The property may relate to a polymorphism at the genomic level, or altered mRNA or altered polypeptide/protein expression. The method may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells (such as blood cells), whereby altered or reduced expression of TRP ion channel and/or ACh receptor is indicative of the subject having the medical condition or symptom thereof. The method may involve, for example, assaying ADR expression in or on cells, whereby altered or reduced ADR is indicative

of the subject having the medical condition or symptom thereof.

[00143] In a thirteenth form, the present invention resides in a method of identifying or diagnosing a subject having a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

[00144] (a) measuring the level of expression of at least one gene marker in a biological sample obtained from the subject that is differentially expressed in the medical condition or symptom thereof; and

[00145] (b) comparing the level of expression of the at least one gene marker in the biological sample relative to a reference, wherein the at least one gene marker is a TRP ion channel, ACh receptor and/or ADR gene, and detection of an alteration in the level of gene expression of the at least one gene marker in the biological sample relative to the reference indicates that the subject has the medical condition or symptom thereof, or is at risk of developing the medical condition or symptom thereof.

[00146] In some embodiments, measuring the level of expression may involve measuring RNA, mRNA or polypeptide/protein expression. Measuring the level of expression may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells, preferably blood cells such as NK, T and/or B cells, whereby altered or reduced expression of TRP ion channel and/or ACh receptor is indicative of the subject having the medical condition or symptom thereof or a predisposition to the medical condition or symptom thereof. Measuring the level of expression may involve, for example, assaying ADR expression in or on cells, whereby altered or reduced ADR is indicative of the subject having the medical condition or symptom thereof or a predisposition to the medical condition or symptom thereof.

[00147] In some embodiments, measuring the level of expression may involve immunocytochemistry and/or flow cytometry.

[00148] In a fourteenth form, the present invention resides in a method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:

[00149] optionally, isolating a biological sample from the subject prior to management of the illness and during and/or after management of the illness;

[00150] measuring the level of expression in the biological samples of at least one gene marker that is differentially expressed in the illness; and

[00151] comparing the level of expression of the gene marker in the biological samples before and during and/or after management of the illness, wherein the at least one gene marker is a TRP ion channel, ACh receptor and/or ADR gene, and a change in the level of expression of the gene marker identifies the subject as having responded to the management of the illness.

[00152] In some embodiments, measuring the level of expression may involve measuring RNA, mRNA or polypeptide/protein expression. In some embodiments, measuring the level of expression may involve, for example, assaying TRP ion channel and/or ACh receptor expression on the surface of cells, preferably blood cells such as NK, T and/or B cells. In some embodiments, measuring the level of expression may involve, for example, assaying ADR expression on or in cells.

[00153] In a fifteenth form, the present invention resides in a TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, or a TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for use in identifying a subject having a medical condition or symptom thereof.

[00154] In a sixteenth form, the present invention resides in a TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for identifying a subject at risk of developing a medical condition or symptom thereof, or a TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for use in identifying a subject at risk of developing a medical condition or symptom thereof.

[00155] In a seventeenth form, the present invention resides in a TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent when used for identifying a subject having, or at risk of developing, a medical condition or symptom thereof.

[00156] In an eighteenth form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterising a TRP ion channel, ACh receptor and/or ADR gene or gene product using a biological sample derived from the subject.

[00157] In a nineteenth form, the present invention resides in a biological sample comprising at least a TRP ion channel, ACh receptor and/or ADR gene or gene product, when isolated for the purpose for testing the biological sample for a medical condition or symptom thereof.

[00158] In a twentieth form, the present invention resides in an array of oligonucleotide probes suitable for determining a TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

[00159] In a twenty-first form, the present invention resides in a microarray comprising oligonucleotide probes suitable for determining a TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

[00160] In a twenty-second form, the present invention resides in a biochip comprising a solid substrate and at least one oligonucleotide probe suitable for determining a TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

[00161] In a twenty-third form, the present invention resides in an article of manufacture comprising: (1) non-naturally occurring polynucleotide, recombinant polynucleotide, oligonucleotide or cDNA form of a TRP ion channel, ACh receptor and/or ADR gene or a fragment thereof; or (2) a polynucleotide or an oligonucleotide that is complementary to the gene of (1) or fragment thereof; or (3) an expression vector, recombinant cell or biological sample, tool, reagent, kit or assay comprising (1) or (2) or fragment thereof.

[00162] In a twenty-fourth form, the present invention resides in a TRP ion channel, ACh receptor and/or ADR gene SNP as shown in a Table, such as any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

[00163] In a twenty-fifth form, the present invention resides in a nucleotide sequence as shown or substantially as shown in Table 35 or Table 36 (SEQ ID Nos. 1 to 64), or a complementary sequence thereof.

[00164] By "substantially as shown", the sequence has sequence identity preferably of between 80-99%, including 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99%.

[00165] The expression "at least one" - context allowing - means 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 , 13, 14, 15, 16, 17, 18, 19, 20 or more.

[00166] Since the inventors have found SNPs in the TRP ion channel, ACh receptor or ADR gene to correlate with calcium metabolic changes/regulation (including calcium ion changes), it follows that those changes can also be used, for example, to identify, screen, diagnose or monitor subjects with, or predisposed to, one or more of the medical conditions or specific symptoms thereof described above.

[00167] It also follows that since the inventors have found SNPs in the TRP ion channel, ACh receptor or ADR gene to correlate with yet other changes, those changes can also be used, for example, to identify, screen, diagnose or monitor subjects with, or predisposed to, one or more of the medical conditions or specific symptoms thereof described above. For the sake of convenience, however, only calcium ion/metabolic changes/testing will be further expanded upon below. Those of skill in the art will appreciate what those other changes/tests could be and may include but not be limited to calcium metabolic co-factors or transcription factors such as calmodulin, calcineurin, nuclear factor of activated T cells (NFAT), IP3, DAG, ORAI, ATPases in all their forms or iso-types.

[00168] Accordingly, in a twenty-sixth form, the present invention resides in the use of calcium metabolism testing for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof. The medical condition or symptom thereof may be attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene, but it need not be attributable to the at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00169] In a twenty-seventh form, the present invention resides broadly in calcium metabolism testing when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00170] In a twenty-eighth form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing or assaying the subject for a change in calcium metabolism. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00171] In a twenty-ninth form, the present invention resides in a method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a change in calcium metabolism. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00172] In a thirtieth form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

[00173] (1) testing the subject for a change in calcium metabolism; and

[00174] (2) managing the subject if the subject has been found to have said change in calcium metabolism. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00175] In a thirty-first form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying calcium metabolic change in the subject. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00176] In a thirty-second form, the present invention resides in a kit or method for testing, screening or managing/treating a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, for any calcium metabolite which may include but not be limited to calcium metabolic co-factors or transcription factors such as calmodulin, calcineurin, nuclear factor of activated T cells (NFAT), IP3, DAG, ORAI, ATPases in all their forms or iso-types. The medical condition or symptom thereof is preferably attributable to at least one SNP in a TRP ion channel, ACh receptor and/or ADR gene.

[00177] The inventors have also discovered that calcium-dependent protein kinase genes may be differentially regulated in patients having particular medical conditions or symptoms thereof compared to healthy individuals. For example, in severe CFS/ME patients, dysfunction in Ca^{2+} dependent protein kinase genes contribute to the pathomechanism of that illness.

[00178] In a thirty-third form, the present invention resides in at least one differentially regulated calcium-dependent kinase gene for use as an indicator of a medical condition or symptom thereof.

[00179] In a thirty-fourth form, the present invention resides in at least one probe, tool or reagent based on or developed from at least one differentially regulated calcium-dependent kinase gene for use as an indicator of a medical condition or symptom thereof.

[00180] In a thirty-fifth form, the present invention resides in the use of at least one

differentially regulated calcium-dependent kinase gene for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof.

[00181] In a thirty-sixth form, the present invention resides in a method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

(a) testing a subject for differential regulation of at least one calcium-dependent kinase gene to obtain a result; and

(b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

[00182] In a thirty-seventh form, the present invention resides in at least one differentially regulated calcium-dependent kinase gene for identifying, screening, diagnosing, monitoring or managing/treating a subject having, or at risk of developing, a medical condition or symptom thereof.

[00183] In a thirty-eighth form, the present invention resides in at least one differentially regulated calcium-dependent kinase gene when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing, monitoring or managing/treating a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

[00184] In a thirty-ninth form, the present invention resides in at least one probe, tool or reagent based on or developed from at least one differentially regulated calcium-dependent kinase gene when used as an indicator of a medical condition or symptom thereof, when used in identifying, screening, diagnosing, monitoring or managing/treating a subject having a medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

[00185] In a fortieth form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

[00186] In a forty-first form, the present invention resides in a method of screening subjects

for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of testing the subjects for at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

[00187] In a forty-second form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) testing the subject for differential regulation of at least one calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof; and

(2) managing the subject if the subject has been found to have the at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

[00188] In a forty-third form, the present invention resides in a method of identifying or diagnosing a subject having a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(a) measuring the level of expression of at least one calcium-dependent kinase gene marker in a biological sample obtained from the subject that is differentially expressed in the medical condition or symptom thereof; and

(b) comparing the level of expression of the at least one gene marker in the biological sample relative to a reference, wherein detection of an alteration in the level of gene expression of the at least one gene marker in the biological sample relative to the reference indicates that the subject has the medical condition or symptom thereof, or is at risk of developing the medical condition or symptom thereof.

[00189] In a forty-fourth form, the present invention resides in a method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:

optionally, isolating a biological sample from the subject prior to management of the illness and during and/or after management of the illness;

measuring the level of expression in the biological samples of at least one calcium-

dependent kinase gene marker that is differentially expressed in the illness; and

comparing the level of expression of the at least one gene marker in the biological samples before and during and/or after management of the illness, wherein a change in the level of expression of the at least one gene marker identifies the subject as having responded to the management of the illness.

[00190] In a forty-fifth form, the present invention resides in at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, or at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for use in identifying a subject having a medical condition or symptom thereof.

[00191] In a forty-sixth form, the present invention resides in at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for identifying a subject at risk of developing a medical condition or symptom thereof, or at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for use in identifying a subject at risk of developing a medical condition or symptom thereof.

[00192] In a forty-seventh form, the present invention resides in at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent when used for identifying a subject having, or at risk of developing, a medical condition or symptom thereof.

[00193] In a forty-eighth form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterizing at least one calcium-dependent kinase gene product using a biological sample derived from the subject.

[00194] In a forty-ninth form, the present invention resides in a biological sample comprising at least at least one calcium-dependent kinase gene or gene product, when isolated for the purpose for testing the biological sample for a medical condition or symptom thereof.

[00195] In a fiftieth form, the present invention resides in an array of oligonucleotide probes suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

[00196] In a fifty-first form, the present invention resides in a microarray comprising

oligonucleotide probes suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

[00197] In a fifty-second form, the present invention resides in a biochip comprising a solid substrate and at least one oligonucleotide probe suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

[00198] In a fifty-third form, the present invention resides in an article of manufacture comprising: (1) non-naturally occurring polynucleotide, recombinant polynucleotide, oligonucleotide or cDNA form of at least one calcium-dependent kinase gene or a fragment thereof; or (2) a polynucleotide or an oligonucleotide that is complementary to the gene of (1) or fragment thereof; or (3) an expression vector, recombinant cell or biological sample, tool, reagent, kit or assay comprising (1) or (2) or fragment thereof.

[00199] In a fifty-fourth form, the present invention resides in the use of calcium metabolism testing for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof, wherein said medical condition or symptom thereof is attributable to differential regulation of at least one calcium-dependent kinase gene.

[00200] In a fifty-fifth form, the present invention resides in calcium metabolism testing when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof, wherein said medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene.

[00201] In a fifty-sixth form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for a change in calcium metabolism, wherein said medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene.

[00202] In a fifty-seventh form, the present invention resides in a method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a change in calcium metabolism, wherein said

medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene.

[00203] In a fifty-eighth form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) testing the subject for a change in calcium metabolism, wherein said medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene; and

(2) managing the subject if the subject has been found to have said change in calcium metabolism.

[00204] In a fifty-ninth form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying calcium metabolic change in the subject, wherein said medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene.

[00205] In a sixtieth form, the present invention resides in a kit or method for testing, screening or treating a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, for any calcium metabolite which may include but not be limited to calcium metabolic co-factors or transcription factors wherein said medical condition or symptom thereof is attributable to at least one differentially regulated calcium-dependent kinase gene.

[00206] For the thirty-third to sixtieth forms of the invention, preferably the medical condition or symptom thereof is: CFS and/or ME; severe CFS and/or ME (subjects are only able to perform minimal necessary hygiene-related tasks and are wheelchair dependent); or very severe CFS and/or ME (subjects are unable to carry out any daily task for themselves and are essentially bedridden) or symptom thereof.

[00207] For the thirty-third to sixtieth forms of the invention, preferably the at least one differentially regulated calcium-dependent kinase gene is a gene selected from a Table, such as Table 31 or Table 32.

[00208] For the thirty-third to sixtieth forms of the invention, preferably the at least one differentially regulated calcium-dependent kinase gene is selected from 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 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 or 92 genes shown in a Table, such as Table 31 or Table 32.

[00209] For clarity, the testing of at least one differentially regulated calcium-dependent kinase gene may involve testing one gene or a group of 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 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 or 92 genes.

[00210] For the thirty-third to sixtieth forms of the invention, preferably the at least one differentially regulated calcium-dependent kinase gene is upregulated in gene expression.

[00211] For the thirty-third to sixtieth forms of the invention, preferably the at least one differentially regulated calcium-dependent kinase gene is downregulated in expression.

[00212] For the thirty-third to sixtieth forms of the invention the calcium-dependent kinase gene may be isolated from or tested in any suitable cell type or tissue. The calcium-dependent kinase gene may be of peripheral blood mononuclear cell original, although this need not be the case. The calcium-dependent kinase gene may be of Natural Killer cell origin.

[00213] For the thirty-third to sixtieth forms of the invention, preferably the biological sample contains peripheral blood mononuclear cells, including Natural Killer cells.

[00214] For the thirty-third to sixtieth forms of the invention, in some embodiments, the testing or measuring involves testing or measuring altered transcription/niRNA expression. In other embodiments, the testing or measuring involves testing or measuring altered translation/protein expression or another property or characteristic of the gene product/RNA/protein. Of course, determining differential regulation of a calcium-dependent kinase gene can be carried out as described for the first to thirty-second forms of the invention.

[00215] The inventors have also discovered that dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway of cells (such as NK cells), including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase

(ERK) l/2 as well as p38, may be used as an indicator that a subject has a particular medical condition or symptom thereof.

[00216] In a sixty-first form, the present invention resides in a method of identifying or diagnosing a subject having a medical condition or symptom thereof, comprising the step of testing a biological sample obtained from the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK) l/2 as well as p38, wherein dysfunctional signalling through the MAPK pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK) l/2 as well as p38, indicates that the subject has the medical condition or symptom thereof.

[00217] In a sixty-second form, the present invention resides in a method of identifying or diagnosing a subject having a medical condition or symptom thereof, said method comprising the steps of:

(a) obtaining at least one biological sample from the subject; and
(b) testing the biological sample for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK 1*12)* and extracellular signal-regulated kinase (ERK) l/2 as well as p38, wherein dysfunctional signalling through the MAPK pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, indicates that the subject has the medical condition or symptom thereof.

[00218] In a sixty-third form, the present invention resides in a method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:

(a) obtaining at least one biological sample from the subject; and
(b) testing the at least one biological sample for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK) l/2 as well as p38, wherein dysfunctional signalling through the MAPK pathway indicates that the subject has the medical condition or symptom thereof, and wherein no or less dysfunctional signalling through the MAPK pathway indicates that the subject is responding to management of the illness.

[00219] In a sixty-fourth form, the present invention resides in at least one probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, said at least one probe, tool or reagent being for assaying or characterising the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, using a biological sample derived from the subject.

[00220] In a sixty-fifth form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterising the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, using a biological sample derived from the subject.

[00221] For the sixtieth to sixty-fifth forms of the invention, the MAPK pathway of any suitable cell or tissue type may be tested/assayed. For example, in some embodiments, the biological sample may contain peripheral blood mononuclear cells. For example, in some embodiments, the biological sample may include Natural Killer cells.

[00222] Testing for dysfunctional signalling may be carried out in any suitable way. For example, dysfunctional signalling may be typified by reduced or increased phosphorylation, so phosphorylation or dephosphorylation may be tested. In other embodiments, the level of gene expression (RNA or protein) may be tested, or a property of the protein or biochemical function may be tested, as described for other forms of the invention as well as elsewhere in this specification.

[00223] For the sixtieth to sixty-fifth forms of the invention, preferably dysfunctional signalling is typified by reduced phosphorylation of ERK1/2. Preferably dysfunctional signalling is typified by increased phosphorylation of MEK1/2 and p38. Preferably dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in conjunction with increased phosphorylation ofMEKl/2 andp38.

[00224] For the sixtieth to sixty-fifth forms of the invention, preferably dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in $CD56^{dim}CD16^{+}$ NK cells in conjunction with increased phosphorylation of MEK1/2 and p38 in $CD56^{bright}CD16^{dim/-}$ NK cells.

[00225] For the sixtieth to sixty-fifth forms of the invention, preferably the medical condition

or symptom is: chronic fatigue syndrome (CFS) or symptom thereof; or, myalgic encephalomyelitis (ME) or symptom thereof. The CFS/ME may or may not be linked to a single nucleotide polymorphism (SNP) in a transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or ADR gene.

[00226] In a sixty-sixth form, the present invention resides in the use of calcium metabolism testing for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK7MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00227] In a sixty-seventh form, the present invention resides in calcium metabolism testing when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00228] In a sixty-eighth form, the present invention resides in a method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for a change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to: at

least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00229] In a sixty-ninth form, the present invention resides in a method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00230] In a seventieth form, the present invention resides in a method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of: (1) testing the subject for a change in calcium metabolism; and (2) managing the subject if the subject has been found to have said change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00231] In a seventy-first form, the present invention resides in a kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying calcium metabolic change in the subject, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00232] In a seventy-second form, the present invention resides in a kit or method for testing, screening or treating a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, for any calcium metabolite, wherein said medical condition or symptom thereof is optionally attributable to: at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene; differential regulation of at least one calcium-dependent kinase gene; and/or dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

[00233] The sixty-fifth to seventy-second forms of the invention can have features as described for the earlier forms of the invention.

[00234] Further features of all forms of the invention, including the first to seventy-second forms of the invention, are explained below.

[00235] Definitions

[00236] The term Oligonucleotide' refers to a single-stranded sequence of ribonucleotide or deoxyribonucleotide bases, known analogues of natural nucleotides, or mixtures thereof. An

oligonucleotide comprises a nucleic-acid based molecule including DNA, RNA, PNA, LNA, UNA or any combination thereof. Oligonucleotides are typically less than about 50 nucleotides in length and may be prepared by direct chemical synthesis or cloning and restriction of appropriate sequences.

[00237] The term 'polynucleotide' refers to a single- or double- stranded polymer of deoxyribonucleotide, ribonucleotide bases or known analogues of natural nucleotides, or mixtures thereof. A polynucleotide comprises a nucleic-acid based molecule including DNA, RNA, PNA, LNA, UNA or any combination thereof. The term includes reference to the specified sequence as well as to the sequence complimentary thereto, unless otherwise indicated. The term 'polynucleotide' includes chemically modified variants, as realised by those skilled in the art.

[00238] The term 'complementary' refers to the ability of two single-stranded nucleotide sequences to base pair, typically according to the Watson-Crick base pairing rules. For two nucleotide molecules to be complementary they need not display 100% complementarity across the base pairing regions, but rather there must be sufficient complementarity to enable base pairing to occur. Thus a degree of mismatching between the sequences may be tolerated and the sequences may still be complementary.

[00239] 'Nucleic acid' as used herein includes 'polynucleotide', 'oligonucleotide', and 'nucleic acid molecule', and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide.

[00240] As used herein, the term 'recombinant' refers to (i) molecules that are constructed outside living cells by joining natural or synthetic nucleic acid segments to nucleic acid molecules that can replicate in a living cell, or (ii) molecules that result from the replication of those described in (i) above. For purposes herein, the replication can be *in vitro* replication or *in vivo* replication.

[00241] The terms 'isolated', 'purified' and 'substantially purified' as used herein mean essentially free of association with other biological components/contaminants, e.g., as a naturally occurring protein that has been separated from cellular and other contaminants by the use of

antibodies or other methods or as a purification product of a recombinant host cell culture.

[00242] 'Probe' as used herein may mean an oligonucleotide capable of binding to a target nucleic acid/RNA of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. Probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. There may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids described herein. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. A probe may be single stranded or partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. Probes may be directly labeled or indirectly labeled such as with biotin to which a streptavidin complex may later bind. Probes may be used for screening and diagnostic methods, as described herein. The probes may be attached or immobilized to a solid substrate or apparatus, such as a biochip.

[00243] 'Target' as used herein (context allowing) can mean an oligonucleotide or portions or fragments thereof, which may be bound by one or more probes under stringent hybridization conditions.

[00244] Subject

[00245] The subject can be any mammal. Mammals include humans, primates, livestock and farm animals (eg. horses, sheep and pigs), companion animals (eg. dogs and cats), and laboratory test animals (eg. rats, mice and rabbits). The subject is preferably human.

[00246] Human subjects having CFS and/or ME can be defined as per the American CDC 1994 case definition [26a] and in the following citations [75a, 76a, 77a, 78a, 79a, 80a, 81a, 82a].

[00247] Non-fatigued/healthy controls/subjects (eg. not having CFS/ME) preferably have no medical history or symptoms of persistent fatigue or illness. Human subjects also preferably exclude individuals who were smokers, pregnant/breast-feeding or immobile, or had autoimmune, thyroid or cardiac related disorders prior to the onset of CFS/ME.

[00248] General techniques overview

[00249] The steps/techniques of isolating a biological sample from a subject, processing a

biological sample, genomic DNA extraction, RNA extraction, polypeptide extraction, DNA detection and characterisation, RNA detection and characterisation, polypeptide detection and characterisation, DNA sequencing, DNA sequence analyses, SNP genotyping studies, RNA location and identification, RNA profiling, RNA screening, RNA sequencing, RNA sequence analyses, measuring a level of expression of RNA, comparing expression levels (differential expression or dysregulation) of an RNA, polypeptide isolation, polypeptide sequencing and characterisation, measuring a level of polypeptide expression, comparing expression levels (differential expression or dysregulation) of a polypeptide, characterisation of dysfunctional signalling through the Mitogen-Activated Protein Kinase pathway of cells (such as PBMCs or NK cells), and detecting changes in calcium-dependent kinase pathways can be carried out in any suitable way.

[00250] It is to be appreciated that methodologies generally described for SNPs, such as differential expression or characterisation of RNA or protein or protein function etc, may equally apply to other forms of the invention, such as testing for changes in calcium metabolism, testing for dysfunctional signalling through the Mitogen-Activated Protein Kinase pathway or detecting changes in calcium-dependent kinase pathways.

[00251] It is also to be appreciated that methodologies generally described for any one form of the invention may equally be applicable to one or more other forms of the invention.

[00252] Biological sample

[00253] Any biological sample that comprises nucleic acid/a polynucleotide (eg. genomic DNA or RNA) from the subject is suitable for use in the methods of the invention. The biological sample can be processed so as to isolate the nucleic acid/polynucleotide. Alternatively, whole cells or other biological samples can be used without isolation of the nucleic acid/polynucleotides contained therein.

[00254] Any biological sample that comprises polypeptide/protein from the subject is suitable for use in the methods of the invention. The biological sample can be processed so as to isolate the polypeptide/protein. Alternatively, whole cells or other biological samples can be used without isolation of the nucleic polypeptide/protein contained therein.

[00255] Some forms of the invention concern a biological sample or a step of isolating one or more biological samples from a subject. Typically, any form of the invention concerning testing of a subject etc. may involve the step of isolating one or more biological samples from the subject and testing that/those. For example, testing for differences in gene expression/gene products may involve isolating more than one biological sample, even from different tissues of

that subject.

[00256] The biological sample can be any suitable sample derived from the subject - obtained either non-invasively or invasively. It can be cellular- or extracellular-derived, or both. For example: 1. Buccal (mouth) cells - obtained by swishing mouthwash in the mouth or by swabbing or brushing the inside of the cheek with a swab or brush; 2. Blood - obtained by pricking the finger and collecting the drops (dried blood spot) or by venepuncture (whole blood); 3. Skin - obtained by a (punch) biopsy; 4. Organ tissue - obtained by biopsy; 5. Plasma - obtained by blood plasma fractionation; 6. Urine - obtained by urination; 7. Faeces - obtained by stool sample; 8. Cerebrospinal fluid - obtained by spinal tap; and 9. Sputum - obtained by expectoration or nasotracheal suctioning.

[00257] Techniques for biological sample collection are well known to skilled persons.

[00258] In some embodiments, the biological sample can be a biofluid such as blood, plasma, serum, other blood isolate/component, urine, sputum, cerebrospinal fluid, milk, or ductal fluid, and can be fresh, frozen or fixed. In some preferred embodiments, for example, biofluid or biological sample comprising plasma or serum can be removed surgically and preferably by extraction, e.g. by hypodermic or other types of needles.

[00259] The biofluid typically will contain at least one SNP/gene/gene product (RNA and/or polpeptide) of interest, and will be relatively stable.

[00260] In some embodiments, plasma harvesting is employed. Plasma harvesting/extraction can be performed in any suitable way, but preferably immediately after peripheral blood collection. Plasma harvesting can involve a centrifugation step so as to separate the plasma from other blood components, and frozen storage of that plasma.

[00261] In some embodiments, different biological samples can be obtained from different tissues from one and the same subject.

[00262] Subject management

[00263] As used herein, the term 'managing' (or 'treating') a subject or 'management' is such that the medical condition or at least one symptom of the medical condition is cured, healed, alleviated, relieved, altered, remedied, ameliorated, or improved. Management can include administering one or more therapeutic compounds in an amount effective to alleviate, relieve, alter, remedy, ameliorate, improve, or affect the illness or a symptom of the illness. The terms

can also refer to providing the subject with a management regime which can comprise, for example, psychological counselling and/or administration of one or more therapeutic compounds by any appropriate route to achieve the desired effect. Administration can include, but is not limited to, oral, sublingual, parenteral (e.g., intravenous, subcutaneous, intracutaneous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional or intracranial injection), transdermal, topical, buccal, rectal, vaginal, nasal, ophthalmic, via inhalation, and implants. [Johnston, S., Staines, D., Brenu, E., & Marshall-Gradisnik, S. (2014). Management of Chronic Fatigue Syndrome: Current Approaches and Future Directions. In Chronic Fatigue Syndrome: Risk Factors, Management and Impacts on Daily Life (pp. 79-90). United States: Nova Science Publishers.]

[00264] In some embodiments, obtainment of the genotype from the biological sample being assayed, the genotype can be evaluated to determine if the subject is predisposed to the medical condition or symptom thereof, or to determine a treatment/management of the subject that is suffering from the medical condition or symptom thereof. In certain embodiments, the obtained genotype may be compared with a reference or control to make a diagnosis.

[00265] When comparing a subject sample to a reference or control, the reference can be any suitable control sample known in the art, such as, for example, a sample from a normal, healthy subject. In some embodiments, the reference can be a sample from the same subject prior to demonstration of illness symptoms or prior to identification with the medical condition or symptom thereof.

[00266] In some embodiments, the reference can be a 'standardised' sample, such as a sample comprising material or data from several samples, preferably also from several subjects.

[00267] Detection of polymorphism overview

[00268] Detection of a target polymorphism (SNP) in a polynucleotide sample derived from an individual can be accomplished by any means known in the art, including, but not limited to, amplification of a sequence with specific primers; determination of the nucleotide sequence of the polynucleotide sample; hybridization analysis; single strand conformational polymorphism analysis; denaturing gradient gel electrophoresis; mismatch cleavage detection; exome sequencing and the like.

[00269] Detection of a target polymorphism can also be accomplished by detecting an alteration in the level of an RNA/mRNA transcript of the gene; aberrant modification of the corresponding gene, e.g., an aberrant methylation pattern; the presence of a non-wild-type splicing pattern of the corresponding transcript/mRNA; an alteration in the expression or translation level of the corresponding polypeptide; an alteration in the length of the corresponding polypeptide; and/or an alteration in corresponding polypeptide activity.

[00270] Polymorphism detection methodologies

[00271] As mentioned, detection of a target polymorphism by analyzing a polynucleotide sample can be conducted in a number of ways. A test nucleic acid sample can be amplified with primers which amplify a region known to comprise the target polymorphism(s). Genomic DNA or mRNA can be used directly. Alternatively, the region of interest can be cloned into a suitable vector and grown in sufficient quantity for analysis. The nucleic acid may be amplified by conventional techniques, such as a polymerase chain reaction (PCR), to provide sufficient amounts for analysis. The use of the polymerase chain reaction is described in a variety of publications, including, e.g., "PCR Protocols (Methods in Molecular Biology)" (2000) J. M.S. Bartlett and D. Stirling, eds, Humana Press; and "PCR Applications: Protocols for Functional Genomics" (1999) Innis, Gelfand, and Sninsky, eds., Academic Press. Once the region comprising a target polymorphism has been amplified, the target polymorphism can be detected in the PCR product by nucleotide sequencing, by Single Strand Conformation Polymorphism (SSCP) analysis, or any other method known in the art. In performing SSCP analysis, the PCR product may be digested with a restriction endonuclease that recognizes a sequence within the PCR product generated by using as a template a reference sequence, but does not recognize a corresponding PCR product generated by using as a template a variant sequence by virtue of the fact that the variant sequence no longer contains a recognition site for the restriction endonuclease.

[00272] PCR can also be used to determine whether a polymorphism is present by using a primer that is specific for the polymorphism. Such methods can comprise the steps of collecting from a subject a biological sample comprising the subject's genetic material as template, optionally isolating template nucleic acid (genomic DNA, mRNA, or both) from the biological sample, contacting the template nucleic acid sample with one or more primers that specifically hybridize with a target polymorphic nucleic acid molecule under conditions such that hybridization and amplification of the template nucleic acid molecules in the sample occurs, and detecting the presence, absence, and/or relative amount of an amplification product and comparing the length to a control sample. Observation of an amplification product of the expected size is an indication that the target polymorphism contained within the target polymorphic primer is present in the test nucleic acid sample. Parameters such as hybridization conditions, polymorphic primer length, and position of the polymorphism within the polymorphic primer can be chosen such that hybridization will not occur unless a polymorphism present in the primer(s) is also present in the sample nucleic acid. Those of ordinary skill in the art are well aware of how to select and vary such parameters. See, e.g., Saiki et al. (1986) Nature 324:163; and Saiki et al (1989) Proc. Natl. Acad. Sci USA 86:6230.

[00273] Alternatively, various methods are known in the art that utilize oligonucleotide ligation as a means of detecting polymorphisms. See, e.g., Riley et al. (1990) Nucleic Acids Res. 18:2887-2890; and Delahunty et al. (1996) Am. J. Hum. Genet. 58:1239-1246.

[00274] A detectable label may be included in an amplification reaction. Suitable labels include fluorochromes, e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'- dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, e.g. ^{32}P, ^{35}S, ^{3}H; etc. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, etc. having a high affinity binding partner, e.g. avidin, specific antibodies, etc., where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

[00275] The sample nucleic acid can be sequenced by a dideoxy chain termination method or other well-known methods. Genomic DNA or mRNA may be used directly. If mRNA is used, a cDNA copy may first be made. If desired, the sample nucleic acid can be amplified using a PCR step. A variety of sequencing reactions known in the art can be used to directly sequence the relevant gene, or a portion thereof in which a specific polymorphism is known to occur, and detect polymorphisms by comparing the sequence of the sample nucleic acid with a reference polynucleotide that contains a target polymorphism. Any of a variety of automated sequencing procedures can be used. See, e.g., WO 94/16101; Cohen et al. (1996) Adv. Chromatography 36:127-162.

[00276] Hybridization with the variant sequence can also be used to determine the presence of a target polymorphism. Hybridization analysis can be carried out in a number of different ways, including, but not limited to Southern blots, Northern blots, dot blots, microarrays, etc. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in U.S. 5,445,934, or in WO 95/35505, may also be used as a means of detecting the presence of variant sequences. Identification of a polymorphism in a nucleic acid sample can be performed by hybridizing a sample and control nucleic acids to high density arrays containing hundreds or thousands of oligonucleotide probes. Cronin et al. (1996) Human Mutation 7:244-255; and Kozal et al. (1996) Nature Med. 2:753-759.

[00277] Single strand conformational polymorphism (SSCP) analysis; denaturing gradient gel electrophoresis (DGGE); mismatch cleavage detection; and heteroduplex analysis in gel matrices can also be used to detect polymorphisms. Alternatively, where a polymorphism creates or

destroys a recognition site for a restriction endonuclease (restriction fragment length polymorphism, RFLP), the sample is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel or capillary electrophoresis, particularly acrylamide or agarose gels. The aforementioned techniques are well known in the art. Detailed description of these techniques can be found in a variety of publications, including, e.g., "Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA" (1997) G. R. Taylor, ed., CRC Press, and references cited therein.

[00278] SNP detection

[00279] As mentioned above, various methods can be used to determine the presence or absence of a SNP in a subject/biological sample. Genotype can be determined, for example, by microarray analysis, sequencing, primer extension, ligation of allele specific oligonucleotides, mass determination of primer extension products, restriction length polymorphism analysis, single strand conformational polymorphism analysis, pyrosequencing, dHPLC or denaturing gradient gel electrophoresis (DGGE). Furthermore, having sequenced nucleic acid of a subject or sample, the sequence information can be retained and subsequently searched without recourse to the original nucleic acid itself. Thus, for example, a sequence alteration or mutation may be identified by scanning a database of sequence information using a computer or other electronic means.

[00280] In general, nucleic acid regions which contain the SNPs of interest (target regions) are preferably subjected to an amplification reaction. Any suitable technique or method may be used for amplification. In general, where multiple SNPs are to be analysed, it is preferable to simultaneously amplify all of the corresponding target regions (comprising the nucleotide variations).

[00281] Some embodiments of the invention can comprise determining the binding of an oligonucleotide probe to a genomic sample. The probe can comprise a nucleotide sequence which binds specifically to a particular SNP. Suitable oligonucleotide probes can be derived based on the SNP and nucleotide sequences of any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, and 34 to 36. The oligonucleotide probe may comprise a label and binding of the probe can be determined by detecting the presence of the label.

[00282] Some embodiments of the invention can comprise hybridising of one, two or more oligonucleotide probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation can be as part of an amplification, e.g. PCR procedure, or as part of a probing procedure not involving amplification, e.g. PCR. An example procedure would

be a combination of PCR and low stringency hybridisation. Any suitable screening procedure can be used to identify successful hybridisation events and isolated hybridised nucleic acid.

[00283] Binding of a probe to target nucleic acid (e.g. DNA) can be measured using any of a variety of techniques. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms, amplification using PCR, RNase cleavage and allele specific oligonucleotide probing. Probing can employ the standard Southern blotting technique. For instance, DNA can be extracted from cells and digested with different restriction enzymes. Restriction fragments can then be separated by electrophoresis on an agarose gel, before denaturation and transferred to a nitrocellulose filter. Labelled probe can be hybridised to the DNA fragments on the filter and binding determined. DNA for probing can be prepared from RNA preparations from cells. Suitable stringency for selective hybridisation, oligonucleotide length, base composition and temperature can be readily determined by the skilled addressee.

[00284] For example, suitable selective hybridisation conditions for oligonucleotides of 17 to 30 bases include hybridization overnight at 42°C in 6X SSC and washing in 6X SSC at a series of increasing temperatures from 42°C to 65°C. Other suitable conditions and protocols are described in Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press and Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

[00285] An oligonucleotide for use in nucleic acid amplification can be about 30 or fewer nucleotides in length (e.g. 18, 20, 22, 24 or 26). Generally, specific primers are upwards of 14 nucleotides in length. Those skilled in the art are well versed in the design of primers for use in processes such as PCR. Suitable oligonucleotides can be designed based on the SNPs or sequences of any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, and 34 to 36. Various techniques for synthesizing oligonucleotide primers are well known in the art, including phosphotriester and phosphodiester synthesis methods. Primers and primer pairs suitable for amplification of nucleic acid regions comprising the sequences in Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34 can be readily developed by those of skill in the art. For examples, see Tables 35 and 36.

[00286] Nucleic acid can also be screened using a variant- or allele-specific probe. Such a probe can correspond in sequence to a region of genomic nucleic acid, or its complement, which contains one or more of the SNPs of interest. Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the sequence alteration in the test nucleic acid. For efficient screening purposes, more than one probe can be used on the same test sample. Suitable probes can be designed based on the SNPs or sequences of any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34 to 36.

[00287] Nucleic acid in a test sample, which can be a genomic sample or an amplified region thereof, can be sequenced to identify or determine the identity of a polymorphic allele. The allele of the SNP in the test nucleic acid can therefore be compared with the SNP as described herein in Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34 to determine whether the test nucleic acid contains one or more alleles which are associated with the medical condition or symptom thereof.

[00288] Since it will not generally be time- or labour-efficient to sequence all nucleic acid in a test sample, a specific amplification reaction such as PCR using one or more pairs of primers can be employed to amplify the region of interest in the nucleic acid, for instance the particular region in which the SNPs of interest occur. The amplified nucleic acid can then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular nucleotide. Nucleic acid for testing can be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

[00289] Sequencing of an amplified product can involve precipitation with isopropanol, resuspension and sequencing using a TaqFS+ Dye terminator sequencing kit. Extension products may be electrophoresed on an ABI 377 DNA sequencer and data analysed using Sequence Navigator software.

[00290] Nucleic acid in a test sample can be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR) (reviewed for instance in "PCR protocols; A Guide to Methods and Applications", Eds. lnnis et al, 1990, Academic Press, New York, Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 5 1 :263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, and Ehrlich et al, Science, 252:1643-1650, (1991)). PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA.

[00291] Other specific nucleic acid amplification techniques include strand displacement activation, the QB replicase system, the repair chain reaction, the ligase chain reaction, rolling circle amplification and ligation activated transcription. Methods of the present invention may therefore comprise amplifying the region in said genomic sample containing the one or more positions of single nucleotide polymorphism of interest.

[00292] Allele-specific oligonucleotides can be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the polymorphism on a denaturing

polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected.

[00293] In some embodiments, the region of genomic sample comprising a polymorphism can be amplified using a pair of oligonucleotide primers, of which the first member of the pair comprises a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' of the position of single nucleotide polymorphism, and the second member of the primer pair comprises a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 3' of the position of single nucleotide polymorphism.

[00294] In other embodiments, the first member of the pair of oligonucleotide primers can comprise a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' or 3' of the polymorphism, and the second member of the pair can comprise a nucleotide sequence which hybridises under stringent conditions to a particular allele of the polymorphism and not to other alleles, such that amplification only occurs in the presence of the particular allele.

[00295] A further aspect of the present invention provides a pair of oligonucleotide amplification primers. A suitable pair of amplification primers according to this aspect can have a first member comprising a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' of a single nucleotide polymorphism and a second member comprising a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 3' of the single nucleotide polymorphism.

[00296] The allele of the at least one polymorphism (i.e. the identity of the nucleotide at the position of single nucleotide polymorphism) can then be determined by determining the binding of an oligonucleotide probe to the amplified region of the genomic sample. A suitable oligonucleotide probe comprises a nucleotide sequence which binds specifically to a particular allele of the at least one polymorphism and does not bind specifically to other alleles of the at least one polymorphism.

[00297] Other suitable pairs of amplification primers can have a first member comprising a nucleotide sequence which hybridises to a complementary sequence which is proximal to and 5' or 3' of a single nucleotide polymorphism and a second member of the pair comprising a nucleotide sequence which hybridises under stringent conditions to a particular allele of the polymorphism and not to other alleles, such that amplification only occurs in the presence of the particular allele.

[00298] PCR primers suitable for amplification of target DNA regions comprising the SNPs in Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34 or sequences of Tables 35 and 36 can be readily prepared by the skilled addressee. A further aspect of the present invention provides an

oligonucleotide which hybridises specifically to a nucleic acid sequence which comprises a particular allele of a polymorphism selected from the group consisting of any one of the single nucleotide polymorphisms shown in Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34, and does not bind specifically to other alleles of the SNP. Hybridisation may be determined under suitable selective hybridisation conditions as described herein.

[00299] Such oligonucleotides may be used in a method of screening nucleic acid.

[00300] In some preferred embodiments, oligonucleotides according to the present invention are at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length. Oligonucleotides may be up to about 100 nucleotides in length, more preferably up to about 50 nucleotides in length, more preferably up to about 30 nucleotides in length. The boundary value 'about X nucleotides' as used above includes the boundary value 'X nucleotides'.

[00301] Approaches which rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH etc.), an oligonucleotide probe will hybridise with a sequence which is not entirely complementary. The degree of base-pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis- match between two annealing nucleic acid molecules. For instance, RNase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresis test nucleic acid to which the relevant probe or probe has annealed and looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full length probe/test hybrid.

[00302] Genotype analysis may be carried out by microarray analysis. Any suitable microarray technology may be used. Preferably the methodology reported in International Patent Application No. PCT/IB2006/00796 filed 12 January 2006 (the contents of which are hereby incorporated by reference) is used. This technology uses a low-density DNA array and hybridisation to allele-specific oligonucleotide probes to screen for SNPs.

[00303] Typically in this technology, nucleic acid regions which contain the SNPs of interest (target regions) may be subjected to an amplification reaction. Any suitable technique or method may be used for amplification. In general, where multiple SNPs are to be analysed, it is preferable to simultaneously amplify all of the corresponding target regions (comprising the variations).

[00304] For example, multiplex PCR may be carried out, using appropriate pairs of oligonucleotide PCR primers. Any suitable pair of primers which allow specific amplification of a target region may be used. In one aspect, the primers allow amplification in the fewest possible

number of PCR reactions.

[00305] Following amplification, the amplified nucleic acid may undergo fragmentation, e.g. by digestion with a suitable nuclease such as DNAse I. Typically the amplified (optionally fragmented) DNA is then labelled. Suitable labels are known in the art.

[00306] A microarray typically comprises a plurality of probes deposited on a solid support. In general the solid support comprises oligonucleotide probes suitable for discrimination between possible nucleotides at each SNP variable to be determined in the method. The microarray typically also comprises additional positive and/or negative controls.

[00307] Typically, for a SNP with the possible alleles A and B, there will be at least one probe which is capable of hybridising specifically to allele A (probe 1) and one probe which is capable of hybridising specifically to allele B (probe 2) under the selected hybridisation conditions. These probes form a probe pair. Typically the probes can be used to discriminate between A and B (e.g. the wildtype and mutant alleles). The probes may examine either the sense or the antisense strand. Typically, probes 1 and 2 examine the same nucleic acid strand (e.g. the sense strand or antisense strand) although in some cases the probes may examine different strands. In one aspect probes 1 and 2 have the same sequence except for the site of the genetic variation.

[00308] In one instance, the probes in a probe pair have the same length. In some aspects, where two or more pairs of probes are provided for analysis of a genetic variation, the probes may all have the same length.

[00309] Preferably more than one probe pair is provided for detection of each genetic variation. Thus, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more probe pairs may be provided per genetic variation. In one aspect, (at least) 2 probe pairs are provided. The aim is to reduce the rate of false positives and negatives in the present methods.

[003 10] For example, for a given genetic variation there may be:

[003 11] Probe 1 which is capable of hybridising to genetic variation A (e.g. a normal allele)

[003 12] Probe 2 which is capable of hybridising to genetic variation B (e.g. a mutant allele)

[003 13] Probe 3 which is capable of hybridising to genetic variation A (e.g. a normal allele)

[003 14] Probe 4 which is capable of hybridising to genetic variation B (e.g. a mutant allele).

[00315] The probes may examine the same or different strands. Thus in one embodiment, probes 3 and 4 are the complementary probes of probes 1 and 2 respectively and are designed to examine the complementary strand. In one aspect it is preferred that the probes provided for detection of each genetic variation examine both strands.

[00316] More than 2 pairs of probes may be provided for analysis of a genetic variation as above. For example, where a genetic variation exists as any one of 4 bases in the same strand

(e.g. there are three mutant possibilities), at least one pair of probes may be provided to detect each possibility. Preferably, at least 2 pairs of probes are provided for each possibility.

[00317] A number of methods are known in the art for designing oligonucleotide probes suitable for use in DNA-chips. These include "standard tiling", "alternative tiling" "block tiling" and "alternative block tiling". Any one or more of these strategies may be used to design probes for the present invention. Preferably standard tiling is used, in particular with 2 pairs of probes e.g. 2 pairs of complementary probes as above. Thus it is preferable that the oligonucleotide sequence is complementary to the target DNA or sequence in the regions flanking the variable nucleotide(s). However, in some cases, one or more mismatches may be introduced. The oligonucleotide probes for use in the present invention typically present the base to be examined (the site of the genetic variation) at the centre of the oligonucleotide.

[00318] In general the probes for use in the present invention comprise or in some embodiments consist (essentially) of 17 to 27 nucleotides, for example, 19, 21, 23, or 25 nucleotides or 18, 20, 22, 24 or 26 nucleotides.

[00319] The probes provided for detection of each genetic variation (as described above) are typically capable of discriminating between genetic variants A and B (e.g. the normal and mutant alleles) under the selected hybridisation conditions. Preferably the discrimination capacity of the probes is substantially 100%. If the discrimination capacity is not 100%, the probes are preferably redesigned. Preferably the melting temperature of the probe/target complexes is in the range of 75-85 ° C.

[00320] In general probes are provided on the support in replicate. Typically, at least 4, 6, 8, 10, 12, 14, 16, 18 or 20 replicates are provided of each probe, in particular, 6, 8 or 10 replicates. Thus for example, the support (or DNA-chip) may comprise or include 10 replicates for each of (at least) 4 probes used to detect each genetic variation (i.e. 40 probes). Alternatively the support (or DNA- chip) may comprise or include 8 replicates for each of (at least) 4 probes used to detect each genetic variation (i.e. 32 probes). Still further the support (or DNA-chip) may comprise or include 6 replicates for each of (at least) 4 probes used to detect each genetic variation (i.e. 24 probes). In general the support also comprises one or more control oligonucleotide probes which are useful as positive and/or negative controls of the hybridisation reactions. These are also provided in replicate as above.

[00321] Typically the chip or array will include positive control probes, e.g., probes known to be complementary and hybridisable to sequences in the target polynucleotide molecules, probes known to hybridise to an external control DNA, and negative control probes, e.g., probes known to not be complementary and hybridizable to sequences in the target polynucleotide molecules. The chip may have one or more controls specific for each target, for example, 2, 3, or more

controls. There may also be at least one control for the array.

[00322] Positive control probes are generally designed to hybridise equally to all target DNA samples and provide a reference signal intensity against which hybridisation of the target DNA (sample) to the test probes can be compared. Negative controls comprise either "blanks" where only solvent (DMSO) has been applied to the support or control oligonucleotides that have been selected to show no, or only minimal, hybridisation to the target, e.g. human, DNA (the test DNA). The intensity of any signal detected at either blank or negative control oligonucleotide features is an indication of non-specific interactions between the sample DNA and the array and is thus a measure of the background signal against which the signal from real probe-sample interactions must be discriminated.

[00323] Desirably, the number of sequences in the array will be such that where the number of nucleic acids suitable for detection of genetic variations is n, the number of positive and negative control nucleic acids is n', where n' is typically from 0.01 to 0.4n.

[00324] One example of a DNA chip/microarray which may be used is Fibrochip.

[00325] A Fibro-chip comprises oligonucleotide probes suitable for detection of some or all of the genetic variations (SNPs) in Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34.

[00326] In general an array comprises a support or surface with an ordered array of binding (e.g. hybridisation) sites or probes. Each probe (i.e. each probe replicate) is located at a known predetermined position on the solid support such that the identity (i.e. the sequence) of each probe can be determined from its position in the array. Preferably, the probes deposited on the support, although they maintain a predetermined arrangement, are not grouped by genetic variation but have a random distribution. Typically they are also not grouped within the same genetic variation. If desired, this random distribution can be always the same. Probes may be arranged on the support in subarrays.

[00327] The support, on which the plurality of probes is deposited, can be any solid support to which oligonucleotides can be attached. For example, the said support can be of a non-porous material, for example, glass, silicon, plastic, or a porous material such as a membrane or filter (for example, nylon, nitrocellulose) or a gel. In one embodiment, the said support is a glass support, such as a glass slide.

[00328] Probes may be attached to the support using conventional techniques for immobilization of oligonucleotides on the surface of the supports.

[00329] In one embodiment, the support is a glass slide and in this case, the probes, in the number of established replicates (for example, 6, 8 or 10) are printed on pre-treated glass slides, for example coated with aminosilanes, using equipment for automated production of DNA-chips by deposition of the oligonucleotides on the glass slides ("micro-arrayer"). Deposition is carried

out under appropriate conditions, for example, by means of crosslinking with ultraviolet radiation and heating (80°C), maintaining the humidity and controlling the temperature during the process of deposition, typically at a relative humidity of between 40-50% and typically at a temperature of 20°C.

[00330] The replicate probes are distributed uniformly amongst the areas or sectors (sub-arrays), which typically constitute a DNA-chip. The number of replicas and their uniform distribution across the DNA-chip minimizes the variability arising from the printing process that can affect experimental results. Likewise, positive and negative hybridisation controls (as described herein) may be printed.

[00331] To control the quality of the manufacturing process of the DNA-chip, in terms of hybridization signal, background noise, specificity, sensitivity and reproducibility of each replica as well as differences caused by variations in the morphology of the spotted probe features after printing, a commercial DNA can be used. For example, as a quality control of the printing of the DNA-chips, hybridization may be carried out with a commercial DNA (e.g. k562 DNA High Molecular Weight, Promega)

[00332] In general, methods for using microarrays for genotyping are known in the art.

[00333] In one aspect the data from the present microarrays may be analysed and used to determine genotype according to the methods in International Patent Application No. PCT/TB2006/00796 filed 12 January 2006, the contents of which are hereby incorporated by reference. Typically, following amplification of the target DNA and optional fragmentation (e.g. by digestion with DNase I), the target DNA is labelled as described herein.

[00334] The labelled DNA may then be hybridised with a microarray under suitable hybridisation conditions which may be determined by the skilled person. For example, an automatic hybridisation station may be used.

[00335] In general the microarray is then scanned and the label intensities at the specific probe positions determined in order to determine which allele is present in the target DNA hybridised to the array.

[00336] In one aspect, following hybridisation, the signal intensity of the label is detected at each probe position on the microarray to determine extent of hybridisation at each position. This may be done by any means suitable for detecting and quantifying the given label. For example, fluorescent labels may be quantified using a confocal fluorescent scanner.

[00337] This signal intensity value is typically corrected to eliminate background noise by means of controls on the array. Where a microarray includes probe pairs and probe replicates as described herein, a hybridisation signal mean can then be calculated for each probe (based on the signals from the probes replicates). The ratio of the hybridisation signal mean of the A allele to

the sum of the hybridisation signal means of the A and B alleles can then be defined for each probe pair used for genotyping of each SNP (ratios 1 and 2).

[00338] The 2 ratio values corresponding to each of the 3 possible genotypes (AA, AB and BB) may be calculated using target DNA from control individuals of each genotype identified previously by, e.g. sequence analysis (at least 10 per genotype).

[00339] By comparison of test DNA results with the control ratios, a genotype may be assigned to a test individual. This may be done using the MG 1.0 software.

[00340] As mentioned above, genotyping may also be carried out using sequencing methods. Typically, nucleic acid comprising the SNPs of interest is isolated and amplified as described herein. Primers complementary to the target sequence are designed so that they are a suitable distance (e.g. 50-400 nucleotides) from the polymorphism. Sequencing is then carried out using conventional techniques. For example, primers may be designed using software that aims to select sequence(s) within an appropriate window which have suitable Tm values and do not possess secondary structure or that will hybridise to non-target sequence.

[00341] Additional references describing various protocols for detecting the presence of a target polymorphism include, but are not limited to, those described in: US Patent Nos. 6,703,228; 6,692,909; 6,670,464; 6,660,476; 6,653,079; 6,632,606; 6,573,049; the disclosures of which are herein incorporated by reference.

[00342] Exome sequencing

[00343] SNPs can be identified and characterised using exome sequencing. Exome sequencing (also known as Whole Exome Sequencing or WES) is a technique for sequencing all the protein-coding genes in a genome (known as the exome). It consists of first selecting only the subset of DNA that encodes proteins (known as exons), and then sequencing that DNA using any high throughput DNA sequencing technology. Different target-enrichment techniques are briefly described below:

[00344] PCR - PCR is technology to amplify specific DNA sequences. It uses a single stranded piece of DNA as a start for DNA amplification. Uniplex PCR uses only one starting point (primer) for amplification and multiplex PCR uses multiple primers.

[00345] Molecular inversion probes (MIP) - Molecular inversion probe uses probes of single stranded DNA oligonucleotides flanked by target-specific ends. The gaps between the flanking sequences are filled and ligated to form a circular DNA fragment. Probes that did not undergo reaction remain linear and are removed using exonucleases.

[00346] Hybrid capture - Microarrays contain single-stranded oligonucleotides with sequences from the human genome to tile the region of interest fixed to the surface. Genomic DNA is sheared to form double-stranded fragments. The fragments undergo end-repair to

produce blunt ends and adaptors with universal priming sequences are added. These fragments are hybridized to oligos on the microarray. Unhybridized fragments are washed away and the desired fragments are eluted. The fragments are then amplified using PCR.

[00347] In-solution capture - To capture genomic regions of interest using in-solution capture, a pool of custom oligonucleotides (probes) is synthesized and hybridized in solution to a fragmented genomic DNA sample. The probes (labeled with beads) selectively hybridize to the genomic regions of interest after which the beads (now including the DNA fragments of interest) can be pulled down and washed to clear excess material. The beads are then removed and the genomic fragments can be sequenced allowing for selective DNA sequencing of genomic regions (e.g., exons) of interest.

[00348] Sequencing - Sequencing platforms include the classical Sanger sequencing, the Roche 454 sequencer, the Illumina Genome Analyzer II and the Life Technologies SOLiD & Ion Torrent - all of which have been used for exome sequencing.

[00349] Sequencing types: Sanger sequencing; SNP sequencing of exome; pyrosequencing; RNA sequencing; and, protein sequencing.

[00350] Expression level detection methodologies

[00351] Biochemical studies may be performed to determine whether a sequence polymorphism in a coding region or control region of interest is associated with the medical condition. Condition-associated polymorphisms may include deletion or truncation of the gene, mutations that alter expression level, that affect the activity of the polypeptide, etc.

[00352] A number of methods are available for determining the expression level of a polymorphic nucleic acid molecule, e.g., RNA/mRNA or a polymorphic polypeptide (protein) in a particular sample. Diagnosis may be performed by a number of methods to determine the absence or presence or altered amounts of normal or abnormal RNA/mRNA or polypeptide in a patient sample.

[00353] Characterisation of RNA expression

[00354] Methods of the subject invention in which the level of (polymorphic) gene expression is of interest will typically involve comparison of the relevant nucleic acid abundance of a sample of interest with that of a control value to determine any relative differences, where the difference may be measured qualitatively and/or quantitatively, which differences are then related to the presence or absence of an abnormal gene expression pattern.

[00355] A variety of different methods for determining the nucleic acid abundance in a sample are known to those of skill in the art, where particular methods of interest include those described in: Pietu et al., Genome Res. (June 1996) 6: 492-503; Zhao et al., Gene (April 24, 1995) 156: 207-213; Soares , Curr. Opin. Biotechnol. (October 1997) 8: 542-546; Raval, J.

Pharmacol Toxicol Methods (November 1994) 32: 125-127; Chalifour et al., Anal. Biochem (February z, 1994) 216: 299-304; Stolz & Tuan, Mol. Biotechnol. (December 19960 6: 225-230; Hong et al., Bioscience Reports (1982) 2: 907; and McGraw, Anal. Biochem. (1984) 143: 298. Also of interest are the methods disclosed in WO 97/27317, the disclosure of which is herein incorporated by reference.

[00356] RNA manipulation techniques are described, for example, in the following references, the entire contents of which are incorporated herein:

[00357] PureLink (Invitrogen), Trizol reagent (Invitrogen), Stratagene (total and small RNA), TRI-Reagent (Sigma-Aldrich), Nucleospin (Machery-Nagel) and RNA-Bee (Tel-test). Reference [89a].

[00358] The degree to which RNA expression differs need only be large enough to quantify via standard characterization techniques such as expression arrays, RT-qPCR, Northern analysis and RNase protection.

[00359] Blotting and hybridization assays: [103a, 104a].

[00360] Microarrays: [105a].

[00361] Next generation assays covering all platforms: [107a].

[00362] Different ways of assaying expression: Real time PCR, Affymetrix, Agilent, Illumina, and Nanostring.

[00363] Profiling methods: Agilent microarray, exiqon array, exiqon microarray, miRCURY LNA ncode array, LC Sciences array ABI Taqman array, affymetrix , illumine array, SOLiD ligation sequencing, Illumina HiSeq and TaqMan miR assay.

[00364] Tools or reagents for assaying for RNA differential expression: SYBR green probes and TaqMan probes.

[00365] Radiolabeled splinted ligation detection: [110a, 111a].

[00366] Preferably, for one or more methods of the present invention, the level of RNA expression or differential expression can be carried out using: Northern analysis and a probe that specifically binds to the RNA; RNase protection; or, reverse transcription-polymerase chain reaction (RT-PCR) using one or more oligonucleotides/primers that will amplify transcribed RNA. A universal primer can be used in combination with the one or more oligonucleotides/primers that will amplify transcribed RNA. Preferably, RT-qPCR is used. Preferably, for one or more methods of the present invention, the method/s can comprise the step of statistical analysis so as to identify differential expression.

[00367] In some embodiments, RNA can be extracted from plasma using a commercially available kit. The size, quantity and quality of the extracted RNA can be assessed using a small RNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

[00368] RNA profiling and sequencing can be carried out in any suitable way. Preferably high throughput sequencing (HTS) is utilised. RNA libraries can be constructed using the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego, CA). RNA samples can be ligated with 5' and 3' adapters, followed by reverse transcription-polymerase chain reaction (RT-PCR) for cDNA library construction and incorporation of index tags. The cDNA library fragments can be separated and size fractioned. cDNA library samples can be pooled in equimolar amounts and used for cluster generation and sequence analysis.

[00369] Sequence data that has been generated can be analysed in any suitable way. In some embodiments, raw FASTQ sequences can be generated.

[00370] In some embodiments, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) may be used for expression and comparison.

[00371] Polypeptide characterisation

[00372] One may screen for polymorphisms at the protein level. Screening for mutations in a polymorphic polypeptide may be based on the functional or antigenic characteristics of the protein. Functional assays include cofactor binding assays, enzyme activity assays, substrate binding assays or surface expression assays. For example, protein truncation assays are useful in detecting deletions that may affect the biological activity of the protein. The activity of the encoded a polymorphic polypeptide may be determined by comparison with a reference polypeptide lacking a specific polymorphism. Alternatively, the three-dimensional structure of the protein may be assayed, for example by fluorescence polarization or circular dichroism spectroscopy, wherein the three-dimensional structure of the encoded a polymorphic polypeptide may be determined by comparison with purified protein carrying the opposing allele of the polymorphism.

[00373] Alternatively, various immunoassays designed to detect polymorphisms in polymorphic polypeptides may be used. The absence or presence of antibody binding to a polymorphic polypeptide may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, etc. Immunocytochemistry and flow cytometry, particularly fluorescence-activated cell sorting (FACS), can be used to evaluate cell-surface expression of proteins on cells, including on the different types of blood cells.

[00374] Detailed descriptions of how to make antibodies, including antibodies that are specific for epitopes, for example, single amino acid substitutions within epitopes, can be found in a variety of publications, including, e.g. "Making and using Antibodies: A Practical Handbook" (2006) G.C. Howard and M.R. Kaser, eds. CRC Press; "Antibody Engineering: Methods and Protocols" (2004) B.K.C. Lo, ed, Humana Press; and US Patent No. 6,054,632, the disclosure of which is herein incorporated by reference.

[00375] Methods for performing protein sequencing include: Edman degradation; peptide mass fingerprinting; mass spectrometry; and, protease digests. For example, detection may utilize staining of cells or histological sections with labeled antibodies, performed in accordance with conventional methods. Cells are permeabilized to stain cytoplasmic molecules. The antibodies of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Alternatively, the secondary antibody conjugated to a fluorescent compound, e.g. fluorescein, rhodamine, Texas red, etc. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase.

[00376] The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, etc. Detailed descriptions of how to make antibodies can be found in a variety of publications, including, e.g. "Making and using Antibodies: A Practical Handbook" (2006) G.C. Howard and M.R. Kaser, eds. CRC Press; "Antibody Engineering: Methods and Protocols" (2004) B.K.C. Lo, ed, Humana Press.

[00377] The techniques described above can be used to assay TRP ion channel, ACh receptor and/or ADR expression in or on the surface of cells, preferably blood cells such as NK, T and/or B cells for TRP and ACh receptor, whereby reduced expression of TRP ion channel, ACh receptor and/or ADR is typically indicative of the subject having the medical condition or symptom thereof or a predisposition to the medical condition or symptom thereof.

[00378] Calcium testing

[00379] Testing for a change in calcium metabolism/calcium metabolic change in a subject can be achieved in any suitable way. For example, all calcium-dependent biochemical processes and genes can be assessed/tested. For example, detection of Ca++ and its signaling mechanisms in all cells and tissues of the body may be utilised. For example, the effects of Ca++ on gene expression may be assessed. For example, the effects of Ca++ on all transcription factors in all cells and tissues of the body and their associated genes can be assessed. For example, testing can include muscle biopsy, blood samples (e.g. immune cells), radiological investigations, cardiac assessments such as exercise testing which manifest Ca++ signaling or disorders of same. For example, Ca++ regulatory mechanisms including IP3, Calcineurin, Calmodulin, ORI, DAG etc, which may be affected can be assessed. For example: assessment of inter- or intra- calcium

wave signaling or other Ca++ signaling mechanisms can be undertaken. [See references lf-6f, for example, as well as other references in the Examples.]

[00380] Preferably, testing for a change in calcium metabolism involves testing for a change in Ca^{2+} cell signaling.

[00381] Testing for a change in calcium metabolism/calcium metabolic change in a subject may involve using calcium channel blockers (CCB), calcium channel antagonists, calcium antagonists or calcium agonists.

[00382] Examples of these include:

[00383] · Amlodipine (Norvasc)

[00384] · Diltiazem (Cardizem, Tiazac), Cardizem CD, Cardizem SR, Dilacor XR, Diltia XT

[00385] · Felodipine

[00386] · Isradipine

[00387] · Nicardipine (Cardene SR)

[00388] · Nifedipine (Procardia)

[00389] · Nisoldipine (Sular)

[00390] · Verapamil (Calan, Verelan, Covera-HS) Calan SR, Covera-HS, Isoptin, Isoptin SR, Verelan PM

[00391] · Cardene, Cardene SR (nicardipine)

[00392] · Sular (nisoldipine)

[00393] · Vascor (bepridil)

[00394] Examples of agonists and antagonists (inhibitors and activators), available from Sigma-Aldrich, include:

[00395] · A-967079 (Product # SML0085)

[00396] · AC-265347 (Product # SML0129)

[00397] · Amiloride hydrochloride hydrate (Product # A74 10)

[00398] · Amiodarone hydrochloride (Product # A8423)

[00399] · Amlodipine besylate (Product # A5605)

[00400] · N-(p-Amylcinnamoyl)anthranilic acid (Product # A8486)

[00401] · AP-18 (Product # A7232)

[00402] · ASP7663 (Product # SML1467)

[00403] · Azelnidipine (Product # A7106)

[00404] · (S)-(-)-Bay K8644 (Product # B 133)

[00405] • (±)-Bay K8644 calcium channel agonist (Product # B 112)

[00406] • Bepridil hydrochloride (Product # B5016)

[00407] • CaCCinh-AOl (Product # SML0916)

[00408] • Caged Ca2+ channel antagonist (Product # C235)

[00409] • Carboxyamidotriazole (Product # SML0408)

[00410] • Cilnidipine (Product # C1493)

[00411] • Cinnarizine (Product # C5270)

[00412] • Daurisoline (Product # SML0597)

[00413] • (+)-cis-Diltiazem hydrochloride (Product # D2521)

[00414] • Efonidipine hydrochloride monoethanolate (Product # E0159)

[00415] • EVP4593 (Product # SML0579)

[00416] • Felodipine (Product # F9677)

[00417] • Fendiline hydrochloride (Product # F7265)

[00418] • Flavoxate hydrochloride (Product # F8304)

[00419] • Flunarizine dihydrochloride (Product # F8257)

[00420] • Fluspirilene (Product # F100)

[00421] • FPL 64176 (Product # F131)

[00422] • Gabapentin (Product # G154)

[00423] • GSK2193874 (Product # SML0942)

[00424] • HA-1077 (Product* H139)

[00425] • HC-030031 (Product # H4415)

[00426] • Isradipine (Product # 16658)

[00427] • KB-R7943 (Product # K4 144)

[00428] • Kurtoxin (Product # K1514)

[00429] • Lacidipine (Product # SML0946)

[00430] • Lanthanum(III) chloride heptahydrate (Product # L413 1)

[00431] • Lercanidipine hydrochloride (Product # L6668)

[00432] • Lidoflazine (Product # L9668)

[00433] • Lomerizine dihydrochloride (Product # L6295)

[00434] • Loperamide hydrochloride (Product # L4762)

[00435] • M8-B hydrochloride (Product # SML0893)

[00436] • 3-MFA (Product # SML0658)

[00437] • Mibefradil dihydrochloride hydrate (Product # M 5441) Ro 40-5967

[00438] • ML204 (Product # SML0400)

[00439] • ML218 (Product # SML0385)

[00440] · ML-SA1 (Product # SML0627)

[00441] · MRS 1845 (Product # M1692)

[00442] · Nateglinide (Product # N3538)

[00443] · Neomycin trisulfate (Product # N1876)

[00444] · Nicardipine hydrochloride (Product # N7510)

[00445] · Nifedipine (Product # N7634)

[00446] · Nifetepimine (Product # SML1372)

[00447] · Nilvadipine (Product # SML0945)

[00448] · Nimodipine (Product # N149)

[00449] · Nisoldipine (Product # N0165)

[00450] · Nitrendipine (Product # N144)

[00451] · NNC 55-0396 hydrate (Product # N0287)

[00452] · ORM-10103 (Product # SML0972)

[00453] · Penfluridol (Product # P3371)

[00454] · PF-05 105679 (Product # PZ0245)

[00455] · Phloretin (Product # P7912)

[00456] · Polygodial (Product # SML0049)

[00457] · Pregabalin (Product # PZ0010)

[00458] · Protopine hydrochloride (Product # P8489)

[00459] · PyrlO (Product # SML1243)

[00460] · Pyr3 (Product # P0032)

[00461] · Pyr6 (Product # SML1241)

[00462] · Ruthenium Red (Product # R2751)

[00463] · N-Salicyloyltryptamine (Product # S6444)

[00464] · SKA-31 (Product # S5576)

[00465] · SKF-96365 (Product # S7809)

[00466] · SNX-482 (Product # S1818)

[00467] · Tetracaine hydrochloride (Product # T7645)

[00468] · Thioridazine hydrochloride (Product # T9025)

[00469] · γ6 TMla trifluoroacetate salt (Product # T2955) pricing

[00470] · Tyrphostin A9 (Product # T1182)

[00471] · (-)-Umbellulone (Product # SML0782)

[00472] · Veratridine (Product # V5754)

[00473] · YM-58483 (Product # Y4895)

[00474] Characterisation of dysfunctional signalling through tissue or cell Mitogen-Activated

Protein Kinase pathway

[00475] Characterisation of dysfunctional signalling through tissue or cell Mitogen-Activated Protein Kinase pathway may be carried out in any suitable manner. For example, cell MAPK phosphorylation studies, including assaying cell cytotoxic activity, cell degranulation, cell lytic proteins and maturation markers and cell cytokines, as well as multiparametric flow cytometry analysis and statistical analysis, may be carried out as previously described [9m, 27m, 29m 38m-45m].

[00476] Protein phosphorylation or dephosphorylation can be determined, measured, quantitiated or assayed in any suitable way, including using antibodies, phospho-specific antibodies, FACS, chemiluminescent detection, immunofluorescence, radioactive ligands and electrophoresis. For example, commercial kits for testing the relative phosphorylation of various kinases are available (eg. R&D Systems).

[00477] In other embodiments, the level of gene expression (RNA or protein) may be tested, or a property of the protein or biochemical function may be tested, as described for other forms of the invention.

[00478] General techniques

[00479] The following general methodologies may be utilised.

[00480] 1. Recombinant technology:

[00481] a. Expression of a recombinant protein (polypeptide) detected by constructing a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing protein expression, then lysing the cells, purifying the protein, and performing SDS-PAGE analysis to verify the presence of the protein.

[00482] b. Protein expression using an inducing agent by a raising of the incubation temperature of the medium or by the addition of an inducing chemical to the culture medium.

[00483] 2. Time-course analysis of protein expression:

[00484] a. To optimize the expression of a given protein construct, a time-course analysis by SDS-PAGE of the level of protein expression could be used. As intracellular protein content is often a balance between the amount of soluble protein in the cells, the formation of inclusion bodies and protein degradation, by checking the protein present at various times after induction, the optimal induction period can be established.

[00485] 3. Protein purification:

[00486] a. The expression and purification of recombinant proteins facilitates production and

detailed characterization of virtually any protein.

[00487] b. Classical purification procedures can be employed, but in most cases recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest; the use of these affinity tags simplifies the purification of the recombinant fusion proteins by employing affinity chromatography methods.

[00488] 4. SDS PAGE:

[00489] a. SDS polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain of interest.

[00490] b. Visualization of proteins in SDS-PAGE gels

[00491] Visualization of protein bands is carried out by incubating the gel with a staining solution, such as Coomassie and silver staining. Silver staining is a more sensitive staining method than Coomassie staining, and is able to detect 2-5 ng protein per band on a gel.

[00492] 5. Western blotting:

[00493] a. Following electrophoresis, proteins in a polyacrylamide gel can be transferred to a positively charged membrane in a buffer-tank-blotting apparatus or by semi-dry electroblotting.

[00494] b. With the semi-dry electroblotting method, the gel and membrane are sandwiched between two stacks of filter paper that have been pre-wet with transfer buffer. The membrane is placed near the anode and the gel is placed near the cathode. SDS-coated, negatively charged proteins are transferred to the membrane when an electric current is applied.

[00495] c. Additionally, a tank-blotting method could be used. This is where a blotting cassette is submerged in a tank for blotting. This can be performed over extended periods since the buffer capacity is far greater than that with semi-dry transfer systems. Results obtained with the tank-blotting method are typically better, with more efficient transfer, particularly of large proteins.

[00496] 6. Acrylamide concentration:

[00497] a. Low acrylamide concentrations are used to separate high molecular weight proteins, while high acrylamide concentrations are used to separate proteins of low molecular weight.

[00498] 7. Dot blots:

[00499] a. Dot blotting is a simple, convenient method for detection of proteins in crude lysates or solutions without the need for separation by SDS-PAGE. This method is especially useful as a simple control because it avoids problems that may be due to the western transfer process. Any components that interfere with binding or bind non-specifically, however, will not

be spatially separated from the protein and will interfere with the intensity of signals.

[00500] 8. Protein detection - Specific antibody-mediated detection of proteins on a membrane/Immimodetection using a chemiluminescent method:

[00501] a. Using primary antibody applied to the membrane to bind to the target protein as well as a secondary antibody that chemically coupled to a reporter, which allows detection and visualization of the antibody and the protein of interest. Fluorescing molecules, or enzymes that produce colored or luminescent reaction products, are typically used as reporter groups.

[00502] b. Importantly a primary antibody chemically coupled to a reporter enzyme (termed a conjugate) can be used for direct detection without the use of a secondary antibody.

[00503] 9. Protein assay -ELISA:

[00504] a. Enzyme-linked immunosorbent assay (ELISA) is a method that is analogous to immunodetection of proteins on a membrane, and is used for the quantitative assay of proteins in solution. In an ELISA, proteins are immobilized on a solid support (e.g., the wells of a 96-well plate) and used as capture molecules to bind the protein that is being assayed. After a wash step to remove nonspecifically bound material, a secondary antibody — specific for the protein being assayed — is added. This secondary antibody is usually conjugated to an enzyme that allows its detection by chromogenic or chemiluminescent methods. ELISA methods can be direct or indirect for the detection of a protein.

[00505] 10. Quantifying proteins using the Bradford method or UV spectrophotometry:

[00506] a. The Bradford method or UV spectrophotometry methods are a quantitative protein assay method, based on the binding of a dye, Coomassie Brilliant Blue, to a protein sample, and comparing this binding to a standard curve generated by the reaction of known amounts of a standard protein, usually BSA.

[00507] 11. Quantification of DNA/mRNA and fragments of proteins using Spectrophotometry and fluorometry:

[00508] a. Spectrophotometry and fluorometry are commonly used to measure both genomic and plasmid DNA concentration. Spectrophotometry can be used to measure microgram quantities of pure DNA samples (i.e., DNA that is not contaminated by proteins, phenol, agarose, or RNA). Fluorometry is more sensitive, allowing measurement of nanogram quantities of DNA, and furthermore, the use of Hoechst 33258 dye allows specific analysis of DNA.

[00509] 12.Ligation of DNA methods:

[00510] a. DNA will be firstly be digested using restriction endonucleases. The individual components of the desired DNA molecule are purified and then combined and treated with DNA ligase. The products of the ligation mixture are introduced into competent *E. coli* cells and transformants are identified by appropriate genetic selection.

[005 11] 13. Analysis of DNA by Southern blotting:

[00512] a. Southern blotting is a widely used technique that allows analysis of specific DNA sequences. DNA is usually first converted into conveniently sized fragments by restriction digestion. The DNA of interest can be identified by hybridization to radioactive or chemiluminescent probes and visualized by autoradiography or stainin.

[00513] 14.PCR, One step and Two Step Real time PCR, Long range PCR, Single Cell PCR, Fast Cycling PCR, Methylation-specific PCR and Differential display PCR methods.

[005 14] 15. Multiplex PCR and RT-PCR and whole transcriptome amplification:

[00515] There are 3 main PCR-based WGA techniques. These are degenerate oligonucleotide PCR (DOP-PCR) (1), primer extension preamplification (PEP) (2) or derivatives thereof, and adaptor-ligation PCR (3). The main difference between the techniques is that PEP uses a preamplification step to add primer binding sites to small DNA fragments for later WGA by PCR, while adaptor-ligation PCR uses adaptors ligated to small DNA fragments to create PCR primer binding sites. PEP utilizes random primers and a low PCR annealing temperature. Less frequently used today, DOP-PCR uses semi-degenerate oligonucleotides and an increasing annealing temperature.

[00516] 16.RAPD: Rapid amplified polymorphic DNA and RACE: Rapid amplification of cDNA ends analysis.

[00517] 17.Ext-generation sequencing, Genotyping using microarrays, Comparative genome hybridization studies (CGH), Single nucleotide polymorphism (SNP) genotyping, Sanger sequencing, STR/micro satellite analysis,

[005 18] 18. Haplotyping , Genotying

[00519] 19.NGS sequencing methods

[00520] 20.Metagenomics

[00521] 21.RNA sequencing

[00522] a. RNA sequencing (RNA-seq) is a method of investigating the transcriptome of an organism using deep-sequencing techniques. The RNA content of a sample is directly sequenced after appropriate library construction, providing a rich data set for analysis. The high level of sensitivity and resolution provided by this technique makes it a valuable tool for investigating the entire transcriptional landscape. The quantitative nature of the data and the high dynamic range of the sequencing technology enables gene expression analysis with a high sensitivity. The single-base resolution of the data provides information on single nucleotide polymorphisms (SNPs), alternative splicing, exon/intron boundaries, untranslated regions, and other elements

[00523] 22.ChIP-Seq:

[00524] a. Chromatin immunoprecipitation (ChIP) is a powerful and versatile method for

understanding the mechanisms of gene regulation by transcription factors and modified histones.

[00525] b. It is used to identify chromatin regions which are bound by transcription factors, co-regulators, modified histones, chromatin remodeling proteins, or other nuclear factors from live cells.

[00526] 23. Flow cytometric analysis:

[00527] Methods using Fluorescence-activated cell sorting.

[00528] a. Fluorophores technology that label a recognised target feature on or in the cell. Use of flurophores may also be attached to a chemical entity with affinity for the cell membrane or another cellular structure.

[00529] b. Quantum dots methods to be in place of traditional fluorophores because of their narrower emission peaks.

[00530] c. Isotope labelling such as Mass cytometry

[00531] 24. UEP results (SNP results):

[00532] a. Unique-event polymorphisms (UEPs) such as SNPs represent haplogroups. STRs represent haplotypes. The results that comprise the full Y-DNA haplotype from the Y chromosome DNA test can be divided into two parts: the results for UEPs, sometimes loosely called the SNP results as most UEPs are single-nucleotide polymorphisms, and the results for microsatellite short tandem repeat sequences (Y-STRs).

[00533] b. The UEP results represent the inheritance of events it is believed can be assumed to have happened only once in all human history. These can be used to identify the individual's Y-DNA haplogroup, his place in the "family tree" of the whole of humanity. Different Y-DNA haplogroups identify genetic populations that are often distinctly associated with particular geographic regions; their appearance in more recent populations located in different regions represents the migrations tens of thousands of years ago of the directpatrilineal ancestors of current individuals.

[00534] 25. Y-STR haplotypes:

[00535] a. Genetic results also include the Y-STR haplotype, the set of results from the Y-STR markers tested. Unlike the UEPs, the Y-STRs mutate much more easily, which allows them to be used to distinguish recent genealogy.

[00536] Using Haplotype technologies also:

[00537] · FAMHAP — FAMHAP is a software for single-marker analysis and, in particular, joint analysis of unphased genotype data from tightly linked markers (haplotype analysis).

[00538] · Fugue — EM based haplotype estimation and association tests in unrelated and nuclear families.

[00539] · HPlus — A software package for imputation and testing of haplotypes in association studies using a modified method that incorporates the expectation-maximization algorithm and a Bayesian method known as progressive ligation.

[00540] · HaploBlockFinder — A software package for analyses of haplotype block structure.

[00541] · Haploscribe — Reconstruction of whole-chromosome haplotypes based on all genotyped positions in a nuclear family, including rare variants.

[00542] · Haploview — Visualisation of linkage disequilibrium, haplotype estimation and haplotype tagging (Homepage).

[00543] · HelixTree — Haplotype analysis software - Haplotype Trend Regression (HTR), haplotypic association tests, and haplotype frequency estimation using both the expectation-maximization (EM) algorithm and composite haplotype method (CHM).

[00544] · PHASE — A software for haplotype reconstruction, and recombination rate estimation from population data.

[00545] · SHAPEIT — SHAPEIT2 is a program for haplotype estimation of SNP genotypes in large cohorts across whole chromosome.

[00546] · SNPHAP — EM based software for estimating haplotype frequencies from unphased genotypes.

[00547] · WHAP- haplotype based association analysis.

[00548] 26.Microfluorimetry

[00549] This is an adaption of fluorimetry for studying the biochemical and biophysical properties of cells by using microscopy to image cell components tagged with fluorescent molecules.

[00550] Kits and assays

[00551] The kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof can comprise one or more probes, tools or reagents, including nucleic acid oligonucleotides or primers, arrays of nucleic acid probes, antibodies to polymorphic polypeptides (e.g., immobilized on a substrate), signal producing system reagents, labelling and detection means, controls and/or other reagents such as buffers, nucleotides or enzymes e.g. polymerase, nuclease or transferase, depending on the particular protocol to be performed. Other examples of reagents include arrays that comprise probes that are specific for one or more of the genes of interest or one or more polymorphisms thereof, and antibodies to epitopes of the proteins encoded by these genes of interest, wherein the epitope may comprise a polymorphism of interest.

[00552] A kit or assay can include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a swab for removing cells from the buccal cavity or a syringe for removing a blood sample (such components generally being sterile).

[00553] In addition to the above components, the kits or assay can further include instructions. These instructions may be present in the subject kits in a variety of forms, one form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another form would be a computer readable medium, e.g., diskette, CD, etc., on which the information has been recorded. Yet another form that may be present is a website address which may be used via the internet to access the information at a removed site.

[00554] It is to be appreciated that one or more components of kits and assays generally described for SNPs/polymorphism detection may also be used for one or more other forms of the invention or may include components as described elsewhere in this specification (such as in the Examples), such as testing for differential regulation of calcium-dependent kinase genes, testing for dysfunctional signalling through the Mitogen-Activated Protein Kinase pathway (eg. for determining protein phosphorylation or dephosphorylation), testing for changes in calcium metabolism, or testing for changes in calcium-dependent kinase pathways. Suitable kit components include: a probe, tool or reagent for detection of a polymorphism at the genomic level, at the transcription level or polypeptide level; a probe, tool or reagent for quantitative or qualitative measurement of RNA transcription or translation; or a probe, tool or reagent such as an antibody or other type of molecule or chemical entity capable of detecting the gene or gene product (RNA or polypeptide) or property of the protein or dysfunctional biochemical signalling or pathway.

[00555] Biochip

[00556] A biochip is also provided. The biochip is an apparatus which, in certain embodiments, comprises a solid substrate comprising an attached probe or plurality of probes/oligonucleotides. The probes may be capable of hybridizing to a target sequence under stringent hybridization conditions. The probes may be attached at spatially defined address on the substrate. More than one probe per target sequence may be used, with either overlapping probes or probes to different sections of a particular target sequence. In an embodiment, two or

more probes per target sequence are used. The probes may be capable of hybridizing to different targets, such as a TRP ion channel and/or ACh receptor gene/allele or gene product.

[00557] The probes may be attached to the biochip in a wide variety of ways, as will be appreciated by those of skill in the art. The probes may either be synthesized first, with subsequent attachment to the biochip, or may be directly synthesized on the biochip.

[00558] The solid substrate may be a material that may be modified to contain discrete individual sites appropriate for the attachment or association of the probes and is amenable to at least one detection method. Representative examples of substrates include glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses and plastics. The substrates may allow optical detection without appreciably fluorescing.

[00559] The substrate may be planar, although other configurations of substrates may be used as well. For example, probes may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Similarly, the substrate may be flexible, such as a flexible foam, including closed cell foams made of particular plastics.

[00560] The biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. For example, the biochip may be derivatized with a chemical functional group including, but not limited to, amino groups, carboxyl groups, oxo groups or thiol groups. Using these functional groups, the probes may be attached using functional groups on the probes either directly or indirectly using linkers. The probes may be attached to the solid support by either the 5' terminus, 3' terminus, or via an internal nucleotide.

[00561] The probe may also be attached to the solid support non-covalently. For example, biotinylated oligonucleotides can be made, which may bind to surfaces covalently coated with streptavidin, resulting in attachment. Alternatively, probes may be synthesized on the surface using techniques such as photopolymerization and photolithography.

[00562] A variety of hybridization conditions may be used, including high, moderate and low stringency conditions as outlined above. The assays may be performed under stringency conditions which allow hybridization of the probe only to the target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not

limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration pH, or organic solvent concentration.

[00563] Hybridization reactions may be accomplished in a variety of ways. Components of the reaction may be added simultaneously, or sequentially, in different orders. In addition, the reaction may include a variety of other reagents. These include salts, buffers, neutral proteins, e.g., albumin, detergents, etc. which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors and anti-microbial agents may also be used as appropriate, depending on the sample preparation methods and purity of the target.

[00564] Exemplary biochips of the present invention include an organized assortment of oligonucleotide probes described above immobilized onto an appropriate platform. Each probe selectively binds a nucleic acid target in a sample.

[00565] In accordance with another embodiment, the biochip of the present invention can also include one or more positive or negative controls. For example, oligonucleotides with randomized sequences can be used as positive controls, indicating orientation of the biochip based on where they are placed on the biochip, and providing controls for the detection time of the biochip.

[00566] Embodiments of the biochip can be made in the following manner. The oligonucleotide probes to be included in the biochip are selected and obtained. The probes can be selected, for example, based on particular SNPs of interest. The probes can be synthesized using methods and materials known to those skilled in the art, or they can be synthesized by and obtained from a commercial source, such as GeneScript USA (Piscataway, N.J.).

[00567] Each discrete probe is then attached to an appropriate platform in a discrete location, to provide an organized array of probes. Appropriate platforms include membranes and glass slides. Appropriate membranes include, for example, nylon membranes and nitrocellulose membranes. The probes are attached to the platform using methods and materials known to those skilled in the art. Briefly, the probes can be attached to the platform by synthesizing the probes directly on the platform, or probe- spotting using a contact or non-contact printing system. Probe-spotting can be accomplished using any of several commercially available systems, such as the GeneMachines(TM) OmniGrid (San Carlos, Calif.).

[00568] Particularly preferred embodiments of the invention are defined in the claims.

[00569] Any of the features described herein can be combined in any combination with any one or more of the other features described herein within the scope of the invention.

[00570] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practise or testing of the present invention.

[00571] Other forms and advantages of the invention will become apparent from a reading of this specification.

EXAMPLES

[00572] The following examples are illustrative only and should not be construed as limiting in any way the general nature of the disclosure of the description throughout this specification.

[00573] **Example 1: The role of the transient receptor potential (TRP) superfamily in CFS.**

[00574] The transient receptor potential (TRP) superfamily in humans comprises 27 cation channels with permeability to monovalent and divalent cations. These channels are widely expressed within humans on cells and tissues, and have significant sensory and regulatory roles in most physiological functions.

[00575] **Methodology**

[00576] *Subjects*

[00577] The study comprised 115 CFS patients (age=48.68+1.06years) and 90 non-fatigued controls (age=46.48+1.22 years). CFS patients were defined in accordance with the 1994 CDC criteria for CFS [20b]. 10 mL of whole blood samples were collected from all participants into EDTA tubes.

[00578] *DNA Extraction*

[00579] Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood miniiit as per manufacturer's instructions (Qiagen). The Nanodrop (Nanodrop) was used to assess the quality and quantity of the DNA extracted. Approximately 2 μg of genomic DNA was used in the SNP assay.

[00580] *SNP Genotyping Studies*

[00581] SNP analysis was performed by Geneworks using the MassARRAY iPLEX Gold

Assay (Sequenom Inc.) as previously described. Customized assays were developed for 240 SNPs across the 21 TRP genes *(TRPA1, TRPC1, TRPC2, TRPC3, TRPC4, TRPC6, TRPC7, TRPM1, TRPM2, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7, TRPM8, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5* and *TRPV6).* Primers and extension primers were created for each of the SNPs using the Assay Designer (Sequenom Inc.) according to the manufacturer's instructions. Briefly, DNA was amplified via PCR under the follow conditions: 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. Amplification products were then treated with shrimp alkaline phosphatase (SAP) at 37°C for 40 min, 85°C for 5 min reaction and a final incubation at 4°C. Extension primers were optimized to control signal-to-noise ratio where un-extended primers (UEPs) were examined on the spectroCHIP and evaluated in Typer 4.0 to enable the division into low mass UEP, medium mass UEP and high mass UEP. To perform the iPLEX extension reaction, a mixture containing iPLEX Gold reaction was carried out using iPLEX Gold Buffer Plus, iPLEX termination mix, iPLEX enzyme and primer mix was prepared. iPLEX reaction was cycled at an initial denaturation of 94°C for 30 s, annealing at 52°C for 5 min, extension at 80°C for 5 min (5 cycles of annealing and extension were performed, however the whole reaction was performed in 40 cycles) and extension again at 72°C for 3 min. Resin beads were used to rinse all iPLEX Gold reaction products. Following iPLEX Gold reaction, MassARRAY was performed using the MassARRAY mass spectrometer, the data generated was analysed using the TyperAnalyzer software.

[00582] *Statistical analysis*

[00583] The PLINK vl.07 [21b] whole genome analysis tool set was used to determine associations between the CFS patients and the non-fatigued control group. A two column $\chi 2$ test was used to determine significance where p value of ≤ 0.05 was determined to be significant.

[00584] **Results**

[00585] *Participants*

[00586] Of the 115 CFS patients (age=48.68±1.06 years), 84 (73.04%) were females and 31 (26.96%) were males. The 90 non-fatigued controls (age=46.48±1.22 years) comprised 59 (65.56%) females and 31 (34.44%) males. All participants in the patient and non-fatigued control groups were of European decent and were all residents of Australia at the time of blood collection.

[00587] *SNP Association Studies*

[00588] Of the 240 SNPs that were examined in the present study, 233 were successfully identified in both participants groups. Of the 233, thirteen were observed to be significantly associated with CFS (Table 1).

[00589] **Table 1:** Analysis of the frequency distribution and significance of TRP Single

Nucleotide Polymorphisms (SNPs) in CFS patients and non-fatigued controls in rank order of significance.

Gene	Chromosome	RefSNP ID	A1	A2	Frequency_A	Frequency_U	χ^2	P
TRPM3	9	rs12682832	A	G	0.444	0.293	8.808	**0.003**
TRPM3	9	rs11142508	C	T	0.445	0.298	8.438	**0.004**
TRPM3	9	rs1160742	A	G	0.470	0.333	7.063	**0.008**
TRPM3	9	rs4454352	C	T	0.240	0.137	6.232	**0.013**
TRPM3	9	rs1328153	C	T	0.240	0.137	6.232	**0.013**
TRPM3	9	rs3763619	A	C	0.440	0.316	5.990	**0.014**
TRPC4	13	rs6650469	T	C	0.505	0.380	5.775	**0.016**
TRPC4	13	rs655207	G	T	0.505	0.381	5.639	**0.018**
TRPA1	8	rs4738202	A	G	0.369	0.253	5.591	**0.018**
TRPM3	9	rs7865858	A	G	0.450	0.331	5.340	**0.021**
TRPA1	8	rs2383844	G	A	0.505	0.398	4.218	**0.040**
TRPM3	9	rs1504401	T	C	0.100	0.173	4.172	**0.041**
TRPM3	9	rs10115622	A	C	0.335	0.435	3.837	**0.050**
TRPM4	19	rs10403114	G	A	0.293	0.390	3.802	0.051
TRPV3	17	rs9909424	G	A	0.115	0.060	3.442	0.064
TRPC4	13	rs612308	A	G	0.439	0.537	3.393	0.065
TRPM3	9	rs7860377	A	C	0.350	0.262	3.314	0.069
TRPC7	5	rs2673930	C	A	0.200	0.280	3.218	0.073
TRPC4	13	rs603955	C	T	0.445	0.536	3.008	0.083
TRPM3	9	rs11142798	C	G	0.135	0.202	2.998	0.083
TRPM3	9	rs4744611	G	A	0.360	0.446	2.843	0.092
TRPM2	21	rs1785452	T	C	0.215	0.289	2.67	0.102
TRPM3	9	rs1566838	G	T	0.460	0.375	2.669	0.102
TRPA1	8	rs1384002	T	C	0.495	0.410	2.664	0.103
TRPM6	9	rs2274924	G	A	0.115	0.175	2.652	0.103
TRPM3	9	rs1394309	G	A	0.030	0.065	2.608	0.106
TRPC4	13	rs2985167	G	A	0.340	0.422	2.577	0.108
TRPM5	11	rs2301698	G	T	0.530	0.446	2.551	0.110
TRPM6	9	rs944857	C	T	0.185	0.125	2.476	0.116
TRPM2	21	rs762426	G	A	0.160	0.223	2.325	0.127

[00590] Nine of these SNPs were associated with *TRPM3* (rs 12682832; p=0.003, rslll42508; p=0.004, rsl 160742; p=0.08, rs4454352; p=0.013, rsl328153; p=0.013, rs3763619; p=0.014, rs7865858; p=0.021, rsl504401; p=0041, rslOl 15622; p=0.050), while the remainder were associated with *TRPA1* (rs2383844; p=0.040, rs4738202; p=0.018) and *TRPC4* (rs6650469; p=0.0 16, rs655207; p=0.0 18).

[00591] **Discussion**

[00592] The purpose of this study was to determine the presence of possible SNP variations in CFS patients with a specific focus on SNPs within the coding sequences of 21 TRP ion

channel genes. Out of the 240 SNPs examined, thirteen alleles were found to be significantly associated with CFS patients compared with the non-fatigued controls. These alleles were located in the gene sequence of one of the canonical TRPs ion channels (TRPC4), one ankyrin (TRPA1) and one melastatin TRP ion channel (TRPM3).

[00593] There is limited information available on the role of these SNPs, however TRPs may mediate the potential onset of CFS. TRPC4 is activated via receptor-dependent activation of the Gqii / PLC β/γ pathway but also via G™ proteins, PI(4,5)P2 proteins and also intracellular Ca^{2+} [22b]. It is mainly involved in vasomotor function, aggregation of platelets and smooth muscle function. Incidentally, Ca^{2+} is known to be required for the regulation of immune cells as Ca^{2+} acts as a second messenger for most cells, particularly T cells and B cells. Intracellular Ca^{2+} increases when lymphocytes receptors are exposed to antigens [23b]. In CFS patients, there are numerous reports on compromises to immune function although there is limited information on the role of Ca^{2+} in these patients. However, dysregulation in TRPCs may affect intracellular calcium concentration and incidentally lymphocyte function. Lymphocytes such as Natural Killer (NK) cells and T cells have been shown to be compromised in CFS. In NK cells, Ca^{2+} enhances cytotoxic activity and its depletion or excessive influx may have severe consequences on NK cells function. In CFS reduced cytotoxic activity has been consistently reported [24b-29b] and this may be related to dysregulation in Ca^{2+}.

[00594] Dysregulation of TRPCs may affect neuronal responses in particular those associated with the stimulation of muscarinic receptors. Following activation of TRPCs by PLCs an influx of Ca^{2+} occurs causing an induction in muscarinic receptors, and maintained incessant neuronal firing [30b, 31b]. Hence, secretion of Ca^{2+} and availability of TRPCs in the neuronal environment is paramount to optimal muscarinic receptor function and overall function of the brain. Importantly, this process is essential for memory, attention, sensory acuity, emotion, pain and motor control [32b, 33b] and occurs in the amygdala, entorhinal cortex, hippocampus and prefrontal cortex [34b]. Neuronal deficits involving memory and attention have been identified in CFS [35b-37b]. Deletion or compromises to TRPC4 may also affect intestinal function. TRPC4 and TRPC6 pair with muscarinic receptors in the intestine activating smooth muscle depolarization, inflow of Ca^{2+} and smooth muscle contraction [38b]. Intestinal dysfunction is a component of CFS [39b], however the extent of damage to the intestinal wall or the exact role of ion channels in the intestine remains to be determined. TRPC4 may be simultaneously regulated by G protein coupled receptors (GPCRs) Gαi and Gαq [40b].

[00595] TRPA1 is a multiple chemical receptor that has been identified on nociceptive sensory neurons (C fibers) and has a role in the regulation of the release of neuropeptides, pain sensation and inflammation [41b]. It may be activated by both exogenous and endogenous

inflammatory agents resulting in inflammation and pain [42b]. GPCRs also activate TRPA1 via PLC signalling sensitising the ion channel to various stimuli [43b]. TRPA1 may be activated and subsequently inactivated in the presence of intracellular and extracellular calcium concentrations [44b, 45b]. TRPA1 gene has been proposed to affect sensitivity to nociceptive stimuli [46b], hence CFS patients expressing SNPs in the TRPA1 gene may increase their sensitivity to nociceptive stimuli. In the CNS astrocytes express TRPA1 channels and these channels are necessary for calcium uptake and neuronal regulation in the astrocytes. Changes in the level of calcium may therefore affect the function of astrocytes and interneuron communication [45b, 47b]. Activation of TRPA1 has been shown to induce acute headache and this may occur through the calcitonin gene related peptide (CGRP) causing vasodilation in the meningeal artery [45b, 48b]. Importantly, headache is a prominent symptom of CFS. TRPA1 is also a key player in migraine, neuropathic, joint and muscle pain which is most often experienced by patients with fibromyalgia [49b, 50b]. TRPA1 forms functional heterotetramers with TRPVl hence variations in the TRPA1 gene may suggest functional deficits to TRPVl that may not be related to polymorphism in nucleotides [51b]. Analgesics and antinociceptive drugs target TRPVl and TRPA1 respectively to alleviate pain sensation [52b-54b] and these drugs are routinely prescribed to CFS patients. Perhaps in CFS these drugs may not be effective due to impairments or variations in these ion channels.

[00596] TRPM channels are mostly permeable to magnesium and calcium. Only TRPM4 and TRPM5 are impermeable for divalent cations. TRPM3 is permeable for cations including Ca^{2+} and Zn^{2+}. However, the permeation profile highly depends on the expressed spliced variant [55b]. No hereditary TRPM3 channelopathy has been described to date. TRPM3 has been implicated in inflammatory pain syndromes, rheumatoid arthritis, and secretion of proinflammatory cytokines. As pancreatic β cells also have a high proportion of TRPM3 channels [45b, 56b-58b], there is the likelihood of perturbations in insulin/glucose regulation in CFS patients. Metabolic disturbance has also long been identified as a cardinal feature of CFS. The most characterised TRPM3 in humans is in the central nervous system (CNS) and eye [55b]. TRPM3 is involved in the detection of heat and in pain transmission. TRPM3-deficient mice exhibit clear deficits in their avoidance responses to noxious heat and in the development of inflammatory heat hyperalgesia [55b]. Dysregulation in thermoregulatory responses has been reported in CFS patients [59b]. Generalised pain is a characteristic of CFS and occurs in the absence of tissue damage and this is suggestive of potential CNS impairments [60b]. As TRPM3 has a role in nociception and thermoregulation, it may have a role in the pathomechansim of CFS. Additionally, TRPM3 is activated by pregnenolone sulfate suggesting that it has neuroendocrine effects [61b, 62b] and might also be involved in the regulation of glutamatergic

signalling in the brain [63b].

[00597] These findings implicate TRP ion channels (predominantly TRPM3) in the aetiology and pathomechanism of CFS. Dysregulation of TRPs, including the TRPM3 family, is likely pertinent in predisposing CFS patients to calcium metabolism perturbations and aligns with symptom presentation. Potentially, dysregulated influx of calcium ions into cells will impact a number of vital components of cell regulatory machinery. These components include calcium sensitive adenylate cyclases (ACs) and hence cAMP expression and function.

[00598] **Example 2: The role of ACh Receptor (nAChRs and mAChRs) SNPs in CFS/ME.**

[00599] **Methodology**

[00600] *Subjects*

[00601] The study comprised 115 CFS/ME patients (age=48.68±1.06years) and 90 non-fatigued controls (age=46.48+1.22 years). CFS patients were defined in accordance with the 1994 CDC criteria for CFS [36c]. Avolume of 10 mL of whole blood was collected from all participants into EDTA tubes.

[00602] *DNA Extraction*

[00603] Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood mini-kit as per manufacturer's instructions (Qiagen). Quality and quantity of the DNA extracted was determined by the Nanodrop (Nanodrop), where approximately 2 μg of genomic DNA was used to perform the SNP assay.

[00604] *SNP Genotyping Studies*

[00605] A total of 464 single nucleotide polymorphisms (SNPs) for nine mammalian ACh receptor genes (Ml, M2, M3, M4, M5, alpha 2, 5, 7 and 10) were examined via the Agena Biosciences iPLEX Gold assay. Geneworks completed the SNP analysis as previously defined (MassARRAY iPLEX Gold Assay) [37c]. Customized assays were developed for 464 SNPs across the 9 mammalian acetylcholine receptor genes (Ml, M2, M3, M4, M5, alpha 2, 5, 7 and 10). Primers and extension primers were created for each of the SNPs using the Assay Designer [37c] according to the manufacturer's instructions. The amplification of the DNA was as previously described. Briefly, DNA was amplified via PCR under the follow conditions 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. Amplification products were then treated with shrimp alkaline phosphatase (SAP) at 37°C for 40 min, 85°C for 5 min reaction and a final incubation at 4°C. Extension primers were optimized to control signal-to-noise ratio where un-extended primers (UEPs) were examined on the spectroCHIP and evaluated in Typer 4.0 to enable the division into low mass UEP, medium mass UEP and high mass UEP. To perform the iPLEX extension reaction, a mixture containing iPLEX Gold reaction

was carried out using iPLEX Gold Buffer Plus, iPLEX termination mix, iPLEX enzyme and primer mix was prepared. iPLEX reaction was cycled at an initial denaturation of 94°C for 30 s, annealing at 52°C for 5 min, extension at 80°C for 5 min (5 cycles of annealing and extension were performed, however the whole reaction was performed in 40 cycles) and extension again at 72°C for 3 min. Resin beads were used to rinse all iPLEX Gold reaction products. Following iPLEX Gold reaction, MassARRAY was performed using the MassARRAY mass spectrometer, the data generated was analysed suing the TyperAnalyzer software.

[00606] *Statistical analysis*

[00607] The PLINK vl.07 [39c] whole genome analysis tool set was implemented to determine associations between the CFS patients and the non-fatigued control group. A two column $\chi 2$ test was used where alpha level of significance was set at p value of <0.05.

[00608] **Results**

[00609] *Participants*

[00610] Of the 115 CFS patients (age=48.68±1.06 years), 84 (73.04%) were females and 31 (26.96%) were males. 90 non-fatigued controls (age=46.48±1.22 years) comprised 59 (65.56%) females and 31 (34.44%) males. All participants in both groups were of European decent. All were residents of Australia at the time of blood collection.

[006 11] *SNP Association Studies*

[00612] Of the 464 SNPs that were examined in the present study, 393 were successfully identified in both participants groups. Of the 393, seventeen were observed to be significantly associated with CFS (Table 2).

[00613] **Table 2:** Analysis of the frequency distribution and significance of acetylcholine receptor Single Nucleotide Polymorphisms (SNPs) in CFS patients and non-fatigued controls in rank order of significance.

Gene	Chromosome	RefSNP ID	Al	Frequency_A	Frequency_U	A2	χ^2	P
mAchM3	1	rs4463655	T	0.3077	0.4671	C	8.932	0.00
mAchM3	1	rs589962	**C**	0.2416	0.3919	T	8.539	0.00
mAchM3	1	rs1072320	G	0.3242	0.1842	A	8.423	0.00
mAchM3	1	rs7543259	A	0.3187	0.1842	G	7.834	0.01
mAchM3	**1**	rs6661621	C	0.3022	0.1711	G	7.755	0.01
nAchαlO	**11**	rs267221 1	C	0.3736	0.2434	T	6.515	0.01
nAchαlO	**11**	rs2672214	**C**	0.3708	0.24	T	6.498	0.01
nAchα5	15	rs951266	T	0.3944	0.2632	**C**	6.382	0.01
nAchalO	**11**	rs2741868	T	0.3693	0.24	A	6.333	0.01
nAchalO	**11**	rs2741870	G	0.3708	0.2434	C	6.195	0.01
nAcha2	8	rs2565048	C	0.0989	0.1933	T	6.034	0.01
mAchM3	**1**	rs7520974	G	0.4205	0.5533	A	5.727	0.02

mAchM3	1	rs726169	G	0.2833	0.4013	A	5.132	0.02
mAchM3	1	rs6669810	G	0.4213	0.5467	C	5.123	0.02
nAchαlO	11	rs2741862	C	0.2857	0.1842	T	4.685	0.03
nAch(x5	15	rs7 180002	T	0.3846	0.2763	A	4.359	0.043
mAchM3	1	rs6429157	G	0.522	0.4079	A	4.327	0.04
nAcha2	8	rs55828312	G	0.2386	0.15 13	A	3.914	0.05
nAcha5	15	rs2175886	C	0.4944	0.3867	T	3.847	0.05
mAchM3	1	rsl2036141	A	0.4121	0.3092	G	3.781	0.05
mAchM3	1	rs6429147	C	0.4444	0.34	G	3.728	0.05
mAchM3	1	rs15945 13	G	0.2198	0.3 133	T	3.722	0.05
nAcha2	8	rsl6891561	T	0.2472	0.1597	c	3.696	0.05
nAchalO	11	rs2672215	A	0.4607	0.36	c	3.399	0.07
nAcha2	8	rs6474413	C	0.2308	0.15 13	T	3.336	0.07
mAchM3	1	rs10926008	G	0.3722	0.277	A	3.333	0.07
nAchα2	8	rs2741343	C	0.5337	0.4324	T	3.317	0.07
nAchα5	15	rs7 178270	G	0.3571	0.4539	c	3.231	0.07
nAcha5	15	rs4243084	G	0.3977	0.3026	c	3.227	0.07
nAchα5	15	rs601079	A	0.3901	0.4868	T	3.155	0.08
nAcha5	15	rs129 11602	C	0.3901	0.4868	T	3.155	0.08
nAcha5	15	rs588765	T	0.3846	0.4803	c	3.095	0.08
nAchα5	15	rs680244	A	0.3846	0.4803	G	3.095	0.08
nAchα5	15	rs6495306	G	0.3895	0.4863	A	3.01	0.08
nAchα5	15	rs6495307	T	0.41 11	0.5068	C	2.997	0.08
mAchM3	1	rsl2093821	A	0.489	0.3947	G	2.979	0.08
mAchM3	1	rsl6838637	G	0.4889	0.3947	A	2.957	0.09
nAchα2	8	rs6997909	A	0.2333	0.1579	G	2.945	0.09
nAchalO	11	rs2672216	C	0.4888	0.3947	T	2.934	0.09
mAchM3	1	rs6429165	A	0.2473	0.171 1	G	2.873	0.09
nAcha2	8	rs891398	C	0.533	0.4392	T	2.872	0.09
nAcha5	15	rs4366683	G	0.3956	0.4868	A	2.802	0.09
nAcha2	8	rs6985052	C	0.2308	0.1579	T	2.774	0.10
nAcha2	8	rs4950	C	0.2308	0.1579	T	2.774	0.10

[00614] Seventeen SNPs were significantly associated with CFS/ME patients compared with the controls. Nine of these SNPs were associated with mAChRM3 (rs4463655; p=0.00, rs589962; p=0.00, rsl072320; p=0.00, rs7543259; p=0.01, rs6661621; p=0.01 rs7520974; p=0.02, rs726169; p=0.02, rsrs6669810; p=0.02, rsrs6429157; p=0.04), while the remainder were associated with nACh alpha 10 (rs2672211; p=0.01, rs2672214; p=0.01, rs2741868; p=0.01, rs2741870; p=0.01, rs2741862; p=0.03) alpha 5 (rs951266; p=0.012; rs7180002, p=0.04) and alpha 2 (rs2565048; p=0.01).

[006 15] **Discussion**

[00616] This study revealed a number of AChR SNP variations in CFS/ME patients. Specifically, within the coding sequences of nine AChR genes out of 464 SNPs examined, 17 significant alleles associated with CFS/ME patients were found compared with the

non-fatigued controls. Moreover these alleles were located in the gene sequence of one of the muscarinic acetylcholine receptors (mAChRM3) and three nicotinic acetylcholine alpha receptors (nAChRa2, nAChRa5 and nAChRalO). Interestingly, in Example 1 the inventors identified a number of SNPs in the TRP family, namely TRPC4. The significance of SNPs in mAChRM3 and TRPC4 is that the latter couples to mAChRM3 and can be activated by ACh [40c-42c].

[00617] There is limited information available on the role of these AChR SNPs, however the role of ACh in calcium (Ca^{2+}) cell signalling suggests these AChRs may mediate, in part, the clinical expression of CFS/ME. Moreover, the inventors have shown in Example 1 significant SNPs in the TRP ion channel family, namely TRPA1, TRPM3 and TRPC4, using the same cohort of CFS/ME patients. These findings suggest the potential for significant aberrations in Ca^{2+} cell signalling possibly reflected in the clinical presentation of CFS/ME patients.

[00618] mAChR receptors are responsible for initiating smooth muscle contraction, such as in the gastrointestinal and genitourinary tracts, as well as effects in immune cells, epithelial, ovarian and ocular skin cells, respiratory and secretory glands [43c-46c, 33c, 34c, 32c, 47c-52c, 35c, 5c]. nAChRs are also reported on T and B lymphocytes [53c, 54c]. Human T lymphocytes express the a3, α4, α7, β2 and β4 receptor subunits [55c] while in the mouse and human thymus mAChR expression has been found to play a role in T lymphocyte development and proliferation [53c, 56c-58c]. The α4 or α7 subunits have also been reported on B lymphocytes and found to stimulate proliferation, while decreasing antibody production [59c]. Such findings provide possible insight regarding the SNPs characterised in this Example noting that previous investigations have reported compromise to immune function in CFS/ME patients. Significantly, changes in numbers and function of lymphocytes such as Natural Killer (NK) lymphocytes, T and B lymphocytes in these studies suggests increased influx of Ca^{2+}.

[00619] The mAChRM3 receptors are located in the gastrointestinal tract and are controlled in part by the parasympathetic nervous system, through the vagus nerve [60c]. Where nerve fibres make synapse within the gut wall, the main neurotransmitter, acetylcholine, usually stimulates GI motility. Moreover, clinical data reports nAChRs are involved in inflammatory bowel disease [61c]. Dysregulation of Ca^{2+} mediated channels such as influx or reduction of Ca^{2} flow could cause significant changes in GI motility. CFS/ME patients often exhibit gastrointestinal associated issues, such as irritable bowel syndrome and constipation [12c, 28c].

[00620] Dysregulation of mAChRM3 receptors may affect metabolic and cardiac responses. In normal pancreas, mAChRM3 receptors play a role in regulating insulin and glucagon secretion [62c, 63c]. Muscarinic acetylcholine receptors expressed by pancreatic β-cells have been reported to play a significant role in maintaining proper insulin release and in

maintaining whole body glucose homeostasis [62c]. Changes in Ca^{2+} mediated channels may result in adverse glucose metabolic outcomes as implied in CFS/ME patients [64c]. AChR SNPs in CFS patients will likely affect Ca^{2+} modulation in intracellular pathways through the influx of Ca^{2+} ions. Pancreatic β-cells rely on a transient decrease in Ca^{24} to initiate the complex sequence of events resulting in insulin secretion following glucose exposure. Hellman et al. [65c] report that elevation of glucose induces transient inhibition of insulin release by lowering cytoplasmic Ca^{2+} below baseline in pancreatic β-cells. This period was found to coincide with increased glucagon release and hence was asserted to be the starting point for anti-synchronous pulses of insulin and glucagon. They conclude that the period of initial decrease of cytoplasmic calcium ion concentration regulates the subsequent β-cell response to glucose. Thus it may be argued that aberrant elevated intracellular Ca^{2+} concentrations through permissive TRP and AChR activity will impede the usual and necessary sequence of events required to initiate insulin response to glucose in CFS patients.

[00621] Cardiac mAChRM3 receptors perform an array of pathological and physiological functions. mAChM2 is not the only muscarinic receptor involved in cardiac function, rather mAChRM3 parasympathetic control of cardiac function is well established [66c]. A report by van Borren et al. [67c] shows the effect of muscarinic AChR stimulation on Ca^{2+} transients, cAMP production and pacemaker frequency in sinoatrial (SA) nodes of the rabbit. They found that the pacemaker slowing effects of muscarinic agonists are augmented by Ca^{2+} transient inhibition, suggesting a negative chronotropic effect of muscarinic agonists is, in part, obtained by Ca^{2+} transient inhibition and subsequent reduction in cAMP. These findings imply that muscarinic agonism will have an effect on SA node function exacerbating disturbances of proper cardio-regulatory mechanisms, particularly in an environment where Ca^{2+} intracellular concentrations are likely to be altered due to direct effects of receptor activity. Clinical consequences such as altered orthostatic cardiovascular responses could be predicted and could align with symptom presentation in CFS/ME [13c, 21c, 25c, 27c, 29c].

[00622] In the vascular system, the endothelium contains nAChRs, including a3, a5, a7, alO, β2, β and β4 [68c, 48c, 69c]. Depending upon the type of smooth muscle a specific subtype of nAChR is present; α3 and α5 are found in arteries, while α′*l* is widespread, although not present in the renal circulatory system. nAChR a5, *al,* β2 and β3 have been found in brain endothelial cells [70c] and are an important component of the blood-brain barrier (BBB). nAChR receptor assembly is important for ion permeability and desensitisation. nAChR *al* subunits are known to desensitise rapidly as well as have a high Ca^{2+}:Na^{+} permeability. A combination of *a′l* with α5 nAChR subunits results in receptors with distinct desensitisation properties and ion permeability relative to the homomeric α7 nAChR [71c, 72c]. More dramatic changes in nAChR channel

kinetics are observed when the a5 nAChR subunit incorporates into receptors with the a3 and β4 nAChR subunits, suggesting subunit conformations may impact on functional properties [73c, 74c] of these receptors. This current Example identified SNPs in α5 and a3 nAChR subunits, implying anomalies of signal transduction in the inventor's patient cohort. nAChRs are reported to be involved in arousal, sleep and fatigue as well as those functions that are responsible for processing of pain, memory and cognition [75c-77c].

[00623] Voltage-gated Ca^{2+}-selective channels (CaVs) and intracellular $Ca2^{+}$ Signalling Networks and nonselective ion channels are known to play a significant role in cell integrity, function and cell cycle. The results in this current Example suggest there is an intrinsic role between SNPs of both TRP and ACh receptors that may underpin CFS/ME pathology.

[00624] Adenylate cyclases (AC) are critical in producing cAMP from ATP through a non-redundant mechanism. Ca^{2+} promotes cAMP production via the Ca^{2+} sensitive AC1 in the guinea pig sinoatrial (SA) node, although the role of the other Ca^{2+}-stimulated AC subtype (AC8), in the guinea pig SA node is uncertain [78c]. The five muscarinic ACh receptors (M(l)-M(5)) are differentially expressed in the brain. M(2) and M(4) are coupled to inhibition of stimulated adenylyl cyclase, while M(l), M(3) and M(5) are mainly coupled to the phosphoinositide pathway [79c]. However as ACh is largely mediated through Ca^{2+} the question is raised as to whether permissive influx of Ca^{2+} occurs through TRP and AChR SNPs and whether this combination of factors may result in dysregulation of AC activity and cAMP/Ca^{2+}interactions. Support for this argument is highlighted where TRPC4 couples to mAChRM3 and is activated by ACh [40c-42c].

[00625] A key component of AC regulation and cAMP production is achieved through two AC stimulating vasoactive neuropeptides, namely vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase activating polypeptide (PACAP). In cardiac neurons which express TRPC transcripts, PACAP activates calcium-permeable non-selective cationic channels, which are likely members of the TRPC family [80c]. Inhibition of intracellular calcium increases by the application of calcium channel blockers indicates that PACAP acts on calcium influx [81c]. Notably it is calcium ion influx, not release from calcium ion stores, which is required for PACAP-induced increase in excitability in guinea pig intra-cardiac neurons. Importantly, the expression of PACAP genes is controlled by calcium and cAMP signals in neurons, suggesting that dysregulated calcium influx into cells will have effects on PACAP expression. The activity-dependent gene expression is jointly controlled by Ca^{2+} and cAMP signals not only at the transcriptional level but also at the post-translational level for the cumulative mRNA expression in neurons [82c]. Earlier research has shown in isolated NK lymphocytes a significant increase in VPAC1R numbers for CFS/ME patients compared with controls [15c]. An increase in

VPAC1R numbers found on these lymphocytes may have occurred to compensate for impaired AC and cAMP signalling.

[00626] In conclusion, the inventors report for the first time the presence of SNPs in receptors for ACh (predominantly M3 and CFS) and in association with TRP SNPs in patients with CFS/ME. Many detrimental consequences for physiological homeostasis are possible through aberrant ACh and TRP function in these patients. These scenarios conceivably are associated with CFS/ME pathomechanisms and symptomatology and require further investigation.

Example 3 - Non-Synonymous Single Nucleotide Polymorphisms in AChR and TRP in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

[00627] In Examples 1 and 2 the inventors identified single nucleotide polymorphisms (SNPs) in genes for transient receptor potential (TRP) ion channels and acetylcholine receptors (AChRs), which have important roles in calcium (Ca^{2+}) and acetylcholine (ACh) signalling. Non-synonymous single nucleotide polymorphisms (nsSNPs) are those SNPs resulting in changes to protein expression of these receptors which may be responsible for aberrant signalling and hence potentially change of function.

[00628] In this Example the inventors determine that nsSNPs are present in those SNPs previously identified in TRP ion channel and AChR genes in CFS/ME patients.

[00629] **Method**

[00630] *Subjects*

[00631] CFS patients were defined in accordance with the 1994 CDC criteria for CFS [32d]. 115 CFS/ME patients (age=48.68±1.06 years) and 90 non-fatigued controls (age=46.48±1.22 years) were examined for nsSNPs in genes for TRP ion channels and AChRs.

[00632] *Blood collection and DNA extraction*

[00633] A volume of 10 mL of whole blood was collected from all participants into EDTA tubes. Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood mini-kit as per manufacturer's instructions (Qiagen). SNP genotyping studies were performed as previously described.

[00634] *nsSNP analysis*

[00635] A total of 81 SNPs were examined in the present study: 53 nsSNPs for four AChR genes (M3, and alpha 2, 5 and 10) and 28 nsSNPs for TRP ion channel genes *(TRPA1, TRPC4, TRPM3 and TRPM4).*

[00636] *nsSNP Statistical analysis*

[00637] All 81 SNPs resulting from the PLINK analysis with p values of < 0.1, were taken and used as input into the Variant Effect Predictor, to determine the effect of the variants. The resulting variants set at an alpha level of $p<0.05$ and their consequences can be found in Table 3-

4 and 5-6 for TRP and AChR, respectively. Analyses were performed at the Australian Genome Research Facility Ltd, The Walter and Eliza Hall Institute, Parkville, Victoria, Australia.

Table 3: Frequency distribution and significance of Transient Receptor Potential (TRP) nsSNPs in CFS/ME patients and non-fatigued controls in rank order of significance.

CHR	SNP	BP	A1	F_A	F_U	A2	P	Location	Allele	Consequence	IMPACT	Gene	Feature_type	Feature	BIOTYPE	HGVSc
9	rs12682832	70605775	A	0.4444	0.2927	G	0.002999	9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.2173-2305N>C
								9:70605775-70605775	G	intron_variant,non_coding_tra	MODIFIER	101927086	Transcript	XR_428546.1	lncRNA	XR_428546.1:n.1265-1283N>G
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.2632-2305N>C
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.2248-2305N>C
								9:70605775-70605775	G	downstream_gene_variant	MODIFIER	101927086	Transcript	XR_242612.2	lncRNA	-
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.2143-2305N>C
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.2209-2305N>C
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.2218-2305N>C
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.2713-2305N>C
								9:70605775-70605775	G	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.2179-2305N>C
9	rs11142508	70616746	C	0.445	0.2976	T	0.003675	9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.1864-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.2323-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.1939-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.1834-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.1900-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.1909-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.2404-671N>A
								9:70616746-70616746	T	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.1870-671N>A
9	rs1160742	70699095	A	0.47	0.3333	G	0.007871	9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.814-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.1273-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.889-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.814-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.814-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.889-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.1354-17517N>C
								9:70699095-70699095	G	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.814-17517N>C
9	rs4454352	70795494	C	0.24	0.1369	T	0.01254	9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.515-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_001007470.1	protein_coding	NM_001007470.1:c.590-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.974-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_206948.2	protein_coding	NM_206948.2:c.515-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.590-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.515-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.515-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.590-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.515-11215N>A
								9:70795494-70795494	T	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.1055-11215N>A

Table 4: Frequency distribution and significance of Transient Receptor Potential (TRP) nsSNPs in CFS/ME patients and non-fatigued controls in rank order of significance.

CHR	SNP	BP	A1	F_A	F_U	A2	P	Location	Allele	Consequence	IMPACT	Gene	Feature_type	Feature	BIOTYPE	HGVSc
13	rs66550469	37793812	T	0.505	0.3795	C	0.01625	13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_016179.2	protein_coding	NM_016179.2:c.-27-10452N>A
								13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_003306.1	protein_coding	NM_003306.1:c.-27-10452N>A
								13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_001135958.1	protein_coding	NM_001135958.1:c.-27-10452N>A
								13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_001135955.1	protein_coding	NM_001135955.1:c.-27-10452N>A
								13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_001135956.1	protein_coding	NM_001135956.1:c.-27-10452N>A
								13:37793812-37793812	T	intron_variant	MODIFIER	7223	Transcript	NM_001135957.1	protein_coding	NM_001135957.1:c.-27-10452N>A
13	rs6655207	37793875	G	0.5051	0.381	T	0.01757	13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_016179.2	protein_coding	NM_016179.2:c.-27-10515N>A
								13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_003306.1	protein_coding	NM_003306.1:c.-27-10515N>A
								13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_001135958.1	protein_coding	NM_001135958.1:c.-27-10515N>A
								13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_001135955.1	protein_coding	NM_001135955.1:c.-27-10515N>A
								13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_001135956.1	protein_coding	NM_001135956.1:c.-27-10515N>A
								13:37793875-37793875	T	intron_variant	MODIFIER	7223	Transcript	NM_001135957.1	protein_coding	NM_001135957.1:c.-27-10515N>A
8	rs4738202	72028626	A	0.3687	0.253	G	0.01806	8:72028626-72028626	G	intron_variant,non_coding_tra	MODIFIER	100132891	Transcript	NR_033652.1	lncRNA	NR_033652.1:n.1029-23913N>G
								8:72028626-72028626	G	intron_variant,non_coding_tra	MODIFIER	100132891	Transcript	NR_033651.1	lncRNA	NR_033651.1:n.434-23913N>G
								8:72028626-72028626	G	intron_variant	MODIFIER	8989	Transcript	NM_007332.2	protein_coding	NM_007332.2:c.2937+1275N>C
9	rs7865858	70589515	A	0.45	0.3313	G	0.02084	9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.2728+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.3187+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.2803+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.2698+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.2764+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.2773+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.3268+1516N>C
								9:70589515-70589515	G	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.2734+1516N>C
								9:70589515-70589515	G	regulatory_region_variant	MODIFIER	-	RegulatoryFeature	ENSR00001471087	promoter_flanking_region	-
8	rs2383844	72049017	G	0.505	0.3976	A	0.03999	8:72049017-72049017	A	intron_variant,non_coding_tra	MODIFIER	100132891	Transcript	NR_033652.1	lncRNA	NR_033652.1:n.1029-3522N>A
								8:72049017-72049017	A	intron_variant,non_coding_tra	MODIFIER	100132891	Transcript	NR_033651.1	lncRNA	NR_033651.1:n.434-3522N>A
								8:72049017-72049017	A	intron_variant	MODIFIER	8989	Transcript	NM_007332.2	protein_coding	NM_007332.2:c.1905+17611N>T
9	rs1504401	71302037	T	0.1	0.1726	C	0.04111	9:71302037-71302037	C	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.183+144616N>G
9	rs10115622	70691635	A	0.335	0.4345	C	0.05014	9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_020952.4	protein_coding	NM_020952.4:c.814-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_001007471.2	protein_coding	NM_001007471.2:c.1273-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_206946.3	protein_coding	NM_206946.3:c.889-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_206944.3	protein_coding	NM_206944.3:c.814-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_024971.5	protein_coding	NM_024971.5:c.814-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_206947.3	protein_coding	NM_206947.3:c.889-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	XM_005252218.2	protein_coding	XM_005252218.2:c.1354-10057N>T
								9:70691635-70691635	A	intron_variant	MODIFIER	80036	Transcript	NM_206945.3	protein_coding	NM_206945.3:c.814-10057N>T
19	rs10403114	49200507	G	0.2929	0.3902	A	0.05119	19:49200507-49200507	G	intron_variant	MODIFIER	54795	Transcript	XM_005259017.1	protein_coding	XM_005259017.1:c.1491+75N>G
								19:49200507-49200507	G	intron_variant	MODIFIER	54795	Transcript	NM_001195227.1	protein_coding	NM_001195227.1:c.2343+75N>G
								19:49200507-49200507	G	intron_variant	MODIFIER	54795	Transcript	XM_005259018.1	protein_coding	XM_005259018.1:c.1170+75N>G
								19:49200507-49200507	G	intron_variant	MODIFIER	54795	Transcript	XM_006723249.1	protein_coding	XM_006723249.1:c.2523+75N>G
								19:49200507-49200507	G	intron_variant	MODIFIER	54795	Transcript	NM_017636.3	protein_coding	NM_017636.3:c.2778+75N>G

Table 5: Frequency distribution and significance of acetylcholine receptor (AChR) nsSNPs in CFS/ME patients and non-fatigued controls in rank order of significance.

CHR	SNP	BP	A1	F_A	F_U	A2	P	Location	Allele	Consequence	IMPACT	Gene	Feature_type	Feature	BIOTYPE	HGVSc
3	rs4463655	239820994	T	0.3077	0.4671	C	0.002803	1:239820994-239820994	C	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-6258T>C
								1:239820994-239820994	C	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-6258T>C
								1:239820994-239820994	C	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-86439T>C
								1:239820994-239820994	C	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-6258T>C
3	rs589962	239826664	C	0.2416	0.3919	T	0.003476	1:239826664-239826664	C	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-588T>C
								1:239826664-239826664	C	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-588T>C
								1:239826664-239826664	C	upstream_gene_variant	MODIFIER	1131	Transcript	XM_005273034.1	protein_coding	-
								1:239826664-239826664	C	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-80769T>C
								1:239826664-239826664	C	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-588T>C
3	rs1072320	239819076	G	0.3242	0.1842	A	0.003704	1:239819076-239819076	G	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-8176A>G
								1:239819076-239819076	G	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-8176A>G
								1:239819076-239819076	G	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-88357A>G
								1:239819076-239819076	G	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-8176A>G
								1:239819076-239819076	G	regulatory_region_variant	MODIFIER	-	RegulatoryFeature	ENSR00000555822	CTCF_binding_site	-
3	rs7543259	239815886	A	0.3187	0.1842	G	0.005128	1:239815886-239815886	A	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-11366G>A
								1:239815886-239815886	A	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-11366G>A
								1:239815886-239815886	A	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-91547G>A
								1:239815886-239815886	A	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-11366G>A
								1:239815886-239815886	A	regulatory_region_variant	MODIFIER	-	RegulatoryFeature	ENSR00000555821	promoter_flanking_region	-
3	rs6661621	239821503	C	0.3022	0.1711	G	0.005358	1:239821503-239821503	C	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-5749G>C
								1:239821503-239821503	C	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-5749G>C
								1:239821503-239821503	C	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-85930G>C
								1:239821503-239821503	C	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-5749G>C
								1:239905329-239905329	C	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-19-2104G>C
11	rs2672211	3669048	C	0.3736	0.2434	T	0.0107	11:3669048-3669048	T	downstream_gene_variant	MODIFIER	417	Transcript	XM_005252933.2	protein_coding	-
								11:3669048-3669048	T	downstream_gene_variant	MODIFIER	417	Transcript	NM_004314.2	protein_coding	-
								11:3669048-3669048	T	intron_variant	MODIFIER	57053	Transcript	NM_020402.2	protein_coding	NM_020402.2:c.362+148G>A
								11:3669048-3669048	T	intron_variant	MODIFIER	417	Transcript	XM_006718236.1	protein_coding	XM_006718236.1:c.886+7635C>T
								11:3669048-3669048	T	downstream_gene_variant	MODIFIER	417	Transcript	XM_006718237.1	protein_coding	-
11	rs2672214	3670282	C	0.3708	0.24	T	0.0108	11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	XM_006718241.1	protein_coding	-
								11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	XM_006718242.1	protein_coding	-
								11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	NM_016320.4	protein_coding	-
								11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	XM_006718240.1	protein_coding	-
								11:3670282-3670282	T	intron_variant	MODIFIER	417	Transcript	XM_006718236.1	protein_coding	XM_006718236.1:c.886+8869C>T
								11:3670282-3670282	T	intron_variant	MODIFIER	57053	Transcript	NM_020402.2	protein_coding	NM_020402.2:c.62-341G>A
								11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	XM_005252950.1	protein_coding	-
								11:3670282-3670282	T	downstream_gene_variant	MODIFIER	4928	Transcript	NM_139132.3	protein_coding	-
11	rs2741868	3668953	T	0.3693	0.24	A	0.01185	11:3668953-3668953	T	downstream_gene_variant	MODIFIER	417	Transcript	XM_005252933.2	protein_coding	-
								11:3668953-3668953	T	downstream_gene_variant	MODIFIER	417	Transcript	NM_004314.2	protein_coding	-
								11:3668953-3668953	T	intron_variant	MODIFIER	57053	Transcript	NM_020402.2	protein_coding	NM_020402.2:c.362+243T>A
								11:3668953-3668953	T	intron_variant	MODIFIER	417	Transcript	XM_006718236.1	protein_coding	XM_006718236.1:c.886+7540A>T
								11:3668953-3668953	T	downstream_gene_variant	MODIFIER	417	Transcript	XM_006718237.1	protein_coding	-
11	rs2741870	3668879	G	0.3708	0.2434	C	0.01281	11:3668879-3668879	G	downstream_gene_variant	MODIFIER	417	Transcript	XM_005252933.2	protein_coding	-
								11:3668879-3668879	G	downstream_gene_variant	MODIFIER	417	Transcript	NM_004314.2	protein_coding	-
								11:3668879-3668879	G	intron_variant	MODIFIER	57053	Transcript	NM_020402.2	protein_coding	NM_020402.2:c.362+317G>C
								11:3668879-3668879	G	intron_variant	MODIFIER	417	Transcript	XM_006718236.1	protein_coding	XM_006718236.1:c.886+7466C>G
								11:3668879-3668879	G	downstream_gene_variant	MODIFIER	417	Transcript	XM_006718237.1	protein_coding	-

Table 6: Frequency distribution and significance of acetylcholine receptor (AChR) nsSNPs in CFS/ME patients and non-fatigued controls in rank order of significance.

CHR	SNP	BP	A1	F_A	F_U	A2	P	Location	Allele	Consequence	IMPACT	Gene	Feature_type	Feature	BIOTYPE	HGVSc
3	rs7520974	239903960	G	0.4205	0.5533	A	0.0167	1:239903960-239903960	A	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-19-3473G>A
								1:239903960-239903960	A	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-19-3473G>A
								1:239903960-239903960	A	intron_variant	MODIFIER	1131	Transcript	XM_005273034.1	protein_coding	XM_005273034.1:c.-19-3473G>A
								1:239903960-239903960	A	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-3473G>A
								1:239903960-239903960	A	upstream_gene_variant	MODIFIER	100873984	Transcript	NR_046582.1	lncRNA	-
								1:239903960-239903960	A	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-19-3473G>A
3	rs6669810	239905329	G	0.4213	0.5467	C	0.02361	1:239905329-239905329	C	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-19-2104G>C
								1:239905329-239905329	C	intron_variant	MODIFIER	1131	Transcript	XM_005273034.1	protein_coding	XM_005273034.1:c.-19-2104G>C
								1:239905329-239905329	C	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-2104G>C
3	rs7180002	78581651	T	0.3846	0.2763	A	0.03682	15:78581651-78581651	T	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-19-2104G>C
								15:78581651-78581651	T	intron_variant	MODIFIER	1138	Transcript	NM_000745.3	protein_coding	NM_000745.3:c.258+689A>T
3	rs6429157	239818343	G	0.522	0.4079	A	0.0375	1:239818343-239818343	G	intron_variant	MODIFIER	1138	Transcript	XM_005254142.1	protein_coding	XM_005254142.1:c.258+689A>T
								1:239818343-239818343	G	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-146-8909A>G
								1:239818343-239818343	G	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-146-8909A>G
								1:239818343-239818343	G	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-89090A>G
								1:239818343-239818343	G	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-146-8909A>G
8	rs55828312	42734459	G	0.2386	0.1513	A	0.04789	8:42734459-42734459	G	intron_variant	MODIFIER	1142	Transcript	NM_000749.3	protein_coding	NM_000749.3:c.1242+1910A>G
3	rs12036141	239902696	A	0.4121	0.3092	G	0.05184	1:239902696-239902696	A	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-19-4737G>A
								1:239902696-239902696	A	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-19-4737G>A
								1:239902696-239902696	A	intron_variant	MODIFIER	1131	Transcript	XM_005273034.1	protein_coding	XM_005273034.1:c.-19-4737G>A
								1:239902696-239902696	A	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-19-4737G>A
								1:239902696-239902696	A	upstream_gene_variant	MODIFIER	100873984	Transcript	NR_046582.1	lncRNA	-
								1:239902696-239902696	A	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-19-4737G>A
3	rs6429147	239631494	C	0.4444	0.34	G	0.05349	1:239631494-239631494	G	intron_variant	MODIFIER	1131	Transcript	XM_005273033.1	protein_coding	XM_005273033.1:c.-249-46692C>G
								1:239631494-239631494	G	intron_variant	MODIFIER	1131	Transcript	XM_005273032.1	protein_coding	XM_005273032.1:c.-312-730C>G
								1:239631494-239631494	G	intron_variant	MODIFIER	1131	Transcript	XM_006711732.1	protein_coding	XM_006711732.1:c.-185-730C>G
								1:239631494-239631494	G	intron_variant	MODIFIER	1131	Transcript	NM_000740.2	protein_coding	NM_000740.2:c.-312-730C>G
8	rs16891561	42724596	T	0.2472	0.1597	C	0.05454	8:42724596-42724596	C	intron_variant	MODIFIER	1142	Transcript	NM_000749.3	protein_coding	NM_000749.3:c.250-5998T>C

[00639] **Results**

[00640] *Participants*

[00641] There were 115 CFS patients (age=48.68+1.06 years), of which 84 (73.04%) were females and 31 (26.96%) were males. There were 90 non-fatigued controls (age=46.48+1.22 years) comprising 59 (65.56%) females and 31 (34.44%) males. All participants in both groups were of European decent and were residents of Australia at the time of blood collection.

[00642] Of 81 SNPs identified in TRP ion channel and AChR genes, 29 nsSNPs were located at intron variants, as well as regulatory region variants, and up-stream and down-stream variants. A total of 12 nsSNPs for TRP ion channel genes *(TRPA1, TRPC4 and TRPM3 and TRPM4)* were identified in the CFS/ME group. Specifically, 7 nsSNPs featured for TRPM3, 2 nsSNPs for TRPC4, 2 nsSNPs for TRPA1 and 1 nsSNP for TRPM4. A total of 17 nsSNPs for AChR were found, where 10 nsSNPs were identified for mAChM3, 4 nsSNPs for nAChalO, 1 nsSNP for nACha5 and 2 nsSNPs for nACh<x2. Tables 3-4 and Tables 5-6 represent the nsSNPs for TRP ion channel and AChR genes, respectively.

[00643] The predominant gene where these nsSNPs for TRP ion channels were reported was gene 80036 as it had 51 significant reportable events (66%) from a total of 77 events. The remaining nsSNPs for TRP ion channels were found in genes 7223, 101927086 and 54795 where each reported 12 (15%), 2 (2%) and 5 (6%) events, respectively.

[00644] Analysis of the nsSNPs for AChRs found the gene 1131 had 44 reportable (59%) events from a total number of 74 events. The remaining nsSNPs for AChR genes were found in genes 417, 4928, 57053, 100873984, 1138 and 1142 where each reported 12 (16%), 6 (8%), 4 (5%), 2 (3%), 2 (3%) and 2 (3%) events, respectively.

[00645] **Discussion**

[00646] This is the first study to report the presence of nsSNP variations in TRP ion channel genes and AChR genes in CFS/ME patients. Collectively, 29 nsSNPs were identified in genes for TRP ion channels and AChRs. A total of 12 nsSNPs were identified for TRP ion channel genes *{TRPA1, TRPC4, TRPM3 and TRPM4)* and 17 nsSNPs were identified for AChR genes (10 nsSNPs for mAChM3, 4 nsSNPs for nAchcdO, 1 nsSNP for nAcha5 and 2 nsSNPs for nAcha2).

[00647] There is limited information available on the role of nsSNPs in these AChR and TRP ion channels in disease. The inventors now report nsSNPs located in intron variants, regulatory region variants and up-stream and down-stream variants of the TRP ion channel and AChR genes in their patient cohort. These variants are likely to be critical in

contributing to perturbations of TRP ion channel and AChR function mediated through altered calcium and ACh signalling and manifested as physiological system compromise. Therefore, the critical role of AChRs and TRP ion channels in Ca^{2+} cell signalling suggests these nsSNPs may contribute to the clinical manifestation of CFS/ME.

[00648] Identification of the genes containing these nsSNPs for both TRP ion channels and AChRs revealed important roles in calcium cell signalling as well as acetylcholine function with additional roles in adenylate cyclase inhibition respectively. Importantly, genes 80036 and 1131 which accounted for the majority of nsSNPs influence these functions. For example, gene 80036 is associated with calcium signalling mechanisms and calcium store depletion via different isoforms which have been identified through alternative splicing [33d]. Gene 1131 codes for muscarinic cholinergic receptors which demonstrate features including binding of acetylcholine as well as adenylate cyclase inhibition, phosphoinositide degeneration, and potassium channel mediation. As noted above muscarinic receptors mediate acetylcholine activity in the central and peripheral nervous systems. The muscarinic cholinergic receptor 3 (mAChRM3), controls smooth muscle contraction and glandular secretion [34d].

[00649] The significance of the inventors' findings is supported by others who suggest that alternate splicing in the coding and also in the non-coding sequences may have significant unexpected outcomes on the splicing mechanism of the gene transcripts [lOd, 1Id]. Splicing genetic variants found in the exons and deep intronic variants, as well as down and up-stream variants have a role in alternative splicing mechanisms resulting in diverse protein isoforms. Such altered protein isoform expression may be an important contributing factor affecting changes in protein function. Incidentally, the human gene has the largest average number of mRNA isoforms per gene [35d] with an average of seven mRNA isoforms per gene [36d, 37d]. Furthermore, the regulatory elements in the intron sequences as well as the assembly of the spliceosome add a significant level of complexity to the splicing mechanism for the correct coding of a protein sequence. Enhancers and silencers that are located either in the exons or introns are integral in recognition of the correct exon sequence [38d]. Additionally, others have shown introns are able to generate active spliceosomes, giving rise to alternative splicing events [39d, 40d]. Importantly, the inventors' data show that the greatest proportion of intron variants as well as regulatory region variants occur in the nsSNPs in TRPM3 and mAChM3 genes and may alter the gene transcripts.

[00650] Research to date highlights the importance of such variants in affecting gene transcripts by causing alternative splicing resulting in anomalies in mRNA and translation

products. Alternative splicing in TRPM3 and mAChM3 genes may result in aberrant Ca^{2+} signalling because of the known secondary pathways involving Ca^{2+} which mediate effects for both TRPM ion channels and AChRs. Changes in AChRs and TRP ion channel signalling may have important physiological implications for CFS/ME patients as these TRP ion channels and AChRs are located on nearly all cells in the body. The predominance of CNS symptoms in CFS/ME may result in part from TRPM3 being substantially distributed in the CNS [41d]. Calcium metabolism and signalling in the context of TRPC ion channel as well as muscarinic receptor function is vital for the function of the CNS. Memory, attention, sensory acuity, emotion, pain and motor control [42d, 43d] are critical functions localised throughout a number of regions in the brain [44d]. These CNS functions have been reported to be significantly impaired in CFS/ME patients [45d-47d]. TRPM3 ion channels also function in the roles of heat detection, nociception and transmission of pain [48d, 49d]. Dysregulation in thermoregulatory responses as well as central and peripheral pain have also been reported in CFS/ME patients [50d], suggesting the nsSNPs reported in this study may contribute to the potential CNS impairments in these patients.

[00651] Interestingly, TRPM3 is the only TRP ion channel discovered so far to have a second embedded channel or Omega pore [51d]. This pore is characterised to have features distinguishing it from the TRPM3 main channel, such as activation and current flow characteristics and permeability to Na^+ and K^+ rather than Ca^{2+}, which may be relevant in signalling. For example, it appears the Omega channel acts to potentiate the signal mediated via the main TRPM3 channel, thus giving TRPM3 unique qualities of magnified signalling, particularly nociception and pain transmission. As there have been a number of previous findings reporting significant changes in inflammatory cytokines from CFS/ME patients [52d-54d] the question is asked if an inflammatory mediator may act on the Omega pore to exert an effect on pore opening and promulgation of a nociceptive signal [55d]. The possibility therefore exists that the reportable nsSNPs for TRPM3 in conjunction with this omega pore may potentiate and amplify pathological signalling of TRPM3 when stimulated by inflammatory or other agents.

[00652] mAChM3 receptors have been documented in the gastrointestinal tract and are controlled in part by the parasympathetic nervous system, through the vagus nerve [56d]. ACh has been shown to mediate gut motility via the nerve fibres that make synapses within the gut wall. Ca^{2+} mediated channel perturbations through excessive influx or reduction of Ca^{2+} flow could cause significant changes in GI motility. It is plausible that nsSNPs' alternative splicing in intron and regulatory regions of mAChM3 genes and TRPC4 genes

may cause irregular gastrointestinal motility through activating smooth muscle depolarization [57d]. Additionally, TRPC4 couples to mAChRM3 in the intestine, activating smooth muscle depolarization, inflow of Ca^{2+} and smooth muscle contraction [57d]. TRPC4 may be simultaneously regulated by G protein-coupled receptors (GPCRs) [58d]. Enhanced cholinergic-mediated increase in the pro-inflammatory cytokines IL-6 and IL-8 has also been reported in patients with irritable bowel syndrome [59d]. CFS/ME patients report intestinal dysfunction or irritable bowel syndrome including diarrhoea [14d, 30d], while other researchers have reported elevated IL-6 and IL-8 in this patient group [60d].

[00653] nsSNPs of intron or regulatory regions of mAChRM3 receptors may affect metabolic and cardiac responses. mAChRM3 receptors, along with TRPM3 ion channels, play a role in regulating insulin and glucagon secretion [61d, 62d]. Muscarinic acetylcholine receptors (mAChRs) expressed by pancreatic β-cells function to maintain homeostasis of whole body glucose [61d]. nsSNPs documented in mAChM3R genes may mediate changes in Ca^{2+} channels thus influencing pancreatic β-cell function and impact glucose metabolism in CFS/ME patients [63d]. Cardiac function via mAChRM3 parasympathetic control is well established [37d] and pacemaker slowing effects of muscarinic agonists are augmented by Ca^{2+} transient inhibition, resulting in altered cardio-regulatory mechanisms. Importantly the nsSNPs (intron variants or regulatory regions) found in mAChM3 may alter intracellular Ca^{2+} concentrations, resulting in changes in insulin response to glucose or other stimuli as well as contributing to orthostatic cardiovascular effects. Both these physiological disturbances are reported in CFS/ME patients [15d, 23d, 27d, 29d, 31d].

[00654] TRPA1 ion channels are reported on astrocytes of the CNS and contribute to calcium uptake and regulation of astrocytes [64d-67d]. TRPA1 ion channels also initiate acute headache as well as mediating pain and migraine in fibromyalgia patients [68d]. Both symptoms are identified in CFS/ME patients, suggesting nsSNPs for TRPA1 may play a role in the pathology of this illness.

[00655] **Conclusion**

[00656] This Example shows a high proportion of nsSNPs (i.e. non-synonymous SNPs) in intronic variants and regulatory variants for TRP ion channels and AChR genes in the inventors' CFS/ME patient cohort. Silent alternative splicing has been suggested to be involved in disease phenotypes, e.g. through exon skipping, alternative splice isoforms of the gene transcript or alternate spliceosomes. The inventors' results suggest such gene variants may result in phenotype anomalies in TRP ion channel expression and AChR expression leading to altered calcium and acetylcholine regulation in CFS/ME and provide a possible

rationale for the development of, or predisposition to this debilitating illness.

[00657] **Example** 4: **Genotype frequencies of TRPM3 ion channels and mAChM3 receptors gene polymorphisms in CFS/ME patients**

[00658] In the Examples above the inventors describe SNPs in genes for TRP ion channels and AChRs, which have important roles in calcium (Ca^{2+}) and acetylcholine (ACh) signalling. The inventors now report from this same cohort of patients additional data showing the prevalence of both melastatin TRP (TRPM3) ion channel and muscarinic acetylcholine receptor (mAChM3R) SNP genotypes in CFS/ME patients.

[00659] Genomic DNA extraction and SNP genotyping studies were performed as previously described. The PLINK vl.07 whole genome analysis toolset and IBM® SPSS® Statistics (version 21) was used to determine the genotype frequency between the CFS patients and the nonfatigued controls. A two column $\chi 2$ test was used, where the alpha level of significance was set at a $p<0.05$ and their consequences can be found in Table 7 for TRPM3 and mAChM3, respectively. Analysis of SNP genotype frequencies in TRPM3 family *(rsl2682832; rslll42508; rs3763619)* and mAChM3R *(rsl2036141; rs589962; rsl072320; rs7543259; rs7520974; rs726169; rsrs6669810; rsrs6429157)* demonstrated high prevalence in this cohort of CFS/ME patients as compared to non-fatigued controls (Table 7).

Table 7: Genotype frequencies of TRPM3 and mAChM3 gene polymorphisms in CFS patients and nonfatigued controls.

Gene	Chromosome	RefSNPID	Genotype	CFS (%)	Non fatigued controls (%)	$\chi 2$	*P*-VALUE	OR
mAchM3	1	rs589962	TT	52 (65%)	28 (35 %)	6.839	0.009	2.286
mAchM3	1	rs1072320	AG	47 (66.2%)	24 (33.8%)	6.825	0.009	2.314
mAchM3	1	rs7543259	AG	46 (65.7%)	24 (34.3%)	6.122	0.013	2.215
mAchM3	1	rs7520974	AA	30 (68.2%)	14 (31.8%)	4.515	0.034	2.178
mAchM3	1	rs726169	AA	49 (67.1%)	24 (32.9%)	8.345	0.004	2.528
mAchM3	1	rs6669810	CC	29 (67.4%)	14 (32.6%)	3.917	0.048	2.071
mAchM3	1	rs6429157	GG	25 (71.4%)	10 (28.6%)	5.123	0.024	2.500
mAchM3	1	rs12036141	AA	15 (75%)	5 (25%)	3.854	0.050	2.803
TRPM3	9	rs12682832	AA	24 (75%)	8 (25%)	5.501	0.019	2.703
TRPM3	9	rs11142508	CC	25 (73.5%)	9 (26.5%)	5.029	0.025	2.500
TRPM3	9	rs3763619	AA	25 (71.4%)	10 (28.6%)	4.028	0.045	2.222

Notes: Data presented for gene TRPM3 (100 CFS/ME patients and 90 controls) and muscarinic M3 (91 CFS patients and 76 controls), chromosome location (CIIR), reference SNP identification (Ref SNP ID), genotype, number and percentage of CFS patients and non-fatigued controls with a genotype, Pearson Chi-Square test was used for genotype frequency (ldf) and p-value for this test was set at a significance of $p < 0.05$, odds ratio (OR).

[00660] mAChRs are involved in autonomic function, particularly parasympathetic and exocrine function, such as in pancreas, exocrine glands and inotropic and chronotropic cardiac regulation. Given AChRs are distributed differentially around the body it is axiomatic that tissues expressing a predominance of AChRs will be affected differentially by SNPs in muscarinic vs nicotinic ACh receptors. Similarly, TRPs are distributed differentially around the body in all tissues. Adding to the complexity is the relative lack of knowledge about interactions between TRP and AChRs in humans. Interestingly, certain muscarinic ACh receptors are antagonists of TRPM3 via e.g. phospholipase C-coupled mAChMlR [21h, 22h]. Given this developing research regarding the interdependence of mAChRs and TRP families, the inventors question whether mAChM3R and TRPM3 SNP genotype combinations in CFS/ME patients contribute to the pathomechanism and phenotypes of this illness.

[00661] Even though the distribution of these receptors varies in peripheral blood mononuclear cells, SNP genotypes such as those identified in this patient cohort are likely to contribute to perturbations of TRP ion channel and AChR function mediated through altered calcium and ACh signalling and manifest as physiological system compromise. The critical role of AChRs and TRP ion channels in Ca^{2+} cell signalling suggests further characterisation of TRPM3 and mAChM3R may elucidate perturbations of second messenger signalling in CFS/ME. Moreover changes in structure of these receptors may contribute to potential autoimmune responses. A recent publication by Loebel et al [23h] suggests a possible autoimmune mechanism in a subgroup of CFS patients affecting muscarinic acetylcholine receptors (mAChR) and β adrenergic receptors (PAdR). However the evidence for an autoimmune pathology is modest as only a minority (29.5%) of patients expressed antibodies against these receptors. Despite multiple Rituximab infusions only 15 of 25 patients responded. However the possibility of some autoimmune mechanisms contributing to pathomechanisms of CFS/ME could be a response to altered structure of SNP affected receptors or ion channels.

[00662] **Example 5: Natural killer cytotoxicity and SNPs in TRP ion channel and AChR genes of isolated natural killer cells in ME/CFS patients**

[00663] NK cells are granular lymphocytes found in peripheral blood, bone marrow, spleen and lymph nodes [lj-4j]. In peripheral blood, NK cells comprise 15% of

lymphocytes and can be grouped into four subtypes according to the surface expression and density of CD56 (neural cell adhesion molecule) and CD16 [Fey III receptor, the low-affinity receptor for immunoglobulin G (IgG)] [lj-3j, 5j, 6j]. These phenotypes include $CD56^{bright}CD16^{-/dim}$, $CD56^{dim}CD16^{bright}$, $CD56^{dim}CD16^{-}$, $CD56^{-}CD16^{bright}$ [lj-3j]. Approximately 90% of NK cells in peripheral blood are $CD56^{dim}CD16^{bright}$ and $CD56^{bright}$ comprise approximately 10% [2j-4j, 7j]. NK cell cytotoxic activity requires a number of regulated processes to ensure apoptosis of the target cell [8j].

[00664] Though little is known about calcium signaling in NK cells, it has been observed that the granule-dependent pathway of apoptosis is calcium dependent whereas the death-receptor pathway is not [9j, 10j]. In this instance lytic protein transport, exocytosis and fusion have clearly shown calcium dependence [**11j**-13j]. Calcium is also required for the reorientation of microtubules and actin skeleton as well as activation of cytokine gene transcription [13j]. Moreover, studies have demonstrated the relationship between calcium mobilisation and the abrogation of degranulation in NK phospholipase C (PLC)-y2-deficient cells [13j-15j].

[00665] Transient receptor potential (TRP) ion channels are expressed on almost all cells and have a significant effect on physiological functions [16j]. Dysregulation in TRPs has been associated with pathological conditions and diseases [17j-21j]. TRP ion channels are activated in the presence of irritants, inflammatory products, and xenobiotic toxins. TRP ion channels have an important role in Ca^{2+} signaling.

[00666] Acetylcholine (ACh) binds to two membrane proteins, namely the muscarinic (mAChR) and nicotinic receptors (nAChR) of which there are multiple isoforms. ACh performs non-neuronal functions, termed the non-neuronal cholinergic system (NNCS), where ACh performs endocrine and paracrine functions of tissue located on smooth muscle, β pancreatic cells, glial cells, lymphocytes, ocular lens cells and brain vascular endothelium [17j-26j] that is mediated through Ca^{2+} signaling. Acetylcholine receptors (AChRs) transmit activation signals in a variety of human tissues including skeletal and smooth muscle, all preganglionic autonomic nerve fibers, post ganglionic autonomic parasympathetic nerves as well as in many locations throughout the central nervous system (CNS) [27j-29j].

[00667] CFS/ME is characterized by significant impairment in physical activity and debilitating fatigue accompanied by impairment in memory, cognition and

concentration, enhanced experience of pain as well as dysregulation of the gastrointestinal, cardiovascular and immune systems [30j-42j]. Importantly, NK cell dysfunction, in particular reduced NK cell cytotoxic activity is a consistent finding in CFS/ME patients [32j-36j, 39j, 43j]. The inventors have described above SNPs in TRP ion channel genes and AChR genes, namely for TRP ion channels TRPM3, TRPA1, TRPC4, the muscarinic receptor mAChRM3 and the nicotinic alpha receptors nAChR alpha 10, alpha 5 and alpha 2 in peripheral blood mononuclear cells from CFS/ME patients. These SNP anomalies in genes for TRP ion channels and AChRs may produce altered receptor proteins, potentially changing TRP ion channel and AChR structures and also functions.

[00668] The aim of the present study was to determine NK cytotoxic activity as well as whether SNPs and their genotypes were present in TRP ion channel and AChR genes in isolated NK cells from CFS/ME patients.

[00669] **Method**

[00670] *Subjects*

[00671] CFS patients were defined in accordance with the 1994 CDC criteria for CFS [45j]. A total of 39 CFS/ME patients and 30 non-fatigued controls were recruited for this study with no medical history or symptoms of prolonged fatigue or illness of any kind [45j].

[00672] *Sample Preparation and Measurements*

[00673] A volume of 80 ml of blood was collected from the antecubital vein of participants into lithium heparinized and EDTA collection tubes between 9 am and 11 am. Routine blood samples were analyzed within 6 hours of collection and analyzed for red blood cell counts, lymphocytes, granulocytes and monocytes using an automated cell counter (ACT Differential Analyzer, Beckman Coulter, Miami, FL). Refer to Table 8.

Table 8: Participant Characteristics for Chronic Fatigue Syndrome and Non Fatigued Controls

Variable	CFS n=39	Non-fatigued controls n=30	p-value
Gender (% F)	71.80%	23.70%	0.228
Mean Age (years)	51.69 ± 2.00	47.60 ± 2.39	0.191

Hemoglobin (g/L)	136.05 ± 2.07	138.80 ± 2.24	0.375
Hematocrit (%)	0.41 ± 0.01	0.41 ± 0.01	0.702
Red Cell Count ($x10^{12}$/L)	4.54 ± 0.07	4.58 ± 0.08	0.697
Mean Corpuscular Volume (fL)	89.97 ± 0.55	90.07 ± 0.70	0.917
White Cell Count ($x10^9$/L)	5.95 ± 0.26	6.38 ± 0.31	0.747
Neutrophils $x10^9$/L)	3.53 ± 0.19	3.96 ± 0.26	0.173
Lymphocytes ($x10^9$/L)	1.91 ± 0.10	1.97 ± 0.08	0.64
Monocytes ($x10^9$/L)	0.34 ± 0.02	0.32 ± 0.02	0.41
Eosinophils ($x10^9$/L)	0.33 ± 0.18	0.37 ± 0.23	0.892
Basophils ($x10^9$/L)	0.20 ± 0.18	0.02 ± 0.00	0.385
Platelets (xlO^9/L)	262.56 ± 8.41	256.79 ± 9.58	0.653

[00674] *NK Cell Isolation*

[00675] Peripheral blood mononuclear cells were isolated from 20 mL of whole blood for NK cells using Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). Enrichment of NK was performed using NK Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched NK purity was examined on the FACS Calibur flow cytometer (BD Bioscience, San Diego, CA) after staining with CD16/CD56 as previously described [35j] (BD Bioscience, San Diego, CA). Flow cytometry and hemocytometer assessment were used to determine the purity of the NK cells isolated. The recovery of isolated cells was calculated based on the observation that NK represent 2% of peripheral blood lymphocytes respectively [46j]. Recovery was expressed as the ratio of percentage of the total number of NK cells isolated to the percentage of cells present in the volume of blood collected. Enriched cells were snap frozen in liquid nitrogen and stored at - 80°C until further assessment.

[00676] *NK Cell Cytotoxicity*

[00677] NK cytotoxic activity was conducted as previously described [36j, 39j]. Briefly, following NK lymphocytes isolation using density gradient centrifugation and labelled with 0.4% PKH-26 (Sigma, St Louis, MO), NK cells were incubated with K562

cells, for 4 hours at 37°C in 95% air, 5% $C0_2$ at an effector to target ratio of 25 (NK cells): 1 (K562). An E:T ratio of 25:1 has been previously been shown by the inventors and other researchers to be the most optimal ratio for assessing cytotoxic activity [36j, 39j]. NK cell lysis was determined following four hours of NK cells with K562 cells, NK lysis was calculated to determine induced tumor cell death or apoptosis [47j]. Fortessa X-20 flow cytometry (BD Bioscience, San Jose, CA), using Annexin V-FITC and 7-AAD reagents (BD Pharmingen, San Diego, CA) was employed. NK cytotoxic activity was performed within 2-4 hours upon receipt of all blood samples.

[00678] *DNA extraction*

[00679] A volume of 40 mL was collected into EDTA tubes for SNP analysis. Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood mini-kit as per manufacturer's instructions (Qiagen). SNP genotyping studies were performed as previously described.

[00680] *SNP analysis*

[00681] A total of 678 SNPs from isolated NK cells were examined for twenty-one mammalian TRP ion channel genes *(TRPA1, TRPC1, TRPC2, TRPC3, TRPC4, TRPC6, TRPC7, TRPM1, TRPM2, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7, TRPM8, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5* and *TRPV6)* and for nine mammalian ACh receptor genes (muscarinic Ml, M2, M3, M4, M5, nicotinic alpha 2, 3, 5, 7, 10 and epsilon) and were examined using MassARRAY iPLEX Gold Assay (Sequenom Inc.). Quality and quantity of the DNA extracted was determined by the Nanodrop (Nanodrop), where approximately 2 μg of genomic DNA was used to perform the SNP analysis. SNP analysis was performed as previously described. Briefly, MassARRAY (MALTI-TOF mass spectrometry platform) was employed to discriminate alleles based on single-base extension of an extension primer of known mass that is designed to attach directly next to the SNP site of interest. Custom multiplexed wells were designed in silico using Agena's Assay Design Suite. The designed multiplexes were then built using custom synthesized oligonucleotides that are pooled together for sample processing. The iPLEX Gold chemistry utilized two multiplexed oligo pools for each genotyping well. These were pooled and balanced prior to running against DNA samples. First a multiplexed PCR pool was utilized to generate short amplicons that include all the genomic markers of interest in that particular well. After PCR and clean-

up steps were undertaken, a secondary PCR 'extension' step was undertaken utilizing pools of extension primers that were designed to attached directly next to the SNP sites of interest. A termination mix was added to the extension phase which allowed these extension primers to be extended by a single base only. As the molecular weight of the extension primer is known, discrimination of the allele was able to be measured using the peak heights of the unextended primer and this primer plus the possible single-base extension possibilities for the SNP.

[00682] *TRP ion channel andAChR SNP assays*

[00683] Primers and extension primers were created for each of the SNPs using the Assay Designer (Sequenom Ine.) according to the manufacturer's instructions. DNA was amplified via polymerase chain reaction (PCR) under the following conditions: 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. Amplification products were then treated with shrimp alkaline phosphatase at 37°C for 40 minutes, 85°C for 5 minutes reaction, and a final incubation at 4°C. Extension primers are optimized to control the signal-to-noise ratio where unextended primers (UEPs) are examined on the spectroCHIP and evaluated in Typer 4.0 to enable the division into low-mass UEP, medium-mass UEP, and high-mass UEP. To perform the iPLEX extension reaction, a mixture containing iPLEX Gold reaction was prepared using iPLEX Gold Buffer Plus, iPLEX termination mix, iPLEX enzyme, and primer mix. The iPLEX reaction was cycled at an initial denaturation of 94°C for 30 seconds, annealing at 52°C for 5 minutes, extension at 80°C for 5 minutes (five cycles of annealing and extension were performed, but the whole reaction was performed in 40 cycles) and extension again at 72°C for 3 minutes. Resin beads were used to rinse all iPLEX Gold reaction products. Following the iPLEX Gold reaction, MassARRAY was performed using the MassARRAY mass spectrometer, and the data generated were analyzed using the TyperAnalyzer software.

[00684] *Statistical analysis*

[00685] Statistical analysis was performed using SPSS software version 22 [IBM Corp]. The experimental data represented in this study are reported as means plus/minus standard error of the mean (+SEM) while all the clinical data are reported as means plus/minus standard deviation (±SD). Comparative assessments among participants (CFS/ME and non-fatigued controls) were performed with the analysis of variance test

(ANOVA) and the criterion for significance was set at $p<0.05$.

[00686] The PLINK vl.07 (http://pngu.mgh.harvard.edu/purcell/plink/) whole genome analysis tool set was used to determine associations between the CFS patients and the non-fatigued control group. A two column $\chi 2$ test was used to examine differences where p value of <0.05 was determined to be significant and the resulting variants and their consequences can be found in Table 9 for TRP and AChR, respectively. Further genotype analysis for differences between CFS and the non-fatigued group was also completed according to a two column $\chi 1$ test with significance of $p <0.05$ and results are presented in Table 10. Analyses were performed at the Australian Genome Research Facility Ltd, The Walter and Eliza Hall Institute, Parkville, Victoria, Australia.

Table 9: Analysis of the frequency, distribution and significance of SNPs in genes for TRP ion channels and AChRs in CFS/ME patients (n=39_) and non-fatigued controls (n=30) in rank order of significance

Gene	CHR	SNP	BP	A1	F_A	F_U	A2	CHISQ	OR	p-value
TRPM8	2	rs17865678	2.34E+08	A	0.4595	0.1667	G	12.88	4.25	0.000332
TRPM8	2	rs11563204	2.34E+08	A	0.3553	0.1167	G	10.18	4.172	0.00142
nAChRβ4	15	rs12441088	78635922	G	0.1795	0.3793	T	6.824	0.358	0.008993
TRPC4	13	rs2985167	37656405	G	0.2821	0.5	A	6.742	0.3929	0.009418
TRPM3	9	rs6560200	71365306	T	0.3974	0.6207	C	6.633	0.4031	0.01001
TRPM3	9	rs1106948	71402258	C	0.3974	0.6167	T	6.521	0.41	0.01066
TRPM8	2	rs6758653	2.34E+08	A	0.2436	0.45	G	6.502	0.3936	0.01078
nAChRα3	15	rs12914385	78606381	T	0.4872	0.2833	C	5.879	2.403	0.01532
nAChRα2	8	rs891398	27467305	C	0.5526	0.3448	T	5.714	2.347	0.01683
TRPM3	9	rs12350232	71417232	G	0.3974	0.6	T	5.571	0.4397	0.01826
nAChRα2	8	rs2741343	27468610	C	0.5526	0.35	T	5.537	2.294	0.01862
TRPM3	9	rs11142822	71427327	T	0.03846	0.15	G	5.314	0.2267	0.02115
nAChRα3	15	rs2869546	78615003	C	0.2763	0.4667	T	5.271	0.4364	0.02168
TRPM3	9	rs1891301	71403580	T	0.5769	0.3833	C	5.085	2.194	0.02414
nAChRα3	15	rs951266	78586199	T	0.4359	0.2586	C	4.536	2.215	0.03319
TRPC2	11	rs7108612	3628856	T	0.1923	0.06667	G	4.509	3.333	0.03372
TRPC2	11	rs6578398	3616831	A	0.3462	0.1833	G	4.506	2.358	0.03378
mAChRM1	11	rs6578398	62920797	A	0.2436	0.1034	G	4.354	2.791	0.03691
mAChRM3	1	rs4620530	2.4E+08	T	0.4744	0.3	G	4.301	2.106	0.03809
mAChRM1	11	rs11823728	62909330	T	0.05263	0.1607	C	4.242	0.2901	0.03943
nAChRα3	15	rs4243084	78619330	G	0.4359	0.2667	C	4.204	2.125	0.04034
nAChRα3	15	rs3743075	78617110	A	0.2821	0.45	G	4.177	0.4802	0.04097
nAChRα3	15	rs3743074	78617138	C	0.2821	0.45	T	4.177	0.4802	0.04097

nAChRe	17	rs339701 19	4901607	A	0.03846	0.1333	G	4.1 6 1	0.26	0.04136
nAChRa5	15	rs7 180002	78581651	T	0.4342	0.2667	A	4.084	2.1 1	0.0433

SNPs of 39 CFS/ME patients and 30 non-fatigued controls. Data presented are included for p<0.05. Data are presented for gene (TRPM3, TRPM8, TRPC2, TRPC4, AChRMl, M3, alpha 2, 3, 5, 10 and epsilon), chromosome location (CHR), reference SNP identification (RefSNPlD), base pair (BP) location of SNP, alleles (Al and A2), allelic frequency A (Frequency_A) of this allele in CFS cases, frequency U (Frequency_U) of this allele in controls, chi-square (χ2) for basic allelic test (1 df), odds ratio (OR) and (*) P-value for this test set at a significance of <0.05.

Table 10: Analysis of the genotype, odds ratio and significance of SNPs in genes for TRP ion channels and AChRs in CFS/ME patients (n=39) and non-fatigued controls (n=30) in rank order of significance

Gene	CHR	SNP	Genotype	CFS (n%)	Non-Fatigued Control (n%)	χ2	OR	p-value
TRPM8	2	rs11563204	GA	23 (82.1%)	5 (17.9%)	12.59	7.19	0
nAChRα2	8	rs891398	CC	11 (91.7%)	1 (8.3%)	7.31	11.39	0.007
nAChRα2	8	rs2741343	CC	11 (91.7%)	1 (8.3%)	7.3	11.39	0.007
TRPC4	13	rs2985167	AA	20 (76.9%)	6 (23.1%)	7.07	4.21	0.008
TRPM3	9	rs6560200	CC	15 (83.3%)	3 (16.7%)	7.12	5.63	0.008
TRPC4	13	rs1570612	GG	30 (68.2%)	14 (31.8%)	6.72	3.81	0.01
nAChRβ4	15	rs12441088	TT	25 (71.4%)	10 (28.6%)	6.42	3.57	0.011
TRPM8	2	rs17865678	AG	22 (73.3%)	8 (26.7%)	6.1	3.56	0.013
TRPC4	13	rs655207	GG	12 (85.7%)	2 (14.3%)	6.09	6.22	0.014
nAChRα3	15	rs12914385	TT	12 (85.7%)	2 (14.3%)	6.09	6.22	0.014
TRPM3	9	rs11142822	GG	36 (63.2%)	21 (36.8%)	5.87	5.14	0.015
TRPM3	9	rs1106948	TT	15 (78.9%)	4 (21.1%)	5.37	4.06	0.021
TRPC2	11	rs7108612	GT	15 (78.9%)	4 (21.1%)	5.37	4.06	0.021

nAChRε	17	rs33970119	GG	36 (62.1%)	22 (37.9%)	4.56	4.36	0.033
TRPM3	9	rs1891301	TT	14 (77.8%)	4 (22.2%)	4.48	3.64	0.034
TRPM3	9	rs12350232	TT	15 (75%)	5 (25%)	3.91	3.13	0.048
Genotype with 39 CFS/ME patients and 30 non-fatigued controls. Data presented are included for $p<0.05$. Data are presented for gene (TRPM3, TRPM8, TRPC2, TRPC4, AChRM3, alpha 2, 3, and epsilon), chromosome location (CHR), reference SNP identification (RefSNPID), genotype percentage of CFS patients with genotype (%), percentage of non-fatigued controls (5), chi-square ($\chi 2$) for basic allelic test (1 df), odds ratio (OR) and (*) P-value for this test set at a significance of <0.05.								

[00687] **Results**

[00688] *Participants*

[00689] There were 39 CFS patients (age=51.69+ 2.00years), of which 72% were females and 18% were males. There were 30 non-fatigued controls (age = 47.60 ± 2.39 years) comprising 24% females and 76% males. All participants in both groups were of European decent and were residents of Australia at the time of blood collection. There were no significant changes in white blood cell counts between CFS/ME patients and the non-fatigued control group. Table 8 outlines participants' characteristics.

[00690] *NK Cell Purity*

[00691] There was no significant difference between groups for levels of NK purity. Figure 1 outlined the high levels of purity (> 93%) of NK cells following isolation and enrichment.

[00692] *NK Cell Cytotoxic Activity*

[00693] There was a significant difference for NK cytotoxic activity between groups at the E:T ratio of 25:1. CFS/ME patients had a significant reduction in NK % lysis (17 ± 4.68) compared with the control group (31 ± 6.78) (Figure 2).

[00694] *SNP Analysis*

[00695] Of 678 SNPs identified in TRP ion channel and AChR genes from isolated NK cells there were 11 SNPs for TRP ion channel genes *(TRPC4, TRPC2, TRPM3 and TRPM8)* significantly associated in the CFS/ME group. Five of these SNPs were associated with *TRPM3* (rs rs6560200; p= 0.010, rsl 106948; p= 0.010, rsl2350232; p= 0.018, rslll42822; p= 0.021, rsl891301; p= 0.024) while the remainder were associated with *TRPM8* (rsl7865678; *p*=0.000, rsl 156320; p=0.001), TRPC2 (rs7108612; p= 0.034, rs6578398; p= 0.0334) and *TRPC4* (rs2985167; *p*=0.00 1, rs655207 ; p=0.018).

[00696] Fourteen SNPs were associated with nicotinic and muscarinic acetylcholine receptor genes, where six were nAChR alpha 3 (rsl2914385; p=0.015, rs2869546; p=0.021, rs951266; p=0.033, rs4243084; p=0.040, rs3743075; p=0.041, rs3743074; p=0.041), while the remainder were associated with nAChR alpha 2 (rs891398; p=0.017, rs2741343; p=0.019), nAChR beta 4 (rsl2441088; p=0.009), nAChR alpha 5 (rs7180002; p=0.043) and nAChR epsilon (rs33970119; p=0.041). Table 9 represents

the SNPs for TRP ion channel and AChR genes isolated from NK cells, respectively.

[00697] *Genotype Analysis*

[00698] There were sixteen genotypes identified from SNPs that were reported significant for TRPM3 (n=5), TRPM8 (n=2), TRPC4 (n=3), TRPC2 (n=l), nAChR epsilon (n=l), nAChR alpha 2 (n=2), nAChR alpha 3 (n=l) and nAChR beta 4 (n=l). Table 10 represents the genotypes for SNPs in TRP and AChR genes from isolated NK cells that were reported as statistically significant between groups. The odds ratio for specific genotypes for SNPs in TRP and AChR genes from isolated NK cells ranged between 3.13 - 11.39 for CFS/ME compared with the non-fatigued control group.

[00699] **Discussion**

[00700] Reduced NK cell cytotoxic activity has previously been reported in CFS/ME and the current investigation supports those findings. The current investigation reports novel findings for a number of SNPs in genes for AChR and TRP variants and genotypes from isolated NK cells from CFS/ME patients. A further novel finding from this investigation is the identification of SNPs in TRPM3 and TRPM8 from isolated NK cells, suggesting TRPM3 and TRPM8 receptors are located on NK cells.

[00701] This investigation reports a significant reduction in NK lysis in CFS/ME patients compared with the non-fatigued controls. TRP ion channels have an important role in Ca^{2+} signaling and immune cells have been documented to express TRPC and TRPM subfamilies, mainly TRPC-1, 3, 5 and TRPM-2, 4, 7 [49j]. These channels are non-selective and permeable to calcium. In NK cells Ca^{2+} plays a key role in lytic granule fusion [llj, 50j, 51j] as well as ensuring lytic granules mobilize to the immune synapse to release perforin and granzymes to kill target cells [llj, 50j, 51j]. Rho-GTPase Miro, provides a link between the mitochondria and the microtubules, where it mediates the Ca^{2+} dependent arrest of mitochondrial motility [52j]. As Rho GTPase Miro modifies mitochondrial polarization, it also may alter lytic granule transport to the immune synapse as well as lytic function due to modulation by cytosolic Ca^{2+}concentration through TRPM and AChR genotypes. Clearly mitochondria play a key role in NK cell function. A recent discovery that mitochondria express a range of AChR subtypes including nicotinic alpha 3, although differentially expressed according to tissue type [53j] suggests that nAChR may impact mitochondrial function and

regulate oxidant stress. Interestingly the inventors have previously reported a significant decrease in respiratory bust function of neutrophils from CFS/ME patients [34j].

[00702] TRPM2 and TRPM3 mobilize Ca^{2+}, where the latter has been shown to mediate Ca^{2+} signaling for cytolytic granule polarization and degranulation [54j]. ADPR targets TRPM2 channels on cytolytic granules resulting in TRPM2-mediated Ca^{24} signaling, subsequently inducing cytolytic granule polarization and degranulation, which results in antitumor activity. Further, NK cells treated with ADPR antagonist had reduced tumor-induced granule polarization, degranulation, granzyme B secretion, and cytotoxicity of NK cells. Interestingly similar findings for NK cell functions have been reported from previous CFS/ME research [32j-36j], potentially suggesting the genotype changes reported in this present study for TRPM3 may also play a similar role for cytolytic granule polarization and degranulation.

[00703] Out of the 678 SNPs examined, eleven variants for TRP ion channels and fourteen variants for AChRs were found to be significantly associated with CFS/ME patients compared with the non-fatigued controls. The variant TRP SNPs were located in the gene sequence of two of the canonical TRP ion channels (TRPC2 and TRPC4) and two melastatin TRP ion channels (TRPM3 and TRPM8). The inventors also report variant SNPs on genes for two of the muscarinic acetylcholine three receptors (mAChRM3), two muscarinic acetylcholine one receptors (mAChRMl), six nicotinic acetylcholine alpha three receptors (nAChRa3), three nicotinic acetylcholine alpha two receptors (nAChRa2), one nicotinic acetylcholine alpha five receptor (nAChRa5) as well as one nicotinic acetylcholine beta four receptor (ηAChη¾34) and one nicotinic acetylcholine epsilon receptor (nAChRe).

[00704] The inventors' current research reports significant SNP associations of genotypes for AChRs in isolated NK cells from CFS/ME patients. Lymphocytes express both muscarinic and nicotinic acetylcholine (ACh) receptors, where T and B cells and monocytes express all five subtypes of mAChRs (M(l)-M(5)), while nAChR are found for 2- 6, 2-4, and 9/10 subunits [55j-58j]. Lymphocytes constitute a cholinergic system that is independent of cholinergic nerves, resulting in the regulation of immune function [55j, 56j]. AChR agonists have been shown to enhance lymphocyte cytotoxicity, increase their intracellular cGMP and inositol- 1,4,5-triphosphate (IP 3) [55j-59j], suggesting the lymphocytic cholinergic system is involved in the regulation of

immune function via AChRs coupled to phospholipase-C (PLC) via changes in [Ca^{2+}] [60j-65j]. Previous research has highlighted the importance of variants in affecting gene transcripts by causing alternative splicing resulting in anomalies in mRNA and translation products [66j]. The inventors have also identified SNPs and genotype in nAChRe in CFS/ME patients. Interestingly, this SNP is located in the 3' untranslated region (3'-UTR), an important coding region that often contains regulatory regions that post-transcriptionally influence gene expression. 3'-UTR is a binding site for regulatory proteins as well as microRNAs (miRNAs) [67j]. Binding to specific sites within the 3'-UTR, miRNAs can decrease gene expression of various mRNAs by either inhibiting translation or directly causing degradation of the transcript. The inventors' previous research has found significant differences in NK cytotoxic activity as well as miRNAs from isolated NK cells from CFS/ME patients [32j].

[00705] Previous investigators suggest that alternate splicing in the coding and also in the non-coding sequences may have significant unexpected outcomes on the splicing mechanism of the gene transcripts [68j, 69j]. Splicing genetic variants located in the exons, introns, as well as the assembly of the spliceosome all contribute to the splicing mechanism for the correct coding of a protein sequence. Moreover, silencers and enhancers located either in the exons or introns are integral in recognition of the correct exon sequence [70j]. Importantly introns are able to generate active spliceosomes, giving rise to alternative splicing events [71j, 72j]. Gene 80036 (TRPM3) is associated with calcium entry and calcium store depletion via different isoforms which have been identified through alternative splicing [Fruhwald, Julia, et al. "Alternative splicing of a protein domain indispensable for function of transient receptor potential melastatin 3 (TRPM3) ion channels." Journal of Biological Chemistry 287.44 (2012): 36663-36672]. The 'indispensable for channel function' (ICF) is an 18 amino acid residue region whose absence renders the channels functionally unable to mediate calcium entry, and is found devoid in a TRPM3 variant [73j]. Co-expression of these TRPM3 ICF variants with functional TRPM3 ion channels additionally show impaired calcium mobilization [73j]. As TRPM3 ICF variants show ubiquitous expression in many tissues and cell types and constitute 15% of all TRPM3 isoforms, expression on NK cells may provide a potential explanation for reduced cytotoxic activity in CFS/ME patients. Additionally, ion selectivity occurs through the selective splicing of exon 24 and results in two

variants, TRPM3al and TRPM3α2 [73j, 74j]. The significance of these two isoforms is highlighted as TRPM3α.l preferentially mediates monovalent cation conduction, while TRPM3a2 shows high and specific permeability towards divalent cations, particularly calcium [73j, 74j]. This alteration in function may be attributed to the introduction of positively charged amino acid residues to the pore region [74j], resulting in increases in electrostatic repulsion of divalent cations, thus promoting increases in monovalent selectivity [74j]. Therefore, particular splice variants such as TRPM3al may potentially be favoured, culminating in a diminished NK cell cytotoxic response as well as heat detection including dysregulation of thermoregulatory responses, nociception and transmission of pain such as central and peripheral pain perception. Moreover, TRPM8 has also been identified to be activated by cold and noxious stimuli [75j-77j], suggesting the genotype changes reported in this investigation align to the clinical presentation of thermoregulatory responses, nociception and transmission of central and peripheral pain perception seen in CFS/ME patients [78j].

[00706] The inventors' results suggest SNP variants and genotypes reported in NK cells may not be exclusive to this immune cell type. Acetylcholine receptors and TRP ion channel receptors are located ubiquitously on multiple cell types and control other functions in body systems. Ca^{2+} signaling in the context of TRP ion channels as well as AChR function is vital for the function of the CNS and there is wide variety in nicotinic receptors expressed in animal and human immune cells [58j]. Inferences regarding differential effects on function between these systems should note limitations depending on sub-types respectively expressed. The endothelium contains nicotinic receptors; nACliRa3, a5 and β4.α3 and a5 are found in arteries [79j-81j] and nAChR α5, a7, β2, and β3 are found in brain endothelial cells [82j], which are important components of the blood-brain barrier. Others have reported various nAChR receptors located on mitochondria, and depending upon tissues, mitochondria express several nicotinic receptor subtypes in a tissue-specific manner; brain and liver mitochondria contain α7β2, α4β2 and less α3β2 nicotinic receptors, while mitochondria from the lung express preferentially α3β4 receptor subtype [53j]. Interestingly this epsilon sub-type has been identified in thymomas from patients with myasthenia gravis [83j]. Of note, nAChRs are reported to be involved in arousal, sleep, and fatigue as well as those functions that are responsible for processing of pain, memory, and cognition all of which are clinical

symptoms reported in CFS/ME patients [84j-86j].

[00707] **Conclusion**

[00708] In this study the inventors identified, for the first time, SNPs in genes for TRPM3 and TRPM8 ion channels on isolated NK cells. The inventors also identified numerous SNPs of nAChRs along with other TRP channels on isolated NK cells, indicating the non-neuronal acetylcholine system has an important role in NK cell function. Anomalies in genotypes for TRP ion channels and AChRs suggest altered calcium would be an important functional consequence not only for NK cells but also depending upon tissue type, susceptibility or predisposition to CFS/ME.

[00709] **Example 6: SNPs and genotypes in TRP ion channel and AChR genes from isolated B lymphocytes in ME/CFS patients**

[00710] The pathomechanism of CFS/ME is unknown. However, a small subgroup of patients has shown muscarinic antibodies and reduced symptom presentation following anti-CD20 intervention. Given the important roles in calcium (Ca^{2+}) and acetylcholine (ACh) signaling in B cell activation and potential antibody development, the inventors' aim in this Example was to determine SNPs and their genotypes from isolated B cells from CFS/ME patients.

[00711] Acetylcholine (Ach) is a neuronal cholinergic neurotransmitter where it performs a vital role through transmitting activation signals to receptors located in the central nervous system (CNS) as well as in skeletal and smooth muscle, all preganglionic autonomic nerve fibers and post ganglionic autonomic parasympathetic nerves as well immune cells and other tissues through the non-neuronal cholinergic system [lx-4x].

[00712] There are two types of membrane proteins that bind ACh known as muscarinic receptors (mAChRs) and nicotinic receptors (nAChRs). Importantly, both receptor proteins (mAChR and nAChR) have multiple isoforms. While muscarinic receptors are metabotropic receptors classified M1-M5, nicotinic receptors are ion channels and, with the exception of homomeric nicotinic alpha 7, are heteromers with various combinations of usually two sub-types (selected from 9 alpha and 3 beta) [5x]. The ratio of subtypes affects signal conducting speed through the receptor [6x]. Importantly one receptor subtype may impact receptor function of the other linked subtype.

[00713] ACh also functions within the non-neuronal cholinergic system (NNCS) where ACh binds AChRs that have been found on immune and other cell types. ACh is produced by lymphocytes where nAChRs have been shown to influence B lymphocyte function including development in the bone marrow as well as regulating B lymphocyte activation and autoantibody response [7x-9x]. ACh also performs endocrine and paracrine functions on tissues such as smooth muscle, beta pancreatic cells, glial cells, lymphocytes, ocular lens cells and brain vascular endothelium [10x-14x]. Calcium signaling is highly important for the activation of cell surface receptors on immune cells. Moreover, these ACh functions are mediated through Ca^{2+} signaling.

[00714] Interestingly, muscarinic acetylcholine receptors have been found to be inhibited by another calcium channel [15x]. Mammalian Transient receptor potential (TRP) ion channels are Ca^{2+} permeable cation channels that when open act as an excitatory signal to induce depolarisation of the cell and cause Ca^{2+} influx which plays a role in intracellular signalling pathways. (TRPs) are comprised of six main groups including the TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) [16x]. TRPs are present on almost all cells and dysregulation in TRPs has been associated with pathological conditions and diseases [17x-22x].

[00715] The inventors have previously described single nucleotide polymorphisms (SNPs) in genes for receptors where Ca^{2+} calcium is an important key component in their function. Additionally, the inventors have shown changes in Ca^{2+} mobilization intracellularly for TRPM3 from NK cells and B lymphocytes. Hence, these SNPs and their genotypes for TRP ion channels and AChRs may produce altered receptor proteins, potentially changing TRP ion channel and AChR structures and functions. A recent study reported a subgroup of CFS/ME patients had muscarinic antibodies and a modest positive response occurred with reduced symptom presentation following anti-CD20 intervention [39x]. Given the important roles in Ca^{2+} and acetylcholine (ACh) signaling in B cell activation as well as the potential for antibody development, the aim of this investigation was to determine SNPs and their genotypes for TRP and AChRs from isolated B cells from CFS/ME patients.

[00716] **Method**

[00717] *Subjects*

[00718] CFS/ME patients were defined in accordance with the 1994 CDC criteria for CFS/ME [40x]. A total of 11 CFS/ME patients and 11 non-fatigued controls were recruited for this study with no medical history or symptoms of prolonged fatigue or illness of any kind [40x].

[00719] *Sample Preparation and Measurements*

[00720] A volume of 40 ml of blood was collected from the antecubital vein of participants into lithium heparinized and EDTA collection tubes between 9 am and 11 am. Routine blood samples were analyzed within 6 hours of collection and analyzed for red blood cell counts, lymphocytes, granulocytes and monocytes using an automated cell counter (ACT Differential Analyzer, Beckman Coulter, Miami, FL). Refer to Table 11.

Table 11: Participant Characteristics for CFS/ME and Non Fatigued Controls.

Descriptive	CFS/ME n=11	Controls n=11	*P*-VALUE
Gender (% F)	8 (72.7%)	7 (63.6%)	0.497
Mean Age (Years)	31.82 (5.50)	33.91 (5.06)	0.783
Haemoglobin (g/L)	133 ± 2.70	134.70 ± 3.85	0.728
Haematocrit (%)	0.36 ± 0.02	0.30 ± 0.02	0.967
Red Cell Count ($x10^{12}$/L)	4.40 ± 0.13	4.50 ± 0.11	0.591
Mean Corposcular Volume (fL)	89.56 ± 1.54	88.20 ± 0.61	0.406
White Cell Count ($x10^9$/L)	7.09 ± 0.69	5.80 ± 0.32	0.097
Neutrophils ($x10^9$/L)	4.15 ± 0.51	3.21 ± 0.21	0.096
Lymphocytes ($x10^9$/L)	2.35 ± 0.23	2.13 ± 0.24	0.549
Monocytes ($x10^9$/L)	0.36 ± 0.02	0.30 ± 0.02	0.043
Eosinophils ($x10^9$/L)	0.19 ± 0.04	0.14 ± 0.03	0.275
Basophils ($x10^9$/L)	0.03 ± 0.00	0.03 ± 0.01	0.752
Platelets ($x10^9$/L)	241.56 ± 19.55	248.10 ± 18.35	0.810

[00721] *B cell isolation*

[00722] A volume of 40 ml of blood was collected from the antecubital vein of participants into EDTA blood collection tubes between 8 am and 11 am. Routine blood samples were analyzed within 6 hours of collection and analyzed for red blood cell

counts, lymphocytes, granulocytes and monocytes using an automated cell counter (ACT Differential Analyzer, Beckman Coulter, Miami, FL). Refer to Table 11.

[00723] Peripheral blood mononuclear (PBMCs) cells were isolated from 40 mL of whole blood for B cell isolation using method previously described Jamies et al. (2004) [69x]. Briefly, PBMCs were isolated by density gradient with Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Subsequently, cells were then washed twice with phosphate-buffered saline (PBS) (Gibco-BRL, Gaithersburg, Md.).

[00724] Cells were then resuspended in autoMACs separation buffer, which contains PBS containing bovine serum albumin, EDTA and 0.09% azide (Miltenyi Biotec, Auburn, Calif.). Immunomagnetic negative selection of B cells was performed with a B-cell isolation kit II (Miltenyi Biotec, Auburn, Calif.), according to the manufacturer's instructions. Briefly, non-B cells, such as T cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells, are indirectly magnetically labeled by using a cocktail of biotin conjugated antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a (Glycophorin A). Consequently, isolation of B cell populations is achieved by depletion of magnetically labeled cells.

[00725] Untouched B-cells were measured with LSR Fortessa X-20 flow cytometry where cells were fluorescently stained with anti-CD19-BV421 and anti-CD3-PerCP. Cell debris and dead cells were excluded from the analysis based on scatter signals. Mean purity was 85.66% ± 9.6% for non-fatigued controls and 76.5% ±13.1% for CFS/ME patients, where there was no significant difference between groups for levels of B lymphocytes.

[00726] *DNA extraction*

[00727] A volume of 40 mL was collected into EDTA tubes for SNP analysis. Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood mini-kit as per manufacturer's instructions (Qiagen). SNP genotyping studies were performed as previously described.

[00728] *SNP analysis*

[00729] A total of 661 SNPs from B cells were examined for twenty-one mammalian TRP ion channel genes *(TRPA1, TRPC1, TRPC2, TRPC3, TRPC4, TRPC6, TRPC7, TRPM1, TRPM2, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7, TRPM8, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5* and *TRPV6)* and for nine mammalian ACh

receptor genes (muscarinic Ml, M2, M3, M4, M5, nicotinic alpha 2, 3, 5, 7, 9, 10, beta 1, 4 and epsilon) and were examined using MassARRAY iPLEX Gold Assay (Sequenom Inc.).

[00730] Quality and quantity of the DNA extracted was conducted as previously described [44x, 48x]. Briefly a Nanodrop (Nanodrop) was used to quantify genomic DNA where approximately 2 μg of genomic DNA was used to perform the SNP analysis. MassARRAY (MALTI-TOF mass spectrometry platform) was employed to discriminate alleles based on single-base extension of an extension primer of known mass that is designed to attach directly next to the SNP site of interest. Custom multiplexed wells were designed in silico using Agena's Assay Design Suite. The designed multiplexes were then built using custom synthesized oligonucleotides that are pooled together for sample processing. The iPLEX Gold chemistry utilized two multiplexed oligo pools for each genotyping well. A multiplexed PCR pool was utilized to generate short amplicons that include all the genomic markers of interest in that particular well. Following PCR and clean-up steps, a secondary PCR 'extension' step was undertaken utilizing pools of extension primers that were designed to attach directly next to the SNP sites of interest. During the extension phase a termination mix was added that enabled these extension primers to be extended by a single base only. Given the molecular weight of the extension primer is known, discrimination of the allele was able to be measured using the peak heights of the unextended primer and this primer plus the possible single-base extension possibilities for the SNP.

[0073 1] *TRP ion channel and AChR SNP assays*

[00732] Primers and extension primers were created for each of the SNPs using the Assay Designer (Sequenom Inc.) according to the manufacturer's instructions and previously described [44x, 45x]. Briefly, DNA was amplified via polymerase chain reaction (PCR) under the following conditions: 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, where the amplification products were then treated with shrimp alkaline phosphatase at 37°C for 40 minutes, 85°C for 5 minutes reaction, and a final incubation at 4°C. Extension primers are optimized to control the signal-to-noise ratio where unextended primers (UEPs) are examined on the spectroCHIP and evaluated in Typer 4.0 to enable the division into low-mass UEP, medium-mass UEP, and high-mass UEP. A mixture containing iPLEX Gold reaction

was prepared using iPLEX Gold Buffer Plus, iPLEX termination mix, iPLEX enzyme, and primer mix to perform the iPLEX extension reaction. This reaction consisted of cycling at an initial denaturation of 94°C for 30 seconds, annealing at 52°C for 5 minutes, extension at 80°C for 5 minutes (five cycles of annealing and extension were performed, but the whole reaction was performed in 40 cycles) and extension again at 72°C for 3 minutes. Resin beads were used to rinse all iPLEX Gold reaction products. Following the iPLEX Gold reaction, MassARRAY was performed using the MassARRAY mass spectrometer, and the data generated were analyzed using the TyperAnalyzer software.

[00733] *Statistical analysis*

[00734] Statistical analysis was performed using SPSS software version 22 [IBM Corp]. The experimental data represented in this study are reported as means plus/minus standard error of the mean (±SEM) while all the clinical data are reported as means plus/minus standard deviation (±SD). Comparative assessments among participants (CFS/ME and non-fatigued controls) were performed with the analysis of variance test (ANOVA) and the criterion for significance was set at $p<0.05$.

[00735] The PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) whole genome analysis tool set was used to determine associations between the CFS/ME patients and the non-fatigued control group. A two column χ^2 test was used to examine differences where p value of <0.05 was determined to be significant and the resulting variants and their consequences can be found in Table 12 for TRP and AChR, respectively.

Table 12: Analysis of the frequency, distribution and significance of SNPs in B cells for TRP ion channels and AChRs in Chronic Fatigue Syndromc/Myalgic Encephalomyelitis patients and non-fatigued controls in rank order of significance

Gene	CHR	SNP	BP	A1	F_A	F_U	A2	CHISQ	P	OR
CHRNA4	20	rs11698563	63360932	A	0.2885	0.7083	C	11.88	0	0.1669
CHRND	2	rs11674608	2.33E+08	G	0.34	0.7778	C	10.23	0	0.1472
CHRNA9	4	rs10009228	40354404	A	0.1786	0.5	G	8.706	0	0.2174
CHRM3	1	rs1867264	2.40E+08	A	0.28	0.6364	T	8.164	0	0.2222
CHRNA9	4	rs4861323	40353797	G	0.1786	0.4583	A	6.792	0.01	0.2569
CHRNA2	8	rs2741341	27472768	C	0.5179	0.2083	T	6.586	0.01	4.081
TRPC6	11	rs11224816	1.02E+08	T	0.5192	0.2083	C	6.511	0.01	4.104
CHRND	2	rs12463989	2.33E+08	C	0.3571	0.6667	T	6.503	0.01	0.2778
CHRND	2	rs2767	2.33E+08	C	0.3571	0.6667	T	6.503	0.01	0.2778
CHRND	2	rs112001880	2.33E+08	D	0.3571	0.6667	I	6.503	0.01	0.2778
CHRNB1	17	rs4151134	7443803	C	0.3214	0.625	T	6.389	0.01	0.2842
CHRM3	1	rs1899616	2.40E+08	A	0.3269	0.6667	G	6.361	0.01	0.2429
CHRNB4	15	rs12440298	78635246	G	0.01786	0.1667	T	6.349	0.01	0.09091
TRPV3	17	rs4790519	3553440	C	0.5556	0.25	T	6.242	0.01	3.75
TRPM3	9	rs1317103	70580786	C	0.3519	0.08333	T	6.089	0.01	5.971
CHRND	2	rs67583510	2.33E+08	A	0.1852	0.4545	G	5.849	0.02	0.2727
CHRM3	1	rs12093821	2.40E+08	A	0.2963	0.5833	G	5.784	0.02	0.3008
CHRM3	1	rs10802802	2.40E+08	A	0.375	0.6667	G	5.749	0.02	0.3
CHRNA9	4	rs4861065	40342377	C	0.3929	0.125	T	5.61	0.02	4.529
CHRNA9	4	rs7669882	40348633	A	0.3929	0.125	G	5.61	0.02	4.529

CHRM3	1	rs6684622	2.40E+08	C	0.38	0.6818	G	5.584	0.02	0.286
CHRM3	1	rs1134	2.40E+08	T	0.3462	0.625	C	5.197	0.02	0.3176
CHRND	2	rs3762529	2.33E+08	C	0.3462	0.625	T	5.197	0.02	0.3176
CHRND	2	rs12466358	2.33E+08	G	0.1731	0.4167	T	5.197	0.02	0.293
CHRND	2	rs3828246	2.33E+08	T	0.1731	0.4167	C	5.197	0.02	0.293
CHRM3	1	rs11585281	2.40E+08	T	0.3889	0.6667	C	5.142	0.02	0.3182
CHRM3	1	rs12029701	2.40E+08	C	0.3889	0.6667	T	5.142	0.02	0.3182
CHRND	2	rs13026409	2.33E+08	T	0.1786	0.4167	C	5.079	0.02	0.3043
CHRNG	2	rs13018423	2.33E+08	T	0.1786	0.4167	C	5.079	0.02	0.3043
CHRM3	1	rs619214	2.40E+08	G	0.3	0.6111	T	5.021	0.03	0.2727
CHRM3	1	rs2165872	2.40E+08	T	0.3148	0.5833	C	5.003	0.03	0.3282
CHRM3	1	rs2083817	2.40E+08	A	0.3148	0.5833	T	5.003	0.03	0.3282
CHRND	2	rs4973537	2.33E+08	G	0.3571	0.625	A	4.898	0.03	0.3333
CHRND	2	rs3791729	2.33E+08	T	0.3571	0.625	C	4.898	0.03	0.3333
TRPV2	17	rs35400274	4900415	A	0.07143	0.25	G	4.898	0.03	0.2308
CHRM3	1	rs16838637	2.40E+08	G	0.3214	0.5833	A	4.802	0.03	0.3383
CHRM3	1	rs1867265	2.40E+08	A	0.3214	0.5833	G	4.802	0.03	0.3383
CHRM3	1	rs7551001	2.40E+08	G	0.3214	0.5833	A	4.802	0.03	0.3383
CHRM5	15	rs603152	34002435	A	0.4643	0.2083	C	4.637	0.03	3.293
CHRM3	1	rs1155612	2.40E+08	G	0.4	0.6667	A	4.616	0.03	0.3333
CHRM2	7	rs1424569	1.37E+08	G	0.4	0.6667	A	4.616	0.03	0.3333
TRPV2	17	rs3514	4898298	C	0.07407	0.25	G	4.601	0.03	0.24
TRPV2	17	rs12942540	4900777	C	0.07407	0.25	G	4.601	0.03	0.24

TRPM4	19	rs11083963	49162082	G	0.2826	0.5417	A	4.534	0.03	0.3333
CHRM2	7	rs1364403	1.37E+08	T	0.4107	0.1667	C	4.475	0.03	3.485
TRPM3	9	rs4620343	71121726	T	0.4107	0.1667	C	4.475	0.03	3.485
CHRM3	i	rs12743042	2.40E+08	C	0.3704	0.6364	T	4.473	0.03	0.3361
CHRM3	1	rs6688537	2.40E+08	A	0.4074	0.6667	C	4.47	0.03	0.3438
CHRM5	15	rs646950	33999458	T	0.4615	0.2083	C	4.461	0.03	3.257
CHRM3	1	rs2163546	2.40E+08	G	0.5385	0.2727	A	4.396	0.04	3.111
CHRM3	1	rs1544170	2.40E+08	A	0.3704	0.625	G	4.355	0.04	0.3529
TRPM3	9	rs3812532	70868677	A	0.3704	0.625	C	4.355	0.04	0.3529
CHRND	2	rs2853457	2.33E+08	A	0.5	0.25	G	4.297	0.04	3
CHRM3	1	rs6429147	2.40E+08	C	0.2963	0.5417	G	4.283	0.04	0.3563
CHRM3	1	rs6700643	2.40E+08	C	0.2963	0.5417	T	4.283	0.04	0.3563
CHRM3	1	rs10925941	2.40E+08	A	0.2963	0.5417	G	4.283	0.04	0.3563
CHRM3	1	rs576386	2.40E+08	C	0.5192	0.25	G	4.24	0.04	3.24
CHRNA9	4	rs10015231	40335548	T	0.1964	0.4167	C	4.209	0.04	0.3422
TRPV2	17	rs33970119	4901606	A	0.03571	0.1667	G	4.153	0.04	0.1852
CHRM3	1	rs1867263	2.40E+08	A	0.3036	0.5417	G	4.063	0.04	0.3688
CHRM5	15	rs511422	33990780	C	0.4464	0.2083	T	4.063	0.04	3.065
TRPM3	9	rs10780950	70578511	T	0.2885	0.08333	C	3.979	0.05	4.459
TRPV2	17	rs2075763	4899389	T	0.03704	0.1667	C	3.932	0.05	0.1923
CHRM3	1	rs685550	2.40E+08	C	0.2222	0.04167	T	3.9	0.05	6.571
CHRM3	1	rs6694220	2.40E+08	G	0.423i	0.6667	A	3.897	0.05	0.3667
TRPV2	17	rs12602006	16433973	G	0.2692	0.5	A	3.885	0.05	0.3684

TRPV2	17	rs7222754	16426430	T	0.4423	0.2083	C	3.863	0.05	3.014
CHRNB1	17	rs3829603	7443722	A	0.2593	0.5	C	3.86	0.05	0.35
CHRM3	1	rs10754677	2.40E+08	G	0.3846	0.625	A	3.819	0.05	0.375
CHRM3	1	rs7513746	2.40E+08	G	0.3889	0.625	A	3.727	0.05	0.3818
CHRM3	1	rs10802795	2.40E+08	C	0.3889	0.625	T	3.727	0.05	0.3818
CHRM3	1	rs3738436	2.40E+08	A	0.3889	0.625	C	3.727	0.05	0.3818
CHRM3	1	rs7511970	2.40E+08	A	0.3889	0.625	G	3.727	0.05	0.3818
CHRM3	1	rs1155611	2.40E+08	T	0.3889	0.625	C	3.727	0.05	0.3818
CHRM3	1	rs1019882	2.40E+08	G	0.3889	0.625	A	3.727	0.05	0.3818
CHRM3	1	rs1416789	2.40E+08	G	0.3889	0.625	A	3.727	0.05	0.3818
CHRM3	1	rs10925964	2.40E+08	A	0.3889	0.625	T	3.727	0.05	0.3818
CHRNB1	17	rs2302767	7447224	C	0.2778	0.5	T	3.725	0.05	0.3846

[00736] Further genotype analysis for differences between CFS/ME and the non-fatigued group was also completed according to a two column $\chi 2$ test with significance of $p < 0.05$ and results are presented in Table 13. Analyses were performed at the Australian Genome Research Facility Ltd, The Walter and Eliza Hall Institute, Parkville, Victoria, Australia.

Table 13: Analysis of the genotype, odds ratio and significance of SMPs in B cell genes for TRP ion channels and AChRs in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients and non-fatigued controls in rank order of significance.

Gene	CHRM	Ref SNP	Genotype	CFS (%)	Non Fatigued Controls (%)	$\chi 2$	OR	*P*-VALUE
CHRNB1	17	rs3829603	CC	8 (72.7%)	1 (9.1%)	9.21	26.67	0.002
CHRNB1	17	rs4151134	TT	7 (63.6%)	1 (9.1%)	7.07	17.50	0.008
CHRNB1	17	rs2302767	TT	7 (63.6%)	1 (9.1%)	7.07	17.50	0.008
CHRNA4	20	rs11698563	CC	6 (54.5%)	1 (9.1%)	5.24	12.00	0.022
CHRNB1	17	rs7210231	CA	7 (63.6%)	2 (18.2%)	4.70	7.88	0.030
TRPM3	9	rs7038646	AG	9 (81.8%)	4 (36%)	4.70	7.88	0.030
TRPC6	11	rs10791504	GG	7 (63.6%)	2 (18.2%)	4.70	7.88	0.030
CHRM3	1	rs1867264	TA	8 (72.7%)	3 (27.3%)	4.55	7.11	0.033
CHRM3	1	rs6688537	CA	8 (72.7%)	3 (27.3%)	4.55	7.11	0.033

[00737] **Results**

[00738] *Participants*

[00739] There were 11 CFS/ME patients (age=31.82 ± 5.50 years) of which 72.7% were females. There were 11 non-fatigued controls (age= 33.91 ± 5.06 years), comprising 63.6% females. All participants in both groups were of European decent and were residents of Australia at the time of blood collection. There were no significant changes in white blood cell counts between CFS/ME patients and the non-fatigued control group. Table 11 outlines participants' characteristics.

[00740] *SNP Analysis*

[00741] Of 661 SNPs identified in TRP ion channel and AChR genes from B cells a total of seventy-seven SNPs were associated with nicotinic and muscarinic acetylcholine receptor genes in CFS/ME patients. A total of thirty-five SNPs for mAChM3 featured, while the remaining predominate SNPs were identified for nAChR delta (n=12), nAChR alpha 9 (n= 5), TRPV2 (n= 7), TRPM3 (n=4), TRPM4 (n=l), mAChRM2 (n=2) and mAChRM5 (n=3). Table 12 represents the SNPs for TRP ion channel and AChR genes in B lymphocytes.

[00742] *Genotype Analysis*

[00743] Nine genotypes were identified from SNPs that reported significant for TRPM3 (n=l), TRPC6 (n=l), mAChRM3 (n=2), nAChR alpha 4 (n=l) and nAChR beta 1 (n=4). Table 13 represents the genotypes for SNPs in TRP and AChR genes from B lymphocytes that were reported as statistically significant between groups. The odds ratio for specific genotypes for SNPs in TRP and AChR genes from B lymphocytes ranged between 7.11 - 26.67 for CFS/ME compared with the non-fatigued control group.

[00744] Genotype with 11 CFS/ME patients and 11 non-fatigued controls. Data presented are included for $p<0.05$. Data are presented for gene (TRPM3, TRPC6, AChRM3, alpha 3,4, 7 and beta 1), chromosome location (CHR), reference SNP identification (RefSNPID), genotype percentage of CFS/ME patients with genotype (%), percentage of non-fatigued controls (5), chi-square ($\chi 2$) for basic allelic test (1 df), odds ratio (OR) and (*) P-value for this test set at a significance of <0.05.

[00745] **Discussion**

[00746] The current investigation reports novel findings for a number of SNPs in genes for AChR and TRP variants and genotypes from B cells from CFS/ME patients. These data are consistent the inventors' findings above in PBMCs and NK cells, showing B cells of high SNP prevalence and genotypes in TRP and AChR genes in CFS/ME patients.

[00747] Intracellular Ca^{2+} levels are substantially modulated by receptor induced alterations and are critical for lymphocyte differentiation and function. Ca^{2+} regulates antigen receptors, co-

receptors, signal transduction, mitochondrial function, transcriptional factors and gene expression [42x-45x]. For example Ca^{2+} entry is regulated by plasma membrane channels, intracellular receptor channels, non-selective cation channels, specific membrane transporters and cell membrane potential [20x, 45x, 46x].

[00748] The immune system is dependent on cholinergic signaling as B and T cells express cholinergic receptors and regulate cytokines in inflammatory responses [47x, 48x] and immune function [49x]. Cholinergic signaling influences both B cell [9x] and T cell [50x] responses and has been found to initiate B cell autoimmunity [51x]. In cholinergic receptor SNPs, mAChM3R featured significantly (45%) which is consistent with the inventors' findings of SNPs and their genotype in NK cells. In this current investigation there were two SNP genotypes reported for mAChM3R. However, given the small sample number as well as noting the inventors' previous results of SNP genotypes from isolated NK cells and PBMCs, other genotypes for this receptor may be present in CFS/ME patients. A recent study has reported a subgroup of CFS/ME patients who had muscarinic antibodies (mAChM3R) and a modest positive response occurred with reduced symptom presentation following anti-CD20 intervention [39x]. As this finding was only reported in a small group of patients and genotype SNPs were not reported, the inventors' current findings, along with their previous SNP genotype findings in isolated NK cells from a larger cohort, suggest these SNP genotype changes and their combinations may play a role in B cell function. Moreover, the ubiquitous distribution of cholinergic receptors throughout the body suggests that anomalies in SNP genotypes and their heterodimer configuration and pattern may contribute to the various clinical symptoms of CFS/ME.

[00749] The inventors have identified SNPs in muscarinic and nicotinic receptors from diverse blood cells, such as PBMC and isolated natural killer cells in larger cohorts of CFS/ME patients, suggesting cholinergic signaling may be impeded in this disorder. Muscarinic signaling has a role in gastrointestinal function [52x] as antibodies to mAChM3Rs have been found to inhibit gastrointestinal motility and cholinergic neurotransmission [53x]. The mAChM3Rs are widely distributed in the heart, where they regulate intracellular phosphoinositide hydrolysis to improve cardiac contraction, haemodynamic function [54x] and provide a protective effect against ischaemia [55x]. The mAChM3Rs are located in the pancreas where they mediate acetylcholine control over insulin secretion and have other important regulatory functions [56x-58x].

[00750] Nicotinic signaling via nAChRs is widely distributed in organisms demonstrating the universal character of cholinergic signaling. Muscle-type nAChRs, such as βï, are similar in all parts of the body [7x]. In the inventors' data, there is high demonstration of SNPs and genotypes in nAChRs, suggesting the extent of SNP genotypes in cholinergic receptors may play a role in B

cell function, as acetylcholine functions as a paracrine/autocrine regulator of immune and other physiological functions [59x]. The present data highlights the SNP genotypes for nAChR beta 1 where SNPs rs3829603 (C/C) and rs4151134 (T/T) are located in the 3' untranslated region and demonstrate significant odds ratio for these genotypes that range between 17.50-26.67 for the CFS/ME group. This location is a regulatory region that post-transcriptionally influences gene expression: 3'-UTR is a binding site for regulatory proteins [60x]. Binding to specific sites within the 3'-UTR may decrease gene expression of various mRNAs by either inhibiting translation or directly causing degradation of the transcript. Additionally, the agonist-binding site of nAChRs is located at the interface between adjacent subunits. Binding of the agonist that is located at the a subunit (al, ***a2,*** a3, a4, a6, α7, or a9), and the binding of the negative agonist-binding site is composed by αï θ, β2, β4, δ, γ, or ε subunit. Importantly α5, βï, and β3 subunits assemble in the receptor complex assumes the fifth subunit position, where they do not directly participate in the formation of the agonist-binding site, however, they form an integral configuration for the binding agonists and ligand selectivity [61x]. Given the number of SNP genotypes for nAChR βï that were located at the 3'UTR, the fifth subunit may alter ligand selectivity. Moreover, various subunit combinations have been shown to result in different nAChR subtypes that vary in the kinetic parameters and selectivity of the ion channels, as well as ligand specificity, signaling pathways and functions that are performed in different tissues [62x]. The density of distribution of AChRs throughout the body means that many tissues are likely to be affected where AChR expression occurs, suggesting a potential loss of function of neuronal and non-neuronal cholinergic signaling pathways in virtually all body tissues. Interestingly, the inventors and others have previously reported changes in B cell phenotypes from CFS/ME patients [26x, 63x] and in a study above the inventors reported a reduction in calcium mobilisation into B cells via TRPM3 where this receptor was identified to have 3'UTR SNP genotypes.

[00751] Cholinergic signaling in the brain is primarily focused on two main loci, the basal forebrain and the pedunculo-pontine area of the hindbrain [64x]. Acute vasoconstriction occurs after removal of the cholinergic parasympathetic input to forebrain cerebral arteries [65x], indicating the critical importance of intact cholinergic signaling in the brain. Both nicotinic and muscarinic cholinergic signaling influence hippocampal synaptic plasticity and processing cholinergic-dependent higher cognitive functions [66x]. Cholinergic and glutamatergic signaling demonstrate interdependence in cortical glial cell function in sleep/wake studies [67x]. Key CNS functions such as memory formation are associated with long term potentiation (LTP) in hippocampal synapses. This memory mechanism is Ca^{2+} dependent through its association with cholinergic signaling [68x].

[00752] **Conclusion**

[00753] These findings of SNP genotypes in cholinergic and TRP receptor genes in B cells, and previously in PBMCs and isolated NK cells, suggest a potential contribution to widespread pathology across all organ systems of the body including immune, CNS, heart, gastrointestinal and hormonal systems. The effects of these SNP genotypes on cholinergic signaling are likely to be particularly important in the central nervous system, peripheral nervous system, autonomic nervous system as well as other organ systems. Taken together, the functional effects of these SNP genotypes and their combinations suggest they may be contributing factors in the aetiology and clinical phenotypes of CFS/ME.

[00754] **Example 7 - Reduction in TRPM3 cell surface expression in NK cells and B lymphocytes from CFS/ME patients as well as decreased intracellular calcium**

[00755] The inventors in the Examples above identify SNPs in TRP ion channels, namely from the TRPM3 family (rsl2682832; rslll42508; rsll60742; rs4454352; rsl328153; rs3763619; rs7865858; rs1504401; rslOl 15622), as well as TRPAl (rs2383844; rs4738202) and TRPC4 (rs6650469; rs655207) in CFS/ME patients, as well as SNPs in ACh receptors, mainly muscarinic M3 receptors (mAChRm3), (rs4463655; rs589962; rsl072320; rs7543259; rs6661621; rs7520974; rs726169; rsrs6669810; rsrs6429157), as well as nicotinic ACh receptors (nAChR) alpha 10 (rs2672211; rs2672214; rs2741868; rs2741870; rs2741862), alpha 5 (rs951266; rs7180002), and alpha 2 (rs2565048; P = 0.01403. These ion channels and receptors are widely expressed in cells and tissues throughout the body and are strongly associated with the symptomatology often reported in CFS/ME. The inventors demonstrated that these are exhibited in 99-100% of n=115 CFS/ME patients compared to 0-1% in healthy controls of n=90 (see Table 7).

[00756] Data presented for gene (TRPM3 and mAChR3), chromosome location (CHR), reference SNP identification (Ref SNP ID), base pair (BP) location of SNP, alleles (Al and A2), chi-square ($\chi 2$) for basic allelic test (1 df), p-value for this test set at a significance of $p < 0.05$, odds ratio (OR), percentage of CFS patients with SNP and percentage of non-fatigue controls with SNPs.

[00757] Recently others have reported muscarinic acetylcholine receptors (mAChR3) have been found to inhibit TRPM3 via the action of phospholipase C [41y]. Given the present inventors found a significant association with SNPs in TRPM3 and mAChR3 in CFS/ME patients and both these receptors mediate calcium mobilization intracellularly for cell function, such as NK lysis, the present inventors investigated TRPM3 surface expression on NK cells and B lymphocytes and determined this phenotype in CFS/ME patients compared to healthy controls.

[00758] In this Example the inventors describe, for the first time, significant reduction in

TRPM3 cell surface expression in NK cells and B lymphocytes from CFS/ME patients as well as decreased intracellular calcium.

[00759] Methods

[00760] Sample preparation and other steps were carried out largely as described in Example 5.

[00761] *TRPM3 immunophenotyping assay*

[00762] PBMCs were incubated in 20µ1 of FCR blocking reagent (Miltenyi Biotech) for 10 minutes at room temperature and washed with phosphate buffer saline (PBS) and centrifuged at 400g for 5 minutes. Supernatant was removed and incubated with primary fluorochrome labelled antibodies (CD19-BV421, CD3-PerCP, CD56-BV421 and CD16-APC Cy7, BD Bioscience) for 30 minutes at room temperature in the dark. Labelled cells were washed and incubated with 10µg final concentration of goat anti-human TRPM3 antibody for 30 minutes, followed by a wash and resuspended in a final concentration of 5% (v/v) of Bovine Serum Albumin (Sigma) for 30 minutes. Cells were washed again and incubated with 5 µg final concentration of donkey anti-goat IgG FITC (Santa Cruz) for 30 minutes. Cells were washed cells and resuspended in 200 µï of staining buffer (BD Bioscience) and acquired at 50, 000 events using LSRFortessa X-20 (BD Bioscience). Lymphocyte populations were identified using forward scatter and side scatter (FSC, SSC) dot plots. Exclusions were $CD3^+$ cells and only $CD3^-$ lymphocytes were further used to characterize B lymphocytes and NK cell subset populations using CD19, CD56 and CD16. Total B cells were identified as $CD19^+$, whereas NK cell subsets were characterized using the expression of $CD56^{Bright}CD16^{Dim-}$ NK cells, $CD56^{Dim}CD16^{Bright+}$ NK cells and $CD56^-CD16^4$ NK cells. NK lysis, degranulation and lytic proteins were conducted as previously described [42y].

[00763] LSRFotessa X-20 Flow cytometry was utilized for sequential determination of cytoplasmic calcium $[Ca2^+]C$ and mitochondrial $[Ca2^+]M$, to help compare cytoplasmic or mitochondrial Ca^{2+} influx kinetics in B lymphocytes and NK cells. Characterizing kinetic measurements using median florescence of Fura-AM or Rhod-2 AM dye were used and smoothing curve method was applied to measure the area under the curve (AUC).

[00764] *Cytoplasmic calcium influx assay*

[00765] Following phenotypic staining, the cells were incubated with 0.5ml staining buffer that contained 0.02% Pluronic® F-127 and IµM Fura-red AM or Rhod-2 AM for 30 minutes in the incubator at 37°C. Stained cells were washed with DPBS without calcium and magnesium. Fura AM stained cells were stimulated after 30 seconds of flow cytometric acquisition in the presence of either a final concentration of 1.4 µg streptavidin, 714ng ionomycin, 50µg 2-APB or 14µg Thapsigargin. Data was recorded over 4 minutes. Rhod-2 AM stain cells were incubated

for a further 12 hours, prior to acquisition. Thapsigargin is a potent inhibitor for Calcium-ATPases receptors and raises cytoplasmic calcium concentration by inhibiting the ability for the cells to pump calcium into the endoplasmic reticulum (ER). 50μg 2-aminoethoxydiphenyl borate (2-APB) was used given its inhibition of ER and IP_3R. NK receptors (NG2DA and NKp46) were identified for cross-linking for calcium influx for activation, co-activation, and co-stimulation of resting human NK cells, whereas, CD19 and complement receptor CR2 (CD21) responsible for signal transduction and activation of Immunoglobulin M (IgM) were identified for cross-linking for induced calcium influx to enhanced activation of CD19+ B cells.

[00766] *Statistical analysis*

[00767] Statistical analysis was performed using IBM SPSS Statistics version 22 software (SPSS, Chicago, USA). Significance was tested by MANOVA ($p<0.05$ for significance) between healthy and CFS/ME groups using parameters including TRPM3, intracellular and calcium influx in B lymphocytes and NK cells. Flowjo was employed to analyze FCS files extracted from FACSDiva 8 software (BD Bioscience). Post Hoc test was performed to determine specifically where the significance was between groups (Control and CFS/ME). Levene test was used to analyze homogeneity of variance between groups.

[00768] **Discussion**

[00769] The inventors have identified, for the first time, TRPM3 on NK cells and B lymphocytes, and also report a significant reduction of TRPM3 surface expression on B lymphocytes and NK cells in CFS/ME patients compared with healthy controls (see Figure 3A and Figure 3B).

[00770] The inventors also report, for the first time, a significant reduction in cytoplasmic calcium ion concentration in $CD19^+$ B lymphocytes during cross-linking between CD21 and IgM following treatment with stepadividin or thapsigargin in CFS/ME patients (Figure 4A) as well as $CD56^{Bright}$ NK cells also had a significant decrease in cytoplasmic calcium in the presence of 2-APB and thapsigargin in CFS/ME patients (Figure 4B). Collectively, these findings suggest TRPM3 play a role in impaired calcium cytoplasmic influx in B lymphocytes and NK cells from CFS/ME patients.

[00771] **Example 8 - Other SNPs and genotypes in TRP ion channel and AChR genes from peripheral blood mononuclear cells (PBMCs), isolated B lymphocytes and NK cells in CFS/ME patients**

[00772] Examples above describe SNPs of TRP ion channel and AChR genes from PBMCs, isolated B lymphocytes and NK cells that scored significantly in a cohort of 115 CFS/ME patients. Using larger cohorts, the inventors believe that other identified SNPs will also score significantly, thus also being useful as probes, tools or reagents for identifying, screening,

diagnosing, monitoring or treating subjects with, or predisposed to, medical conditions (or symptoms thereof), such as chronic fatigue syndrome (CFS), myalgic encephalomyelitis (ME), Gulf war syndrome (GWS), irritable bowel syndrome (IBS), multiple chemical sensitivity (MCS), fibromyalgia, and migraine, as well as some medical conditions caused by dysregulation in calcium, acetylcholine and TRP, and dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary and immune systems.

[00773] Identified SNPs that have p values of 0.05 to 0.1, which the inventors believe may score significantly in a larger cohort of patients, are listed in the tables below (Tables 14 to 17).

Table 14: Analysis of the frequency distribution and significance of AChR gene SNPs in PBMCs in CFS/ME patients and non-fatigued controls that were not significant in n=115, in rank order of significance.

Gene	Chromosome	RefSNP ID	A1	Frequency_A	Frequency_U	A2	χ^2	P
nAchalO	11	rs2672215	A	0.4607	0.36	C	3.399	0.07
nAcha2	8	rs6474413	C	0.2308	0.1513	T	3.336	0.07
mAchM3	3	rsl0926008	G	0.3722	0.277	A	3.333	0.07
nAcha2	8	rs2741343	C	0.5337	0.4324	T	3.317	0.07
nAchcd	15	rs7178270	G	0.3571	0.4539	**C**	3.231	0.07
nAcha5	15	rs4243084	G	0.3977	0.3026	**C**	3.227	0.07
nAchα5	15	rs601079	A	0.3901	0.4868	T	3.155	0.08
nAcha5	15	rsl291 1602	C	0.3901	0.4868	T	3.155	0.08
nAcha5	15	rs588765	T	0.3846	0.4803	**C**	3.095	0.08
nAcha5	15	rs680244	A	0.3846	0.4803	G	3.095	0.08
nAchα5	15	rs6495306	G	0.3895	0.4863	A	3.01	0.08
nAcha5	15	rs6495307	T	0.4111	0.5068	C	2.997	0.08
mAchM3	3	rs12093821	A	0.489	0.3947	G	2.979	0.08
mAchM3	3	rsl6838637	G	0.4889	0.3947	A	2.957	0.09
nAcha2	8	rs6997909	A	0.2333	0.1579	G	2.945	0.09
nAchalO	11	rs2672216	C	0.4888	0.3947	T	2.934	0.09
mAchM3	3	rs6429165	A	0.2473	0.1711	G	2.873	0.09
nAcha2	8	rs891398	C	0.533	0.4392	T	2.872	0.09
nAcha5	15	rs4366683	G	0.3956	0.4868	A	2.802	0.09
nAcha2	8	rs6985052	C	0.2308	0.1579	T	2.774	0.10
nAcha2	8	rs4950	C	0.2308	0.1579	T	2.774	0.10

[00775] **Table 15:** Analysis of the frequency distribution and significance of TRP receptor gene SNPs in PBMCs in CFS/ME patients and non-fatigued controls that were not significant in n=l 15, in rank order of significance.

Gene	Chromosome	RefSNP ID	A1	Frequency_A	Frequency_U	A2	$\chi 2$	P
TRPM4	19	rsl04031 14	G	A	0.293	0.390	3.802	0.051
TRPV3	17	rs9909424	G	A	0.115	0.060	3.442	0.064
TRPC4	13	rs612308	A	G	0.439	0.537	3.393	0.065
TRPM3	9	rs7860377	A	C	0.350	0.262	3.3 14	0.069
TRPC7	5	rs2673930	C	A	0.200	0.280	3.218	0.073
TRPC4	13	rs603955	C	T	0.445	0.536	3.008	0.083
TRPM3	9	rsl 1142798	**C**	G	0.135	0.202	2.998	0.083
TRPM3	9	rs474461 1	G	A	0.360	0.446	2.843	0.092
TRPM2	2 1	rsl785452	T	C	0.215	0.289	2.67	0.102
TRPM3	9	rsl566838	G	T	0.460	0.375	2.669	0.102
TRPA1	8	rsl384002	T	**C**	0.495	0.410	2.664	0.103
TRPM6	9	rs2274924	G	A	0.115	0.175	2.652	0.103
TRPM3	9	rsl 394309	G	A	0.030	0.065	2.608	0.106
TRPC4	13	rs2985167	G	A	0.340	0.422	2.577	0.108
TRPM5	11	rs2301698	G	T	0.530	0.446	2.55 1	0.1 10
TRPM6	9	rs944857	C	T	0.185	0.125	2.476	0.1 16
TRPM2	2 1	rs762426	G	A	0.160	0.223	2.325	0.127

Table 16: Analysis of the frequency distribution and significance of AChR and TRP gene SNPs in isolated NK cells in CFS/ME patients (n=39) and non-fatigued controls (n=30) that were not signif cant, in rank order of signi ficance.

Gene	CHR	SNP	BP	MAF	A1	F_A	F_U	A2	CHISQ	P	OR
CHRM5	15	rs623941	34060377	0.362221	C	0.4211	0.25	A	3.76	0.05249	2.182
CHRNA3	15	rs615470	78593646	0.291134	T	0.2895	0.45	C	3.749	0.05285	0.4979
CHRNA3	15	rs7182583	78606868	0.271565	C	0.2895	0.45	G	3.749	0.05285	0.4979
CHRM3	1	rs536071	2.4E+08	0.352236	C	0.4615	0.3	T	3.715	0.05391	2
CHRM3	1	rs693948	2.4E+08	0.446486	G	0.4605	0.3	A	3.633	0.05665	1.992
TRPM3	9	rs4620343	71121727	0.428115	T	0.3718	0.5345	C	3.571	0.05879	0.5155
CHRNA5	15	rs495956	78577588	0.308307	G	0.2949	0.45	A	3.532	0.06019	0.5111
CHRNA5	15	rs692780	78584163	0.5	G	0.2949	0.45	C	3.532	0.06019	0.5111
CHRNA5	15	rs11637635	78584808	0.254593	A	0.2949	0.45	G	3.532	0.06019	0.5111
CHRNA3	15	rs17408276	78589276	0.208866	C	0.2949	0.45	T	3.532	0.06019	0.5111
CHRNA3	15	rs660652	78595490	0.256989	A	0.2949	0.45	G	3.532	0.06019	0.5111
CHRNA3	15	rs472054	78595652	0.256989	T	0.2949	0.45	C	3.532	0.06019	0.5111
TRPC4	13	rs6650469	37793812	0.399561	T	0.5256	0.3667	C	3.454	0.06308	1.914
CHRNB4	15	rs1316971	78638168	0.442492	A	0.141	0.2667	G	3.402	0.06513	0.4515
CHRNA3	15	rs4887070	78623845	0.339457	C	0.2692	0.4167	T	3.317	0.06855	0.5158
CHRM5	15	rs8035849	34058132	0.34385	A	0.359	0.2167	C	3.289	0.06976	2.025
CHRM3	1	rs606709	2.4E+08	0.347444	T	0.4342	0.2833	C	3.283	0.07	1.941
TRPC2	11	rs2898934	3623827	0.14996	C	0.1795	0.07143	A	3.273	0.07042	2.844
TRPM8	2	rs10170647	2.34E+08	0.207867	G	0.1053	0.2167	T	3.187	0.07423	0.4253
CHRNA7	15	rs2337980	32151995	0.375	T	0.3974	0.55	C	3.174	0.07482	0.5397
CHRNA3	15	rs514743	78591885	0.241214	T	0.3026	0.45	A	3.132	0.07676	0.5304
CHRND	2	rs2853446	2.33E+08	0.480232	C	0.5526	0.4	T	3.127	0.077	1.853
CHRND	2	rs2245601	2.33E+08	0.483427	T	0.5513	0.4	C	3.107	0.07795	1.843
CHRM3	1	rs6701181	2.4E+08	0.491014	T	0.3846	0.5333	C	3.031	0.08167	0.5469
TRPV4	12	rs3825394	1.1E+08	0.245607	A	0.3846	0.5333	C	3.031	0.08167	0.5469
TRPV4	12	rs1861809	1.1E+08	0.239617	T	0.3846	0.5333	C	3.031	0.08167	0.5469

CHRND	2	rs2278478	2.33E+08	0.272564	C	0.1974	0.3276	T	2.946	0.0861	0.5047
TRPC4	13	rs655207	37793875	0.388179	G	0.5128	0.3667	T	2.928	0.08707	1.818
CHRNE	17	rs2075763	4899390	0.10623	T	0.05128	0.1333	C	2.876	0.08993	0.3514
TRPM3	9	rs10123815	71068915	0.1248	G	0	0.03571	A	2.828	0.09264	0
TRPC6	11	rs6578397	3614380	0.427516	T	0.3846	0.25	A	2.797	0.09447	1.875
CHRM3	1	rs12036141	2.4E+08	0.473442	A	0.3553	0.5	G	2.779	0.09551	0.551
TRPV4	12	rs10850783	1.1E+08	0.273163	A	0.3947	0.5333	C	2.596	0.1071	0.5707

Table 17: Analysis of the frequency distribution and significance of AChR and TRP gene SNPs in isolated B lymphocytes in CFS/ME patients (n=111) and non-fatigued controls (n=111) that were not significant, in rank order of significance.

Gene	CHR	SNP	BP	A1	F_A	F_U	A2	CHISQ	P	OR
CHRM3	1	rs10754677	239669799	G	0.3846	0.625	A	3.819	0.05066	0.375
CHRM3	1	rs7513746	239699110	G	0.3889	0.625	A	3.727	0.05353	0.3818
CHRM3	1	rs10802795	239707474	C	0.3889	0.625	T	3.727	0.05353	0.3818
CHRM3	1	rs3738436	239709192	A	0.3889	0.625	C	3.727	0.05353	0.3818
CHRM3	1	rs7511970	239719954	A	0.3889	0.625	G	3.727	0.05353	0.3818
CHRM3	1	rs1155611	239734526	T	0.3889	0.625	C	3.727	0.05353	0.3818
CHRM3	1	rs1019882	239735555	G	0.3889	0.625	A	3.727	0.05353	0.3818
CHRM3	1	rs1416789	239738344	G	0.3889	0.625	A	3.727	0.05353	0.3818
CHRM3	1	rs10925964	239739213	A	0.3889	0.625	T	3.727	0.05353	0.3818
TRPV2	17	rs8079010	16425640	C	0.4815	0.25	T	3.68	0.05508	2.786
CHRM3	1	rs6429154	239713965	G	0.3929	0.625	A	3.642	0.05634	0.3882
CHRNB1	17	rs2302767	7447224	C	0.2778	0.5	T	3.625	0.05691	0.3846
TRPM3	9	rs7038646	70822907	A	0.4808	0.25	G	3.621	0.05706	2.778
CHRNA2	8	rs2741342	27472578	A	0.1786	0.375	G	3.579	0.0585	0.3623
CHRND	2	rs4973536	232527170	C	0.3571	0.5909	G	3.536	0.06004	0.3846
C17orf107	17	rs33978919	4899033	A	0.07407	0.2273	G	3.514	0.06085	0.272
TRPM3	9	rs1891301	71403579	T	0.5577	0.3333	C	3.309	0.06892	2.522
CHRM3	1	rs12406493	239689804	C	0.5556	0.3333	A	3.284	0.06995	2.5
CHRM3	1	rs6429152	239690836	G	0.5556	0.3333	A	3.284	0.06995	2.5

CHRM3	1	rs2355237	239694223	A	0.5556	0.3333	G	3.284	0.06995	2.5
CHRM3	1	rs988231	239696189	C	0.5556	0.3333	T	3.284	0.06995	2.5
CHRM3	1	rs717227	239719298	C	0.5556	0.3333	T	3.284	0.06995	2.5
TRPM3	9	rs1106948	71402257	T	0.5536	0.3333	C	3.262	0.07092	2.48
CHRNA2	8	rs2565048	27472614	C	0.125	0.2917	T	3.232	0.0722	0.3469
CHRNB1	17	rs2302762	7455541	T	0.2778	0.5	C	3.222	0.07267	0.3846
CHRM3	1	rs1431719	239717902	G	0.4038	0.625	A	3.221	0.07268	0.4065
AVEN	15	rs2702282	34023860	G	0.4643	0.25	T	3.214	0.073	2.6
CHRM3	1	rs6693851	239678896	C	0.3182	0.5455	T	3.173	0.07486	0.3889
CHRM3	1	rs2278642	239703842	T	0.4074	0.625	G	3.155	0.07569	0.4125
CHRM3	1	rs12751235	239706520	T	0.4074	0.625	C	3.155	0.07569	0.4125
CHRM3	1	rs6663632	239714420	A	0.4074	0.625	C	3.155	0.07569	0.4125
CHRM3	1	rs665159	239798701	C	0.4074	0.625	T	3.155	0.07569	0.4125
TRPM4	19	rs12461216	49160966	C	0.07143	0.2083	G	3.154	0.07575	0.2923
CHRM3	1	rs714803	239631122	T	0.3704	0.5833	A	3.065	0.08001	0.4202
CHRM3	1	rs2120241	239645190	T	0.2885	0.5	A	3.035	0.08146	0.4054
AC009264.1	7	rs1455858	136946955	A	0.5	0.2917	G	2.963	0.08519	2.429
AC009264.1	7	rs1378646	136950253	G	0.5	0.2917	A	2.963	0.08519	2.429
AC009264.1	7	rs1158586	136952388	G	0.5	0.2917	A	2.963	0.08519	2.429
AC009264.1	7	rs1455857	136955193	A	0.5	0.2917	G	2.963	0.08519	2.429
CHRM3	1	rs685548	239831605	T	0.5	0.2917	G	2.933	0.0868	2.429
CHRM3	1	rs16839070	239900649	T	0.3571	0.1667	A	2.902	0.08844	2.778
CHRNG	2	rs2697782	232542805	C	0.3571	0.1667	G	2.902	0.08844	2.778
CHRNB2	1	rs2072660	154576244	T	0.1964	0.375	C	2.857	0.09097	0.4074
TRPM7	15	rs4775894	50611402	T	0.4375	0.2273	C	2.856	0.09105	2.644
CHRM3	1	rs6657343	239728210	T	0.537	0.3333	A	2.765	0.09634	2.32
CHRM3	1	rs891700	239718625	A	0.5357	0.3333	G	2.759	0.09669	2.308
AC018890.6	2	rs2600685	174762319	G	0.4464	0.25	A	2.731	0.09841	2.419
AVEN	15	rs1685119	33984851	G	0.4464	0.25	A	2.731	0.09841	2.419
AVEN	15	rs489832	33985705	A	0.4464	0.25	G	2.731	0.09841	2.419
AC009264.1	7	rs2113550	136881785	G	0.2963	0.5	A	2.657	0.1031	0.4211

AC009264.1	7	rs6944132	136885528	T	0.4444	0.25	A	2.654	0.1033	2.4
CHRNA10	11	rs2672214	3670281	C	0.4259	0.2273	T	2.651	0.1035	2.523
CHRM3	1	rs658842	239785334	T	0.4259	0.625	A	2.636	0.1045	0.4452
TRPV2	17	rs8121	16422653	C	0.375	0.1667	T	2.619	0.1056	3

[00776] **Example 9 - Exome sequencing for determining SNPs and genotypes in TRP ion channel and AChR genes from isolated B lymphocytes in CFS/ME patients**

[00777] Example 6 above describes SNPs and genotypes in TRP ion channel and AChR genes from isolated B lymphocytes in ME/CFS patients. In this Example the inventors utilise exome sequencing to characterise SNPs and genotypes in TRP ion channel and AChR genes from isolated B lymphocytes in ME/CFS patients.

[00778] **Methods**

[00779] For details of the subjects and sample preparation, see Example 6.

[00780] *DNA extraction*

[00781] Genomic DNA was extracted from all whole blood samples using the Qiagen DNA blood mini-kit as per manufacturer's instructions (Qiagen). The Nanodrop (Nanodrop) was used to assess the quality and quantity of the DNA extracted. Approximately 2μg of genomic DNA was used in the SNP assay.

[00782] *DNA quantification and qualification*

[00783] DNA degradation and contamination was monitored on 1% agarose gels.

[00784] (1) DNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA).

[00785] (2) DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA).

[00786] (3) Fragment distribution of DNA library was measured using the DNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

[00787] *Library preparation for sequencing*

[00788] A total amount of 1μg genomic DNA per sample was used as input material for the DNA sample preparation. Sequencing libraries were generated using Agilent SureSelect Human All ExonV5 kit (Agilent Technologies, CA, USA) following manufacturer's recommendations and x index codes were added to attribute sequences to each sample. Briefly, fragmentation was carried out by hydrodynamic shearing system (Covaris, Massachusetts, USA) to generate 180-280bp fragments. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and enzymes were removed. After adenylation of 3' ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with ligated adapter molecules on both ends were selectively enriched in a PCR reaction. After PCR reaction, the library was hybridized with Liquid phase with biotin labeled probe, then magnetic beads with streptomycin were used to capture the 334,378 exons in 20, 965 genes. Captured libraries were enriched in a PCR reaction to add index tags to prepare for hybridization. Products were purified using AMPure XP system (Beckman Coulter, Beverly, USA) and quantified using the Agilent

high sensitivity DNA assay on the Agilent Bioanalyzer 2100 system.

[00789] *Clustering and sequencing*

[00790] If library qualifies, the clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia, San Diego, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 125 bp paired-end reads were generated.

[00791] *Analysis Result*

[00792] *Raw data*

[00793] The original raw data obtained from high throughput sequencing platforms (e.g. illumina platform) was transformed to sequenced reads by base calling and recorded in FASTQ file (which contains sequence information (reads) and corresponding sequencing quality information) as explained in Section 4.1 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.ip/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .

[00794] *Quality control*

[00795] *Sequencing data filtration*

[00796] The steps of data processing undertaken were as explained in Section 4.2.1 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.jp/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .

[00797] *Sequencing error rate distribution*

[00798] A Phred score of a base (Phred score, Qphred) was calculated as explained in Section 4.2.2 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.ip/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .

[00799] *Sequencing quality distribution*

[00800] Sequence quality distribution was carried out as explained in Section 4.2.4 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.jp/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .To ensure downstream analysis, most base quality is required to be greater than Q20. According to sequencing feature, base quality in sequence end is usually lower than that in sequence beginning.

[00801] *Statistics summary of sequencing quality*

[00802] According to the illumina platform sequencing feature, for PE data the average

percentage of Q20 was required to be above 90%, Q30 was required to be above 80%, average error rate was required to be below 0.1%. (See Section 4.2.5 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.ip/Product/Bioscience5-seq/Cancer_WGS_report_Vl.l.pdf .

[00803] **Table 18** - Overview of data production quality

Sample name	Library	Lane	Raw reads	Raw data(G)	Raw depth(x)	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
SEV1114047	DHE01587	HCWL3CCXX_L6	25814640	7.74	153.6	98.92	0.04	94.23	86.82	48.55
SEV1114046	DHE01595	HF5KJCCXX_L5	27668541	8.3	164.71	98.96	0.03	96.29	90.78	48.45
SEV1114065	DHE01585	HCWL3CCXX_L3	21195532	6.36	126.21	98.59	0.04	94.82	88.25	48.64
SEV1114064	DHE01594	HF5KJCCXX_L5	21146920	6.34	125.82	98.93	0.03	96.25	90.69	48.89
SEV1114001	DHE01605	HF5KJCCXX_L4	27647015	8.29	164.51	98.87	0.03	96.36	90.97	48.77
SEV1114066	DHE01602	HF5KJCCXX_L7	22154417	6.65	131.97	98.71	0.03	95.08	88.41	49.27
SEV1114006	DHE01584	HCWL3CCXX_L6	20859072	6.26	124.23	98.80	0.04	94.28	86.84	48.28
SEV1114049	DHE01582	HCWL3CCXX_L4	23731273	7.12	141.3	98.98	0.04	95.06	88.68	48.86
SEV1114020	DHE01601	HF5KJCCXX_L5	25066003	7.52	149.23	98.37	0.03	96.44	91.08	49.22
SEV1114022	DHE01598	HF5KJCCXX_L5	20484764	6.15	122.05	98.87	0.03	96.26	90.68	48.52
SEV1114025	DHE01591	HF5KJCCXX_L5	25343055	7.6	150.82	98.87	0.03	96.3	90.8	49.0
SEV1114026	DHE01593	HF5KJCCXX_L5	24210778	7.26	144.07	99.06	0.03	96.18	90.57	48.98
SEV1114029	DHE01604	HF5KJCCXX_L4	22825090	6.85	135.94	98.77	0.03	96.22	90.69	47.81
SEV1114052	DHE01592	HF5KJCCXX_L5	22862429	6.86	136.14	98.98	0.03	96.13	90.41	48.09
SEV1114054	DHE01586	HCWL3CCXX_L6	18507970	6.76	134.15	98.85	0.05	94.03	86.41	48.08
SEV1114054	DHE01586	H3LLHBBXX_L2	4044682			98.82	0.03	95.16	89.02	47.95
SEV1114055	DHE01603	HF5KJCCXX_L4	28499810	8.55	169.67	98.80	0.03	96.43	91.09	49.28
SEV1114056	DHE01590	HCWL3CCXX_L3	22338448	6.7	132.96	98.92	0.04	93.91	86.43	48.62
SEV1114033	DHE01588	HCWL3CCXX_L6	24351060	7.31	145.07	98.82	0.07	92.06	83.24	48.48
SEV1114017	DHE01580	HCWL3CCXX_L3	19811908	6.79	134.75	98.65	0.04	94.84	88.22	48.42
SEV1114017	DHE01580	H3LLHBBXX_L2	2841615			98.65	0.03	95.22	89.11	48.33

SEV11140 36	DHE015 99	HF5KJCCXX_ L5	292496 82	8.77	174.04	98.76	0.03	96.5	91.19	49.01
SEV11140 13	DHE015 97	HF5KJCCXX_ L5	207993 94	6.24	123.83	98.85	0.03	96.31	90.81	48.97
SEV11140 38	DHE015 83	HCWL3CCXX _L6	227370 75	6.82	135.34	98.35	0.04	94.27	86.93	44.61
SEV11140 35	DHE016 00	HF5KJCCXX_ L5	238642 53	7.16	142.09	98.86	0.03	95.94	90.01	49.42
SEV11140 18	DHE015 96	HF5KJCCXX_ L5	260462 85	7.81	154.99	98.89	0.03	96.34	90.87	48.28
SEV11140 19	DHE015 81	HCWL3CCXX _L6	228476 21	6.85	135.94	98.75	0.04	94.37	87.01	48.7
SEV11140 67	DHE015 89	HCWL3CCXX _L6	296362 40	8.89	176.42	99.09	0.05	94.06	86.52	48.18

[00804] Note:

[00805] Sample name: Sample name

[00806] Library: Library name

[00807] Lane: The flowcell ID and lane number of the sequencing machine

[00808] Raw reads: The number of sequencing reads pairs; According to the format of FASTQ, four lines will be considered as one unit

[00809] Raw data: The original sequence data

[00810] Raw depth: The original sequence depth

[00811] Effective: The percentage of clean reads in all raw reads

[00812] Error: The average error rate of all bases on readl and read2; the error rate of a base is obtained from equation No. 1

[00813] Q20, Q30: Percentage of reads with average quality>Q20 and percentage of reads with average quality>Q30

[00814] GC: Percentage of G and C in the total bases

[00815] *Sequence alignment*

[00816] Sequence alignment was carried out as explained in Section 4.3 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.jp/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .

[00817] *Sequencing depth, coverage distribution*

[00818] Sequence depth, coverage and distribution was carried out as explained in Section 4.3.1 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.jp/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf .

[00819] *Statistics of coverage*

[00820] **Table 19-** Mapping rate and coverage:

Sample:	SEV111400 1	SEV111400 6	SEV111401 3	SEV111401 7	SEV111401 8	SEV111401 9	SEV111402 0	SEV111402 2	SEV111402 5	SEV111402 6	SEV111402 9	SEV111403 3	SEV111403 5	SEV111403 6	SEV111403 8	SEV111404 6	SEV111404 7	SEV111404 9	SEV111405 2	SEV111405 4	SEV111405 5	SEV111405 6	SEV111406 4	SEV111406 5	SEV111406 6	SEV111406 7
Total:[1]	546701 94 (100%)	412157 50 (100%)	411194 12 (100%)	446966 26 (100%)	515149 00 (100%)	451244 48 (100%)	493148 48 (100%)	405074 28 (100%)	501140 94 (100%)	479682 66 (100%)	450886 96 (100%)	481260 88 (100%)	471844 02 (100%)	577763 30 (100%)	447220 48 (100%)	547632 44 (100%)	510732 68 (100%)	469781 30 (100%)	452566 24 (100%)	445826 98 (100%)	563180 06 (100%)	441925 64 (100%)	418418 78 (100%)	417922 96 (100%)	437353 54 (100%)	587329 10 (100%)
Duplicate:[2]	413581 2 (7.57%)	351131 7 (8.55%)	294107 6 (7.16%)	349660 5 (7.84%)	394012 1 (7.65%)	426919 0 (9.48%)	414423 1 (8.41%)	263517 7 (6.51%)	461491 4 (9.22%)	370111 5 (7.72%)	329197 7 (7.31%)	422744 5 (8.81%)	372894 4 (7.91%)	574677 7 (9.95%)	454185 6 (10.17%)	464163 2 (8.48%)	529150 0 (10.38%)	488366 9 (10.43%)	325147 8 (7.19%)	333634 6 (7.50%)	517513 0 (9.20%)	339699 3 (7.69%)	302175 0 (7.23%)	326034 7 (7.81%)	284932 8 (6.53%)	558668 5 (9.53%)
Mapped:[3]	546027 97 (99.88%)	410652 84 (99.63%)	410863 92 (99.92%)	446493 55 (99.89%)	514778 39 (99.93%)	450170 96 (99.83%)	492794 59 (99.93%)	404770 86 (99.93%)	500747 92 (99.92%)	479243 35 (99.91%)	450314 44 (99.67%)	479957 58 (99.73%)	471435 56 (99.91%)	577361 93 (99.93%)	446470 68 (99.83%)	547133 03 (99.91%)	509851 63 (99.83%)	468399 75 (99.71%)	452192 71 (99.92%)	445010 53 (99.82%)	562512 45 (99.86%)	441527 52 (99.91%)	418090 15 (99.92%)	417568 03 (99.92%)	436677 50 (99.65%)	586318 91 (99.80%)
Properly mapped:[4]	543605 18 (99.43%)	408133 20 (99.02%)	408570 02 (99.36%)	443670 34 (99.26%)	511947 50 (99.38%)	448034 32 (99.29%)	490263 66 (99.42%)	402331 88 (99.32%)	498244 58 (99.42%)	476501 22 (99.34%)	448109 18 (99.38%)	476320 54 (98.97%)	468543 08 (99.30%)	573798 32 (99.31%)	444251 84 (99.34%)	543943 98 (99.33%)	506846 34 (99.24%)	465414 88 (99.07%)	449830 62 (99.40%)	442168 64 (99.18%)	559744 40 (99.39%)	439386 22 (99.43%)	415626 58 (99.33%)	415091 40 (99.32%)	434281 92 (99.30%)	582606 38 (99.20%)
PE mapped:[5]	545635 48 (99.80%)	410409 22 (99.58%)	410584 50 (99.85%)	446196 16 (99.83%)	514477 68 (99.87%)	450226 18 (99.77%)	492502 78 (99.87%)	404505 60 (99.86%)	500436 72 (99.86%)	478909 28 (99.84%)	449967 12 (99.80%)	479349 94 (99.60%)	471073 62 (99.84%)	577031 92 (99.87%)	446221 26 (99.78%)	546756 38 (99.84%)	509551 30 (99.77%)	468081 18 (99.64%)	451877 52 (99.85%)	444708 94 (99.75%)	562123 94 (99.81%)	441213 56 (99.84%)	417810 42 (99.85%)	417321 06 (99.86%)	436281 76 (99.75%)	585947 70 (99.76%)
SE mapped:[6]	784982 4 (0.14%)	487241 4 (0.12%)	558840 74 (0.13%)	594780 22 (0.13%)	601422 0 (0.12%)	487761 8 (0.11%)	583622 4 (0.12%)	530522 0 (0.13%)	622401 42 (0.12%)	668141 70 (0.14%)	694641 28 (0.15%)	121528 88 (0.25%)	723880 2 (0.15%)	660020 84 (0.11%)	498840 22 (0.11%)	753300 68 (0.14%)	600650 14 (0.12%)	637140 60 (0.14%)	630380 62 (0.14%)	604060 74 (0.14%)	777020 92 (0.14%)	627920 46 (0.14%)	559460 42 (0.13%)	533940 86 (0.13%)	791480 42 (0.18%)	742420 38 (0.13%)
With mate mapped to a different chr:[7]	142924 6 (0.26%)	149738 6 (0.36%)	154072 74 (0.37%)	170022 8 (0.38%)	183192 6 (0.36%)	162664 6 (0.36%)	162730 3 (0.33%)	172036 2 (0.42%)	154142 1 (0.31%)	174070 6 (0.36%)	134364 0 (0.30%)	165888 4 (0.34%)	192974 1 (0.40%)	260964 5 (0.45%)	140022 1 (0.31%)	194068 5 (0.35%)	192980 8 (0.38%)	176660 8 (0.38%)	139118 1 (0.31%)	193716 3 (0.43%)	163956 9 (0.29%)	132692 0 (0.30%)	159952 8 (0.38%)	164858 9 (0.38%)	142734 3 (0.33%)	234308 0 (0.40%)
With mate mapped to a different chr ((mapQ>=5)):[8]	101328 (0.19%)	112180 (0.27%)	117932 (0.29%)	124944 (0.28%)	137490 (0.27%)	118673 (0.26%)	119873 (0.24%)	135940 (0.34%)	107675 (0.21%)	129625 (0.27%)	947 40 (0.21%)	119379 (0.25%)	153442 (0.30%)	207140 (0.36%)	103050 (0.23%)	147351 (0.27%)	142995 (0.28%)	125435 (0.27%)	100054 (0.22%)	148414 (0.33%)	116431 (0.26%)	874 72 (0.20%)	119588 (0.28%)	125123 (0.30%)	101128 (0.23%)	174256 (0.30%)
Initial_bases_on_target:[9]	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01	503906 01
Initial_bases_near_target:[10]	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22	739022 22
Initial_bases_on_or_near_target:[11]	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823	124292 823
Total_effective_reads:[12]	547243 46	411654 46	411841 55	447688 06	516059 73	451637 55	493975 15	405736 17	502034 11	480471 47	451415 93	481118 10	472490 31	578792 27	447544 48	548445 66	511150 02	469654 50	453300 42	446161 03	563867 08	442669 81	419175 20	418659 66	437775 39	587870 66
Total_effective_yield (Mb):[13]	813535	610514	612439	663470	767035	669731	734294	603160	745936	713771	670770	711019	702335	860359	663776	815246	757903	695951	673617	660726	838018	656825	622801	621075	648911	871250
Effective_sequences_on_target(Mb):[14]	503778	370462	385415	407238	471159	410186	468735	379841	462048	436711	414025	429055	444267	548755	395817	500231	482123	416687	412572	400335	523893	407161	384098	375007	406000	520635
Effective_sequences_near_target(Mb):[15]	20096	13906	14536	14779	17955	14986	17288	14286	17406	17317	16462	16501	16578	19727	15244	19260	17332	15932	16163	15148	19855	15474	14673	14280	15614	20916
Effective_sequences_on_or_near_target(Mb):[16]	703873	509468	530651	554417	651014	559971	640923	522627	636754	609828	578687	594357	610049	746382	548472	692691	635455	576619	574235	552282	722438	561909	530831	518503	562114	729650
Fraction_of_effective_bases_on_target:[17]	61.9%	60.7%	63.0%	61.4%	61.4%	61.2%	63.8%	63.0%	61.9%	61.2%	61.7%	60.3%	63.3%	63.8%	59.6%	61.4%	61.0%	59.9%	61.2%	60.6%	62.5%	62.1%	61.7%	60.5%	62.6%	59.8%
Fraction_of_effective_bases_on_or_near_target:[18]	86.5%	83.4%	86.7%	83.6%	84.9%	83.6%	87.3%	86.6%	85.4%	85.4%	86.3%	83.6%	86.9%	86.8%	82.6%	85.0%	83.8%	82.9%	85.2%	83.6%	86.2%	85.6%	85.2%	83.5%	86.6%	83.7%
Average_sequencing_depth_on_target:[19]	99.97	73.52	76.49	80.82	93.50	81.49	93.02	75.38	91.69	86.57	82.16	85.15	88.16	108.90	78.55	99.27	91.71	82.69	81.27	79.45	103.97	80.80	76.22	74.56	80.57	103.32
Average_sequencing_depth_near_target:[20]	27.08	18.81	19.65	19.92	24.34	20.27	23.30	19.32	23.64	23.43	22.31	22.37	22.43	26.74	20.66	26.04	23.45	21.64	21.88	20.56	26.87	20.94	19.85	19.32	21.12	26.28
Mismatch_rate_in_target_region:[21]	0.42%	0.71%	0.42%	0.66%	0.42%	0.70%	0.41%	0.43%	0.43%	0.44%	0.43%	1.01%	0.46%	0.40%	0.73%	0.42%	0.72%	0.64%	0.44%	0.73%	0.41%	0.79%	0.43%	0.67%	0.54%	0.75%
Mismatch_rate_in_all_effective_sequence:[22]	0.36%	0.60%	0.37%	0.56%	0.36%	0.58%	0.36%	0.37%	0.37%	0.38%	0.37%	0.83%	0.40%	0.35%	0.60%	0.37%	0.60%	0.54%	0.38%	0.61%	0.36%	0.65%	0.37%	0.56%	0.46%	0.62%
Base_covered_on_target:[23]	503390 57	502722 95	502892 69	502807 56	502880 70	502829 58	502936 85	502783 28	503397 39	502831 55	502769 05	502808 17	502770 32	502871 29	498626 20	502862 41	502832 19	502803 22	502845 17	502750 77	502828 39	502814 52	503302 19	502949 71	503364 02	502824 37
Coverage_of_target_region:[24]	99.9%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	99.9%	99.8%	99.8%	99.8%	99.8%	99.8%	99.0%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	99.9%	99.8%	99.9%	99.8%
Base_covered_near_target:[25]	723765 06	705677 61	709908 93	703876 26	717493 73	704914 62	713576 22	707712 33	716844 33	720938 97	717590 95	715478 79	714334 93	714098 86	675442 45	721143 59	713305 39	714944 31	718117 77	709388 98	721241 65	713230 56	712522 28	710680 79	717388 89	723738 69

Coverage_of_flanking_region:[28]	97.9%	95.5%	96.1%	95.2%	97.1%	95.4%	96.6%	95.8%	97.0%	97.6%	97.1%	96.8%	96.7%	96.6%	91.4%	97.6%	95.5%	96.7%	97.2%	96.0%	97.6%	96.5%	96.4%	96.2%	97.1%	97.9%
Fraction_of_target_covered_with_at_least_20x:[27]	97.9%	95.5%	96.0%	96.5%	97.6%	96.8%	97.5%	95.8%	97.5%	97.3%	96.6%	96.7%	97.1%	96.3%	81.7%	98.0%	97.4%	96.9%	96.8%	96.3%	98.2%	96.8%	96.1%	95.9%	96.3%	98.0%
Fraction_of_target_covered_with_at_least_10x:[26]	99.4%	98.8%	99.0%	99.1%	99.3%	99.2%	99.3%	98.9%	99.4%	99.3%	99.1%	99.0%	99.2%	99.4%	89.0%	99.3%	99.2%	99.2%	99.1%	99.0%	99.4%	99.1%	99.1%	99.0%	99.2%	99.3%
Fraction_of_target_covered_with_at_least_4x:[29]	99.8%	99.6%	99.6%	99.6%	99.7%	99.7%	99.7%	99.6%	99.8%	99.7%	99.6%	99.6%	99.7%	99.7%	95.7%	99.7%	99.7%	99.7%	99.6%	99.6%	99.7%	99.7%	99.7%	99.6%	99.8%	99.7%
Fraction_of_flanking_region_covered_with_at_least_20x:[30]	46.1%	33.5%	35.3%	34.9%	41.8%	35.8%	40.2%	34.6%	41.0%	41.6%	39.4%	39.2%	39.4%	43.9%	33.7%	44.3%	40.4%	38.4%	38.9%	36.4%	45.2%	37.4%	35.7%	34.7%	37.7%	47.2%
Fraction_of_flanking_region_covered_with_at_least_10x:[31]	67.4%	54.7%	56.7%	55.0%	62.5%	56.0%	60.7%	55.9%	61.8%	63.6%	61.3%	60.7%	60.4%	63.1%	51.2%	65.1%	60.8%	59.8%	60.9%	57.3%	65.9%	58.6%	57.4%	56.3%	59.8%	68.0%
Fraction_of_flanking_region_covered_with_at_least_4x:[32]	87.7%	78.6%	80.3%	78.0%	84.2%	78.7%	82.6%	79.4%	83.6%	85.6%	84.0%	83.4%	82.7%	83.4%	72.5%	86.2%	82.6%	82.7%	83.9%	80.3%	86.5%	81.5%	81.0%	80.2%	83.2%	88.0%

[0082 1] (1) Total: The number of total clean reads

[00822] (2) Duplicate: The number of duplication reads

[00823] (3) Mapped: The number of total reads that mapped to the reference genome (percentage)

[00824] (4) Properly mapped: The number of reads that mapped to the reference genome and the direction is right

[00825] (5) PE mapped: The number of pair-end reads that mapped to the reference genome (percentage)

[00826] (6) SE mapped: The number of single-end reads that mapped to the reference genome

[00827] (7) With mate mapped to a different chr: The number of mate reads that mapped to the different chromosomes

[00828] (8) With mate mapped to a different chr (mapQ>=5): The number of mate reads that mapped to the different chromosomes and the MAQ >5

[00829] (9) Initial_bases_on_target: Total bases mapped to the target region(exonic region we capture)

[00830] (10) Initial_bases_near_target: Total based mapped to the flanking region(The region nearby target upstream and downstream 200bp)

[00831] (11) Initial_bases_on_or_near_target: Total length of target region and flanking region

[00832] (12) Total_effective_reads: The number of valid reads that mapped to the reference genome

[00833] (13) Total_effective_yield(Mb): Total effective yield

[00834] (14) Effective_sequences_on_target(Mb): Total reads that mapped to the reference genome target region

[00835] (15) Effective_sequences_near_target(Mb): Total reads that mapped to the reference

genome flanking region

[00836] (16) Effective_sequences_on_or_near_target(Mb): Total reads that mapped to the reference genome target region and flanking region

[00837] (17) Fraction_of_effective_bases_on_target: The percentage of the mapped reads in target region to the reads in reference genome

[00838] (18) Fraction_of_effective_bases_on_or_near_target: The percentage of the mapped reads in target region and flanking region to the reads in reference genome

[00839] (19) Average_sequencing_depth_on_target: The average sequencing depth that mapped to the reference genome target region

[00840] (20) Average_sequencing_depth_near_target: The average sequencing depth that mapped to the reference genome flanking region

[00841] (21) Mismatch_rate_in_target_region: The percentage of mismatch reads in reference genome target region

[00842] (22) Mismatch_rate_in_all_effective_sequence: The percentage of mismatch reads in reference genome

[00843] (23) Base_covered_on_target: The coverage length of target region

[00844] (24) Coverage_of_target_region: The percentage of target region coveraged

[00845] (25) Base_covered_near_target: The coverage length of flanking region

[00846] (26) Coverage_of_flanking_region: The percentage of flanking region coveraged

[00847] (27) Fraction_of_target_covered_with_at_least_20x: The percentage of bases with depth >20X in target region

[00848] (28) Fraction_of_target_covered_with_at_least_10x: The percentage of bases with depth >10X in target region

[00849] (29) Fraction_of_target_covered_with_at_least_4x: The percentage of bases with depth >4X in target region

[00850] (30) Fraction_of_flanking_region_covered_with_at_least_20x: The percentage of bases with depth >20X in flanking region

[00851] (31) Fraction_of_flanking_region_covered_with_at_least_10x: The percentage of bases with depth >10X in flanking region

[00852] (32) Fraction_of_flanking_region_covered_with_at_least_4x: The percentage of bases with depth >4X in flanking region

[00853] (Source: Novogene Bioinformatics Technology Co., Ltd - www.filgen.ip/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf)

[00854] *Variation detection result*

[00855] *SNV detection result*

[00856] *SNV statistical result*

[00857] Generally, the whole genome of human has about 3.6M SNV. Most (above 95%) SNVs with high frequency (the allele frequency in population is above 5%) have records in dbSNP (Sherry S T, Ward M H, Kholodov M, et al. dbSNP: the NCBI database of genetic variationfj]. Nucleic acids research, 2001, 29(1): 308-3 11.(dbSNP)). The ration of Ts/Tv can reflect the accuracy of sequencing. Generally, the ratio in genome is about 2.2 and in coding region is about 3.2.

[00858] GATK was used to detect SNV, and the statistics of SNVs are as follows:

[00859] **Table 20** - The number of SNV in different genomic region

Sample	exonic	intronic	UTR3	UTR5	intergenic	ncRNA_exonic	ncRNA_intronic	upstream	downstream	splicing	ncRNA_UTR3	ncRNA_UTR5	ncRNA_splicing
SEV1114055	22693	108836	5125	3010	629300	2703	7604	4137	1766	2499	103	44	100
SEV1114029	22152	96990	4598	2716	49336	2425	6679	3335	1342	2438	109	47	90
SEV1114001	21959	107581	4943	2928	61021	2619	7373	3865	1674	2431	103	43	92
SEV1114025	22104	103872	4819	2855	59388	2490	7256	3721	1600	2457	94	46	79
SEV1114052	21397	98878	4689	2713	51860	2476	6558	3347	1462	2382	105	48	90
SEV1114026	21834	105544	4910	2943	59627	2576	7014	3867	1628	2417	107	35	84
SEV1114064	21987	94806	4651	2745	49648	2520	6280	3401	1437	2340	97	35	72
SEV1114046	21913	110289	5051	2900	67019	2473	7607	3834	1758	2400	111	59	73
SEV1114018	22132	106056	4890	2852	64134	2555	7639	3749	1621	2475	123	41	104
SEV1114013	22038	90748	4549	2652	44070	2419	5745	3054	1233	2491	106	42	92
SEV1114022	23040	91171	4521	2698	44429	2502	5940	3002	1349	2507	107	46	87
SEV1114036	21900	103186	4820	2839	61614	2609	7300	3630	1564	2427	100	43	87
SEV1114035	22105	96007	4666	2790	49280	2455	6280	3428	1477	2448	108	42	77
SEV1114020	22306	98238	4682	2902	52087	2621	6606	3514	1460	2436	113	44	101
SEV1114006	21961	92358	4428	2630	50616	2495	6364	3136	1503	2393	88	43	84
SEV1114019	22256	97165	4501	2662	56449	2494	6485	3359	1458	2443	98	41	91
SEV1114033	21903	100337	4680	2726	56302	2445	6731	3484	1538	2351	107	46	85
SEV1114038	19932	88855	3826	1478	55433	1984	6182	1913	1322	2197	83	18	79
SEV1114047	22493	104441	4784	2836	62484	2606	7244	3553	1709	2460	100	45	82

SEV11 14049	223 47	103 536	47 41	28 81	6194 1	2452	7110	3724	1665	239 7	102	50	86
SEV11 14056	220 15	927 77	46 62	27 43	4793 3	2498	6141	3273	1397	244 0	99	41	91
SEV11 14065	223 24	959 24	49 85	27 29	5280 0	2535	6526	3306	1458	241 5	133	43	85
SEV11 14067	221 92	117 546	51 70	29 29	7583 5	2611	8118	4029	1901	248 4	117	42	93
SEV11 14066	219 87	954 49	45 21	27 79	4679 6	2429	5993	3388	1281	238 8	111	45	77
SEV11 14017	225 04	974 98	53 57	27 52	5892 9	2584	6815	3291	1630	243 2	181	46	94
SEV11 14054	223 57	986 72	50 01	27 02	5837 9	2684	6810	3221	1464	241 6	118	35	84

[00860] Note:

[00861] Sample: Sample name

[00862] exonic: The number of SNV in exonic region

[00863] intronic: The number of SNV in intronic region

[00864] UTR3 : The number of SNV in 3'UTR region

[00865] UTR5: The number of SNV in 5'UTR region

[00866] intergnic: The number of SNV in intergenic region

[00867] ncRNA_exonic: The number of SNV in non-coding RNA exonic region

[00868] ncRNA_intronic: The number of SNV in non-coding RNA intronic region

[00869] upstream: The number of SNV in the Ikb upstream region of transcription start site

[00870] downstream: The number of SNV in the Ikb downstream region of transcription ending site

[00871] splicing: The number of SNV in 4bp splicing junction region

[00872] ncRNA_UTR3 : The number of SNV in 3'UTR of non-coding RNA

[00873] ncRNA_UTR5: The number of SNV in 5'UTR of non-coding RNA

[00874] ncRNA_splicing: The number of SNV in 4bp splicing junction of non-coding RNA

[00875] **Table 21** - The number of SNV of different types in coding region

Sample	synonymous_SNV	missense_SNV	stopgain	stoploss	unknown
SEV1 114055	11508	10703	87	12	383
SEV1 114029	11321	10376	71	9	375
SEV1 114001	11228	10281	74	9	367
SEV1 114025	11277	10402	77	13	335
SEV1 114052	11076	9883	74	12	352
SEV1 114026	11164	10185	64	10	411
SEV1 114064	11267	10285	67	11	357
SEV1 114046	11145	10315	78	12	363
SEV1 114018	11353	10329	73	14	363

SEV1 114013	11349	10251	70	14	354
SEV1 114022	11776	10774	84	8	398
SEV1 114036	11200	10259	76	8	357
SEV1 114035	11280	10358	72	7	388
SEV1 114020	11415	10422	70	9	390
SEV1 114006	11330	10205	71	11	344
SEV1 114019	11471	10294	67	12	412
SEV1 114033	11298	10185	64	10	346
SEV1 114038	10235	9282	70	10	335
SEV1 114047	11492	10555	76	10	360
SEV1 114049	11425	10451	81	12	378
SEV1 114056	11364	10207	70	10	364
SEV1 114065	11436	10387	75	12	414
SEV1 114067	11357	10378	76	8	373
SEV1 114066	11248	10272	77	15	375
SEV1 114017	11769	10275	79	11	370
SEV1 114054	11398	10524	69	15	351

[00876] Note:

[00877] Sample: Sample name

[00878] synonymous_SNV: A single nucleotide change that does not cause an amino acid change

[00879] missense_SNV: A single nucleotide change that cause an amino acid change

[00880] stopgain: A nonsynonymous SNV that lead to the immediate creation of stop codon at the variant site

[00881] stoploss: A nonsynonymous SNV that lead to the immediate elimination of stop codon at the variant site

[00882] unknown: Unknown function (due to various errors in the gene structure definition in the database file)

[00883] *InDel detection result*

[00884] *Indel statistical result*

[00885] See Section 4.4.2 of Novogene Bioinformatics Technology Co., Ltd's document entitled "Novogene - Cancer Project Report (2014, 11), which is accessible at www.filgen.jp/Product/Bioscience5-seq/Cancer WGS report Vl.l.pdf . Generally, the genome of human has about 350K InDel (insertion and deletion, less than 50bp insertion and deletion).

[00886] The InDel in coding region or splicing site may change the protein translation. Frameshift mutation, in which the number of inserted or deleted bases is not an integral multiple of three, may lead to the change of the whole reading frame. Compared to non-frameshift mutation, frameshift mutation is more limited by selective pressure.

[00887] GATK was used to detect Indel, and obtained InDel result is as follows:

[00888] **Table 22** - The number of InDel in different genomic regions

Sample	exonic	intronic	UTR3	UTR5	intergenic	ncRNA_exonic	ncRNA_intronic	upstream	downstream	splicing	ncRNA_UTR3	ncRNA_UTR5	ncRNA_splicing
SEV1114055	672	17873	794	492	10953	299	1306	780	288	558	9	9	13
SEV1114029	683	15858	733	434	9117	269	1156	601	223	557	13	11	23
SEV1114001	647	17626	813	459	10382	293	1235	685	282	528	20	9	11
SEV1114025	691	16993	792	499	10461	230	1274	672	266	527	12	13	14
SEV1114052	641	16193	724	435	9494	273	1111	612	258	525	14	13	14
SEV1114026	674	16902	815	502	10158	285	1153	689	275	520	10	13	12
SEV1114064	684	15395	712	433	8833	291	1039	621	237	516	14	11	13
SEV1114046	669	17948	816	475	11903	287	1307	700	302	537	11	14	20
SEV1114018	714	17488	764	465	11739	295	1318	636	289	523	15	9	19
SEV1114013	655	14728	702	437	8107	283	982	592	202	546	9	12	12
SEV1114022	701	14623	682	416	8114	270	1022	539	235	542	13	8	16
SEV1114036	710	17213	773	458	11080	280	1308	661	268	480	11	10	15
SEV1114035	694	15320	703	451	8775	276	1056	601	229	509	16	11	12
SEV1114020	688	16166	747	470	9501	305	1124	675	278	531	14	12	10
SEV1114006	681	14416	665	403	8968	265	1014	549	235	506	7	9	20
SEV1114019	632	15739	669	445	10080	288	1130	614	242	535	12	10	9
SEV1114033	646	14906	670	415	9164	262	1074	586	231	479	14	12	17
SEV1114038	525	14784	614	184	10648	216	1120	306	239	475	16	3	17
SEV1114047	667	16312	727	447	10515	293	1206	632	281	524	9	12	17
SEV1114049	653	16413	716	451	10636	274	1142	621	230	491	10	14	15
SEV1114056	642	14451	748	432	8209	277	1002	578	228	501	13	12	14
SEV1114065	680	14902	798	427	9262	253	1051	590	255	521	14	11	13
SEV1114067	662	18057	772	454	11955	276	1297	705	296	535	12	10	15
SEV1114066	654	14929	728	495	8035	266	969	618	225	531	10	14	10

SEV1114017	680	15847	932	432	10707	269	1167	565	297	530	38	14	15
SEV1114054	661	15406	766	440	10098	289	1121	561	247	495	11	7	11

[00889] Note:

[00890] Sample: Sample name

[00891] exonic: The number of InDel in exonic region

[00892] intronic: The number of InDel in intronic region

[00893] UTR3 : The number of InDel in 3'UTR region

[00894] UTR5: The number of InDel in 5'UTR region

[00895] intergnic: The number of InDel in intergenic region

[00896] ncRNA_exonic: The number of InDel in non-coding RNA exonic region

[00897] ncRNA_intronic: The number of InDel in non-coding RNA intronic region

[00898] upstream: The number of InDel in the lkb upstream region of transcription start site

[00899] downstream: The number of InDel in the lkb downstream region of transcription ending site

[00900] splicing: The number of InDel in 4bp splicing junction region

[00901] ncRNA_UTR3 : The number of InDel in 3'UTR of non-coding RNA

[00902] ncRNA_UTR5 : The number of InDel in 5'UTR of non-coding RNA

[00903] ncRNA_splicing: The number of InDel in 4bp splicing junction of non-coding RNA

[00904] **Table 23** - The number of different type InDel in coding regions

Sample	frameshift_deletion	frameshift_insertion	nonframeshift_deletion	nonframeshift_insertion	stoploss	stopgain	unknown
SEV1114055	106	104	203	158	1	6	94
SEV1114029	131	101	196	155	1	7	92
SEV1114001	110	90	199	152	0	5	91
SEV1114025	123	100	203	168	0	11	86
SEV1114052	107	90	190	156	1	7	90
SEV1114026	119	84	204	169	2	5	91
SEV1114064	135	100	183	170	0	6	90
SEV1114046	117	94	192	168	0	10	88
SEV1114018	135	105	201	174	0	9	90
SEV1114013	123	87	192	157	1	6	89
SEV1114	134	95	200	176	1	6	89

022							
SEV1114 036	125	110	207	166	0	10	91
SEV1114 035	134	99	189	172	0	9	91
SEV1114 020	116	107	206	157	1	9	92
SEV1114 006	120	103	200	157	1	10	90
SEV1114 019	102	86	174	175	0	4	91
SEV1114 033	113	84	193	161	1	7	87
SEV1114 038	99	90	139	113	0	7	76
SEV1114 047	126	89	196	158	0	6	92
SEV1114 049	109	93	200	157	1	5	88
SEV1114 056	114	89	183	160	1	7	87
SEV1114 065	124	94	192	169	0	7	94
SEV1114 067	115	88	196	166	0	7	90
SEV1114 066	112	89	199	161	1	6	86
SEV1114 017	119	97	200	167	1	7	89
SEV1114 054	123	85	187	168	1	7	89

[00905] Note:

[00906] Sample: Sample name

[00907] frameshift_deletion: A deletion of one or more nucleotides that cause frameshift changes in protein coding sequence. the deletion length is not multiple of 3

[00908] frameshift_insertion: An insertion of one or more nucleotides that cause frameshift changes in protein coding sequence.the insertion length is not multiple of 3

[00909] nonframeshift_deletion: Non-frame shift deletion, does not change coding protein frame deletion, the deletion length is multiple of 3

[00910] nonframeshift_insertion: Non-frame shift insertion, does not change coding protein frame insertion: the insertion length is multiple of 3

[00911] stopgain: Frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that lead to the immediate creation of stop codon at the variant site

[00912] stoploss: Frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that lead to the immediate elimination of stop codon at the variant site

[00913] unknown: Unknown function (due to various errors in the gene structure definition in the database file)

[009 14] **Table 24** - InDel and genotype distribution

Sample	all	genotype.Het	genotype.Hom	novel	novel_proportion
SEV1114055	34046	11817	22229	7444	0.218645362
SEV1114029	29678	10651	19027	6444	0.217130534
SEV1114001	32990	11595	21395	7354	0.222916035
SEV1114025	32444	11106	21338	7158	0.220626310
SEV1114052	30307	10586	19721	6403	0.211271323
SEV1114026	32008	10399	21609	6806	0.212634341
SEV1114064	28799	9604	19195	6237	0.216570020
SEV1114046	34989	11712	23277	7625	0.217925634
SEV1114018	34274	11635	22639	7404	0.216023808
SEV1114013	27267	9951	17316	5958	0.218505886
SEV1114022	27181	10092	17089	5743	0.211287296
SEV1114036	33267	11523	21744	7358	0.221180148
SEV1114035	28653	10361	18292	6293	0.219627962
SEV1114020	30521	10454	20067	6567	0.215163330
SEV1114006	27738	8998	18740	5688	0.205061648
SEV1114019	30405	9881	20524	6407	0.210721921
SEV1114033	28476	9479	18997	5693	0.199922742
SEV1114038	29147	8841	20306	6029	0.206848046
SEV1114047	31642	10710	20932	6516	0.205928829
SEV1114049	31666	10356	21310	6603	0.208520179
SEV1114056	27107	9451	17656	5797	0.213856200
SEV1114065	28777	9653	19124	6094	0.211766341
SEV1114067	35046	12015	23031	7438	0.212235348
SEV1114066	27484	9915	17569	5826	0.211977878
SEV1114017	31493	10055	21438	7195	0.228463468
SEV1114054	30113	9919	20194	6486	0.215388703

[00915] Note:

[00916] Sample: Sample name

[009 17] all: The total number of InDel

[00918] genotype.Het: The genotype of heterozygote

[009 19] genotype.Hom: The genotype of homozygote

[00920] novel: InDel not in dbSNP

[00921] novel_proportion: Is calculated as novel Indel/total number of Indel

[00922] *Software usedfor analysis*

[00923] The softwares which were applied in the bioinformatic analysis are listed as below:

[00924] **Table 25** - The list of exome analysis software

Analytical content	Software	Comments	Version

Quality control	in-house	Quality control	1.0
Alignment	BWA	Map the sequencing reads to the reference genome and the BAM file was obtained	0.7.8-r455
	SAMtools	Sort bam	1.0
	Picard	Merge the bam file from the same sample and mark the duplicate reads	1.111
SNP/INDEL detection	GATK	Detect and filter SNP, InDel	v3.1
Functional annotation	ANNOVAR	Annotate variation site	2013Aug23

[00925] Analysis was conducted using association software such as PLINK.

[00926] *Statistical analysis*

[00927] The PLINK v1.0721 whole genome analysis tool set was used to determine associations between the CFS patients and the non-fatigued control group. A two column χ^2 test was used to determine significance where p value of <0.05 was determined to be significant. Data analysis was performed by the Australian Genome Research Facility.

[00928] **Results**

[00929] Exome sequencing identified SNP variants in the TRP channel and AChR genes TRPVl, TRPC6, TRPV4, TRPCl, TRPM8, TRPC4, TRPV2, TRPV5, TRPC5, TRPM6, TRPC7, TRPM5, TRPC3, PKD1, TRPV6, PKD2, TRPA1, TRPM7, TRPM2, TRPM4, TRPM3, TRPV3, and CHRNA7, CHRM3, CHRNA4, CHRNA3, CHRNB4, CHRNB2 and CHRNE. These, together with their annotated consequences, are described in Tables 26, 27 and 28 below

Table 26: SNP variants of TRP channel or ACh receptors (TRP family, TRPVl, TRPC6, TRPV4, TRPCl, TRPM8, TRPC4, TRPV2, TRPV5, TRPC5, TRPM6, TRPC7, TRPM5, TRPC3, PKD1, TRPV6, PKD2, TRPA1, TRPM7, TRPM2, TRPM4, TRPM3, TRPV3, and CHRNA7, CHRM3, CHRNA4, CHRNA3, CHRNB4, CHRNB2 and CHRNE) annotated with their consequences.

Chromosome	Location	Reference allele	Alternative allele	Consequences (intronic or exonic)
15	32322929	G	A	ExonicFunc=synonymous_SNV
1	240070784	T	C	ExonicFunc=synonymous_SNV
1	240070944	G	A	ExonicFunc=missense_SNV
20	61981104	C	T	ExonicFunc=synonymous_SNV
20	61981134	G	A	ExonicFunc=synonymous_SNV
20	61981253	C	T	ExonicFunc=missense_SNV
20	61981362	G	A	ExonicFunc=synonymous_SNV
20	61981411	G	A	ExonicFunc=missense_SNV
20	61981536	A	G	ExonicFunc=synonymous_SNV

20	61981554	C	A	ExonicFunc=synonymous_SNV
20	61982085	A	G	ExonicFunc=synonymous_SNV
20	61982124	A	G	ExonicFunc=synonymous_SNV
20	61990939	G	A	ExonicFunc=synonymous_SNV
20	61992467	C	T	ExonicFunc=synonymous_SNV
20	61992509	T	C	ExonicFunc=synonymous_SNV
15	78880752	G	A	ExonicFunc=missense_SNV
15	78882925	G	A	ExonicFunc=missense_SNV
15	78885574	T	A	ExonicFunc=missense_SNV
15	78894339	G	A	ExonicFunc=synonymous_SNV
15	78894357	G	T	ExonicFunc=synonymous_SNV
15	78909452	T	C	ExonicFunc=synonymous_SNV
15	78911181	T	C	ExonicFunc=synonymous_SNV
15	78911230	C	T	ExonicFunc=missense_SNV
15	78913131	G	A	ExonicFunc=synonymous_SNV
15	78917399	A	G	ExonicFunc=synonymous_SNV
15	78921762	G	A	ExonicFunc=synonymous_SNV
15	78922194	G	A	ExonicFunc=synonymous_SNV
15	78922229	T	C	ExonicFunc=missense_SNV
15	78922240	C	T	ExonicFunc=missense_SNV
15	78923505	G	A	ExonicFunc=missense_SNV
17	4796274	T	C	ExonicFunc=missense_SNV
17	4796286	C	T	ExonicFunc=missense_SNV
17	4797305	G	A	ExonicFunc=missense_SNV
17	4797910	G	A	ExonicFunc=missense_SNV
17	4802317	T	C	ExonicFunc=synonymous_SNV
17	4802329	G	A	ExonicFunc=synonymous_SNV
17	4802829	G	A	ExonicFunc=synonymous_SNV
17	4803711	G	A	ExonicFunc=stopgain
17	4804902	G	A	ExonicFunc=synonymous_SNV
17	4805777	C	G	ExonicFunc=missense_SNV
17	4806052	C	A	ExonicFunc=missense_SNV
17	3475490	C	T	ExonicFunc=synonymous_SNV
17	3476990	G	A	ExonicFunc=synonymous_SNV
17	3480433	G	C	ExonicFunc=missense_SNV
17	3480447	T	C	ExonicFunc=missense_SNV
17	3480910	A	G	ExonicFunc=synonymous_SNV
17	3486702	G	A	ExonicFunc=missense_SNV
17	3493200	C	G	ExonicFunc=missense_SNV
17	3494361	G	A	ExonicFunc=synonymous_SNV
17	3495374	G	A	ExonicFunc=missense_SNV
17	3495465	C	T	ExonicFunc=synonymous_SNV
11	101323770	C	T	ExonicFunc=synonymous_SNV
11	101325788	G	A	ExonicFunc=synonymous_SNV
11	101342958	G	A	ExonicFunc=synonymous_SNV
11	101347093	A	G	ExonicFunc=synonymous_SNV

11	101359750	G	A	ExonicFunc=missense_SNV
11	101454192	G	A	ExonicFunc=missense_SNV
12	110222146	C	G	ExonicFunc=synonymous_SNV
12	110226379	G	A	ExonicFunc=synonymous_SNV
12	110230597	C	T	ExonicFunc=missense_SNV
12	110238481	G	A	ExonicFunc=synonymous_SNV
12	110238487	A	G	ExonicFunc=synonymous_SNV
12	110240838	T	G	ExonicFunc=synonymous_SNV
12	110240848	G	A	ExonicFunc=synonymous_SNV
12	110252547	G	A	ExonicFunc=missense_SNV
3	142443441	G	A	ExonicFunc=missense_SNV
3	142503605	G	A	ExonicFunc=synonymous_SNV
3	142523349	G	A	ExonicFunc=synonymous_SNV
3	142524858	G	A	ExonicFunc=synonymous_SNV
2	234854540	G	C	ExonicFunc=missense_SNV
2	234854547	A	T	ExonicFunc=synonymous_SNV
2	234854550	G	C	ExonicFunc=synonymous_SNV
2	234854552	A	G	ExonicFunc=missense_SNV
2	234858645	C	T	ExonicFunc=missense_SNV
2	234863788	G	A	ExonicFunc=missense_SNV
2	234875354	G	A	ExonicFunc=synonymous_SNV
2	234905078	C	T	ExonicFunc=synonymous_SNV
2	234915540	C	G	ExonicFunc=synonymous_SNV
13	38211105	T	C	ExonicFunc=missense_SNV
13	38211313	T	C	ExonicFunc=synonymous_SNV
13	38237564	A	G	ExonicFunc=synonymous_SNV
13	38357384	G	A	ExonicFunc=synonymous_SNV
20	33585437	C	T	ExonicFunc=synonymous_SNV
20	33586193	C	T	ExonicFunc=synonymous_SNV
20	33587198	G	C	ExonicFunc=missense_SNV
20	33587596	G	A	ExonicFunc=synonymous_SNV
20	33589107	G	A	ExonicFunc=synonymous_SNV
20	33657126	G	A	ExonicFunc=synonymous_SNV
20	33665969	C	T	ExonicFunc=synonymous_SNV
17	16320994	C	T	ExonicFunc=synonymous_SNV
17	16321032	G	C	ExonicFunc=missense_SNV
17	16325968	A	G	ExonicFunc=synonymous_SNV
17	16326005	A	C	ExonicFunc=synonymous_SNV
17	16326990	C	G	ExonicFunc=missense_SNV
17	16336992	C	G	ExonicFunc=synonymous_SNV
7	142609749	C	T	ExonicFunc=missense_SNV
7	142622714	G	A	ExonicFunc=synonymous_SNV
7	142625249	T	C	ExonicFunc=synonymous_SNV
7	142625258	G	A	ExonicFunc=synonymous_SNV
7	142625882	G	A	ExonicFunc=synonymous_SNV
7	142625933	G	A	ExonicFunc=synonymous_SNV

7	142626549	C	T	ExonicFunc=missense_SNV
7	142626656	C	T	ExonicFunc=synonymous_SNV
7	142630534	G	A	ExonicFunc=missense_SNV
X	111078236	G	C	ExonicFunc=synonymous_SNV
9	77376633	A	G	ExonicFunc=synonymous_SNV
9	77376647	T	C	ExonicFunc=missense_SNV
9	77376652	A	C	ExonicFunc=missense_SNV
9	77377410	C	T	ExonicFunc=missense_SNV
9	77407636	C	T	ExonicFunc=synonymous_SNV
9	77415284	A	C	ExonicFunc=synonymous_SNV
9	77416972	C	T	ExonicFunc=synonymous_SNV
9	77436641	G	A	ExonicFunc=synonymous_SNV
9	77448950	A	G	ExonicFunc=synonymous_SNV
9	77502160	G	A	ExonicFunc=missense_SNV
5	135692575	G	A	ExonicFunc=synonymous_SNV
5	135692743	C	A	ExonicFunc=synonymous_SNV
11	2423913	A	C	ExonicFunc=missense_SNV
11	2424105	A	G	ExonicFunc=missense_SNV
11	2424541	C	G	ExonicFunc=missense_SNV
11	2424684	A	C	ExonicFunc=missense_SNV
11	2427291	A	C	ExonicFunc=synonymous_SNV
11	2432666	C	T	ExonicFunc=missense_SNV
11	2432964	T	C	ExonicFunc=synonymous_SNV
11	2434402	C	T	ExonicFunc=synonymous_SNV
11	2435946	A	G	ExonicFunc=synonymous_SNV
11	2435956	C	T	ExonicFunc=missense_SNV
11	2436464	C	T	ExonicFunc=missense_SNV
11	2438963	C	A	ExonicFunc=missense_SNV
11	2439542	A	G	ExonicFunc=missense_SNV
11	2439767	T	C	ExonicFunc=missense_SNV
11	2442364	G	A	ExonicFunc=synonymous_SNV
11	2444188	C	T	ExonicFunc=missense_SNV
4	122800987	T	C	ExonicFunc=synonymous_SNV
4	122824052	C	T	ExonicFunc=synonymous_SNV
4	122854116	G	C	ExonicFunc=synonymous_SNV
4	122872719	G	A	ExonicFunc=synonymous_SNV
7	47835027	A	G	ExonicFunc=missense_SNV
7	47840310	C	G	ExonicFunc=missense_SNV
7	47840387	C	T	ExonicFunc=missense_SNV
7	47851578	G	A	ExonicFunc=missense_SNV
7	47851623	C	T	ExonicFunc=missense_SNV
7	47852837	C	T	ExonicFunc=missense_SNV
7	47854956	C	T	ExonicFunc=synonymous_SNV
7	47869038	T	C	ExonicFunc=synonymous_SNV
7	47872845	A	G	ExonicFunc=synonymous_SNV
7	47874630	G	A	ExonicFunc=missense_SNV

7	47876567	G	A	ExonicFunc=synonymous_SNV
7	47879049	G	A	ExonicFunc=missense_SNV
7	47892745	A	G	ExonicFunc=missense_SNV
7	47913560	G	T	ExonicFunc=missense_SNV
7	47913579	T	C	ExonicFunc=missense_SNV
7	47913580	G	A	ExonicFunc=synonymous_SNV
7	47917087	C	T	ExonicFunc=synonymous_SNV
7	47917126	T	C	ExonicFunc=synonymous_SNV
7	47920345	G	A	ExonicFunc=synonymous_SNV
7	47921682	A	T	ExonicFunc=synonymous_SNV
7	47925331	C	G	ExonicFunc=missense_SNV
7	47927744	C	T	ExonicFunc=missense_SNV
7	47930148	C	T	ExonicFunc=synonymous_SNV
7	47930280	C	T	ExonicFunc=synonymous_SNV
7	47968927	C	A	ExonicFunc=missense_SNV
7	47970707	G	A	ExonicFunc=missense_SNV
7	47971575	A	G	ExonicFunc=synonymous_SNV
7	47971626	G	A	ExonicFunc=synonymous_SNV
16	2138269	T	C	ExonicFunc=synonymous_SNV
16	2138584	G	C	ExonicFunc=synonymous_SNV
16	2139814	G	A	ExonicFunc=missense_SNV
16	2139935	G	A	ExonicFunc=synonymous_SNV
16	2140010	A	G	ExonicFunc=synonymous_SNV
16	2140321	G	A	ExonicFunc=synonymous_SNV
16	2140454	T	C	ExonicFunc=synonymous_SNV
16	2140554	G	A	ExonicFunc=missense_SNV
16	2140680	T	C	ExonicFunc=missense_SNV
16	2140912	G	C	ExonicFunc=synonymous_SNV
16	2141454	G	A	ExonicFunc=synonymous_SNV
16	2144176	G	A	ExonicFunc=missense_SNV
16	2144182	G	A	ExonicFunc=missense_SNV
16	2147421	C	T	ExonicFunc=missense_SNV
16	2152387	A	G	ExonicFunc=missense_SNV
16	2152388	C	G	ExonicFunc=synonymous_SNV
16	2156021	A	G	ExonicFunc=synonymous_SNV
16	2158871	C	A	ExonicFunc=synonymous_SNV
16	2159405	C	T	ExonicFunc=synonymous_SNV
16	2159522	C	T	ExonicFunc=synonymous_SNV
16	2159750	G	A	ExonicFunc=synonymous_SNV
16	2159996	G	A	ExonicFunc=synonymous_SNV
16	2160494	C	T	ExonicFunc=synonymous_SNV
16	2160503	T	G	ExonicFunc=synonymous_SNV
16	2160973	A	G	ExonicFunc=missense_SNV
16	2161113	C	T	ExonicFunc=missense_SNV
16	2161150	G	A	ExonicFunc=missense_SNV
16	2161489	C	A	ExonicFunc=missense_SNV

16	2161793	G	A	ExonicFunc=synonymous_SNV
16	2161796	G	A	ExonicFunc=synonymous_SNV
16	2162955	A	G	ExonicFunc=missense_SNV
16	2164808	C	T	ExonicFunc=missense_SNV
16	2167970	G	A	ExonicFunc=synonymous_SNV
16	71967886	G	A	ExonicFunc=unknown
16	71967927	C	T	ExonicFunc=unknown
16	71983772	G	C	ExonicFunc=unknown
16	71986946	A	G	ExonicFunc=unknown
16	71988106	C	T	ExonicFunc=unknown
16	72001110	G	A	ExonicFunc=unknown
16	72001136	G	A	ExonicFunc=unknown
16	72003952	G	C	ExonicFunc=unknown
16	72007232	G	A	ExonicFunc=unknown
16	72007399	C	T	ExonicFunc=unknown
16	72011162	G	C	ExonicFunc=unknown
16	72011181	G	T	ExonicFunc=unknown
16	72011193	A	C	ExonicFunc=unknown
16	72011261	A	G	ExonicFunc=unknown
16	72012239	C	G	ExonicFunc=unknown
16	72013797	G	C	ExonicFunc=unknown
16	72020134	T	C	ExonicFunc=unknown
16	72020294	G	A	ExonicFunc=unknown
16	72020323	A	G	ExonicFunc=unknown
16	72027191	T	A	ExonicFunc=unknown
16	72032221	G	A	ExonicFunc=unknown
16	72032231	T	A	ExonicFunc=unknown
16	72033801	G	T	ExonicFunc=unknown
16	81129822	G	A	ExonicFunc=missense_SNV
16	81134860	C	G	ExonicFunc=unknown
16	81142257	T	C	ExonicFunc=unknown
16	81145807	C	G	ExonicFunc=unknown
16	81145976	C	T	ExonicFunc=unknown
16	81151122	A	C	ExonicFunc=unknown
16	81151123	C	G	ExonicFunc=unknown
16	81157324	G	A	ExonicFunc=unknown
16	81157353	G	A	ExonicFunc=unknown
16	81157385	G	T	ExonicFunc=unknown
16	81161552	C	T	ExonicFunc=unknown
16	81161569	T	G	ExonicFunc=unknown
16	81161571	T	C	ExonicFunc=unknown
16	81161578	G	A	ExonicFunc=unknown
16	81161608	T	C	ExonicFunc=unknown
16	81161635	C	A	ExonicFunc=unknown
16	81173136	T	C	ExonicFunc=unknown
16	81173193	C	T	ExonicFunc=unknown

16	81174978	A	G	ExonicFunc=unknown
16	81174992	G	T	ExonicFunc=unknown
16	81174999	A	G	ExonicFunc=unknown
16	81175103	G	A	ExonicFunc=unknown
16	81180988	C	G	ExonicFunc=unknown
16	81180995	T	C	ExonicFunc=unknown
16	81181066	G	A	ExonicFunc=unknown
16	81181097	G	T	ExonicFunc=unknown
16	81181783	T	C	ExonicFunc=unknown
16	81181821	T	C	ExonicFunc=unknown
16	81181869	T	C	ExonicFunc=unknown
16	81183325	T	A	ExonicFunc=unknown
16	81183492	T	G	ExonicFunc=unknown
16	81185412	C	T	ExonicFunc=unknown
16	81185416	A	G	ExonicFunc=unknown
16	81185419	G	C	ExonicFunc=unknown
16	81187685	G	A	ExonicFunc=unknown
16	81190598	T	C	ExonicFunc=unknown
16	81190601	T	C,A	ExonicFunc=unknown
16	81190613	A	G	ExonicFunc=unknown
16	81193321	C	T	ExonicFunc=unknown
16	81193358	C	G	ExonicFunc=unknown
16	81194382	T	C,A	ExonicFunc=unknown
16	81197218	G	A	ExonicFunc=unknown
16	81198306	C	A	ExonicFunc=unknown
16	81199468	G	C	ExonicFunc=unknown
16	81199520	T	C	ExonicFunc=unknown
16	81199538	T	C	ExonicFunc=unknown
16	81199544	G	A	ExonicFunc=unknown
16	81199554	C	T	ExonicFunc=unknown
16	81199555	A	G	ExonicFunc=unknown
16	81201620	C	A	ExonicFunc=unknown
16	81201625	G	A	ExonicFunc=unknown
16	81204396	G	A	ExonicFunc=synonymous_SNV
16	81204635	G	C	ExonicFunc=synonymous_SNV
16	81208515	G	A	ExonicFunc=missense_SNV
16	81209234	C	T	ExonicFunc=synonymous_SNV
16	81211496	C	A	ExonicFunc=missense_SNV
16	81211548	G	A	ExonicFunc=synonymous_SNV
16	81211587	T	C	ExonicFunc=synonymous_SNV
16	81213378	A	G	ExonicFunc=missense_SNV
16	81213381	A	C	ExonicFunc=missense_SNV
16	81219187	C	T	ExonicFunc=missense_SNV
16	81232275	G	A	ExonicFunc=missense_SNV
16	81232294	T	C	ExonicFunc=missense_SNV
16	81232336	T	C	ExonicFunc=missense_SNV

16	81232564	T	G	ExonicFunc=missense_SNV
16	81241098	C	T	ExonicFunc=synonymous_SNV
16	81241100	G	C	ExonicFunc=missense_SNV
16	81242102	G	A	ExonicFunc=missense_SNV
16	81242107	T	C	ExonicFunc=missense_SNV
16	81242151	T	C	ExonicFunc=synonymous_SNV
16	81242194	T	C	ExonicFunc=missense_SNV
16	81242198	G	A	ExonicFunc=stopgain
16	81248716	C	T	ExonicFunc=missense_SNV
16	81248745	A	G	ExonicFunc=missense_SNV
16	81249927	C	T	ExonicFunc=missense_SNV
16	81249954	T	A	ExonicFunc=missense_SNV
16	81253745	C	G	ExonicFunc=missense_SNV
16	81253759	A	G	ExonicFunc=missense_SNV
16	81253917	A	G	ExonicFunc=missense_SNV
7	142565385	G	A	ExonicFunc=synonymous_SNV
7	142565776	G	A	ExonicFunc=synonymous_SNV
7	142568070	G	A	ExonicFunc=missense_SNV
7	142569556	A	G	ExonicFunc=synonymous_SNV
7	142569596	A	G	ExonicFunc=missense_SNV
7	142569701	C	T	ExonicFunc=missense_SNV
7	142570142	T	C	ExonicFunc=synonymous_SNV
7	142570217	C	T	ExonicFunc=synonymous_SNV
7	142572304	G	A	ExonicFunc=synonymous_SNV
7	142572908	T	C	ExonicFunc=missense_SNV
7	142573263	C	T	ExonicFunc=synonymous_SNV
7	142573614	G	A	ExonicFunc=missense_SNV
7	142573644	A	T	ExonicFunc=missense_SNV
7	142574913	A	G	ExonicFunc=missense_SNV
4	88928968	G	C	ExonicFunc=missense_SNV
4	88929305	G	A	ExonicFunc=synonymous_SNV
4	88929453	G	A	ExonicFunc=missense_SNV
4	88964586	C	T	ExonicFunc=synonymous_SNV
5	137244517	G	A	ExonicFunc=missense_SNV
5	137259179	T	C	ExonicFunc=missense_SNV
5	137278682	T	C	ExonicFunc=missense_SNV
10	102046380	T	G	ExonicFunc=missense_SNV
10	102048208	G	T	ExonicFunc=missense_SNV
10	102050242	C	A	ExonicFunc=missense_SNV
10	102056745	C	T	ExonicFunc=missense_SNV
10	102089663	C	T	ExonicFunc=missense_SNV
8	72936145	T	C	ExonicFunc=missense_SNV
8	72948588	C	T	ExonicFunc=synonymous_SNV
8	72951118	T	C	ExonicFunc=synonymous_SNV
8	72964965	G	A	ExonicFunc=synonymous_SNV
8	72966002	G	A	ExonicFunc=synonymous_SNV

8	72975801	T	G	ExonicFunc=missense_SNV
8	72977703	C	T	ExonicFunc=missense_SNV
8	72981318	G	A	ExonicFunc=synonymous_SNV
8	72981327	A	G	ExonicFunc=synonymous_SNV
8	72984041	C	G	ExonicFunc=missense_SNV
8	72987638	G	A	ExonicFunc=missense_SNV
15	50867082	G	A	ExonicFunc=synonymous_SNV
15	50867142	C	T	ExonicFunc=synonymous_SNV
15	50878630	G	A	ExonicFunc=missense_SNV
15	50888568	A	G	ExonicFunc=synonymous_SNV
15	50897114	A	G	ExonicFunc=synonymous_SNV
21	45811343	T	G	ExonicFunc=missense_SNV
21	45820196	C	T	ExonicFunc=missense_SNV
21	45825799	C	T	ExonicFunc=missense_SNV
21	45833864	C	T	ExonicFunc=missense_SNV
21	45844751	A	G	ExonicFunc=missense_SNV
21	45855100	G	T	ExonicFunc=missense_SNV
19	49657613	G	T	ExonicFunc=missense_SNV
19	49658084	G	A	ExonicFunc=synonymous_SNV
19	49658209	A	C	ExonicFunc=missense_SNV
19	49658367	C	T	ExonicFunc=missense_SNV
19	49658390	T	C	ExonicFunc=synonymous_SNV
19	49671214	A	G	ExonicFunc=missense_SNV
19	49671281	G	A	ExonicFunc=synonymous_SNV
19	49675017	G	T	ExonicFunc=synonymous_SNV
19	49699866	C	T	ExonicFunc=synonymous_SNV
9	73150873	T	G	ExonicFunc=missense_SNV
9	73150918	C	T	ExonicFunc=missense_SNV
9	73150984	C	T	ExonicFunc=missense_SNV
9	73151715	C	T	ExonicFunc=synonymous_SNV
9	73151970	C	T	ExonicFunc=synonymous_SNV
9	73240431	T	G	ExonicFunc=synonymous_SNV
9	73255554	G	A	ExonicFunc=synonymous_SNV
9	73461337	T	A	ExonicFunc=synonymous_SNV
17	3417253	A	G	ExonicFunc=synonymous_SNV
17	3422032	G	A	ExonicFunc=synonymous_SNV
17	3422073	C	T	ExonicFunc=missense_SNV
17	3422077	G	A	ExonicFunc=synonymous_SNV
17	3436080	C	T	ExonicFunc=synonymous_SNV
17	3436209	T	C	ExonicFunc=synonymous_SNV
17	3445901	T	G	ExonicFunc=synonymous_SNV
17	3446885	T	C	ExonicFunc=missense_SNV
17	3447914	C	T	ExonicFunc=synonymous_SNV
17	3458072	T	C	ExonicFunc=missense_SNV

Table 27: Frequency distribution and significance of Transient Receptor Potential (TRP) SNPs in CFS/ME patients (n=14) and non-fatigued controls (n=11) from isolated B cells in rank order of significance.

Chr	Position	A1	F_A	F_U	A2	CHISQ	P	OR	ExonicFunc	Gene
4	122,872,719	G	0.1	0.45	A	6.144	0.01318	0.1358	synonymous_SNV	Gene=NM_001130698
16	81,253,759	A	0.0625	0.3333	G	3.8	0.05124	0.1333	missense_SNV	Gene=NM_001076780,NM_052892
16	81,253,917	A	0.0625	0.3333	G	3.8	0.05124	0.1333	missense_SNV	Gene=NM_001076780,NM_052892
9	73,151,715	C	0.2308	0.04545	T	3.285	0.0699	6.3	synonymous_SNV	Gene=NM_001007471,NM_020952,NM_024971, NM_206944,NM_206945,NM_206946,NM_206947
11	2,439,542	A	0.3846	0.15	G	3.069	0.07979	3.542	missense_SNV	Gene=NM_014555
11	2,435,946	A	0.2917	0.1111	G	1.992	0.1582	3.294	synonymous_SNV	Gene=NM_014555
17	3,493,200	C	0.3077	0.1364	G	1.98	0.1594	2.815	missense_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
12	110,226,379	G	0.5	0	A	1.714	0.1904	NA	synonymous_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204
17	16,325,968	A	0.25	0.5	G	1.699	0.1924	0.3333	synonymous_SNV	Gene=NM_016113
17	16,326,005	A	0.25	0.5	C	1.699	0.1924	0.3333	synonymous_SNV	Gene=NM_016113
7	47,913,579	T	0.2308	0.09091	C	1.678	0.1951	3	missense_SNV	Gene=NM_138295
16	81,249,954	T	0.125	0.3125	A	1.646	0.1995	0.3143	missense_SNV	Gene=NM_001076780,NM_052892
17	3,446,885	T	0.5	0.2857	C	1.429	0.232	2.5	missense_SNV	Gene=NM_001258205,NM_145068
8	72,975,801	T	0.3636	0.2	G	1.375	0.241	2.286	missense_SNV	Gene=NM_007332
8	72,981,327	A	0.3636	0.2	G	1.375	0.241	2.286	synonymous_SNV	Gene=NM_007332
9	73,150,873	T	0.5	0.25	G	1.25	0.2636	3	missense_SNV	Gene=NM_001007471,NM_020952,NM_024971,NM_206944, NM_206945,NM_206946,NM_206947
9	73,150,918	C	0.5	0.25	T	1.25	0.2636	3	missense_SNV	Gene=NM_001007471,NM_020952,NM_024971,NM_206944, NM_206945,NM_206946,NM_206947
8	72,966,002	G	0.3889	0.2143	A	1.117	0.2905	2.333	synonymous_SNV	Gene=NM_007332
16	81,241,098	C	0.08333	0.1818	T	0.9816	0.3218	0.4091	synonymous_SNV	Gene=NM_001076780,NM_052892
16	81,242,194	T	0.0833	0.1818	C	0.9816	0.3218	0.409	missense_SNV	Gene=NM_001076780,NM_052892

			3						1		
17	16,336,992	C	0.3125	0.5	G	0.9141	0.339	0.454 5	synonymous_SNV	Gene=NM_016113
17	3,480,447	T	0.35	0.5	C	0.765	0.3818	0.538 5	missense_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
11	101,347,093	A	0.5	0.3	G	0.7481	0.3871	2.333	synonymous_SNV	Gene=NM_004621
17	3,422,032	G	0.25	0.3889	A	0.7466	0.3876	0.523 8	synonymous_SNV	Gene=NM_001258205,NM_145068
11	2,432,964	T	0.2778	0.4	C	0.6288	0.4278	0.576 9	synonymous_SNV	Gene=NM_014555
15	50,878,630	G	0.25	0.5	A	0.625	0.4292	0.333 3	missense_SNV	Gene=NM_017672
16	2,160,973	A	0.25	0.5	G	0.5333	0.4652	0.333 3	missense_SNV	Gene=NM_000296,NM_001009944
17	3,436,080	C	0.4375	0.3125	T	0.5333	0.4652	1.711	synonymous_SNV	Gene=NM_001258205,NM_145068
17	3,494,361	G	0.25	0.5	A	0.5333	0.4652	0.333 3	synonymous_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
7	47,872,845	A	0.2083	0.3	G	0.4889	0.4844	0.614	synonymous_SNV	Gene=NM_138295
11	2,427,291	A	0.1667	0.25	C	0.4656	0.495	0.6	synonymous_SNV	Gene=NM_014555
16	81,211,496	C	0.1667	0.3333	A	0.4444	0.505	0.4	missense_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	81,211,548	G	0.1667	0.3333	A	0.4444	0.505	0.4	synonymous_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
3	142,443,441	G	0.25	0.5	A	0.375	0.5403	0.333 3	missense_SNV	Gene=NM_001251845,NM_003304
7	47,913,580	G	0.2	0.3333	A	0.3556	0.551	0.5	synonymous_SNV	Gene=NM_138295
7	47,920,345	G	0.2	0.3333	A	0.3556	0.551	0.5	synonymous_SNV	Gene=NM_138295
8	72,987,638	G	0.3333	0.5	A	0.3429	0.5582	0.5	missense_SNV	Gene=NM_007332
15	50,888,568	A	0.3333	0.5	G	0.3429	0.5582	0.5	synonymous_SNV	Gene=NM_017672
17	3,486,702	G	0.375	0.5	A	0.3429	0.5582	0.6	missense_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
16	81,232,275	G	0.0833 3	0.1364	A	0.3332	0.5638	0.575 8	missense_SNV	Gene=NM_001076780,NM_052892
16	81,232,564	T	0.0833 3	0.1364	G	0.3332	0.5638	0.575 8	missense_SNV	Gene=NM_001076780,NM_052892
12	110,240,838	T	0.3	0.3889	G	0.3326	0.5641	0.673 5	synonymous_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204

17	3,422,077	G	0.375	0.5	A	0.3208	0.5711	0.6	synonymous_SNV	Gene=NM_001258205,NM_145068
5	137,244,517	G	0.25	0.1818	A	0.3136	0.5755	1.5	missense_SNV	Gene=NM_001258448,NM_014386
3	142,523,349	G	0.375	0.25	A	0.2909	0.5896	1.8	synonymous_SNV	Gene=NM_001251845,NM_003304
3	142,524,858	G	0.375	0.25	A	0.2909	0.5896	1.8	synonymous_SNV	Gene=NM_001251845,NM_003304
16	81,208,515	G	0.4	0.3	A	0.2871	0.5921	1.556	missense_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
9	77,415,284	A	0.3889	0.5	C	0.2801	0.5966	0.6364	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
2	234,854,550	G	0.375	0.5	C	0.254	0.6143	0.6	synonymous_SNV	Gene=NM_024080
16	81,242,102	G	0.3182	0.25	A	0.2386	0.6252	1.4	missense_SNV	Gene=NM_001076780,NM_052892
9	77,502,160	G	0.3889	0.3	A	0.2212	0.6381	1.485	missense_SNV	Gene=NM_001177311
2	234,905,078	C	0.5	0.4	T	0.22	0.639	1.5	synonymous_SNV	Gene=NM_024080
3	142,503,605	G	0.4	0.3	A	0.2198	0.6392	1.556	synonymous_SNV	Gene=NM_001251845,NM_003304
7	47,876,567	G	0.5	0.3333	A	0.1778	0.6733	2	synonymous_SNV	Gene=NM_138295
9	73,150,984	C	0.2222	0.1667	T	0.1773	0.6737	1.429	missense_SNV	Gene=NM_001007471,NM_020952,NM_024971,NM_206944,NM_206945,NM_206946,NM_206947
2	234,915,540	C	0.375	0.5	G	0.1714	0.6788	0.6	synonymous_SNV	Gene=NM_024080
11	2,438,963	C	0.375	0.5	A	0.1714	0.6788	0.6	missense_SNV	Gene=NM_014555
4	88,928,968	G	0.25	0.3333	C	0.1587	0.6903	0.6667	missense_SNV	Gene=NM_000297
7	47,925,331	C	0.35	0.2857	G	0.1555	0.6933	1.346	missense_SNV	Gene=NM_138295
8	72,936,145	T	0.5	0.4	C	0.1524	0.6963	1.5	missense_SNV	Gene=NM_007332
9	73,151,970	C	0.2222	0.2778	T	0.1481	0.7003	0.7429	synonymous_SNV	Gene=NM_001007471,NM_020952,NM_024971,NM_206944,NM_206945,NM_206946,NM_206947
9	77,436,641	G	0.4	0.3333	A	0.1422	0.7061	1.333	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
11	2,439,767	T	0.4286	0.5	C	0.1327	0.7157	0.75	missense_SNV	Gene=NM_014555
4	122,854,116	G	0.5	0.4	C	0.1167	0.7327	1.5	synonymous_SNV	Gene=NM_001130698,NM_003305
16	81,242,198	G	0.4167	0.5	A	0.1125	0.7373	0.7143	stopgain	Gene=NM_001076780,NM_052892
9	73,461,337	T	0.3333	0.2857	A	0.0928	0.7607	1.25	synonymous_SNV	Gene=NM_001007470,NM_001007471,NM_020952,NM_024971,NM_206944,NM_206945,NM_206946,NM_206947,NM_206948
17	3,445,901	T	0.2692	0.3125	G	0.09087	0.7631	0.8105	synonymous_SNV	Gene=NM_001258205,NM_145068
16	81,241,100	G	0.3182	0.2778	C	0.07696	0.7815	1.213	missense_SNV	Gene=NM_001076780,NM_052892

16	81,242,151	T	0.3182	0.2778	C	0.07696	0.7815	1.213	synonymous_SNV	Gene=NM_001076780,NM_052892
7	47,971,575	A	0.2727	0.3125	G	0.07124	0.7895	0.825	synonymous_SNV	Gene=NM_138295
16	81,253,745	C	0.2857	0.3333	G	0.06878	0.7931	0.8	missense_SNV	Gene=NM_001076780,NM_052892
16	2,140,010	A	0.375	0.4286	G	0.06044	0.8058	0.8	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,160,503	T	0.375	0.4286	G	0.06044	0.8058	0.8	synonymous_SNV	Gene=NM_000296,NM_001009944
15	50,867,082	G	0.3182	0.3571	A	0.05844	0.809	0.84	synonymous_SNV	Gene=NM_017672
4	122,824,052	C	0.4	0.4375	T	0.05143	0.8206	0.857 1	synonymous_SNV	Gene=NM_001130698,NM_003305
16	81,213,378	A	0.0833 3	0.1	G	0.03667	0.8481	0.818 2	missense_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	2,159,996	G	0.375	0.4167	A	0.03472	0.8522	0.84	synonymous_SNV	Gene=NM_000296,NM_001009944
16	81,248,716	C	0.3333	0.3	T	0.02794	0.8673	1.167	missense_SNV	Gene=NM_001076780,NM_052892
11	2,435,956	C	0.4444	0.4167	T	0.02262	0.8804	1.12	missense_SNV	Gene=NM_014555
17	3,447,914	C	0.2308	0.25	T	0.02019	0.887	0.9	synonymous_SNV	Gene=NM_001258205,NM_145068
16	2,140,454	T	0.4	0.4286	C	0.01959	0.8887	0.888 9	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,140,680	T	0.4	0.4286	C	0.01959	0.8887	0.888 9	missense_SNV	Gene=NM_000296,NM_001009944
7	47,921,682	A	0.375	0.4	T	0.01625	0.8986	0.9	synonymous_SNV	Gene=NM_138295
16	81,248,745	A	0.3571	0.3333	G	0.01618	0.8988	1.111	missense_SNV	Gene=NM_001076780,NM_052892
7	142,626,549	C	0.3571	0.375	T	0.00701 5	0.9332	0.925 9	missense_SNV	Gene=NM_019841
7	47,968,927	C	0.3182	0.3125	A	0.00138 4	0.9703	1.027	missense_SNV	Gene=NM_138295
2	234,854,540	G	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_024080
2	234,854,552	A	0.5	0.5	G	0	1	1	missense_SNV	Gene=NM_024080
2	234,863,788	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_024080
4	88,929,305	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_000297
5	135,692,575	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_001167576,NM_001167577,NM_020389
7	47,840,387	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_138295
7	47,851,623	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_138295
7	47,852,837	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_138295
7	47,854,956	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_138295

7	47,869,038	T	0.5	0.5	C	0	1	1	synonymous_SNV	Gene=NM_138295
7	47,874,630	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_138295
7	47,879,049	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_138295
7	47,913,560	G	0.3333	0.3333	T	0	1	1	missense_SNV	Gene=NM_138295
7	47,917,087	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_138295
7	47,927,744	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_138295
7	47,930,148	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_138295
7	47,971,626	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_138295
7	142,569,596	A	0.5	0.5	G	0	1	1	missense_SNV	Gene=NM_018646
7	142,570,142	T	0.5	0.5	C	0	1	1	synonymous_SNV	Gene=NM_018646
7	142,572,304	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_018646
7	142,572,908	T	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_018646
7	142,573,263	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_018646
7	142,574,913	A	0.5	0.5	G	0	1	1	missense_SNV	Gene=NM_018646
7	142,622,714	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_019841
7	142,625,249	T	0.5	0.5	C	0	1	1	synonymous_SNV	Gene=NM_019841
7	142,625,258	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_019841
7	142,625,882	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_019841
7	142,625,933	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_019841
7	142,626,656	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_019841
8	72,977,703	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_007332
8	72,981,318	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_007332
8	72,984,041	C	0.5	0.5	G	0	1	1	missense_SNV	Gene=NM_007332
9	73,240,431	T	0.5	0.5	G	0	1	1	synonymous_SNV	Gene=NM_024971,NM_206945
9	77,376,633	A	0.5	0.5	G	0	1	1	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
9	77,376,647	T	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_001177310,NM_001177311,NM_017662
9	77,377,410	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_001177310,NM_001177311,NM_017662
9	77,407,636	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
9	77,416,972	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
10	102,048,208	G	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_001253837,NM_016112

10	102,050,242	C	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_001253837,NM_016112
10	102,056,745	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_001253837,NM_016112
10	102,089,663	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_001253837,NM_016112
11	2,432,666	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_014555
11	101,323,770	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_004621
11	101,359,750	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_004621
12	110,238,487	A	0.5	0.5	G	0	1	1	synonymous_SNV	Gene=NM_001177431,NM_021625,NM_147204
13	38,211,105	T	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_001135955,NM_001135956,NM_001135957, NM_001135958,NM_003306,NM_016179
13	38,357,384	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_001135955,NM_001135956,NM_001135957, NM_001135958,NM_003306,NM_016179
16	2,140,321	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,140,554	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_000296,NM_001009944
16	2,144,176	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_000296,NM_001009944
16	2,144,182	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_000296,NM_001009944
16	2,159,405	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_000296,NM_001009944
16	81,219,187	C	0.3333	0.3333	T	0	1	1	missense_SNV	Gene=NM_001076780,NM_052892
16	81,232,336	T	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_001076780,NM_052892
16	81,249,927	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_001076780,NM_052892
17	3,475,490	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	3,476,990	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	3,480,910	A	0.5	0.5	G	0	1	1	synonymous_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	3,495,374	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	16,321,032	G	0.5	0.5	C	0	1	1	missense_SNV	Gene=NM_016113
19	49,671,281	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_001195227,NM_017636
19	49,675,017	G	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_001195227,NM_017636
19	49,699,866	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_017636
20	33,657,126	G	0.25	0.25	A	0	1	1	synonymous_SNV	Gene=NM_015638,NM_199368
20	33,665,969	C	0.5	0.5	T	0	1	1	synonymous_SNV	Gene=NM_015638,NM_199368
21	45,811,343	T	0.5	0.5	G	0	1	1	missense_SNV	Gene=NM_003307
21	45,820,196	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_003307

2	234,854,547	A	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_024080
2	234,858,645	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_024080
2	234,875,354	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_024080
4	88,929,453	0	NA	0	A	NA	NA	NA	missense_SNV	Gene=NM_000297
4	88,964,586	C	NA	0.5	T	NA	NA	NA	synonymous_SNV	Gene=NM_000297
4	122,800,987	T	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_001130698,NM_003305
5	135,692,743	C	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_001167576,NM_001167577,NM_020389
5	137,259,179	0	0	0	C	NA	NA	NA	missense_SNV	Gene=NM_001258448,NM_001258449,NM_014386
7	47,840,310	C	0.5	NA	G	NA	NA	NA	missense_SNV	Gene=NM_138295
7	47,851,578	G	0.5	NA	A	NA	NA	NA	missense_SNV	Gene=NM_138295
7	47,892,745	A	0.5	NA	G	NA	NA	NA	missense_SNV	Gene=NM_138295
7	47,917,126	T	NA	0.5	C	NA	NA	NA	synonymous_SNV	Gene=NM_138295
7	47,930,280	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_138295
7	47,970,707	G	0.5	NA	A	NA	NA	NA	missense_SNV	Gene=NM_138295
7	142,569,556	A	0.5	NA	G	NA	NA	NA	synonymous_SNV	Gene=NM_018646
7	142,569,701	C	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_018646
7	142,570,217	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_018646
7	142,573,614	G	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_018646
7	142,573,644	A	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_018646
7	142,609,749	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_019841
7	142,630,534	G	0.5	NA	A	NA	NA	NA	missense_SNV	Gene=NM_019841
8	72,948,588	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_007332
8	72,951,118	T	NA	0.5	C	NA	NA	NA	synonymous_SNV	Gene=NM_007332
8	72,964,965	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_007332
9	73,255,554	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_001007471,NM_020952,NM_024971,NM_206944, NM_206945,NM_206946,NM_206947
9	77,376,652	A	NA	0.5	C	NA	NA	NA	missense_SNV	Gene=NM_001177310,NM_001177311,NM_017662
9	77,448,950	A	0.5	NA	G	NA	NA	NA	synonymous_SNV	Gene=NM_001177310,NM_001177311,NM_017662
11	2,434,402	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_014555
11	2,436,464	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_014555

11	2,442,364	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_014555
11	2,444,188	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_014555
11	101,325,788	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_004621
11	101,342,958	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_004621
11	101,454,192	G	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_004621
12	110,222,146	C	0.5	NA	G	NA	NA	NA	synonymous_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204
12	110,230,597	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204
12	110,238,481	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_001177431,NM_021625,NM_147204
12	110,240,848	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204
12	110,252,547	G	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_001177428,NM_001177431,NM_001177433, NM_021625,NM_147204
13	38,211,313	T	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_001135955,NM_001135956,NM_001135957, NM_001135958,NM_003306,NM_016179
13	38,237,564	A	NA	0.5	G	NA	NA	NA	synonymous_SNV	Gene=NM_001135955,NM_001135956,NM_001135957, NM_001135958,NM_003306,NM_016179
15	50,867,142	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_017672
15	50,897,114	A	0.5	NA	G	NA	NA	NA	synonymous_SNV	Gene=NM_017672
16	2,139,814	G	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,139,935	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,140,912	G	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,141,454	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,152,387	0	NA	0	G	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,152,388	0	NA	0	G	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,156,021	A	0.5	NA	G	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,158,871	C	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,159,522	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,159,750	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,160,494	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,161,113	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,161,150	G	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944

16	2,161,489	C	NA	0.5	A	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,161,793	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,161,796	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	2,162,955	0	0	NA	G	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,164,808	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_000296,NM_001009944
16	2,167,970	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000296,NM_001009944
16	81,204,396	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_001076780,NM_001278423
16	81,204,635	G	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	81,209,234	C	NA	0.5	T	NA	NA	NA	synonymous_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	81,211,587	T	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	81,213,381	A	NA	0.5	C	NA	NA	NA	missense_SNV	Gene=NM_001076780,NM_001278423,NM_001278425,NM_052892
16	81,232,294	T	0.5	NA	C	NA	NA	NA	missense_SNV	Gene=NM_001076780,NM_052892
16	81,242,107	T	0.5	NA	C	NA	NA	NA	missense_SNV	Gene=NM_001076780,NM_052892
17	3,417,253	A	NA	0.5	G	NA	NA	NA	synonymous_SNV	Gene=NM_001258205,NM_145068
17	3,422,073	C	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_001258205,NM_145068
17	3,436,209	T	NA	0.5	C	NA	NA	NA	synonymous_SNV	Gene=NM_001258205,NM_145068
17	3,458,072	0	0	0	C	NA	NA	NA	missense_SNV	Gene=NM_001258205,NM_145068
17	3,480,433	G	NA	0.5	C	NA	NA	NA	missense_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	3,495,465	C	NA	0.5	T	NA	NA	NA	synonymous_SNV	Gene=NM_018727,NM_080704,NM_080705,NM_080706
17	16,320,994	C	NA	0.5	T	NA	NA	NA	synonymous_SNV	Gene=NM_016113
17	16,326,990	C	0.5	NA	G	NA	NA	NA	missense_SNV	Gene=NM_016113
19	49,671,214	A	NA	0.5	G	NA	NA	NA	missense_SNV	Gene=NM_001195227,NM_017636
21	45,825,799	C	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_003307
21	45,833,864	C	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_003307
21	45,844,751	0	0	0	G	NA	NA	NA	missense_SNV	Gene=NM_003307
21	45,855,100	G	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_003307

Table 28: Frequency distribution and significance of AChR SNPs in CFS/ME (n=14) patients and non-fatigued controls (n=l 1) from isolated B cells in rank order of significance.

Chr	Position	A1	F_A	F_U	A2	CHISQ	P	OR	ExonicFunc	Gene
20	61981554	C	0.04167	0.1818	A	2.327	0.1271	0.1957	synonymous_SNV	Gene=NM_000744,NM_001256573
20	61982124	A	0.04167	0.1818	G	2.327	0.1271	0.1957	synonymous_SNV	Gene=NM_000744,NM_001256573
20	61981134	G	0.2727	0.4	A	0.7636	0.3822	0.5625	synonymous_SNV	Gene=NM_000744,NM_001256573
15	78909452	T	0.2083	0.3182	C	0.7183	0.3967	0.5639	synonymous_SNV	Gene=NM_000743,NM_001166694
20	61981104	C	0.2917	0.4	T	0.5698	0.4503	0.6176	synonymous_SNV	Gene=NM_000744,NM_001256573
15	78894339	G	0.3889	0.3	A	0.2212	0.6381	1.485	synonymous_SNV	Gene=NM_000743,NM_001166694
15	78911181	T	0.4	0.3333	C	0.181	0.6706	1.333	synonymous_SNV	Gene=NM_000743,NM_001166694
20	61982085	A	0.07692	0.09091	G	0.03051	0.8613	0.8333	synonymous_SNV	Gene=NM_000744,NM_001256573
20	61981536	A	0.03846	0.04545	G	0.01459	0.9038	0.84	synonymous_SNV	Gene=NM_000744,NM_001256573
15	78917399	A	0.2083	0.2222	G	0.01178	0.9136	0.9211	synonymous_SNV	Gene=NM_001256567
15	32322929	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_001190455
15	78911230	C	0.5	0.5	T	0	1	1	missense_SNV	Gene=NM_000743,NM_001166694
15	78923505	G	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_000750,NM_001256567
17	4802329	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_000080
17	4806052	C	0.5	0.5	A	0	1	1	missense_SNV	Gene=NM_000080
20	61990939	G	0.5	0.5	A	0	1	1	synonymous_SNV	Gene=NM_000744
1	240070784	T	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_000740
1	240070944	G	0.5	NA	A	NA	NA	NA	missense_SNV	Gene=NM_000740
15	78894357	G	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_000743,NM_001166694
15	78913131	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000743,NM_001166694
15	78921762	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000750
15	78922194	0	0	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000750
15	78922229	T	NA	0.5	C	NA	NA	NA	missense_SNV	Gene=NM_000750
15	78922240	C	0.5	NA	T	NA	NA	NA	missense_SNV	Gene=NM_000750
17	4802317	T	NA	0.5	C	NA	NA	NA	synonymous_SNV	Gene=NM_000080
17	4802829	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000080

17	4804902	G	NA	0.5	A	NA	NA	NA	synonymous_SNV	Gene=NM_000080
17	4805777	C	NA	0.5	G	NA	NA	NA	missense_SNV	Gene=NM_000080
20	61981253	C	NA	0.5	T	NA	NA	NA	missense_SNV	Gene=NM_000744,NM_001256573
20	61981362	G	0.5	NA	A	NA	NA	NA	synonymous_SNV	Gene=NM_000744,NM_001256573
20	61981411	G	0.5	NA	A	NA	NA	NA	missense_SNV	Gene=NM_000744,NM_001256573
20	61992467	C	0.5	NA	T	NA	NA	NA	synonymous_SNV	Gene=NM_000744
20	61992509	T	0.5	NA	C	NA	NA	NA	synonymous_SNV	Gene=NM_000744

[00930] Further information on these SNPs can be found at http://www.ncbi.nlm.nih.gov/projects/SNP/ .

[00931] The SNPs and genotypes of Tables 26, 27 and 28 are consistent with those identified for isolated B lymphocytes in Example 6.

[00932] PLINK analysis highlighted missense, synonomous and genes for SNPs of the TRP families and PKD1L2.

[00933] 81,253,759 and 81,253,917 SNPs were identified as missense SNPs for the exon sequence for PKDlL2:NM_001076780:exonl:c.T217C:p.W73R and PKDlL2:NM_001076780:exonl:c.T59C:p.V20A. SNP 122,872,719, a TRPC3 receptor, was also found to be significantly associated with CSF/ME patients compared to controls from isolated B cells.

[00934] These finds are significant as TRPC3 has shown a direct association with PKCbeta that is required for downstream activation in B cells [43y]. Addtionally, TRPP subunits can be divided into two subcategories depending on structural similarity. The first group, polycystic kidney disease 1 (PKDl)-like, contains polycystin-1 (Previously known as TRPP1), PKDREJ, PKD1L1, PKD1L2, and PKD1L3.

[00935] **Example 10 - ERK1/2, MEK1/2 and p38 Downstream Signalling Molecules Impaired in CD56^{dim}CD16^{+} and CD56^{bright}CD16$^{-/-}$ Natural Killer Cells in Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis Patients**

[00936] Natural Killer (NK) cells are innate immune cells which comprise approximately 10-15% of lymphocytes circulating in the peripheral blood [lm]. Two predominant NK cell phenotypes identified by the surface expression of cluster of differentiation (CD) 56 and CD16 and an absence of CD3 provide host immunity through the production of immunoregulatory cytokines and the cytotoxic lysis of target cells [2m-4m].

[00937] Ten percent of peripheral NK cells are CD56^{bright}CD16$^{dim/-}$ NK cells which constitutively express receptors for monocyte derived cytokines (monokines) [5m, 6m]. Monokine receptor ligation rapidly stimulates CD56^{bright}CD16$^{dim/-}$ NK cells to produce cytokines including interferon gamma (IFN-γ), tumour necrosis factor alpha and beta (TNF-α and β), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10 and IL-13 [5m, 6m]. CD56^{bright}CD16$^{dim/-}$ NK cell cytokine production provides an early source of cytokines which augments NK cell cytotoxic activity and regulates the function of other lymphocytes [2m, 5m, 4m]. Approximately 90% of peripheral NK cells are cytotoxic CD56^{dim}CD16^{+} NK cells [7m, 5m]. Cytotoxic NK cells contain high numbers of secretory granules which constitutively

express apoptotic inducing lytic proteins perforin, Granzyme A and Granzyme B [3m, 8m].

[00938] Following $CD56^{dim}CD16^{+}$ NK cell recognition of a target cell, the lytic proteins are released by a process known as degranulation to induce cytotoxic lysis and subsequent removal of target cells infected with viruses, bacteria or cells which have been malignantly transformed [9m, 10m].

[00939] Unlike T and B lymphocytes, the effector function of NK cells is governed by a myriad of surface receptors which integrate activating or inhibiting signals into intracellular signalling cascades [11m- 13m]. After NK cell receptor ligation, intracellular activation signals are propagated through protein phosphorylation cascades by mitogen-activated protein kinases (MAPKs) [14m- 16m]. Three main subgroups of MAPKs include extracellular signal-regulated kinases (ERK) 1/2, p38 MAPK (p38) and the c-Jun N-terminal kinase (JNK) [14m- 16m]. In response to extra cellular stimuli, the MAPK signalling pathways transduce signals to specific intracellular targets to mediate cellular responses including gene expression, mitosis, motility, cell survival, apoptosis and differentiation [17m]. Within the NK cells, phosphorylation of MEK1/2 and p38 regulate cytokine production and ERK1/2 phosphorylation polarises the secretory granule towards the immune synapse for degranulation [16m, 18m]. In addition to MAPK signalling for normal cellular responses, impairments in MAPK signalling have been suggested to contribute to the pathology of disease processes relating to leukaemia, diabetes, Alzheimer's and Parkinson's disease, atherosclerosis, arthritis and airway inflammation [19m-25m].

[00940] Longitudinal reports of significantly reduced NK cell cytotoxic activity in Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis (CFS/ME) patients suggests the presence of an NK cell functional deficiency which may contribute to the illness pathogenesis [26m-34m]. Current investigations into NK cell phenotypes, receptors and lytic proteins in CFS/ME have reported equivocal findings and importantly, intracellular signalling by MAPKs in NK cells remains to be examined [27m, 35m, 36m]. Therefore, the purpose of the present study was to investigate NK cell phosphorylation of the MAPK signalling cascade, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK) 1/2 as well as p38, cytotoxic activity, degranulation, lytic proteins and cytokine production in $CD56^{dim}CD16^{+}$ and $CD56^{bright}CDi6^{dim/-}$ NK ceUs from CFS/ME patients.

[00941] **Methods**

[00942] *Participant recruitment and inclusion criteria*

[00943] CFS/ME patients and non-fatigued controls (NFC) were recruited from a participant database at the National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland. All participants completed an online questionnaire based on the 1994

Fukuda definition for fatigue and symptom presentation to determine suitability for study inclusion [37m]. From the questionnaire responses, CFS/ME patients meeting the 1994 Fukuda definition and NFC were included. All participants were screened for exclusionary conditions such as epilepsy, thyroid conditions, psychosis, diabetes, cardiac disorders, smoking, pregnant or breastfeeding and immunological, inflammatory or autoimmune diseases.

[00944] *Blood collection and cell isolation*

[00945] Forty millilitres of sodium heparin blood was collected by venepuncture from the antecubital vein of each participant. To avoid the influence of circadian variation, all blood samples were collected in the morning between 7:30- 10am. Laboratory analysis commenced within four hours of blood collection to maintain cell viability. Routine blood parameters including a full blood count, erythrocyte sedimentation rate, electrolytes and high sensitivity C-reactive protein were assessed on each participant sample by Queensland Pathology. The whole blood samples were diluted with unsupplemented Roswell Park Memorial Institute medium (RPMI) 1640 media (Life Technologies, Carlsbad, USA) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Hypaque (GE Health Care, Uppsala, UP).

[00946] *NK cell MAPK phosphorylation*

[00947] Phosphorylation of signalling proteins in the MAPK pathway including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, was examined under two stimulatory conditions using phospho-specific antibodies as previously described [38m-40m]. Following isolation, the PBMCs in RPMI 1640 media supplemented with ten percent FBS were incubated for a minimum of two hours at 37°C with five percent CO_2 to reduce background phosphorylation. After resting, the PBMCs were stained with mAbs for CD56-APC (Miltenyi Biotech, Cologne, BG) or CD56-phycoerythrin-cyanine (PE-Cy)7, CD16- brilliant violet (BV)711 and CD3-BV510 (BD Biosciences, San Diego, USA) for 25 minutes and subsequently washed. The PBMCs were stimulated with either K562 cells (E:T of 25:1) or PMA (50ng/ml) plus ionomycin (I, 0.5μg/ml) as a positive control for 15 minutes in a water bath at 37°C. A parallel sample of unstimulated (US) cells in RPMI media alone was used to determine basal levels of phosphorylation. BD Phosflow fix buffer 1 (San Diego, USA) containing 4.2% formaldehyde was pre-warmed to 37°C and added to the PBMCs, incubated for ten minutes at 37°C and subsequently washed off. The cells were then incubated in BD perm/wash buffer 1 (San Diego, USA) containing FBS and saponin for ten minutes which was followed by staining with phosospecific mAbs including signal transducer and activator of transcription (Stat)-3 (pS727)- alexa fluor (AF) 488, MEK1 (pS218)/MEK2 (pS222)-AF488, p38 (pT180/pY182)-PerCP Cy5.5, ERK1/2 (pT202/pY204)-BV421, nuclear factor kappa beta (NF-

κβ, pS529)-AF488, inhibitory kappa beta (Iicp)-AF647, PKCα (pT497)-AF488 and JNK (pT183/pY185)-AF647 for 30 minutes and subsequent flow cytometry analysis.

[00948] *NK cell cytotoxic activity*

[00949] Flow cytometry was used to measure NK cell cytotoxic activity against the human chronic myelogenous leukaemia K562 cell line as previously described [41m, 29m]. Briefly, K562 cells (Sigma-Aldrich, St Louis, USA) were cultured in RPMI 1640 media (Life Technologies, Carlsbad, USA) supplemented with ten percent fetal bovine serum (FBS) (Life Technologies, Carlsbad, USA). Following isolation, the PBMCs were stained with Paul Karl Horan-26 fluorescent cell linker dye (Sigma-Aldrich, St Louis, USA) and washed with RPMI supplemented with ten percent FBS. The concentrations of the PBMCs and K562 cells were adjusted to 2.5xl0 ^{6}cells/ml and lxl0 ^{5}cells/ml respectively and combined at three effector to target (E:T) ratios including 25:1, 12.5:1 and 6.25:1. A control sample of only K562 cells was also included to determine K562 cells undergoing apoptosis not induced by NK cell cytotoxic activity. The PBMCs and K562 cells were incubated for four hours at 37°C with five percent CO_2 and then stained with fluorescein isothiocyanate (FITC) annexin V and 7-aminoactinomycin (Becton Dickinson [BD] Pharminogen, San Diego, USA) for flow cytometric analysis on a BD Calibur (BD Biosciences, San Diego, USA) dual laser four colour flow cytometer. NK cytotoxic activity was calculated as percent specific death of the K562 cells for the three E:T ratios as previously described [41m].

[00950] *NK cell degranulation*

[00951] NK cell surface expression of CD107a and CD107b was measured as a marker for NK cell degranulation as previously reported [9m]. PBMCs in the presence of mAbs for CD107a-PE and CD107b-FITC (BD Biosciences, San Diego, USA) were stimulated with either K562 cells (E:T of 25: 1) or PMA (50ng/ml) plus ionomycin (0.5ug/ml) for one hour at 37°C with five percent CO_2. Monensin (BD Biosciences, San Diego, USA) was added to the PBMCs and the cells were then incubated for an additional three hours. An unstimulated control sample included PBMCs incubated in only RPMI 1640 media. Post four hours incubation, the cells were washed and incubated with mAbs against CD56-APC, CD16-BV711 and CD3-BV510 (BD Biosciences, San Diego, USA) for 25 minutes which was followed by flow cytometric analysis.

[00952] *NK cell lytic proteins and maturation marker*

[00953] Intracellular staining was used to measure the lytic proteins perforin, granzyme A and granzyme B contained within the secretory granules of NK cells [27m, 42m]. Surface expression of CD57 was measured as a marker for NK cell maturation [43m]. The PBMCs were incubated with mAbs for CD56-PE-Cy7, CD16-BV711, CD3-BV510 and CD57-PE-cyanin-based fluorescent dye (CF)594 for 25 minutes. The PBMCs were then permeabilised with BD

fixation/permeabilisation solution for 20 minutes, washed in BD perm/wash buffer and then incubated with mAbs including perforin-APC (Miltenyi Biotec, Cologne, BG), granzyme A-FITC and granzyme B-V450 (BD Biosciences, San Diego, USA) for 30 minutes which was followed by flow cytometric analysis.

[00954] *NK cell cytokines*

[00955] NK cell production of the cytokines IFN-γ, TNF-α and GM-CSF was determined by intracellular staining under two stimulatory conditions as described previously [9m, 44m]. After isolation, PBMCs were incubated in the presence of either K562 cells (E:T of 25:1) or phorbol-12-myristate-13-acetate (PMA, 50ng/ml) (Sigma-Aldrich, St Louis, USA) plus ionomycin (I, 0.5pg/ml) (Sigma-Aldrich, St Louis, USA) for one hour at 37°C with five percent **CO2.** Brefeldin A (BD Biosciences, San Diego, USA) was added to prevent cytokine secretion during stimulation and the cells were incubated for an additional five hours [9m, 44m]. PBMCs incubated in RPMI 1640 media alone served as the unstimulated control sample. Following six hours incubation, the PBMCs were washed and incubated with monoclonal antibodies (mAbs) for CD56-PE-Cy7, CD16-BV711 and CD3-BV510 (BD Biosciences, San Diego, USA) for 25 minutes. The PBMCs were subsequently washed, incubated in BD fixation/permeabilisation solution (BD Biosciences, San Diego, USA) for 20 minutes, washed in BD perm/wash buffer (BD Biosciences, San Diego, USA) and then incubated for 30 minutes with mAbs against IFN-γ-allophycocyanin (APC), TNF-a- peridinin chlorophyll protein-cyanine (PerCP-Cy)-5.5 (BD Biosciences, San Diego, USA) and GM-CSF-PE (Biolegend, San Diego, USA) for flow cytometric detection of intracellular cytokines.

[00956] *Multiparametric flow cytometry analysis*

[00957] Data were collected on a 14-parameter LSR-Fortessa X20 flow cytometer (BD Biosciences, San Diego, USA). Cell signalling technology beads (BD Biosciences, San Diego, USA) were run on a daily basis to ensure optimal flow cytometry performance and application settings were employed to standardise target values for the duration of the experiments. A total of 2500 to 5000 CD56 positive events were acquired. Data generated for NK cell cytokines, degranulation, lytic proteins and cell maturation was analysed on FlowJo (version 10.0.8) and phosphorylation data were analysed on Cytobank (version 5.0) [45m]. NK cell analysis was performed on cells which fell within the lymphocyte population according to forward and side scatter properties. $CD56^{+}CD3^{-}$ NK cells were gated to determine total NK cells which was extrapolated to a plot of CD56 and CD16 to identify $CD56^{brjgh_t}CD16^{dim/-}$ and $CD56^{dim}CD16^{*}$NK cells for the analysis of each marker for cytokines, degranulation, phosphorylation, lytic proteins and cell maturation. A combination of appropriate fluorescence minus one controls, isotype controls matched to antibody concentrations and unstimulated samples were used to determine

NK cell gating for each analysis.

[00958] *Statistical Analysis*

[00959] Statistical analysis of the data was performed on the Statistical Package for the Social Sciences (version 22) and GraphPad Prism (version 6). All data sets were tested for normality using the Shapiro-Wilk test. The independent Mann-Whitney U-test was used to identify any significant differences in the NK cell parameters between the CFS/ME and NFC groups. A Kraskal-Wallis multiple comparisons test was used to identify significant differences in NK cell parameters before and after stimulation within the CFS/ME and NFC cohorts. Significance was set at $p<0.05$ and the data is presented as median ± interquartile range unless otherwise stated.

[00960] *Abbreviations*

[00961] APC: allophycocyanin, AF: alexa fluor, BD: Becton Dickinson, BV: brilliant violet, CD: cluster of differentiation, CF: cyanin-based fluorescent dye, ERK: extracellular signal-regulated kinases, E:T: effector to target, FBS: fetal bovine serum, FITC: fluorescein isothiocyanate, GM-CSF: granulocyte-macrophage colony-stimulating factor, Iκβ: inhibitory kappa beta, I: ionomycin , IFN-γ: interferon gamma, IL: interleukin, JNK: Jun N-terminal kinase, mABs: monoclonal antibodies, MAPK: mitogen-activated protein kinase, MFI: median fluorescence intensity, NK: Natural killer, NFC: non-fatigued control, NF-κβ: nuclear factor kappa beta, PBMCs: peripheral, blood mononuclear cells, PE: phycoerythrin, PE-Cy: phycoerythrin-cyanine, PerCP-Cy: peridinin chlorophyll protein-cyanine, PMA: phorbol-12-myristate-13-acetate, p38: p38 mitogen-activated protein kinase, RPMI: Roswell Park Memorial Institute, Stat: signal transducer and activator of transcription, TNF: tumour necrosis factor, US: unstimulated.

[00962] **Results**

[00963] *Participant inclusion, blood parameters and NK cell phenotypes*

[00964] 14 CFS/ME patients meeting the 1994 Fukuda definition (mean age [years] ± standard error of the mean (SEM) = 53.5 ± 2.17) and 11 NFC (mean age [years] ± SEM = 48.82 ± 3.46) were included in this study. Comparison of the group ages and blood parameters including erythrocyte sedimentation rate, high sensitivity C-reactive protein and full blood counts of white and red blood cells between CFS/ME and the NFC revealed no significant differences (Table 29). Total NK cells were compared according to two phenotype populations, which were $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ - between CFS/ME and NFC cohorts and no significant differences were observed (See Figure 5).

[00965] **Table 29:** CFS/ME and NFC blood parameters.

		CFS/ME (n= 14)	NFC (n=11)	P value
	ESR (mm/Hr)	7.85 ± 0.77	8.45 ± 1.44	0.700
	High sensitivity C-reactive protein (mg/L)	0.99 ± 0.30	0.91 ± 0.41	0.873
White and red blood cells	White blood cells (10^9/L)	5.16 ± 0.38	5.26 ± 0.41	0.860
	Lymphocytes (10^9/L)	1.67 ± 0.15	1.67 ± 0.13	1.000
	Monocytes (10^9/L)	0.32 ± 0.03	0.27 ± 0.03	0.258
	Neutrophils (10^9/L)	2.96 ± 0.24	3.15 ± 0.28	0.610
	Eosinophils (10^9/L)	0.17 ± 0.03	0.15 ± 0.03	0.647
	Basophils (10^9/L)	0.03 ± 0.001	0.03 ± 0.001	1.000
	Platelets (10^9/L)	238.54 ± 15.50	248.00 ± 18.01	0.693
	Red blood cells (10^{12}/L)	4.55 ± 0.12	4.61 ± 0.15	0.755
	Haemoglobin (g/L)	138.85 ± 3.82	138.82 ± 4.26	0.996
	Haematocrit	0.42 ± 0.01	0.41 ± 0.01	0.493
	Mean cell volume (fL)	91.62 ± 0.94	89.27 ± 0.93	0.094
Electrolytes	Sodium (mmol/L)	137.92 ± 0.46	137.09 ± 0.53	0.249
	Potassium (mmol/L)	4.10 ± 0.10	4.16 ± 0.12	0.702
	Chloride (mmol/L)	100.69 ± 0.61	101.64 ± 0.65	0.301
	Bicarbonate (mmol/L)	28.62 ± 0.63	27.27 ± 0.45	0.112
	Anion gap (mmol/L)	8.54 ± 0.63	8.36 ± 0.64	0.845

[00966] *ERKl/2 significantly reduced in $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients*

[00967] After incubation with K562 cells at an E:T ratio of 25: 1, ERKl/2 was significantly reduced in $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients when compared to NFC. (See Figure 5.) PMA/I induced a significant increase in ERKl/2 phosphorylation in $CD56^{dim}CD16^{+}$ NK cells compared to the US and K562 stimulated cells from CFS/ME and NFC participants. Comparison of ERKl/2 in $CD56^{bright}CD16^{dim/-}$ NK cells revealed no significant differences between CFS/ME and NFCs. (See Figure 6.)

[00968] *MEK1/2 and p38 significantly increased $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients*

[00969] In CFS/ME patients, phosphorylation of MEK1/2 and p38 was significantly increased in $CD56^{bright}CD16^{dim/-}$ NK cells following incubation with K562 cells at an E:T ratio of 25:1 compared to the NFC. (See Figure 6.) Stimulation with PMA/I induced a significant increase in MEK1/2 and p38 compared to US and K562 stimulated cells in both CFS/ME and NFC cohorts. Comparison of MEK1/2 and p38 in $CD56^{dim}CD16^{+}$ NK cells from CFS/ME and NFC revealed no significant differences. (See Figures 7 and 8.) Measurement of additional MAPK proteins including Stat-3, NF-κβ, Iκβ, protein kinase c-a and JNK revealed no significant differences between CFS/ME and the NFC cohorts. (See Figures 9-13.)

[00970] *NK cell cytotoxic activity reduced in CFS/ME*

[00971] In both CFS/ME patients and NFC, NK cell cytotoxic activity at 25:1 was significantly increased compared to 12.5:1 and 6.25:1 ratios. Compared to NFC, CFS/ME was reduced at 25:1 and 12.5 ratios, although this was not statistically significant. (See Figure 14.)

[00972] *CD107a and CD107b increased on $CD56^{dim}CD16^{+}$ NK cells after stimulation*

[00973] Surface expression of CD107a and CD107b on $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells was significantly increased following stimulation with PMA/I and K562 cells in both CFS/ME and NFC. (See Figures 15 and 16.) Comparison of CD107a and CD107b expression between CFS/ME and the NFC under each stimulatory condition revealed no significant differences. $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients displayed increased CD107a following K562 stimulation, although this increase was not significant.

[00974] *No significant differences in NK cell lytic proteins from CFS/ME patients*

[00975] NK cell lytic proteins perforin, granyzme A and granzyme B were measured in $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients and NFC. Comparison between the two groups revealed no significant differences. Surface expression of CD57 was measured as a marker for NK cell maturation on $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells and no significant differences were observed between the CFS/ME patients and the NFC. (See Figures 17 and 18.)

[00976] *$CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cell cytokine production increased after PMA/I stimulation*

[00977] $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cell cytokine production was measured under two stimulatory conditions with PMA/I or K562 cells. INF-γ, TNF-a and GM-CSF production in $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells increased following stimulation with PMA/I in both the NFC and CFS/ME patients. (See Figures 19-21.) Comparisons of $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cell cytokine production between CFS/ME patients

and the NFC under the different stimulatory conditions revealed no significant differences between groups.

[00978] **Discussion**

[00979] This is the first study to investigate ERKl/2 and MEK1/2 MAPK intracellular signalling in $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cell phenotypes in CFS/ME. The inventors report novel and significant findings of reduced ERKl/2 in $CD56^{dim}CD16^{+}$ NK cells in conjunction with increased MEK1/2 and p38 in $CD56^{bright}CD16^{dim/-}$ NK cells. Further investigation of other extracellular signal regulated kinases will contribute to the understanding of the role of dysregulated MAPK signalling and reduced cytotoxic function of NK cells in CFS/ME. The synergistic functions of both $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells are required for clearance of target cells and dysfunctional signalling through the MAPK pathway in CFS/ME patients may compromise efficient removal of target cells.

[00980] $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients had a significant decrease in ERKl/2 which has been identified as an important component for cytotoxic activity due to substrate targeting of paxillin, a cytoskeletal protein kinase [46m, 47m]. Downstream activation of ERKl/2 is the result of intracellular signalling networks propagating activating signals through phosphorylation cascades [48m, 49m]. Sequential phosphorylation of MAPK kinase kinase (MAPKKK) and MAPK kinase (MAPKK/MEKl/2) activates ERKl/2 through dual phosphorylation of threonine and tyrosine residues [48m, 49m]. Phosphorylation of ERKl/2 induces a significant conformational change which is required for NK cell cytotoxic activity as it increases substrate accessibility to phosphorylate paxillin [50m, 51m]. Paxillin is an adaptor protein which provides a docking site for regulatory proteins such as ERKl/2 and structural proteins including microtubules and actin cytoskeleton [50m, 51m]. Colocalisation of phosphorylated ERK2 and paxillin to the microtubules and the microtubule organising centre (MTOC) facilitates polarisation of the secretory granules towards the immune synapse [14m, 15m, 46m, 51m, 52m]. In CFS/ME patients, abnormal signalling through ERKl/2 may interfere with and delay release of the lytic proteins to induce cytotoxic lysis of target cells

[00981] NK cell cytotoxic activity was reduced in the CFS/ME cohort compared to the NFC. The significant reduction of ERKl/2 in $CD56^{dim}CD16^{+}$ NK cells may disrupt intracellular signalling required for secretory granule polarisation through the MAPK pathway. As the MAPK cascade integrates signals received from the cell surface, the pathway is subject to complex regulatory and feedback mechanisms which may contribute to the reduction observed in ERKl/2 from CFS/ME patients [46m, 53m]. ERKl/2 is under constant regulation which also functions to determine specificity of ERKl/2 to target the secretory granules in cytotoxic NK cells [14m, 15m, 46m, 53m]. Regulatory mechanisms of ERKl/2 include phosphatases MKP3 and MKPX

which dephosphorylate protein tyrosine kinases to inhibit activation [46m, 53m]. Receptor desensitisation and dissociation of the receptor-ligand interaction changes the strength and duration of activation signals [46m, 53m]. Scaffold proteins and subcellular localisation of the cascade regulate phosphorylation by directing ERKl/2 to target substrates in the cytoplasm or nucleus [46m, 53m]. The integration and crosstalk of ERKl/2 with other signalling pathways also acts as a feedback mechanism to regulate phosphorylation levels [46m, 53m]. As ERKl/2 is subject to a number of distinct mechanisms of regulation, further investigations in $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients are required to determine if these regulatory mechanisms contribute to reduce ERKl/2 phosphorylation.

[00982] Degranulation of cytotoxic NK cells was measured to investigate if potential impairments in intracellular signalling through ERKl/2 contribute to reduced cytotoxic activity in CFS/ME patients. Whilst no significant differences were observed in NK cell surface expression of CD107a and CD107b, $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients displayed increased CD107a following K562 stimulation. In support of this current finding, the inventors previously reported a significant increase in CD107a on NK cells following K562 stimulation in a larger cohort of CFS/ME patients [27m]. This finding suggests that the reduction in ERKl/2 may delay movement of the secretory granule and MTOC towards the immune synapse but does not prevent degranulation [14m, 15m]. Increased degranulation of $CD56^{dim}CD16^{+}$ NK cells from CFS/ME patients suggests the cells may be under a continuum of activation due to an inability to induce cytotoxic lysis and subsequent removal of the target cells [27m].

[00983] Continual activation of NK cells in CFS/ME patients may be the result of prolonged contact with target cells. Kinetic priming facilitated by sustained NK cell contact with target cells retains convergence of the secretory granules and the MTOC at the plasma membrane [54m]. This mechanism is known as 'serial killing' as subsequent lysis of target cells is more rapid due to pre-docking of the secretory granules, bypassing the need for ERKl/2 to initiate polarisation of the secretory granules towards the immune synapse for degranulation [55m]. Further investigations are required to determine if the secretory granule completely fuses with the NK cell membrane to release the entire lytic protein content or if deficiencies in the lytic proteins may contribute to reduced target cell lysis in CFS/ME patients [27m, 36m, 56m]. Reduced perforin and granzyme B has been reported in NK cells from CFS/ME patients which may be a consequence of 'serial killing' [27m, 36m]. Whilst it has been identified that NK cells from CFS/ME patients are degranulating, the inability of NK cells to eliminate target cells by cytotoxic activity suggests that the NK cells may be highly activated through a potential mechanism of inefficient 'serial killing'.

[00984] NK cell production of cytokines including IFN-γ and TNF-a has been identified as

an integral part of NK cell cytotoxic activity and increased production of IFN-γ has previously been reported in CFS/ME [27m, 29m, 57m]. NK cells differentiate and mature from $CD56^{bright}CD16^{dim/-}$ to $CD56^{dim}CD16^{+}$ NK cells with predominant cytokine or cytotoxic effector function [6m, 58m-60m]. This differentiation process suggests that together $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$ NK cells function to optimise an efficient NK cell response which may be impaired in CFS/ME patients [6m, 58m-60m]. NK cell production of IFN-γ has been reported to augment cytotoxic activity by up-regulating expression of the adhesion molecule ICAM-1 on tumour target cells through the NF-κβ pathway which improves conjugate formation and adherence with cytotoxic NK cells [60m]. Conversely, it has also been reported that IFN-γ treatment of tumour cells with high basal levels of ICAM-1, such as K562 cells, up-regulates major histocompatibility class I which acts as a ligand for inhibitory receptors on NK cells and reduces NK cytotoxic activity [61m, 62m, 60m]. In CFS/ME patients, further investigations are required to determine if increased IFN-γ may contribute to the proposed inefficient mechanism of 'serial killing' resulting in increased degranulation or if IFN-γ desensitises K562 cells to NK cell mediated cytotoxic activity.

[00985] Phosphorylation of MEK1/2 and p38 has been implicated in the pathogenesis of many chronic inflammatory diseases and increased production of IFN-γ may be a result of increased MEK1/2 and p38 in $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients [27m, 46m, 63m]. Receptor ligation through environmental stress or innate proinflammatory cytokines including IL-12 and IL-18 initiate MAPK intracellular signalling cascades [17m, 64m-67m]. Similar to ERK1/2 activation, phosphorylation of MEK1/2 and p38 is the result of a tiered protein phosphorylation cascade [17m, 64m-67m]. Activated MEK1/2 in turn phosphorylates ERK1/2, resulting in the formation of ERK2-MEK1 chimera [46m, 66m, 67m]. This chimera is released from its cytoplasmic anchors to undergo a cyto-nuclear shift to initiate IFN-γ production in the nucleus [46m, 66m, 67m]. The phosphorylated ERK2-MEK1 chimera activates c-Fos transcription factor and the activating protein (AP)-l heterodimer which regulates the IFN-γ gene promoter and subsequent cytokine production [66m, 67m]. Increased phosphorylation of MEK1/2 may therefore result in increased production of IFN-γ from NK cells in CFS/ME patients as we have previously reported [27m, 29m, 57m]. In contrast to targeting IFN-γ transcription factors, phosphorylated p38 translocates into the nucleus to mediate cytokine production by regulating the half-life of adenylate/uridylate (AU)-rich IFN-γ gene which stabilises and prevents degradation of IFN-γ mRNA [64m, 65m]. In $CD56^{bright}CD16^{dim/-}$ NK cells from CFS/ME patients, an increase in p38 may prolong transcription and translation of IFN-γ [27m, 65m, 64m].

[00986] Cytokine synthesis by MEK1/2 and p38 is tightly controlled and each tier of the

MAPK signalling cascade is subject to regulation which may be impaired in CFS/ME patients [46m, 53m]. Phosphatase MKP1 is located in the nucleus and downregulates MEK1/2 and p38 activity by dephosphorylating threonine and tyrosine residues, attenuating cytokine production [53m, 46m]. Further investigations into the regulation of MEK1/2 and p38 in $CO56^{bright}CO16^{dim/-}$ NK cells from CFS/ME patients are required to determine if a regulatory mechanism such as MPK1 may contribute to increased MEK1/2 and p38 activity and IFN-γ cytokine production.

[00987] Investigations into the MAPK intracellular signalling pathway in NK cells from CFS/ME patients has revealed novel findings which may explain previous reports of reduced NK cell cytotoxic activity and increased cytokine production. To the inventors' knowledge, this is the first study to report significant differences in $CD56^{dim}CD16^{+}$ NK cell ERK1/2 from CFS/ME patients. $CD56^{dim}CD16^{4}$ NK cell cytotoxic activity is dependent on synergistic action of $CD56^{bright}CD16^{dim/-}$ NK cell cytokine production. Consequently, increased MEK1/2 and p38 may increase IFN-γ production which in turn may desensitise K562 cells against NK cell cytotoxic activity in CFS/ME patients. The novel, preliminary findings of this study provide a rationale for further investigations into a larger cohort and into particular clinical subgroups of CFS/ME including severity to elucidate the cause of reduced NK cytotoxic activity.

[00988] **Conclusions**

[00989] The results from this study highlight the importance of intracellular signalling through the MAPK pathway for synergistic function of $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$ NK cells to ensure efficient clearance of target cells in CFS/ME patients. Importantly, this intracellular signalling through the MAPK pathway is likely to be a mechanism operating in other cell/tissue types, including peripheral blood mononuclear cells.

[00990] **Example 11 - Dysregulation of Ca^{2+} Dependent Protein Kinase Gene Expression in NK Cells from Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Patients**

[00991] In this Example, mRNA expression of 528 Ca^{2+} dependent protein kinase genes in isolated NK cells was analysed from moderate and severe CFS/ME patients. The expression of 92 Ca^{2+} dependent protein kinase genes was significantly different in the severe CFS/ME group compared with non-fatigued controls. Among these, 37 Ca^{2+} dependent protein kinase genes were significantly upregulated and 55 Ca^{2+} dependent protein kinase genes were significantly downregulated in severe CFS/ME patients compared to non-fatigued controls. In severe CFS/ME patients, dysfunction in Ca^{2+} dependent protein kinase genes may contribute to impairments in NK cell intracellular signalling and effector function. Similar changes in Ca^{2+} dependent protein kinase genes may be present in other cells, potentially contributing to the pathomechanism of this illness.

[00992] **Introduction**

[00993] Calcium (Ca^{24}) ions play an integral role in intracellular signalling. Calcium controls a diverse range of cellular processes, such as gene transcription, muscle contraction and cell proliferation [lu]. The effects of Ca^{2+} ions are mediated by the Ca^{2+} binding protein calmodulin, which activates a number of different protein kinases. Ca^{2+}/calmodulin-dependent protein kinase includes myosin light chain kinase which signals muscle contraction and members of the CaM kinase family which phosphorylate a number of different proteins, including metabolic enzymes, ion channels, and transcription factors [2u].

[00994] In natural killer (NK) cells, Ca^{2+} signaling plays an important role in the granule dependent pathway of apoptosis [3u]. Ca^{2+} is required for inducing cytolytic granule polarization, cytokine gene transcription and degranulation in NK cells [4u, 5u]. Ca^{2+} also regulates lytic granule fusion [5u-7u] as well as lytic granule mobilization to the immune synapse to release perforin and granzymes to kill target cells [3u, 8u, 9u]. Furthermore, the downstream intracellular signals that occur in NK cells upon target cell ligation to trigger target lysis are governed by the Ca^{2+} regulated mitogen-activated protein kinase (MAPK) pathway [10u]. Intracellular Ca^{2+} concentrations can either stimulate or inhibit the MAPK cascade, and thereby play an important role in the regulation of MAPK dependent cellular process. The effector functions of NK cells are regulated by three specific MAPK subgroups [llu-13u] which include the p38 MAPK (p38) and the C-Jun terminal kinase (INK) which regulate cytokine production and extracellular signal regulated kinases (ERK1/2). These kinases regulate the mobilization and redistribution of cytoplasmic perforin and granzyme B towards the contact zone with target cells [14u]. Interestingly, the inventors' earlier findings (described in Example 10) demonstrate impairments in MAPK signalling as well as decreased intracellular Ca^{2+} concentration in NK cells as well as isolated B cells from CFS/ME patients. Collectively, these anomalies may contribute to NK cell dysfunction in particular the reduced NK cell cytotoxic activity which is consistently reported in CFS/ME patients [17u-25u].

[00995] Given the importance of Ca^{2+} signalling in regulating NK cell function, the present study aimed to examine the role of Ca^{2+} dependent protein kinase genes in isolated NK cells from CFS/ME patients. This investigation explores the association of functionally important NK intracellular signalling alterations with CFS/ME.

[00996] **Methodology**

[00997] *Participants*

[00998] Participants were recruited from the National Centre for Neuroimmunology and Emerging Diseases (NCNED) research database for CFS/ME. All participants completed an online questionnaire regarding their medical history and symptoms based on the 2011

International Consensus Criteria (ICC) to determine suitability for the study [26u]. This requires the presence of debilitating post-exertional fatigue, accompanied further by neurological, immune, and autonomic symptoms. CFS/ME patients meeting the 2011 ICC symptoms and non-fatigued healthy controls were included in this study. Severity of CFS/ME was defined according to the Dr Bell's diability scale that ranges from 100% (no symptoms) to 0% (severe symptoms) [27u]. Patients categorized as moderate CFS/ME scored >30%. Severe CFS/ME scored <30% and were considered housebound or bedridden. Patients disability was further characterized by self-reporting scales which included the Fatigue Severity Scale (FSS), and the SF-36 [28u, 29u]. Participants were excluded if they were previously diagnosed or had a history of any alternative disease that would explain symptoms including autoimmune disorder, multiple sclerosis, psychosis, major depression, heart disease or thyroid-related disorders or if they were pregnant, breast feeding, smokers.

[00999] *Blood collection*

[001000] Forty millilitres of EDTA blood was collected from the antecubital vein of participants into EDTA blood collection tubes. All the laboratory analysis was performed within six hours of blood collection to maintain the cell viability. Routine pathology testing parameters including full blood count, erythrocyte sedimentation rate, electrolytes and high sensitivity C reactive protein were also assessed on each participant sample by Queensland pathology.

[001001] *NK cell isolation*

[001002] NK cells were isolated from 40 mL of whole blood using negative selection with RosetteSep Human Natural Killer Cell Enrichment Cocktail (STEMCELL Technologies Australia Pty. Ltd, Victoria, Australia) according to manufacturer instructions. The isolated NK cells were incubated in unsupplemented Rosewell Park Memorial Institute (RPMI)-1640 culture media (Life technologies, Carlsbad, USA) and were counted using Trypan blue. Briefly following NK cell isolation (Figure 22), the NK cell purity was also assessed on the LSR-Fortessa X-20 flow cytometer (BD Biosciences, San Diego, USA) after labelling with CD3 and CD56 as previously described (BD Biosciences, San Diego, USA). The isolated NK cells were lysed at a concentration 10,000 cells/μï of RLT buffer (Cat No: 79216, QIAGEN, Australia) and were snap-frozen in liquid nitrogen and stored at -80 ° C until further Ca^{2+} dependent protein kinase genes assessment.

[001003] *Ca^{2+} Dependent Protein Kinase Gene Expression*

[001004] Gene expression was directly measured via counts of corresponding messenger RNA (mRNA) in each sample using an nCounter (Nanostring, Seattle, WA) GX human kinase kit v2 (XT), which is a multiplex assay for 528 genes known to be differentially expressed in the human kinome [31u]. The nCounter system allows for direct detection and counting of nucleic

acid via reporter probes appended with multiple fluorophore barcodes and biotinylated capture-probes that attach to microscopic beads, which are then affixed to lanes in a translucent cartridge and read in an optical scanner. Batches of 12 separate samples at one time were prepared as per manufacturer's instructions, with NK cell lysate hybridized with probes at 65°C for 16-18 hours before being placed into the automated nCounter Prep Station (Nanostring) in which samples were affixed to cartridges. Cartridges were then immediately placed into the nCounter Digital Analyzer (Nanostring) optical scanner and read at a goal resolution of 550 Fields of View (FOV), which is the maximum resolution for this instrument.

[001005] *Statistical analysis*

[001006] Statistical analysis was performed using SPSS software version 22 and GraphPad Prism version 6. Data were compared among the three participant groups (control, moderate CFS/ME and severe CFS/ME) with statistical analysis performed based on the distribution. Shapiro-Wilk normality tests were performed on all the data sets to test for Gaussian distribution. ANOVA was used to examine parametric data and the Kruskal Wallis test of independent was performed for non-parametric data when appropriate, with statistical significance set at an alpha criterion at $p < 0.05$. Gene expression was directly measured via counts of corresponding messenger RNA (mRNA) in each sample using DESeq R package software [32u], where alpha level of significance was set at a p value of < 0.05.

[001007] **Results**

[001008] *Participants*

[001009] Table 30 summarises participant clinical characteristics. The study included 11 moderate CFS/ME (age 54.9 ± 10.3; 83.3% female) and 12 severe CFS/ME (age 47.5 ± 8.0; 75.0% female), and 11 non-fatigued controls (age 50.0 ± 12.3 years; 72.5 % female). There was no significant difference between mean age and sex between groups. All participants in the study were of European decent and were residents of Australia at the time of blood collection. Fatigue severity was highest among severe CFS/ME compared with moderate CFS/ME and non-fatigued controls ($p<0.05$). Furthermore, severe CFS/ME reported significantly greater impairment across all SF-36 scales compared with moderate CFS/ME and non-fatigued controls ($p<0.05$), except for general mental health ($p=0.11$). Comparison of the group age and blood parameters including erythrocyte sedimentation rate, high sensitivity C-reactive protein and full blood counts for white and red blood cells between participant groups showed no significant difference. Table 30 outlines participant characteristics.

[001010] **Table 30** Clinical characteristics between non-fatigued controls, moderate CFS/ME and severe CFS/ME

Variable	Moderate CFS/ME n=ll	Severe CFS/ME n=12	Control n= 11	P-value
Mean age (years)	54.9 ± 10.3	47.5 ± 8.0	50.0 ± 12.3	0.286
Sex (% Female)	83.3	75	72.5	0.813
FSS	5.2 ± 1.3	7.0 +0.3	2.1 ± 1.4	<0.05
SF-36				
Physical functioning	47.5 ± 27.8	15.3 ± 12.3	98.2 ± 4.8	<0.05
Physical role	15.9 ± 29.4	3.3 ± 8.8	98.7 ± 5.7	<0.05
Bodily pain	48.3 ± 28.1	36.0 ± 22.6	94.2 ± 13.0	<0.05
General health	31.1 ± 20.5	19.7 ± 10.8	83.9 ± 4.5	<0.05
Vitality	25.2 ± 10.6	7.2 ± 6.3	80.2 ± 4.3	<0.05
Emotional role	96.3 ± 5.4	92.3 ± 6.4	100 ± 0	<0.05
Social functioning	47.5 ± 24.8	25.0 ± 17.6	96.9 ± 5.4	<0.05
Mental health	85.6 ± 22.0	72.8 ± 18.6	87.0 ± 6.8	0.11
Pathology				
White Cell Count (x 10^9/L)	5.8 ± 1.9	5.3 ± 1.2	6.2 ± 0.9	0.362
Neutrophils (x 10^9/L)	3.4 ± 1.5	3.2 ± 1.0	3.7 ± 0.8	0.738
Lymphocytes (x 10^9/L)	2.0 ± 0.5	1.7 +0.4	2.0 ± 0.5	0.147
Monocytes (x 10^9/L)	0.3 ± 0.1	0.3 ± 0.6	0.3 ± 0.1	0.926
Eosinophils (x 10^9/L)	0.1 ± 0.14	0.1 ± 0.1	0.2 ± 0.2	0.375
Basophils (x 10^9/L)	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.707
Platelets (x 10^9/L)	262.0 ± 67.5	242.6 ± 50.0	257.5 ± 43.0	0.537
Haemoglobin (g/L)	136.3 ± 9.6	135.6 ± 13.1	137.6 ± 16.2	0.975
Haematocrit (	0.4 ± 0.02	0.8 ± 1.2	0.4 ± 0.03	0.844
Red Cell Count (x 10^12/L)	4.3 ± 1.1	4.6 ± 0.5	4.7 ± 0.5	0.775

MCV (fL)	90.1 ± 4.3	88.5 ± 3.4	87.3 ± 2.8	0.446
Sodium (mmol/L)	137.3 ± 3.04	138.0 ± 2.1	137.6 ± 1.3	0.769
Potassium (mmol/L)	4.2 ± 0.4	3.9 ± 0.2	4.1 ± 0.1	0.357
Chloride (mmol/L)	102.4 ± 3.3	102.9 ± 2.5	102.2 ± 2.7	0.730
Bicarbonate (mmol/L)	27.4 ± 2.2	26.5 ± 1.9	27.5 ± 2.4	0.303
Anion Gap (mmol/L)	7.3 ± 1.4	7.3 ± 1.4	7.8 ± 1.4	0.546
ESR (mm/Hr)	19.0 ± 17.1	14.5 ± 15.2	11.1 ± 4.2	0.341
C-Reactive Protein (mg/L)	3.8 ± 4.5	2.8 ± 6.4	1.0 ± 0.6	0.188

[001011] Data represented as mean ± standard deviation. CFS, Chronic fatigue syndrome; ME, myalgic encephalomyelitis; FSS, Fatigue severity scale; SF-36, Short form 36 item health survey; WHODAS, World health organisation disability adjustment schedule.

[001012] *Ca^{2+} Dependent Protein Kinase Gene Expression*

[001013] Microarray analysis of the 528 kinase genes revealed there were 92 genes which were significantly associated with severe CFS/ME patients compared with non-fatigued controls. Of the 92 genes, 37 genes were significantly upregulated (Table 31) and 55 genes were significantly downregulated (Table 32) in severe CFS/ME patients compared to non-fatigued controls. A heat map of gene expression with clustering using spearman correlation in severe CFS/ME patients and non-fatigued controls is shown in Figure 23. There was no significant alteration in the expression of kinase genes in moderate CFS/ME patients compared with non-fatigued controls.

[001014] **Table 31.** List of calcium-dependent kinase genes significantly upregulated in severe CFS/ME group compared with non-fatigued controls.

Gene name	**Log2 (fold change)**	**P value**
CDK9	0.418505235	1.44786E-09
MAPKAPK2	0.458988465	3.17148E-08
CSNK1G3	0.354351365	1.68531E-06
CAMK1D	0.322970895	1.65639E-05
MST4	0.281702517	2.2541E-05
PRKACA	0.294878003	3.20405E-05
STK39	0.316411907	6.97134E-05
ADRBK1	0.233462724	7.80645E-05
MAP4K5	0.325434189	0.000148478
EIF2AK3	0.548932354	0.000156029

YES1	0.586095547	0.00019027
PRPF4B	0.330804207	0.00024688
CAMKK2	0.459516611	0.000307664
TAOK3	0.207997286	0.000476049
CDK7	0.500189253	0.000494901
RJPK2	0.484439203	0.0006405
RIOK3	0.550401811	0.000630622
STK4	0.261572944	0.000663088
CLK3	0.257415278	0.000730866
CLK4	0.249497083	0.000975821
ADRBK2	0.75043569	0.000997031
MAP3K8	0.474587427	0.001133225
LMTK2	0.414585483	0.001318789
BRD4	0.161675211	0.001674596
RPS6KA5	0.300257315	0.001883647
HPRT1	0.235063603	0.002985669
ABL1	0.473436245	0.00506836
INSR	0.625855881	0.005335724
SNRK	0.249400839	0.005940401
ERN1	0.414439855	0.006022066
MAP3K3	0.191248184	0.006262361
PDK1	0.281715195	0.006628024
C21orf7	0.611100452	0.006700917
SIK1	0.593133707	0.006828076
RJPK1	0.235813317	0.00781539
STK32C	0.571056394	0.010416118

[001015] **Table 32.** List of calcium-dependent kinase genes significantly downregulated in severe CFS/ME group compared with non-fatigued controls.

Gene name	**Log2 (fold change)**	**P value**
TNK2	-0.5929763	7.8935E-17
TGFBR1	-0.951662	4.4321E-15
CSNK1G2	-0.5948012	2.5963E-13
STK11	-0.6196713	8.9479E-12

CSK	-0.5855852	5.6434E-10
STK10	-0.3506819	5.5896E-09
CSNK2A1	-0.5130831	2.1444E-08
PRKD2	-0.5883312	5.7048E-08
DYRK1B	-0.6287082	8.4794E-08
SBK1	-0.7100798	1.2219E-07
FES	-0.9457073	1.4352E-07
STK25	-0.4783125	1.4354E-07
SDHA	-0.5258195	2.5036E-07
ADCK4	-0.571751	8.0054E-07
TUBES	-0.4055129	1.0585E-06
NEK9	-0.3498812	2.1641E-06
STK35	-0.6132598	3.9862E-06
IKBKE	-0.6077292	5.3859E-06
EIF2AK1	-0.4001172	6.3883E-06
VRK3	-0.32799	2.4521E-05
RIOK1	-0.558951	2.8792E-05
MTOR	-0.389873	3.1103E-05
NEK6	-0.5891655	5.3333E-05
RPS6KA4	-0.4077403	6.6127E-05
STRADA	-0.3801652	6.9352E-05
SCYL2	-0.2888867	7.9998E-05
CDK5	-0.6222848	8.9234E-05
MATK	-0.4030913	0.00012599
MKNK2	-0.3089365	0.00018668
IRAK4	-0.2873769	0.00024716
MAP4K1	-0.3408634	0.00023847
FGR	-0.4531449	0.0002525
STRADB	-0.4698047	0.00041397
PDK2	-0.3431478	0.00074842
STK38	-0.3263961	0.00076823
CSNK1G1	-0.3344949	0.00075652
LCK	-0.3682158	0.00084175
RPS6KB2	-0.3343033	0.00087291

ADCK1	-0.5383807	0.00103763
PSKH1	-0.3536634	0.00137302
DMPK	-0.6344797	0.00173864
ZAP70	-0.2369642	0.00217501
CASK	-0.613404	0.00231632
SCYL1	-0.2825376	0.00258513
CSNK1E	-0.2937711	0.00270961
SRPK1	-0.2377855	0.0037189
GSK3A	-0.2383325	0.00476715
MAPK3	-0.3200085	0.0048246
MAP2K7	-0.2578818	0.00538244
FLJ25006	-0.4910571	0.00567251
NUAK2	-0.4855813	0.0061345
MAP2K2	-0.1521624	0.00689828
TESK1	-0.3332313	0.00752948
TLK2	-0.1859512	0.00846469
MAST3	-0.3216447	0.00968208

[001016] Ninety-two genes associated with severe CFS/ME patients were analysed using MetaCore pathway analysis. The gene signatures were associated with seventy-seven significant process networks including NK cytotoxicity, IFN-γ, IL-17 of immune cells, immune cell function, physiological processes, signal transduction and translation in CFS/ME patients (Table 33).

[001017] **Table 33.** Significant calcium-dependent kinase gene networks associated with the severe CFS/ME patients compared to non-fatigued controls.

Network	**p-value**	**no. of genes**	**gene name**
Translation_Regulation of initiation	2.247E-16	18	Insulin receptor, MEK2, p70 S6 kinase2, Casein kinase Π, alpha chain (CSNK2A1), MSK1/2 (RPS6KA5/4), GSK3 alpha, ERK1 (MAPK3), MSK1, GSK3 alpha, MNK2(GPRK7), mTOR, MEK2(MAP2K2), TGF-beta receptor type I

Signal transduction_WNT signaling	2.025E-08	13	PKA-cat (cAMP-dependent), Casein kinase II, alpha chain (CSNK2A1), Casein kinase I epsilon, ERK1 (MAPK3), PKC-alpha, MKK7 (MAP2K7), Casein kinase I gamma-3, TGF-beta receptor type I
Cell cycle_Gl-S Growth factor regulation	6.448E-08	13	MEK2, GSK3 alpha, ERK1 (MAPK3), PKC-alpha, c-Fes, MKK7 (MAP2K7), MKK7, MATK, MEK2(MAP2K2), TGF-beta receptor type I
Reproduction_Spermatogenesis, motility and copulation	4.017E-07	13	Insulin receptor, Casein kinase II, alpha chains, PKA-cat (cAMP-dependent), ERK1 (MAPK3), MSK1, TESK1, STK39, TGF-beta receptor type I, GRK2
Cell adhesion_Cell junctions	6.161E-07	11	Casein kinase II, alpha chains, YES, Casein kinase II, alpha chain (CSNK2A1), ERK1 (MAPK3), Tubulin beta, ZAP70
Reproduction_Male sex differentiation	8.338E-07	13	Insulin receptor PKA-cat (cAMP-dependent), ERK1 (MAPK3), MSK1, TLK2, STK39, Casein kinase II,TGF-beta receptor type I, GRK2
Inflammationjnflammasonic	2.704E-06	9	eIF2AK3, IRAK4, RIPK2, eIF2AKl, IKK-epsilon, ERK1 (MAPK3), RIPK1
Translation_Translation initiation	8.046E-06	10	eIF2AK3, eIF2AKl, Casein kinase II, alpha chain (CSNK2A1), Casein kinase I epsilon, Casein kinase I gamma 2
Development_Hemopoi	8.741E-06	9	MEK2, ERK1 (MAPK3), MSK1,

esis, Erythropoietin pathway			PKC-alpha, c-Fes, MEK2(MAP2K2)
Immune response_TCR signaling	9.388E-06	10	Csk, MEK2, Lck, ERKl (MAPK3), ZAP70, MKK7 (MAP2K7), MKK7, MEK2(MAP2K2),TPL2(MAP3K 8)
Signal Transduction_Cholecy stokinin signaling	1.093E-05	8	MAPKAPK2, PKA-cat (cAMP-dependent), MEK2, MSK1/2 (RPS6KA5/4), MSK1, PKC-alpha, MEK2(MAP2K2), ERK1
Inflammation_MIF signaling	1.107E-05	9	Casein kinase II, alpha chains, PKA-cat (cAMP-dependent), Casein kinase II, alpha chain (CSNK2A1), ERKl (MAPK3), CDK5
Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT	1.206E-05	10	PKA-cat (cAMP-dependent), MSK1, PKC-alpha, MKK7 (MAP2K7),CDK5, MEK2(MAP2K2), ERKl
Cell adhesion_Cadherins	1.266E-05	10	c-Abl, Casein kinase II, alpha chains, YES, Casein kinase II, alpha chain (CSNK2A1), Casein kinase I epsilon, PKC-alpha
Neurophysiological process_Long-term potentiation	1.798E-05	7	PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), PKC-alpha,mTOR
Inflammation_IL-4 signaling	1.992E-05	8	c-Abl, MEK2, ERKl (MAPK3), c-Fes, MEK2(MAP2K2)
Reproduction_FSH-beta signaling pathway	3.234E-05	9	PKA-cat (cAMP-dependent), MEK2, p70 S6 kinase2, ERKl (MAPK3), mTOR, MEK2(MAP2K2),TGF-beta

			receptor type I, GRK2
Immune response_BCR pathway	7.036E-05	8	MEK2, Casein kinase II, alpha chain (CSNK2A1), GSK3 alpha, ERKl (MAPK3), mTOR, MEK2(MAP2K2)
Development_Neuromuscular junction	1.156E-04	8	c-Abl, MEK2, ERKl (MAPK3), PKC-alpha, MKK7 (MAP2K7), MEK2(MAP2K2)
Signal transduction_NOTCH signaling	1.281E-04	10	MEK2, p70 S6 kinase2, Lck, ERKl (MAPK3), MKK7 (MAP2K7), mTOR, MEK2(MAP2K2), TGF-beta receptor type I
Cell adhesion_Amyloid proteins	1.504E-04	9	c-Abl, Casein kinase II, alpha chains, CASK, ERKl (MAPK3),PKC-alpha
Proliferation_Lymphocyte proliferation	2.536E-04	9	p70 S6 kinase2, eIF2AKl, ERKl (MAPK3), PKC-alpha, ZAP70, MKK7 (MAP2K7), mTOR, MEK2(MAP2K2)
Reproduction_Feeding and Neurohormone signaling	2.723E-04	9	Casein kinase II, alpha chains, PKA-cat (cAMP-dependent), p70 S6 kinase2, ERKl (MAPK3), PKC-alpha, CDK5, mTOR, TGF-beta receptor type I
Neurophysiological process_Transmission of nerve impulse	2.820E-04	9	PKA-cat (cAMP-dependent), MEK2, CASK, ERKl (MAPK3), PKC-alpha, MEK2(MAP2K2),PKA-cat alpha
Development_Keratinocyte differentiation	2.923E-04	5	PKC-alpha, MKK7 (MAP2K7), MEK2(MAP2K2), ERKl, TGF-beta receptor type I
Cell adhesionjntegrin-	3.024E-04	9	MEK2, ERKl (MAPK3), Tubulin beta, PKC-alpha,

mediated cell-matrix adhesion			MEK2(MAP2K2)
Cell cycle_Gl-S Interleukin regulation	3.078E-04	7	GSK3 alpha, ERKl (MAPK3), c-Fes, MKK7 (MAP2K7), MEK2(MAP2K2)
Signal transduction_ESRl-membrane pathway	3.111E-04	6	PKA-cat (cAMP-dependent), GSK3 alpha, GSK3 alpha, MEK2(MAP2K2)
Signal transductionjnsulin signaling	3.409E-04	8	Insulin receptor, PKA-cat (cAMP-dependent), p70 S6 kinase2, GSK3 alpha, mTOR, MEK2(MAP2K2), ERKl
Proliferation_Positive regulation cell proliferation	3.837E-04	9	PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), c-Fes, MATK, MEK2(MAP2K2)
Development_Regulation of angiogenesis	4.100E-04	9	MAPKAPK2, ERKl (MAPK3), RIPK1, PKC-alpha, MEK2(MAP2K2),TGF-beta receptor type I
Signal transduction_CREM pathway	4.645E-04	6	PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), MEK2(MAP2K2)
Cytoskeleton_Regulation of cytoskeleton rearrangement	5.170E-04	8	ACK1, Tubulin beta 1, ERKl (MAPK3), Tubulin beta, GRK2
Inflammation_IL-2 signaling	6.380E-04	6	MEK2, Lck, ERKl (MAPK3), PKC-alpha, MEK2(MAP2K2)
Reproduction_Gonadotropin regulation	8.987E-04	8	PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), PKC-alpha, MKK7 (MAP2K7), MEK2(MAP2K2), GRK2
Signal transduction_ERBB-family signaling	9.624E-04	5	ERKl (MAPK3), MKK7 (MAP2K7), MEK2(MAP2K2)

Neurophysiological process_Circadian rhythm	1.022E-03	5	PKA-cat (cAMP-dependent), Casein kinase I epsilon, MEK2(MAP2K2)
Cell adhesion_Leucocyte chemotaxis	1.090E-03	8	Lck, ERKl (MAPK3), Tubulin beta, ZAP70
Cell cycle_G2-M	1.125E-03	8	MAPKAPK2, PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), CDK7, MEK2(MAP2K2) PKA-cat alpha
Inflammation_NK cell cytotoxicity	1.352E-03	7	MEKl/2, Lck, ERKl (MAPK3), PKC-alpha, ZAP70, MEK2(MAP2K2)
Reproduction_GnRH signaling pathway	1.451E-03	7	PKA-cat (cAMP-dependent), MEKl/2, ERKl (MAPK3), PKC-alpha, MEK2(MAP2K2), GRK2
Neurophysiological process_Corticoliberin signaling	1.505E-03	4	PKA-cat (cAMP-dependent), PKC-alpha, GRK3
Signal transduction_Oxytocin signaling	1.780E-03	5	MEK2(MAP2K2), ERKl
InflamniationJgE signaling	2.652E-03	6	MEK2, ERKl (MAPK3), MKK7 (MAP2K7), MKK7, MEK2(MAP2K2)
Inflamniation_Jak-STAT Pathway	2.775E-03	7	Insulin receptor, MEK2, ERK1 (MAPK3), MEK2(MAP2K2)
Inflammation_TREMl signaling	3.519E-03	6	MEK2, ERKl (MAPK3), ZAP70,MEK2(MAP2K2)
Signal transduction_Leptin signaling	4.606E-03	5	PKA-cat (cAMP-dependent), GSK3 alpha, LKB1, ERKl
Signal transduction_Neurope	4.880E-03	6	PKA-cat (cAMP-dependent), MEK2, ERKl (MAPK3), PKC-

ptide signaling pathways			alpha, MEK2(MAP2K2)
Inflammationjnterferon signaling	5.179E-03	5	MEK2, ERKl (MAPK3), MEK2(MAP2K2)
Cell adhesion_Glycoconjugates	6.040E-03	6	ERKl (MAPK3), PKC-alpha, ZAP70
Signal transduction_Androgen receptor signaling cross-talk	6.125E-03	4	PKA-cat (cAMP-dependent), mTOR, ERKl, TGF-beta receptor type I
Immune response_IL-5 signalling	6.691E-03	3	MEK2(MAP2K2), ERKl
Signal transduction_ESR2 pathway	7.755E-03	4	Insulin receptor, MNK2(GPRK7), MEK2(MAP2K2), ERKl
Development_EMT_Regulation of epithelial-to-mesenchymal transition	7.831E-03	7	c-Abl, MKK7 (MAP2K7), mTOR, MEK2(MAP2K2), ERKl, TGF-beta receptor type I
DNA damage_Checkpoint	8.536E-03	5	c-Abl, ERKl (MAPK3), TLK2, LKB1
Cell adhesion_Attractive and repulsive receptors	8.714E-03	6	c-Abl, PKA-cat (cAMP-dependent), ERKl (MAPK3), c-Fes, CDK5
Development_Neurogenesis_Axonal guidance	8.791E-03	7	c-Abl, PKA-cat (cAMP-dependent), c-Fes, CDK5, MEK2(MAP2K2), ERKl
Immune responsejnnate immune response to RNA viral infection	1.092E-02	4	IRAK4, IKK-epsilon, RIPK1
Signal transduction_Nitric	1.229E-02	4	MKK7 (MAP2K7), MEK2(MAP2K2), ERKl

oxide signaling			
Apoptosis_Endoplasmic reticulum stress pathway	1.277E-02	4	IRE1, JIK, eIF2AK3
Immune response_T helper cell differentiation	1.395E-02	5	IRAK4, Lck, ZAP70, MEK2(MAP2K2), ERKl
Development_Melanocyte development and pigmentation	1.425E-02	3	PKA-cat (cAMP-dependent), ERKl
Development_Hedgehog signaling	1.464E-02	7	PKA-cat (cAMP-dependent), Casein kinase I epsilon, ERKl (MAPK3), MEK2(MAP2K2), PKA-cat alpha, GRK2
Apoptosis_Apoptosis stimulation by external signals	1.559E-02	5	RIPK1, MKK7 (MAP2K7), MEK2(MAP2K2), ERKl, TGF-beta receptor type I
Apoptosis_Anti-Apoptosis mediated by external signals by Estrogen	1.591E-02	4	PKC-alpha, MEK2(MAP2K2), ERKl
Cardiac development_Wnt_beta-catenin, Notch, VEGF, IP3 and integrin signaling	1.830E-02	5	Casein kinase II, alpha chains, PKA-cat (cAMP-dependent), GSK3 alpha, PKC-alpha, GRK2
Autophagy_Autophagy	1.839E-02	3	Insulin receptor, mTOR
Inflammation_Histamine signaling	2.127E-02	6	PKA-cat (cAMP-dependent), ERKl (MAPK3), PKC-alpha, MEK2(MAP2K2)PKA-cat alpha
Development_ERK5 in cell proliferation and neuronal survival	2.461E-02	2	Lck, MAP3K3
Cell adhesion_Integrin	2.575E-02	4	MEK2, ERKl (MAPK3),

priming			MEK2(MAP2K2)
Development_Blood vessel morphogenesis	2.854E-02	6	MAPKAPK2, ERK1 (MAPK3), PKC-alpha, MEK2(MAP2K2)
DNA damage_DBS repair	3.052E-02	4	Casein kinase II, alpha chain (CSNK2A1)
Cardiac development_BMP_TGF_beta_signaling	3.136E-02	4	Casein kinase II, alpha chains, GSK3 alpha, PKC-alpha, TGF-beta receptor type I
Inflammation_Amphoterin signaling	3.222E-02	4	MEK2, ERK1 (MAPK3), MEK2(MAP2K2)
Inflammation_IL-6 signaling	3.309E-02	4	MEK2, ERK1 (MAPK3), MEK2(MAP2K2),
Regulation of metabolisni_Bile acid regulation of lipid metabolism and negative FXR-dependent regulation of bile acids concentration	3.571E-02	3	Insulin receptor, GSK3 alpha
Cytoskeleton_Intermediate filaments	4.963E-02	3	Tubulin beta, CDK5

[001018] **Discussion**

[001019] This study reports, for the first time, the differential expression of Ca^{2+} dependent protein kinase genes from isolated NK cells in severe CFS/ME patients compared with non-fatigued controls. Thirty seven Ca^{2+} dependent protein kinase genes were significantly upregulated and 55 Ca^{2+} dependent protein kinase genes were significantly downregulated in severe CFS/ME patients compared to non-fatigued controls. As this current investigation was undertaken in isolated NK cells, the Ca^{2+} dependent protein kinase genes that are reported will be discussed in the context of intracellular pathways involved in JNK, STAT and NFkappa beta (NF-κβ) activity and NK cell lysis.

[001020] The results from this current investigation highlight significant down regulation of Ca^{2+} dependent protein kinases, namely Lck and ZAP70, between the severe CFS/ME patients compared to non- fatigued controls. NK cells contain a zeta chain, associated to the Fc receptor CD16 (FcgRIIIA), where Zap-70 phosphorylates as well as the associated transducing gamma

chain [33u]. The cytoplasmic tails of adhesion molecules and activating receptors of the NK cells recruit Src family of kinases to phosphorylate ITAMs or ITSMs [34u-37u]. Subsequently the signalling molecules including Lck, Zap70, linker activation for T cells (LAT) and SH2 domain-containing Leukocyte Protein of 7f5kDa (SLP-76) are phosphorylated which continue to phosphorylate and mobilize multiple downstream signalling proteins which results in the activation of NK cells and the initiation of granule dependent exocytosis [38u-40u]. The significant reduction in ZAP70 and Lck expression may affect the phosphorylation of NK cell activating receptors that contain immunoreceptor tyrosine-based activation motifs (ITAMs) to phosphorylate and mobilize multiple downstream signalling proteins which results in the activation of NK cells and the initiation of granule dependent exocytosis. Given the significant reduction of both ZAP70 and Lck, these being Ca^{2+} dependent protein kinases, the intracellular downstream effect may be significant for effector functions of NK cells. The present inventors and others have previously described significant reduction in NK lysis, changes in cytokine production and mobilization and redistribution of cytoplasmic perforin and granzyme B towards the contact zone with target cells [14u, 20u, 41u-45u].

[001021] The significant reduction in Ca^{2+} dependent protein kinases ERKl/2 and MEK1/2 reported in this current study aligns to the inventors' previous investigation that reported a significant decrease in ERKl/2 in $CD56^{dim}CD16^{+}$ NK cells compared to the non-fatigued controls [see the Example above]. A significant reduction in MEK1/2 suggests further compromises in the effector NK cell functions. Following the activation of triggers, a signalling cascade via sequential phosphorylation of MAPK, MEK and ERK results in the lytic granule polarization mediated by TUBB [47u] which regulates the reorientation of the microtubule and microtubule organizing centre (MTOC) towards the target cells to release perforin and granzymes. Activation of ERKl/2 facilitates polarisation of cytotoxic granules towards the microtubule organising centre (MTOC) [48u, 49u]. MAPK intracellular signals activate reorganisation and polarisation of the actin cytoskeleton which facilitates movement of the cytotoxic granules along the MTOC microtubules towards the immune synapse [48u, 49u]. As a critical threshold of signalling of MAPK, MEK and ERK are required for NK cells to mount an effector cell response. A significant reduction in the expression of MEK2, ERK1 and TUBB, as reported in this current investigation, may disrupt these distal events that lead ultimately to reduced NK cytotoxicity. This reduced NK cytotoxicity may be due to a reduction in the ERKl/2 phosphorylation, reducing the polarisation of the secretory granule towards the immune synapse for degranulation 13u, 50u] in severe CFS/ME patients. Importantly, the inventors have reported reduced ERKl/2 from isolated NK cells. Also, the inventors and other researchers have reported a significant reduction in lytic granules, such as granzyme B from CFS/ME patients

[14u, 20u, 41u-45u].

[001022] Binding of NK cells to target cells triggers phosphatidylinositol (PI)-3 kinase (PI3K) to be rapidly activated by Src-family tyrosine kinases (SETKs) and/or SYK leading to calcium influx [51u] and protein kinase C (PKC) activation. In this present investigation the inventors report significant increases in PKC alpha in the severe CFS/ME group compared with the non-fatigued control group. Importantly, PKC-alpha, a member of protein kinase C (PKC) family of Ca^{2+} and/or lipid-activated serine/threonine kinases, functions downstream of many membrane-associated signal transduction pathways [52u]. The activation of PKC alpha triggers a signalling cascade via sequential phosphorylation of MAPK, MEK, ERK and JNK pathways. Calcium ions, magnesium ions, and diacylglycerols (DAGs) are the most important molecules for regulating PKC-a activity as low concentrations of these molecules increase the PKC-alpha activity. Hence the present study highlights the importance of Ca^{2+} transport ion channels in this context.

[001023] As described in earlier Examples, the inventors investigated the role of transient receptor potential melastatin 3 (TRPM3) cation channels and intracellular calcium levels in isolated NK and B cells and found significant reductions in intracellular calcium from each of the cell types as well as significant reduction in cell surface TRPM3 receptors. These findings suggest that the significant reduction in intracellular calcium from these cell types may result in significant increases in calcium-dependent kinase PKC-alpha. Consequently, the downstream effect of this increased gene expression suggest increased p38 and subsequently NF-κβ activation and the production of inflammatory mediators [54u]. Interestingly, the inventors have reported in an earlier Example a significant increase in isolated NK cells, of MAPK (p38) from CFS/ME patients. Other researchers have reported significantly increased NF-κβ production as well as increased pro-inflammatory factors, such as IL-6, IFN gamma, and anti-inflammatory IL10 products from CFS/ME patients [56u-62u].

[001024] Moreover, activation of C-Iun terminal kinase (JNK) is activated by PKC-alpha, where JNK modifies the activity of numerous proteins located in the mitochondria or activates inflammation and pro-inflammatory cytokines such as IL-2, IL-6 and TNF-a. Increased activation of PKC alpha may provide possible explanation for the increase in JNK along p38, resulting in proinflammatory cytokine production such as IFNy, TNF alpha, IL-2 and IL-6, from NK cells [63u]. The significant increase in PKC-alpha may suggest a shift towards a Thl/pro-inflammatory immune response. Previous researchers report significant increases in IFN gamma, IL-2, TNF alpha and IL-6 in CFS/ME patients [56u-62u]. Moreover, anti-inflammatory IL-10 exerts inhibitory effects on cytokine secretion and impedes pro-inflammatory cytokine secretion

by multiple cells including NK cells (IFN-γ and TNF-a) [63u]. A decrease in IL-10 favours an increase in pro-inflammatory responses and this may increase the prevalence of Thl-like cytokines. Importantly the inventors and others have reported significant reductions in IL-10 from CFS/ME patients [64u, 65u].

[001025] During inflammation, NK cells are recruited to lymph nodes where they are activated by trans-presentation of IL-15 by IL-15Ra expressed on dendritic cells [66u]. Engagement of IL-15R on NK cells causes auto-phosphorylation and activation of Janus kinases (JAK1 and JAK3). Subsequently this induces Ras-Raf-MEK, PI3K-AKT-mTOR, and signal transduction and activation of transcription (STAT) 5 pathways [67u, 68u]. Studies have shown that IL-15 activates NK cells to become equipped with cytotoxic granules and sensitize them to secondary stimuli. Furthermore, previous researchers have reported mTOR pathway is central to the IL-15-induced activation of vital NK cell functions. Hence a significant reduction in mTOR reported in this investigation suggests reduced NK effector function of the production of lytic granules and reduced cell lysis as previously described in CFS/ME patients [14u, 20u, 41u-45u]. Stat5 proteins are activated by a wide variety of cytokines and growth factors, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15 and granulocyte-macrophage colony-stimulating factors. Importantly previous investigations found IL-2, IL-4 and IL-15 were tightly associated in CFS/ME and less centred about any individual cytokine. Importantly the authors also highlight IL-2, 4 and 15 belong to a family of cytokines that also includes IL-7, IL-9 and are initiated by STAT5 [69u].

[001026] The kinase genes identified in this study control a large number of process networks within cells affecting synaptic function, signal transduction, inflammation pathways, apoptosis, muscle contraction, microtubule cytoskeleton spindle assembly, circadian rhythm, calcium transport and nitric oxide signalling. Metabolic effects, predominantly insulin gene expression pathways were identified. Protein phosphorylation and protein modification pathways predominated in gene association analysis. Thus this study revealed multiple gene, metabolic and signalling pathway perturbations manifest in calcium-sensitive kinase genes. Kinase pathways control or regulate numerous physiologies including cardiovascular, urogenital, gastrointestinal, neurological, and respiratory systems. Kinase perturbations suggest the likely demonstration of an inflammatory profile along with other dysregulated physiological mechanisms, adding to widespread inflammatory mechanism dysregulation in virtually all cells [70u]. Furthermore, Ca^{2+} dysregulation is an important consequence of altered membrane receptor signalling and likely to have effects in neuronal function, such as impulse transmission [71u], as well as muscle contraction [72u]. Impaired neurological and motor control are common symptoms associated with CFS/ME [26u]. Therefore, it is suggested that Ca^{2+} and kinase signalling dysregulation be

further investigated in the central nervous system given the high dependence on Ca^{2+} signalling for glial and neuronal cell functioning and their potential role in the pathomechanism of CFS/ME.

[001027] **Conclusion**

[001028] This study identifies, for the first time, *92* calcium-dependent kinase genes differentially regulated in NK cells of CFS/ME patients compared with healthy non-fatigued controls. Specifically 37 genes were upregulated and 55 genes were downregulated that are involved in numerous cell signalling and metabolic pathways including inflammation. While primarily indicating functional impairment in NK cytotoxic activity and immunological dysfunction, kinases are located throughout cells in the body and may be associated with other clinical manifestations reported in CFS/ME.

[001029] **Example 12 - A targeted genome association study examining transient receptor potential (TRP) ion channels, acetylcholine receptors (AChRs), and adrenergic receptors (ADRs) in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME)**

[001030] In this Example the inventors identify and characterise a SNP in adrenergic receptor α1 *(ADRA1A)* that is a potential cellular marker for CFS/ME.

[001031] **Introduction**

[001032] Biological processes responsible for the varied symptoms reported for CFS/ME may involve several ion channels and receptors that are located on cells throughout the body. Transient receptor potential ion channels (TRPs) are widely expressed on tissues and cells and are activated and regulated by various stimuli in the cellular environment such as pain, temperature, taste, pressure, and vision [5p]. There are six subfamilies including TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPV (vanilloid) [6p]. Most consist of non-selective channels permeable to cations such as calcium (Ca^{2+}), sodium (Na^{+}), and magnesium (Mg^{+}). This has an important role in maintaining homeostasis for a number of physiological requirements. Accordingly, dysregulation of these channels has been found to have a role in pathological conditions such as chronic pain, overactive bladder, diabetes, chronic obstructive pulmonary disease, cardiac hypertrophy, familial Alzheimer's disease, skin diseases, skeletal dysplasia's, neuropathy, and cancer [7p-12p].

[001033] In addition to TRPs, acetylcholine receptors (AChRs) are of particular interest due to their role in neurological and neuromuscular transmission [13p,14p]. Their function may have a role in difficulties processing information and short term memory loss reported in CFS/ME [1ρ.15ρ]. AChRs consist of two types that bind with acetylcholine and transmit its signal. Nicotinics (nAChRs) are ligand-gated ion channels and are involved in fast synaptic interactions

of neurotransmitters [16p]. Muscarinics (mAChRs) consist of 17 different subunits and are G-protein coupled receptors that facilitate slow metabolic responses through secondary messenger cascades [17p].

[001034] Moreover, adrenergic receptors (ADRs) are another class of G-protein coupled receptors which have catecholamine ligands [18p]. This binding is associated with stimulation of the sympathetic nervous system, commonly known for the fight or flight response in which energy is mobilised and blood flow is diverted from non-essential organs to skeletal muscle. There are 3 types of receptor; al, which is primarily involved in intracellular Ca^{2+} and subsequent smooth muscle contractions [19p]. The a2 receptors have a role in inhibition of neurotransmitters, decreased cAMP and decreased smooth muscle contraction. Beta receptors have 3 subtypes and alternatively increase cAMP activity resulting in heart muscle contractions, smooth muscle relaxation and glycogenolysis [20p,21p].

[001035] In the earlier Examples the inventors identified significant SNPs and genotypes in TRPs and AChRs in peripheral blood mononuclear cells in CFS/ME patients compared with healthy controls. Specifically, 13 significant SNPs in TRPs and 17 significant SNPs in AChRs were identified (9 mAChRs; 8 nAChRs). CFS/ME is largely characterised as a heterogeneous illness. The above ion channels and receptors were chosen as targets in a genome-wide association study due to their wide expression in cells and their involvement in numerous physiological processes. Hence, the purpose of this investigation was to identify whether association between SNPs for TRPs, AChRs, and ADRs are observed in patients with CFS/ME compared with healthy controls.

[001036] **Methodology**

[001037] *Participants*

[001038] Participants were from the National Centre for Neuroimmunology and Emerging Diseases (NCNED) research database for CFS/ME. Participants aged between 18 and 65 years were recruited from community support networks in the South East Queensland and Northern New South Wales region of Australia. All participants completed a screening questionnaire reporting their sociodemographic details, medical history, and symptoms. CFS/ME patients were classified according to Fukuda criteria (1). This required the presence of fatigue that significantly impacts with daily activities for at least 6 months. This should not be due to ongoing exertion or other medical conditions and accompanied by at least four of the following symptoms: post-exertional malaise, unrefreshing sleep, impairment of short-term memory or concentration, muscle pain, joint pain, headaches, tender lymph nodes, and/or sore throat. Healthy controls reported no evidence of disease. Exclusions were participants not meeting the above criteria or with other medical diagnoses that would exclude CFS/ME for example autoimmune disorder,

multiple sclerosis, psychosis, major depression, cardiovascular disease. Participants were also excluded if they were pregnant, breast feeding, smokers or had a history of substance abuse.

[001039] *DNA extraction*

[001040] Peripheral blood mononuclear cells were collected into ethylenadiaminetetraacetic acid tubes. Routine pathology was performed for screening of any abnormal parameters including full blood count, erythrocyte sedimentation rate, and high sensitivity C reactive protein by Pathology Queensland. The Qiagen DNA blood mini-kit was used to extract approximately 2μg of genomic DNA as per manufacturer instructions. To assess the quality and quantity of DNA, the nCounter Digital Analyzer (Nanostring, United States of America) optical scanner was used. Whole genome genotyping was performed using the HumanOmniExpress BeadChip array (Illumina, South Korea).

[001041] *Statistical analysis*

[001042] Statistical analysis was performed using PLINK vl.07 (http://pngu.mgh.harvard.edu/purcell/plink/) whole genome analysis software [Purcell, 2007p] to identify the frequency of SNPs. For quality control, a major allele frequency filter of <1% was applied. Further, SNPs with a variance lower than 2% were removed. Sample heterozygosity was also applied as a quality control measure and calculated as the proportion of heterozygous genotypes in relation to all genotypes at the SNP and sample levels. Data were compared between CFS/ME patients and healthy controls using R (R Core Team, 2013). Fisher's exact probability test was used to examine significant genotype association for each individual SNP, and a Bonferroni correction for multiple test correction was applied as post hoc analysis ($p<0.05$).

[001043] **Results**

[001044] *Demographic characteristics*

[001045] The majority of participants in this study were of Caucasian descent (97.8%). Of the 172 participants, 95 met criteria for CFS/ME and 77 met criteria for healthy controls, and the mean age and proportion female was 45.8 ± 8.9 (69% female) and 42.3 ± 10.3 (63% female) respectively. Potential confounding factors for analysis such as age, sex and ethnicity were analysed for interaction with genes of interest and no outliers were identified, hence no adjustments were required.

SNP association study

[001046] A total of 950 SNPs were included for analysis after quality control measures were applied. These are listed in Table 34a below.

Table 34a: 26: SNP variants of TRP channel, ACh receptors or ADR annotated with their consequence.

SNP	Location	Consequence	Gene
rs2072660	1:154576245-154576245	3_prime_UTR_variant	CHRNB2
rs38 1 1450	1:154578556-154578556	3_prime_UTR_variant	CHRNB2
rs726 168	1:23963 1064-239631064	intron_variant	CHRM3
rs12037424	1:239635 112-239635 112	intron_variant	CHRM3
rs1867263	1:239644620-239644620	intron_variant	CHRM3
rs16832 152	1:239648409-239648409	intron_variant	CHRM3
rs6691263	1:239648803-239648803	intron_variant	CHRM3
rsl0925941	1:239649238-239649238	intron_variant	CHRM3
rs12090480	1:239650653-239650653	intron_variant	CHRM3
rs4659550	1:239656203-239656203	intron_variant	CHRM3
rs1202 1900	1:239661754-239661754	intron_variant	CHRM3
rs10802789	1:239669380-239669380	intron_variant	CHRM3
rs10754677	1:239669800-239669800	intron_variant	CHRM3
rsl867266	1:239676005-239676005	intron_variant	CHRM3
rs66927 11	1:239683080-239683080	intron_variant	CHRM3
rs12406493	1:239689805-239689805	intron_variant	CHRM3
rs4145784	1:239694692-239694692	intron_variant	CHRM3
rs2278642	1:239703843-239703843	intron_variant	CHRM3
rs10802794	1:239707321 -239707321	intron_variant	CHRM3
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rs 15 14690	11:36358 17-3635817	dcwnstream_gene_variant	ART5
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rs660652	15:78595490-78595490	3_prime_UTR_variant	CHRNA3
rs578776	15:78596058-78596058	3_prime_UTR_variant	CHRNA3
rs6495307	15:78597979-78597979	intron_variant	CHRNA3
rsl05 1730	15:78601997-78601997	synonymous_variant	CHRNA3
rs3743077	15:78602554-78602554	intron_variant	CHRNA3
rsl2914385	15:7860638 1-78606381	intron_variant	CHRNA3
rs6495308	15:78615314-78615314	intiOn_variant	CHRNA3
rs3743074	15:78617138-78617138	splice_region_variant, intron_variant	CHRNA3
rs8040868	15:786 18839-78618839	synonymous_variant	CHRNA3
rs8 192475	15:786 18888-78618888	missense_variant	CHRNA3
rsl948	15:78625057-78625057	3_prime_UTR_variant	CHRNB4
rs950776	15:78633676-78633676	intiOn_variant	CHRNB4
rsl3 16971	15:78638 168-78638168	intiOn_variant	CHRNB4
rs72088 l 1	17:3513261 -3513261	3_prime_UTR_variant	TRPV3
rs7219780	17:35 15260-3515260	intron_variant	TRPV3
rs9909424	17:35 15304-3515304	intron_variant	TRPV3
rs808 1785	17:3516395-3516395	intron_variant	TRPV3
rs72 17270	17:351 8 18 1-3518181	intton_variant	TRPV3
rs17763099	17:3520145-3520145	intron_variant	TRPV3
rs72 12403	17:3526009-3526009	intron_variant	TRPV3
rs4790145	17:3528392-3528392	intron_variant	TRPV3
rs395357	17:3532786-3532786	synonymous_variant	TRPV3
rs401643	17:3536160-3536160	intron_variant	TRPV3
rs322942	17:3540949-3540949	intron_variant	TRPV3

rs11078458	17:3542607-3542607	synonymous_variant	TRPV3
rsl0395_19	17:3544620-3544620	synonymous_variant	TRPV3
rs991 1213	17:3545 140-3545 140	intron_variant	TRPV3
rsl2453_105	17:3548453-3548453	intron_variant	TRPV3
rsl699_138	17:3549024-3549024	intron_variant	TRPV3
rs322962	17:3555838-3555838	intron_variant	TRPV3
rs4790522	17:3566559-3566559	3_prime_UTR_variant	TRPV1
rsl6953_163	17:3567945-3567945	intron_variant	TRPV1
rs224546	17:3569577-3569577	intron_variant	TRPV1
rs11655540	17:3570651 -3570651	intron_variant	TRPV1
rs877610	17:3572196-3572196	synonymous_variant	TRPV1
rs990258_1	17:3579005-3579005	intron_variant	TRPV1
rs150908	17:358 1074-3581074	intron_variant	TRPV1
rs224534	17:3583408-3583408	missense_variant	TRPV1
rs17706630	17:3583848-3583848	intron_variant	TRPV1
rs222745	17:3585577-3585577	intron_variant	TRPV1
rs150846	17:3591574-3591574	intron_variant	TRPV1
rs222749	17:3592080-3592080	missense_variant	TRPV1
rs72 17945	17:3594276-3594276	intron_variant	TRPV1
rs2277675	17:3597216-3597216	intron_variant	TRPV1
rs17707155	17:3601939-3601939	intron_variant	TRPV1
rsl61373	17:3602749-3602749	intron_variant	TRPV1
rs222741	17:3605586-3605586	intron_variant	TRPV1
rs460716	17:36085 14-3608514	5_prime_UTR_variant	TRPV1
rsl053754	17:4897993-4897993	3_prime_UTR_variant	CHRNE
rs4790235	17:4902757-4902757	missense_variant	CHRNE
rs2302767	17:7447225-7447225	intron_variant	CHRNB l
rs2302765	17:7447656-7447656	splice_region_variant, intron_variant	CHRNB 1
rs12452047	17:74485 17-7448517	intron_variant	CHRNB 1
rs2302761	17:7455201 -7455201	intron_variant	CHRNB l
rs2302763	17:7455958-7455958	intron_variant	CHRNB l
rs2302764	17:7456791 -7456791	3_prime_UTR_variant	CHRNB l
rs3855924	17:7457004-7457004	3_prime_UTR_variant	CHRNB l
rs38 13769	17: 16415618-16415618	5_prime_UTR_variant	TRPV2
rs8079271	17: 16419050-16419050	intron_variant	TRPV2
rs8 121	17: 16422654-16422654	synonymous_variant	TRPV2
rs4792742	17: 16428506-16428506	intron_variant	TRPV2
rs12602006	17: 16433974-16433974	intron_variant	TRPV2
rs12979689	19:49160532-49 160532	intron_variant	TRPM4
rs3760666	19:49164094-49 164094	intron_variant	TRPM4
rsl477363	19:4917 1238-49 17 1238	intron_variant	TRPM4
rs2287923	19:49171976-49171976	intron_variant	TRPM4
rs909010	19:49188565-49188565	intron_variant	TRPM4
rsl 175803	19:49193800-49193800	intron_variant	TRPM4
rs17 16274	19:49203479-49203479	intron_variant	TRPM4
rs2229169	2:961 14968-961 14968	synonymous_variant	ADRA2B

rs2646165	2:174756754-174756754	intron_variant,non_coding_transcript_variant	AC018890.6
rs1376865	2:174759869-174759869	intron_variant,non_coding_transcript_variant	AC018890.6
rs2245601	2:232526227-232526227	synonymous_variant	CHRND
rs2767	2:232535364-232535364	3_prime_UTR_variant	CHRND
rs2289080	2:232541468-232541468	missense_variant	CHRNG
rsl88_1492	2:232542288-232542288	intron_variant	CHRNG
rs2853462	2:232542410-232542410	intron_variant	CHRNG
rs2099489	2:232545584-232545584	upstream_gene_variant	EIF4E2
rsl7862921	2:233920475-233920475	intron_variant	TRPM8
rs1003757	2:233921224-233921224	intron_variant	TRPM8
rs1003756	2:233921255-233921255	intron_variant	TRPM8
rs643_1648	2:233924812-2339248_12	intron_variant	TRPM8
rsl0803665	2:233925989-233925989	intron_variant	TRPM8
rs11563220	2:233926525-233926525	splice_region_variant,intron_variant	TRPM8
rsl_l563219	2:233926795-233926795	intron_variant	TRPM8
rs735552	2:233928621 -233928621	intron_variant	TRPM8
rs758277	2:233929092-233929092	intron_variant	TRPM8
rsl2473889	2:233936301 -233936301	intron_variant	TRPM8
rs7577157	2:233940259-233940259	intron_variant	TRPM8
rsl7868387	2:233945908-233945908	missense_variant	TRPM8
rs12466401	2:233948374-233948374	intron_variant	TRPM8
rsl0169266	2:233952992-233952992	intron_variant	TRPM8
rs4663990	2:233953396-233953396	intron_variant	TRPM8
rs10490013	2:233954593-233954593	intron_variant	TRPM8
rs7593557	2:233955 144-233955144	missense_variant	TRPM8
rs9 17435	2:233958869-233958869	intron_variant	TRPM8
rs10929320	2:233959741 -233959741	intron_variant	TRPM8
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rs28948671	2:233963 105-233963105	intron_variant	TRPM8
rsl2185625	2:233966789-233966789	intron_variant	TRPM8
rsl3401339	2:233967393-233967393	intron_variant	TRPM8
rs28902_187	2:233970784-233970784	intron_variant	TRPM8
rs67193 11	2:233974736-233974736	intron_variant	TRPM8
rsl3414162	2:233975300-233975300	intron_variant	TRPM8
rsl_1685673	2:233976386-233976386	intron_variant	TRPM8
rs10803667	2:233977209-233977209	intron_variant	TRPM8
rs17864755	2:233977719-233977719	intron_variant	TRPM8
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rs4663995	2:233983483-233983483	intron_variant	TRPM8
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rsl7864768	2:233989074-233989074	intron_variant	TRPM8
rsl_1563057	2:233989825-233989825	intron_variant	TRPM8

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rs2362294	2:23399 1869-233991869	intron_variant	TRPM8
rs11563056	2:233992526-233992526	intron_variant	TRPM8
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rs11563208	2:233996434-233996434	synonymous_variant	TRPM8
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rs6723922	2:233999852-233999852	intron_variant	TRPM8
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rs6721761	2:2340040 17-2340040 17	intron_variant	TRPM8
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rsl 1563204	2:234008733-234008733	intron_variant	TRPM8
rsl 04900 18	2:234009088-234009088	intron_variant	TRPM8
rs17864777	2:2340 10474-2340 10474	intron_variant	TRPM8
rsl7865678	2:234010670-234010670	intron_variant	TRPM8
rs17865679	2:234010944-234010944	intron_variant	TRPM8
rs3732214	2:234014480-234014480	intron_variant	TRPM8
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rs8 183794	20:4229801 -4229801	intron_variant	ADRAID
rs6 116268	20:4230793-4230793	intron_variant	ADRA1D
rs9461 88	20:4234669-4234669	intron_variant	ADRA1D
rs48 15670	20:4236217-4236217	intron_variant	ADRAID
rs48 15675	20:4242807-4242807	intron_variant	ADRAID
rs6052456	20:4244926-4244926	intron_variant	ADRAID
rs946189	20:4246877-4246877	intron_variant	ADRAID
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rs606015 1	20:35006423-35006423	downstream_ gene_variant	MYH7B
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rs6088678	20:35019748-35019748	intron_variant	TRPC4AP
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rsl3042358	20:35046676-35046676	intron_variant	TRPC4AP
rs8 117847	20:35054677-35054677	intron_variant	TRPC4AP
rsl998233	20:35069323-35069323	synonymous_variant	TRPC4AP
rs6090378	20:63344026-63344026	3_prime_UTR_variant	CHRNA4
rs1044394	20:63350733-63350733	synonymous_variant	CHRNA4
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rs1785469	21:44398726-44398726	intron_variant	TRPM2

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rs2238722	21:44420722-44420722	intron_variant	TRPM2
rs2003775	2 1:44434503-44434503	intron_variant	TRPM2
rs 1403725	3:142725291 -142725291	intron_variant	TRPC1
rs 13094259	3:142725783-142725783	intron_variant	TRPC1
rs953239	3:142727363-142727363	intron_variant	TRPC1
rs2177398	3:142768058-142768058	intron_variant	TRPC1
rs9836269	3:142772883-142772883	intron_variant	TRPC1
rs 13086677	3:142784353-142784353	intron_variant	TRPC1
rs7621642	3:142784763-142784763	synonymous_variant	TRPC1
rsl6852615	3:142797984-142797984	intron_variant	TRPC1
rs382 1647	3:142804507-142804507	synonymous_variant	TRPC1
rs4627	3:142807752-142807752	downstream_gene_variant	TRPC1
rs 10022491	4:40335891 -40335891	synonymous_variant	CHRNA9
rsl0021263	4:40340601 -40340601	intron_variant	CHRNA9
rs4861065	4:40342378-40342378	intron_variant	CHRNA9
rs486 1307	4:40343222-40343222	intron_variant	CHRNA9
rs 100293 13	4:40348 130-40348130	intron_variant	CHRNA9
rs7669882	4:40348634-40348634	intron_variant	CHRNA9
rs 10029872	4:40348746-40348746	intron_variant	CHRNA9
rs 10009228	4:40354405-40354405	missense_variant	CHRNA9
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rs 11726 196	4:12 1885073-121885073	intron_variant	TRPC3
rsl2502635	4:121892304-121892304	intron_variant	TRPC3
rs2 135976	4:121895026-121895026	intron_variant	TRPC3
rs 11732666	4:121902897-121902897	synonymous_variant	TRPC3
rsl75 17624	4:121903436-121903436	intron_variant	TRPC3
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rs950574	4:12 1943086-121943086	intron_variant	TRPC3
rs970349	4:121950477-121950477	intron_variant	TRPC3
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rs10427 18	5:148827354-148827354	synonymous_variant	ADRB2
rs3729604	5:159917454-159917454	synonymous_variant	ADRA1B
rs2030373	5:159920474-159920474	intron_variant	ADRA1B
rs6884105	5:159921436-159921436	intron_variant	ADRA1B
rs17455628	5:159921891 -159921891	intron_variant	ADRA1B
rs756275	5:159924453-159924453	intron_variant	ADRA1B
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rs105 15805	5:159938560-159938560	intron_variant	ADRA1B
rs4921242	5:159939691 -159939691	intron_variant	ADRA1B
rs6888306	5:159940107-159940107	intron_variant	ADRA1B
rs77 18362	5:159940858-159940858	intron_variant	ADRA1B
rsl 1743425	5:159941279-159941279	intron_variant	ADRA1B
rs7737796	5:159942422-159942422	intron_variant	ADRA1B
rs34467921	5:159950714-159950714	intron_variant	ADRA1B
rsl3 171967	5:15995 1152-159951 152	intron_variant	ADRA1B
rs3896275	5:159954519-159954519	intron_variant	ADRA1B
rsl2653825	5:159956264-159956264	intron_variant	ADRA1B
rs952037	5:159963870-159963870	intron_variant	ADRA1B
rs7636	7:100892456-100892456	upstream_gene_variant	UFSP1
rs1799805	7:100893 176-100893176	upstream_gene_variant	UFSP1
rs3735028	7:13687341 1-13687341 1	intron_variant	CHRM2
rs10954565	7:136875501 -136875501	intron_variant	CHRM2
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rs2113550	7:13688 1786-136881786	intron_variant	CHRM2
rs1424569	7:136884669-136884669	intron_variant	CHRM2
rs17494846	7:136885565-136885565	intron_variant	CHRM2
rsl 0242 108	7:136886960-136886960	intron_variant	CHRM2
rs4475425	7:136890452-136890452	intron_variant	CHRM2
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rsl2535371	7:136899328-136899328	intron_variant	CHRM2

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rs324594	7:136970576-136970576	intron_variant	CHRM2
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rs4208 17	7:137002656-137002656	intron_variant	CHRM2
rs324640	7:137004249-137004249	intron_variant	CHRM2
rs10488602	7:137005756-137005756	intron_variant	CHRM2
rs4987682	7:142871843-142871843	downstream_ gene_variant	EPHB6
rs4987668	7:142874896-142874896	downstream_ gene_variant	EPHB6
rs4987667	7:142875 155-142875155	downstream_ gene_variant	EPHB6
rs4987665	7:142875510-1428755 10	dcwnstream_ gene_variant	EPHB6
rs4252499	7:1429 12583-1429 12583	missense_variant	TRPV5
rs4252460	7:142920130-142920130	intron_variant	TRPV5
rs4252448	7:142922613-142922613	intron_variant	TRPV5
rs4252435	7:142925619-142925619	synonymous_variant	TRPV5
rs4252424	7:142927238-142927238	intron_variant	TRPV5
rs4252417	7:142928 163-142928 163	synonymous_variant	TRPV5
rs4252416	7:142928322-142928322	intron_variant	TRPV5
rs4252407	7:142929561 -142929561	synonymous_variant	TRPV5
rs4252402	7:142930043-142930043	intron_variant	TRPV5
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rs1442341	8:26752910-26752910	intron_variant	ADRA1A
rs4732853	8:26753 134-26753134	intron_variant	ADRA1A
rs17055923	8:26754329-26754329	intron_variant	ADRA1A

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rs4236678	8:26760849-26760849	intron_variant	ADRA1 A
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rsl 7060568	9:74828508-74828508	intron_variant	TRPM6
rs7867868	9:7483 1958-74831958	inrrc>n_variant	TRPM6
rsl475717	9:74839224-74839224	inti on_variant	TRPM6
rsl2378991	9:74857150-74857150	intron_variant	TRPM6
rs6560417	9:74860672-74860672	intron_variant	TRPM6
rs21841 18	9:7486 1470-74861470	intron_variant	TRPM6
rs9650770	9:74871543-74871543	intron_variant	TRPM6
rs7858012	9:74879240-74879240	intton_variant	TRPM6
rsl333343	9:74887406-74887406	intiOn_variant	TRPM6
rs3027744	X:1 11822103-1 11822103	intron_variant	TRPC5
rsl0521536	X:1 11894324-1 11894324	intron_variant	TRPC5
rs7050529	X:l 11912005-1 11912005	intron_variant	TRPC5
rs4893416	X:l 11913443-1 11913443	intron_variant	TRPC5
rs2238999	X:1 11916179-1 11916179	intron_variant	TRPC5

rs5985655	X:1 11917973-1 11917973	intron_variant	TRPC5
rs5943223	X:1 11922316-1 11922316	intron_variant	TRPC5
rs767034	X:1 11925601-1 11925601	intron_variant	TRPC5
rs5943226	X:1 11935514-1 11935514	intron_variant	TRPC5
rs7876872	X:l 11943013-1 11943013	intron_variant	TRPC5
rs17222629	X:111972464-1 11972464	intron_variant	TRPC5
rs7063059	X:111972495-1 11972495	intron_variant	TRPC5
rs1009560	X:1 11991013-1 11991013	intron_variant	TRPC5
rs16986729	X:1 12005891-1 12005891	intron_variant	TRPC5
rs6642976	X:1 12025020-1 12025020	intron_variant	TRPC5
rsl6986741	X:1 12027326-1 12027326	intron_variant	TRPC5
rs16986742	X:112030800- 112030800	intron_variant	TRPC5
rs7060180	X:112042274- 112042274	intron_variant	TRPC5
rs16986746	X:1 12042431-1 1204243 1	intron_variant	TRPC5

[001047] The distribution of these SNPs per chromosome is summarised in Figure 24. Accordingly, the majority of SNPs were observed on chromosome 9 (204 SNPs).

[001048] Figure 25 demonstrates a Manhattan plot of results of Fisher's exact test. Blue line (ie. the lower line between 1 and 2 on the log axis) corresponds to the significant threshold without any adjustment (raw p-values). Prior to Bonferroni correction, 60 significant SNPs were associated with CFS/ME compared with healthy controls. The red line (ie. the upper line between four and five on the log axis) corresponds to the significant threshold after Bonferroni correction.

[001049] The raw p-values of the top 10 SNPs identified are summarised in Table 34b. The corresponding frequencies in CFS/ME compared with healthy controls are shown in Figure 26.

[001050] **Table 34b.** Results of Fisher's exact test for top 10 SNPs prior to Bonferroni corrections

SNP name	raw p-value	padj FDR	padj Bonferroni
"rs2322333 "	"6.2e-05"	"0.059"	"0.059"
"xs4779524"	"0.002"	"0.788"	"1"
"rs11787707"	"0.004"	"0.788"	"1"
"Tsi04€7S9e"	"0.005"	"0.788"	"1"
"rs10118380"	"0.01"	"0.788 "	"1"
"rs7022747 "	"0.011"	"0.78S"	"1"
"rs13i697!"	"0.013"	"0.7SS'	"1"
"rs526302"	"0.013"	"0.788"	"1"
"rs6719311"	"0.013"	"0.788"	"1"
"rsli?82i5S*	"0.016"	"0.TBS"	"1"

[001051] Following adjustment using Bonferroni correction, the association with adrenergic α1A *(ADRA1A)* SNP *rs2322333* located on chromosome 8 was almost significant (p=0.058)

(Figure 27). The proportion of CFS/ME patients being homozygous major (GG) for this SNP was higher compared with healthy controls. Moreover, the genotype class that was homozygous minor (AA) was much lower in CFS/ME patients compared with healthy controls (4.2% vs. 24.7) (Figure 27).

[001052] **Discussion**

[001053] This study is the first to identify *ADRA1A* as a novel candidate gene for CFS/ME according to whole genome analysis. After stringent corrections for multiple testing were applied, the *ADRA1A* SNP remained predominant. Moreover, the proportion of patients that were homozygous minor, AA was much lower in CFS/ME compared with healthy controls. These results specifically suggest that patients exhibiting this allele marker may have a decreased risk of development of CFS/ME.

[001054] The specific physiological implications of *ADRA1A* are mainly involved in smooth muscle contraction [20p]. This is required for vasoconstriction of blood vessels throughout the body including the skin, gastrointestinal system, genitourinary system, kidney and brain. It is also involved in the glyogenolysis and gluconeogenesis of adipose tissue in the liver, in addition to secretions from sweat glands [24p, 25p, 26p]. These above processes have been commonly reported in the symptomatology of CFS/ME [3p,4p]. Hence, the differential expression of *ADRA1A* may explain particular clinical phenotypes of CFS/ME.

[001055] *ADRA1A* are members of the superfamily for G protein-coupled receptors [27p]. When activated, heterotrimeric G protein (G_g) in turn activates phospholipase (PLC). PLC cleaves phosphatidylinositol 4,5-biphosphate (PIP2), which leads to an increase in inositol triphosphate (IP3) and diacyglycerol (DAG) (REFS). IP3 acts as a secondary messenger and is a soluble molecule that is able to diffuse through the cytoplasm to the endoplasmic reticulum of cells (or sarcoplasmic reticulum in muscle cells) to stimulate Ca^{2+} influx. This involves the binding of IP3 ligand to IP3 sensitive Ca^{2+} channels that result in the release of Ca^{2+} into the cytoplasm [28p,29p]. This contributes to a number of cellular processes, including a slow after depolarizing current (sADP) in neurons [30p].

[001056] As described in an earlier Example, the inventors investigated the dysregulation of Ca^{2+} dependent kinase genes in isolated Natural Killer (NK) cells from CFS/ME patients [Chacko et al. 2016p]. Compared with healthy controls, reduced NK cytotoxic activity is consistently reported in CFS/ME patients [31p-39p]. In NK cells, Ca^{2+} signaling has a vital role in the granule dependent pathway of apoptosis [40p]. Ca^{2+} is required for inducing cytolytic granule polarisation, cytokine gene transcription and degranulation in NK cells [41p,42p]. The inventors found that 92 significant Ca^{2+} dependent protein kinase genes were differentially expressed in a clinically severe (housebound or bedridden) CFS/ME group compared with non-

fatigued controls. These may contribute downstream to impairments in intracellular signalling networks and effector function. Accordingly, the inventors have also demonstrated significant impairments in the MAPK signalling pathway, as well as observed decreased intracellular Ca^{2+} concentration in NK cells as well as isolated B cells from CFS/ME patients.

[001057] In addition to adrenergic receptors, this study selected genes for TRPs, AChRs, and acetylcholinesterase due their role in neurological, sensory and motor function that feature as symptoms of CFS/ME. Although these did not remain significant following post-hoc analysis, additional genes that were observed at a higher frequency in CFS/ME patients included *TRPC1, TRPM1, TRPM3, TRPM6, TRPM8* and *CHRNB4.* Previously in the Examples above, the inventors examined 678 SNPs in isolated NK cells in CFS/ME patients and identified 11 significant TRP ion channel genes for *TRPC4, TRPC2, TRPM3, and TRPM8,* as well as 14 significant AChR genes including *CHRNA2, CHRNA2, CHRNB4, CHRNA5,* and *CHRNE* ($p<0.05$). Importantly the present study examined an additional 950 SNP in which there were only 80 overlapping with the previous studies in the earlier Examples. *TRPM3* in particular is known to have a vital role in Ca^{2+} signalling and was prominent across the inventors' analyses. Hence, the inventors have also previously investigated and reported a significantly decreased surface expression of *TRPM3* on NK and B cells.

[001058] It is not known whether the associations observed in this study may be involved in the underlying biological mechanism of CFS/ME. Of particular interest is if the functional role of the SNP *rs2322333* identified in this study is involved in the regulation of further genes. This SNP is located within the intron of *ADRA1A,* some GWAS studies have indicated that intronic genes may regulate the transcription of a nearby gene by specific chromatin looping [47p]. Furthermore, the results of this study are indicative that a larger cohort should be examined to determine if being homozygous minor for various allele markers have a protective effect from CFS/ME.

[001059] This study is the first genome-wide association study conducted on an Australian cohort with CFS/ME. A particular strength of this study was a considerable association with *ADRA1A* being detected among a preliminary cohort of patients, when strict statistical considerations were applied.

[001060] **Conclusion**

[001061] In conclusion, this study demonstrated that *ADRAIA* is a potential cellular marker for CFS/ME. It is recommended that future studies examine their functional role in the variation of further genes to further elucidate whether these allele markers have a potential protective role against CFS/ME.

[001062] **Example 13 - AchR, TRP and ADR Gene and Gene Product-Based Probes,**

Tools and Reagents as well as Other Types of Tools and Reagents

[001063] The Examples above explain how TRP, AchR and ADR SNPs can be used as 'tools' for identifying subjects with, or predisposed to, CFS/ME as well as other medical conditions or symptoms thereof. This key SNP finding enables the inventors to develop TRP ion channel, ACh receptor or ADR gene/allele-based and gene product-based probes, tools, reagents, methods and assays for identifying, screening, diagnosing, monitoring and/or treating subjects with, or predisposed to, those medical conditions/symptoms.

[001064] One of skill in the art could readily design, produce or manufacture a wide range of TRP ion channel, ACh receptor or ADR gene/allele-based and gene product-based probes, tools, reagents, methods and assays based on the information of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, and 34.

[001065] Generally speaking, such TRP ion channel, ACh receptor or ADR gene/allele-based or gene product-based probes, tools, reagents, methods and assays can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to, those medical conditions.

[001066] Generally speaking, such probes, tools or reagents based on or developed from a TRP ion channel, ACh receptor or ADR gene or gene product can, for example, specifically bind, detect, identify, characterise or quantify the gene or part of the gene, the RNA gene product or part of the RNA gene product, the polypeptide gene product or part of the polypeptide gene product.

[001067] Generally speaking, such probe, tool or reagent can be for detection of a polymorphism at the genomic level, at the transcription level or polypeptide level.

[001068] Generally speaking, such probe, tool or reagent can be for quantitative or qualitative measurement of RNA transcription or translation.

[001069] Generally speaking, such probe, tool or reagent can also be an antibody or other type of molecule or chemical entity capable of detecting the gene or gene product (RNA or polypeptide).

[001070] More specifically, probes, tools and reagents of particular interest include, but are not limited to, the following:

[001071] 1. An isolated, purified, synthetic or recombinant form of TRP, AchR or ADR, or a fragment thereof, including a fragment containing a SNP of interest - single stranded or double stranded.

[001072] 2. A non-naturally occurring polynucleotide, recombinant polynucleotide, oligonucleotide or cDNA form of TRP, AchR or ADR, or a fragment thereof, including a fragment containing a SNP of interest - single stranded or double stranded.

[001073] 3. An expression product (mRNA) of TRP, AchR or ADR, or a fragment thereof, including a fragment containing a SNP of interest. Depending on the SNP, the mRNA may differ from an expression product in a healthy individual. The expression product may be unlabelled or labelled with a detectable moiety.

[001074] 4. A polynucleotide, oligonucleotide, probe or primer (unlabelled or labelled with a detectable moiety) for specifically binding to, annealing to, detecting, isolating or amplifying (eg. by PCR) TRP, AchR or ADR, or a fragment thereof, including a SNP of interest.

[001075] 5. A polynucleotide, oligonucleotide, probe or primer (unlabelled or labelled with a detectable moiety) for specifically binding to, annealing to, detecting, isolating or amplifying (eg. by PCR) the expression product of 3.

[001076] 6. An expression vector, recombinant cell or biological sample comprising the nucleic acid or polynucleotide of 1, 2, 3, 4 or 5.

[001077] 7. An expression product (polypeptide/protein) of TRP, AchR or ADR, or a fragment thereof, including a fragment containing a SNP of interest. Depending on the SNP, the polypeptide may differ from a polypeptide in a healthy individual. The polypeptide may be unlabelled or labelled with a detectable moiety or for isolation (eg. tagged at the C- or N-terminus).

[001078] 8. A monoclonal or polyclonal antibody capable of binding to the expression product of 7.

[001079] Yet other probes, tools and reagents are described in the specification section entitled "Detailed Description".

[001080] The key SNP finding also enables the inventors to develop kits, assays, microarrays, biochips and methods for identifying, screening, diagnosing, monitoring and/or treating subjects with, or predisposed to, the medical conditions/symptoms described in this specification.

[001081] Generally speaking, the kit, assay, microarray, biochip or method for identifying, screening, diagnosing, monitoring and/or treating subjects with, or predisposed to, the medical conditions/symptoms, can comprise one or more materials of any one of 1-8. This may be, for example, for genotyping, or identifying or measuring gene product expression or lack of expression.

[001082] Yet other kits, assays, microarrays, biochips and methods are described in the specification section entitled "Detailed Description".

[001083] Examples of preferred polynucleotides, oligonucleotides, probes or primers for specifically binding to, annealing to, detecting, isolating or amplifying (eg. by PCR) the SNPs of TRP or AchR are shown in Table 35. An example of a preferred polynucleotide, oligonucleotide, probe or primer for specifically binding to, annealing to, detecting, isolating or amplifying (eg.

by PCR) a SNP of ADR is shown in Table 36.

[001084] **Table 35:** Preferred polynucleotides, oligonucleotides, probes or primers for detecting the SNPs of TRP or AChR. (On next page.)

Gene	RefSNP ID	SNP ID	Forward Primer ID	Forward Primer Sequence	Reverse Primer ID	Reverse Primer Sequence	Extended Primer ID	Extended Primer Sequence
TRPM3	rs12682832	rs12682832_W3	rs12682832_W3_F	ACGTTGGATGAGCCTCCTTCTGACTTGAAC (SEQ ID No. 1)	rs12682832_W3_R	ACGTTGGATGCATTTCACCTACAAGTGATG (SEQ ID No. 2)	rs12682832_W3_E	cGATGGAATTTGACCCAAC (SEQ ID No. 3)
TRPM3	rs11142508	rs11142508_W9	rs11142508_W9_F	ACGTTGGATGGCTCCGTATGTGCTGAGAG (SEQ ID No. 4)	rs11142508_W9_R	ACGTTGGATGAGAAATACAGCGCTGGCTTC (SEQ ID No. 5)	rs11142508_W9_E	aGGGGCTTGTGTGTAA (SEQ ID No. 6)
TRPM3	rs1160742	rs1160742_W6	rs1160742_W6_F	ACGTTGGATGTTCTCACAGTTAAGGCCTTG (SEQ ID No. 7)	rs1160742_W6_R	ACGTTGGATGGCTGCTAATGATAGAGGCTG (SEQ ID No. 8)	rs1160742_W6_E	TACATGGGGATTTACATAG ACTA (SEQ ID No. 9)
TRPM3	rs4454352	rs1160742_W6	rs1160742_W6_F	ACGTTGGATGTTCTCACAGTTAAGGCCTTG (SEQ ID No. 10)	rs1160742_W6_R	ACGTTGGATGGCTGCTAATGATAGAGGCTG (SEQ ID No. 11)	rs1160742_W6_E	TACATGGGGATTTACATAG ACTA (SEQ ID No. 12)
TRPM3	rs1328153	rs1160742_W6	rs1160742_W6_F	ACGTTGGATGTTCTCACAGTTAAGGCCTTG (SEQ ID No. 13)	rs1160742_W6_R	ACGTTGGATGGCTGCTAATGATAGAGGCTG (SEQ ID No. 14)	rs1160742_W6_E	TACATGGGGATTTACATAG ACTA (SEQ ID No. 15)
TRPM3	rs3763719	rs3763619_W9	rs3763619_W9_F	ACGTTGGATGCTCAGGCAAAGGGTATTCA C (SEQ ID No. 16)	rs3763619_W9_R	ACGTTGGATGAGAACCTAAGAACCCAAGG C (SEQ ID No. 17)	rs3763619_W9_E	gggaAGAGATTTAGAGGTTG TACC (SEQ ID No. 18)
TRPC4	rs6650469	rs6650469_W4	rs6650469_W4_F	ACGTTGGATGTTGCTGGTGGTGGCTTAAA C (SEQ ID No. 19)	rs6650469_W4_R	ACGTTGGATGCTAGGGTGAACAACTTGAA C (SEQ ID No. 20)	rs6650469_W4_E	ggggACCTTTCAAAAGAGTG ATAC (SEQ ID No. 21)
TRP	rs65520	rs655207_	rs655207_W3	ACGTTGGATGAAGGTTCAAGTTGTTCACCC	rs655207_W3	ACGTTGGATGTTACCTGCCTTTTACCACAC	rs655207_W3_	cCCTCCTTCCAGGAACTTA

C4	7	W3	_F	(SEQ ID No. 22)	_R	(SEQ ID No. 23)	E	C (SEQ ID No. 24)
TRPA1	rs4738202	rs4738202_W8	rs4738202_W8_F	ACGTTGGATGAGTGTTCCAATCGCTCTGTG (SEQ ID No. 25)	rs4738202_W8_R	ACGTTGGATGAATCAACTGAGAACCATTC (SEQ ID No. 26)	rs4738202_W8_E	cttcTAATATACAGCCATGTCATAGA (SEQ ID No. 27)
TRPM3	rs7865858	rs7865858_W7	rs7865858_W7_F	ACGTTGGATGGGAAAAACAATTTCTTGGGG (SEQ ID No. 28)	rs7865858_W7_R	ACGTTGGATGCCCACCTATGACCATTTTCC (SEQ ID No. 29)	rs7865858_W7_E	GACCATTTTCCTCAGAGA (SEQ ID No. 30)
TRPA1	rs2383844	rs2383844_W7	rs2383844_W7_F	ACGTTGGATGCATCAAGACAGATTTCAAC (SEQ ID No. 31)	rs2383844_W7_R	ACGTTGGATGCCTACATCTCATCAAAGGAC (SEQ ID No. 32)	rs2383844_W7_E	ggTACAGAATAAGAAAGTTTGAGATTA (SEQ ID No. 33)
TRPM3	rs1504401	rs1504401_W6	rs1504401_W6_F	ACGTTGGATGCGTTTGTGTTTATGCCCCTC (SEQ ID No. 34)	rs1504401_W6_R	ACGTTGGATGGGAGTTTGCTATATTATTCCC (SEQ ID No. 35)	rs1504401_W6_E	ggggcACCATTACAGGTAATTTCCA (SEQ ID No. 36)
TRPM3	rs10115622	rs10115622_W4	rs10115622_W4_F	ACGTTGGATGTTTTCCCTTATTCCTCCCAC (SEQ ID No. 37)	rs10115622_W4_R	ACGTTGGATGACCTCTAGCCTCTGAATTGC (SEQ ID No. 38)	rs10115622_W4_E	GGAGGAGAAACAAACTCCAG (SEQ ID No. 39)
TRPM4	rs10403114	rs10403114_W7	rs10403114_W7_F	ACGTTGGATGAAAGTGGGCGGGGACATAG (SEQ ID No. 40)	rs10403114_W7_R	ACGTTGGATGAAAAACACGCCCCATTGCT C (SEQ ID No. 41)	rs10403114_W7_E	AAGTCACGCCCCTTC (SEQ ID No. 42)
TRPV3	rs9909424	rs9909424_W6	rs9909424_W6_F	ACGTTGGATGGAAATGATGCTTTCCACGG G (SEQ ID No. 43)	rs9909424_W6_R	ACGTTGGATGAACTGCCTGAGCCTACAGA C (SEQ ID No. 44)	rs9909424_W6_E	ctgcaTGAGCCTACAGACCACCTTCT (SEQ ID No. 45)
TRPC4	rs612308	rs612308_W8	rs612308_W8_F	ACGTTGGATGGAGGCTTTTAATCAACTCCC (SEQ ID No. 46)	rs612308_W8_R	ACGTTGGATGGATAATTTTTCTGTGACAGA C (SEQ ID No. 47)	rs612308_W8_E	gacacTGTCTTTCATTTGACTTGT (SEQ ID No. 48)

TRP M3	rs7860377	rs7860377_W9	rs7860377_W9_F	ACGTTGGATGCTGGTGGGAGAATGCAAGTC (SEQ ID No. 49)	rs7860377_W9_R	ACGTTGGATGGGCTAATAGTCCCTTTTACC (SEQ ID No. 50)	rs7860377_W9_E	gggGTCATGTTTTTCCATTGTCA (SEQ ID No. 51)
TRP C7	rs2673930	rs2673930_W6	rs2673930_W6_F	ACGTTGGATGTGTCAACCTAGTAGACGAGC (SEQ ID No. 52)	rs2673930_W6_R	ACGTTGGATGTGGAGATGCATCCTCTAGGC (SEQ ID No. 53)	rs2673930_W6_E	AGGCGAAAGCTCTAATT (SEQ ID No. 54)
TRP C4	rs603955	rs603955_W9	rs603955_W9_F	ACGTTGGATGACCATCTGCAGGACTTTAGG (SEQ ID No. 55)	rs603955_W9_R	ACGTTGGATGCTTTTGGGGCTGAGTTTAAG (SEQ ID No. 56)	rs603955_W9_E	ccccgCTCTTCCTTCAAAACTATCTTG (SEQ ID No. 57)
TRP M3	rs11142798	rs11142798_W9	rs11142798_W9_F	ACGTTGGATGGGGTAAAAGAATTACACAAG (SEQ ID No. 58)	rs11142798_W9_R	ACGTTGGATGTGCCTGAATTATGCAATAG (SEQ ID No. 59)	rs11142798_W9_E	TATGCAATAGAATCACTTGGT (SEQ ID No. 60)
TRP M3	rs4744611	rs4744611_W9	rs4744611_W9_F	ACGTTGGATGTCTTCTCCAGTGTCTAAGGG (SEQ ID No. 61)	rs4744611_W9_R	ACGTTGGATGCCCAATGTTACATGGCTTCC (SEQ ID No. 62)	rs4744611_W9_E	AGGCTACAGAGCTGA (SEQ ID No. 63)

[001085] **Table 36:** A preferred polynucleotide, oligonucleotide, probe or primer for detecting a SNP of ADR.

Gene: *ADRA1A*
Sequence: CTCATCCTGTCTTTGCAGGAGATTCTGGGTATATAGTTCCTCCAGAGACA (SEQ ID No. 64)

[001086] One or more Examples above explain how calcium metabolism testing can be used for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof - particularly CFS/ME. This key finding by the inventors allows one of skill in the art to develop probes, tools, reagents, methods and assays for calcium metabolism testing, as also described elsewhere.

[001087] Example 11 above explains how a differentially regulated calcium-dependent kinase gene can be used as an indicator of a medical condition or symptom thereof - particularly severe CFS/ME. This key finding by the inventors allows one of skill in the art to develop probes, tools, reagents, methods and assays for detecting the differentially regulated calcium-dependent kinase gene, as also described elsewhere.

[001088] Example 10 above explains how Natural Killer (NK) cells (and other cell types or tissues) can be tested in a subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, whereby dysfunctional signalling indicates that the subject has the medical condition or symptom thereof - particularly CFS/ME. This key finding by the inventors allows one of skill in the art to develop probes, tools, reagents, methods and assays for assaying or characterising the cell Mitogen-Activated Protein Kinase pathway, as also described elsewhere.

[001089] **Example 14 - AchR, TRP and ADR SNPs; Differentially Regulated Calcium-dependent Kinase Genes; and Dysfunctional Signalling Through the MAPK Pathway, as Indicators of Medical Conditions**

[001090] Based on Examples 1 to 9 and 12, the skilled person will appreciate that the SNPs listed in the earlier Tables, such as Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34, can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to, CFS or specific symptoms thereof as well as ME or specific symptoms thereof.

[001091] The skilled person will also appreciate that the SNPs listed in the earlier Tables, such as Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34, can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to, other medical

conditions or specific symptoms thereof, such as: IBS; MCS; non-allergic rhinitis; fibromyalgia; migraine; rheumatoid arthritis.

[001092] The skilled person will also appreciate that the SNPs listed in the earlier Tables, such as Tables 1 to 7, 9, 10, 12 to 17, 26 to 28 and 34, can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to, other medical conditions or specific symptoms thereof: caused by dysregulation in calcium (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in acetylcholine (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in TRP (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine); caused by dysregulation in ADR; caused by dysregulation of the gastrointestinal, cardiovascular, neurological and immune systems (especially in respect of CFS, ME, GWS, IBS, MCS, non-allergic rhinitis, fibromyalgia or migraine).

[001093] Specific symptoms of CFS or ME include: neuromuscular fatigue, particularly fatigue upon exertion; memory and concentration difficulties; muscle and joint pain; altered blood pressure, particularly postural orthorstatic tachycardia syndrome; headache; immunological dysregulation; sore throat; swollen lymph nodes/glands; gastrointestinal symptoms including IB, diarrhoea, constipation and abdominal pain; chemical sensitives; and intolerances to drugs and chemicals.

[001094] MCS conditions/symptoms include: headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; or respiratory symptoms.

[001095] Medical conditions caused by dysregulation in calcium, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation such as: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and immunological "allergic" sensitivities.

[001096] Medical conditions caused by dysregulation in acetylcholine, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation such as: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness;

memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems (immunological "allergic" sensitivities).

[001097] Medical conditions caused by dysregulation in TRP are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; dysregulation of the gastrointestinal, cardiovascular and immune systems; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and dysregulation of the gastrointestinal, cardiovascular and immune systems (immunological "allergic" sensitivities).

[001098] Medication conditions caused by dysregulation in ADR are typified by specific symptoms such as respiratory difficulties including shortness or breath, air hunger, colds and nasalpharynx congestion, cardiovascular conditions such as as hypertension, and palpitations, gastrointestinal illness, kidney disease, diabetes, and autonomic function including sweating episodes.

[001099] Medical conditions caused by dysregulation of the gastrointestinal, cardiovascular and immune systems, (especially in respect of CFS, ME, GWS, IBS, MCS, fibromyalgia or migraine), are typified by specific symptoms or dysregulation, including: significant impairment in physical activity; debilitating fatigue accompanied by impairment in memory, cognition and concentration; enhanced experience of pain; headache; fatigue; confusion; depression; shortness of breath; arthralgia; myalgia; nausea; dizziness; memory problems; gastrointestinal symptoms; respiratory symptoms; and immunological "allergic" sensitivities.

[001100] The inventors note that up to 45% of patients with CFS have IBS. M3 muscarinic drugs are being used to target IBS. The inventors have identified abnormalities in the Ach receptors in patients with CFS.

[001101] The inventors also note that many patients with CFS have headache and chemical smell sensitivity. The TRPV1 receptor has been reported to be increased in this condition.

[001102] Based on Example 10, the skilled person will appreciate that testing cells (such as NK cells) for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to a medical condition described above.

[001103] Based on Example 11, the skilled person will appreciate that one or more

differentially regulated calcium-dependent kinase genes as listed in Tables 31 and 32 can be used for identifying, screening, diagnosing, monitoring or treating/managing subjects with, or predisposed to a medical condition described above.

[001104] In the present specification and claims, the word 'comprising' and its derivatives including 'comprises' and 'comprise' include each of the stated integers but do not exclude the inclusion of one or more further integers.

[001105] Reference throughout this specification to ‘one embodiment' or 'an embodiment' means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearance of the phrases 'in one embodiment' or 'in an embodiment' in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more combinations.

[001106] The articles 'a' and 'an' are used herein to refer to one or to more than one of the article.

[001 107] The term 'about' is to be understood as referring to a range of numbers that a person of skill in the art would consider equivalent to the recited value in the context of achieving the same function or result.

[001 108] In compliance with the statute, the invention has been described in language more or less specific to structural or methodical features. It is to be understood that the invention is not limited to specific features shown or described since the means herein described comprises preferred forms of putting the invention into effect. The invention is, therefore, claimed in any of its forms or modifications within the proper scope of the appended claims appropriately interpreted by those skilled in the art.

CITATION LIST

[The entire contents of which are incorporated herein by way of cross-reference.]

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CLAIMS

1. At least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene for use as an indicator of a medical condition or symptom thereof.

2. At least one probe, tool or reagent based on or developed from at least one TRP ion channel, ACh receptor and/or ADR gene or gene product for use as an indicator of a medical condition or symptom thereof.

3. Use of at least one SNP of at least one TRP ion channel, ACh receptor and/or ADR gene for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof.

4. A method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

   (a) genotyping said subject for at least one polymorphism in at least one TRP ion channel, ACh receptor and/or ADR gene to obtain a result; and

   (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

5. A method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

   (a) testing said subject for at least one TRP ion channel, ACh receptor and/or ADR gene product to obtain a result; and

   (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

6. Use of at least one probe, tool or reagent based on or developed from at least one TRP ion channel, ACh receptor and/or ADR gene or gene product for identifying, screening, diagnosing, monitoring or treating a subject having, or at risk of developing, a medical condition or symptom thereof.

7. At least one SNP of at least one TRP ion channel, ACh receptor and/or ADR gene when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or

symptom thereof.

8. At least one probe, tool or reagent based on or developed from at least one TRP ion channel, ACh receptor and/or ADR gene or gene product when used as an indicator of a medical condition or symptom thereof, when used in identifying, screening, diagnosing, monitoring or treating a subject having a medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

9. A method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for at least one SNP of at least one TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

10. A method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of assaying the subject for a property of at least one TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

11. A method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of testing the subjects for at least one SNP of at least one TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

12. A method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a property of at least one TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

13. A method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) testing the subject for at least one SNP of at least one TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof; and

(2) managing the subject if the subject has been found to have the at least one SNP of the

at least one TRP ion channel, ACh receptor and/or ADR gene known to correlate with the medical condition or symptom thereof.

14. A method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) assaying the subject for a property of at least one TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof; and

(2) managing the subject if the subject has been found to have the property of the at least one TRP ion channel, ACh receptor and/or ADR gene or gene product known to correlate with the medical condition or symptom thereof.

15. A method of identifying or diagnosing a subject having a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(a) measuring the level of expression of at least one gene marker in a biological sample obtained from the subject that is differentially expressed in the medical condition or symptom thereof; and
(b) comparing the level of expression of the gene marker in the biological sample relative to a reference, wherein the at least one gene marker is at least one TRP ion channel, ACh receptor and/or ADR gene, and detection of an alteration in the level of gene expression of the gene marker in the biological sample relative to the reference indicates that the subject has the medical condition or symptom thereof, or is at risk of developing the medical condition or symptom thereof.

16. A method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:

optionally, isolating a biological sample from the subject prior to management of the illness and during and/or after management of the illness;

measuring the level of expression in the biological samples of at least one gene marker that is differentially expressed in the illness; and

comparing the level of expression of the gene marker in the biological samples before and during and/or after management of the illness, wherein the at least one gene marker is at least one TRP ion channel, ACh receptor and/or ADR gene, and a change in the

level of expression of the gene marker identifies the subject as having responded to the management of the illness.

17. At least one TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, or at least one TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for use in identifying a subject having a medical condition or symptom thereof.

18. At least one TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for identifying a subject at risk of developing a medical condition or symptom thereof, or at least one TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent for use in identifying a subject at risk of developing a medical condition or symptom thereof.

19. At least one TRP ion channel, ACh receptor and/or ADR gene-based or gene-product-based probe, tool or reagent when used for identifying a subject having, or at risk of developing, a medical condition or symptom thereof.

20. A kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterising at least one TRP ion channel, ACh receptor and/or ADR gene or gene product using a biological sample derived from the subject.

21. A biological sample comprising at least one TRP ion channel, ACh receptor and/or ADR gene or gene product, when isolated for the purpose for testing the biological sample for a medical condition or symptom thereof.

22. An array of oligonucleotide probes suitable for determining at least one TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

23. A microarray comprising oligonucleotide probes suitable for determining at least one TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

24. A biochip comprising a solid substrate and at least one oligonucleotide probe suitable for determining at least one TRP ion channel, ACh receptor and/or ADR gene/allele or gene product in a biological sample.

25. An article of manufacture comprising: (1) non-naturally occurring polynucleotide, recombinant polynucleotide, oligonucleotide or cDNA form of at least one TRP ion channel, ACh receptor and/or ADR or a fragment thereof; or (2) a polynucleotide or an oligonucleotide that is complementary to the gene of (1) or fragment thereof; or (3) an expression vector, recombinant cell or biological sample, tool, reagent, kit or assay comprising (1) or (2) or fragment thereof.

26. At least one TRP ion channel, ACh receptor or ADR gene SNP as shown in any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

27. A nucleotide sequence as shown or substantially as shown in Table 35 (SEQ ID Nos. 1 to 63) or Table 36 (SEQ ID No. 64), or a complementary sequence thereof.

28. At least one differentially regulated calcium-dependent kinase gene for use as an indicator of a medical condition or symptom thereof.

29. At least one probe, tool or reagent based on or developed from at least one differentially regulated calcium-dependent kinase gene for use as an indicator of a medical condition or symptom thereof.

30. Use of at least one differentially regulated calcium-dependent kinase gene for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof.

31. A method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

    (a) testing a subject for differential regulation of at least one calcium-dependent kinase gene to obtain a result; and

    (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

32. Use of at least one differentially regulated calcium-dependent kinase gene for identifying, screening, diagnosing, monitoring or treating a subject having, or at risk of developing, a medical condition or symptom thereof.

33. At least one differentially regulated calcium-dependent kinase gene when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

34. At least one probe, tool or reagent based on or developed from at least one differentially regulated calcium-dependent kinase gene when used as an indicator of a medical condition or symptom thereof, when used in identifying, screening, diagnosing, monitoring or treating a subject having a medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof.

35. A method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

36. A method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of testing the subjects for at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

37. A method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

    (1) testing the subject for differential regulation of at least one calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof; and

    (2) managing the subject if the subject has been found to have the at least one differentially regulated calcium-dependent kinase gene known to correlate with the medical condition or symptom thereof.

38. A method of identifying or diagnosing a subject having a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

    (a) measuring the level of expression of at least one calcium-dependent kinase gene marker in a biological sample obtained from the subject that is differentially expressed in the medical condition or symptom thereof; and
    (b) comparing the level of expression of the at least one gene marker in the biological sample relative to a reference, wherein detection of an alteration in the level of gene expression of the at least one gene marker in the biological sample relative to the reference indicates

that the subject has the medical condition or symptom thereof, or is at risk of developing the medical condition or symptom thereof.

39. A method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:
optionally, isolating a biological sample from the subject prior to management of the illness and during and/or after management of the illness;
measuring the level of expression in the biological samples of at least one calcium-dependent kinase gene marker that is differentially expressed in the illness; and
comparing the level of expression of the at least one gene marker in the biological samples before and during and/or after management of the illness, wherein a change in the level of expression of the at least one gene marker identifies the subject as having responded to the management of the illness.

40. At least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, or at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for use in identifying a subject having a medical condition or symptom thereof.

41. At least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for identifying a subject at risk of developing a medical condition or symptom thereof, or at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent for use in identifying a subject at risk of developing a medical condition or symptom thereof.

42. At least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent when used for identifying a subject having, or at risk of developing, a medical condition or symptom thereof.

43. A kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterizing at least one calcium-dependent kinase gene product using a biological sample derived from the subject.

44. A biological sample comprising at least at least one calcium-dependent kinase gene or gene product, when isolated for the purpose for testing the biological sample for a medical condition or symptom thereof.

45. An array of oligonucleotide probes suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

46. A microarray comprising oligonucleotide probes suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

47. A biochip comprising a solid substrate and at least one oligonucleotide probe suitable for determining at least one calcium-dependent kinase gene product in a biological sample.

48. An article of manufacture comprising: (1) non-naturally occurring polynucleotide, recombinant polynucleotide, oligonucleotide or cDNA form of at least one calcium-dependent kinase gene or a fragment thereof; or (2) a polynucleotide or an oligonucleotide that is complementary to the gene of (1) or fragment thereof; or (3) an expression vector, recombinant cell or biological sample, tool, reagent, kit or assay comprising (1) or (2) or fragment thereof.

49. A method of evaluating a subject for a medical condition or symptom thereof, or predisposition to a medical condition or symptom thereof, said method comprising:

    (a) testing the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38; and

    (b) employing said result to provide an evaluation of the subject for the medical condition or symptom thereof.

50. A method of identifying or diagnosing a subject having a medical condition or symptom thereof, comprising the step of testing the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, wherein dysfunctional signalling through the MAPK pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, indicates that the subject has the medical condition or symptom thereof.

51. A method of identifying or diagnosing a subject having a medical condition or symptom thereof, said method comprising the steps of:

    (a) obtaining at least one biological sample from the subject; and

    (b) testing the biological sample for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, wherein dysfunctional signalling through the MAPK pathway, including signalling via the

MAPK kinase (MAPKKVMEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, indicates that the subject has the medical condition or symptom thereof.

52. A method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of testing the subjects for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK 1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, wherein said dysfunctional signalling is known to correlate with the medical condition or symptom thereof.

53. A method of identifying whether a subject having a medical condition or symptom thereof ("illness") is responding to management of that illness, said method comprising the steps of:

(a) obtaining at least one biological sample from the subject; and

(b) testing the at least one biological sample for dysfunctional signalling through the Mitogen-Activated Protein Kinase **(MAPK)** pathway, including signalling via the **MAPK** kinase **(MAPKK** /MEK1/2) and extracellular signal-regulated kinase **(EPvK)l/2** as well as p38, wherein dysfunctional signalling through the **MAPK** pathway indicates that the subject has the medical condition or symptom thereof, and wherein no or less dysfunctional signalling through the **MAPK** pathway indicates that the subject is responding to management of the illness.

54. A method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) testing the subject for dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK 1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, wherein said dysfunctional signalling is known to correlate with the medical condition or symptom thereof; and

(2) managing the subject if the subject has been found to have said dysfunctional signalling.

55. At least one probe, tool or reagent for identifying a subject having a medical condition or symptom thereof, said at least one probe, tool or reagent being for assaying or characterising the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEKl/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, using a biological sample derived from the subject.

56. A kit or assay for identifying a subject having a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying or characterising the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38, using a biological sample derived from the subject.

57. Use of calcium metabolism testing for identifying, screening, diagnosing or monitoring a subject having, or at risk of developing, a medical condition or symptom thereof, wherein said medical condition or symptom thereof is optionally attributable to:

    (a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

    (b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

    (c) differential regulation of at least one calcium-dependent kinase gene; and/or

    (d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK7MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

58. Calcium metabolism testing when used as an indicator of a medical condition or symptom thereof, when used for identifying, screening, diagnosing or monitoring a subject having the medical condition or symptom thereof, or when used for identifying a subject at risk of developing a medical condition or symptom thereof, wherein said medical condition or symptom thereof is optionally attributable to:

    (a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

    (b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

    (c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

59. A method of identifying a subject at risk of developing, or diagnosing a subject having, a medical condition or symptom thereof, said method comprising the step of testing the subject for a change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to:

(a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

60. A method of screening subjects for a prevalence of a medical condition or symptom thereof, or a method of identifying subjects at risk of developing a medical condition or symptom thereof, said method comprising the step of assaying each of the subjects for a change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to:

(a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

61. A method of managing a subject with a medical condition or symptom thereof, or at risk of developing a medical condition or symptom thereof, said method comprising the steps of:

(1) testing the subject for a change in calcium metabolism; and

(2) managing the subject if the subject has been found to have said change in calcium metabolism, wherein said medical condition or symptom thereof is optionally attributable to:

(a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK7MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

62. A kit or assay for identifying a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, said kit or assay comprising one or more probes, tools or reagents for assaying calcium metabolic change in the subject, wherein said medical condition or symptom thereof is optionally attributable to:

(a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

63. A kit or method for testing, screening or treating a subject having a medical condition or symptom thereof or at risk of developing a medical condition or symptom thereof, for any calcium metabolite, wherein said medical condition or symptom thereof is optionally attributable to:

(a) at least one SNP of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(b) a polymorphism at the genomic level, altered RNA expression, altered polypeptide/protein expression, or an altered biological function of at least one transient receptor potential (TRP) ion channel, acetylcholine receptor (AchR) and/or adrenergic receptor (ADR) gene;

(c) differential regulation of at least one calcium-dependent kinase gene; and/or

(d) dysfunctional signalling through the Mitogen-Activated Protein Kinase (MAPK) pathway, including signalling via the MAPK kinase (MAPKK/MEK1/2) and extracellular signal-regulated kinase (ERK)l/2 as well as p38.

64. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, or the biological sample of claim 21, wherein the medical condition or symptom thereof is: chronic fatigue syndrome (CFS) or symptom thereof; myalgic encephalomyelitis (ME) or symptom thereof; Gulf war syndrome (GWS) or symptom thereof; irritable bowel syndrome (IBS) or symptom thereof; multiple chemical sensitivity (MCS) or symptom thereof; fibromyalgia or symptom thereof; migraine; a medical condition caused by dysregulation in calcium, acetylcholine, TRP or ADR; or dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary or immune systems.

65. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of

claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, or the biological sample of claim 21, wherein the medical condition or symptom thereof is chronic fatigue syndrome (CFS) or symptom thereof.

66. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25,

67. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the at least one TRP ion channel, ACh receptor an/or ADR gene has a SNP as shown in any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

68. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the TRP ion channel is selected from one or more of the following: TRPC4, TRPA1 (ankyrin), TRPM3 (melastatin) and TRPM4.

69. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the TRP

ion channel gene is selected from one or more of the following genes: Gene ID 80036, 7223, 101927086 and 54795.

70. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the at least one SNP of a TRP ion channel gene is one or more of the following SNPs: rs12682832, rsl 1142508, rsll60742, rs4454352, rsl328153, rs3763619, rs7865858, rsl504401 or rsl0115622 of *TRPM3;* rs2383844 or rs4738202 of *TRPA1;* or rs6650469 or rs655207 of *TRPC4.*

71. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the ACh receptor is selected from one or more of the following: muscarinic acetylcholine receptor, especially mAChRM3; and nicotinic acetylcholine alpha receptors, especially nAChRa2, nAChRa5 ornAChRalO.

72. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the AChR gene is selected from one or more of the following genes: Gene ID 1131, 417, 4928, 57053, 100873984, 1138 and 1142.

73. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or

assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the at least one SNP of an ACh receptor gene is one or more of the following SNPs: rs4463655, rs589962, rsl072320, rs7543259, rs6661621, rs7520974, rs726169, rsrs6669810 or rsrs6429157 of mAChRM3; rs2672211, rs2672214, rs2741868, rs2741870 or rs2741862 of nACh alpha 10; rs951266 or rs7 180002 of nACh alpha 5; or rs2565048 of nACh alpha 2.

74. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the ADR is adrenergic receptor αl (ADRA1A)

75. The at least one SNP of claim 1, the at least one probe, tool or reagent of claim 2, the use of claim 3, the method of claim 4 or 5, the use of claim 6, the at least one SNP of claim 7, the at least one probe, tool or reagent of claim 8, the method of any one of claims 9 to 16, the gene-based or gene-product-based probe, tool or reagent of any one of claims 17 to 19, the kit or assay of claim 20, the biological sample of claim 21, the array of claim 22, the microarray of claim 23, the biochip of claim 24, or the article of manufacture of claim 25, wherein the at least one SNP of the ADRA1A gene is rs2322333.

76. The method of claim 5, wherein the subject is tested for altered mRNA or altered polypeptide/protein expression.

77. The method of any one of claims 10, 12 and 14, wherein the property relates to a polymorphism at the genomic level, or altered RNA or altered polypeptide/protein expression.

78. The method of claim 15 or claim 16, wherein measuring the level of expression involves measuring RNA or polypeptide/protein expression.

79. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe,

tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the medical condition or symptom thereof is: chronic fatigue syndrome (CFS) or symptom thereof; myalgic encephalomyelitis (ME) or symptom thereof; Gulf war syndrome (GWS) or symptom thereof; irritable bowel syndrome (IBS) or symptom thereof; multiple chemical sensitivity (MCS) or symptom thereof; fibromyalgia or symptom thereof; migraine; a medical condition caused by dysregulation in calcium, acetylcholine, TRP or ADR; or dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary or immune systems.

80. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the medical condition or symptom thereof is: severe chronic fatigue syndrome (CFS) or symptom thereof; or, severe myalgic encephalomyelitis (ME) or symptom thereof.

81. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, the biological sample of claim 44, the array of oligonucleotide probes of claim 45, the microarray of claim 46, the biochip of claim 47, or the article of manufacture of claim 48, wherein the at least one differentially regulated calcium-dependent kinase gene is at least one gene selected from Table 31 or Table 32.

82. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, the biological sample

of claim 44, the array of oligonucleotide probes of claim 45, the microarray of claim 46, the biochip of claim 47, or the article of manufacture of claim 48, wherein the at least one differentially regulated calcium-dependent kinase gene is selected from 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 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 or 92 genes shown in Table 31 and/or Table 32.

83. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the at least one differentially regulated calcium-dependent kinase gene is upregulated in expression.

84. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the at least one differentially regulated calcium-dependent kinase gene is downregulated in expression.

85. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the biological sample contains peripheral blood mononuclear cells.

86. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the

use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein the biological sample contains Natural Killer cells.

87. The at least one differentially regulated calcium-dependent kinase gene of claim 28, the at least one probe, tool or reagent of claim 29, the use of claim 30, the method of claim 31, the use of claim 32, the at least one differentially regulated calcium-dependent kinase gene of claim 33, the at least one probe, tool or reagent of claim 34, the method of any one of claims 35-39, the at least one calcium-dependent kinase gene-based or gene-product-based probe, tool or reagent of any one of claims 40-42, the kit or assay of claim 43, or the biological sample of claim 44, wherein said testing or measuring involves testing or measuring altered RNA expression.

88. The method of any one of claims 49-54, the at least one probe, tool or reagent of claim 55, or the kit or assay of claim 56, wherein the medical condition or symptom thereof is: chronic fatigue syndrome (CFS) or symptom thereof; myalgic encephalomyelitis (ME) or symptom thereof; Gulf war syndrome (GWS) or symptom thereof; irritable bowel syndrome (IBS) or symptom thereof; multiple chemical sensitivity (MCS) or symptom thereof; fibromyalgia or symptom thereof; migraine; a medical condition caused by dysregulation in calcium, acetylcholine, TRP or ADR; or dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary or immune systems.
89. The method of any one of claims 49-54, the at least one probe, tool or reagent of claim 55, or the kit or assay of claim 56, wherein the medical condition or symptom thereof is: severe chronic fatigue syndrome (CFS) or symptom thereof; or, severe myalgic encephalomyelitis (ME) or symptom thereof.
90. The method of any one of claims 49-54, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2.
91. The method of any one of claims 49-54, wherein said dysfunctional signalling is typified by increased phosphorylation of MEK1/2 and p38.
92. The method of any one of claims 49-54, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in conjunction with increased phosphorylation of MEK1/2 and p38 in peripheral blood mononuclear cells.
93. The method of any one of claims 49-54, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in CD56⁺CD16⁺ NK cells in conjunction with

increased phosphorylation of MEK1/2 and p38 in $CD56^{bright}CD16^{dim/-}$ NK cells.

94. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the medical condition or symptom thereof is: chronic fatigue syndrome (CFS) or symptom thereof; myalgic encephalomyelitis (ME) or symptom thereof; Gulf war syndrome (GWS) or symptom thereof; irritable bowel syndrome (IBS) or symptom thereof; multiple chemical sensitivity (MCS) or symptom thereof; fibromyalgia or symptom thereof; migraine; a medical condition caused by dysregulation in calcium, acetylcholine, TRP or ADR; or dysregulation in the gastrointestinal, cardiovascular, neurological, genitourinary or immune systems.

95. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the medical condition or symptom thereof is: severe chronic fatigue syndrome (CFS) or symptom thereof; or, severe myalgic encephalomyelitis (ME) or symptom thereof.

96. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one TRP ion channel, ACh receptor an/or ADR gene has a SNP as shown in any one of Tables 1 to 7, 9, 10, 12 to 17, 26 to 28, 34a and 34b.

97. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the TRP ion channel is selected from one or more of the following: TRPC4, TRPA1 (ankyrin), TRPM3 (melastatin) and TRPM4.

98. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the TRP ion channel gene is selected from one or more of the following genes: Gene ID 80036, 7223, 101927086 and 54795.

99. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one SNP of a TRP ion channel gene is one or more of the following SNPs: rsl2682832, rslll42508, rsll60742, rs4454352, rsl328153, rs3763619, rs7865858, rsl504401 or rslOl 15622 of *TRPM3;* rs2383844 or rs4738202 of *TRPA1;* or rs6650469 or rs655207 of *TRPC4.*

100. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, the ACh receptor is selected from one or more of the following: muscarinic acetylcholine receptor, especially mAChRM3; and nicotinic acetylcholine alpha receptors, especially nAChRa2, nAChRa5 or nAChRalO.

101. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the AChR gene is selected from one or more of the following genes: Gene ID 1131, 417, 4928, 57053, 100873984, 1138 and 1142.

102. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one SNP of an ACh receptor gene is one or more of the following SNPs: rs4463655, rs589962, rsl072320, rs7543259, rs6661621, rs7520974, rs726169, rsrs6669810 or rsrs6429157 of mAChRM3; rs2672211, rs2672214, rs2741868, rs2741870 or rs2741862 of nACh alpha 10; rs951266 or rs7180002 of nACh alpha 5; or rs2565048 of nACh alpha 2.

103. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the ADR is adrenergic receptor αl (ADRA1A)

104. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one SNP of the ADRA1A gene is rs2322333.

105. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one differentially regulated calcium-dependent kinase gene is at least one gene selected from Table 31 or Table 32.

106. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein the at least one differentially regulated calcium-dependent kinase gene is selected from 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 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 or 92 genes shown in Table 31 and/or Table 32.

107. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2.

108. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein said dysfunctional signalling is typified by increased phosphorylation of MEK1/2 and p38.

109. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in conjunction with increased phosphorylation of MEK1/2 and p38 in peripheral blood mononuclear cells.

110. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein said dysfunctional signalling is typified by reduced phosphorylation of ERK1/2 in $CD56^{dim}CD16^{+}$ NK cells in conjunction with increased phosphorylation of MEK1/2 and p38 in $CD56^{bright}CD16^{dim/-}$ NK cells.

111. The method of any one of claims 49-54, the at least one probe, tool or reagent of claim 55, or the kit or assay of claim 56, wherein testing or assaying of dysfunctional signalling involves testing or assaying for protein phosphorylation and/or dephosphorylation.

112. The use of claim 57, the testing of claim 58, the method of any one of claims 59-61, or the kit or assay of claim 62 or 63, wherein testing or assaying of dysfunctional signalling involves testing or assaying for protein phosphorylation and/or dephosphorylation.

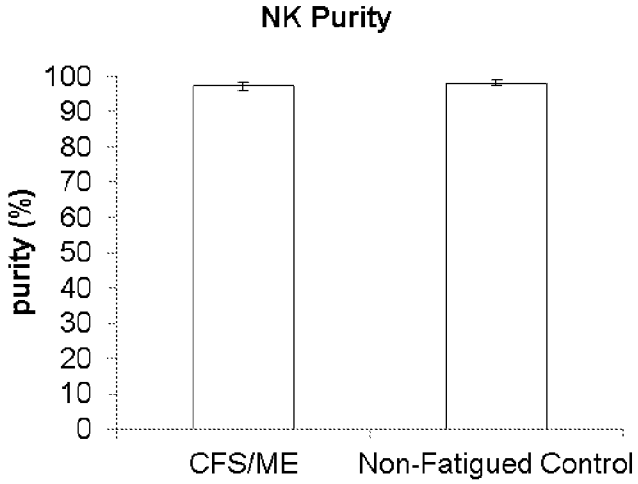


Figure 1

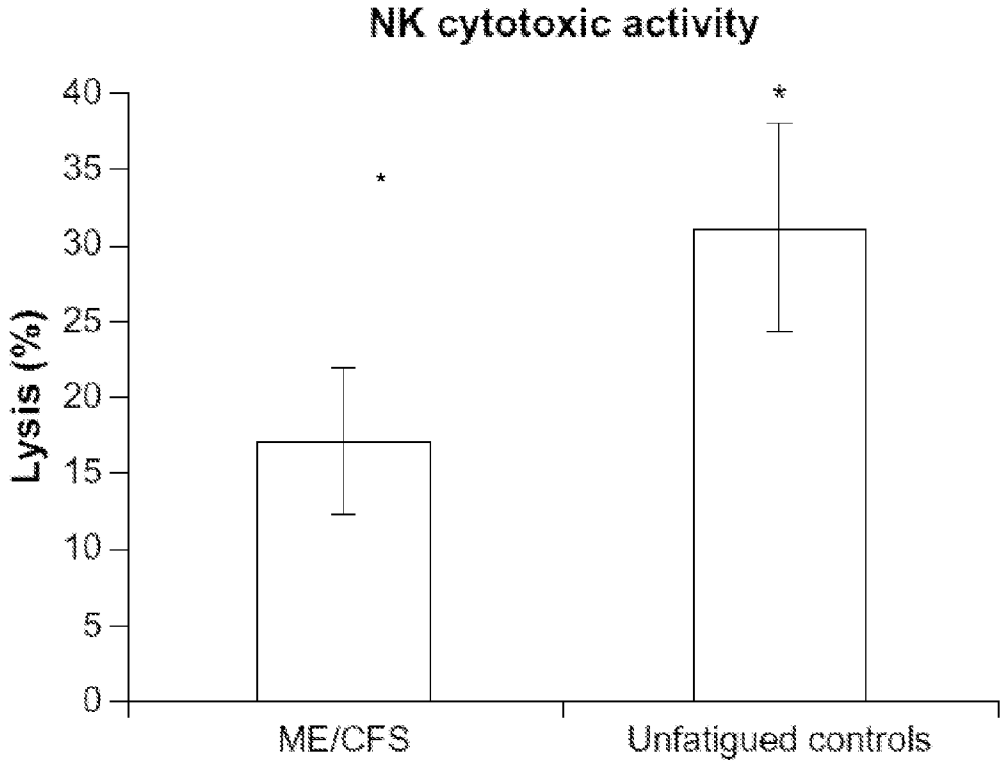


Figure 2

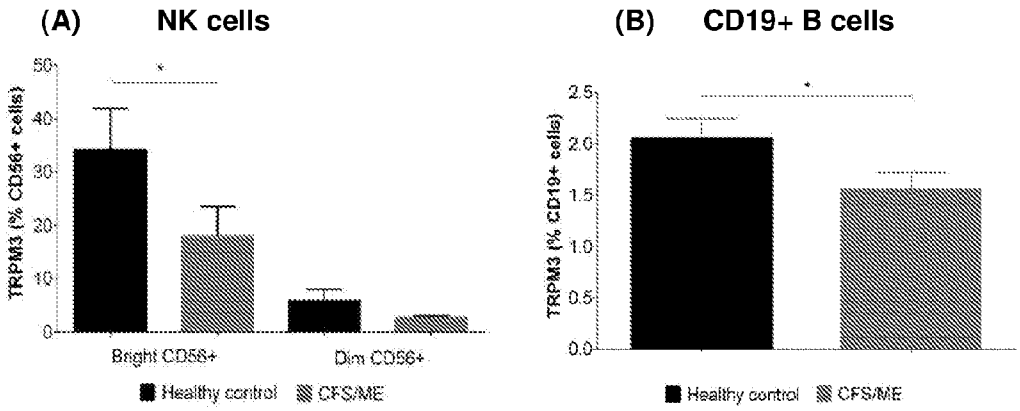


Figure 3

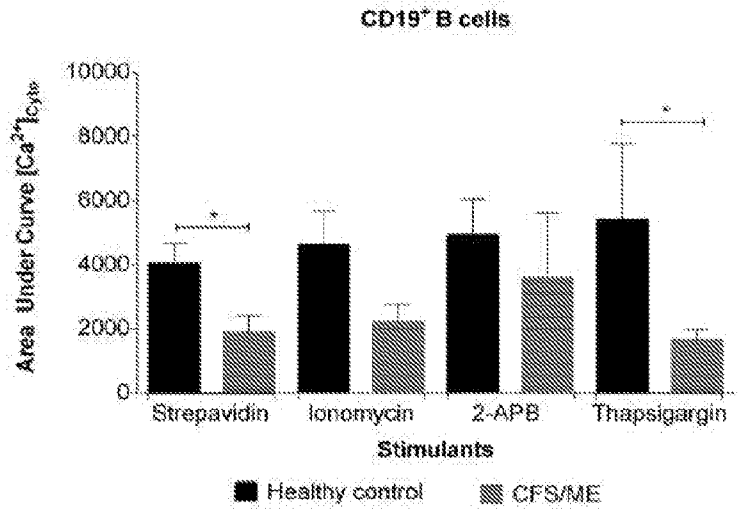


Figure 4 (A) CD19$^+$ B cells

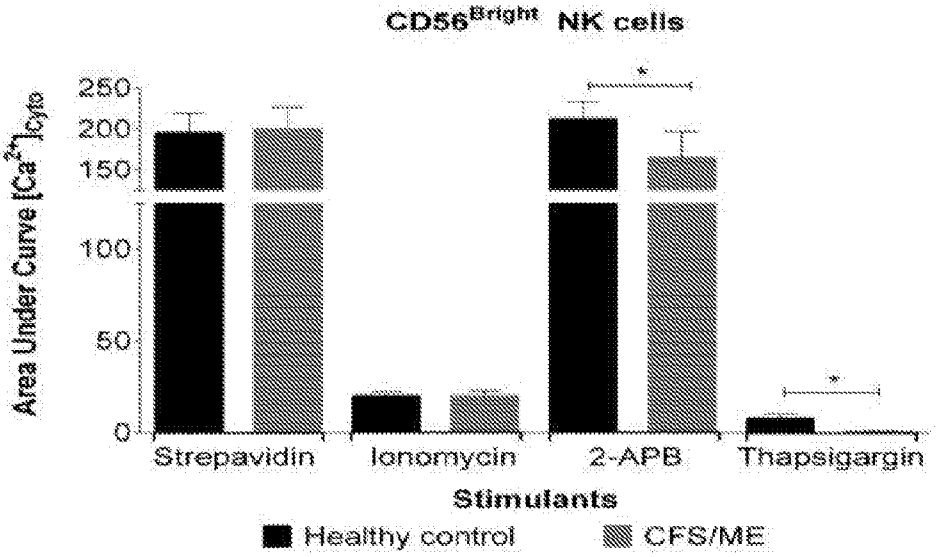


Figure 4 (B) CD56^{Bright} NK cells

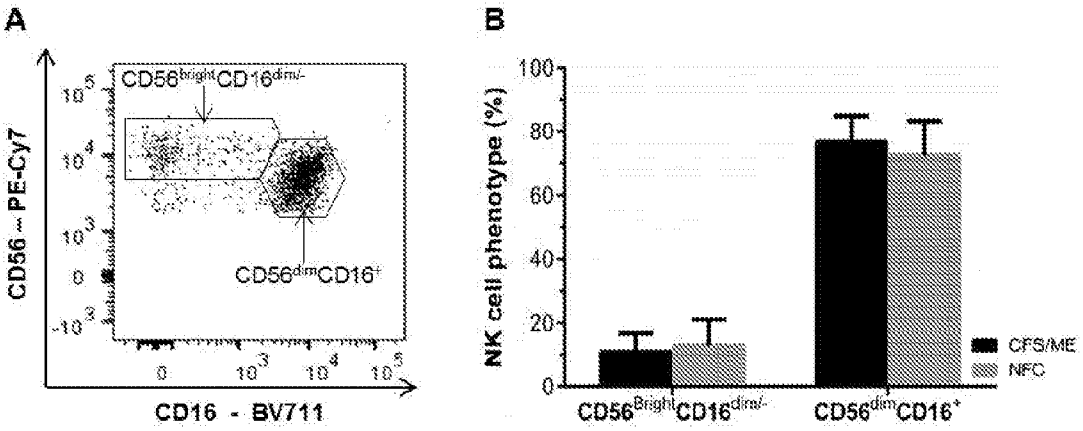


Figure 5

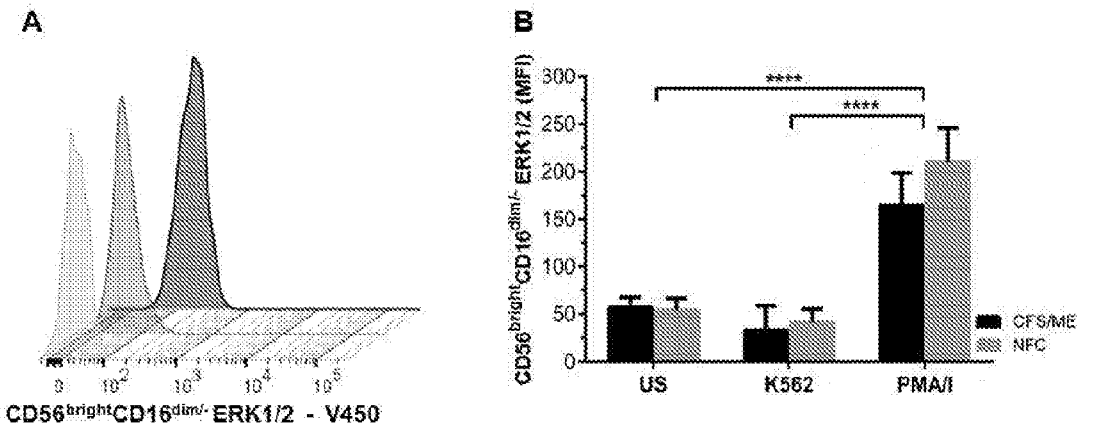


Figure 6

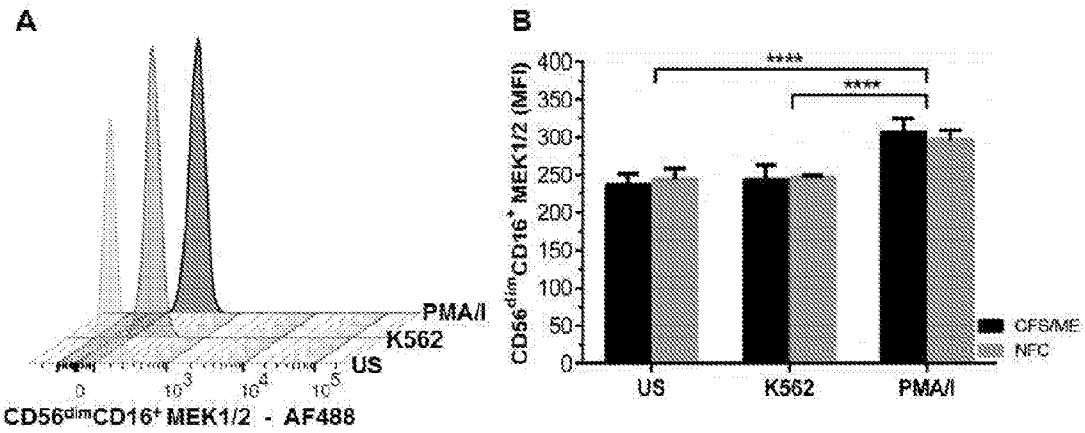


Figure 7

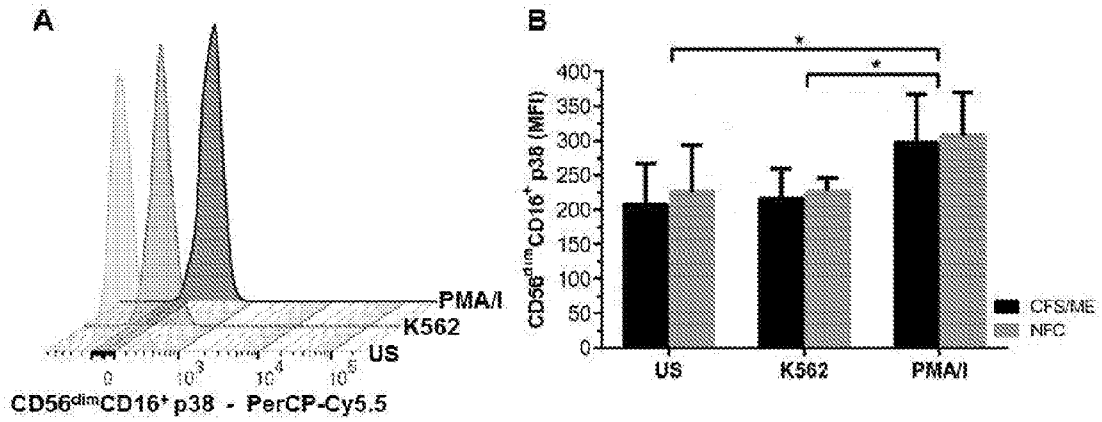


Figure 8

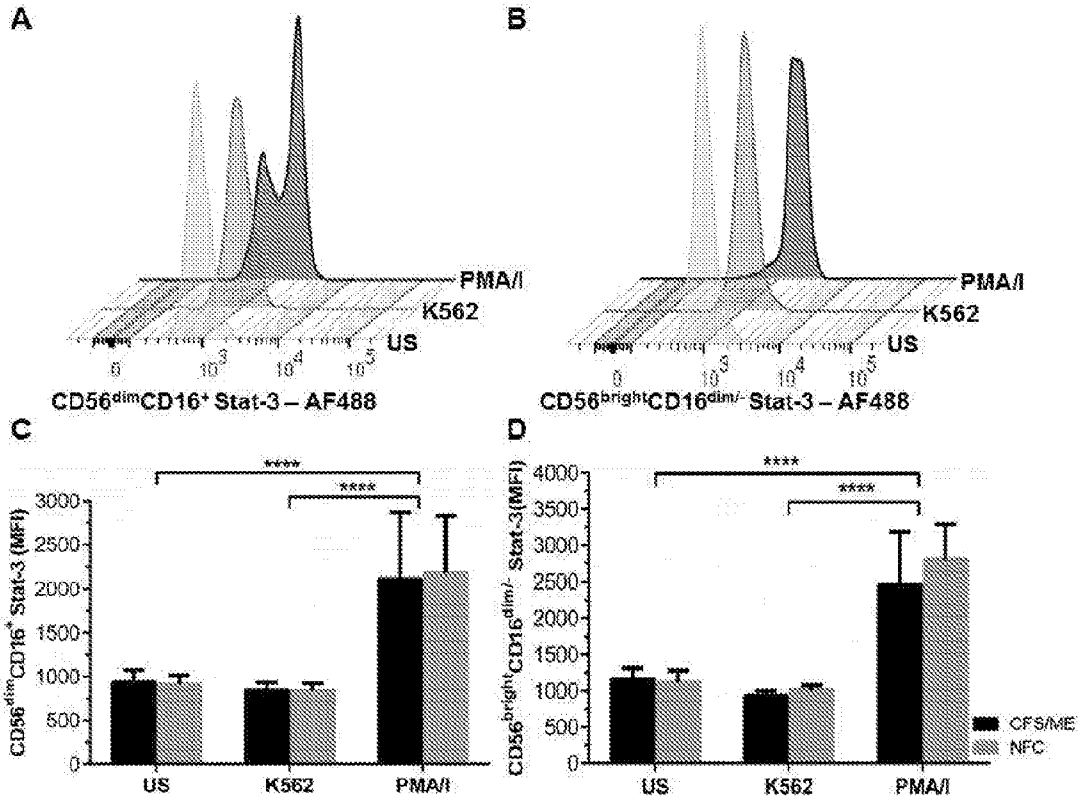

A
B
PMA/I
K562
US
CD56dimCD16+ Stat-3 – AF488
CD56brightCD16dim/- Stat-3 – AF488
C
D
CD56dimCD16+ Stat-3 (MFI)
CD56brightCD16dim/- Stat-3(MFI)
****
US
K562
PMA/I
CFS/ME
NFC

Figure 9

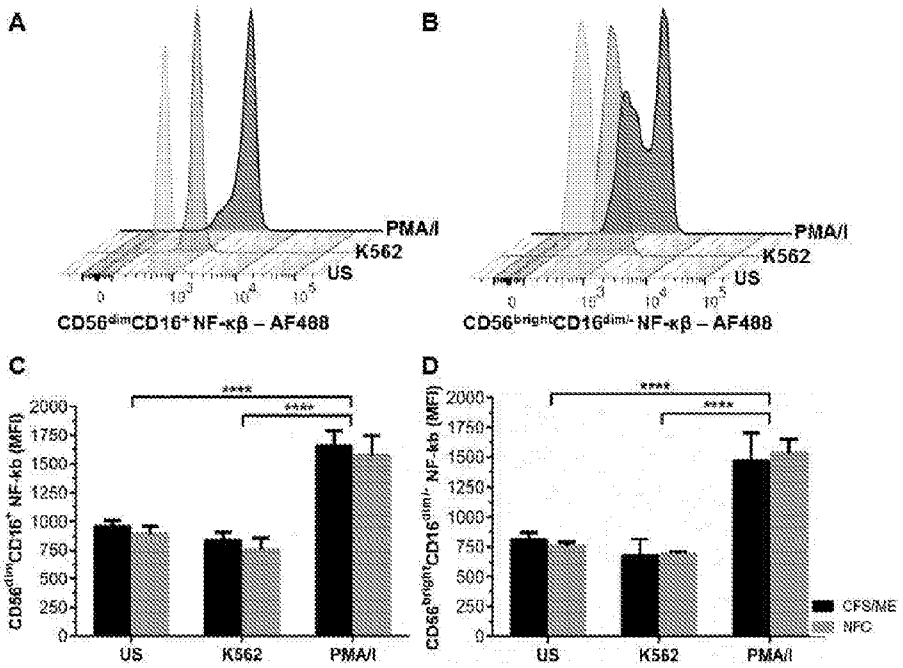


Figure 10

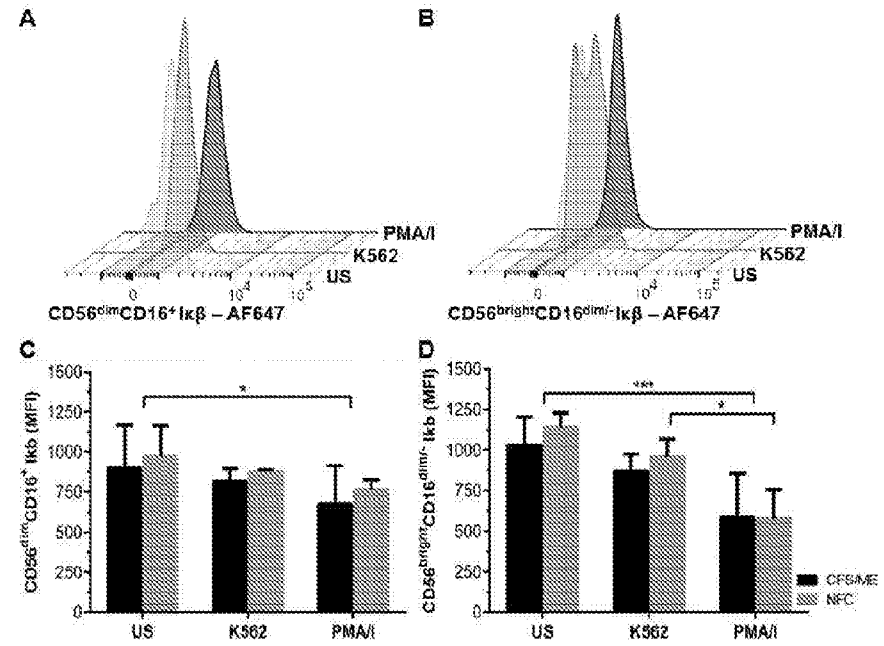


Figure 11

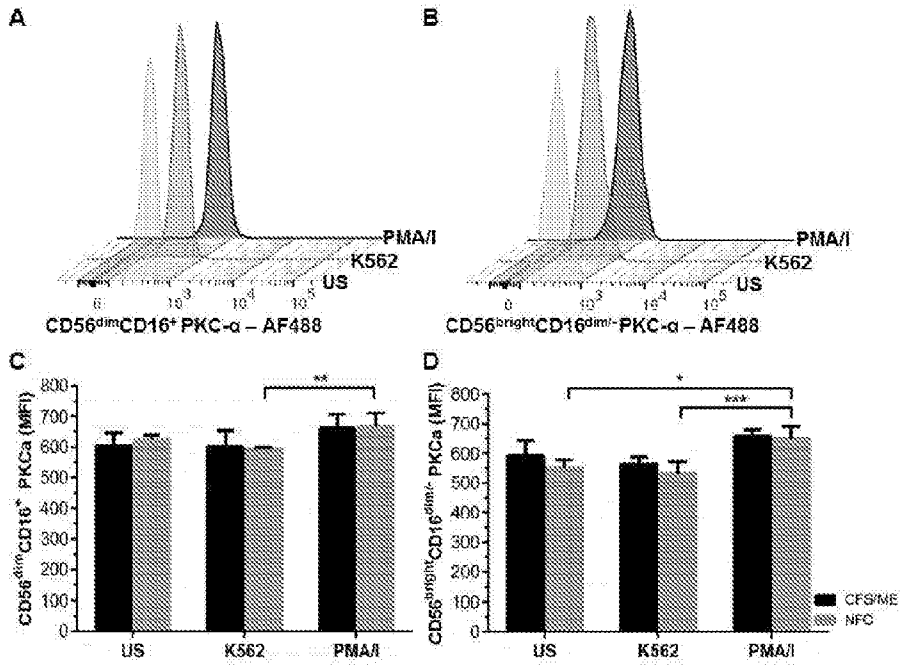


Figure 12

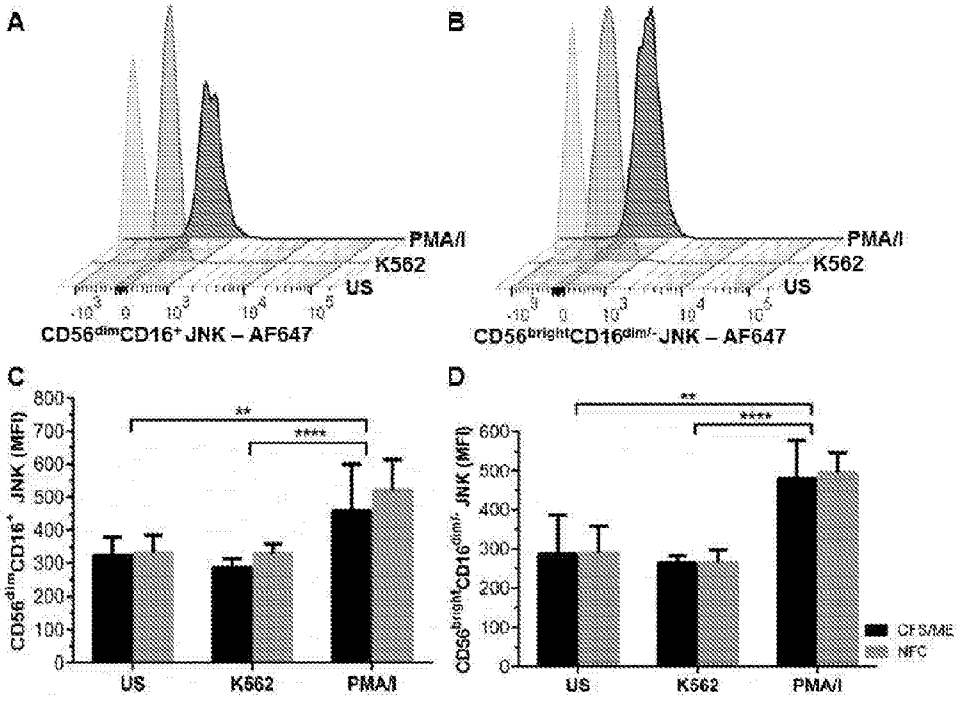


Figure 13

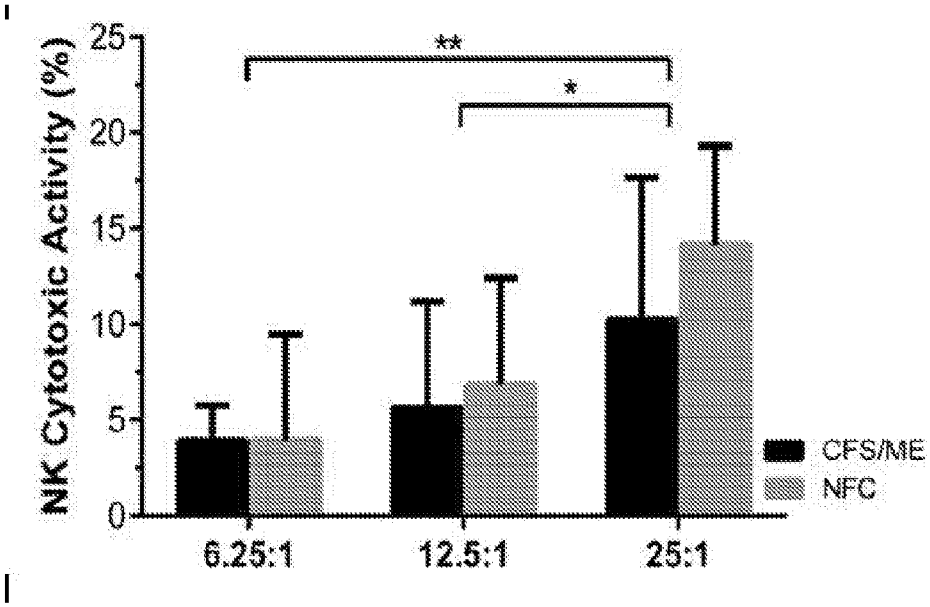


Figure 14

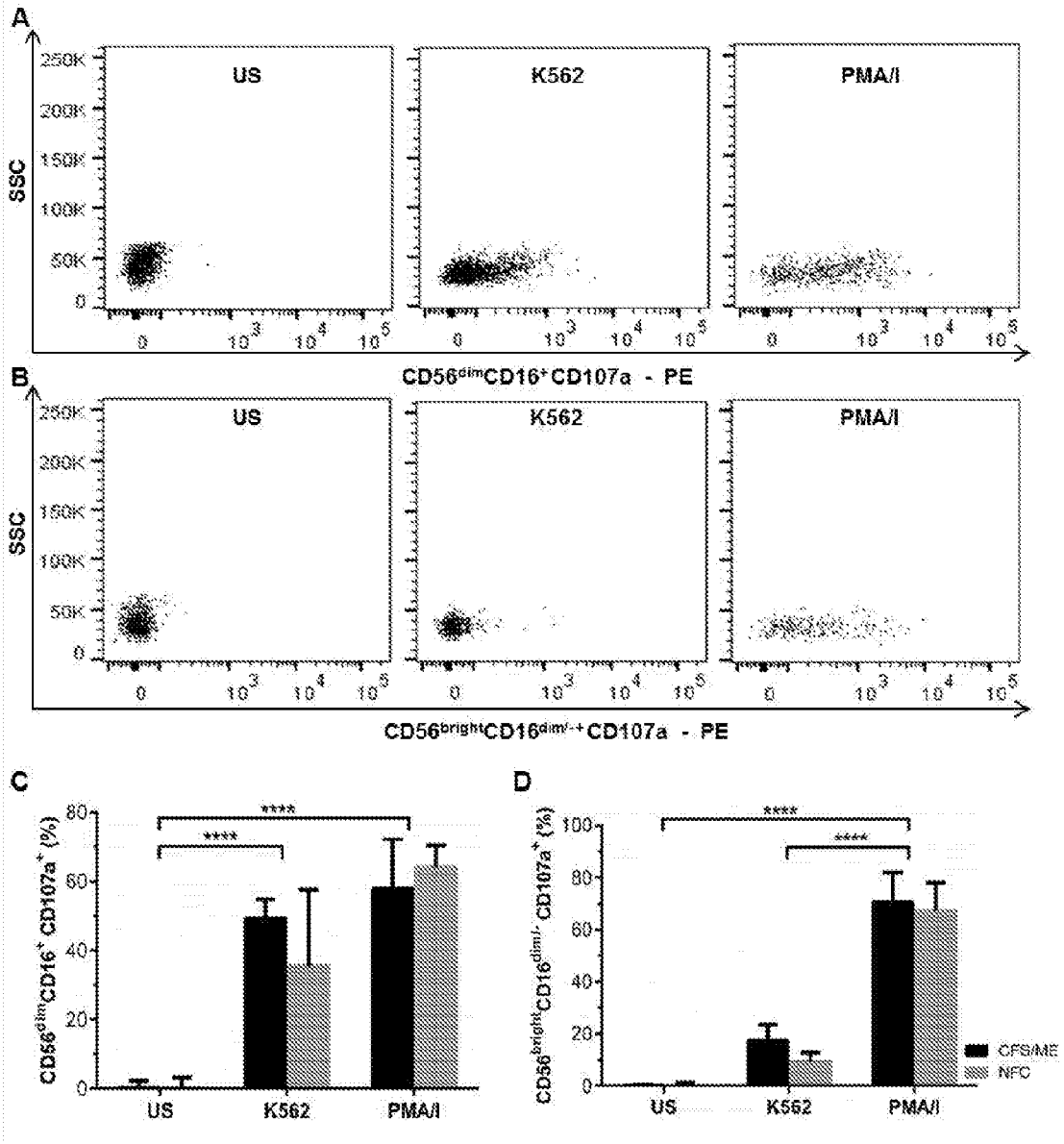

A
US
K562
PMA/I
SSC
250K
200K
150K
100K
50K
0
10^3
10^4
10^5
CD56dimCD16+CD107a - PE
B
CD56brightCD16dim/-+CD107a - PE
C
CD56dimCD16+ CD107a+ (%)
****
D
CD56brightCD16dim/- CD107a+ (%)
CFS/ME
NFC

Figure 15

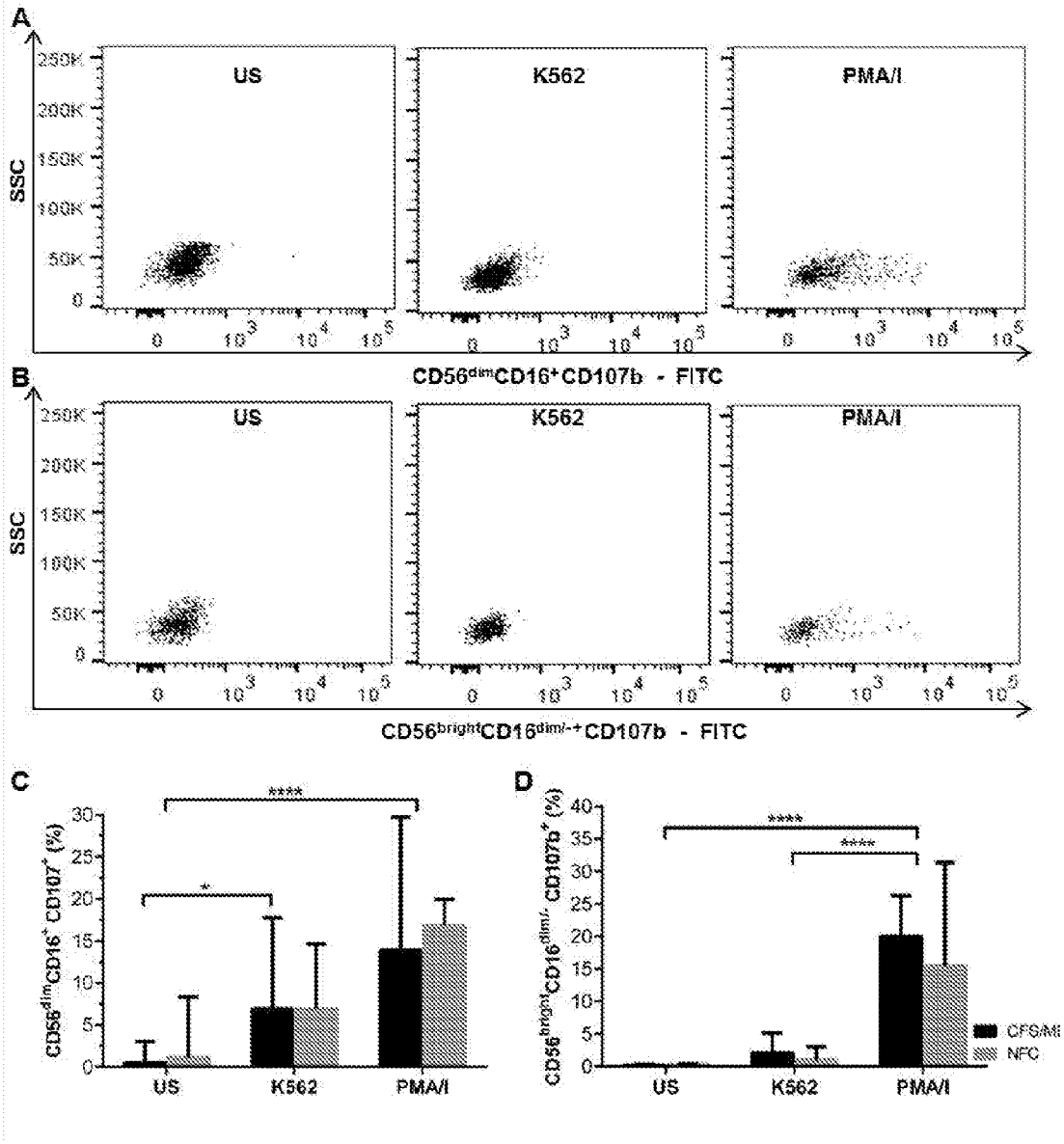

A
US
K562
PMA/I
SSC
CD56dimCD16+CD107b - FITC
B
US
K562
PMA/I
SSC
CD56brightCD16dim/-+CD107b - FITC
C
CD56dimCD16+CD107+ (%)
US
K562
PMA/I
D
CD56brightCD16dim/- CD107b+ (%)
CFSME
NFC
US
K562
PMA/I

Figure 16

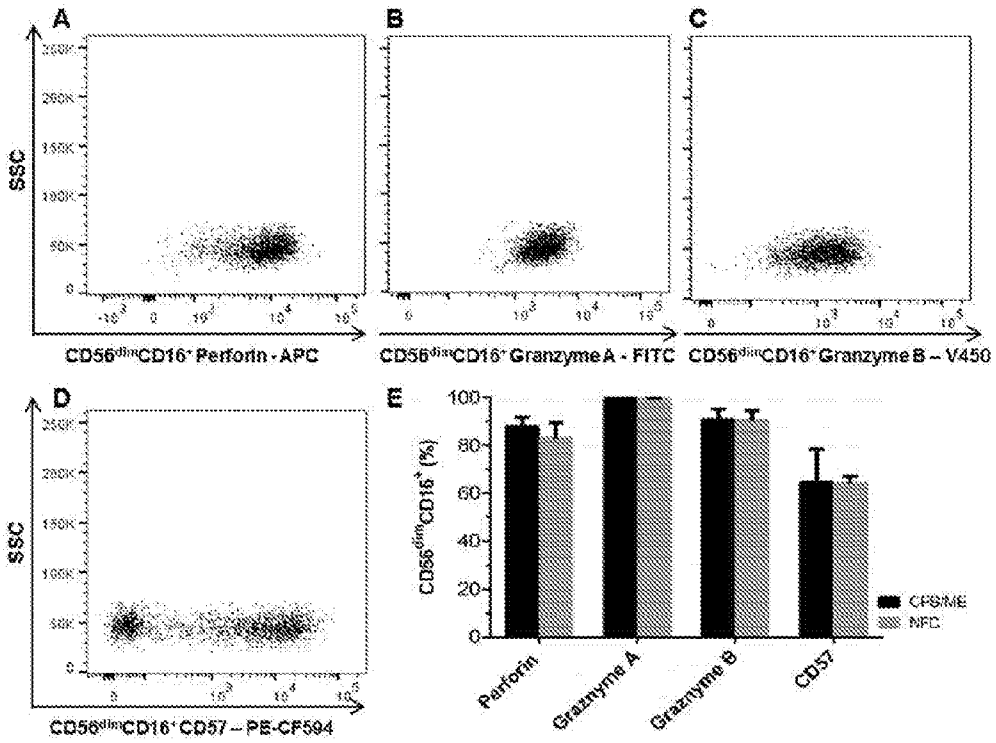


Figure 17

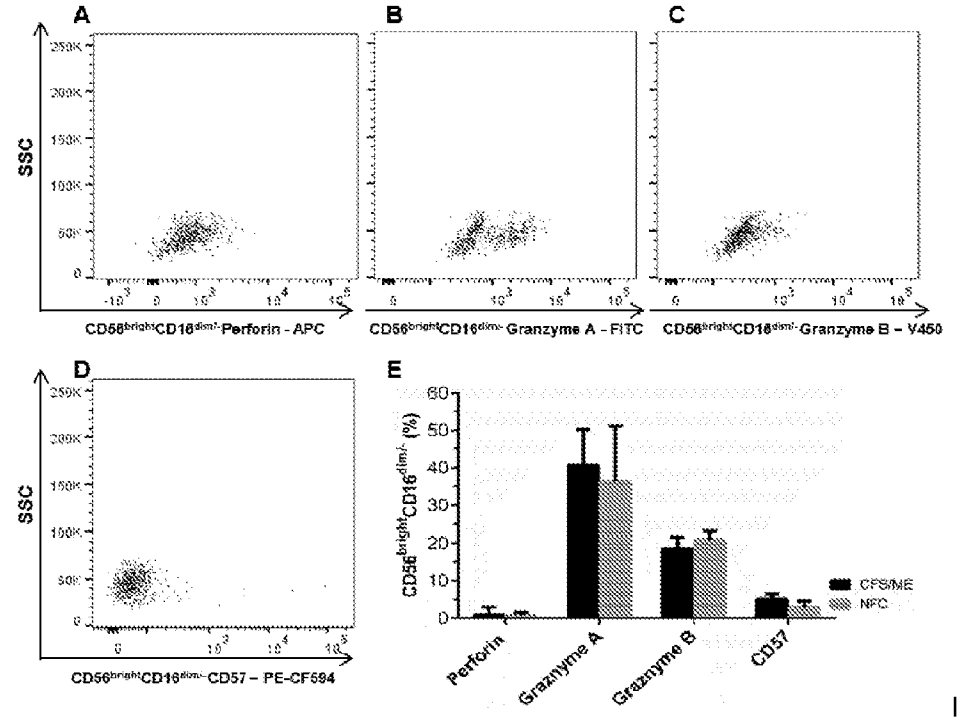


Figure 18

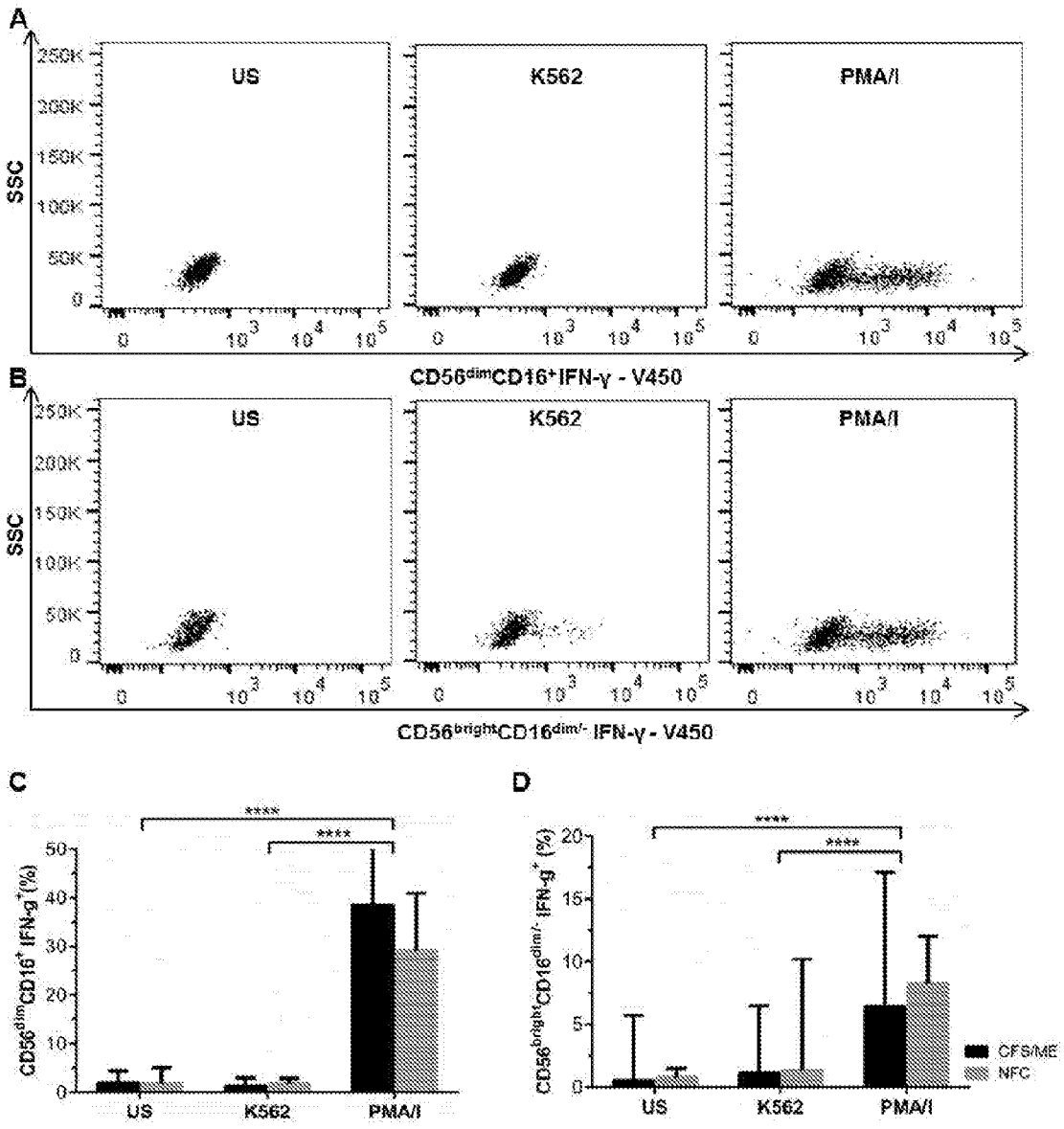
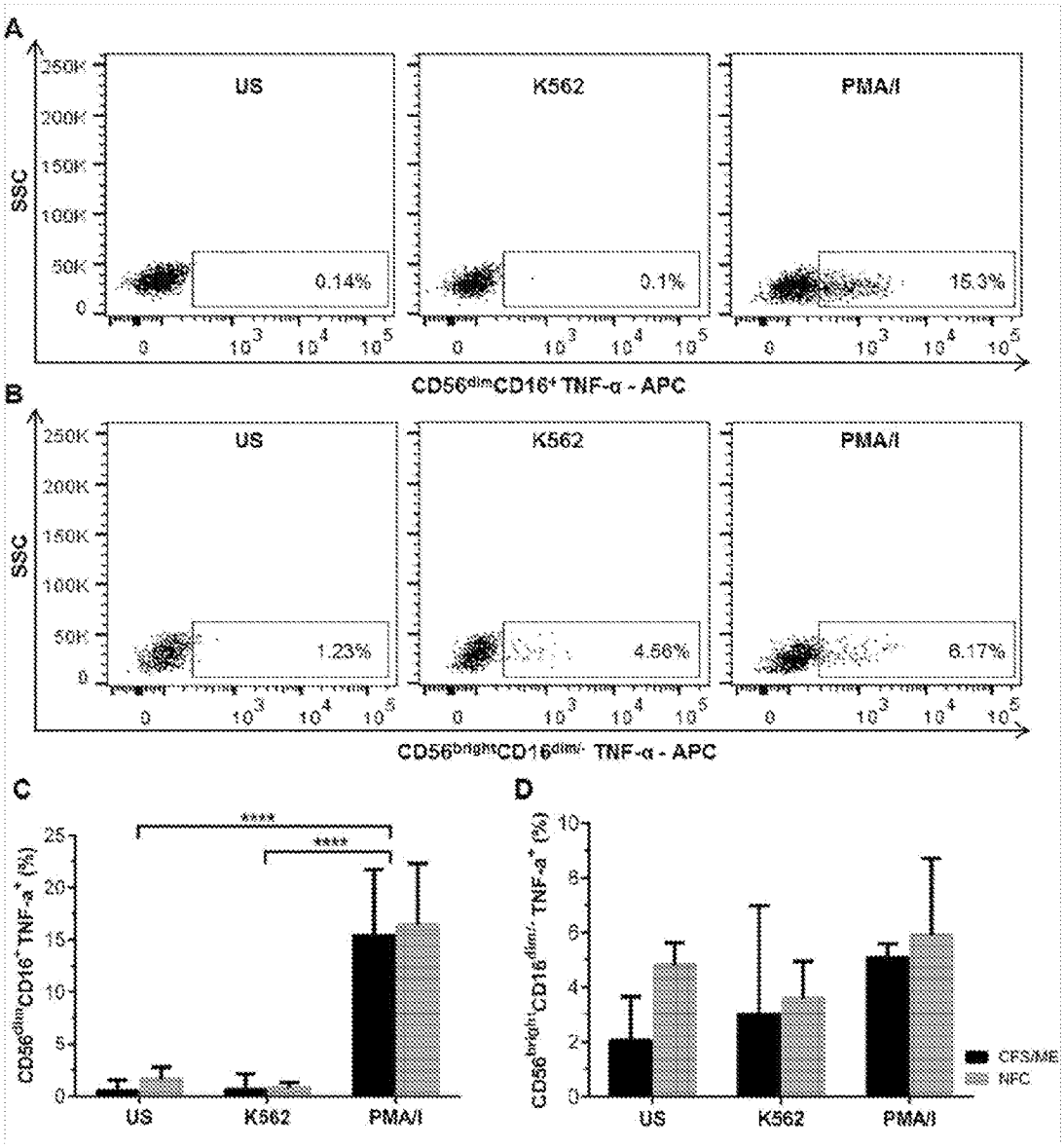

A
US
K562
PMA/I
SSC
250K
200K
150K
100K
50K
0
0
10^3
10^4
10^5
CD56^dimCD16^+IFN-γ - V450
B
US
K562
PMA/I
SSC
CD56^brightCD16^dim/- IFN-γ - V450
C
****
****
CD56^dimCD16^+ IFN-g^+(%)
50
40
30
20
10
0
US
K562
PMA/I
D
****
****
CD56^brightCD16^dim/- IFN-g^+ (%)
20
15
10
5
0
US
K562
PMA/I
CFS/ME
NFC

Figure 19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SSC
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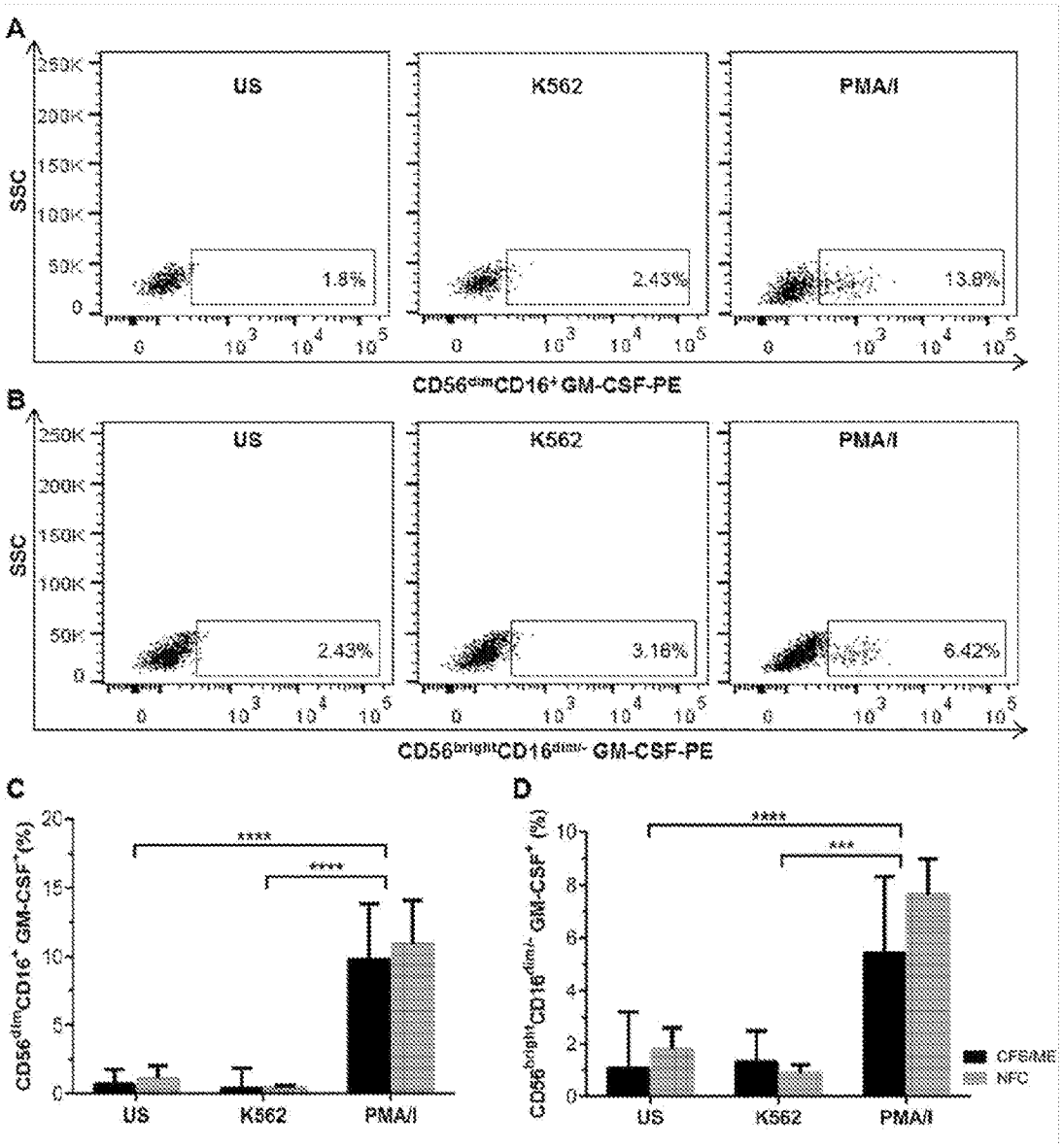
0.1%
15.3%
CD56dimCD16+ TNF-α - APC
B
US
K562
PMA/I
SSC
1.23%
4.56%
6.17%
CD56brightCD16dim/- TNF-α - APC
C
CD56dimCD16+ TNF-α+ (%)
****
****
US
K562
PMA/I
D
CD56brightCD16dim/- TNF-α+ (%)
CFS/ME
NFC
US
K562
PMA/I

Figure 20


A
US
K562
PMA/I
SSC
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2.43%
13.8%
CD56dimCD16+ GM-CSF-PE
B
US
K562
PMA/I
SSC
2.43%
3.16%
6.42%
CD56brightCD16dim/- GM-CSF-PE
C
CD56dimCD16+ GM-CSF+(%)
****
****
US
K562
PMA/I
D
CD56brightCD16dim/- GM-CSF+(%)
****
***
US
K562
PMA/I
CFSME
NFC

Figure 21

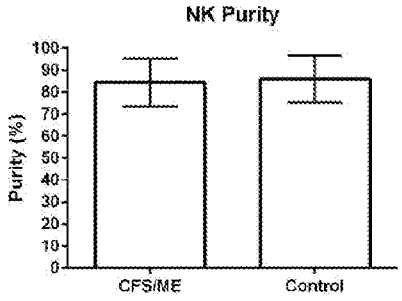


Figure 22

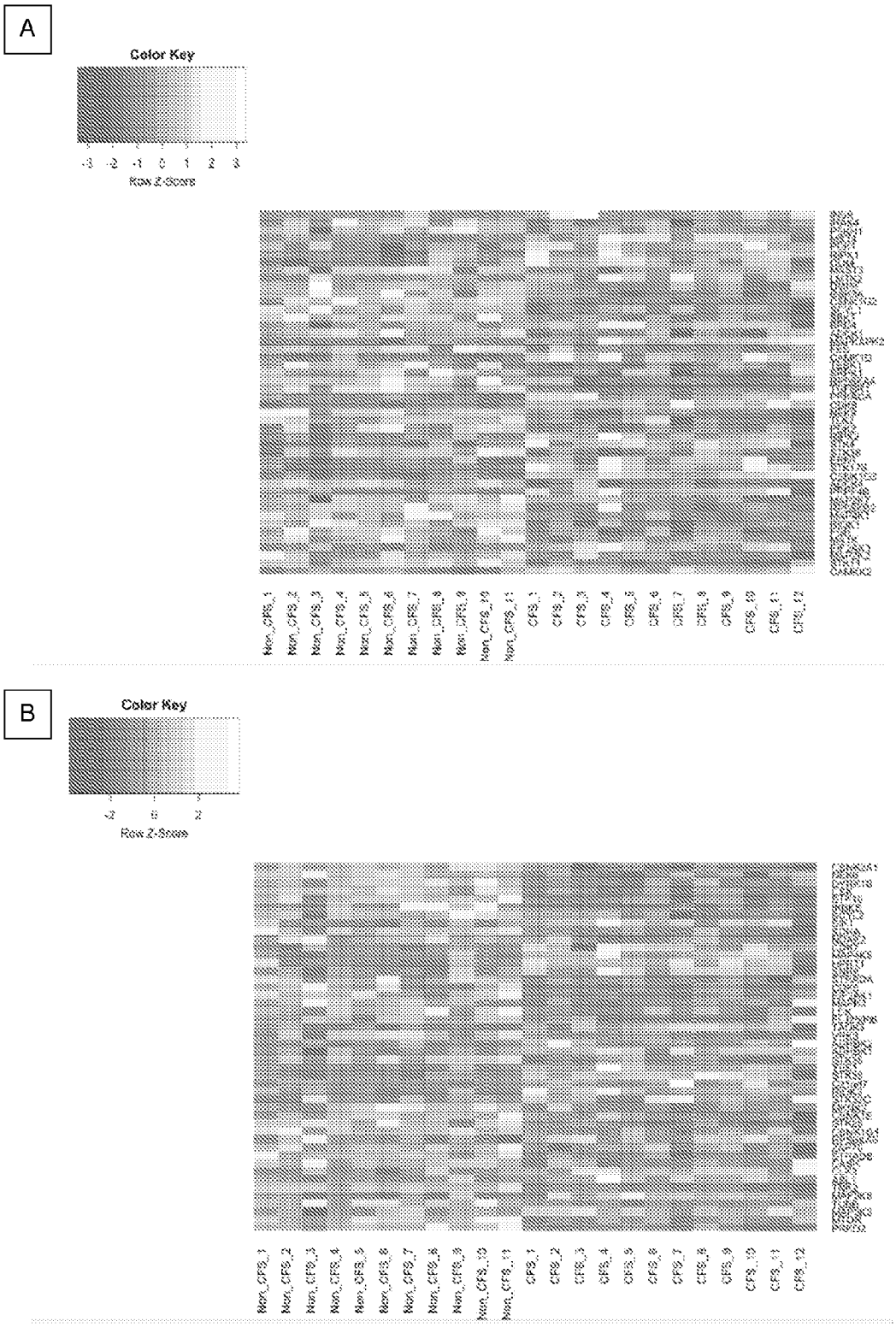


Figure 23

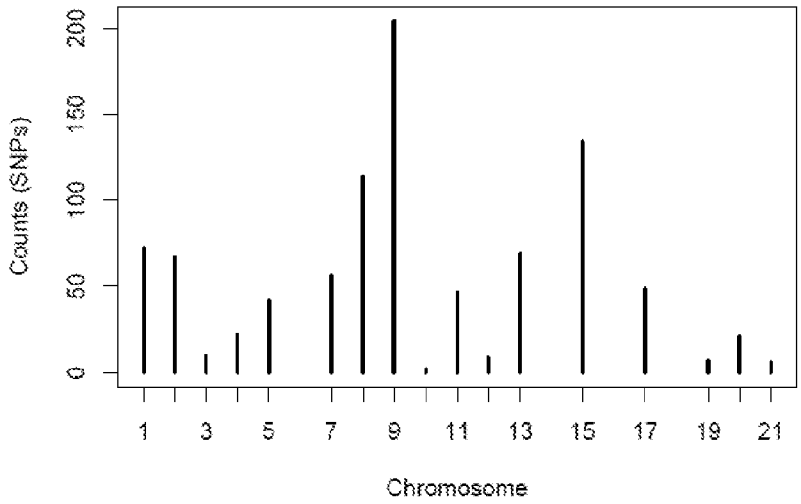


Figure 24

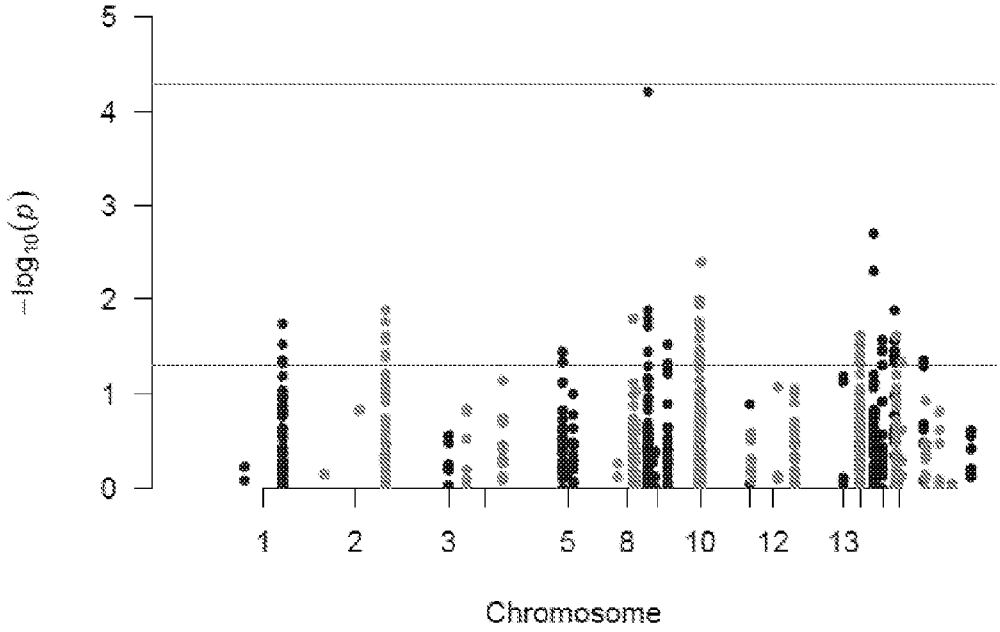
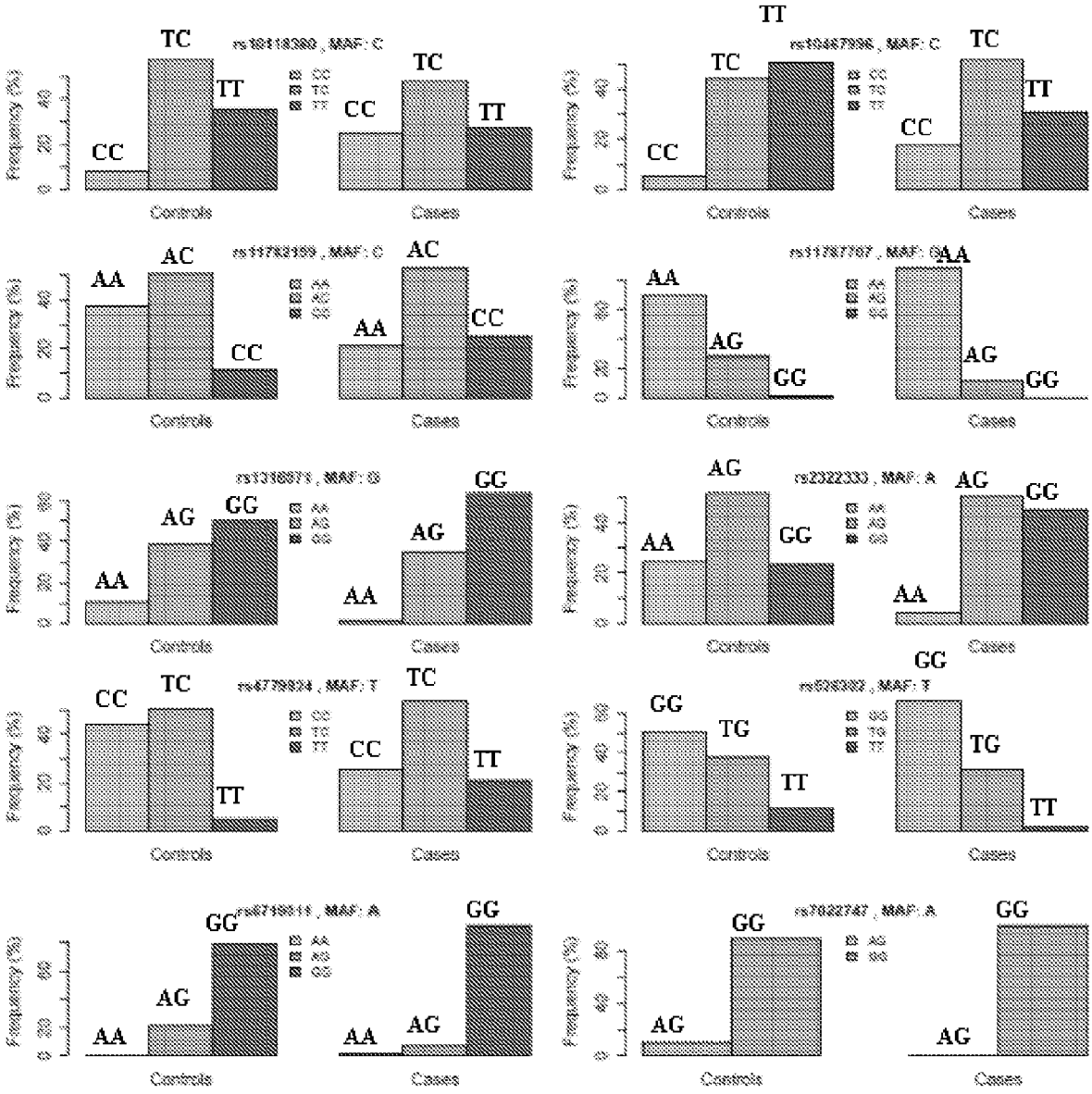


Figure 25


CC
TC
TT
CC
TC
TT
TT
TC
CC
TC
TT
CC
AA
AC
CC
AA
AC
CC
AA
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AA
AG
GG
AA
AG
GG
AA
AG
GG
CC
TC
TT
CC
TC
TT
GG
TG
TT
GG
TG
TT
AA
AG
GG
AA
AG
GG
AG
GG
AG
GG
Controls
Cases
Frequency (%)

Figure 26

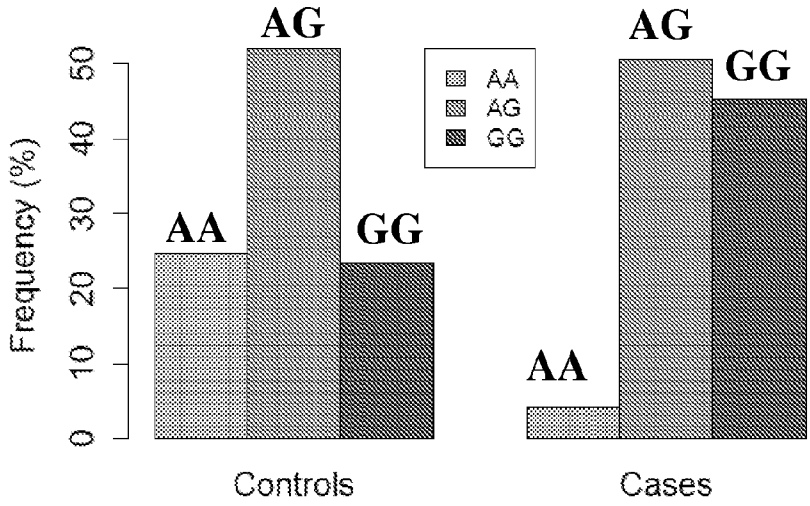
AG
AG
GG
AA
GG
AA
AA
AG
GG
Frequency (%)
0
10
20
30
40
50
Controls
Cases

Figure 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050313

A. CLASSIFICATION OF SUBJECT MATTER

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: EPODOC, WPIAP, TXTE, MEDLINE, CAPLUS, BIOSIS, EMBASE. Searched for keywords: TRP, TRANSIENT RECEPTOR CHANNEL, CHRONIC FATIGUE, MYALGIC ENCEPHALOMYELITIS, SNP, POLYMORPHISM and like terms.

Databases: EPODOC, WPIAP, TXTE, MEDLINE, CAPLUS, BIOSIS, EMBASE, GOOGLE SCHOLAR. Searched for TRP SNP identification (rs) numbers.

Database: GENOMEQUEST. Searched for SEQ ID NOs 1-63 at 100% sequence identity.

Databases: THE LENS, PUBMED, GOOGLE SCHOLAR and internal databases provided by IP Australia. Searched for applicant and inventor names.

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	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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

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

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2016	16 August 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Kelly Hitchens AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 262223604

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2016/050313**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHU, G., et al., "Association of TRPV4 gene polymorphisms with chronic obstructive pulmonary disease", Human Molecular Genetics, 2009, vol. 18, no. 11, pages 2053-2062. Abstract, Page 2059 column 2 paragraph 3	1-25, 64-66, 76-78
L	SOMMERFELDT, L., et al., "Polymorphisms of adrenergic cardiovascular control genes are associated with adolescent chronic fatigue syndrome", Acta Paediatrica, 2011, vol. 100, no. 2, pages 293-298. Unity	
L	FINN, S., et al., "Expression microarray analysis of papillary thyroid carcinoma and benign thyroid tissue: emphasis on the follicular variant and potential markers of malignancy", Virchows Archiv, 2007, vol. 450, no. 3, pages 249-260. Unity	
L	LANDMARK-H0YVIK, H., et al., "Alterations of gene expression in blood cells associated with chronic fatigue in breast cancer survivors", The Pharmacogenomics Journal, 2009, vol. 9, no. 5, pages 333-340. Unity	
L	KIM, S.-C, at al, "Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PAC1", Blood, 1999, vol. 93, no. 11, pages 3893-3899. Unity	
X	LIGHT, A., et al, "Gene expression alterations at baseline and following moderate exercise in patients with Chronic Fatigue Syndrome and Fibromyalgia Syndrome", Journal of Internal Medicine, 2012, vol. 271, no. 1, pages 64-81. Tables 2-3, Figures 2-3, Page 68 column 2 paragraph 3 - page 69 column 1, Page 79 column 2 paragraph 1	2, 5-6, 8, 10, 12, 14-25, 64-66, 76-78
X	WHITE, A., et al., "Differences in metabolite-detecting, adrenergic, and immune gene expression following moderate exercise in chronic fatigue syndrome, multiple sclerosis and healthy controls", Psychosomatic Medicine, 2012, vol. 74, no. 1, pages 46-54. Abstract, Figures 2 and 5, 'mRNA Extraction and Analysis' page 47, 'Conclusion' page 53	2, 5-6, 8, 10, 12, 14-25, 64-66, 76-78
X	VON SPICZAK, S., et al., "Association study of TRPC4 as a candidate gene for generalized epilepsy with photosensitivity", Neuromolecular Medicine, 2010, vol. 12, no. 3, pages 292-299. Abstract, Table 2, Page 293 column 2 paragraph 1	1-25, 64-66, 68-69, 76-78
X	CARRENO, O., et al., "SNP variants within the vanilloid TRPVl and TRPV3 receptor genes are associated with migraine in the Spanish population", American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2012, vol. 159, no. 1, pages 94-103. Abstract, Table 1, Page 96 column 1 paragraph 2	1-26, 64-67, 76-78
X	WO 2010/001419 A2 (DECODE GENETICS EHF) 07 January 2010 Abstract, Table 8 page 90, Page 18 lines 1 - 18, Page 27 lines 32-34, , Page 48 lines 12-15, Page 55 lines 1 - 12, Page 102 lines 16-26	1-26, 64-70, 76-78
	WO 2010/120746 A1 (OREGON HEALTH AND SCIENCE UNIVERSITY) 21 October 2010	

INTERNATIONAL SEARCH REPORT	International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2016/050313**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Claim 1, Tables 2-3, Page 15 lines 27-31, Page 16 lines 16-18, Page 29 line 31 - page 30 line 2, Page 33 lines 25-26, Page 38 lines 12-16	1-25, 64-66, 76-78
X	US 7888497 B2 (BENTWICH et al.) 15 February 2011 Abstract, Sequence listing (SEQ ID NO 83470)	27
X	US 2006/0057564 A1 (WANG) 16 March 2006 Abstract, Sequence listing (SEQ ID NOs 4829667, 5702727, 19061 74, 481 6350, 5702729, 6084837, 4792847, 4828243, 5449880, 6099712, 4763466, 4777334)	27
P,X	YIM, Y.-R., et al., "Polymorphisms of Transient Receptor Potential Vanilloid (TRPV) 2 and TRPV3 Gene Polymorphisms Were Associated with Fibromyalgia in a Korean Population", Arthritis & Rheumatology, 29 September 2015, vol. 67, supplement 10, abstract 2300. Abstract	1-25, 64-66 and 76-78
P,X	PARK, D.-J., et al., "Polymorphisms of the TRPV2 and TRPV3 genes associated with fibromyalgia in a Korean population", Rheumatology, 2016 (epub date 13 April 2016), article kewl80. Abstract, Page 1520 column 1 paragraph 3	1-25, 64-66 and 76-78
P,X	IACOB, E., et al., "Gene expression factor analysis to differentiate pathways linked to fibromyalgia, chronic fatigue syndrome, and depression in a diverse patient sample", Arthritis Care & Research, 2016 (epub date 21 December 2015), vol. 68, no. 1, pages 132-140. Abstract, Figure 1, Page 134 column 1 paragraph 3	2, 5-6, 8, 10, 12, 14-25, 64-66 and 76-78
P,X	MARSHALL-GRADISNIK, S., et al., "Examination of single nucleotide polymorphisms (SNPs) in transient receptor potential (TRP) ion channels in chronic fatigue syndrome patients", Immunology and Immunogenetics Insights, 2015 (epub date 10 May 2015), vol. 7, page 1-6. Abstract, Table 1	1-27, 64-70 and 76-78
P,X	MARSHALL-GRADISNIK, S., et al., "Genotype Frequencies of Transient Receptor Potential Melastatin M3 Ion Channels and Acetylcholine Muscarinic M3 Receptor Gene Polymorphisms in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Patients", Immunology and Immunogenetics Insights, 2016 (epub date 14 February 2016), vol. 8, pages 1-2. Table 1	1-26, 64-70 and 76-78
P,X	MARSHALL-GRADISNIK, S., et al., "Natural killer cells and single nucleotide polymorphisms of specific ion channels and receptor genes in myalgic encephalomyelitis/chronic fatigue syndrome", The Application of Clinical Genetics, 31 March 2016, vol. 9, article 39. Abstract, Tables 2-3	1-27, 64-70 and 76-78

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050313

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. Ill 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.:

1-27, 64-70, 76-78 (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.

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 4 major groupings, comprising of 68 inventions, based on the following features that separate the claims into distinct groups:

Group A*

Invention 1*. Claims 1-27, 64-70 and 76-78 (in part) insofar as they relate to TRPs, in particular detecting a SNP, measuring the level of expression, testing for the gene or a gene product, or property thereof to indicate, determine a predisposition to, identify, screen for, diagnose, monitor, manage, or determine risk of developing a medical condition or symptom thereof.

Invention 2*. Claims 1-26 and 64-78 (in part) insofar as they relate to AChRs, in particular detecting a SNP, measuring the level of expression, testing for the gene or a gene product, or property thereof to indicate, determine a predisposition to, identify, screen for, diagnose, monitor, manage, or determine risk of developing a medical condition or symptom thereof.

Invention 3*. Claims 1-27 and 64-78 (in part) insofar as they relate to ARDs, in particular detecting a SNP, measuring the level of expression, testing for the gene or a gene product, or property thereof to indicate, determine a predisposition to, identify, screen for, diagnose, monitor, manage, or determine risk of developing a medical condition or symptom thereof.

Group B

Invention 4. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated ABL1 to indicate, identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 5. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated ADCK1 or ADCK4 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 6. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated ADRBK1 or ADRBK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 7. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated BRD4 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 8. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated C21orf7 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 9. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CAMK1D or CAMKK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 10. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CASK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 11. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CDK5, CDK7 or CDK9 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 12. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CLK3 or CLK4 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 13. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CSK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 14. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated CSNK1E, CSNK1G1, CSNK1G2, CSNK1G3 or CSNK2A1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Supplemental Box

Invention 15. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated DMPK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 16. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated DYRK1B to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 17. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated EIF2AK1 or EIF2AK3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 18. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated ERN1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 19. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated FES to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 20. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated FGR to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 21. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated FLJ25006 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 22. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated GSK3A to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 23. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated HPRTl to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 24. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated IKBKE to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 25. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated INSR to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 26. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated IRAK4 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 27. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated LCK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 28. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated LMTK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 29. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAP2K2 or MAP2K7 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 30. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAP3K3 or MAP3K8 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 31. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAP4K1 or MAP4K5 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 32. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAPK3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 33. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAPKAPK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 34. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MAST3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Supplemental Box

Invention 35. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MATK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 36. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MKNK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 37. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MST4 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 38. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated MTOR to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 39. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated NEK6 or NEK9 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 40. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated NUAK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 41. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated PDKlor PDK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 42. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated PRKACA or PRKD2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 43. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated PRPF4B to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 44. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated PSKH1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 45. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated RIOK1 or RIOK3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 46. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated RIPK1 or RIPK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 47. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated RPS6KA4, RPS6KA5 or RPS6KB2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 48. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SBK1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 49. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SCYL1 or SCYL2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 50. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SDHA to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 51. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SIK1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 52. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SNRK to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 53. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated SRPK1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Supplemental Box

Invention 54. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated STK4, STKIO, STK11, STK25, STK32C, STK35, STK38 or STK39 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 55. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated STRADA or STRADB to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 56. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TAOK3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 57. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TESK1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 58. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TGFBR1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 59. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TLK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 60. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TNK2 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 61. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated TUBB to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 62. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated VRK3 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 63. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated YES1 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof.

Invention 64. Claims 28-48 and 79-87 (in part) insofar as they relate to detecting differentially regulated ZAP70 to identify, screen for, diagnose, monitor, or determine risk of developing a medical condition or symptom thereof

Group C

Invention 65. Claims 49-56, 88-89 and 91-94 (in part) insofar as they relate to detecting a dysfunctional signalling through the MAPK pathway via MEK1/2 to evaluate for, determine a predisposition to, identify, diagnose, screen for or manage a medical condition or symptom thereof.

Invention 66. Claims 49-56, 88-90 and 92-94 (in part) and claim 90 (in full) insofar as they relate to detecting a dysfunctional signalling through the MAPK pathway via ERK1/2 to evaluate for, determine a predisposition to, identify, diagnose, screen for or manage a medical condition or symptom thereof.

Invention 67. Claims 49-56, 88-89 and 91-94 (in part) insofar as they relate to detecting a dysfunctional signalling through the MAPK pathway via p38 to evaluate for, determine a predisposition to, identify, diagnose, screen for or manage a medical condition or symptom thereof.

Group D

Invention 68. Claims 57-63 and 95-112 insofar as they relate to detecting calcium metabolism to indicate to identify, screen for, diagnose, monitor, determine risk of developing or managing a medical condition or symptom thereof.

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.

Supplemental Box

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 is detecting a biomarker to diagnose, monitor, manage, or determine risk of developing a medical condition or symptom thereof. Further, within the major invention groupings the only unifying features are: a gene or gene product (Group A); a calcium-dependent kinase gene (Group B); or dysfunctional signalling through the MAPK pathway (Group C). However, these features do not make a contribution over the prior art because they are disclosed in:

D1 (ZHU, G, ET AL., Human Molecular Genetics, 2009, vol. 18, no. 11, pages 2053-2062) discloses seven SNPs in TRPV4 are associated with Chronic obstructive pulmonary disease (Abstract).

D2 (SOMMERFELDT, L., ET AL., Acta Paediatrica, 2011, vol. 100, no. 2, pages 293-298) discloses SNPs in the adrenergic receptors associated with chronic fatigue syndrome (Abstract, Table 3).

D3 (FINN, S., ET AL., Virchows Archiv, 2007, vol. 450, no. 3, pages 249-260) discloses differentially expressed genes in malignant thyroid tissue, in comparison to benign tissue, including the calcium-dependent kinases MST4 and CAMK1 (Abstract, Table 2).

D4 (LANDMARK-H0YVIK, H., ET AL., The Pharmacogenomics Journal, 2009, vol. 9, no. 5, pages 333-340) discloses 103 genes, including MAPK3, that are differentially regulated in breast cancer survivors suffers suffering from chronic fatigue syndrome (Abstract, Table 8).

D5 (KIM, S.-C, ET AL., Blood, 1999, vol. 93, no. 11, pages 3893-3899) discloses constitutive activation (phosphorylation) of ERK and MEK kinases in bone marrow derived CD34+ cells from patients with acute leukemia (Abstract).

Therefore these common features cannot be special technical features. Hence there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

Due to the lack of unity only Invention 1 has been searched and examined.

*Please note, at national phase, depending on the jurisdiction, a further lack of unity within each of the Group A inventions may apply with regard to concepts of SNPs, level of a gene expression, testing for a gene or a gene product, or assaying a property thereof, and/or within the gene families claimed, and/or within the concept of any medical condition or symptom thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050313

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item l.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   SEQ ID NOs 1-63 were used for search purposes.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/050313**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/050313**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
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Patent Document/s Cited in Search Report		Patent Family Member/s	
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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End of Annex

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