Title: BIOMARKERS FOR DIAGNOSING POST TRAUMATIC STRESS DISORDER

Abstract: The invention relates to methods of determining if a subject is at risk of developing post-traumatic stress disorder (PTSD), with the methods comprising determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) and comparing the MCP-4/MCP-1 ratio (MMR) to normal MMR. An elevation in the MMR over normal ratios is indicative that the subject has an increased risk of suffering from PTSD.
BIOMARKERS FOR DIAGNOSING POST TRAUMATIC STRESS DISORDER

Statement Regarding Federally Sponsored Research or Development

[0001] Part of the work performed during development of this invention utilized U.S. Government funds through grant number CDMRP-PTSD (PTO74415). The U.S. Government has certain rights in this invention.

Reference to Sequence Listing

[0002] A computer readable text file, entitled “044508-5052-WO-SequenceListing.txt,” created on or about October 8, 2015 with a file size of about 5 kb contains the sequence listing for this application and is hereby incorporated by reference in its entirety.

Background of the Invention

Field of the Invention

[0003] The invention relates to methods of determining if a subject is at risk of developing post-traumatic stress disorder (PTSD), with the methods comprising determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) and comparing the MCP-4/MCP-1 ratio (MMR) a normal MMR. An elevation in the MMR over normal ratios is indicative that the subject has an increased risk of suffering from PTSD.

Background of the Invention

[0004] Post-traumatic stress disorder (PTSD) is a psychiatric disease, which occurs following exposure to traumatic events. PTSD may be acute or chronic, and can have a waxing and waning course of symptoms that can persist for months, years or decades [2,3]. Patients with chronic PTSD have changes in their immune systems which have been interpreted as due to sustained activation of the hypothalamic-pituitary-adrenal (HPA) axis, and resultant sympatho-adrenal-medullary (SAM) stress [4-6],[7]. The difference between a chronic PTSD patient and a resilient healthy control is that for a stress experience that resolves normally, the initially high levels of norepinephrine from the adrenal medulla, via the SAM, are eventually reduced by transient elevation of cortisol from the HPA. However, some studies have found chronic PTSD patients to have intrinsically low levels of circulating cortisol. Failure of feedback inhibition has been conjectured to be a driver for excessive levels of cell-mediated and proinflammatory cytokine expression [8-10]. For example, cytokines such as TNF-alpha and IL-6 are known to cross the blood brain barrier and to regulate the immune...
response through stimulation of the HPA axis [10,11]. Consistently, it has been reported that IL-1beta [12], TNF-alpha [13], and IL-6 [14,15] are elevated in the serum of PTSD patients. Furthermore, in the cerebrospinal fluid (CSF) of PTSD patients, both IL-6 [16] and norepinephrine [17] are reported to be higher in PTSD patients than in normal controls. The transition from a conventional high cortisol state to a low cortisol/high norepinephrine state has also been documented in a cohort of pediatric PTSD patients [16].

Patients with a diagnosis of PTSD often have co-morbid conditions such as major depressive disorder (MDD), as well as addiction to alcohol and other drugs [6,19]. Interestingly, while PTSD patients who are co-morbid with MDD have higher levels of serum IL-6 [20], those PTSD patients without co-morbid MDD have serum IL-6 levels that are identical to normal controls [20]. Furthermore, in a carefully controlled study of CSF from the NIMH cohort of civilian chronic PTSD patients, all with smaller dorsal hippocampus and relatively free from MDD, concentrations of Corticotrophin Releasing Factor (CRF), IL-6, BDNF, IGF-1 and Substance P were identical to levels in CSF from normal controls [1]. These results seem to suggest that cytokines or chemokines may not be involved in PTSD.

Summary of the Invention

The invention relates to methods of determining if a subject is at risk of developing post-traumatic stress disorder (PTSD), with the methods comprising determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) and comparing the MCP-4/MCP-1 ratio (MMR) to a normal MMR. An elevation in the MMR over normal ratios is indicative that the subject has an increased risk of suffering from PTSD.

The invention relates to methods of monitoring the progression of post-traumatic stress disorder (PTSD) in a subject, with the methods comprising determining the MMR in the subject on at least two different days and comparing the MMRs over time to determine if the subject’s MMR is changing over time. An increase in the subject’s MMR over time is indicative that the PTSD is progressing in the subject.

The invention relates to methods of diagnosing post-traumatic stress disorder (PTSD) in a subject, with the methods comprising determining the MMR in the subject and comparing the MMR to a normal MMR. An elevation in the MMR over a normal ratio is indicative that the subject has or is suffering from PTSD.

Brief Description of the Drawings
FIGURE 1 depicts dot-plot distributions of MCP-4, MCP-1 and the MCP-4/MCP-1 ratio in PTSD and healthy control plasma at 2 AM (a) and 9 AM (b), and in CSF at 9 AM (c). (a,b,c). MCP-1 (Monocyte Chemoattractant Protein-1, CCL2) is lower in PTSD plasma. (d,e,f). MCP-4 (Monocyte Chemoattractant Protein-4, CCL13) is higher in PTSD plasma. Levels of MCP-4 are vanishingly low in the CSF and cannot be accurately measured. (g,h,i). MCP-4/MCP-1 ratio is elevated in the 2 AM PTSD plasma (about 20%, p = 0.02) and in 9 AM PTSD plasma (about 66%, p = 0.004). All p values are two-tailed. Dotted horizontal lines are lower limits of detection (LLOD).

FIGURE 2 depicts the influence of PTSD on the circadian rhythms for the MCP-4/MCP-1 ratio, and individually for MCP-4 and MCP-1. (a). Circadian rhythm for the MCP-4/MCP-1 ratio in PTSD patients and healthy controls. The dotted contours are +/- 2 standard deviations of the data at each time point. (b). Circadian rhythm data for the MCP-4/MCP-1 ratio in which the data have been normalized, patient by patient, and filtered for high frequency variation. (c). Circadian rhythm data for MCP-4 alone, in which the data have been normalized, patient by patient, and filtered for high frequency variation. (d). Circadian rhythm data for MCP-1 alone, in which the data have been normalized, patient by patient, and filtered for high frequency variation. PTSD seems to affect both the phase and the amplitude of the MCP-4/MCP-1 ratio, as well as its component analytes.

FIGURE 3 depicts the tracking of MCP-4 and MCP-1 in healthy controls and PTSD patients. (a) healthy controls; (b) PTSD patients.

FIGURE 4 depicts the plasma concentrations of MCP-4 and MCP-1 in PTSD patients and healthy controls. (a) absolute concentrations; (b) average concentrations

FIGURE 5 (A-D) depicts area under the curve (AUC) for receiver operator curves (ROCs) for embodiments of the present invention.

FIGURE 6 depicts the AUC for ROCs for various chemokines.

FIGURE 7 (A-H) depicts circadian dependence of plasma chemokines in PTSD and controls.

FIGURE 8 (A-D) depicts the distribution of MCP-4/MCP-1 ratio in plasma over a circadian interval for patients with PTSD and healthy controls.

**Detailed Description of the Invention**

The invention relates to methods of determining if a subject is at risk of developing post-traumatic stress disorder (PTSD), with the methods comprising determining the ratio of monocyte
chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) and comparing the MCP-4/MCP-1 ratio (MMR) a normal MMR. An elevation in the MMR over normal ratios is indicative that the subject has an increased risk of suffering from PTSD.

[0018] Both MCP-4(CCL13) and MCP-1(CCL2) share 67% sequence homology and function as molecular attractants (“chemokines”) for monocytes, and, to a lesser extent, for lymphocytes and basophils. Both chemokines also share CCR2 as a common receptor [29,30]. Segman et al (2005) [31] studied peripheral blood mononuclear cells (PBMCs) in survivors of terror attacks in Israel. Yehuda et al (2009) [32] studied whole blood expression patterns in survivors of the World Trade Center attack in New York. Neylan et al (2011) [33] studied purified CD14+ monocytes, from men and women with PTSD, and other co-morbidities. In all three cases, a common observation was suppression of gene expression. The timing of blood collection was not mentioned or suggested in these publications.

[0019] The ratio of two monocyte chemokines, MCP-4 (CCL13) and MCP-1 (CCL2), constitute a time-independent, bivariate plasma biomarker for PTSD. Furthermore, in plasma from PTSD patients there is a disordered circadian pattern that is superimposed on the quantitatively elevated MCP-4/MCP-1 ratio, and its component individual analytes. These results suggest that the biomarker comprising the MCP-4/MCP-1 ratio is independent of an additional defect in circadian biology which may also characterize PTSD patients.

[0020] Recent studies, however, indicate that circulating monocytes exhibit a circadian oscillation, coinciding with endogenous MCP-1 expression that is driven by an autonomous circadian clock, and for which the time-dependent variation is independent of infection or metabolic stress [34,35]. In humans, nocturnal peak blood levels, encompassing the time period between 1 AM and 3 AM, can be observed for circulating monocytes, T lymphocytes and B lymphocytes [36]. Nocturnal monocytes activate the expression of MCP-1 [34]. Beginning at about 4 AM, levels of circulating monocytes, B cells and T cells begin to decline [36]. Coincidently, monocyte expression of MCP-1 is blocked by the transcription factors CLOCK, BMAL1 and EZH2 [34]. MCP-1 plasma levels of healthy controls also significantly drop from 2 AM to 9 AM by about 70% (p = 0.001) (Table 1 below and Figure 1). In the case of PTSD patients, MCP-1 levels also drop, but by a greater proportion, about 90%, and with greater significance (p = 2 X 10^-6). This process is also seen in greater detail in the circadian pattern. It is therefore possible that this disease-specific difference is the dynamic basis for MCP-1 contributing to the lower denominator portion of the PTSD-specific MCP-4/MCP-1 biomarker.

Table 1
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Healthy Controls</th>
<th>PTSD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio (CSF/plasma)</td>
<td>p-value</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↑ 1.3 ± 0.4</td>
<td>0.406</td>
</tr>
<tr>
<td>IL-10</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>~LLOD</td>
<td>0.857</td>
</tr>
<tr>
<td>IL-1β</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-2</td>
<td>↑ 2.4 ± 0.6</td>
<td>0.099</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑ 1.6 ± 0.5</td>
<td>0.211</td>
</tr>
<tr>
<td>IL-8</td>
<td>↑ 20.4 ± 5.5</td>
<td>2E-06</td>
</tr>
<tr>
<td>TNF-α</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>Eotaxin-1</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>≈ 1.1±0.1</td>
<td>0.649</td>
</tr>
<tr>
<td>IP-10</td>
<td>↑ 3.6 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>MCP-1</td>
<td>↑ 9.0 ± 1.3</td>
<td>2E-07</td>
</tr>
<tr>
<td>MCP-4</td>
<td>~LLOD</td>
<td>n.a.</td>
</tr>
<tr>
<td>MDC</td>
<td>↑ 18.6 ± 5.6</td>
<td>1E-04</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>↑ 5.1 ± 1.0</td>
<td>1E-05</td>
</tr>
<tr>
<td>TARC</td>
<td>↑ 10.1 ± 8.6</td>
<td>0.939</td>
</tr>
<tr>
<td>plasma MCP-1 vs CSF IL-6</td>
<td>↑ 7.5 ± 1.9</td>
<td>4E-04</td>
</tr>
</tbody>
</table>

↑ PTSD>HC; ↓ PTSD<HC
~LLOD: (viz, the analyte was too low in either the AM plasma or 9 AM CSF to calculate accurately. n.a.: not available

[0021] Less is known about the genetics or possible circadian variation of plasma MCP-4. MCP-4 plasma levels, however, also significantly decrease from 2 AM to 9 AM, by about 60% in Healthy Controls (p =0.004), compared to a significant decrease of only about 40% in PTSD patients (p =0.01). This process is also seen in greater detail in the circadian pattern (see Figure 2c). Thus the PTSD patients appear to express relatively reduced amounts of both MCP-1 and MCP-4 as the wake period begins, with a greater reduction in MCP-1 than for MCP-4. Thus the elevation of the MCP-4/MCP-1 ratio in PTSD patients may be due to PTSD-dependent modifications in synthesis rates of both of these analytes.

[0022] Sleep disturbance is a hallmark feature of PTSD [21,22]. It is therefore possible that sleep deprivation might be the root cause of the disordered circadian profile for MCP-4 and MCP-1. In a comprehensive study of ten fully instrumented normal males, however, Born et al (1997) [36] reported that following a 24 hour sleep deprivation experience, the succeeding 24 hours were characterized only by a blunting of the circadian changes in immune cell numbers, including monocytes, as well as in TNFα, IL-1β, and IL-6 levels. Born et al also reported that there were no changes in phase or appearance of multimodality. Thus the circadianopathy observed for PTSD patients for plasma MCP-4 and MCP-1 appears to be disease-specific. Furthermore, because there
were no observed PTSD-specific variations at the one available time point in any of the cytokines and chemokines in the CSF, the data disclosed herein may argue for a PTSD contribution to specific deficits in monocyte or immune cell biology. This conclusion is consistent with results mentioned above for PBMC [31] and whole blood [32], respectively, and for CD14+ mononuclear cells [33].

[0023] The central clock mechanism in the brain is run by light exposure and activation of CLOCK/BMAL1 signaling in the suprachiasmatic nucleus [37]. Subsidiary clocks in the periphery take their cue from this central mechanism, via the hypothalamic-Pituitary-Adrenal (HPA) axis, and adapt the exact subsidiary timing to their own requirements. In healthy controls, the circadian clock program is intrinsically plastic and can reversibly change in response to alterations in, for example, metabolism or sunrise time[28]. By contrast, chronic neuropsychiatric disorders have been associated with conditions in which the central clock appears to permanently “lose track of time” [38]. In the case of PTSD, however, these patients exhibit phase-shifted peripheral clocks for MCP-1 and MCP-4 and, at least in the case of MCP-4, a different pattern altogether.

[0024] For example, Figure 2c shows that MCP-4 levels in healthy controls have a monomodal distribution over 24 hours. In contrast MCP-4 distribution in PTSD patients transitions to four discrete peaks over 24 hours. In the case of MCP-1, Figure 2d shows MCP-1 in healthy controls also distributes in a monomodal distribution, peaking in the late night hours and lagging MCP-4 by about 2 hours (see Figure 3). MCP-1 in PTSD patients, however, lags the major late night peak by about 6 hours, and appears to “catch up” somewhat to lower levels in the late afternoon and evening.

[0025] Data presented here show that few PTSD-specific changes in CSF can be detected from simply screening cytokines and chemokines at 9 AM. In fact, the only detectable change was a small reduction in IL-8. These data are consistent with data reported by Bonne et al (2011) for a subset of cytokines in the same CSF samples.

[0026] In the meantime, there are other concentration gradients between 9 AM CSF and plasma. The highest elevation in the gradients was for IL-8 (elevated about 14-fold); for MCP-1 (elevated about 7-fold); for IP-10 (elevated about 7-fold); for MIP-1β and for Eotaxin 3 (both elevated about 4-fold). The most reduced analytes were MCP-4 (reduced about 80-100-fold); Eotaxin 1 (reduced about 18-fold); MDC (reduced about 25-fold); TARC (reduced about 9-fold); and TNFα (reduced about 6-fold).

[0027] Based on the data herein, the MCP-4/MCP-1 ratio constitutes a quantitative candidate biomarker for PTSD, which has the benefit of being measurable at any hour blood can be drawn. Furthermore, the circadian pattern of expression for both the ratio, and its individual components,
MCP-4 and MCP-1, are phase-shifted and multimodal compared to healthy controls. Thus, the relative MCP-4 and MCP-1 levels in plasma, accessed through the MCP-4/MCP-1 ratio, can be used as a functional biomarker for PTSD. Moreover, these data suggest that the MCP-4/MCP-1 ratio is independent of any defect in circadian biology which could also affect PTSD patients.

In addition, the circadian rhythms for plasma MCP-4 and MCP-1 are disordered in PTSD patients. Thus it cannot be ruled out that circadian dysfunction, possibly involving monocytes, or immune cells in general, may play an important role in the behavioral problems afflicting this class of psychiatric patients.

MCP-1 or CCL2, UniProt Accession No. P13500, is a well-known C-C motif chemokine and is tethered to endothelial cells via proteoglycans. MCP-1 is a protein of 99 amino acids, with residues 1-23 being the signal sequence. MCP-1 typically binds to the CCR2 and CCR4 receptors. As used herein, the term MCP-1 is not limited to a specific amino acid sequence but instead is used to mean a chemokine found in the blood of a subject that would be readily characterized as MCP-1 in any standard or commercially available analysis, e.g., ELISA assay. Thus any proteins detected as MCP-1, e.g., ability to bind to an antibody raised against a known MCP-1 protein, would be considered MCP-1 for the purposes of the present invention. In some embodiments, the term MCP-1 means the mature (full length polypeptide without the signal sequence) form of the MCP-1 protein.

In specific embodiments, the term MCP-1 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:1. In other embodiments, the term MCP-1 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:2.

MKVSAALCL LLIAATFIPQ GLAQPDAINA PVTCCYNFTN RKISVQRLAS YRRITSSKCP KEAVIFKTIV AKEICADPKQ KWVQDSMDHL DKQTQTPKT (SEQ ID NO: 1)

QPDAINAPVT CCYNFTNKRQ SVQRASYRR ITSSKCPKEA VIFKTIVAKE ICADPKQKWV QDSMDHLDKQ QTQPKT (SEQ ID NO: 2)

MCP-4 or CCL 13, UniProt Accession No. Q99616, is a well-known C-C motif chemokine and is widely expressed in small intestine, thymus, colon, lung, trachea, stomach, lymph nodes and pulmonary artery smooth muscle cells. MCP-4 is a protein of 98 amino acids, with residues 1-16 being the signal sequence. The mature or “long chain” MCP-4 protein (full length protein without
the signal sequence) can be further processed or cleaved into two additional chains, known as medium and short chains, which are shown below. MCP-4 typically binds to the CCR2B and CCR3 receptors and is a chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils. As used herein, the term MCP-4 is not limited to a specific amino acid sequence but instead is used to mean a chemokine found in the blood of a subject that would be readily characterized as MCP-4 in any standard or commercially available analysis, e.g., ELISA assay. Thus any proteins detected as MCP-4, e.g., ability to bind to an antibody raised against a known MCP-4 protein, would be considered MCP-4 for the purposes of the present invention. In specific embodiments, the term MCP-4 means only the mature MCP-4 chain, the medium MCP-4 chain version and the shorter MCP-4 chain version of the protein. In other specific embodiments, the term MCP-4 means only the medium MCP-4 chain version and the shorter MCP-4 chain version of the protein. In addition embodiments, the term MCP-4 means only the shorter MCP-4 chain version of the protein.

[0032] In specific embodiments, the term MCP-4 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:3 (full length). In other embodiments, the term MCP-4 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:4 (long chain MCP-4). In other embodiments, the term MCP-4 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:5 (medium chain MCP-4). In other embodiments, the term MCP-4 means a protein with an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:6 (short chain MCP-4).

MKVSAVLLCL LLMTAAFPQ GLAQPDALNV PSTCCFTSSS KKLISLQRLKS YVITTSRCPQ KAVIFRTKLG KEICADPEK EKVQNYMKHLG RKAHTLKT (SEQ ID NO:3)

FNPQGLAQPD ALNVSTCCF TFSKKLQSLQ RLKSYVITTS RCPQKAVIFRTKLGKEICAD PKEKQVQNYMKKHLRKAHTLKT (SEQ ID NO:4)

LAQPDALNVFP STCCFTSSK KISLQRLKSY VITTSRCPQK AVIFRTKLGKEICADPEK KVEQNYMKHLGR KAHTLKT (SEQ ID NO:5)
A polypeptide having an amino acid sequence at least, for example, about 95% “identical” to a reference amino acid sequence, e.g., SEQ ID NO:2, is understood to mean that the amino acid sequence of the polypeptide is identical to the reference sequence except that the amino acid sequence may include up to about five modifications per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a peptide having an amino acid sequence at least about 95% identical to a reference amino acid sequence, up to about 5% of the amino acid residues of the reference sequence may be deleted or substituted with another amino acid or a number of amino acids up to about 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These modifications of the reference sequence may occur at the N-terminus or C-terminus positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

As used herein, “identity” is a measure of the identity of nucleotide sequences or amino acid sequences compared to a reference nucleotide or amino acid sequence. In general, the sequences are aligned so that the highest order match is obtained. “Identity” per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g., Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York (1988); Biocomputing: Informatics And Genome Projects, Smith, D. W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); von Heinje, G., Sequence Analysis In Molecular Biology, Academic Press (1987); and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York (1991)). While there are several methods to measure identity between two polynucleotide or polypeptide sequences, the term “identity” is well known to skilled artisans (Carillo, H. & Lipton, D., Siam J Applied Math 48:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego (1994) and Carillo, H. & Lipton, D., Siam J Applied Math 48:1073 (1988). Computer programs may also contain methods and algorithms that calculate identity and similarity. Examples of computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., Nucleic Acids Research 12(i):387 (1984)), BLASTP, ExPASy, BLASTN, FASTA (Atschul, S. F., et al., J Molec Biol 215:403 (1990)) and FASTDB. Examples of methods to determine identity and similarity are

[0035] In one embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is BLASTP. In another embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is FASTDB, which is based upon the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990), incorporated by reference). In a FASTDB sequence alignment, the query and reference sequences are amino sequences. The result of sequence alignment is in percent identity. In one embodiment, parameters that may be used in a FASTDB alignment of amino acid sequences to calculate percent identity include, but are not limited to: Matrix=PAM, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject amino sequence, whichever is shorter.

[0036] If the reference sequence is shorter or longer than the query sequence because of N-terminus or C-terminus additions or deletions, but not because of internal additions or deletions, a manual correction can be made, because the FASTDB program does not account for N-terminus and C-terminus truncations or additions of the reference sequence when calculating percent identity. For query sequences truncated at the N- or C-termini, relative to the reference sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminus to the reference sequence that are not matched/aligned, as a percent of the total bases of the query sequence. The results of the FASTDB sequence alignment determine matching/alignment. The alignment percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score can be used for the purposes of determining how alignments “correspond” to each other, as well as percentage identity. Residues of the reference sequence that extend past the N- or C-termini of the query sequence may be considered for the purposes of manually adjusting the percent identity score. That is, residues that are not matched/aligned with the N- or C-termini of the comparison sequence may be counted when manually adjusting the percent identity score or alignment numbering.

[0037] For example, a 90 amino acid residue query sequence is aligned with a 100 residue reference sequence to determine percent identity. The deletion occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment does not show a match/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the reference sequence (number of residues at the N- and C-termini not matched/total number of residues in the reference
sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched (100% alignment) the final percent identity would be 90% (100% alignment – 10% unmatched overhang). In another example, a 90 residue query sequence is compared with a 100 reference sequence, except that the deletions are internal deletions. In this case the percent identity calculated by FASTDB is not manually corrected, since there are no residues at the N- or C-termini of the subject sequence that are not matched/aligned with the query. In still another example, a 110 amino acid query sequence is aligned with a 100 residue reference sequence to determine percent identity. The addition in the query occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment may not show a match/alignment of the first 10 residues at the N-terminus. If the remaining 100 amino acid residues of the query sequence have 95% identity to the entire length of the reference sequence, the N-terminal addition of the query would be ignored and the percent identity of the query to the reference sequence would be 95%.

[0038] As used herein, the terms “correspond(s) to” and “corresponding to,” as they relate to sequence alignment, are intended to mean enumerated positions within the reference protein, e.g., wild-type MCP-1, and those positions in a mutant or related MCP-1 that align with the positions on the reference protein. Thus, when the amino acid sequence of a subject peptide is aligned with the amino acid sequence of a reference MCP-1, e.g., SEQ ID NO:2, the amino acids in the subject sequence that “correspond to” certain enumerated positions of the reference sequence are those that align with these positions of the reference sequence, e.g., SEQ ID NO:2, but are not necessarily in these exact numerical positions of the reference sequence. Methods for aligning sequences for determining corresponding amino acids between sequences are described herein. Accordingly, embodiments of the present invention comprise detecting or determining levels (or ratios thereof) of biomarkers, with the biomarkers corresponding to MCP-1 and/or MCP-4.

[0039] As used herein, the term subject or “test subject” indicates a mammal, in particular a human or non-human primate. The test subject may or may not be in need of an assessment of a predisposition to PTSD. For example, the test subject may or may not have a recollection of an experience that could be associated with PTSD prior to applying the methods of the present invention. In another embodiment, the test subject has not been identified as a subject that may have had an experienced that could lead to PTSD prior to applying the methods of the present invention.

[0040] As used herein, PTSD is used as it is commonly understood in the art. In select embodiments, PTSD in the subject is assessed using the Structured Clinical Interview for DSM-IV
(SCID) as is understood in the art. As is also understood in the art, the severity of PTSD can range from mild to severe. In select embodiments of the present invention, the severity of the PTSD can be assessed at any time, i.e., before or after determining the MMR in the subject. The severity of the PTSD can be assessed in any manner, such as, but not limited to, the Clinician-Administered PTSD scale (CAPS).

[0041] As used herein, the term means “increased risk” is used to mean that the test subject has an increased chance of developing or acquiring PTSD compared to a normal individual. The increased risk may be relative or absolute and may be expressed qualitatively or quantitatively. For example, an increased risk may be expressed as simply determining the subject’s MMR and placing the patient in an “increased risk” category, based upon previous population studies. Alternatively, a numerical expression of the subject’s increased risk may be determined based upon the MMR. As used herein, examples of expressions of an increased risk include but are not limited to, odds, probability, odds ratio, p-values, attributable risk, relative frequency, positive predictive value, negative predictive value, and relative risk.

[0042] For example, the correlation between a subject’s MMR and the likelihood of suffering from PTSD may be measured by an odds ratio (OR) and by the relative risk (RR). If $P(R^+)$ is the probability of developing PTSD for individuals with the risk profile (R) and $P(R^-)$ is the probability of developing PTSD for individuals without the risk profile, then the relative risk is the ratio of the two probabilities: $RR = P(R^+)/P(R^-)$.

[0043] In case-control studies, however, direct measures of the relative risk often cannot be obtained because of sampling design. The odds ratio allows for an approximation of the relative risk for low-incidence diseases and can be calculated: $OR = (F^+/(1-F^-))/((F^-/(1-F^+))$, where $F^+$ is the frequency of a risk profile in cases studies and $F^-$ is the frequency of risk profile in controls. $F^+$ and $F^-$ can be calculated using the MMR frequencies of the study.

[0044] The attributable risk (AR) can also be used to express an increased risk. The AR describes the proportion of individuals in a population exhibiting PTSD due to a specific member of the MMR. AR may also be important in quantifying the role of individual components (specific member, e.g., MCP-4 or MCP-1) in condition etiology and in terms of the public health impact of the individual marker. The public health relevance of the AR measurement lies in estimating the proportion of cases of PTSD in the population that could be prevented if the MMR or individual component were considered normal. AR may be determined as follows: $AR = P_e(1 RR - 1)/(P_e(1 RR - 1) + 1)$, where $AR$ is the risk attributable to an MMR value or individual component of the MMR, and $P_e$ is
the frequency of exposure to an MMR or individual component of the MMR within the population at large. RR is the relative risk, which can be approximated with the odds ratio when the MMR or individual component of the MMR under study has a relatively low incidence in the general population.

[0045] In one embodiment, the increased risk of a patient can be determined from p-values that are derived from association studies. Specifically, associations with specific MMRs can be performed using regression analysis by regressing the MMR with PTSD. In addition, the regression may or may not be corrected or adjusted for one or more factors. The factors for which the analyses may be adjusted include, but are not limited to age, sex, weight, ethnicity, geographic location, fasting state, state of pregnancy or post-pregnancy, menstrual cycle, general health of the subject, alcohol or drug consumption, caffeine or nicotine intake and circadian rhythms, and the subject’s genotype to name a few.

[0046] Increased risk can also be determined from p-values that are derived using logistic regression. Binomial (or binary) logistic regression is a form of regression which is used when the dependent is a dichotomy and the independents are of any type. Logistic regression can be used to predict a dependent variable on the basis of continuous and/or categorical independents and to determine the percent of variance in the dependent variable explained by the independents; to rank the relative importance of independents; to assess interaction effects; and to understand the impact of covariate control variables. Logistic regression applies maximum likelihood estimation after transforming the dependent into a “logit” variable (the natural log of the odds of the dependent occurring or not). In this way, logistic regression estimates the probability of a certain event occurring. These analyses are conducted with the program SAS.

[0047] Techniques to assay levels of individual components of the MMR from test samples are well known to the skilled technician, and the invention is not limited by the means by which the components are assessed. In one embodiment, levels of the individual components of the MMR are assessed using mass spectrometry in conjunction with ultra-performance liquid chromatography (UPLC), high-performance liquid chromatography (HPLC), and UPLC to name a few. Other methods of assessing levels of the individual components include biological methods, such as but not limited to ELISA assays.

[0048] The assessment of the levels of the individual components of the MMR can be expressed as absolute or relative values and may or may not be expressed in relation to another component, a standard an internal standard or another molecule of compound known to be in the sample. If the
levels are assessed as relative to a standard or internal standard, the standard may be added to the test sample prior to, during or after sample processing.

[0049] The subject’s MMR is compared to an MMR that is deemed to be a normal MMR. To establish the MMR of a normal individual, an individual or group of individuals may be first assessed for PTSD to establish that the individual or group of individuals is not suffering from PTSD. Once established, the MMR of the individual or group of individuals can then be determined to establish a “normal MMR.” In one embodiment, a normal MMR can be ascertained from the same subject when the subject is deemed to not be suffering from or exhibiting sign (clinical or otherwise) of PTSD. In one embodiment, a “normal” MMR is assessed in the same subject from whom the sample is taken prior to the onset of measurable, perceivable or diagnosed PTSD. That is, the term “normal” with respect to an MMR can be used to mean the subject’s baseline MMR prior to the onset of PTSD. The MMR can then be reassessed periodically and compared to the subject’s baseline MMR. Thus, the present invention also include methods of monitoring the progression of PTSD in a subject (including monitoring the effectiveness of a treatment of PTSD), with the methods comprising determining the subject’s MMR more than once over a period of at least more than one day. As used herein, a “day” is a 24-hour time period, not necessarily a different calendar day. For example, some embodiments of the methods of the present invention will comprise determining the subject’s MMR two, three, four, five, six, seven, eight, nine, 10 or even more times over a period of time, such as a year, two years, three, years, four years, five years, six years, seven years, eight years, nine years or even 10 years or longer. The methods of monitoring a subject’s risk of suffering from PTSD would also include embodiments in which the subject’s MMR is assessed during and after treatment of PTSD. In other words, the present invention also includes methods of monitoring the efficacy of treatment of PTSD by assessing the subject’s MMR over the course of the treatment and after the treatment. The treatment may be any treatment designed to treat the symptoms or root cause of MMR.

[0050] In another embodiment, a normal MMR is assessed in a sample from a different subject or patient (from the subject being analyzed) and this different subject does not have or is not suspected of having PTSD. In still another embodiment, the normal MMR is assessed in a population of healthy individuals, the constituents of which display no signs of PTSD. Thus, the subject’s MMR can be compared to a normal MMR generated from a single normal sample or a MMR generated from more than one normal sample.

[0051] The invention also relates to methods of monitoring the progression of post-traumatic stress disorder (PTSD) in a subject, with the methods comprising determining the MMR in the subject on at
least two different days and comparing the MMRs over time to determine if the subject’s MMR is changing over time. An increase in the subject’s MMR over time is indicative that the PTSD is progressing in the subject.

[0052] The invention also relates to methods of diagnosing post-traumatic stress disorder (PTSD) in a subject, with the methods comprising determining the MMR in the subject and comparing the MMR to a normal MMR. An elevation in the MMR over a normal ratio is indicative that the subject has or is suffering from PTSD.

[0053] The invention also relates to methods of treating a subject with post-traumatic stress disorder (PTSD), with the methods comprising determining that the subject has PTSD by using the methods of the invention described herein and administering to the subject with PTSD a therapeutic regimen to treat the PTSD. A therapeutic regimen used to treat PTSD includes but is not limited to, cognitive behavioral therapy (CBT), administration of one or more selective serotonin reuptake inhibitors (SSRIs), administration of one or more anti-anxiety medications and administration of one or more anti-insomnia medications. Specifically, the methods of treatment comprise determining the MMR in a sample obtained from the subject, and comparing the MMR in the sample to a normal MMR to determine if the subject’s MMR is altered compared to the normal MMR, where a change in the subject’s MMR compared to those defined as having a normal MMR is indicative that the subject is suffering from PTSD. Subsequent to this determination the subject is administered a therapeutic regimen for treating PTSD.

[0054] In select embodiments, the SSRIs that are administered to subject suffering from PTSD are selected from the group consisting of citalopram, dapoxetine, escitalopram, fluoxetine, fluvoxamine, indalpine, paroxetine, sertraline and zimelidine.

[0055] The invention also relates to kits that can be used in the methods of the present invention. Specifically, the present invention provides kits for the diagnosis, monitoring and/or treatment of PTSD, with the kits comprising one or more sets of antibodies that are immobilized onto a solid substrate and specifically bind to at least one of MCP-1 and MCP-4. In specific embodiments, the kits comprise at least two sets of antibodies immobilized onto a solid substrate, with one set of antibodies directed towards MCP-1 and the second set of antibodies being directed towards MCP-4.

[0056] The antibodies that are immobilized onto the substrate may or may not be labeled. For example, the antibodies may be labeled, e.g., bound to a labeled protein, in such a manner that binding of the specific protein may displace the label and the presence of the marker in the sample is marked by the absence of a signal. In addition, the antibodies that are immobilized onto the
substrate may be directly or indirectly immobilized onto the surface. Methods for immobilizing proteins, including antibodies, are well-known in the art, and such methods may be used to immobilize a target protein, e.g., MCP-4, or another antibody onto the surface of the substrate to which the antibody directed to the specific biomarker can then be specifically bound. In this manner, the antibody directed to the specific biomarker is immobilized onto the surface of the substrate for the purposes of the present invention.

[0057] The kits of the present invention may or may not include containers for collecting samples from the subject and one or more reagents, e.g., purified target biomarker such as MCP-1 or MCP-4 for preparing a calibration curve. The kits may or may not include additional reagents such as wash buffers, labeling reagents and reagents that are used to detect the presence (or absence) of the label.

Examples

[0058] Example 1 – Methods and Materials

[0059] Twelve medication-free outpatients with chronic civilian PTSD (median age 8 years old, 8 women/4 men) and eleven non-traumatized, healthy subjects (median age 29.5 years old, 5 women, 5 men) were selected from the original cohort study.

[0060] The healthy control subjects were chosen to match PTSD patients as closely as possible with respect to age, sex and BMI. In the subset of PTSD patients studied here, prodromal PTSD traumas were prepubertal in 5 subjects and as adults for 7 subjects. Time elapsed from trauma exposure was 26±4 years in pre-pubertal trauma, and 10.1±8.8 years in adult exposure. Patients were otherwise physically healthy, did not meet criteria for alcohol or substance abuse, or dependence, for at least six months prior to the study and were not receiving psychotropic medication for at least three weeks prior to lumbar puncture and concomitant venipuncture. The required medication-free period for PTSD patients, however, was extended to six weeks for patients previously taking fluoxetine or other SSRI. In addition, four patients (three female and one male) were included who had a history of trauma but without a follow-on history of PTSD. Although these latter patients were not included in the study, the data for these “trauma controls” were not statistically different from the healthy controls.

[0061] Psychiatric diagnoses were established using the Structured Clinical Interview for DSM-IV (SCID). The severity of PTSD was determined using the Clinician-Administered PTSD Scale (CAPS). Severity of depressive, anxiety and overall symptoms was assessed using the Inventory of Depressive
Symptomatology (IDS), Hamilton Anxiety Rating Scale (HAMA) and Clinical Global Impression-Severity scale (CGI-S), respectively. Individuals with PTSD and controls did not differ with regard to age, gender distribution, race, or body mass index (BMI). The severity of PTSD was moderate, with a CAPS score of 73.1 ± 10.3. The severity of depression (IDS 16.4 ± 8.2), anxiety (HAMA 13.1 ± 6.8) and overall symptoms (CGI-S 4 ± 1.2) were moderate as well.

[0062] Blood samples were collected from PTSD and healthy control patients. For this study, patients were implanted with indwelling intravenous catheters, and blood was collected each hour over a 27-hour period. There were at least two 9 AM time points in the entire plasma collection process, and samples from the second 9 AM time point were chosen for analysis. Care was taken to ensure that blood samples, drawn by hand from the indwelling catheter, were immediately anticoagulated with sodium citrate, and plasma collected by centrifugation. Care was also taken to ensure that nighttime sampling was done without disturbing the patient. Following immediate centrifugation, supernatant solutions were split and stored at -80°C.

[0063] Lumbar puncture (LP) was performed between 8:00 and 9:00 AM by an experienced physician. A 20-gauge introducer needle was inserted and approximately 15 cc of CSF was withdrawn, centrifuged at 4,000 RPM and frozen in aliquots at -80°C. The LPs were drawn on different days than the plasma collection.

[0064] Two multiplexed assays for cytokines and chemokines were used for analysis of patient and control plasma samples on the SECTOR® Imager 6000 instrument (Meso Scale Discovery, Gaithersburg, MD) The first of these assays was the Human ProInflammatory 9 Plex Assay for the measurement of IL-2, IL-8, IL-12p70, IL-1β, GM-CSF, IFN-γ, IL-6, IL-10 and TNF-α. (MesoScale catalog #K15007C-4). The second of these assays was the Human Chemokine 9 Plex Assay for the measurement of Eotaxin 1, MIP-1β, Eotaxin-3, TARC, IP-10, IL-8, MCP-1 (CCL2), MDC, and MCP-4 (CCL13) (catalog #K15001C-1). The samples were added to plates that were pre-coated with capture antibodies for the specific cytokines. The plates was sealed and shaken at room temperature for two hours. The plates were the washed in PBS + 0.05% Tween-20 and detection antibody solution (1 X or 1 µg/mL) was then added. The plates were sealed and shaken at room temperature for two hours. The plate was then washed in PBS + 0.05% Tween-20. Read buffer was added at a 2X concentration, and the plate was read on the SECTOR® 6000 Imager.

[0065] Data points were characterized on the basis of reproducible technical replicates, low % coefficient of variation (CV < 5%) present within the linear portion of the standard curve and a value above the lower limit of detection (LLOD). The differences between PTSD samples and normal
controls were calculated using a 2-tailed t-test and were taken as significant at the p ≤ 0.05 level or more than 2 standard deviations from the mean (SD ≥2.0), as appropriate. Values for all analytes from CSF for healthy controls were statistically indistinguishable from data published by others who had also used the industry-standard MesoScale electrochemiluminescence Sector 6000 platform for analysis [23-26]. For analysis of the relative circadian variation, values for individual patients were normalized to their individual 24 hour means, and changes for each patient were calculated as a percentage. The percentages were added for each hour to create composite profiles for PTSD and healthy control patients. Times were standardized to the sunrise time on the day blood sampling began (Z=0, the Zeitgeber). Circadian rhythms were modeled by fitting data to a cosine function.

[0066] *Example 2 – Results*

[0067] Table 2 below shows measurements of cytokines and chemokines in plasma collected from both PTSD and healthy control patients at the 9 AM time point. MCP-4 was significantly elevated by about 43%. The p value is 0.01 and the area under the curve ("AUC") is 0.82. In contrast, MCP-1 was reduced by about 20%. The AUC value is also 0.82. The MCP-1 and MCP-4 data thus stratify in opposite directions.

[0068] As shown in the last row in Table 2, by dividing these two inversely directional classifiers, the MCP-4/MCP-1 ratio was elevated 84% in PTSD plasma, and provides a highly significant candidate metric for PTSD plasma collected at 9 AM. The difference is significant, based on both a low p value of 0.004 and a high AUC value of 0.84. This PTSD-specific metric has a higher value at 9 AM than 2 AM (see below), indicating that the signal may be diurnal.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>PTSD (^1)</th>
<th>HC (^1)</th>
<th>p-value (^2)</th>
<th>Ratio (^3)</th>
<th>AUC (^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.79 ± 0.24</td>
<td>0.69 ± 0.32</td>
<td>0.21</td>
<td>≥1.15 ± 0.60</td>
<td>0.69</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.08 ± 0.02</td>
<td>0.18 ± 0.04</td>
<td>0.04</td>
<td>≥2.12 ± 0.63</td>
<td>0.71</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.15 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>0.72</td>
<td>≥0.93 ± 0.38</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.47 ± 0.04</td>
<td>0.77 ± 0.16</td>
<td>0.15</td>
<td>≥1.62 ± 0.33</td>
<td>0.74</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.67 ± 0.56</td>
<td>1.98 ± 0.59</td>
<td>0.67</td>
<td>≥0.84 ± 0.37</td>
<td>0.52</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.57 ± 0.41</td>
<td>1.57 ± 0.22</td>
<td>0.03</td>
<td>≥1.64 ± 0.44</td>
<td>0.76</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>503.45 ± 37.95</td>
<td>496.61 ± 37.29</td>
<td>0.91</td>
<td>≥1.01 ± 0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>6.90 ± 0.55</td>
<td>6.42 ± 0.38</td>
<td>0.58</td>
<td>≥1.07 ± 0.10</td>
<td>0.63</td>
</tr>
<tr>
<td>IP-10</td>
<td>226.06 ± 30.53</td>
<td>149.66 ± 13.57</td>
<td>0.04</td>
<td>≥1.51 ± 0.34</td>
<td>0.73</td>
</tr>
<tr>
<td>MCP-1</td>
<td>171.11 ± 17.01</td>
<td>207.10 ± 17.49</td>
<td>0.14</td>
<td>≥0.83 ± 0.11</td>
<td>0.82</td>
</tr>
<tr>
<td>MCP-4</td>
<td>298.35 ± 27.09</td>
<td>288.62 ± 23.94</td>
<td>0.01</td>
<td>≥1.43 ± 0.26</td>
<td>0.77</td>
</tr>
<tr>
<td>MDC</td>
<td>1850.02 ± 155.12</td>
<td>1680.60 ± 167.21</td>
<td>0.40</td>
<td>≥1.10 ± 0.14</td>
<td>0.61</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>65.85 ± 8.12</td>
<td>55.58 ± 4.77</td>
<td>0.36</td>
<td>≥1.18 ± 0.17</td>
<td>0.66</td>
</tr>
<tr>
<td>TARC</td>
<td>85.52 ± 13.86</td>
<td>67.32 ± 10.24</td>
<td>0.30</td>
<td>≥1.27 ± 0.28</td>
<td>0.70</td>
</tr>
<tr>
<td>MCP1/MCP4</td>
<td>0.61 ± 0.06</td>
<td>1.12 ± 0.18</td>
<td>4E-03</td>
<td>≥1.84 ± 0.33</td>
<td>0.84</td>
</tr>
<tr>
<td>MCP4/MCP1</td>
<td>1.82 ± 0.19</td>
<td>1.10 ± 0.16</td>
<td>4E-03</td>
<td>≥1.66 ± 0.28</td>
<td>0.84</td>
</tr>
</tbody>
</table>

[0069] Several other individual cytokines and chemokines were also significantly different in the PTSD 9 AM plasmas compared to healthy control plasmas. Significantly different cytokines and chemokines included IL-1β (reduced more than 2-fold; \( p = 0.04; \) AUC = 0.71); TNF-α (elevated about 64%; \( p = 0.03; \) AUC = 0.76); and IP-10 (elevated about 50%; \( p = 0.04; \) AUC = 0.73). Nonetheless, while these differences were significant, they were relatively modest, and the AUC values calculated from the receiver operation condition (ROC) were also modest.

[0070] Measurement of cytokines and chemokines in PTSD plasma at 2 AM

[0071] Table 3 below shows measurements of cytokines and chemokines in plasma collected from both PTSD and healthy control patients at 2 AM. The table indicates that the two analytes, MCP-1 and MCP-4, still varied in opposite directions, and each was among the highest AUC values on the list. For the case of PTSD, the last row in Table 3 shows that the MCP-4/MCP-1 ratio was elevated 34% in plasma from PTSD patients compared to healthy controls. The \( P \) value, based on a two-tailed
t-test, was 0.02, and the ROC curve has an AUC of 0.75. The multiparameter analysis thus also identified the MCP-4/MCP-1 ratio as a candidate binary classifier for PTSD and healthy controls in both 9 AM and 2 AM plasma samples.

Table 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>PTSD$^1$</th>
<th>HC$^1$</th>
<th>p-value$^2$</th>
<th>Ratio$^3$</th>
<th>AUC$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>0.39 ± 0.06</td>
<td>0.49 ± 0.09</td>
<td>0.50</td>
<td>0.81 ± 0.18</td>
<td>0.63</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.01 ± 0.15</td>
<td>1.01 ± 0.15</td>
<td>0.96</td>
<td>1.09 ± 0.20</td>
<td>0.56</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.45 ± 0.64</td>
<td>1.76 ± 0.56</td>
<td>0.22</td>
<td>1.14 ± 0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>1.21 ± 0.37</td>
<td>0.67 ± 0.15</td>
<td>0.46</td>
<td>1.82 ± 0.66</td>
<td>0.53</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.16 ± 0.03</td>
<td>0.22 ± 0.04</td>
<td>0.14</td>
<td>1.35 ± 0.36</td>
<td>0.68</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.31 ± 0.07</td>
<td>0.40 ± 0.06</td>
<td>0.11</td>
<td>1.29 ± 0.34</td>
<td>0.78</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.58 ± 0.31</td>
<td>2.09 ± 0.43</td>
<td>0.17</td>
<td>1.32 ± 0.36</td>
<td>0.68</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.63 ± 0.65</td>
<td>2.52 ± 0.41</td>
<td>0.84</td>
<td>1.05 ± 0.30</td>
<td>0.57</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.28 ± 0.52</td>
<td>3.91 ± 0.61</td>
<td>0.41</td>
<td>1.18 ± 0.21</td>
<td>0.55</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>529.42 ± 68.32</td>
<td>520.09 ± 90.82</td>
<td>0.71</td>
<td>1.02 ± 0.21</td>
<td>0.52</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>5.54 ± 0.75</td>
<td>6.61 ± 1.01</td>
<td>0.25</td>
<td>0.84 ± 0.17</td>
<td>0.61</td>
</tr>
<tr>
<td>IP-10</td>
<td>287.02 ± 49.92</td>
<td>197.54 ± 37.57</td>
<td>0.07</td>
<td>1.48 ± 0.34</td>
<td>0.70</td>
</tr>
<tr>
<td>MCP-1</td>
<td>292.69 ± 35.35</td>
<td>333.08 ± 49.15</td>
<td>0.47</td>
<td>0.88 ± 0.16</td>
<td>0.67</td>
</tr>
<tr>
<td>MCP-4</td>
<td>375.52 ± 48.01</td>
<td>320.24 ± 59.16</td>
<td>0.22</td>
<td>1.17 ± 0.25</td>
<td>0.65</td>
</tr>
<tr>
<td>MDC</td>
<td>3807.36 ± 656.11</td>
<td>2962.75 ± 470.42</td>
<td>0.24</td>
<td>1.29 ± 0.29</td>
<td>0.61</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>78.53 ± 10.84</td>
<td>93.43 ± 15.21</td>
<td>0.34</td>
<td>0.84 ± 0.17</td>
<td>0.61</td>
</tr>
<tr>
<td>TARC</td>
<td>87.96 ± 14.02</td>
<td>108.58 ± 19.72</td>
<td>0.38</td>
<td>0.81 ± 0.19</td>
<td>0.59</td>
</tr>
<tr>
<td>MCP1/MCP4</td>
<td>0.74 ± 0.09</td>
<td>1.02 ± 0.15</td>
<td>0.02</td>
<td>1.38 ± 0.25</td>
<td>0.74</td>
</tr>
<tr>
<td>MCP4/MCP1</td>
<td>1.20 ± 0.15</td>
<td>0.90 ± 0.14</td>
<td>0.02</td>
<td>1.34 ± 0.26</td>
<td>0.75</td>
</tr>
</tbody>
</table>

1 average ± sem, pg/ml
2 two-tailed t-test
3 † PTSD≠HC; ‡ PTSD×HC
4 Area Under the Curve of the ROC Curve
~LLOD: (viz, the analyte was too low in either the plasma or the CSF to calculate accurately
n.a.: not available.

[0072] Table 4 shows measurements of cytokines and chemokines in CSF, which were collected from both PTSD and healthy control patients at the 9 AM time point. Of the complete set of analytes, only reduction of IL-8 was able to approach significance in PTSD CSF. While IL-8 in PTSD CSF was reduced by about 25%, the p value (two tailed) was 0.06. Surprisingly, the MCP-4 and MCP-1 levels in the 9 AM CSF samples were the reverse of those found in plasma, and independent of PTSD. Specifically, in healthy control CSF, the MCP-1 levels were about 7-fold higher than in plasma, while the MCP-4 levels were about 100-fold lower. Furthermore, the MCP-4/MCP-1 ratio in CSF did
not significantly discriminate between PTSD and healthy control patients, as it does in the 2 AM or 9 AM plasma samples.

Table 4

<table>
<thead>
<tr>
<th>Analyte</th>
<th>PTSD³</th>
<th>HC³</th>
<th>p-value⁴</th>
<th>Ratio⁴</th>
<th>AUC⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.79 ± 0.24</td>
<td>0.69 ± 0.32</td>
<td>0.21</td>
<td>1.15 ± 0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>~LLOD</td>
<td>~LLOD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.08 ± 0.02</td>
<td>0.18 ± 0.04</td>
<td>0.94</td>
<td>2.12 ± 0.61</td>
<td>0.71</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.15 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>0.72</td>
<td>0.93 ± 0.38</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.47 ± 0.04</td>
<td>0.77 ± 0.16</td>
<td>0.13</td>
<td>1.62 ± 0.33</td>
<td>0.74</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.67 ± 0.56</td>
<td>1.98 ± 0.59</td>
<td>0.67</td>
<td>0.94 ± 0.32</td>
<td>0.52</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.57 ± 0.41</td>
<td>1.57 ± 0.22</td>
<td>0.91</td>
<td>1.01 ± 0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>503.45 ± 37.95</td>
<td>496.61 ± 37.29</td>
<td>0.58</td>
<td>1.07 ± 0.19</td>
<td>0.63</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>6.80 ± 0.55</td>
<td>6.42 ± 0.38</td>
<td>0.58</td>
<td>1.01 ± 0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>IF-10</td>
<td>226.06 ± 30.53</td>
<td>149.66 ± 13.37</td>
<td>0.94</td>
<td>1.51 ± 0.24</td>
<td>0.73</td>
</tr>
<tr>
<td>MCP-1</td>
<td>717.11 ± 17.01</td>
<td>207.10 ± 17.49</td>
<td>0.14</td>
<td>3.43 ± 0.13</td>
<td>0.82</td>
</tr>
<tr>
<td>MCP-4</td>
<td>298.35 ± 27.00</td>
<td>208.82 ± 23.94</td>
<td>0.14</td>
<td>1.45 ± 0.20</td>
<td>0.77</td>
</tr>
<tr>
<td>MDC</td>
<td>1830.02 ± 155.12</td>
<td>1680.69 ± 167.21</td>
<td>0.40</td>
<td>1.19 ± 0.14</td>
<td>0.61</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>65.85 ± 8.12</td>
<td>55.58 ± 4.77</td>
<td>0.16</td>
<td>1.18 ± 0.17</td>
<td>0.66</td>
</tr>
<tr>
<td>TARC</td>
<td>85.52 ± 13.86</td>
<td>67.52 ± 10.24</td>
<td>0.30</td>
<td>1.27 ± 0.23</td>
<td>0.70</td>
</tr>
<tr>
<td>MCP1/MCP4</td>
<td>0.61 ± 0.06</td>
<td>1.12 ± 0.18</td>
<td>1.0E-03</td>
<td>1.84 ± 0.23</td>
<td>0.84</td>
</tr>
<tr>
<td>MCP1/MCP1</td>
<td>1.82 ± 0.19</td>
<td>1.10 ± 0.16</td>
<td>1.0E-03</td>
<td>1.66 ± 0.28</td>
<td>0.84</td>
</tr>
</tbody>
</table>

³ average ± sem, pg/ml  
⁴ two-tailed t-test  
⁵ PTSD=HC  
⁶ AUC Under the Curve of the ROC Curve  
⁷ LLOD: the analyte was too low in either the plasma or the CSF to calculate accurately  
n.a.: not available

[0073] Figure 1 shows the dot-plot distributions of MCP-1 (Figure 1: a, b and c), MCP-4 (Figure 1: c, d and e) and the MCP-4/MCP-1 ratio (Figure 1: f, g and h) in PTSD and healthy control plasma at 2 AM and 9 AM, and in CSF at 9 AM, respectively. The MCP-1 levels in the 2 AM (Figure 1a) and the 9 AM (Figure 1b) plasma samples trend lower than levels for the healthy controls.

[0074] In contrast, in the 9 AM CSF (Figure 1c), the MCP-1 levels were similar for both PTSD and healthy controls, and were about 7 fold greater than MCP-1 levels in the parallel 9 AM plasma samples. On the other hand, Figure 1d (plasma 2 AM) and Figure 1e (plasma 9 AM) show that MCP-4 was elevated in PTSD plasma. However, levels of MCP-4 were similar in both PTSD and healthy controls in 9 AM CSF (Figure 1f), and contained about 100-fold less MCP-4 than in parallel 9 AM plasma samples.
plasma from either cohort. The MCP-4/MCP-1 ratio at 2 AM (Figure 1g) and 9 AM (Figure 1h) were elevated in PTSD plasma, but was low in the 9 AM CSF (Figure 1i).

[0075] Figure 4 also shows a difference plot for ([MCP-4] – [MCP-1]) from the 9 AM plasma samples. The statistical significance for the difference was the same as for the ratio (p= 0.004).

[0076] Figure 2a shows the 24 hour profile for the MCP-4/MCP-1 ratio in plasma from 5 PTSD and 5 healthy controls. The data indicate that across the entire 24 hour time period, the scale-free MCP-4/MCP-1 ratio for PTSD patients remained approximately twice that of healthy controls. Thus the ratio itself constitutes a viable metric that can be computed at any time a blood collection is made. Normalized and filtered data in Figure 2b shows that the MCP4/MCP-1 ratio in healthy controls peaks once in the late night hours. The PTSD patients, on the other hand, have two peaks, coinciding with the late night healthy control peak, and another occurring later in the morning.

[0077] Figure 2c also shows that MCP-4 peaks four times throughout the day in PTSD patients, compared to only once in healthy controls. Furthermore, the late night MCP-4 peak in PTSD patients trails the peak times healthy control by almost three hours. Similar but unique temporal disorder appears to also characterize the MCP-1 profile. For example, Figure 2d shows that there is a late night peak in MCP-1 concentration in PTSD patients and healthy controls, but the late night peak in PTSD patients trails by nearly six hours.

[0078] To further verify the specificity and significance of the MCP-4 and MCP-1 signals for PTSD, the circadian pattern of expression of seven other chemokines was tested for significance between PTSD patients and healthy controls across the entire 24 hour time period. Although there was an average difference between PTSD and healthy controls for some of these analytes, the variance among patients, shows substantial overlap in levels in all chemokines tested except for MCP-4. Figure 3 plots the p-values for the hourly differences between PTSD and healthy controls for all chemokines tested, versus the MCP-4/MCP-1 ratio, across the circadian 24 hour time period. The P-values for, MCP-4 and the MCP-4/MCP-1 ratio are in the range of between p = 0.01 to 0.0001. The best statistics for the MCP-4/MCP-1 ratio are at 3-4 hours past Z=0 (8-10 AM). In contrast to MCP-4, the other analytes were clustered individually in the statistical space occupied by p values between 0.1 and 1.0.

[0079] However, the noisy properties of individual circadian rhythm components are well known in the chronobiology literature. An alternative solution has been to compare differences in circadian rhythm for specific analytes by (i) filtering (“smoothing out”) the variation from hour to hour by making a moving average of every three hours; (ii) averaging the results for 5 individuals; (iii) and
ratio’ing all data to the mean for the entire 24 hour period [28]. Using these methods, Figure 7(A-H) shows that for the different chemokines tested, the circadian rhythms are disordered for PTSD patients, although significant, quantitative, time-independent differences can only be detected for the MCP-4/MCP-1 ratio. It is indeed not typical that a ratio of two unrelated markers would be useful for identifying a subject having a specific disease or condition.

[0080] Figure 8A shows the differences between average log plasma MCP-4/MCP-1 ratio in female PTSD patients vs female healthy controls. PTSD females are greater than healthy control females at every hour and the differences are significant, or trend to being significant at each hour (p < 0.05), across circadian time. Figure 8B shows the differences between average log plasma MCP-4/MCP-1 ratio in male PTSD patients vs male healthy controls. PTSD males are significantly greater than Healthy Control males at every hour (p < 0.05). Figure 8C shows the differences between average log plasma MCP-4/MCP-1 ratio in all PTSD patients vs all healthy controls. All PTSD patients are greater than all Healthy Controls at every hour and the differences are significant (p < 0.05). P values for each of the differences (Figure 8A-8C) at each hour are shown in Figure 8D. Error bars are +/- SEM (standard error of the mean).

[0081] All references herein are incorporated by reference.


What is Claimed is:

1. A method of determining if a subject is at risk of developing post-traumatic stress disorder (PTSD), the method comprising

   a) determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) in at least one sample obtained from the subject, and

   b) comparing the MCP-4/MCP-1 ratio (MMR) in the at least one sample to a normal MMR to determine if the subject’s MMR is altered compared to the normal MMR,

   wherein a change in the subject’s MMR is indicative that the subject has an increased risk of suffering from PTSD.

2. The method of claim 1, wherein the normal MMR comprises the subject’s MMR prior to the onset of PTSD.

3. The method of claim 1, wherein the normal MMR comprises an MMR generated from a population of individuals that do not suffer from PTSD.

4. The method of any of claims 1-3, wherein the at least one sample is a plasma sample obtained from whole blood taken from the subject.

5. The method of claim 4, wherein the whole blood is taken from the subject at one or more time points.

6. The method of claim 5, wherein the whole blood is taken from the subject at about 2 am (0200) and/or at about 9 am (0900).

7. The method of any of claims 1-6, wherein the subject was diagnosed with a depressive disorder prior to determining the MMR is the at least one sample obtained from the subject.

8. The method of claim 7, wherein the subject is receiving treatment for the depressive disorder, prior to determining the MMR is the at least one sample obtained from the subject.

9. The method of claim 8, wherein the treatment for the depressive disorder comprises administering at least one selective serotonin reuptake inhibitor (SSRI) to the subject.
10. The method of claim 9, wherein the SSRI is selected from the group consisting of citalopram, dapoxetine, escitalopram, fluoxetine, fluvoxamine, indalpine, paroxetine, sertraline and zimelidine.

11. The method of any of claims 1-6, wherein the subject has not been diagnosed with a depressive disorder.

12. A method of diagnosing post-traumatic stress disorder (PTSD) in a subject suspected of having PTSD, the method comprising

   a) determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) in a sample obtained from the subject, and

   b) comparing the MCP-4/MCP-1 ratio (MMR) in the sample to a normal MMR to determine if the subject’s MMR is altered compared to the normal MMR,

   wherein a change in the subject’s MMR compared to those defined as having a normal MMR is indicative that the subject is suffering from PTSD.

13. The method of claim 12, wherein the normal MMR comprises the subject’s MMR prior to the onset of PTSD.

14. The method of claim 12, wherein the normal MMR comprises an MMR generated from a population of individuals that do not suffer from PTSD.

15. The method of any of claims 12-14, wherein the at least one sample is a plasma sample obtained from whole blood taken from the subject.

16. The method of claim 15, wherein the whole blood is taken from the subject after midnight and before noon.

17. The method of claim 16, wherein the whole blood is taken from the subject at about 2 am (0200) and/or at about 9 am (0900).

18. The method of any of claims 12-17, wherein the subject was diagnosed with a depressive disorder prior to determining the MMR is the at least one sample obtained from the subject.
19. The method of claim 18, wherein the subject is receiving treatment for the depressive disorder, prior to determining the MMR is the at least one sample obtained from the subject.

20. The method of claim 19, wherein the treatment for the depressive disorder comprises administering at least one selective serotonin reuptake inhibitor (SSRI) to the subject.

21. The method of claim 20, wherein the SSRI is selected from the group consisting of citalopram, dapoxetine, escitalopram, fluoxetine, fluvoxamine, indalpine, paroxetine, sertraline and zimelidine.

22. A method of monitoring the progression of post traumatic stress disorder (PTSD) in a subject, the method comprising
   a) analyzing at least two samples from a subject with each sample taken on different days to determine the ratios of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1), and
   b) comparing the MCP-4/MCP-1 ratios (MMRs) over time to determine if the subject’s MMR is changing over time,

   wherein an increase in the subject’s MMR over time is indicative that the PTSD is progressing in the subject, and wherein a decrease or no change in the subject’s MMR over time is indicative that the PTSD is not progressing in the subject.

23. The method of claim 22, wherein the at least two samples are plasma samples obtained from whole blood taken from the subject.

24. The method of claim 23, wherein the whole blood is taken from the subject after midnight and before noon each day on which the samples are obtained.

25. The method of claim 23, wherein the whole blood is taken from the subject at about 2 am (0200) and/or at about 9 am (0900) each day on which the samples are obtained.

26. The method of any of claims 22-25, wherein the subject was diagnosed with a depressive disorder prior to determining the MMR in the subject.

27. The method of claim 26, wherein the subject is receiving treatment for the depressive disorder, prior to determining the MMR in the subject.
28. The method of claim 27, wherein the treatment for the depressive disorder comprises administering at least one selective serotonin reuptake inhibitor (SSRI) to the subject.

29. The method of claim 28, wherein the SSRI is selected from the group consisting of citalopram, dapoxetine, escitalopram, fluoxetine, fluvoxamine, indalpine, paroxetine, sertraline and zimelidine.

30. The method of any of claims 22-25, wherein the subject has not been diagnosed with a depressive disorder.

31. The method of any one of claims 22-30, wherein the subject is receiving treatment for the PTSD.

32. A method of treating a subject with post-traumatic stress disorder (PTSD), the method comprising

   a) determining the ratio of monocyte chemoattractant protein 4 (MCP-4) to monocyte chemoattractant protein 1 (MCP-1) in a sample obtained from the subject, and

   b) comparing the MCP-4/MCP-1 ratio (MMR) in the sample to a normal MMR to determine if the subject’s MMR is altered compared to the normal MMR, wherein a change in the subject’s MMR compared to those defined as having a normal MMR is indicative that the subject is suffering from PTSD, and

   c) administering to the subject with PTSD a therapeutic regimen to treat the PTSD, wherein the therapeutic regimen is selected from the group consisting of cognitive behavioral therapy (CBT), administration of one more selective serotonin reuptake inhibitors (SSRIs), administration of one or more anti-anxiety medications and administration of one or more anti-insomnia medications.

33. The method of claim 32, wherein the normal MMR comprises the subject’s MMR prior to the onset of PTSD.

34. The method of claim 32, wherein the normal MMR comprises an MMR generated from a population of individuals that do not suffer from PTSD.

35. The method of any of claims 32-34, wherein the at least one sample is a plasma sample obtained from whole blood taken from the subject.
36. The method of claim 35, wherein the whole blood is taken from the subject after midnight and before noon.

37. The method of claim 36, wherein the whole blood is taken from the subject at about 2 am (0200) and/or at about 9 am (0900).

38. The method of claim 32, wherein the SSRI is selected from the group consisting of citalopram, dapoxetine, escitalopram, fluoxetine, fluvoxamine, indalpine, paroxetine, sertraline and zimelidine.

39. A kit for detecting or diagnosing post-traumatic stress disorder (PTSD) comprising at least one set of antibodies that specifically bind to at least one of monocyte chemoattractant protein 4 (MCP-4) or monocyte chemoattractant protein 1 (MCP-1), wherein the antibodies are immobilized onto a solid surface.

40. The kit of claim 39, wherein the kit comprises two sets of antibodies, with each set being able to specifically bind to MCP-1 and MCP-4, respectively.
FIG. 3

SIGNIFICANCE OF DIFFERENCE BETWEEN CONTROLS AND PTSD PATIENTS

LOG P-VALUE

Z, (HOURS, RELATIVE TO SUNRISE)

[MCP4/MCP1]
[IL-8]
[EOTAXIN]
[EOTAXIN-3]
[IP-10]
[MCP-1]
[MCP-4]
[MDC]
[MIP-1β]
FIG. 4A

PLASMA [MCP-4] - [MCP-1]

PG/mL

PTSD

HEALTHY

FIG. 4B
MCP4/MCP1, -7.75 HRS AFTER SUNRISE,
AUC=0.879

FIG. 5A
FIG. 5B

FIG. 5C
FIG. 5D
FIG. 7A
FIG. 7B
FIG. 7C
FIG. 7D
FIG. 7E
FIG. 7F
FIG. 7H
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/68 (2015.01)
CPC - G01N 33/6896 (2015.12)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61B 5/00, 5/145; G01N 33/68 (2015.01)
CPC - A61B 5/145, 5/14507; G01N 33/6893, 33/6896, 2333/4709, 2440/14, 2800//2828, 2800/2871, 2800/40, 2800/50 (2015.12)

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

CPC - A61B 5/145, 5/14507; G01N 33/6893, 33/6896, 2333/4709, 2440/14, 2800/2828, 2800/2871, 2800/40, 2800/50 (2015.12)
(keyword delimited); USPC - 2/468; 435/7.1, 7.92; 436/86, 501; 506/9; 600/300, 302, 309, 310

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

Oracle, Google Patents, Google Scholar.

Search terms used: "post-traumatic stress disorder" OR PTSD "monocyte chemoattractant protein" mcP-1 mcP-4 ratio depression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US 2003/0059791 A1 (ROKUTAN et al) 27 March 2003 (27.03.2003) entire document</td>
<td>1-6, 12-17, 22-30, 32-40</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
03 December 2015

Date of mailing of the international search report
12 JAN 2016

Name and mailing address of the ISA/
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer
Blaine Copenhaver
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774
**INTERNATIONAL SEARCH REPORT**

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

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

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

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

3. ☒ Claims Nos.: 7-11, 18-21, 31
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

**Remark on Protest**

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

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

☐ No protest accompanied the payment of additional search fees.

*Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)*