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(57) Abstract: Disclosed are phospholipid biomarkers and method for predicting the sensitivity or propensity of a patient to relapse after treatment for an addiction.



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LIPID BIOMARKERS OF ADDICTION AND RELAPSE RISK**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims benefit of U.S. Provisional Application No. 61/782,188, filed March 14, 2014, which is hereby incorporated herein by reference in its entirety.

BACKGROUND

Drug abuse and addiction costs exceed 500 billion dollars each year in the United States in health, crime, and lost productivity (NIDA Info Facts, June 2008). In fact, the overall cost of addiction to prescription pain killers is about \$70 billion a year. Furthermore, each addict that “shops doctors” can cost prescription insurers \$10,000-\$15,000 apiece. Although huge strides have been taken over the past twenty years towards understanding the neurobiological basis for the state of addiction, translating these findings to the clinical setting in the form of effective therapeutics has enjoyed limited success. For example, there is currently no FDA approved pharmacotherapeutic approach for the treatment of cocaine addiction.

With the rates of prescription drug abuse skyrocketing, a need exists for a means to predict the likelihood of patient’s propensity for drug seeking behavior. However, the individual risk of becoming addicted to a particular drug is affected by genetic, developmental and environmental factors. As a consequence, despite similar initial exposure levels to a given drug of abuse, only approximately 15% of individuals progress to the addicted state (Warner LA, et al. Arch Gen Psychiatry. 1995 52(3):219-29). The recognition of this susceptible subpopulation for addiction provides strong impetus for incorporating the evaluation of individual responses into treatments whenever/wherever practical.

Although protocols themselves vary, treatment for addicted patients tends to be uniform across the patient population. This increases expenses for health care providers and employers in terms of lost work hours, increased treatment costs including costs from repeated rehabilitation and potentially unnecessary treatment sessions. Even a 10% improvement in the management of this disease would save the US economy tens of billions of dollars annually. A biochemical-based quantitative test is therefore needed to predict a patient’s likelihood to resume drug-seeking behavior and allow for the personalization of therapy protocols with regards to addiction treatment.

SUMMARY

Lipidomics was used to identify biomarkers and lipid fingerprints that are induced following drug-exposure that can be used to predict subsequent behavioral response in individual

patients. These biomarkers can be identified using a ELISA-based (or similar) screening assay, employing antibodies designed to recognize specific phospholipids demonstrated to be altered in blood or urine in correlation with increased drug-seeking behavior. Such a test would allow clinicians, therapists and employers to identify individuals in need of additional support. This would allow for personalization of treatment therapy, resulting in decreased risk of relapse and for efficient allocation of treatment resources.

Disclosed is a method for predicting the sensitivity or propensity of a patient to relapse after treatment for an addiction. The method can involve assaying a biological sample, such as blood or urine, from the subject for the levels of one or more phospholipids, comparing the levels of the one or more phospholipids to control values to produce a lipid profile; and analyzing the lipid profile to calculate a risk score. In these methods, an elevated risk score can be an indication that the patient will relapse after treatment for the addiction. The phospholipid levels can be assayed by any suitable technique. For example, the biological sample can be assayed by immunoassay or electrospray ionization mass spectrometry (ESI/MS) or electrospray ionization tandem mass spectrometry (ESI-MS/MS).

A risk score can be determined using standard statistical methods, such as multivariate analysis. In some embodiments, the risk score is a regression value, where a regression value of about 1 is an indication that the patient will relapse after treatment. For example, the lipid profile may be analyzed by multivariate regression analysis (e.g., determined by linear regression) or principal component analysis to derive a risk score. In other embodiments, specific lipid biomarkers are used to calculate the risk score. For example, in some embodiments, levels of 20:3 lysophosphatidylcholine (LPC) relative to control values are negatively correlated to risk score.

The disclosed methods may be used to predict the sensitivity or propensity of a patient to relapse after treatment for any type of addiction. In some embodiments, the patient is being treated for a substance addiction, such as a dependence on one or more drugs of abuse. Non-limiting examples of drugs of abuse include cocaine, methamphetamine, nicotine, ethanol, opiates, or any combination thereof.

The lipid profile of the disclosed method preferably comprises levels of at least six distinct molecular species of phospholipids. Non-limiting examples of phospholipids that can be used to produce the lipid profile include choline glycerophospholipids, phosphatidylglycerols, lysophosphatidylcholine, phosphatidic acids, lysophosphatidic acids, sphingomyelins, and oxidized derivatives thereof. In addition, the method may further involve assaying the biological sample from the subject for the levels of one or more cholesterol, free fatty acids, or a

combination thereof, and comparing the levels of the one or more cholesterol, free fatty acids, or a combination thereof, to a control and including these values in the lipid profile.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A and 1B show a treatment and training schedule used for rat cocaine conditioning. On Day 2, rats were randomly separated into two groups and placed into recording chambers for 30 minutes prior to injection with either saline or 10 mg/kg cocaine. The animals then underwent 4 conditioning sessions where they received either saline (controls) or 15 mg/kg cocaine for 4 days (Fig. 1A and 1B). Rats were challenged on Day 13 with either cocaine or saline (Fig. 1A) and sensitization was determined based on movement in the recording chamber. Brain tissues and blood were then isolated 7 days later for lipidomic analysis.

Figures 2A-2C show the effect of cocaine exposure on locomotor (LM) activity (total horizontal counts) following initial exposure (Day 2) and after sensitization (Day 13). Figures 2A and 2B are plots demonstrating horizontal counts in rats receiving 10 mg/kg cocaine on Day 2 (Fig. 2A) or Day 13 (Fig. 2B). Note the increased density of horizontal counts in Fig. 2B suggesting sensitization. Figure 2C is a graph showing quantitation of horizontal counts during the initial training session prior to injection (10-30 minutes) and after injection with either saline or cocaine at Day 2 (filled symbols) or saline or cocaine on day 13 (open symbols or challenge). Note the increased horizontal counts in rats injected with cocaine at 40 minutes on Day 2 and after challenge indicating increased initial response and sensitization. Data in Fig. 2C are represented as the mean $\pm$ the SEM of 6 rats.

Figures 3A-3C show the structures and nomenclature of commonly studied phospholipids. Figure 3A shows the basic structure of a glycerophospholipid. Figure 3B shows the basic structure of sphingomyelin. Figure 3C shows the structure and abbreviations for phospholipids determined to be altered by cocaine exposure in rat brain and blood.

Figures 4A-4C show the effect of cocaine re-exposure on the expression of phospholipids in the blood. Rats were treated with either saline control or cocaine using a standardized conditioning protocol that increased sensitization. Seven days after the final treatment with cocaine (Day 19) whole blood isolated from rats and protein precipitated using PCA and the resulting supernatant was subjected to Bligh-Dyer extraction and analyzed by ESI-MS. Figure 4A represents positive ion ESI-MS spectra from control rats for the indicated tissues, while

Figure 4B represents spectra from cocaine exposed rats. Figure 4C is a bar graph showing changes in the expression of select phospholipids in cocaine treated rats (open bars) as compared to controls (filled bars), and are presented at the mean $\pm$ the SEM of at least 6 different rats.

DETAILED DESCRIPTION

5 The study of the lipid classes in their natural environment is termed lipidomics, which focuses on what role specific lipids play in normal physiology and in disease states. Lipidomics was used herein to identify biomarkers and lipid fingerprints that are induced following drug-exposure and that can be used to predict subsequent behavioral response in individual patients. In particular, lipidomics was used to demonstrate that lipid profiles observed in bodily samples
10 are informative biomarkers for individual responsiveness including addiction, abstinence, and relapse.

 While brain and blood lipid profiles have been shown to be altered in patients who have abused drugs compared to control patients (Ross BM, et al. (2002) Drug and alcohol dependence 67(1):73-9), the ability of these lipids to be used as biomarkers or prognostic indicators of drug-
15 seeking, relapse, or other types of drug related behaviors was not previously known.

 Provided is a translational bridge between the neuroadaptations that occur following exposure to drugs of abuse and the practical means for monitoring and assessing the occurrence and progression of these neuroadaptations in the whole organism by measuring putative peripheral lipids biomarkers in samples from a subject.

20 Methods

 Methods are provided for determining the risk that a subject will relapse after treatment of an addiction based using phospholipid biomarkers. The method involves assaying a sample from the subject for one or more phospholipid levels and comparing these phospholipid levels to a control to produce a lipid profile. The lipid profile can then be analyzed to derive a risk score,
25 such as a regression value derived from the lipid profile as a weighted function of the quantified lipidome.

 In some embodiments, the addiction is a substance addiction. However, any addictive behavior that can results in a relapse behavior may be examined using the disclosed lipid biomarkers. Addiction is the continued behavior or use of a substance despite adverse
30 dependency consequences, or a neurological impairment leading to such behaviors. Addictions can include, but are not limited to, substance abuse, exercise abuse, sexual activity and gambling. Classic hallmarks of addiction include impaired control over substances or behavior, preoccupation with substance or behavior, continued use despite consequences, and denial.

Habits and patterns associated with addiction are typically characterized by immediate gratification (short-term reward), coupled with delayed deleterious effects (long-term costs).

Physiological dependence occurs when the body has to adjust to the substance by incorporating the substance into its “normal” functioning. This state creates the conditions of tolerance and withdrawal. Tolerance is the process by which the body continually adapts to the substance and requires increasingly larger amounts to achieve the original effects. Withdrawal refers to physical and psychological symptoms experienced when reducing or discontinuing a substance that the body has become dependent on. Symptoms of withdrawal generally include but are not limited to anxiety, irritability, intense cravings for the substance, nausea, hallucinations, headaches, cold sweats, and tremors.

Substance dependence can be diagnosed with physiological dependence, evidence of tolerance or withdrawal, or without physiological dependence. Examples of substance dependencies include: alcohol dependence; opioid dependence; sedative, hypnotic, or anxiolytic dependence (including benzodiazepine dependence and barbiturate dependence); cocaine dependence; cannabis dependence; amphetamine dependence (or amphetamine-like); hallucinogen dependence; inhalant dependence; polysubstance dependence; phencyclidine (or phencyclidine-like) dependence; and nicotine dependence.

Biological samples suitable for lipid profiling include blood, plasma, cerebral spinal fluid (CSF), brain tissue, fractionated cells or cell lysates, a tissue biopsy, surgical specimen, urine, and autopsy material. A skilled artisan will recognize that the use of each biological sample is associated with advantages and disadvantages and the appropriate biological sample type is dependent upon the intended use of the method. For example, while a blood or plasma sample can be obtained with little discomfort and risk, the lipids in the blood or plasma sample may be from other parts of the body. Using CSF allows the analysis of lipids directly linked to brain lipid metabolism, but samples taken using spinal tap are uncomfortable and include moderate risks. Likewise, brain tissue samples offer the most direct measurement of the brain lipid profile, but require very invasive methods to obtain the samples and are associated with very high risks (most brain tissue samples are taken post-mortem).

Historically, lipid profiles have referred to LDL and HDL and the like. However, the disclosed lipid fingerprints include the use of phospholipid biomarkers. Exemplary phospholipids include glycerophospholipids, their metabolites (lysophospholipids), sphingomyelins, and cardiolipins. The term does not include free fatty acids or cholesterol. However, the disclosed methods may further include the use of these as biomarkers to increase sensitivity and selectivity.

The major groups of lipids are based on chemical properties and chemical structure. Two major groups of lipids include saponifiable and nonsaponifiable lipids. Saponifiable lipids include those having long-chain fatty acids esterified to a backbone molecule such as phospholipids. Nonsaponifiable lipids include lipids of the cholesterol, carbon-ring compound, and isoprene derivative classes.

The human plasma lipidome is described in Quehenberger O, et al. J Lipid Res. 2010 Nov;51(11):3299-305, which is incorporated by reference for the teaching of the the lipids found in human plasma.

Exemplary lipid classes include choline glycerophospholipid (all subclasses), ethanolamine glycerophospholipid (all subclasses), phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, lyso-glycerophospholipid, lysoethanolamine glycerophospholipid, phosphatidic acid, lyso-phosphatidic acid, sphingomyelin, galactosylceramide, glucosylceramide, gangliosides, sulfatide, triacylglycerol, diacylglycerol, monoacylglycerols, acyl-CoA, acylcarnitine, cholesterol, cholesterol esters, oxysterols, prostaglandins, ceramide, cardiolipin, and sphingoid base-1-phosphate classes. Each class includes multiple lipid molecular species.

In some embodiments, the phospholipid biomarkers are choline-based glycerophospholipids (PtdCho), glycerophosphocholines (GPCho), phosphatidylglycerols (PtdGro), lysophosphatidylcholines (LPC), glycerophosphatidic acids (GPA), sphingomyelins (SM), or any combination thereof.

Phospholipids may be identified using shorthand designation of its constituent fatty acid. The shorthand includes two numbers separated by a colon, the number before the colon specifying the number of carbon atoms, and the number after the colon specifying the number of double bonds, in the fatty acid chain.

Examples of choline-based glycerophospholipids (PtdCho) include 28:0, 28:1, 28:2, 30:0, 30:1, 30:2, 32:0, 32:1, 32:2, 32:3, 32:4, 34:0, 34:1, 34:2, 34:3, 34:4, 36:0, 36:1, 36:2, 36:3, 36:4, 36:5, 36:6, 38:0, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6, 40:0, 40:1, 40:2, 40:3, 40:4, 40:5, 40:6, 42:0, 42:1, 42:2, 42:3, 42:4, 42:5, and 42:6

Examples of glycerophosphocholines (GPCho) include 26:0, 26:1, 26:2, 26:3, 27:0, 27:1, 27:2, 27:3, 28:0, 28:1, 28:2, 28:3, 30:0, 30:1, 30:2, and 30:3

Examples of phosphatidylglycerols (PtdGro) include 20:1, 20:2, 20:3, 20:4, 22:0, 22:1, 22:2, 22:3, 22:4, 24:0, 24:1, 24:2, 24:3, 24:4, 26:0, 26:1, 26:2, 26:3, 26:4, 27:0, 27:1, 27:2, 27:3, 27:4, 28:0, 28:1, 28:2, 28:3, 28:4, 30:1, 30:2, 30:3, and 30:4

Examples of lysophosphatidylcholines (LPC) include 14:0, 14:1, 14:2, 14:3, 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 20:4, 22:0, 22:1, 22:2, 22:4

Examples of glycerophosphatidic acids (GPA) include 28:0, 28:1, 28:2, 28:3, 28:4, 30:0, 30:1, 30:2, 30:3, 30:4, 32:0, 32:1, 32:2, 32:3, 32:4, 34:0, 34:1, 34:2, 34:3, 34:4, 34:6, 36:0, 36:1, 36:2, 36:3, 36:4, 36:5, 36:6, 38:0, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6, 40:0, 40:1, 40:2, 40:3, 40:4, 40:5, and 40:6.

Examples of sphingomyelins (SM) include 18:0/18:0, 18:0/18:1, 18:0/18:2, 18:0/18:3, 18:1/18:0, 18:1/18:2, 18:1/18:3, 18:2/18:0, 18:2/18:1, 18:2/18:2/ 18:2/18:3, 18:3/18:0, 18:3/18:1, 18:3/18:3, 18:0/20:0, 18:0/20:1, 18:0/20:2, 18:0/20:3, 18:0/20:4, 18:1/20:0, 18:1/20:1, 18:1/20:2, 18:1/20:3, 18:1/20:4, 18:0/22:0, 18:1/22:0, 18:2/20:0, 18:2/20:1, 18:2/20:2, 18:2/20:3, 18:2/20:4, 18:1/22:0, 18:1/24:0, 18:1/24:1, 18:1/24:2, 18:2/24:0, and 18:2/24:2.

The lipid biomarkers do not include free fatty acids. However, in some embodiments, the method further comprises the use of free fatty acids (FA) as biomarkers. Examples of free FAs include 14:0, 14:1, 16:0, 16:1, 16:2, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 20:4, 22:0, 22:1, 22:2, and 22:3. For example, the levels of polyunsaturated fatty acids (PUFAs) have been shown to play a role in the pathophysiology of a large number of psychiatric disorders, including relapse (Buydens-Branchey L, et al. Psychiatry Res. 2003 Aug 30;120(1):29-35).

The phospholipid levels can be assayed by any suitable technique. For example, the biological sample can be assayed by immunoassay or by a chromatographic method.

The steps of various useful immunodetection methods have been described in the scientific literature, such as, e.g., Maggio et al., Enzyme-Immunoassay, (1987) and Nakamura, et al., Enzyme Immunoassays: Heterogeneous and Homogeneous Systems, Handbook of Experimental Immunology, Vol. 1: Immunochemistry, 27.1-27.20 (1986), each of which is incorporated herein by reference in its entirety and specifically for its teaching regarding immunodetection methods. Immunoassays, in their most simple and direct sense, are binding assays involving binding between antibodies and antigen. Many types and formats of immunoassays are known and all are suitable for detecting the disclosed biomarkers. Examples of immunoassays are enzyme linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), radioimmune precipitation assays (RIPA), immunobead capture assays, Western blotting, dot blotting, gel-shift assays, Flow cytometry, protein arrays, multiplexed bead arrays, magnetic capture, *in vivo* imaging, fluorescence resonance energy transfer (FRET), and fluorescence recovery/localization after photobleaching (FRAP/ FLAP).

Chromatographic methods for measuring phospholipids in biological samples are well known in the art and described in Peterson BL and Cummings BS. Biomed Chromatogr. 2006 Mar;20(3):227-43, which is incorporated by reference for the teaching of these methods.

The first step in any analysis of phospholipids will involve separating lipids from the biological milieu. These extraction procedures are necessary to remove any other constituents such as proteins, sugars or other small molecules that would interfere with the chromatographic steps. There are several methods for extracting lipids from samples including liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

After successful extraction of phospholipids from their source, analysis can be performed for the detection of specific phospholipids using, for example, chromatographic methods. All of the systems of chromatography consist of a stationary and mobile phase. A sample is placed on a stationary phase, which is either a solid or a liquid, and then the mobile phase, a gas or a liquid, is allowed to pass through the system. The components of the sample will be separated based on their varying physical and chemical properties, imparting different affinities for the two phases.

Thin-layer chromatography (TLC) was the earliest chromatographic method used to assess phospholipids, and is frequently used today. Gas chromatography (GC) has applications for identifying individual fatty acids present in phospholipids, but is generally not used to assess phospholipids. HPLC is the most popular technique used currently for separating lipid classes. However, advances in liquid chromatography-mass spectrometry (LC-MS), especially those tied to electrospray ionization (ESI), have resulted in a decrease in studies using HPLC to assess phospholipids. With the exception of TLC, there is a variety of detectors that can be coupled to the above chromatographic methods including refractive index (RI), ultraviolet (UV), fluorescence and evaporative light scattering detection (ELSD).

The resultant lipid extracts may then be analyzed by mass spectrometric techniques commonly known in the art (Han, X., et al. (2004) Towards fingerprinting cellular lipidomes directly from biological samples by two-dimensional electrospray ionization mass spectrometry, Anal. Biochem. 330, 317-331 and incorporated herein by reference). Mass spectrometry determines the masses of molecules in which an electrical charge is placed on the molecule and the resulting ions are separated by their mass to charge ratio (m/z). There are numerous types of mass spectrometers available that vary in the type of ionization source and mass analyzer that is used. Non-limiting examples of mass spectrometric techniques that may be used to create a lipid profile include gas chromatography-mass spectrometry, electrospray ionization (ESI) mass spectrometry (ESI-MS) in the positive or negative modes, tandem ESI-MS, multi-dimensional mass spectrometry, and MALDI mass spectrometry.

In some embodiments, the phospholipids are detected in a biological sample using shotgun lipidomics or shotgun sphingolipidomics. Shotgun lipidomics and shotgun sphingolipidomics allow the identification and quantification directly from lipid extracts of more than about 95% of a subject's total lipid mass, representing about a thousand individual molecular species. Shotgun lipidomics methods are known in the art (Han, X., et al. (2006) Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples, J Lipid Res 47, 864-879; and Jiang, X., and Han, X. (2006) Characterization and direct quantitation of sphingoid base-1-phosphates from lipid extracts: A shotgun lipidomics approach, J. Lipid Res. 47, 1865-1873, both incorporated herein by reference). The majority of lipid classes are identifiable using shotgun lipidomics and shotgun sphingolipidomics. For example, some of the classes of lipids that can be detected by using shotgun lipidomics include choline glycerophospholipid (GPCCho), ethanolamine glycerophospholipid (GPEtn), phosphatidylinositol (GPIlns), phosphatidylglycerol (GPGro), phosphatidylserine (GPSer), lysoGPCCho, lysoGPEtn, phosphatidic acid (GPA), lysoGPA, sphingomyelin (SM), galactosylceramide (GalCer), glucosylceramide, sulfatide, free fatty acid, triacylglycerol (TG), acylcarnitine, cholesterol and cholesterol esters, ceramide, cardiolipin, sphingosine-1-phosphate (S1P), dihydrosphingosine-1-phosphate (DHS1P), sphingosine, and lysoSM, as well as those listed in Tables 1-25. Shotgun lipidomics and sphingolipidomics allow all of the molecular species of lipids present in a biological sample to be screened in an unbiased fashion.

Multiple - and often interactive - factors can increase the likelihood of relapse. Therefore, the disclosed methods can further involve the use of biological, behavioral, and socio-demographic factors to predict the risk of relapse. For example, "reminder" cues (sights, sounds, smells, dreams) tightly linked to substance or behavior can trigger craving and relapse. Negative mood states or stress as well as positive mood states or celebrations can trigger relapse. Of course, actual sampling of a substance or behavior itself, even in very small amounts, can trigger relapse behavior. Clinical factors and subjective and behavioral measures such as depressive symptoms, stress, and drug craving are all somewhat predictive of future relapse risk. In some cases, women and older, married and better-educated individuals may experience better short-term outcomes. Likewise, those with social resources, especially supportive relationships with family members and friends, may have a lower likelihood of relapse.

In some embodiments, the patient's lipid profile is used to derive a risk score that predicts the likelihood of relapse behavior. A lipid profile contains numerous data points that are best managed and stored in a computer readable form. Therefore, in preferred embodiments, the risk score is a regression value derived from the lipid profile as a weighted function of the

quantified lipidome. The weighted function can be derived from linear regression analysis of experimental results comparing lipid profiles of normal subjects versus those with relapse behavior. Each lipid molecular species can be multiplied by a weighting constant and summed.

Generally speaking, a regression value is a single value that is sensitive to changes in abundance of lipid molecular species of a lipid profile, with a regression value of about 1 being indicative of a high risk of relapse. For example, a regression value of about 0 is indicative of no relapse tendencies, while a regression value of about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, and more may be indicative of a risk of relapse. The changed lipid species of the lipid profile includes at least 3 or more lipid molecular species. An embodiment of the invention comprises a lipid profile inclusive of the lipid molecular species of at least one lipid class. Another embodiment comprises a lipid profile including the lipid molecular species of about 2 or more lipid classes. An additional embodiment comprises a lipid profile including about 20 or more lipid classes or about 100 or more individual lipid molecular species. Further, an embodiment comprises a lipid profile that includes the entire lipidome of a biological sample.

Prior to analysis, the data in each dataset can be collected by measuring the values for each lipid biomarker, usually in duplicate or triplicate or in multiple replicates. The data may be manipulated, for example raw data may be transformed using standard curves, and the average of replicate measurements used to calculate the average and standard deviation for each patient. These values may be transformed before being used in the models, e.g. log-transformed, Box-Cox transformed, etc. This data can then be input into an analytical process with defined parameter.

The analytic classification process may be any type of learning algorithm with defined parameters, or in other words, a predictive model. In general, the analytical process will be in the form of a model generated by a statistical analytical method such as those described below.

Examples of such analytical processes may include a linear algorithm, a quadratic algorithm, a polynomial algorithm, a decision tree algorithm, or a voting algorithm.

Using any suitable learning algorithm, an appropriate reference or training dataset can be used to determine the parameters of the analytical process to be used for classification, i.e., develop a predictive model. The reference or training dataset to be used will depend on the desired classification to be determined. The dataset may include data from two, three, four or more classes.

The number of features that may be used by an analytical process to classify a test subject with adequate certainty is 2 or more. In some embodiments, it is 3 or more, 4 or more, 10 or more, or between 10 and 200. Depending on the degree of certainty sought, however, the number

of features used in an analytical process can be more or less, but in all cases is at least 2. In one embodiment, the number of features that may be used by an analytical process to classify a test subject is optimized to allow a classification of a test subject with high certainty.

Suitable data analysis algorithms are known in the art. In one embodiment, a data analysis algorithm of the disclosure comprises Classification and Regression Tree (CART), Multiple Additive Regression Tree (MART), Prediction Analysis for Microarrays (PAM), or Random Forest analysis. Such algorithms classify complex spectra from biological materials, such as a blood sample, to distinguish subjects as normal or as possessing biomarker levels characteristic of a particular condition (e.g., relapse behavior). In other embodiments, a data analysis algorithm of the disclosure comprises ANOVA and nonparametric equivalents, linear discriminant analysis, logistic regression analysis, nearest neighbor classifier analysis, neural networks, principal component analysis, hierarchical cluster analysis, quadratic discriminant analysis, regression classifiers and support vector machines.

As will be appreciated by those of skill in the art, a number of quantitative criteria can be used to communicate the performance of the comparisons made between a test marker profile and reference marker profiles. These include area under the curve (AUC), hazard ratio (HR), relative risk (RR), reclassification, positive predictive value (PPV), negative predictive value (NPV), accuracy, sensitivity and specificity, Net reclassification Index, Clinical Net reclassification Index. In addition, other constructs such as receiver operator curves (ROC) can be used to evaluate analytical process performance.

Kits

The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. In particular, kits are disclosed for predicting the sensitivity or propensity of a patient to relapse after treatment for an addiction. For example, the kit can be an immunoassay comprising a plurality of antibodies that selectively bind three or more phospholipids, including 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more phospholipids.

For example, the immunoassay can be an enzyme linked immunoassay (ELISA), electrophoretic mobility shift assay (EMSA), or lateral flow immunoassay. Examples of phospholipids that can be detected by the antibodies in this assay are disclosed above, and include choline glycerophospholipids, phosphatidylglycerols, lysophosphatidylcholine, phosphatidic acids, lysophosphatidic acids, sphingomyelins, and oxidized derivatives thereof.

The kit can further comprise containers, such as tubes, cuvettes, vacutainers, and syringes for collecting, storing, and/or assaying bodily fluids. The kit can also include reagents, such as storage and reaction buffers for use in preparing and/or assaying the bodily fluid sample. For example, the kit can contain reagents for extracting phospholipids from the bodily fluid.

Definitions

The term “subject” refers to any individual who is the target of administration or treatment. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient. The term “patient” refers to a subject under the treatment of a clinician, e.g., physician.

The term “sample from a subject” refers to a tissue (e.g., tissue biopsy), organ, cell (including a cell maintained in culture), cell lysate (or lysate fraction), biomolecule derived from a cell or cellular material (e.g. a polypeptide or nucleic acid), or body fluid from a subject. Non-limiting examples of body fluids include blood, urine, plasma, serum, tears, lymph, bile, cerebrospinal fluid, interstitial fluid, aqueous or vitreous humor, colostrum, sputum, amniotic fluid, saliva, anal and vaginal secretions, perspiration, semen, transudate, exudate, and synovial fluid.

The term “treatment” refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

The term “phospholipid” refers to a lipid containing a diglyceride or sphingosine linked to one or two fatty acids, respectively, a phosphate group, and a polar molecule, such as choline.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. For Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Example 1:

Methods

5 *Simultaneous induction of sensitization & CPP:* Among rodent models of drug reward and addiction, conditioned place preference (CPP) and locomotor sensitization (“sensitization”) are two commonly employed behavioral assays that involve experimenter administration of drug via the i.p. route. Since surgical preparation (i.e. catheterization) of the subject is not required, larger numbers of animals can be incorporated into the experimental design, and animals can be
10 tested over long periods of time without concern of losing catheter patency. A cocaine conditioning protocol has been developed in which CPP and sensitization can be induced and assessed in the same rat, allowing both of these behavioral phenomena to be monitored in the same individual (Seymour CM, et al. (2008) Brain research 1213:57-68).

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were housed in pairs in clear
15 plastic cages and maintained on a 12h light/dark cycle (0700/1900h). Food and water was available *ad libitum* except during the behavioral sessions. Animals were allowed to adapt to the lab conditions for a week before behavioral testing begins. Behavioral sessions were conducted daily between 0900 and 1600h.

The apparatus and measurement of activity have been described in detail elsewhere
20 (Gosnell BA (2005) Brain research 1031 (2): 194-201; Seymour CM, et al. (2008) Brain research 1213:57-68). Briefly, locomotor (LM) activity was measured in four 43.2 x 43.2cm chambers with clear plastic walls and a solid smooth floor (Med Associates, St. Albans, VT, USA). The chambers were individually housed in sound-attenuating cubicles equipped with a house light and a ventilation fan. Two banks of 16 infrared photobeams and detectors, mounted at right
25 angles 3.5cm above the floor, detect horizontal activity. Activity Monitor software (Med Associates) was used to count photobeam breaks. For the conditioned place preference (CPP) experiments, a two compartmental insert (Med Associates) was used to modify the open field activity chamber. The insert was divided into two equal-sized compartments by a black partition containing a guillotine door which can be removed to allow access to both compartments, or
30 inserted to confine the animal's movements to one side. The compartments differed in floor type (grid vs. rods) and by ceiling color (clear vs. black).

Animals were concurrently analyzed for CPP and behavioral sensitization over a period of thirteen days (Figure 1). In the CPP pre-test session, animals were given free access to both compartments of the CPP insert for 30 minutes (Protocol day 1). The next day, animals were

placed in the center of the open field activity chamber for 30 minutes to establish a baseline LM activity for each animal (Protocol day 2). At that time, they were given an i.p. injection of either 10 mg/kg cocaine or saline and returned to the chamber for an additional 60 minutes of monitoring. During conditioning sessions (Protocol days 3-6), animals will be injected i.p. with either cocaine (15 mg/kg) or 0.9% saline and then confined in one of the two compartments for 15 minutes. Four hours later the animals were injected with either cocaine or saline and confined to the opposite compartment. The order of the injections was rotated daily to prevent the animal from developing an association between the drug and the time of day. The day after the last conditioning session the animals were tested for CPP on Protocol day 7. Six days after the CPP test (Protocol day 13) animals were LM challenged with cocaine or saline in the open field activity chamber as described above for Protocol day 2.

Isolation of Blood: Rats were anesthetized with halothane and trunk blood was collected following decapitation. Blood samples (0.5ml) were stored at -80°C in glass vials containing methanol/water (2:1, 1 ml). Lipids were then extracted as described below.

Bligh-Dyer lipid extraction: Phospholipids were extracted using chloroform and methanol according to the method of Bligh and Dyer (Bligh and Dyer 1959). For isolation blood or urine 1 ml of either was added to 3 ml of methanol: water (2.0:0.8 v/v) in a glass test tube and 1.25 ml chloroform was added. Tubes were vortexed for 30 seconds and allowed to sit for 10 minutes on ice. Tubes were centrifuged at 213 g for 1 minute and the bottom chloroform layer was transferred to a new test tube. The extraction steps were repeated a second time and the chloroform layers combined. The collected chloroform layers were dried under argon, reconstituted with 50 µl of methanol:chloroform (2: 1 v/v), and stored at -20°C until analysis.

Lipid phosphorus assay: Lipid phosphorus was quantified using malachite green (Zhou and Arthur 1992). Lipid extract (10 µl) was dried down under argon in a test tube and 200 µl of perchloric acid was added to the tube, which was then heated at 130°C for 2-3 hours. After this time, 1 ml of dH₂O was added to the tube while vortexing, 1.5 ml of reagent C (4.2 grams Ammonium Molybdate Tetrahydrate in 100 ml 5 N HCl and 0.15 grams Malachite Green Oxalate in 300 ml dd H₂O) was then added followed by vortexing and addition of 200 µl of 1.5% v/v Tween 20. After 25 minutes of sitting at room temperature, a 200 µl aliquot was used to measure the absorbance at 590 nm.

Characterization and quantitation of phospholipids using electrospray ionization-mass spectrometry (ESI-MS): Lipid extract samples (500 pmol/µl) were prepared by reconstituting in chloroform: methanol (2:1, v/v). Mass spectrometry was performed as described previously (Taguchi R, et al. (2000) J Mass Spectrom 35(8):953-66). Samples were analyzed using a LCT

Premier time of flight mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source. Sample (5 µl) were introduced by means of a flow injector into the ESI chamber at a rate of 0.2 ml/min. The elution solvent was acetonitrile: methanol:water (2:3:1, v/v/v) containing 0.1 % (w/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the positive scanning mode. The flow rate of nitrogen drying gas was 10 L/min at 80°C. The capillary and cone voltages were set at 2.5 kV and 30 V respectively. As previously described (Taguchi R, et al. (2000) J Mass Spectrom 35(8):953-66), qualitative identification of individual phospholipid molecular species was based on their calculated theoretical monoisotopic mass values and quantification was done by comparison to the most abundant phospholipid in each sample, which corresponded to a m/z ratio of 760 [(34:1 (16:0-18:1)] PtdCho. MSⁿ fragmentation was performed on an Agilent Trap XCT ion-trap mass spectrometer equipped with an ESI source. The analyte was introduced by direct injection from the HPLC system. The nitrogen drying gas flow-rate was 8.0 L/min at 350°C. The ion source and ion optic parameters were optimized with respect to the positive molecular ion of interest.

Statistical Analysis of CPP results: Statistical analysis for CPP data were compiled using SigmaStat for windows version 3.11 (SPSS Science, Chicago, IL). Two-way ANOVA with Holm-Sidak post-hoc analysis was used to analyze the percentage of time spent in the drug-paired compartment and the shift in preference for the CPP results. Conditioning days and baseline and post-injection activity for the behavioral sensitization experiment was analyzed using a two-way Repeated Measures ANOVA followed by Holm-Sidak post-hoc analysis. Behavioral sensitization data was analyzed by combining the ambulatory counts and stereotypic counts to get the total horizontal counts (i.e. the total number of horizontal beam breaks) for each animal.

Statistical analysis of lipid results: Statistical analysis for lipids were compiled using SigmaStat for windows version 3.11 (SPSS Science, Chicago, IL). Lipids isolated from one animal source (blood, urine, tissue) equaled an n of 1. Experiments were carried out until an n of at least 4 was reached with the goal being 6. Data were analyzed using a Two-way ANOVA with Holm-Sidak post-hoc analysis and differences in the intensity, relative to the control, of individual lipids (normalized to most abundant phospholipid) were compared for all peaks. This method is standard for lipidomic analysis (Peterson B, et al. (2008) Chem Biol Interact 174(3):163-76). Correlations between lipids and CPP were determined using linear regression analysis in Sigma Stat.

Results

Induction of locomotor sensitization: Using the protocol described above, locomotor sensitization to cocaine was assessed in rats by measuring increases in movement after initial dosing (Day 2) and one week after 4 subsequent doses with cocaine (Day 12). As previously reported (Seymour CM, et al. (2008) Brain research 1213:57-68), initial exposure to cocaine (10mg/kg i.p.) increased movement compared to rats exposed to a saline injection (saline vs. cocaine, Figure 2C). After administering 4 consecutive daily doses of cocaine (15mg/kg i.p.), followed by 1 week abstinence, the challenge dose at day 13 (simulating relapse) increased movement as compared to the saline-treated (i.e. non-sensitized) group of rats (Figure 2). In addition, the magnitude of cocaine-induced movement observed on the challenge test day was significantly increased as compared with that observed on the initial activity test day (i.e. the rats exhibited locomotor sensitization). This enhanced movement is indicative of sensitization, and has been correlated with a reinstatement of drug-seeking behavior in self-administration experiments (De Vries TJ, et al. (1998) The European journal of neuroscience 10(11):3565-71).

Seven days after the final exposure to cocaine or saline, rats were euthanized and blood isolated and prepared for lipidomic analysis as explained above. Over 1000 *m/z* values were analyzed using ESI-MS, representing multiple phospholipid classes. Figure 3 shows the basic structures of phospholipids. Glycerophospholipids differ in terms of the numbers of carbons and double bonds and polar head groups. Typically, the nomenclature used to identify these traits is X:Y, where X is the number of carbons and Y is the number of double bonds; hence, 34:2 would indicate a lipid with 34 carbons and 2 double bonds. The polar head groups are indicated by abbreviations, which are also defined in Figure 3.

Analysis of spectra derived from the blood of rats exposed to cocaine demonstrated several differences compared to saline exposed rats (Figure 4A and B). The intensities for some *m/z* values increased, while others decreased. Increases were detected in 40:0 PlsCho, 40:0 PtdGro and 20:3 LPC (Figure 4C). The level of 40:0 PlsCho in cocaine treated rats was almost 3-fold higher than controls, while 40:0 PtdGro and 20:3 LPC were only increased about 1.5- and 1.2-fold, respectively. The rest of the lipids identified as significantly changing in cocaine treated animals, compared to controls, decreased 15-40% compared to controls. A majority of the lipids that decreased corresponded to PtdCho; however, significant decreases were detected for several sphingomyelins (SM), including 18:1/22:0, 18:1/24:1 and 18:1/24:0 SM. This is first study to show that cocaine exposure selectively alters the phospholipid profile in blood.

Correlations of changes in phospholipids to behavior: Simply identifying that lipids change after cocaine exposure in a test group is not enough to determine if these changes correlate to behavior. To address this limitation the relationship in individual rats between either

the initial response (the amount of movement after the first exposure of animals to cocaine on day 2) or sensitization (the amount of movement after the challenge dose to cocaine on day 12) to changes in phospholipid expression was determined (Table 1). The correlation coefficient (R²) for these comparisons was then calculated. The greatest fold-changes were seen for 40:0 PlsCho and 40:0 PtdGro. Despite these large increases, there was little correlation between these specific phospholipids and initial response to cocaine or sensitization to cocaine, based on R² values (Table 1). Changes in the levels of 20:3 LPC did not correlate well to the initial response to cocaine, but did negatively correlate to sensitization to cocaine with a R² of -0.75. In contrast, changes in the levels of 32:0, 40:6, 32:2 and 34:1 and 36:2 PtdCho correlated to changes in the initial response to cocaine, with R² values ranging from 0.64, to 0.89; however, these correlations did not translate to changes in sensitization, which were all below 0.6, with the exception of 34:1 PtdCho. Changes in the levels of SM also correlated to changes in the initial response to cocaine, with R² values of 0.89 and 0.77 for 18:1/22:0 and 18:1/24:0 SM respectively. Once again, these correlations did not hold for changes in sensitization.

Discussion

This study showed strong correlations between select blood phospholipids and behavior changes induced by cocaine using an established model of locomotor sensitization in rats. In some instances, correlations between sensitization or initial response to cocaine and specific phospholipids were almost 0.9. Such high correlations suggest that these lipids could be used as biomarkers to indicate behavioral responses after cocaine re-exposure.

As would be expected, not all phospholipid changes correlated to changes in behavior. Changes in intensities of over a 1,000 *m/z* values, representing as many lipids, were assessed and significant changes were detected only in approximately 1 % of these lipids. This suggests phospholipid changes induced by cocaine are specific and not systemic.

These data support the idea that lipid biomarkers can be used to predict a treatment-seeking patient's behavioral response. For example, the phospholipids identified in the data above can be used with high correlations to either initial response or sensitization to cocaine to determine the likelihood to resume drug-taking. .

Although phospholipid profiles following cocaine exposure was evaluated in our supporting data, fatty acids can also be used as contributing factor to determine a "lipid fingerprint", or lipidomic profile. In addition, the applicability of this technology would not necessarily be exclusive to cocaine, as other drugs of abuse may also result in lipid fingerprint that could be used to predict individual response to therapy.

The m/z values listed in **Table 2** may serve as an initial fingerprint used to predict drug behavior. In addition, m/z values corresponding to select fatty acids may also be used in combination to predict response. These are listed in **Table 2** as well.

Table 1. Correlation between Initial Response and Sensitization to Cocaine and Changes in the Expression of Select Glycerophospholipids in Rat Blood^a

m/z	Probable Lipid ^b	R ² Initial Response	R ² Sensitization
546.6	20:3 LPC	0.44	-0.75
734.5	32:0 PtdCho	0.75	0.66
735.5	40:6 PtCho	0.83	0.23
758.8	32:2 PtdCho	0.76	0.57
760.6	34:1 PtdCho	0.64	0.63
782.3	36:4 PtdCho	0.52	0.13
786.6	36:2 PtdCho	0.89	0.23
787.6	18:1/22:0 SM	0.79	0.50
813.6	18:1/24:1 SM	0.59	0.53
815.3	18:1/24:0 SM	0.77	0.63
832.4	40:0 PlsCho	-0.36	-0.48
834.6	40:0 PtdGro	0.08	-0.39

^a As determined by comparing the percent change in lipid to initial response to cocaine

^b Based on theoretical m/z reported in Lipids Maps (www.lipidmaps.org).

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Table 2. Blood and urine m/z values, and the proposed phospholipids, proposed for use in finger printing biomarkers of behavioral response to cocaine and possibly other drugs of abuse.

	m/z	Probable Lipid	R ² Initial Response	R ² Sensitization
Phospholipid	546.6	20:3 LPC	0.44	-0.75
	734.5	32:0 PtdCho	0.75	0.66
	735.5	40:6 PtCho	0.83	0.23
	758.8	32:2 PtdCho	0.76	0.57
	760.6	34:1 PtdCho	0.64	0.63
	786.6	36:2 PtdCho	0.89	0.23
	787.6	18:1/22:0 SM	0.79	0.50
	815.3	18:1/24:0 SM	0.77	0.63
Fatty Acids	279.5	18:2	ND ^a	ND ^a
	303.5	20:4	ND ^a	ND ^a

^a Not determined. Data is from the literature in humans (Buyden-Branchy et al., Psychiatry Research, 120:29-35,2003).

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method for predicting the sensitivity or propensity of a patient to relapse after treatment for an addiction, comprising
 - (a) assaying a biological sample from the subject for the levels of one or more phospholipids;
 - (b) comparing the levels of the one or more phospholipids to control values to produce a lipid profile; and
 - (c) analyzing the lipid profile to calculate a risk score,wherein an elevated risk score is an indication that the patient will relapse after treatment for the addiction.
2. The method of claim 1, wherein the biological sample is blood or urine.
3. The method of claim 1, wherein the biological sample is assayed for phospholipid levels using an immunoassay comprising antibodies that selectively bind the one or more phospholipids.
4. The method of claim 3, wherein the immunoassay comprises an enzyme linked immunoassay (ELISA), electrophoretic mobility shift assay (EMSA), or lateral flow immunoassay.
5. The method of claim 1, wherein the biological sample is assayed for phospholipid levels using electrospray ionization mass spectrometry (ESI/MS) or electrospray ionization tandem mass spectrometry (ESI-MS/MS).
6. The method of claim 1, wherein the risk score comprises a regression value, wherein a regression value of about 1 is an indication that the patient will relapse after treatment.
7. The method of claim 6, wherein the lipid profile is analyzed by multivariate regression analysis or principal component analysis.
8. The method of claim 1, wherein the patient is being treated for a substance addiction.
9. The method of claim 8, wherein substance addiction comprises a dependence on one or more drugs of abuse.
10. The method of claim 9, wherein the one or more drugs of abuse comprises cocaine, methamphetamine, nicotine, ethanol, opiates, or any combination thereof.
11. The method of claim 1, wherein the one or more phospholipids are selected from the group consisting of choline glycerophospholipids, phosphatidylglycerols,

lysophosphatidylcholine, phosphatidic acids, lysophosphatidic acids, sphingomyelins, and oxidized derivatives thereof.

12. The method of claim 1, further comprising assaying the biological sample from the subject for the levels of one or more cholesterol, free fatty acids, or a combination thereof, and comparing the levels of the one or more cholesterol, free fatty acids, or a combination thereof, to a control and including these values in the lipid profile.

13. The method of claim 1, wherein the lipid profile comprises levels of at least three distinct molecular species of phospholipids.

14. The method of claim 1, wherein a decrease in 20:3 lysophosphatidylcholine (LPC) levels compared to the control values is an indication of a high risk score.

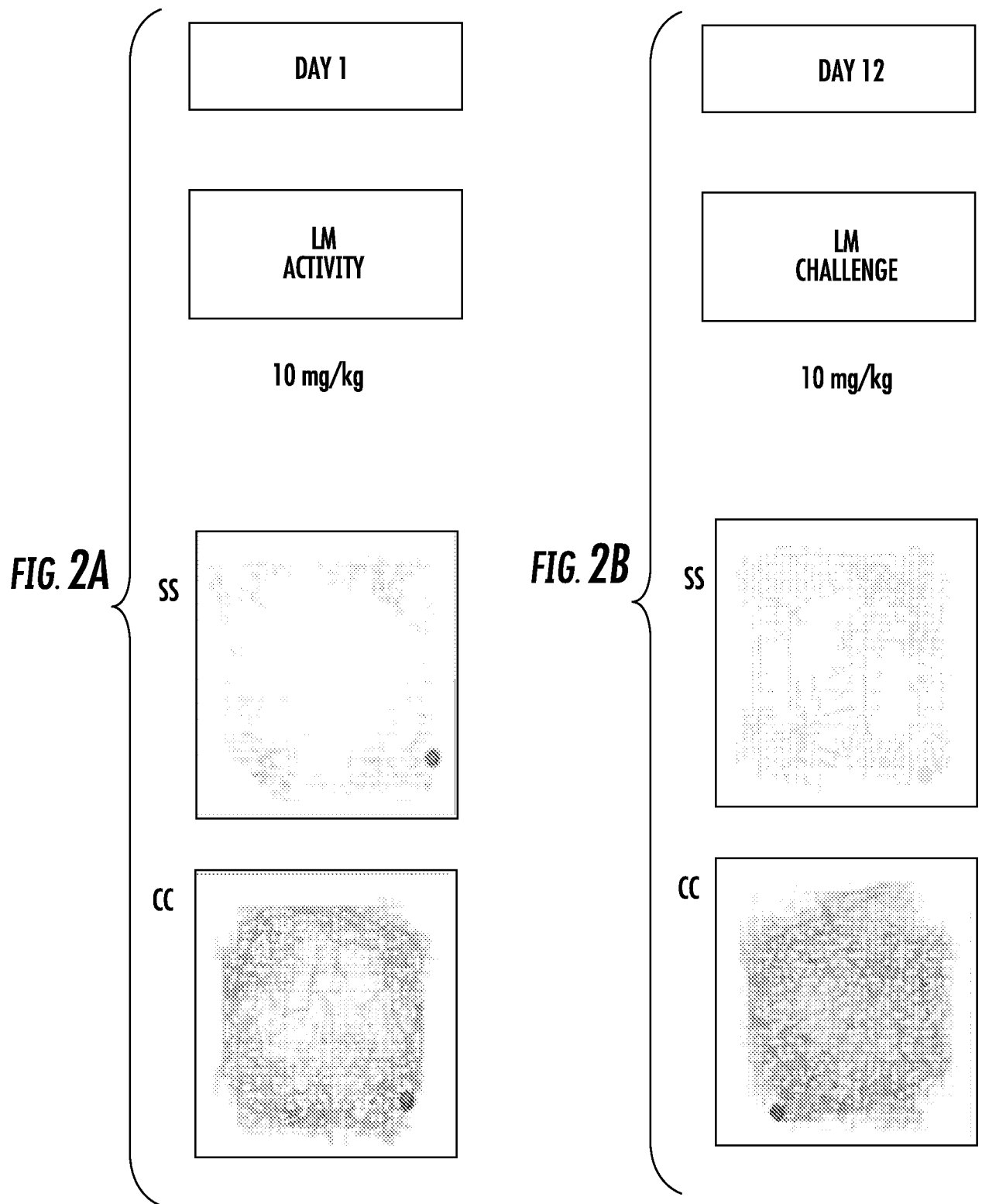
PROTOCOL DAY

1		2		3		4		5		6		7		13	
CPP PRE-TEST (0 mg/kg)	LM ACTIVITY (10 mg/kg)														
		CONDITIONING/SENSITIZING SESSIONS													
		1	2	3	4										
		(15 mg/kg)	(15 mg/kg)	(15 mg/kg)	(15 mg/kg)										
									</						

FIG. 1A

DAY	AM	PM
3	DRUG/VEHICLE	SALINE
4	SALINE	DRUG/VEHICLE
5	DRUG/VEHICLE	SALINE
6	SALINE	DRUG/VEHICLE

FIG. 1B



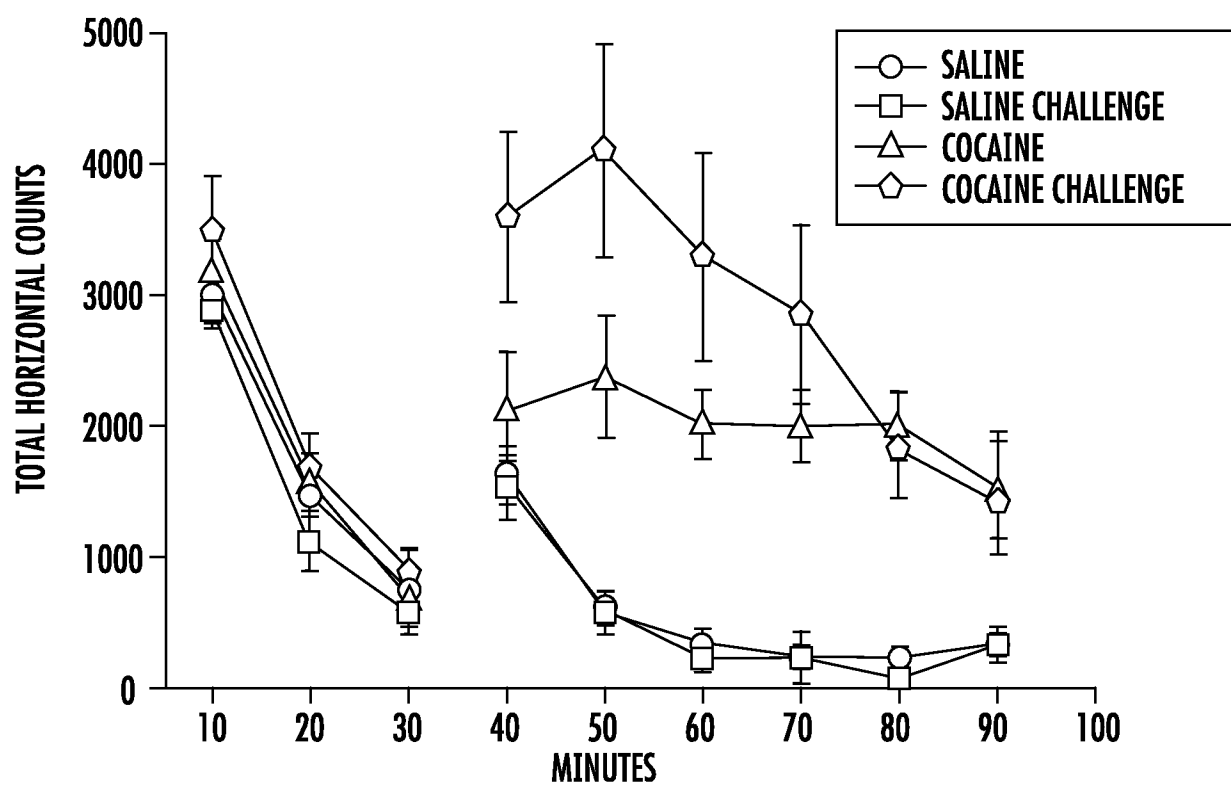


FIG. 2C

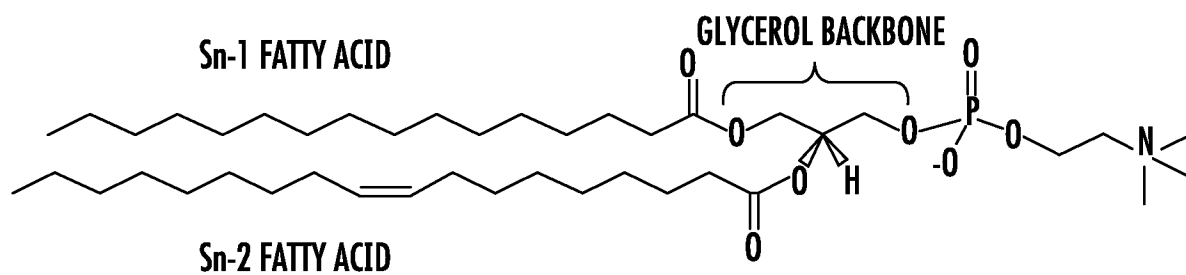
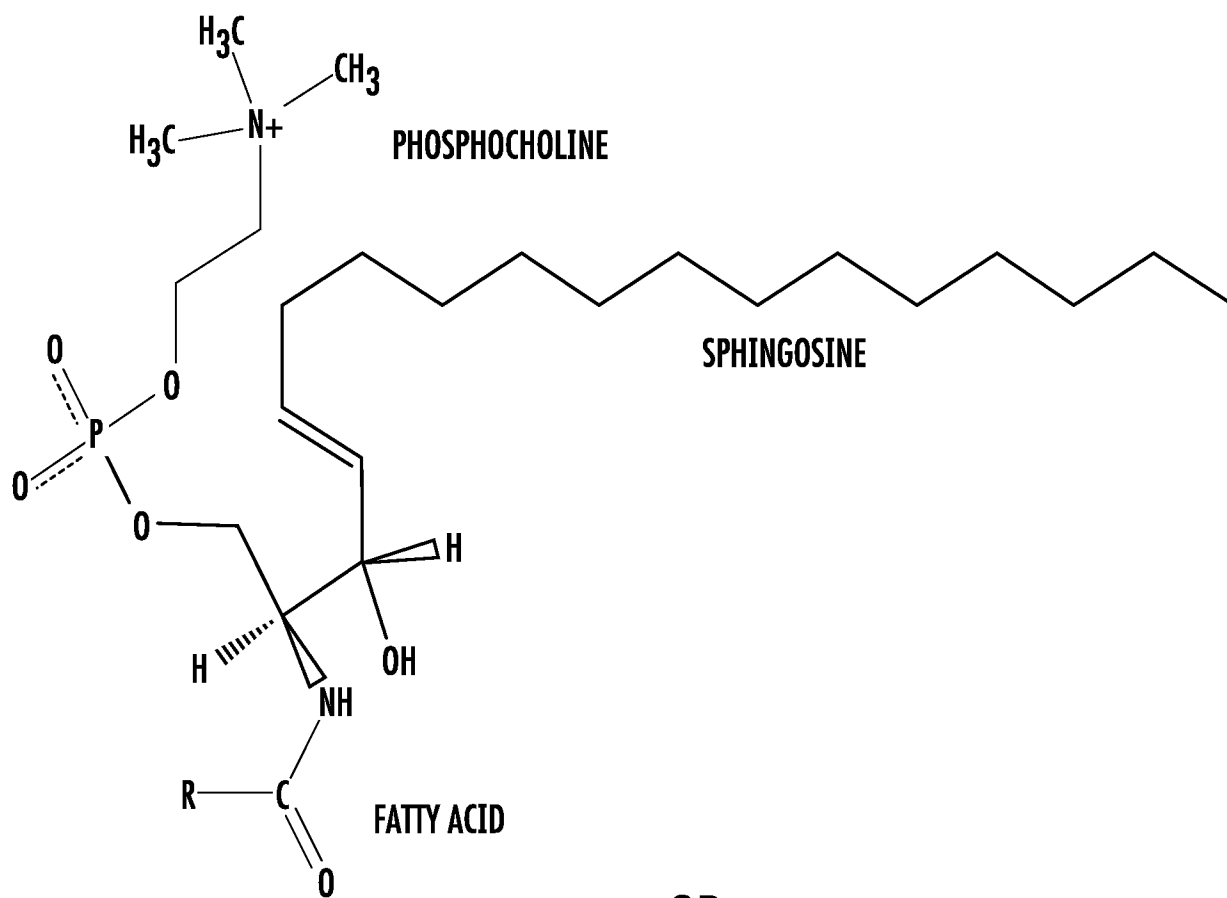
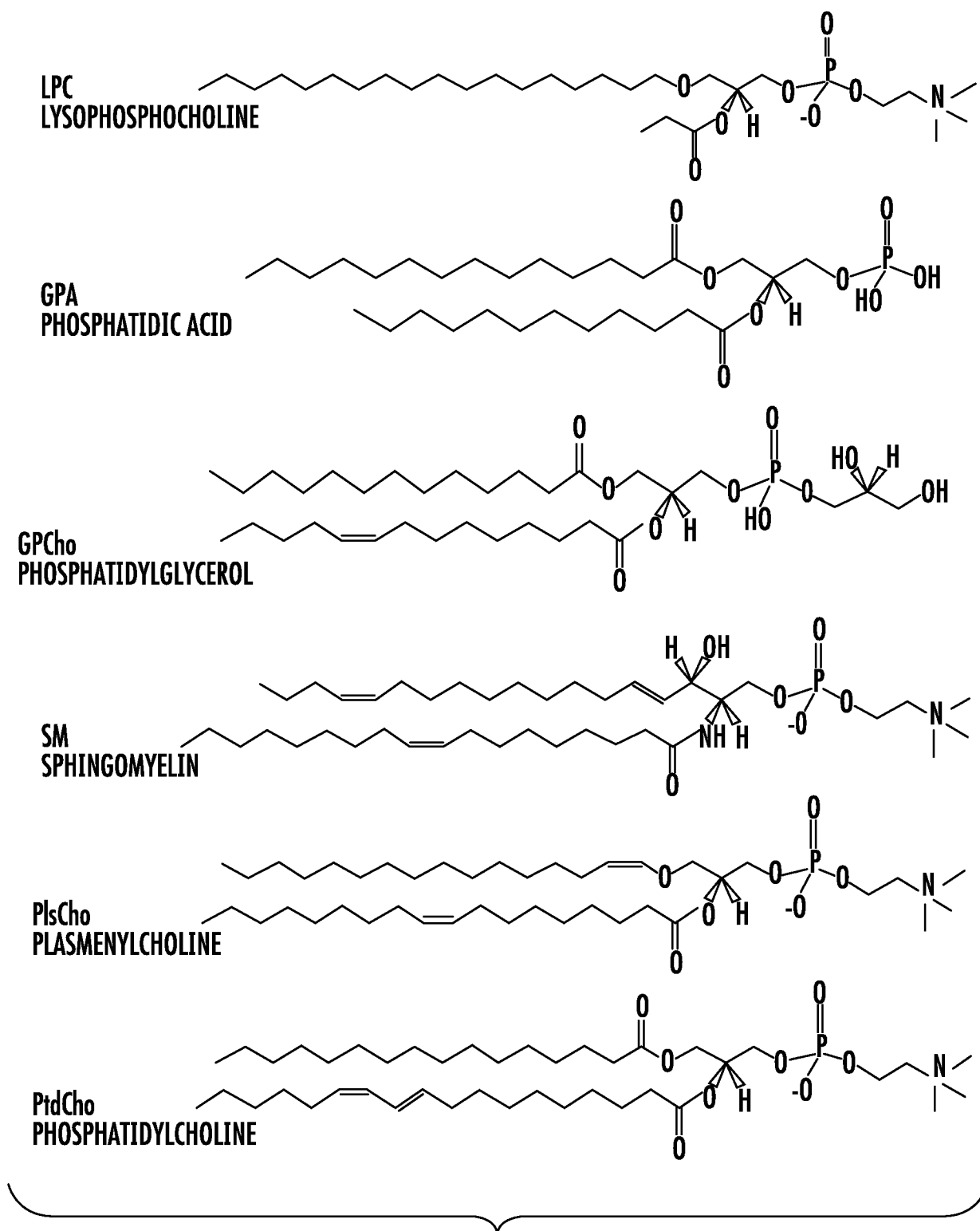


FIG. 3A

**FIG. 3B**

**FIG. 3C**

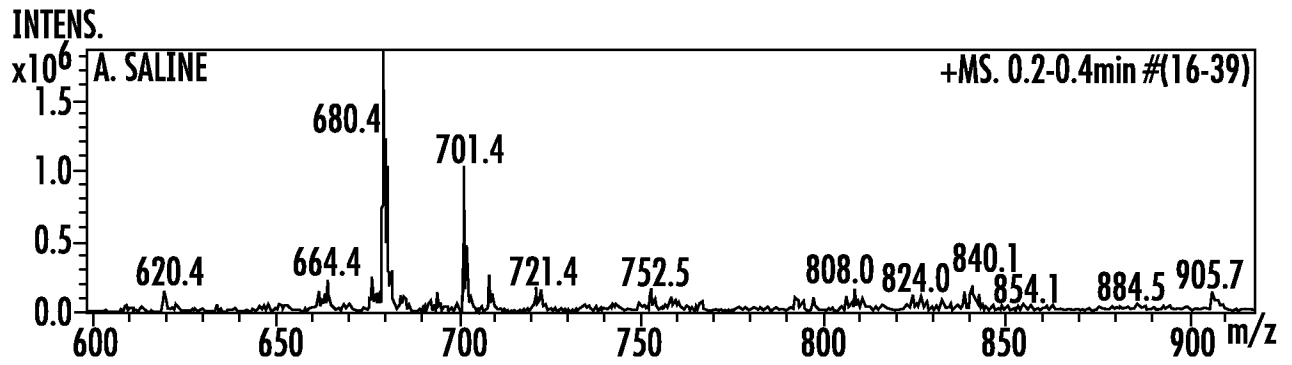


FIG. 4A

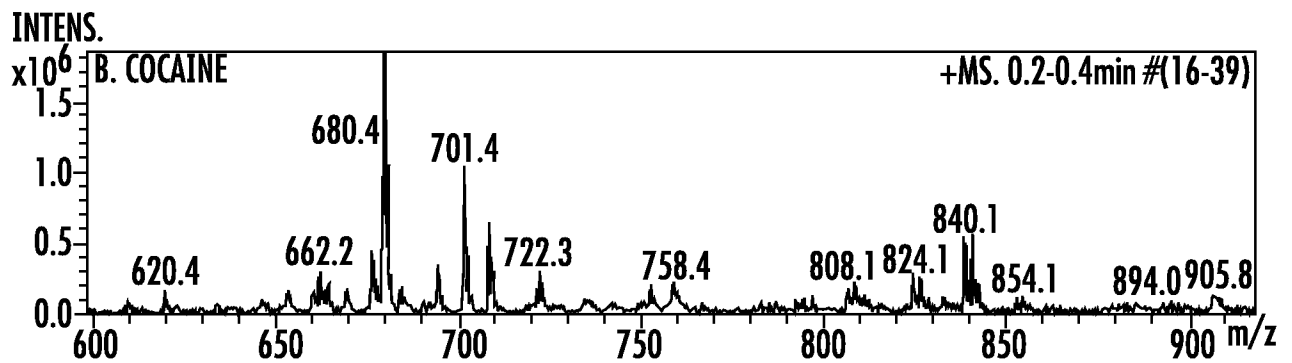


FIG. 4B

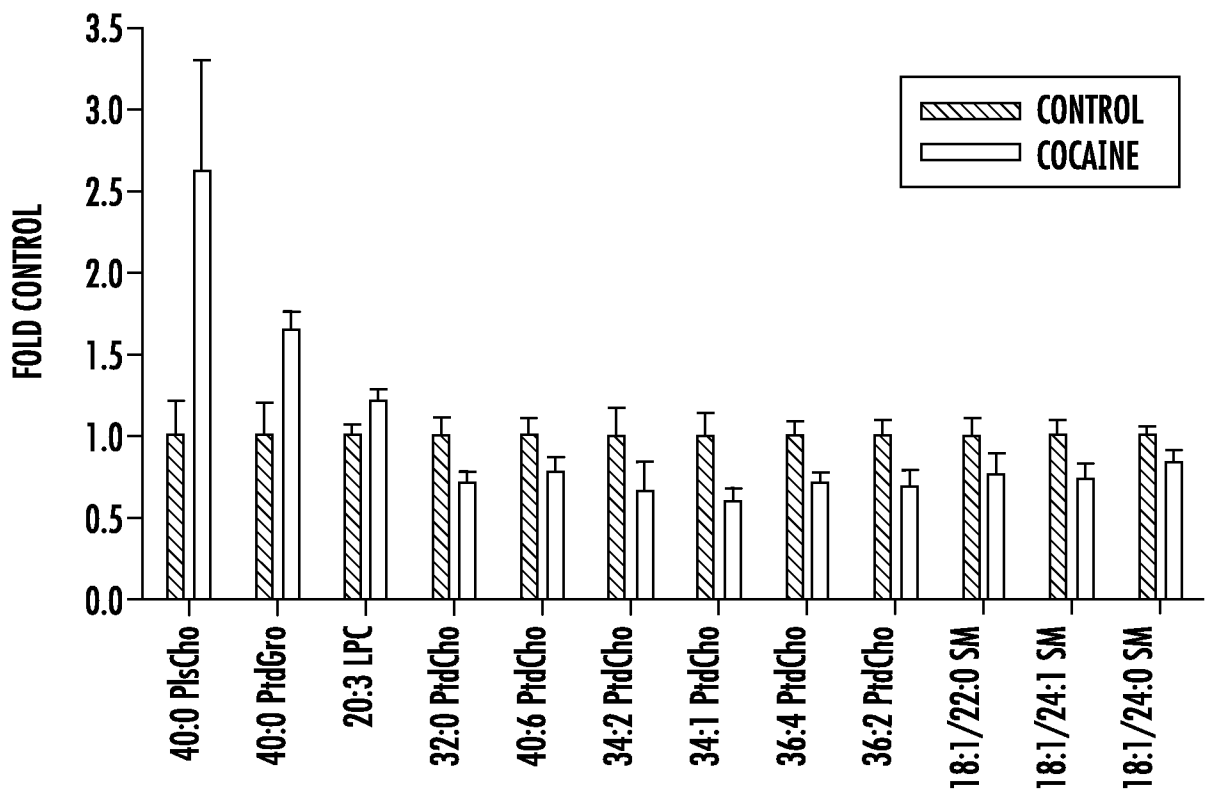


FIG. 4C

INTERNATIONAL SEARCH REPORT

47022900 00-00-2014

International application No.

PCT/US 14/22966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G06Q 10/00; G01N 33/48; G06G 7/58 (2014.01)

USPC - 705/2;702/19;703/11

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) - G06Q 10/00; G01N 33/48; G06G 7/58 (2014.01)

USPC - 705/2;702/19;703/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Text search (see terms below)

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

PatBase, Google Scholar, Search Terms: predicting sensitivity assaying biological sample phospholipids lipid profile analyzing risk score

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0305323 A1 (KADDURAH-DAOUK et al.) 10 December 2009 (10.12.2009) entire document, especially Abstract, paras [0014]; [0016]; [0018]; [0072]; [0125]; [0128]; [0151]; [0157]; [0166]	1-2, 6-14 --- 3-5
Y	US 2007/0111316 A1 (SHI et al.) 17 May 2007 (17.05.2007) entire document, especially paras para [0067]; [0111]; [0152]	3-5

☐ Further documents are listed in the continuation of Box C.

* 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

17 May 2014 (17.05.2014)

Date of mailing of the international search report

06 JUN 2014

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