

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
31 May 2007 (31.05.2007)

PCT

(10) International Publication Number  
**WO 2007/061995 A2**

(51) International Patent Classification:

A61K 31/22 (2006.01) A61K 31/47 (2006.01)  
A61K 31/366 (2006.01) A61K 31/505 (2006.01)  
A61K 31/40 (2006.01) A61P 9/00 (2006.01)  
A61K 31/405 (2006.01) G01N 33/50 (2006.01)

(21) International Application Number:

PCT/US2006/044987

(22) International Filing Date:

20 November 2006 (20.11.2006)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/738,631 21 November 2005 (21.11.2005) US

(71) Applicant (for all designated States except AT, US): **NOVARTIS AG** [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH).

(71) Applicant (for AT only): **NOVARTIS PHARMA GMBH** [AT/AT]; Brunner Strasse 59, A-1230 Vienna (AT).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **LI, Muhua** [CN/US]; 15 Mount Ararat Road, Short Hills, NJ 07078 (US).

(74) Agent: **PRINCE, John, T.**; NOVARTIS, Corporate Intellectual Property, One Health Plaza, Bldg. 104, East Hanover, NJ 07936-1080 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

**Published:**

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **BIOMARKERS FOR STATIN-INDUCED MYOPATHY OR RHABDOMYOLYSIS**

(57) Abstract: The invention provides genes as predictive biomarkers of statin-induced muscle toxicity. Following statin administration, three types of skeletal muscle (soleus, gastrocnemius and extensor digitorum lateralis (EDL)), including fast-twitching and slow-twitching, were profiled and analyzed. Remarkably, for samples exhibiting myopathy, the three muscle types showed highly similar gene expression profiles. Genes involved in oxidation, apoptosis, and ubiquitin-dependent protein catabolism and proteolysis were significantly changed, suggesting extensive protein degradation. In addition, significant induction of genes involved in the regulation of glycolysis and fatty acid oxidation, such as PDK4, 6-phosphofructo-2-kinase, and acetyl-Coenzyme A carboxylase beta, were also observed. PDK4 phosphorylates and inactivates the pyruvate dehydrogenase complex, and is the key inducer of the metabolic switch from glycolysis to fatty acid oxidation.



WO 2007/061995 A2

## BIOMARKERS FOR STATIN-INDUCED MYOPATHY OR RHABDOMYOLYSIS

### FIELD OF THE INVENTION

[01] This invention relates generally to the analytical testing of tissue samples *in vitro*, and more particularly to aspects of gene expression induced by statin administration.

### BACKGROUND OF THE INVENTION

[02] Statins (3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitors) reduce cholesterol production by reducing the synthesis of mevalonate, a critical intermediary in the cholesterol pathway. Statins are the most effective medications for managing elevated concentrations of low-density lipoprotein cholesterol (LDL-C). Various statins have been developed, including fluvastatin (Lescol®, Novartis Pharmaceuticals), lovastatin (Mevacor®, Merck & Co. Inc.), simvastatin (Zocor®, Merck & Co. Inc.), atorvastatin (Lipitor®, Pfizer Inc.), pravastatin (Pravacol®, Bristol Myers Squibb Co.) and rosuvastatin (Crestor, AstraZeneca). A novel statin - pitavastatin, also known as NKS104, NK-104, itavastatin, or nisvastatin - is currently under clinical investigations.

[03] Statin-induced myopathy and rhabdomyolysis are rare adverse drug reactions. For the class of statins, myopathy and rhabdomyolysis has become the most feared adverse effect. The epidemiological studies of incidences of muscle effects with statins are incomplete, but allow estimating that the incidences have a range of 0.1-1% for all muscular pathologies (elevated creatine phosphokinase (CPK), myalgia, muscle weakness) in monotherapy, rising to 2.5% for combination therapy with fibrates. Rhabdomyolysis appears with one order of magnitude less, but has, when undetected, a significant mortality. Hodel C, *Toxicol Lett* 128(1-3):159-68 (2002). For the class of statins including pitavastatin (NKS104), myopathy and rhabdomyolysis, although rare, has become the most feared adverse drug reactions.

[04] Despite the numerous papers describing statin-induced myopathy and rhabdomyolysis, the mechanism of statin-induced myopathy and rhabdomyolysis has not been elucidated.

[05] Published international patent application WO 2005/056837, entitled "Genetic polymorphisms associated with cardiovascular disorders and drug response and methods for their detection and use and diagnostic biomarkers", provides some genetic polymorphisms that are associated d. with cardiovascular disorders, particularly acute coronary events such as

myocardial infarction and stroke. The patent application also provides genetic polymorphisms that are associated with responsiveness of an individual to treatment of cardiovascular disorders with statin. Among 517 transcript and encoded protein sequences and 321 genomic sequences, there are 12,159 transcript-based context polymorphisms and 71,576 genomic-based context polymorphisms.

[06] Published international patent application WO 2003/072813, entitled "Single nucleotide polymorphism genotyping to predict adverse drug reactions and efficacy of statin therapy in patients with cardiovascular disease", provides single nucleotide polymorphism genotyping for prediction of adverse drug reactions and medication efficacy of statin therapy in patients with cardiovascular disease. An analysis of phenotype-associated genes, cDNA and promoter sequences was performed to identify allele variations associated with cardiovascular disease.

[07] However, there continues to be a need in the art for biomarkers for statin-induced myopathy and for methods of using biomarkers for a better understanding of statin therapy in individual patients.

#### SUMMARY OF THE INVENTION

[08] The invention provides genes as predictive biomarkers of statin-induced muscle toxicity. Following statin administration, three types of skeletal muscle (soleus, gastrocnemius and extensor digitorum lateralis (EDL)), including fast-twitching and slow-twitching, were profiled and analyzed. Remarkably, for samples exhibiting myopathy, the three muscle types showed highly similar gene expression profiles. Genes involved in oxidation, apoptosis, and ubiquitin-dependent protein catabolism and proteolysis were significantly changed, suggesting extensive protein degradation. In addition, significant induction of genes involved in the regulation of glycolysis and fatty acid oxidation, such as PDK4, 6-phosphofructo-2-kinase, and acetyl-Coenzyme A carboxylase beta, were also observed. PDK4 phosphorylates and inactivates the pyruvate dehydrogenase complex, and is the key inducer of the metabolic switch from glycolysis to fatty acid oxidation.

[09] Accordingly, in one embodiment, the invention provides for the use of statin in the manufacture of a medicament for the treatment of a cardiovascular disorder in a selected patient population. The patient population is selected on the basis of the gene expression of

biomarkers of statin-induced muscle toxicity by the patients following administration of statin to the patients.

[10] In another embodiment, the invention provides a method for treating cardiovascular disease in a subject, by determining the gene expression pattern of the subject, to whom statin has been administered. Then, the statin therapy is continued if the gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates a lack of muscle toxicity. Otherwise, the statin therapy is stopped or reduced if the gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates muscle toxicity.

[11] In yet another embodiment, the invention provides a method diagnosing a propensity for muscle toxicity in a subject, by determining the gene expression pattern of the subject, to whom statin has been administered. The gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates a propensity for muscle toxicity. Thus, the invention provides a diagnostic tool for individual susceptibility for statin-induced myopathy and an important tool for differentiating the current statins on the market.

[12] In another embodiment, the invention provides a method diagnosing a propensity for muscle toxicity in a subject, by determining the genetic polymorphism of the subject that affect the affinity and/or the activity of transporters, such as OATP-C and OATP-B.

[13] In another embodiment, the invention provides a method for avoiding fibrate and statin-induced rhabdomyolysis by improving the time schedule of medication and the doses of the drugs.

[14] In one aspect, the invention provides a new hypothesis of how statin leads to myopathy. Briefly, statins, by inhibition of its target HMGCR, decreases availability of geranylgeranyl pyrophosphate, which leads to decreased activity of small GTPase (Rho, Rab, Ras). Decreased activity of small GTPase, acting through AKT and forkhead transcription factors, leads to the induction of PDK4 and ubiquitin ligases related to muscle atrophy. PDK4 induction leads to metabolic switching and extensive protein degradation, which can lead to myopathy and rhabdomyolysis.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[15] The drawing figures depict preferred embodiments by way of example, not by way of limitations. In the figures, like reference numerals refer to the same or similar elements.

[16] FIG 1 is a workflow diagram of a Veloce genomics analysis.

- [17] FIG. 2 is a bar graph showing the relative potency ranking of statins based on pathway genes.
- [18] FIG. 3 is a flow diagram of the metabolic switching hypothesis that is the first part of the full hypothesis on statin induced-myopathy.
- [19] FIG. 4 is a diagram showing the detailed pathway and impact of PDK4 induction.
- [20] FIG. 5 is a diagram showing the full hypothesis on statin-induced myopathy

#### DETAILED DESCRIPTION OF THE INVENTION

[21] Pharmacogenomics is an emerging scientific discipline that examines the genetic basis for individual variations in response to therapeutics and may lead to the stratification of diseases into genetically defined categories. Pharmacogenomics is useful to aid in target discovery and validation, prioritize and optimize lead compounds, evaluate preclinical efficacy and safety, stratify patients enrolled in clinical trials, and to create predictive and diagnostic tests.

[22] In order to compare pitavastatin against atorvastatin and rosuvastatin at the molecular level and to investigate the molecular mechanism of statin-induced rhabdomyolysis or myopathy, a genomics gene expression (Veloce) study was conducted in cynomolgus monkeys where pitavastatin, atorvastatin and rosuvastatin, were administered each at low and high doses daily for 4 weeks. Veloce is a method for genomics analyses aiming to investigate biomarkers and mechanisms of an administered compound's action or toxicity in a rapid way utilizing gene expression profiling technologies. The workflow of Veloce genomics studies is briefly captured in FIG. 1. See also, WO 2005/045044.

[23] The present analysis had three goals: (1) to profile pitavastatin against atorvastatin and rosuvastatin at the molecular level; (2) to suggest hypothesis on the molecular mechanism of statin-induced myopathy; and (3) to identify candidate genes as potential predictive genetic markers for the development of muscle toxicity.

[24] In the liver, the induction of genes involved in the cholesterol biosynthesis and fatty acid metabolism pathways signify a clear pharmacological signature for the statin effect. Based on the magnitude of induction of cholesterol biosynthesis and fatty acid metabolism pathway genes (total number 27), pitavastatin was ranked as more potent than rosuvastatin and atorvastatin.

[25] Three types of skeletal muscle (soleus, gastrocnemius and extensor digitorum lateralis (EDL)), including fast-twitching and slow-twitching, were profiled and analyzed. Remarkably, for samples exhibiting myopathy, the three muscle types showed highly similar gene expression profiles. Genes involved in oxidation, apoptosis, and ubiquitin-dependent protein catabolism and proteolysis were significantly changed, suggesting extensive protein degradation. In addition, significant induction of genes involved in the regulation of glycolysis and fatty acid oxidation, such as PDK4, 6-phosphofructo-2-kinase, and acetyl-Coenzyme A carboxylase beta, were also observed. PDK4 phosphorylates and inactivates the pyruvate dehydrogenase complex, and is the key inducer of the metabolic switch from glycolysis to fatty acid oxidation.

[26] The result from skeletal muscle analysis, combined with the vast amount of knowledge in literature on these pathways, led us to propose a new hypothesis of how statin leads to myopathy. Briefly, statins, by inhibition of its target HMGCR, decreases availability of geranylgeranyl pyrophosphate, which leads to decreased activity of small GTPase (Rho, Rab, Ras). Decreased activity of small GTPase, acting through AKT and forkhead transcription factors, leads to the induction of PDK4 and ubiquitin ligases related to muscle atrophy. PDK4 induction leads to metabolic switching and extensive protein degradation, which can lead to myopathy and rhabdomyolysis.

[27] It is to be appreciated that certain aspects, modes, embodiments, variation and features of the invention are described below in various levels of detail in order to provide a substantial understanding of the present invention. In general, such disclosure provides useful biomarkers for the diagnosis and treatment of subjects in need thereof. Accordingly, the various aspects of the present invention relate to diagnostic/theranostic methods and kits to identify individuals predisposed to disease or to classify individuals with regard to drug responsiveness, side effects, or optimal drug dose. The methods and kits are useful for studying the aetiology of diseases, studying the efficacy of drug targeting, predicting individual susceptibility to diseases, and predicting individual responsiveness to drugs targeting the gene product. Accordingly, various particular embodiments that illustrate these aspects follow.

[28] *Definitions.* The definitions of certain terms as used in this specification are provided below. Definitions of other terms may be found in the glossary provided by the U.S. Department of Energy, Office of Science, Human Genome Project

([http://www.ornl.gov/sci/techresources/Human\\_Genome/glossary/](http://www.ornl.gov/sci/techresources/Human_Genome/glossary/)). In practicing the present invention, many conventional techniques in molecular biology, microbiology and recombinant DNA are used. These techniques are well-known and are explained in, *e.g.*, *Current Protocols in Molecular Biology*, Vols. I-III, Ausubel, ed. (1997); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, Second Edition* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989); *DNA Cloning: A Practical Approach*, Vols. I and II, Glover D, ed. (1985); *Oligonucleotide Synthesis*, Gait, ed. (1984); *Nucleic Acid Hybridization*, Hames & Higgins, eds. (1985); *Transcription and Translation*, Hames & Higgins, eds. (1984); *Animal Cell Culture*, Freshney, ed. (1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); Perbal, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymol.* (Academic Press, Inc., 1984); *Gene Transfer Vectors for Mammalian Cells*, Miller & Calos, eds. (Cold Spring Harbor Laboratory, New York, 1987); and *Methods in Enzymology*, Vols. 154 and 155, Wu & Grossman, and Wu, Eds., respectively.

[29] As used herein, the term "antibody" includes, but is not limited to, *e.g.*, polyclonal antibodies, monoclonal antibodies, humanized or chimaeric antibodies and biologically functional antibody fragments sufficient for binding of the antibody fragment to the protein.

[30] The term "biological sample" is intended to include, but is not limited to, *e.g.*, tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. In particular, EXAMPLE shows the use of liver and skeletal muscle samples as biological samples.

[31] The term "clinical response" means any or all of the following: a quantitative measure of the response, no response, and adverse response (*i.e.*, side effects).

[32] The term "clinical trial" means any research study designed to collect clinical data on responses to a particular treatment, and includes, but is not limited to phase I, phase II and phase III clinical trials. Standard methods are used to define the patient population and to enrol subjects.

[33] The term "effective amount" of a compound is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, for example, an amount which results in the prevention of, or a decrease in the symptoms associated with, a disease that is being treated. The amount of compound administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease.

The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Effective doses of statins are known to those in the medical arts. See, *e.g.*, Maron *et al.*, "Current Perspectives on Statins", *Circulation* 101:207 (2000).

[34] As used herein, "expression" includes but is not limited to one or more of the following: transcription of the gene into precursor mRNA; splicing and other processing of the precursor mRNA to produce mature mRNA; mRNA stability; translation of the mature mRNA into protein (including codon usage and mRNA availability); and glycosylation and/or other modifications of the translation product, if required for proper expression and function.

[35] The term "gene" means a segment of DNA that contains all the information for the regulated biosynthesis of an RNA product, including promoters, exons, introns, and other untranslated regions that control expression.

[36] The term "genotype" means an unphased 5' to 3' sequence of nucleotide pairs found at one or more polymorphic sites in a locus on a pair of homologous chromosomes in an individual. As used herein, genotype includes a full-genotype and/or a sub-genotype.

[37] The term "locus" means a location on a chromosome or DNA molecule corresponding to a gene or a physical or phenotypic feature.

[38] The term "isogene" means the different forms of a given gene that exist in the population.

[39] The term "mutant" means any heritable variation from the wild-type that is the result of a mutation, *e.g.*, single nucleotide polymorphism. The term "mutant" is used interchangeably with the terms "marker", "biomarker", and "target" throughout the specification.

[40] The term "medical condition" includes, but is not limited to, any condition or disease manifested as one or more physical and/or psychological symptoms for which treatment is desirable, and includes previously and newly identified diseases and other disorders.

[41] The term "nucleotide pair" means the nucleotides found at a polymorphic site on the two copies of a chromosome from an individual.

[42] The term "polymorphic site" means a position within a locus at which at least two alternative sequences are found in a population, the most frequent of which has a frequency of no more than 99%.

[43] The term “population” may be any group of at least two individuals. A population may include, *e.g.*, but is not limited to, a reference population, a population group, a family population, a clinical population, and a same sex population.

[44] The term “phased” means, when applied to a sequence of nucleotide pairs for two or more polymorphic sites in a locus, the combination of nucleotides present at those polymorphic sites on a single copy of the locus is known.

[45] The term “polymorphism” means any sequence variant present at a frequency of >1% in a population. The sequence variant may be present at a frequency significantly greater than 1% such as 5% or 10% or more. Also, the term may be used to refer to the sequence variation observed in an individual at a polymorphic site. Polymorphisms include nucleotide substitutions, insertions, deletions and microsatellites and may, but need not, result in detectable differences in gene expression or protein function.

[46] The term “polynucleotide” means any RNA or DNA, which may be unmodified or modified RNA or DNA. Polynucleotides include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, RNA that is mixture of single- and double-stranded regions, and hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons.

[47] The term “polypeptide” means any polypeptide comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. Polypeptide refers to both short chains, commonly referred to as peptides, glycopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. Polypeptides include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques that are well-known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature.

[48] The term “reference standard population” means a population characterized by one or more biological characteristics, *e.g.*, drug responsiveness, genotype, haplotype, phenotype, *etc.*

[49] The term “reference standard gene expression profile” is the pattern of expression of one or more gene observed in either a reference standard population or a single subject prior to administration of a compound.

[50] The term “statin” means any of a class of lipid-lowering drugs that reduce serum cholesterol levels by inhibiting hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), a liver enzyme involved in the biosynthesis of cholesterol. Pitavastatin, atorvastatin and rosuvastatin are statins.

[51] The term “subject” means that preferably the subject is a mammal, such as a human, but can also be an animal, including but not limited to, domestic animals (*e.g.*, dogs, cats and the like), farm animals (*e.g.*, cows, sheep, pigs, horses and the like) and laboratory animals (*e.g.*, monkeys such as cynomolgus monkeys, rats, mice, guinea pigs and the like).

[52] The term “test sample” means a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue, or isolated nucleic acid or polypeptide derived therefrom. In the EXAMPLE, a test sample can be a liver sample or a skeletal muscle sample, in particular soleus, extensor digitorum lateralis (EDL) and gastrocnemius muscle samples.

[53] The administration of an agent or drug to a subject or patient includes self-administration and the administration by another. It is also to be appreciated that the various modes of treatment or prevention of medical conditions as described are intended to mean “substantial”, which includes total but also less than total treatment or prevention, and wherein some biologically or medically relevant result is achieved.

[54] The details of one or more embodiments of the invention are set forth in the accompanying description below. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description and the claims. In the specification and the appended claims, the singular forms include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which

this invention belongs. All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[55] *Amplifying a Target Gene Region.* The target regions may be amplified using any oligonucleotide-directed amplification method, including but not limited to polymerase chain reaction (PCR). (U.S. Pat. No. 4,965,188), ligase chain reaction (LCR) (Barany *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:189-193 (1991); published PCT patent application WO 90/01069), and oligonucleotide ligation assay (OLA) (Landegren *et al.*, *Science*, 241:1077-1080 (1988)). Oligonucleotides useful as primers or probes in such methods should specifically hybridize to a region of the nucleic acid that contains or is adjacent to the polymorphic site. Other known nucleic acid amplification procedures may be used to amplify the target region including transcription-based amplification systems. (U.S. Pat. No. 5,130,238; EP 0 329 822; U.S. Pat. No. 5,169,766, published PCT patent application WO 89/06700) and isothermal methods (Walker *et al.*, *Proc. Natl. Acad. Sci., USA* 89:392-396 (1992)).

[56] *Hybridizing Allele-Specific Oligonucleotide to a Target Gene.* Hybridization of an allele-specific oligonucleotide to a target polynucleotide may be performed with both entities in solution, or such hybridization may be performed when either the oligonucleotide or the target polynucleotide is covalently or noncovalently affixed to a solid support. Attachment may be mediated, for example, by antibody-antigen interactions, poly-L-Lysine, streptavidin or avidin-biotin, salt bridges, hydrophobic interactions, chemical linkages, UV cross-linking, baking, *etc.* Allele-specific oligonucleotide may be synthesized directly on the solid support or attached to the solid support subsequent to synthesis. Solid-supports suitable for use in detection methods of the invention include substrates made of silicon, glass, plastic, paper and the like, which may be formed, for example, into wells (as in 96-well plates), slides, sheets, membranes, fibres, chips, dishes, and beads. The solid support may be treated, coated or derivatised to facilitate the immobilization of the allele-specific oligonucleotide or target nucleic acid.

[57] The genotype or haplotype for the gene of an individual may also be determined by hybridization of a nucleic sample containing one or both copies of the gene to nucleic acid arrays and subarrays such as described in WO 95/11995. The arrays would contain a battery

of allele-specific oligonucleotides representing each of the polymorphic sites to be included in the genotype or haplotype.

[58] See, also, *Molecular Cloning A Laboratory Manual, Second Ed.*, Sambrook, Fritsch & Maniatis, ed. (Cold Spring Harbor Laboratory Press, 1989); *DNA Cloning, Volumes I and II*, Glover DN ed. (1985); *Oligonucleotide Synthesis*, Gait MJ ed. (1984); *Nucleic Acid Hybridization*, Hames BD & Higgins SJ, eds., 1984).

[59] In the EXAMPLE below, all GeneChip experiments were conducted as recommended by the manufacturer of the GeneChip system. Affymetrix, *Expression Analysis Technical Manual* (Affymetrix, Santa Clara, California, 2005).

[60] *Computer System for Storing or Displaying Gene Expression or Polymorphism Data*. The invention also provides a computer system for storing and displaying data determined for the gene. Polymorphism data is information that includes, but is not limited to, e.g., the location of polymorphic sites; sequence variation at those sites; frequency of polymorphisms in one or more populations; the different genotypes and/or haplotypes determined for the gene; frequency of one or more of these genotypes and/or haplotypes in one or more populations; any known associations between a trait and a genotype or a haplotype for the gene. The computer system comprises a computer processing unit, a display, and a database containing the polymorphism data. The polymorphism data includes the polymorphisms, the genotypes and the haplotypes identified for a given gene in a reference population. In a preferred embodiment, the computer system is capable of producing a display showing gene expression pattern organized according to their evolutionary relationships.

[61] In addition, the computer may execute a program that generates views (or screens) displayed on a display device and with which the user can interact to view and analyze large amounts of information, relating to the gene and its genomic variation, including chromosome location, gene structure, and gene family, gene expression data, polymorphism data, genetic sequence data, and clinical data population data (e.g., data on ethnogeographic origin, clinical responses, and gene expression pattern for one or more populations). The polymorphism data described herein maybe stored as part of a relational database (e.g., an instance of an Oracle database or a set of ASCII flat files). These polymorphism data may be stored on the computer's hard drive or may, for example, be stored on a CD-ROM or on one or more other

storage devices accessible by the computer. For example, the data may be stored on one or more databases in communication with the computer *via* a network.

[62] In the EXAMPLE below, data analysis was performed with the Silicon Genetics software package GeneSpring version 6.1. Various filtering and clustering tools in these programs were used to explore the datasets and identify transcript level changes that inform on altered cellular and tissue functions that can be used to establish working hypotheses on the modes of action of the compound. The information content of these data sets is a conjunction of numerical changes and biological information. The decision to consider a specific gene relevant was based on a conjunction of numerical changes identified by comparative and statistical algorithms and the relationship to other modulated genes that point to a common biological theme. The value of that relationship was assessed by the analyst through a review of the relevant scientific literature.

[63] *Kits of the Invention.* It is to be understood that the methods of the invention described herein generally may further comprise the use of a kit according to the invention. The invention provides nucleic acid and polypeptide detection kits useful for haplotyping and/or genotyping the gene in an individual. Such kits are useful to classify subjects. Generally, the methods of the invention may be performed *ex-vivo*, and such *ex-vivo* methods are specifically contemplated by the present invention. Also, where a method of the invention may include steps that may be practised on the human or animal body, methods that only comprise those steps which are not practised on the human or animal body are specifically contemplated by the present invention.

[64] The kits of the invention are useful for detecting the presence of a polypeptide or nucleic acid corresponding to a marker of the invention in a biological sample, *e.g.*, any body fluid including, but not limited to, *e.g.*, serum, plasma, lymph, cystic fluid, urine, stool, cerebrospinal fluid, acitic fluid or blood and including biopsy samples of body tissue. For example, the kit can comprise a labelled compound or agent capable of detecting a polypeptide or an mRNA encoding a polypeptide corresponding to a marker of the invention in a biological sample and means for determining the amount of the polypeptide or mRNA in the sample *e.g.*, an antibody which binds the polypeptide or an oligonucleotide probe which binds to DNA or mRNA encoding the polypeptide.

[65] For antibody-based kits, the kit can comprise, *e.g.*, (1) a first antibody, *e.g.*, attached to a solid support, which binds to a polypeptide corresponding to a marker or the

invention; and, optionally; (2) a second, different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable label.

[66] For oligonucleotide-based kits, the kit can comprise, *e.g.*, (1) an oligonucleotide, *e.g.*, a detectably-labelled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to a marker of the invention; or (2) a pair of primers useful for amplifying a nucleic acid molecule corresponding to a marker of the invention.

[67] The kit can also comprise, *e.g.*, a buffering agent, a preservative or a protein-stabilizing agent. The kit can further comprise components necessary for detecting the detectable-label, *e.g.*, an enzyme or a substrate. The kit can also contain a control sample or a series of control samples, which can be assayed and compared to the test sample. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit. In a preferred embodiment, such kit may further comprise a DNA sample collecting means. The kits of the invention may contain a written product on or in the kit container. The written product describes how to use the reagents contained in the kit, *e.g.*, to use the biomarkers of the present invention in determining a strategy for preventing or treating a medical condition in a subject. In several embodiments, the use of the reagents can be according to the methods of the invention. In one embodiment, the reagent is a gene chip for determining the gene expression of relevant genes.

[68] *Correlating a Subject to a Standard Reference Population.* To deduce a correlation between clinical response to a treatment and a gene expression pattern, it is necessary to obtain data on the clinical responses exhibited by a population of individuals who received the treatment, *i.e.*, a clinical population. This clinical data maybe obtained by retrospective analysis of the results of clinical trials. Alternatively, the clinical data may be obtained by designing and carrying out one or more new clinical trials. The analysis of clinical population data is useful to define standard reference populations which, in turn, are useful to classify subjects for clinical trial enrolment or for selection of therapeutic treatment. In a preferred embodiment, the subjects included in the clinical population have been graded for the existence of the medical condition of interest. Grading of potential subjects can include, *e.g.*, a standard physical exam or one or more lab tests. Alternatively, grading of subjects can include use of a gene expression pattern. For example, gene expression pattern is useful as grading criteria where there is a strong correlation between gene expression pattern

and disease susceptibility or severity. Such standard reference population comprising subjects sharing gene expression pattern profile characteristics. For example, biomarker gene expression characteristics, are useful in the methods of the present invention to compare with the measured level of one or more gene expression product in a given subject. This gene expression products useful in the methods of the present invention include, but are not limited to, *e.g.*, characteristic mRNA associated with that particular genotype group or the polypeptide gene expression product of that genotype group. In one embodiment, a subject is classified or assigned to a particular genotype group or class based on similarity between the measured levels of a one or more biomarkers in the subject and the level of the one or more biomarkers observed in a standard reference population.

[69] In one embodiment of the invention, a therapeutic treatment of interest is administered to each subject in a trial population, and each subject's response to the treatment is measured using one or more predetermined criteria. It is contemplated that in many cases, the trial population will exhibit a range of responses, and that the investigator will choose the number of responder groups (*e.g.*, low, medium, high) made up by the various responses. In addition, the gene for each individual in the trial population is genotyped and/or haplotyped, which may be done before or after administering the treatment.

[70] Statistical analysis methods, which may be used, are described in L.D. Fisher & G. vanBelle, *Biostatistics: A Methodology for the Health Sciences* (Wiley-Interscience, New York, 1993). This analysis may also include a regression calculation of which polymorphic sites in the gene contribute most significantly to the differences in phenotype.

[71] An alternative method for finding correlations between haplotype content and clinical responses uses predictive models based on error-minimizing optimization algorithms, one of which is a genetic algorithm (Judson R, "Genetic Algorithms and Their Uses in Chemistry" in *Reviews in Computational Chemistry*, Vol. 10, pp 1-73, Lipkowitz KB and Boyd DB, eds, (VCH Publishers, New York, 1997). Simulated annealing (Press *et al.*, *Numerical Recipes in C: The Art of Scientific Computing*, Ch. 10 (Cambridge University Press, Cambridge, 1992), neural networks (Rich E & Knight K, *Artificial Intelligence*, 2nd Edition, Ch.10 (McGraw-Hill, New York, 1991), standard gradient descent methods (Press *et al.*, *supra* Ch. 10), or other global or local optimization approaches can also be used.

[72] Correlations may also be analyzed using analysis of variation (ANOVA) techniques to determine how much of the variation in the clinical data is explained by

different subsets of the polymorphic sites in the gene. ANOVA is used to test hypotheses about whether a response variable is caused by, or correlates with, one or more traits or variables that can be measured. See, L.D. Fisher & G. vanBelle, *Biostatistics: A Methodology for the Health Sciences* (Wiley-Interscience, New York, 1993) Ch. 10.

[73] After both the clinical and polymorphism data have been obtained, correlations between individual response and genotype or haplotype content are created. Correlations may be produced in several ways. In one method, individuals are grouped by their genotype or haplotype (or haplotype pair) (also referred to as a polymorphism group), and then the averages and standard deviations of clinical responses exhibited by the members of each polymorphism group are calculated.

[74] The skilled artisan can construct a mathematical model that predicts clinical response as a function of genotype or haplotype from the analyses described above. The identification of an association between a clinical response and a genotype or haplotype (or haplotype pair) for the gene may be the basis for designing a diagnostic method to determine those individuals who will or will not respond to the treatment, or alternatively, will respond at a lower level and thus may require more treatment, *i.e.*, a greater dose of a drug. The diagnostic method may take one of several forms: for example, a direct DNA test (*i.e.*, genotyping or haplotyping one or more of the polymorphic sites in the gene), a serological test, or a physical exam measurement. The only requirement is that there be a good correlation between the diagnostic test results and the underlying genotype or haplotype. In a preferred embodiment, this diagnostic method uses the predictive haplotyping method described above.

[75] *Predictive Medicine.* The invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to treat prophylactically a subject. Accordingly, one aspect of the invention relates to diagnostic assays for determining biomarker molecule expression as well as biomarker molecule activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant biomarker molecule expression or activity.

[76] The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with biomarker molecule

expression or activity. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with a biomarker polypeptide.

[77] The levels of certain polypeptides in a particular tissue (or in the blood) of a subject may be indicative of the toxicity, efficacy, rate of clearance or rate of metabolism of a given drug when administered to the subject. The methods described herein can also be used to determine the levels of such polypeptides in subjects to aid in predicting the response of such subjects to these drugs. Another aspect of the invention provides methods for determining mutant polypeptide activity in an individual to thereby select appropriate therapeutic or prophylactic compounds for that individual. Methods of the present invention allow for the selection of compounds (*e.g.*, drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (*e.g.*, the genotype of the individual examined to determine the ability of the individual to respond to a particular compound.)

[78] *Prognostic Assays.* The binding of a prognostic compound to a biomarker molecule, *e.g.*, biomarker polypeptide or nucleic acid encoding a biomarker polypeptide, can be utilized to identify a subject having or at risk of developing a disorder associated with biomarker polypeptide expression or activity (which are described above). A prognostic compound is any compound which binds to or associates with a biomarker molecule, including, but not limited to, *e.g.*, anti-biomarker polypeptide antibody, small molecule, nucleic acid, polypeptide, oligosaccharide, lipid, or combination thereof. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing the disease or disorder. Thus, the invention provides a method for identifying a disease or disorder associated with biomarker expression or activity in which a test sample is obtained from a subject and prognostic compound binding or activity is detected, wherein the presence of an alteration of prognostic compound binding or activity is diagnostic for a subject having, or at risk of developing, a disease or disorder associated with biomarker expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue, or isolated nucleic acid or polypeptide derived therefrom.

[79] Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered a compound (*e.g.*, an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate)

to treat a biomarker-associated disease or disorder. As used herein, the administration of a compound to a subject or patient includes self-administration and the administration by another. In one embodiment, the prognostic assays described herein are used to determine if a subject will be responsive to a compound. For example, such methods can be used to determine whether a subject can be effectively treated with a therapeutic compound for a biomarker-associated disorder (*i.e.*, biomarker-associated medical condition). Thus, the invention provides methods for determining whether a subject can be effectively treated with a compound for a disorder associated with biomarker expression or activity in which a test sample is obtained and biomarker molecule is detected using prognostic compound (*e.g.*, wherein the presence, or altered level of expression of, the biomarker molecule compared with the level of expression of the biomarker in a reference is diagnostic for a subject that can be administered the compound to treat a biomarker-associated disorder.

[80] There are a number of diseases in which the degree of overexpression (or underexpression) of certain biomarker molecules, *i.e.*, biomarker-associated disease or medical condition, is known to be indicative of whether a subject will develop a disease. Thus, the method of detecting a biomarker in a sample can be used as a method of predicting whether a subject will develop a disease. The level of a one or more biomarkers in a suitable tissue or blood sample from a subject at risk of developing the disease is determined and compared with a suitable control, *e.g.*, the level in subjects who are not at risk of developing the disease. The degree to which the one or more biomarkers is overexpressed (or underexpressed) in the sample compared with the control may be predictive of likelihood that the subject will develop the disease. The greater the overexpression (or underexpression) relative to the control, the more likely the subject will develop the disease.

[81] The methods described herein can be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe reagent, *e.g.*, anti-biomarker polypeptide antibody described herein, which can be conveniently used, *e.g.*, in clinical setting to diagnose patients exhibiting symptoms or family history of a disease or illness involving a biomarker of the invention. Furthermore, any cell type or tissue in which a biomarker of the invention is expressed can be utilized in the prognostic assays described herein.

[82] *Monitoring Clinical Efficacy.* Monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of a biomarker (*e.g.*, the ability to modulate aberrant

cell proliferation and/or differentiation) can be applied in basic drug screening and in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase biomarker gene expression, protein levels, or upregulate biomarker activity, can be monitored in clinical trials of subjects exhibiting decreased biomarker gene expression, protein levels, or downregulated biomarker activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease biomarker gene expression, protein levels, or downregulate biomarker activity, can be monitored in clinical trials of subjects exhibiting increased biomarker gene expression, protein levels, or upregulated biomarker activity. In such clinical trials, the expression or activity of a biomarker and, preferably, other genes that have been implicated in, for example, a proliferative disorder and cancers, can be used as a “read out” or marker of the responsiveness of a particular cell.

[83] For example, genes, including genes encoding a biomarker of the invention, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) that modulates a biomarker activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of a biomarker and other genes implicated in the disorder. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of a gene or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

[84] *Gene Expression and Subject Classification.* Standard control levels of a gene expression product are determined by measuring gene expression in different control groups. The control group gene expression levels are then compared with the measured level of a gene expression product in a given subject. This gene expression product could be the characteristic mRNA associated with that particular genotype group or the polypeptide gene expression product of that genotype group. The subject can be classified or assigned to a particular genotype group based on how similar the measured levels were compared to the control levels for a given group.

[85] As one of skill in the art will understand, there will be a certain degree of uncertainty involved in making this determination. Therefore, the standard deviations of the control group levels can be used to make a probabilistic determination and the method of this invention are applicable over a wide range of probability-based genotype group determinations. Thus, for example, and not by way of limitation, in one embodiment, if the measured level of the gene expression product falls within 2.5 standard deviations of the mean of any of the control groups, then that individual may be assigned to that genotype group. In another embodiment if the measured level of the gene expression product falls within 2.0 standard deviations of the mean of any of the control groups then that individual may be assigned to that genotype group. In still another embodiment, if the measured level of the gene expression product falls within 1.5 standard deviations of the mean of any of the control groups then that individual may be assigned to that genotype group. In yet another embodiment, if the measured level of the gene expression product is 1.0 or less standard deviations of the mean of any of the control groups levels then that individual may be assigned to that genotype group.

[86] Thus, this process allows determination, with various degrees of probability, which group a specific subject should be placed in, and such assignment to a genotype group would then determine the risk category into which the individual should be placed.

[87] *Detection of Biomarker Gene Expression.* An exemplary method for detecting the presence or absence of mutant polypeptide or nucleic acid of the invention in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound, or a compound capable of detecting mutant polypeptide or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes mutant polypeptide of the invention, such that the presence of mutant gene is detected in the biological sample. A compound for detecting mutant mRNA or mutant genomic DNA is a labelled nucleic acid probe capable of hybridizing to mutant mRNA or mutant genomic DNA. The nucleic acid probe can be, for example, a full-length mutant nucleic acid or a portion thereof, such as an oligonucleotide of at least 5, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to mutant mRNA or mutant genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein. An example of a compound for detecting a mutant polypeptide of the invention is an antibody raised against mutant polypeptide of the invention, capable of binding to the mutant

polypeptide, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')<sub>2</sub>) can be used. The term "labelled", with regard to the probe or antibody, is intended to encompass direct labelling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labelling of the probe or antibody by reactivity with another compound that is directly labelled. Examples of indirect labelling include detection of a primary antibody using a fluorescently-labelled secondary antibody and end-labelling of a DNA probe with biotin such that it can be detected with fluorescently-labelled streptavidin. That is, the detection method of the invention can be used to detect mutant mRNA, polypeptide, or genomic DNA of the invention in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of mutant mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of mutant polypeptide of the invention include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, and immunofluorescence. *In vitro* techniques for detection of mutant genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of mutant polypeptide include introducing into a subject a labelled anti-mutant polypeptide antibody. For example, the antibody can be labelled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. In one embodiment, the biological sample contains polypeptide molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

[88] In practicing the present invention, many conventional techniques in molecular biology, protein biochemistry, cell biology, immunology, microbiology and recombinant DNA are used. These techniques are well-known and are explained in, *e.g.*, *Current Protocols in Molecular Biology*, Vols. I-III, Ausubel, Ed. (1997); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, Second Edition* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989); *DNA Cloning: A Practical Approach*, Vols. I and II, Glover, Ed. (1985); *Oligonucleotide Synthesis*, Gait, Ed. (1984); *Nucleic Acid Hybridization*, Hames & Higgins, Eds. (1985); *Transcription and Translation*, Hames & Higgins, Eds. (1984); *Animal Cell Culture*, Freshney, ed. (1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); Perbal,

*A Practical Guide to Molecular Cloning*; the series, *Meth. Enzymol.*, (Academic Press, Inc., 1984); *Gene Transfer Vectors for Mammalian Cells*, Miller & Calos, eds. (Cold Spring Harbor Laboratory, New York, 1987); and *Meth. Enzymol.*, Vols. 154 and 155, Wu & Grossman, and Wu, eds., respectively. Methods to detect and measure mRNA levels (*i.e.*, gene transcription level) and levels of polypeptide gene expression products (*i.e.*, gene translation level) are well-known in the art and include the use of nucleotide microarrays and polypeptide detection methods involving mass spectrometers and/or antibody detection and quantification techniques. See also, Strachan & Read, *Human Molecular Genetics*, Second Edition. (John Wiley and Sons, Inc., New York, 1999).

[89] Techniques for the detection of gene expression of the genes described by this invention include, but are not limited to Northern blots, RT-PCT, real time PCR, primer extension, RNase protection, RNA expression profiling and related techniques. Techniques for the detection of gene expression by detection of the protein products encoded by the genes described by this invention include, but are not limited to, *e.g.*, antibodies recognizing the protein products, western blots, immunofluorescence, immunoprecipitation, ELISAs and related techniques. These techniques are well known to those of skill in the art. Sambrook J. *et al.*, *Molecular Cloning: A Laboratory Manual*, Third Edition (Cold Spring Harbor Press, Cold Spring Harbor, New York, 2000). In one embodiment, the technique for detecting gene expression includes the use of a gene chip. The construction and use of gene chips are well known in the art. See, U.S. Pat Nos. 5,202,231; 5,445,934; 5,525,464; 5,695,940; 5,744,305; 5,795,716 and 5,800,992. See also, Johnston M, *Curr. Biol.*, 8:R171-174 (1998); Iyer VR *et al.*, *Science*, 283:83-87 (1999) and Elias P, "New human genome 'chip' is a revolution in the offing" *Los Angeles Daily News* (October 3, 2003).

[90] *Determination of Marker Gene Transcription.* The determination of the level of the expression product of a marker gene in a biological sample, *e.g.*, the tissue or body fluids of an individual, may be performed in a variety of ways. Many expression detection methods use isolated RNA. For *in vitro* methods, any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from cells. See, *e.g.*, Ausubel *et al.*, ed., *Curr. Prot. Mol. Biol.* (John Wiley & Sons, NY, 1987-1999).

[91] In one embodiment, the level of the mRNA expression product of a marker gene is determined. Methods to measure the level of a specific mRNA are well-known in the art and include Northern blot analysis, reverse transcription PCR and real time quantitative PCR or

by hybridization to a oligonucleotide array or microarray. In other more preferred embodiments, the determination of the level of expression may be performed by determination of the level of the protein or polypeptide expression product of the gene in body fluids or tissue samples including but not limited to blood or serum.

[92] In a particular embodiment, the level of mRNA corresponding to a marker can be determined both by in situ and by in vitro formats in a biological sample using methods known in the art. Additionally, large numbers of tissue samples can readily be processed using techniques well-known to those of skill in the art, such as, *e.g.*, the single-step RNA isolation process of U.S. Pat. No. 4,843,155.

[93] The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, PCR analyses and probe arrays. One preferred diagnostic method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, *e.g.*, a full-length cDNA, or a portion hereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to an mRNA or genomic DNA encoding a marker of the present invention. Other suitable probes for use in the diagnostic assays of the invention are described herein. Hybridization of an mRNA with the probe indicates that the marker in question is being expressed.

[94] In one format, the mRNA is immobilized on a solid surface and contacted with a probe, for example, by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probes are immobilized on a solid surface and the mRNA is contacted with the probes, for example, in an Affymetrix gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the markers of the present invention.

[95] An alternative method for determining the level of mRNA corresponding to a marker of the present invention in a sample involves the process of nucleic acid amplification, *e.g.*, by RT-PCR (the experimental embodiment set forth by Mullis, U.S. Pat. No. 4,683,232); ligase chain reaction, Barany (1991), *supra*; self-sustained sequence replication, Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874-1878 (1990); transcriptional amplification system, Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173-1177 (1989); Q-Beta Replicase, Lizardi *et*

*al.*, *Biol. Technolog*, 6: 1197 (1988); rolling circle replication, U.S. Pat. No. 5,854,033; or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of the nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or *vice-versa*) and contain a short region in between. In general, amplification primers are from about 10-30 nucleotides in length and flank a region from about 50-200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

[96] As noted above, RT-PCR (real-time quantitative PCR) is one way to assess gene expression levels, *e.g.*, of genes of the invention (*e.g.*, those containing SNPs and polymorphisms of interest). The RT-PCR assay utilizes an RNA reverse transcriptase to catalyze the synthesis of a DNA strand from an RNA strand, including an mRNA strand. The resultant DNA may be specifically detected and quantified and this process may be used to determine the levels of specific species of mRNA. One method for doing this is known under the Trademark TAQMAN (PE Applied Biosystems, Foster City, CA) and exploits the 5' nuclease activity of AMPLITAQ GOLD™ DNA polymerase to cleave a specific form of probe during a PCR reaction. This is referred to as a TAQMAN™ probe. See Luthra *et al.*, *Am. J. Pathol.*, 153: 63-68 (1998)). The probe consists of an oligonucleotide (usually ≈20 mer) with a 5'-reporter dye and a 3' -quencher dye. The fluorescent reporter dye, such as FAM (6-carboxyfluorescein), is covalently linked to the 5' end of the oligonucleotide. The reporter is quenched by TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) attached *via* a linker arm that is located at the 3' end. See Kuimelis *et al.*, *Nucl. Acids Symp. Ser.*, 37: 255-256 (1997) and Mullah *et al.*, *Nucl. Acids Res.*, 26(4):1026-1031 (1998)). During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter.

[97] The accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. See Heid *et al.*, *Genome Res.*, 6(6): 986-994 (1996). Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed

number of cycles. The higher the starting copy number of nucleic acid target, the sooner a significant increase in fluorescence is observed, (Gibson *et al.*, *Genome Res.*, 6: 995-1001. (1996)).

[98] When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer. See Lakowicz *et al.*, *J. Biol. Chem.*, 258:4794-4801 (1983)). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'-3' nucleolytic activity of the AMPLITAQ GOLD™ DNA polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

[99] The passive reference is a dye included in the TAQMAN™ buffer and does not participate in the 5' nuclease assay. The passive reference provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

[100] Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio defined as the  $R_n$  (normalized reporter) for a given reaction tube.

[101] The threshold cycle or  $C_t$  value is the cycle at which a statistically significant increase in  $\Delta R_n$  is first detected. On a graph of  $R_n$  vs. cycle number, the threshold cycle occurs when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product.

[102] To perform quantitative measurements, serial dilutions of a cRNA (standard) are included in each experiment in order to construct a standard curve necessary for the accurate and fast mRNA quantification. In order to estimate the reproducibility of the technique, the amplification of the same cRNA sample may be performed multiple times.

[103] Other technologies for measuring the transcriptional state of a cell produce pools of restriction fragments of limited complexity for electrophoretic analysis, such as methods combining double restriction enzyme digestion with phasing primers (see, *e.g.*, EP 0 534858

Al), or methods selecting restriction fragments with sites closest to a defined mRNA end.

See, *e.g.*, Prashar *et al.*, *Proc. Natl. Acad. Sci.*, USA, 93(2):659-663 (1996)).

[104] Other methods statistically sample cDNA pools, such as by sequencing sufficient bases, *e.g.*, 20-50 bases, in each of multiple cDNAs to identify each cDNA, or by sequencing short tags, *e.g.*, 9-10 bases, which are generated at known positions relative to a defined mRNA end pathway pattern. See, *e.g.*, Velculescu, *Science*, 270:484-487 (1995). The cDNA levels in the samples are quantified and the mean, average and standard deviation of each cDNA is determined using by standard statistical means well-known to those of skill in the art. Bailey NTJ, *Statistical Methods In Biology, Third Edition* (Cambridge University Press, 1995).

[105] For *in situ* methods, mRNA does not need to be isolated from the cells prior to detection. In such methods, a cell or tissue sample is prepared or processed using known histological methods. The sample is then immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the marker. As an alternative to making determinations based on the absolute expression level of the marker, determinations may be based on the normalized expression level of the marker. Expression levels are normalized by correcting the absolute expression level of a marker by comparing its expression to the expression of a gene that is not a marker, *e.g.*, a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes, such as the actin gene or epithelial cell-specific genes. This normalization allows the comparison of the expression level in one sample, *e.g.*, a patient sample, to another sample or between samples from different sources.

[106] *Determination of Biomarker Gene Translation.* In another embodiment of the present invention, a polypeptide corresponding to a marker is detected. The detection of the biomarker polypeptide (*a.k.a.*, biomarker, marker, marker protein or marker polypeptide) expression product of the biomarker gene in body fluids or tissues can be used to determine the presence or absence of the polymorphism, and the relative level of the biomarker polypeptide expression product can be used to determine if the polymorphism is present in a homozygous or heterozygous state (and hence the risk category of the individual). That is, in another embodiment of the present invention, a polypeptide corresponding to a marker (*i.e.*, biomarker polypeptide) is detected. The level of this biomarker polypeptide gene expression product in body fluids or tissue sample may be determined by any means known in the art.

[107] *Immunological Detection Methods*. Expression of the protein encoded by the genes of the invention can be detected by a probe which is detectably labelled, or which can be subsequently labelled. Generally, the probe is an antibody that recognizes the expressed protein. A variety of formats can be employed to determine whether a sample contains a biomarker protein that binds to a given antibody. Immunoassay methods useful in the detection of biomarker polypeptides of the present invention include, but are not limited to, *e.g.*, dot blotting, western blotting, protein chips, competitive and non-competitive protein binding assays, enzyme-linked immunosorbant assays (ELISA), immunohistochemistry, fluorescence activated cell sorting (FACS), and others commonly used and widely-described in scientific and patent literature, and many employed commercially. A skilled artisan can readily adapt known protein/antibody detection methods for use in determining whether cells express a marker of the present invention and the relative concentration of that specific polypeptide expression product in blood or other body tissues. Proteins from individuals can be isolated using techniques that are well-known to those of skill in the art. The protein isolation methods employed can, *e.g.*, be such as those described in Harlow & Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor, New York, 1988)).

[108] An intact antibody, or a fragment thereof, *e.g.*, Fab or F(ab')<sub>2</sub> can be used. Antibody fragments, which recognize specific epitopes, may be generated by known techniques. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (see Huse *et al.*, *Science*, 246:1275-1281 (1989)), to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

[109] The term "labelled", with regard to the probe or antibody, is intended to encompass direct-labelling of the probe or antibody by coupling, *i.e.*, physically linking, a detectable substance to the probe or antibody, as well as indirect-labelling of the probe or antibody by reactivity with another reagent that is directly-labelled. Examples of indirect labelling include detection of a primary antibody using a fluorescently-labelled secondary antibody and end-labelling of a DNA probe with biotin such that it can be detected with fluorescently-labelled streptavidin.

[110] Monoclonal antibodies (mAbs), which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler & Milstein, *Nature*, 256:495-497 (1975); and U.S. Pat. No. 4,376,110; the human B-cell hybridoma technique of Kosbor *et al.*, *Immunol. Today*, 4:72 (1983); Cole *et al.*, *Proc. Natl. Acad. Sci., USA*, 80:2026-2030 (1983); and the EBV-hybridoma technique, Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96 (Alan R. Liss, Inc., 1985). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgG and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titres of mAbs *in vivo* makes this the presently preferred method of production.

[111] In one format, antibodies or antibody fragments can be used in methods, such as Western blots or immunofluorescence techniques, to detect the expressed proteins. In such uses, it is generally preferable to immobilize either the antibody or proteins on a solid support. Suitable solid phase supports or carriers include any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite.

[112] The extent to which the known proteins are expressed in a biological sample is determined by immunoassay methods that utilize the antibodies described above. Particularly preferred, for ease of detection, is the sandwich ELISA, of which a number of variations exist, all of which are intended to be used in the methods and assays of the present invention. For example, in a typical forward assay, unlabeled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule after a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen binary complex. At this point, a second antibody, labelled with a reporter molecule capable of inducing a detectable signal, is then added and incubated, allowing time sufficient for the formation of a ternary complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include the simultaneous assay, in which both sample and antibody are added simultaneously to the bound antibody, or a reverse assay in

which the labelled antibody and sample to be tested are first combined, incubated and added to the unlabelled surface bound antibody. These techniques are well-known to those skilled in the art, and the possibility of minor variations will be readily apparent. As used herein, "sandwich assay" is intended to encompass all variations on the basic two-site technique. For the immunoassays of the present invention, the only limiting factor is that the labelled antibody must be an antibody that is specific for the protein expressed by the gene of interest.

[113] *Two-Dimensional Gel Electrophoresis.* Proteins can be separated by two-dimensional gel electrophoresis systems and then identified and/or quantified. Two-dimensional gel electrophoresis is well-known in the art and typically involves isoelectric focusing along a first dimension followed by SDS PAGE electrophoresis along a second dimension. (See, *e.g.*, Hames *et al.*, *Gel Electrophoresis of Proteins: A Practical Approach* (IRL Press, NY, 1990); Shevchenko *et al.*, *Proc Natl. Acad. Sci. USA*, 93:14440-14445 (1996); Sagliocco *et al.*, *Yeast*, 12:1519-1533 (1996); and Lander, *Science* 274: 536-539 (1996)). The resulting electropherograms can be analyzed by numerous techniques, including mass spectrometric techniques, western blotting and immunoblot analysis using polyclonal and monoclonal antibodies, and internal and N-terminal micro-sequencing. Using these techniques, it is possible to identify a substantial fraction of all the proteins produced under given physiological conditions, including in cells, *e.g.*, in yeast, exposed to a drug, or in cells modified by, *e.g.*, deletion or over-expression of a specific gene.

[114] *Mass Spectroscopy.* The identity and the expression level of biomarker polypeptide can both be determined using mass spectroscopy technique (MS). MS-based analysis methodology is use for analysis of isolated biomarker polypeptide as well as analysis of biomarker polypeptide in a biological sample. MS formats for use in analyzing a biomarker polypeptide include ionization (I) techniques, such as, but not limited to, MALDI, continuous or pulsed ESI and related methods, such as ionspray or thermospray, and massive cluster impact (MCI). Such ion sources can be matched with detection formats, including linear or non-linear reflectron TOF, single or multiple quadrupole, single or multiple magnetic sector, Fourier transform ion cyclotron resonance (FTICR), ion trap and combinations thereof such as ion-trap/TOF. For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed. Sub-attomole levels of protein have been detected, *e.g.*, using ESI MS (Valaskovic *et al.*, *Science*, 273:1199-1202 (1996)) and MALDI MS (Li *et al.*, *J. Am. Chem. Soc.*, 118:1662-1663 (1996)).

[115] For MS analysis, the biomarker polypeptide can be solubilized in an appropriate solution or reagent system. The selection of a solution or reagent system, *e.g.*, an organic or inorganic solvent, will depend on the properties of the biomarker polypeptide and the type of MS performed, and is based on methods well-known in the art. See, *e.g.*, Vorm *et al.*, *Anal. Chem.*, 61:3281 (1994) for MALDI; and Valaskovic *et al.*, *Anal. Chem.*, 67:3802 (1995), for ESI. MS of peptides also is described, *e.g.*, in International PCT Application No. WO 93/24834 and U.S. Pat. No. 5,792,664. A solvent is selected that minimizes the risk that the biomarker polypeptide will be decomposed by the energy introduced for the vaporization process. A reduced risk of biomarker polypeptide decomposition can be achieved, *e.g.*, by embedding the sample in a matrix. A suitable matrix can be an organic compound such as a sugar, *e.g.*, a pentose or hexose, or a polysaccharide such as cellulose. Such compounds are decomposed thermolytically into CO<sub>2</sub> and H<sub>2</sub>O such that no residues are formed that can lead to chemical reactions. The matrix can also be an inorganic compound, such as nitrate of ammonium, which is decomposed essentially without leaving any residue. Use of these and other solvents is known to those of skill in the art. See, *e.g.*, US. Pat. No. 5,062,935.

[116] Electrospray MS has been described by Fenn *et al.*, *J. Phys. Chem.*, 88:4451-4459 (1984); and in PCT Application No. WO 90/14148; and current applications are summarized in review articles. See Smith *et al.*, *Anal. Chem.*, 62:882-89 (1990); and Ardrey, *Spectroscopy*, 4:10-18 (1992). With ESI, the determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks, all of which can be used for mass calculation.

[117] Matrix Assisted Laser Desorption (MALDI) is one preferred method among the MS methods herein. Methods for performing MALDI are well-known to those of skill in the art. Numerous methods for improving resolution are also known. For example, resolution in MALDI-TOF-MS can be improved by reducing the number of high energy collisions during ion extraction. See, *e.g.*, Juhasz *et al.*, *Analysis, Anal. Chem.*, 68:941-946 (1996); see also, *e.g.*, U.S. Pat. No. 5,777,325; 5,742,049; 5,654,545; 5,641,959; 5,654,545, and 5,760,393 for descriptions of MALDI and delayed extraction protocols. MALDI-TOF: MS has been described by Hillenkamp *et al.*, Burlingame & McCloskey, eds., pp. 49-60 (Elsevier Science Publ., 1990).

[118] In a preferred embodiment, the level of the biomarker protein in a biological sample, *e.g.*, body fluid or tissue sample, maybe measured by means of mass spectrometric

(MS) methods including, but not limited to, those techniques known in the art as matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) and surfaces enhanced for laser desorption/ionization, time-of-flight mass spectrometry (SELDI-TOF-MS) as further detailed below.

[119] *MASLDI-TOF-MS Protein Detection Technique*. In some preferred embodiments, the detection of specific proteins or polypeptide gene expression products in a biological sample, *e.g.*, body fluid or tissue sample, is performed by means of MS, especially matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MASLDI-TOF-MS). These techniques have been used to analyze macromolecules, such as proteins or biomolecules and utilize sample probe surface chemistries that enable the selective capture and desorption of analytes, including intact macromolecules, directly from the probe surface into the gas (vapour phase), and in the most preferred embodiments without added chemical matrix.

[120] In other embodiments a variety of other techniques for marker detection using mass spectroscopy can be used. See *Bordeaux Mass Spectrometry Conference Report*, Hillenkamp, ed., pp. 354-362 (1988); *Bordeaux Mass Spectrometry Conference Report*, Karas & Hillenkamp, Eds., pp. 416-417 (1988); Karas & Hillenkamp, *Anal. Chem.*, 60:2299-2301 (1988); and Karas *et al.*, *Biomed Environ Mass Spectrum*, 18:841-843 (1989). The use of laser beams in TOF-MS is shown, *e.g.*, in U.S. Pat. Nos., 4,694,167; 4,686,366; 4,295,046; and 5,045,694, which are incorporated herein by reference in their entireties. Other MS techniques allow the successful volatilization of high molecular weight biopolymers, without fragmentation, and have enabled a wide variety of biological macromolecules to be analyzed by mass spectrometry.

[121] *Surfaces Enhanced for Laser Desorption/Ionization (SELDI)*. In a preferred embodiment of the present invention, other techniques are used which employ new MS probe element compositions with surfaces that allow the probe element to actively participate in the capture and docking of specific analytes, described as Affinity Mass Spectrometry (AMS). Several types of new MS probe elements have been designed with Surfaces Enhanced for Affinity Capture (SEAC). See Hutchens & Yip, *Rapid Commun. Mass Spectrom.*, 7:576-580 (1993). SEAC probe elements have been used successfully to retrieve and tether different classes of biopolymers, particularly proteins, by exploiting what is known about protein surface structures and biospecific molecular recognition.

[122] In another preferred embodiment of the present invention, the method of detection to be used with the methods of this invention uses a general category of probe elements, *i.e.*, sample presenting means with surfaces enhanced for laser desorption/ionization (SELDI). See SELDI patents U.S. Pat. Nos. 5,719,060; 5,894,063; 6,020,208; 6,027,942; 6,124,137; and US. Patent Application No. U.S. 2003/0003465.

[123] A polypeptide of interest can be attached directly to a support *via* a linker. Any linkers known to those of skill in the art to be suitable for linking peptides or amino acids to supports, either directly or *via* a spacer, may be used. For example, the polypeptide can be conjugated to a support, such as a bead, through means of a variable spacer. Linkers, include, Rink amide linkers (see, *e.g.*, Rink, *Tetrahedron Lett.*, 28:3787 (1976)); trityl chloride linkers (see, *e.g.*, Leznoff, *Acc Chem. Res.* 11:327 (1978)); and Merrifield linkers. (See, *e.g.*, Bodansky *et al.*, *Peptide Synthesis, Second Edition* (Academic Press, New York, 1976)). For example, trityl linkers are known. (See, *e.g.*, U.S. Pat. Nos. 5,410,068 and 5,612,474). Amino trityl linkers are also known, (See, *e.g.*, U.S. Pat. No. 5,198,531). Other linkers include those that can be incorporated into fusion proteins and expressed in a host cell. Such linkers may be selected amino acids, enzyme substrates or any suitable peptide. The linker may be made, *e.g.*, by appropriate selection of primers when isolating the nucleic acid. Alternatively, they may be added by post-translational modification of the protein of interest.

[124] A linker can provide a reversible linkage such that it is cleaved under the select conditions. In particular, selectively cleavable linkers are useful, including photocleavable linkers, acid cleavable linkers, acid-labile linkers and heat sensitive linkers. A linker can be, *e.g.*, a photo-cleavable bond, such as a charge transfer complex or a labile bond formed between relatively stable organic radicals. The conjugation can be directly cleavable, *e.g.*, through a photocleavable linkage, such as a streptavidin or avidin to biotin interaction. Alternatively, the linkage can indirectly cleavable through a photocleavable linker (U.S. Pat. No. 5,643,722) or an acid labile linker, heat sensitive linker, enzymatically cleavable linker or other such linker. Combinations of any linkers are also contemplated herein. For example, a linker that is cleavable under MS conditions, such as a silyl linkage or photocleavable linkage, can be combined with a linker, such as an avidin biotin linkage, that is not cleaved under these conditions, but may be cleaved under other conditions.

[125] *Other Aspects of the Biological State.* In various embodiments of the present invention, aspects of the biological activity state, or mixed aspects can be measured in order

to obtain drug and pathway responses. The activities of proteins relevant to the characterization of cell function can be measured, and embodiments of this invention can be based on such measurements. Activity measurements can be performed by any functional, biochemical or physical means appropriate to the particular activity being characterized. Where the activity involves a chemical transformation, the cellular protein can be contacted with natural substrates, and the rate of transformation measured. Where the activity involves association in multimeric units, *e.g.*, association of an activated DNA binding complex with DNA, the amount of associated protein or secondary consequences of the association, such as amounts of mRNA transcribed, can be measured. Also, where only a functional activity is known, *e.g.*, as in cell cycle control, performance of the function can be observed. However known and measured, the changes in protein activities form the response data analyzed by the methods of this invention. In alternative and non-limiting embodiments, response data may be formed of mixed aspects of the biological state of a cell. Response data can be constructed from, *e.g.*, changes in certain mRNA abundances, changes in certain protein abundances and changes in certain protein activities.

[126] The following EXAMPLE is presented in order to more fully illustrate the preferred embodiments of the invention. This EXAMPLE should in no way be construed as limiting the scope of the invention, as defined by the appended claims.

## EXAMPLE

MICROARRAY GENE EXPRESSION ANALYSIS IN LIVER AND SKELETAL MUSCLE  
INVESTIGATING MECHANISM OF RHABDOMYOLYSIS

[127] *Introduction.* Pitavastatin, atorvastatin and rosuvastatin were administered once daily p.o. to cynomolgus monkeys for 4 weeks. Pitavastatin was dosed at a lower level than the other statins since it is considered 5 times more potent.

TABLE 1

Experimental animals, study design, animal allocation and test item dosages

| <u>Treatment</u> | <u>Dose (mg/kg/day)</u> | <u># of Males</u> | <u># of Female</u> |
|------------------|-------------------------|-------------------|--------------------|
| Vehicle          | 0                       | 3                 | 3                  |
| pitavastatin     | 10                      | 3                 | 3                  |
| pitavastatin     | 50                      | 3                 | 3                  |
| atorvastatin     | 50                      | 3                 | 3                  |
| atorvastatin     | 250                     | 3                 | 3                  |
| rosuvastatin     | 50                      | 3                 | 3                  |
| rosuvastatin     | 250                     | 3                 | 3                  |

[128] All the biological and tissue samples were frozen in liquid nitrogen. All selected tissues for gene expression profiling were examined histopathologically.

[129] *RNA extraction and purification.* A set of tissues was selected for gene expression profiling. These set included samples from kidney, bone, muscle, duodenum, pituitary and liver. Briefly, total RNA was obtained by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol, Invitrogen Life Technologies) from each frozen tissue section and the total RNA was then purified on an affinity resin (Rneasy, Qiagen) according to the manufacturer's instructions. Total RNA was quantified by the absorbance at  $\lambda = 260$  nm ( $A_{260nm}$ ), and the purity was estimated by the ratio  $A_{260nm}/A_{280nm}$ . Integrity of the RNA molecules was confirmed by non-denaturing agarose gel electrophoresis. RNA was stored at approximately -80°C until analysis. One part of each individual RNA sample was kept for the analysis of critical genes by means of Real-time PCR.

[130] *GeneChip experiment.* All GeneChip experiments were conducted as recommended by the manufacturer of the GeneChip system. Affymetrix, *Expression Analysis Technical Manual* (Affymetrix, Santa Clara, California, 2005). Genome U95Av2 expression probe array set (Affymetrix, Inc., San Diego, California, USA) were used. Raw data were converted to expression levels using a "target intensity" of 150. The data were checked for

quality and loaded in the GeneSpring software 4.2.4 (Silicon Genetics, California, USA) for analysis.

[131] *Data analysis.* Data analysis was performed with the Silicon Genetics software package GeneSpring version 6.1. Various filtering and clustering tools in these programs were used to explore the datasets and identify transcript level changes that inform on altered cellular and tissue functions that can be used to establish working hypotheses on the modes of action of the compound. The information content of these data sets is a conjunction of numerical changes and biological information. The decision to consider a specific gene relevant was based on a conjunction of numerical changes identified by comparative and statistical algorithms and the relationship to other modulated genes that point to a common biological theme. The value of that relationship was assessed by the analyst through a review of the relevant scientific literature. Increased and decreased expression reported here are referring to the RNA expression levels unless specifically stated.

[132] *Pharmacological signature for statins at gene expression level.* Cholesterol biosynthesis pathway and fatty acid metabolism pathway genes were found to be up-regulated in a dose-dependent manner in response to drug exposure, providing a clear pharmacological signature for NKS-104 and the other two statins. Gene changes in the cholesterol biosynthesis pathway and fatty acid metabolism pathways are summarized in TABLE 2.

**TABLE 2A**  
**NKS104 and the other two statins up-regulate cholesterol biosynthesis and fatty acid**  
**metabolism pathway genes**

| <u>Probe id</u> | <u>Mean of</u><br><u>controls</u> | <u>Std dev</u><br><u>of</u><br><u>controls</u> | <u>FC of</u><br><u>pitava-</u><br><u>statin</u><br><u>10mg</u> | <u>FC of</u><br><u>pitava-</u><br><u>statin</u><br><u>50mg</u> | <u>FC of</u><br><u>atorva-</u><br><u>statin</u><br><u>50mg</u> | <u>FC of</u><br><u>atorva-</u><br><u>statin</u><br><u>250mg</u> | <u>FC of</u><br><u>rosuva-</u><br><u>statin</u><br><u>50mg</u> | <u>FC of</u><br><u>rosuva-</u><br><u>statin</u><br><u>250mg</u> |
|-----------------|-----------------------------------|------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------------------|
| 202539_<br>s_at | 33                                | 14                                             | 16.2                                                           | 65.8                                                           | 22.9                                                           | 35.1                                                            | 16.5                                                           | 50.7                                                            |
| 205822_<br>s_at | 266                               | 63                                             | 8.7                                                            | 23.3                                                           | 10.7                                                           | 11.0                                                            | 7.6                                                            | 21.1                                                            |
| 202540_<br>s_at | 111                               | 26                                             | 5.7                                                            | 15.7                                                           | 6.9                                                            | 9.9                                                             | 5.4                                                            | 12.7                                                            |
| 221750_<br>at   | 570                               | 95                                             | 5.3                                                            | 21.0                                                           | 7.3                                                            | 10.2                                                            | 5.0                                                            | 12.4                                                            |
| 201790_<br>s_at | 30                                | 20                                             | 4.3                                                            | 10.5                                                           | 4.3                                                            | 3.0                                                             | 2.0                                                            | 14.8                                                            |
| 216607_<br>s_at | 1860                              | 604                                            | 3.2                                                            | 5.8                                                            | 3.5                                                            | 3.6                                                             | 3.2                                                            | 5.0                                                             |
| 213577_<br>at   | 68                                | 33                                             | 3.2                                                            | 4.6                                                            | 3.3                                                            | 3.5                                                             | 4.3                                                            | 4.6                                                             |
| 201791_<br>s_at | 52                                | 31                                             | 2.9                                                            | 7.7                                                            | 3.3                                                            | 4.6                                                             | 3.3                                                            | 7.1                                                             |
| 204256_<br>at   | 848                               | 164                                            | 2.9                                                            | 9.4                                                            | 4.2                                                            | 6.0                                                             | 4.1                                                            | 8.5                                                             |
| 234312_<br>s_at | 964                               | 280                                            | 2.8                                                            | 6.7                                                            | 3.6                                                            | 3.9                                                             | 3.2                                                            | 5.3                                                             |
| 209218_<br>at   | 437                               | 100                                            | 2.6                                                            | 5.5                                                            | 3.0                                                            | 3.7                                                             | 2.6                                                            | 4.6                                                             |
| 220081_<br>x_at | 537                               | 93                                             | 2.6                                                            | 4.6                                                            | 2.7                                                            | 2.7                                                             | 2.2                                                            | 3.6                                                             |
| 209279_<br>s_at | 280                               | 203                                            | 2.4                                                            | 4.9                                                            | 2.3                                                            | 1.8                                                             | 1.8                                                            | 4.5                                                             |
| 210950_<br>s_at | 618                               | 126                                            | 2.4                                                            | 7.0                                                            | 2.8                                                            | 3.3                                                             | 2.2                                                            | 5.2                                                             |
| 209146_<br>at   | 1238                              | 255                                            | 2.3                                                            | 6.0                                                            | 3.9                                                            | 4.2                                                             | 3.6                                                            | 5.6                                                             |
| 202314_<br>at   | 218                               | 26                                             | 2.3                                                            | 5.1                                                            | 2.7                                                            | 3.5                                                             | 2.8                                                            | 4.4                                                             |
| 201275_<br>at   | 989                               | 138                                            | 2.2                                                            | 4.6                                                            | 2.8                                                            | 2.5                                                             | 2.4                                                            | 3.9                                                             |
| 211423_<br>s_at | 430                               | 194                                            | 2.1                                                            | 4.0                                                            | 2.2                                                            | 3.4                                                             | 2.4                                                            | 4.3                                                             |
| 208962_<br>s_at | 918                               | 351                                            | 2.0                                                            | 3.4                                                            | 2.2                                                            | 1.9                                                             | 1.7                                                            | 3.6                                                             |
| 202245_<br>at   | 191                               | 97                                             | 1.8                                                            | 4.3                                                            | 2.2                                                            | 2.3                                                             | 1.9                                                            | 3.6                                                             |
| 208963_<br>x_at | 274                               | 75                                             | 1.7                                                            | 2.4                                                            | 1.8                                                            | 1.7                                                             | 1.7                                                            | 2.2                                                             |
| 200862_<br>at   | 1739                              | 203                                            | 1.6                                                            | 3.1                                                            | 2.0                                                            | 2.0                                                             | 1.8                                                            | 2.6                                                             |
| 202735_<br>at   | 1471                              | 151                                            | 1.6                                                            | 2.7                                                            | 1.7                                                            | 1.6                                                             | 1.7                                                            | 2.0                                                             |
| 217869_<br>at   | 464                               | 65                                             | 1.5                                                            | 2.2                                                            | 1.7                                                            | 1.9                                                             | 1.8                                                            | 2.4                                                             |

-36-

TABLE 2A

NKS104 and the other two statins up-regulate cholesterol biosynthesis and fatty acid

| Probe id    | Mean of<br>controls | Std dev<br>of<br>controls | metabolism pathway genes           |                                    |                                    |                                     |                                    |                                     |
|-------------|---------------------|---------------------------|------------------------------------|------------------------------------|------------------------------------|-------------------------------------|------------------------------------|-------------------------------------|
|             |                     |                           | FC of<br>pitava-<br>statin<br>10mg | FC of<br>pitava-<br>statin<br>50mg | FC of<br>atorva-<br>statin<br>50mg | FC of<br>atorva-<br>statin<br>250mg | FC of<br>rosuva-<br>statin<br>50mg | FC of<br>rosuva-<br>statin<br>250mg |
| 200832_s_at | 817                 | 390                       | 1.4                                | 3.7                                | 1.8                                | 1.7                                 | 1.8                                | 3.3                                 |
| 200831_s_at | 863                 | 312                       | 1.3                                | 3.1                                | 1.5                                | 1.2                                 | 1.7                                | 3.6                                 |
| 208964_s_at | 888                 | 138                       | 1.3                                | 2.1                                | 1.6                                | 1.9                                 | 1.4                                | 1.9                                 |
| 206210_s_at | 831                 | 252                       | 1.0                                | 0.1                                | 0.6                                | 0.3                                 | 0.5                                | 0.4                                 |

TABLE 2B

NKS104 and the other two statins up-regulate cholesterol biosynthesis and fatty acid

| Probe id    | Description                                                                                                               | metabolism pathway genes |  |  |  |  |  |
|-------------|---------------------------------------------------------------------------------------------------------------------------|--------------------------|--|--|--|--|--|
|             |                                                                                                                           |                          |  |  |  |  |  |
| 202539_s_at | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase                                                                           |                          |  |  |  |  |  |
| 205822_s_at | Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (HMGCS1), mRNA.                                   |                          |  |  |  |  |  |
| 202540_s_at | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase                                                                           |                          |  |  |  |  |  |
| 221750_at   | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)                                                                |                          |  |  |  |  |  |
| 201790_s_at | 7-dehydrocholesterol reductase                                                                                            |                          |  |  |  |  |  |
| 216607_s_at | Human lanosterol 14-alpha demethylase (CYP51P2) processed pseudogene, complete cds.                                       |                          |  |  |  |  |  |
| 213577_at   | squalene epoxidase                                                                                                        |                          |  |  |  |  |  |
| 201791_s_at | 7-dehydrocholesterol reductase                                                                                            |                          |  |  |  |  |  |
| 204256_at   | ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)                            |                          |  |  |  |  |  |
| 234312_s_at | acetyl-Coenzyme A synthetase 2 (ADP forming)                                                                              |                          |  |  |  |  |  |
| 209218_at   | squalene epoxidase                                                                                                        |                          |  |  |  |  |  |
| 220081_x_at | hydroxysteroid (17-beta) dehydrogenase 7                                                                                  |                          |  |  |  |  |  |
| 209279_s_at | NAD(P) dependent steroid dehydrogenase-like                                                                               |                          |  |  |  |  |  |
| 210950_s_at | farnesyl-diphosphate farnesyltransferase 1                                                                                |                          |  |  |  |  |  |
| 209146_at   | sterol-C4-methyl oxidase-like                                                                                             |                          |  |  |  |  |  |
| 202314_at   | cytochrome P450, family 51, subfamily A, polypeptide 1                                                                    |                          |  |  |  |  |  |
| 201275_at   | farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase) |                          |  |  |  |  |  |
| 211423_s_at | sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like                                                       |                          |  |  |  |  |  |
| 208962_s_at | fatty acid desaturase 1                                                                                                   |                          |  |  |  |  |  |
| 202245_at   | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)                                                                |                          |  |  |  |  |  |
| 208963_x_at | fatty acid desaturase 1                                                                                                   |                          |  |  |  |  |  |
| 200862_at   | 24-dehydrocholesterol reductase                                                                                           |                          |  |  |  |  |  |
| 202735_at   | emopamil binding protein (sterol isomerase)                                                                               |                          |  |  |  |  |  |
| 217869_at   | hydroxysteroid (17-beta) dehydrogenase 12                                                                                 |                          |  |  |  |  |  |
| 200832_s_at | stearoyl-CoA desaturase (delta-9-desaturase)                                                                              |                          |  |  |  |  |  |
| 200831_s_at | stearoyl-CoA desaturase (delta-9-desaturase)                                                                              |                          |  |  |  |  |  |
| 208964_s_at | fatty acid desaturase 1                                                                                                   |                          |  |  |  |  |  |
| 206210_s_at | cholesteryl ester transfer protein (CETP)                                                                                 |                          |  |  |  |  |  |

[133] The target enzyme for statins, HMG CoA reductase, is up-regulated. Other up-regulated genes include HMG CoA synthase, squalene epoxidase, farnesyl pyrophosphate synthetase, lanosterol synthase, stearyl-CoA desaturase, *etc.* These genes are sterol regulatory element binding protein (SREBP)-responsive genes, suggesting that the mechanism of up-regulation of cholesterol biosynthesis and fatty acid metabolism pathways is through SREBP, which regulate lipid homeostasis in vertebrate cells. SREBPs are a family of membrane-bound transcription factors that directly activate the expression of genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids, as well as the NADPH cofactor required to synthesize these molecules. SREBPs are activated through proteolytic processing by SCAP (SREBP cleavage-activating protein). SCAP senses cholesterol level in a cell through its membranous sterol-sensing domain. Hence, the statin-induced strong up-regulation of cholesterol and fatty acid pathway genes, is a result of the feed-back control loop of intracellular cholesterol levels regulated through SREBP and SCAP.

[134] In summary, the gene expression changes in the cholesterol and fatty acid pathways provide a clear pharmacological signature for statins' effect.

[135] *Relative potency ranking of statins based on pathway genes.* A statistical method has been developed for relative potency ranking of compounds based on relevant pathway genes. Since the induction of cholesterol biosynthesis and fatty acid metabolism pathways provided a clear pharmacological signature for the statins, these 27 pathway genes (TABLE 1) were used for the relative potency ranking of the tested statins in this study. Based on the magnitude of pathway induction of cholesterol biosynthesis and fatty acid metabolism, pitavastatin was ranked as more potent than rosuvastatin and atorvastatin. See, FIG. 2.

[136] *The CETP gene was down-regulated by high-dose statin treatment.* The CETP gene (cholesteryl ester transfer protein) was down-regulated in all treatment groups except low dose of pitavastatin treatment. See, TABLE 2A, above. CETP is a plasma glycoprotein that facilitates the transfer of cholesteryl esters from HDL cholesterol to apolipoprotein B-containing lipoproteins. Humans with CETP deficiency due to molecular defects in the *CETP* gene have markedly elevated plasma levels of HDL cholesterol and apolipoprotein A-I, leading to the concept that CETP inhibition might increase HDL cholesterol levels. Therefore, down-regulation of CETP gene may be the mechanism of the observed moderate elevation of HDL with high-dose statin treatment. Interestingly, Brousseau ME *et al.*, *New Eng J Med*; 350:1505-15 (2004) noted that decreased HDL cholesterol levels constitute a

major risk factor for coronary heart disease, and investigated the effect of a novel CETP inhibitor, torcetrapib, on plasma lipoproteins in patients with low HDL cholesterol levels. They found that in patients with low HDL cholesterol levels, CETP inhibition with torcetrapib markedly increased HDL cholesterol levels and also decreased LDL cholesterol levels, both when administered as monotherapy and when administered in combination with a statin.

[137] *Muscle data analysis.* Three types of muscle were profiled on gene chips and each analyzed in detail and compared. These samples include soleus, extensor digitorum lateralis (EDL), and gastrocnemius.

[138] Overall, four animals in the study showed high and consistent CK elevation in all three muscle types. Three of these animals were in the pitavastatin 50 mg/kg/day group, and one was in the rosuvastatin 250 mg/kg/day group. Among these four animals, two animals showed necrosis in muscle samples visible by histopathology and molecular profiles. We focused on the two animals with CK elevation and concomitant necrosis profiles in their muscle samples (animal D60909 and D60958, both in the pitavastatin 50 mg/kg/day high-dose group). These two animals were compared to the rest of animals in the same dosing group.

[139] Differentially expressed genes were identified. These differentially expressed gene lists were generated for all three types of muscles. Remarkably, the three types of muscles showed highly similar gene expression profiles. See, TABLES 4-9. The differentially expressed gene lists were analyzed to assign the pathways and cellular processes they are involved in.

[140] *Hypothesis formation on statin-induced myopathy.* Increased expression of genes associated with apoptosis, oxidation and proteolysis suggest extensive protein degradation. Genes involved in oxidation, apoptosis, ubiquitin-dependent protein catabolism and proteolysis were significantly changed in the muscle samples with myopathy in all three muscle types. TABLES 3-4.

**TABLE 3A**  
Apoptosis and oxidation-related genes in the three types of muscles

| <u>Probe id</u> | <u>EDL Mean</u><br><u>of samples</u><br><u>with NO</u><br><u>myotoxic-</u><br><u>ity</u> | <u>EDL Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxic-</u><br><u>ity</u> | <u>Gastrocnem</u><br><u>ius Mean of</u><br><u>samples</u><br><u>with NO</u><br><u>myotoxicity</u> | <u>Gastrocnem</u><br><u>ius Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxicity</u> | <u>Soleus</u><br><u>Mean of</u><br><u>samples</u><br><u>with NO</u><br><u>myotoxicity</u> | <u>Soleus Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxicity</u> |
|-----------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| 204326_x_at     | 395                                                                                      | 24.0                                                                                                   | 716                                                                                               | 11.3                                                                                                            | 344                                                                                       | 24.6                                                                                          |
| 206461_x_at     | 595                                                                                      | 14.6                                                                                                   | 862                                                                                               | 15.5                                                                                                            | 642                                                                                       | 13.4                                                                                          |
| 204745_x_at     | 554                                                                                      | 11.4                                                                                                   | 766                                                                                               | 11.6                                                                                                            | 515                                                                                       | 11.2                                                                                          |
| 212185_x_at     | 1170                                                                                     | 11.3                                                                                                   | 1903                                                                                              | 9.7                                                                                                             | 1097                                                                                      | 11.3                                                                                          |
| 208581_x_at     | 1055                                                                                     | 10.2                                                                                                   | 1776                                                                                              | 11.3                                                                                                            | 953                                                                                       | 10.6                                                                                          |
| 215223_s_at     | 825                                                                                      | 8.1                                                                                                    | 2466                                                                                              | 12.4                                                                                                            | 1657                                                                                      | 8.5                                                                                           |
| 211456_x_at     | 1396                                                                                     | 7.3                                                                                                    | 2753                                                                                              | 6.5                                                                                                             | 1306                                                                                      | 6.4                                                                                           |
| 217165_x_at     | 934                                                                                      | 7.1                                                                                                    | 1329                                                                                              | 5.6                                                                                                             | 980                                                                                       | 6.4                                                                                           |
| 217889_s_at     | 131                                                                                      | 4.0                                                                                                    | 118                                                                                               | 12.1                                                                                                            | 72                                                                                        | 12.5                                                                                          |
| 210564_x_at     | 117                                                                                      | 3.4                                                                                                    | 127                                                                                               | 4.6                                                                                                             | 77                                                                                        | 4.0                                                                                           |
| 201848_s_at     | 1720                                                                                     | 3.3                                                                                                    | 4701                                                                                              | 4.0                                                                                                             | 2606                                                                                      | 3.7                                                                                           |
| 216841_s_at     | 243                                                                                      | 3.2                                                                                                    | 265                                                                                               | 23.3                                                                                                            | 378                                                                                       | 8.6                                                                                           |
| 225262_at       | 209                                                                                      | 3.1                                                                                                    | 411                                                                                               | 2.6                                                                                                             | 469                                                                                       | 2.5                                                                                           |
| 218880_at       | 229                                                                                      | 2.9                                                                                                    | 448                                                                                               | 2.6                                                                                                             | 581                                                                                       | 2.0                                                                                           |
| 213629_x_at     | 453                                                                                      | 2.9                                                                                                    | 299                                                                                               | 2.1                                                                                                             | 451                                                                                       | 2.1                                                                                           |
| 212859_x_at     | 451                                                                                      | 2.8                                                                                                    | 724                                                                                               | 1.7                                                                                                             | 375                                                                                       | 3.1                                                                                           |
| 211548_s_at     | 471                                                                                      | 2.5                                                                                                    | 322                                                                                               | 4.7                                                                                                             | 387                                                                                       | 2.6                                                                                           |
| 1556069_s_at    | 69                                                                                       | 2.5                                                                                                    | 143                                                                                               | 1.0                                                                                                             | 64                                                                                        | 2.4                                                                                           |
| 211862_x_at     | 200                                                                                      | 2.4                                                                                                    | 230                                                                                               | 3.3                                                                                                             | 213                                                                                       | 3.3                                                                                           |
| 203815_at       | 143                                                                                      | 2.4                                                                                                    | 179                                                                                               | 1.6                                                                                                             | 112                                                                                       | 3.1                                                                                           |
| 203914_x_at     | 812                                                                                      | 2.4                                                                                                    | 606                                                                                               | 3.5                                                                                                             | 701                                                                                       | 2.0                                                                                           |
| 242281_at       | 117                                                                                      | 2.4                                                                                                    | 71                                                                                                | 2.1                                                                                                             | 126                                                                                       | 2.1                                                                                           |
| 208485_x_at     | 213                                                                                      | 2.4                                                                                                    | 273                                                                                               | 3.5                                                                                                             | 259                                                                                       | 2.9                                                                                           |
| 210563_x_at     | 248                                                                                      | 2.3                                                                                                    | 302                                                                                               | 3.0                                                                                                             | 297                                                                                       | 2.3                                                                                           |
| 237367_x_at     | 82                                                                                       | 2.3                                                                                                    | 85                                                                                                | 3.3                                                                                                             | 100                                                                                       | 2.8                                                                                           |
| 208928_at       | 211                                                                                      | 2.2                                                                                                    | 286                                                                                               | 3.2                                                                                                             | 300                                                                                       | 2.1                                                                                           |
| 201849_at       | 1292                                                                                     | 2.0                                                                                                    | 1248                                                                                              | 3.6                                                                                                             | 2032                                                                                      | 2.4                                                                                           |

TABLE 3B  
Apoptosis and oxidation-related genes in the three types of muscles

| <u>Probe id</u> | <u>Description</u>                                                                                                                                                      |
|-----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 204326_x_at     | synonyms: MT1, MT1R; metallothionein 1R; Homo sapiens metallothionein 1L (MT1L), mRNA.                                                                                  |
| 206461_x_at     | metallothionein 1H                                                                                                                                                      |
| 204745_x_at     | metallothionein 1G                                                                                                                                                      |
| 212185_x_at     | metallothionein 2A                                                                                                                                                      |
| 208581_x_at     | metallothionein 1X                                                                                                                                                      |
| 215223_s_at     | superoxide dismutase 2, mitochondrial                                                                                                                                   |
| 211456_x_at     | MT-1H-like protein; mutant as compared to wild-type sequence MT-1H in GenBank Accession Number X64834; Homo sapiens metallothionein 1H-like protein mRNA, complete cds. |
| 217165_x_at     | human metallothionein-If; Human metallothionein-If gene (hMT-If).                                                                                                       |
| 217889_s_at     | cytochrome b reductase 1                                                                                                                                                |
| 210564_x_at     | CASP8 and FADD-like apoptosis regulator                                                                                                                                 |
| 201848_s_at     | Homo sapiens BCL2/adenovirus E1B 19kD-interacting protein 3 (BNIP3) mRNA, complete cds.                                                                                 |
| 216841_s_at     | superoxide dismutase 2, mitochondrial                                                                                                                                   |
| 225262_at       | FOS-like antigen 2                                                                                                                                                      |
| 218880_at       | FOS-like antigen 2                                                                                                                                                      |
| 213629_x_at     | metallothionein 1F (functional)                                                                                                                                         |
| 212859_x_at     | metallothionein 2A                                                                                                                                                      |
| 211548_s_at     | hydroxyprostaglandin dehydrogenase 15-(NAD)                                                                                                                             |
| 1556069_s_at    | hypoxia inducible factor 3, alpha subunit                                                                                                                               |
| 211862_x_at     | CASP8 and FADD-like apoptosis regulator                                                                                                                                 |
| 203815_at       | glutathione S-transferase theta 1                                                                                                                                       |
| 203914_x_at     | hydroxyprostaglandin dehydrogenase 15-(NAD)                                                                                                                             |
| 242281_at       | glutamate-ammonia ligase (glutamine synthase)                                                                                                                           |
| 208485_x_at     | CASP8 and FADD-like apoptosis regulator                                                                                                                                 |
| 210563_x_at     | CASP8 and FADD-like apoptosis regulator                                                                                                                                 |
| 237367_x_at     | CASP8 and FADD-like apoptosis regulator                                                                                                                                 |
| 208928_at       | P450 (cytochrome) oxidoreductase                                                                                                                                        |
| 201849_at       | BCL2/adenovirus E1B 19kDa interacting protein 3                                                                                                                         |

[141] Superoxide dismutase 2 (mitochondrial, SOD2) is highly up-regulated (8-12 fold) in samples exhibiting myopathy (TABLE 3). The SOD2 gene encodes an intramitochondrial free radical scavenging enzyme that is the first line of defence against superoxide produced as a by-product of oxidative phosphorylation.

[142] A number of metallothionein (MT) genes are highly up-regulated (10-24 fold) in samples exhibiting myopathy (TABLE 3). The antioxidant function of metallothionein (MT) was first suggested in the early 1980s. *In vitro* studies have revealed that metallothionein reacts directly with reactive oxygen species, including superoxide and hydroxyl radicals and hydrogen peroxide. Both pharmacologic and genetic studies have shown that metallothionein functions in protection against oxidative injury *in vivo*. Kang YJ, *Proc. Soc. Exp. Biol. Med.* 222(3):263-73 (1999).

[143] Glutamine synthetase is up-regulated by two to three fold in samples with myopathy (TABLE 3). Glutamine synthetase, also called glutamate-ammonia ligase (GLUL), has been used as a biomarker for oxidative stress. Liu J *et al.*, *J. Appl. Physiol.* 89(1):21-8 (2000).

[144] CASP8 and FADD-like apoptosis regulator, FOS-like antigen 2, and BCL2-interacting protein 3 (BNIP3) are up-regulated by two to five fold in samples with myopathy (TABLE 3).

[145] Multiple genes involved in the proteasome 26S subunit. The proteasome regulatory particle are up-regulated by two to six fold (TABLE 4).

TABLE 4A  
Proteolysis and ubiquitin proteasome pathways in the three types of muscles

| <u>Probe id</u> | <u>EDL Mean<br/>of samples<br/>with NO<br/>myotoxic-<br/>ity</u> | <u>EDL Fold<br/>change of<br/>samples<br/>with<br/>myotoxic-<br/>ity</u> | <u>Gastrocnem<br/>ius Mean of<br/>samples<br/>with NO<br/>myotoxicity</u> | <u>Gastrocnem<br/>ius Fold<br/>change of<br/>samples<br/>with<br/>myotoxicity</u> | <u>Soleus<br/>Mean of<br/>samples<br/>with NO<br/>myotoxicity</u> | <u>Soleus Fold<br/>change of<br/>samples<br/>with<br/>myotoxicity</u> |
|-----------------|------------------------------------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-------------------------------------------------------------------|-----------------------------------------------------------------------|
| 241763_s_at     | 162                                                              | 13.0                                                                     | 197                                                                       | 29.3                                                                              | 272                                                               | 13.4                                                                  |
| 225801_at       | 54                                                               | 10.7                                                                     | 162                                                                       | 9.1                                                                               | 93                                                                | 7.4                                                                   |
| 225803_at       | 1807                                                             | 6.2                                                                      | 2837                                                                      | 6.8                                                                               | 2366                                                              | 5.0                                                                   |
| 225328_at       | 2338                                                             | 4.4                                                                      | 3359                                                                      | 5.3                                                                               | 2635                                                              | 4.1                                                                   |
| 232729_at       | 173                                                              | 3.8                                                                      | 232                                                                       | 5.1                                                                               | 158                                                               | 5.6                                                                   |
| 225345_s_at     | 293                                                              | 3.4                                                                      | 590                                                                       | 3.1                                                                               | 339                                                               | 3.8                                                                   |
| 236972_at       | 2602                                                             | 3.3                                                                      | 2497                                                                      | 7.8                                                                               | 2719                                                              | 3.8                                                                   |
| 212220_at       | 722                                                              | 3.1                                                                      | 799                                                                       | 6.0                                                                               | 889                                                               | 3.7                                                                   |
| 212219_at       | 1085                                                             | 3.1                                                                      | 1826                                                                      | 3.8                                                                               | 1387                                                              | 3.4                                                                   |
| 212222_at       | 2240                                                             | 3.0                                                                      | 4292                                                                      | 3.1                                                                               | 2936                                                              | 2.6                                                                   |
| 219935_at       | 104                                                              | 2.6                                                                      | 78                                                                        | 2.0                                                                               | 67                                                                | 2.9                                                                   |
| 200820_at       | 557                                                              | 2.5                                                                      | 563                                                                       | 5.0                                                                               | 637                                                               | 2.7                                                                   |
| 201387_s_at     | 226                                                              | 2.5                                                                      | 532                                                                       | 6.2                                                                               | 408                                                               | 2.1                                                                   |
| 202038_at       | 1127                                                             | 2.5                                                                      | 1271                                                                      | 3.0                                                                               | 1134                                                              | 2.6                                                                   |
| 208777_s_at     | 1965                                                             | 2.4                                                                      | 3118                                                                      | 3.5                                                                               | 2750                                                              | 2.3                                                                   |
| 201114_x_at     | 723                                                              | 2.4                                                                      | 1229                                                                      | 2.3                                                                               | 883                                                               | 2.3                                                                   |
| 201945_at       | 301                                                              | 2.4                                                                      | 570                                                                       | 2.4                                                                               | 330                                                               | 3.3                                                                   |
| 202753_at       | 1956                                                             | 2.2                                                                      | 2453                                                                      | 2.7                                                                               | 2465                                                              | 2.3                                                                   |
| 208776_at       | 450                                                              | 2.1                                                                      | 667                                                                       | 2.9                                                                               | 672                                                               | 2.6                                                                   |
| 204845_s_at     | 162                                                              | 0.4                                                                      | 159                                                                       | 0.6                                                                               | 203                                                               | 0.3                                                                   |
| 202966_at       | 225                                                              | 0.1                                                                      | 100                                                                       | 0.3                                                                               | 163                                                               | 0.3                                                                   |

**TABLE 4B**  
Proteolysis and ubiquitin proteasome pathways in the three types of muscles

| <u>Probe id</u> | <u>Description</u>                                                                                          |
|-----------------|-------------------------------------------------------------------------------------------------------------|
| 241763_s_at     | F-box only protein 32                                                                                       |
| 225801_at       | F-box only protein 32                                                                                       |
| 225803_at       | F-box only protein 32                                                                                       |
| 225328_at       | F-box only protein 32                                                                                       |
| 232729_at       | F-box only protein 32                                                                                       |
| 225345_s_at     | F-box only protein 32                                                                                       |
| 236972_at       | ring finger protein 28                                                                                      |
| 212220_at       | proteasome (prosome, macropain) activator subunit 4                                                         |
| 212219_at       | proteasome (prosome, macropain) activator subunit 4                                                         |
| 212222_at       | proteasome (prosome, macropain) activator subunit 4                                                         |
| 219935_at       | a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2) |
| 200820_at       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 8                                                  |
| 201387_s_at     | ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)                                           |
| 202038_at       | ubiquitination factor E4A (UFD2 homolog, yeast)                                                             |
| 208777_s_at     | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11                                                 |
| 201114_x_at     | proteasome (prosome, macropain) subunit, alpha type, 7                                                      |
| 201945_at       | furin (paired basic amino acid cleaving enzyme)                                                             |
| 202753_at       | proteasome regulatory particle subunit p44S10                                                               |
| 208776_at       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11                                                 |
| 204845_s_at     | glutamyl aminopeptidase (aminopeptidase A)                                                                  |
| 202966_at       | calpain 6                                                                                                   |

[146] Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) and ubiquitination factor E4A are up-regulated by two to six fold (TABLE 4). More significantly, RNF28 and FBXO32, which both encode muscle-specific ubiquitin ligases that function to conjugate ubiquitin to protein substrates, are highly induced in samples with myopathy (TABLE 4). These gene changes indicate the activation of proteolysis and ubiquitin proteasome pathways in the muscle samples exhibiting myopathy.

[147] These broad-based gene expression changes in oxidation, apoptosis, and proteolysis suggest extensive protein degradation in the muscle samples with myopathy. Extensive protein degradation has been reported to be a general characteristic of muscle myopathy.

[148] *The trigger for extensive protein degradation--PDK4 induction and a hypothesis of metabolic switching.* We then proceeded to determine the possible trigger of such extensive protein degradation. The observation of PDK4 induction in samples with myopathy provided the first clue (TABLE 5). PDK4 phosphorylates and inactivates pyruvate dehydrogenase complex which catalyzes irreversible decarboxylation of pyruvate to acetyl-CoA. Significant induction of two other genes involved in glucose and fatty acid oxidation, 6-

phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and acetyl-Coenzyme A carboxylase beta, was also observed (TABLE 5). These gene expression changes affecting the regulation of glycolysis and fatty acid oxidation led us to propose a metabolic switching hypothesis for the onset of statin-induced myopathy.

TABLE 5A

Metabolic switching and fatty acid oxidation related gene changes in all three muscle types

| <u>Probe id</u> | <u>EDL Mean of samples with NO myotoxicity</u> | <u>EDL Fold change of samples with myotoxicity</u> | <u>Gastrocnemius Mean of samples with NO myotoxicity</u> | <u>Gastrocnemius Fold change of samples with myotoxicity</u> | <u>Soleus Mean of samples with NO myotoxicity</u> | <u>Soleus Fold change of samples with myotoxicity</u> |
|-----------------|------------------------------------------------|----------------------------------------------------|----------------------------------------------------------|--------------------------------------------------------------|---------------------------------------------------|-------------------------------------------------------|
| 202464_s_at     | 972                                            | 7.9                                                | 3610                                                     | 3.6                                                          | 2098                                              | 2.9                                                   |
| 1552616_a_at    | 284                                            | 2.9                                                | 459                                                      | 6.5                                                          | 306                                               | 5.3                                                   |
| 221928_at       | 364                                            | 2.7                                                | 697                                                      | 2.5                                                          | 516                                               | 2.2                                                   |
| 225207_at       | 628                                            | 2.6                                                | 794                                                      | 2.3                                                          | 687                                               | 3.6                                                   |
| 205960_at       | 3703                                           | 2.5                                                | 5242                                                     | 2.5                                                          | 3808                                              | 2.4                                                   |
| 205364_at       | 357                                            | 0.5                                                | 402                                                      | 0.3                                                          | 390                                               | 0.5                                                   |

TABLE 5B

Metabolic switching and fatty acid oxidation related gene changes in all three muscle types

| <u>Probe id</u> | <u>Description</u>                                    |
|-----------------|-------------------------------------------------------|
| 202464_s_at     | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 |
| 1552616_a_at    | acetyl-Coenzyme A carboxylase beta                    |
| 221928_at       | acetyl-Coenzyme A carboxylase beta                    |
| 225207_at       | pyruvate dehydrogenase kinase, isoenzyme 4            |
| 205960_at       | pyruvate dehydrogenase kinase, isoenzyme 4            |
| 205364_at       | acyl-Coenzyme A oxidase 2, branched chain             |

[149] The first part of the hypothesis of the invention is the following: Statin effects in muscle can rapidly induce PDK4 gene expression, leading to inactivation of pyruvate dehydrogenase. This in turn induces a metabolic switch to limit oxidative fuel to fatty acids. Statin-induced decreased availability of triglycerides and fatty acids forces the muscle to use amino acids from proteins as an energy source. Extensive protein degradation result in damage to the muscle, leading to acute myopathy or rhabdomyolysis. This metabolic switching may also explain the higher blood lactate/pyruvate ratios in patients treated by statins. The scheme is depicted in FIG. 3. The detailed regulation of glucose and fatty acid oxidation by PDK4 and statin's effect on this regulation is depicted in FIG. 4.

[150] Thus, there is a concurrent decreased availability of triglycerides and fatty acids induced by statin. The mechanism for statin alone to induce decreased availability of triglycerides and fatty acids may be through statin-induced activation of PPAR $\alpha$  via inhibition of the Rho-signalling pathway. Martin G *et al.*, *J. Clin. Invest.* 107(11):1423-32 (2001).

[151] *What triggers PDK4 induction then? --links from PDK4 to statin.* We then investigated the link between PDK4 induction and statin exposure. We observed a highly correlated expression pattern linking PDK4 and the transcription factors, forkhead box O1A (rhabdomyosarcoma) and forkhead box O3A. Forkhead box transcription factors have been shown in *C. elegans* to bind directly to the promoter of PDK4 and induce PDK4 gene expression in skeletal muscle during energy deprivation. Furuyama T *et al.*, *Biochem J.* 375(Pt 2):365-71 (2003).

[152] Moreover, the fact that two muscle atrophy-related genes, F-box only protein 32 and ring finger protein 28 are concurrently induced with PDK4 at gene expression level in the samples with myopathy. F-box only protein 32 and ring finger protein 28 have recently been reported to be down-stream genes regulated by forkhead box transcription factors through IGF-1/PI3K/Akt. Stitt TN *et al.*, *Mol. Cell.* 14(3):395-403 (2004).

[153] Furthermore, many small GTPase, Rho/Ras/Rab related genes, show significant changes in the samples with myopathy (TABLE 7, below). These three clues in conjunction with the vast amount of knowledge in the literature on these pathways led us to propose the following hypothesis of the invention of how statin leads to myopathy.

[154] Very briefly, statin, by inhibiting its target HMGCR, decreases availability of geranylgeranyl pyrophosphate, which in turn leads to decreased activity of small GTPase, and finally leads to PDK4 induction through AKT and forkhead transcription factors. See, FIG. 5.

[155] *Metabolic switching and fatty acid oxidation related gene expression changes.* PDK4 (pyruvate dehydrogenase kinase 4) gene expression is up-regulated by two to three fold in samples with myopathy (TABLE 5). PDK4 phosphorylates and inactivates pyruvate dehydrogenase complex which catalyzes irreversible decarboxylation of pyruvate to acetyl-CoA. Inactivation of the complex limits oxidation of glucose and three-carbon compounds to maintain blood glucose levels and promotes fatty acid oxidation. This is an adaptive mechanism for conditions of starvation. PDK4 is the most highly expressed isoform of PDKs in skeletal muscle.

[156] PFKFB3 (6-phosphofructo-2-kinase) is up-regulated by three to eight fold in samples with myopathy (TABLE 5). PFKFB3 regulates the steady-state concentration of fructose-2,6-bisphosphate, a potent activator of a key regulatory enzyme of glycolysis, phosphofructokinase.

[157] Acetyl-Coenzyme A carboxylase beta (ACACB) is up-regulated by two to six fold in samples with myopathy (TABLE 5). In muscle tissue, ACC-beta is thought to control fatty acid oxidation by means of the ability of malonyl-CoA to inhibit carnitine-palmitoyl-CoA transferase I, the rate-limiting step in fatty acid uptake and oxidation by mitochondria.

[158] *Muscle growth, differentiation, and degradation related gene expression changes.* Forkhead box O1A and forkhead box O3A are up-regulated by two to three fold in samples with myopathy (TABLE 6). Forkhead box transcription factors have been shown in *C. elegans* to bind directly to the promoter of PDK4 and induces PDK4's gene expression in skeletal muscle during energy deprivation. Furuyama T *et al.*, *Biochem. J.* 375(Pt 2):365-71 (2003). Forkhead box transcription factors have recently been shown to be inhibited by the IGF-1/PI3K/AKT pathway to prevent the expression of muscle atrophy-induced ubiquitin ligases. Stitt TN *et al.*, *Mol. Cell.* 14(3):395-403 (2004).

-46-

**TABLE 6A**  
Muscle growth differentiation and degradation genes in all three muscle types

| <u>Probe id</u> | <u>EDL Mean</u><br><u>of samples</u><br><u>with NO</u><br><u>myotoxic-</u><br><u>ity</u> | <u>EDL Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxic-</u><br><u>ity</u> | <u>Gastrocnem</u><br><u>ius Mean of</u><br><u>samples</u><br><u>with NO</u><br><u>myotoxicity</u> | <u>Gastrocnem</u><br><u>ius Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxicity</u> | <u>Soleus</u><br><u>Mean of</u><br><u>samples</u><br><u>with NO</u><br><u>myotoxicity</u> | <u>Soleus</u><br><u>Fold change</u><br><u>of samples</u><br><u>with</u><br><u>myotoxicity</u> |
|-----------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| 241763_s_at     | 162                                                                                      | 13.0                                                                                                   | 197                                                                                               | 29.3                                                                                                            | 272                                                                                       | 13.4                                                                                          |
| 225801_at       | 54                                                                                       | 10.7                                                                                                   | 162                                                                                               | 9.1                                                                                                             | 93                                                                                        | 7.4                                                                                           |
| 225803_at       | 1807                                                                                     | 6.2                                                                                                    | 2837                                                                                              | 6.8                                                                                                             | 2366                                                                                      | 5.0                                                                                           |
| 225328_at       | 2338                                                                                     | 4.4                                                                                                    | 3359                                                                                              | 5.3                                                                                                             | 2635                                                                                      | 4.1                                                                                           |
| 232729_at       | 173                                                                                      | 3.8                                                                                                    | 232                                                                                               | 5.1                                                                                                             | 158                                                                                       | 5.6                                                                                           |
| 225345_s_at     | 293                                                                                      | 3.4                                                                                                    | 590                                                                                               | 3.1                                                                                                             | 339                                                                                       | 3.8                                                                                           |
| 236972_at       | 2602                                                                                     | 3.3                                                                                                    | 2497                                                                                              | 7.8                                                                                                             | 2719                                                                                      | 3.8                                                                                           |
| 202723_s_at     | 663                                                                                      | 2.8                                                                                                    | 809                                                                                               | 3.3                                                                                                             | 888                                                                                       | 2.2                                                                                           |
| 210655_s_at     | 393                                                                                      | 2.7                                                                                                    | 497                                                                                               | 1.9                                                                                                             | 460                                                                                       | 2.0                                                                                           |
| 235261_at       | 867                                                                                      | 0.4                                                                                                    | 1346                                                                                              | 0.5                                                                                                             | 1159                                                                                      | 0.5                                                                                           |
| 204179_at       | 5424                                                                                     | 0.3                                                                                                    | 9585                                                                                              | 0.3                                                                                                             | 6052                                                                                      | 0.3                                                                                           |
| 1553071_a_at    | 364                                                                                      | 0.3                                                                                                    | 286                                                                                               | 0.3                                                                                                             | 271                                                                                       | 0.2                                                                                           |
| 207145_at       | 249                                                                                      | 0.2                                                                                                    | 264                                                                                               | 0.0                                                                                                             | 286                                                                                       | 0.2                                                                                           |
| 206657_s_at     | 425                                                                                      | 0.2                                                                                                    | 624                                                                                               | 1.3                                                                                                             | 866                                                                                       | 0.5                                                                                           |
| 213955_at       | 773                                                                                      | 0.1                                                                                                    | 519                                                                                               | 0.0                                                                                                             | 442                                                                                       | 0.2                                                                                           |

**TABLE 6B**  
Muscle growth differentiation and degradation genes in all three muscle types

| <u>Probe id</u> | <u>Description</u>                  |
|-----------------|-------------------------------------|
| 241763_s_at     | F-box only protein 32               |
| 225801_at       | F-box only protein 32               |
| 225803_at       | F-box only protein 32               |
| 225328_at       | F-box only protein 32               |
| 232729_at       | F-box only protein 32               |
| 225345_s_at     | F-box only protein 32               |
| 236972_at       | ring finger protein 28              |
| 202723_s_at     | forkhead box O1A (rhabdomyosarcoma) |
| 210655_s_at     | forkhead box O3A                    |
| 235261_at       | cardiomyopathy associated 4         |
| 204179_at       | myoglobin                           |
| 1553071_a_at    | myozenin 3                          |
| 207145_at       | growth differentiation factor 8     |
| 206657_s_at     | myogenic factor 3                   |
| 213955_at       | myozenin 3                          |

[159] F-box only protein 32 (FBXO32) is up-regulated by over thirteen fold in samples with myopathy (TABLE 6). More significantly, six different probe sets representing different regions of the gene on the Affymetrix chip showed highly similar pattern of up-regulation (TABLE 6). This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of the ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which

function in phosphorylation-dependent ubiquitination. This protein is highly expressed during muscle atrophy, whereas mice deficient in this gene were found to be resistant to atrophy.

[160] Ring finger protein 28 (RNF28) is up-regulated by three to eight fold in samples with myopathy (TABLE 6). This gene encodes a member of the RING zinc finger protein family found in striated muscle and iris. The product of this gene is localized to the Z-line and M-line lattices of myofibrils, where titin's N-terminal and C-terminal regions respectively bind to the sarcomere. RNF28 has been shown to be a ubiquitin ligase and that it is expressed selectively in cardiac and skeletal muscle. RNF28 and FBXO32, both encode ubiquitin ligases, which function to conjugate ubiquitin to protein substrates, have been shown to be biomarkers of atrophy.

[161] *Small GTPase Ras/Rho/Rab related gene expression changes and the IGF-1/PI3K/Akt signalling pathway.* The inhibition of HMG-CoA reductase by statins leads to decreased synthesis of cholesterol and associated precursors, which are isoprenoid products of mevalonate. These isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), provide lipophilic anchors that are essential for both membrane attachment and biological activity of small GTP-binding proteins from the Ras/Rho/Rab family.

[162] Protein kinase Akt is an important regulator of various cellular processes, including cell metabolism, proliferation, and apoptosis. It has been reported that statins can activate Akt. Kureishi Y *et al.*, *Nat. Med.* 6(9):1004-10 (2000). Furthermore, inhibitors of PI3 kinase, such as wortmannin, block the effects of statins on Akt activation. *Id.*

[163] Recently, it has been shown that the IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO (forkhead) transcription factors. Stitt TN *et al.*, *Mol. Cell.* 14(3):395-403 (2004).

-48-

TABLE 7A  
Rho/Ras/Rab in three types of skeletal muscles

| <u>Probe id</u> | <u>EDL</u><br><u>Mean</u><br><u>of</u><br><u>sampl</u><br><u>es</u><br><u>with</u><br><u>NO</u><br><u>myoto</u><br><u>xic-ity</u> | <u>EDL</u> <u>Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxic-</u><br><u>ity</u> | <u>Gastrocnemius</u><br><u>Mean of</u><br><u>samples with</u><br><u>NO</u><br><u>myotoxicity</u> | <u>Gastrocnemius</u><br><u>Fold change of</u><br><u>samples with</u><br><u>myotoxicity</u> | <u>Soleus</u><br><u>Mean of</u><br><u>samples</u><br><u>with NO</u><br><u>myotoxicity</u> | <u>Soleus</u> <u>Fold</u><br><u>change of</u><br><u>samples</u><br><u>with</u><br><u>myotoxicity</u> |
|-----------------|-----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| 227481_at       | 123                                                                                                                               | 3.7                                                                                                           | 124                                                                                              | 3.3                                                                                        | 168                                                                                       | 2.6                                                                                                  |
| 228003_at       | 210                                                                                                                               | 2.5                                                                                                           | 225                                                                                              | 2.1                                                                                        | 239                                                                                       | 2.2                                                                                                  |
| 206530_at       | 398                                                                                                                               | 2.3                                                                                                           | 502                                                                                              | 2.2                                                                                        | 294                                                                                       | 3.0                                                                                                  |
| 217762_s_at     | 185                                                                                                                               | 2.3                                                                                                           | 38                                                                                               | 2.3                                                                                        | 167                                                                                       | 2.1                                                                                                  |
| 203885_at       | 468                                                                                                                               | 2.0                                                                                                           | 502                                                                                              | 1.2                                                                                        | 430                                                                                       | 2.7                                                                                                  |
| 1552731_at      | 1341                                                                                                                              | 0.5                                                                                                           | 1653                                                                                             | 0.4                                                                                        | 1350                                                                                      | 0.4                                                                                                  |
| 212651_at       | 248                                                                                                                               | 0.5                                                                                                           | 321                                                                                              | 0.2                                                                                        | 328                                                                                       | 0.2                                                                                                  |
| 226507_at       | 820                                                                                                                               | 0.4                                                                                                           | 1022                                                                                             | 0.4                                                                                        | 1085                                                                                      | 0.5                                                                                                  |
| 207455_at       | 112                                                                                                                               | 0.4                                                                                                           | 78                                                                                               | 0.8                                                                                        | 111                                                                                       | 0.3                                                                                                  |
| 225171_at       | 405                                                                                                                               | 0.4                                                                                                           | 346                                                                                              | 0.3                                                                                        | 306                                                                                       | 0.4                                                                                                  |
| 225274_at       | 159                                                                                                                               | 0.4                                                                                                           | 188                                                                                              | 0.7                                                                                        | 158                                                                                       | 0.5                                                                                                  |
| 202241_at       | 1083                                                                                                                              | 0.2                                                                                                           | 1038                                                                                             | 0.4                                                                                        | 1068                                                                                      | 0.3                                                                                                  |

TABLE 7B  
Rho/Ras/Rab in three types of skeletal muscles

| <u>Probe id</u> | <u>Description</u>                                              |
|-----------------|-----------------------------------------------------------------|
| 227481_at       | membrane associated guanylate kinase interacting protein-like 1 |
| 228003_at       | RAB30, member RAS oncogene family                               |
| 206530_at       | RAB30, member RAS oncogene family                               |
| 217762_s_at     | RAB31, member RAS oncogene family                               |
| 203885_at       | RAB21, member RAS oncogene family                               |
| 1552731_at      | striated muscle activator of Rho-dependent signalling           |
| 212651_at       | Rho-related BTB domain containing 1                             |
| 226507_at       | p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)        |
| 207455_at       | purinergic receptor P2Y, G-protein coupled, 1                   |
| 225171_at       | Rho GTPase activating protein 18                                |
| 225274_at       | prenylcysteine oxidase 1                                        |
| 202241_at       | phosphoprotein regulated by mitogenic pathways                  |

[164] Multiple genes involved in the Ras/Rho/Rab signalling pathways are changed by more than two fold in the muscle samples with myopathy (TABLE 7). These gene expression changes may reflect the impact of statin on these pathways.

[165] *IGFBP (insulin-like growth factor binding protein) and IGF signalling-related gene expression changes.* Genes encoding IGFBP 2, 3, and 5 show significant changes in samples with myopathy. Additionally, insulin receptor substrate 2 and leptin receptor are up-regulated by two to three fold in samples with myopathy (TABLE 8). These gene changes may reflect the impact of statin on small GTPase proteins and the IGF-1/PI3K/Akt signalling pathway.

TABLE 8A

IGFBP and IGF signalling-related gene expression changes in the three types of muscle

| <u>Probe id</u> | <u>EDL Mean of samples with NO myotoxicity</u> | <u>EDL Fold change of samples with myotoxicity</u> | <u>Gastrocnemius Mean of samples with NO myotoxicity</u> | <u>Gastrocnemius Fold change of samples with myotoxicity</u> | <u>Soleus Mean of samples with NO myotoxicity</u> | <u>Soleus Fold change of samples with myotoxicity</u> |
|-----------------|------------------------------------------------|----------------------------------------------------|----------------------------------------------------------|--------------------------------------------------------------|---------------------------------------------------|-------------------------------------------------------|
| 1555997_s_at    | 289                                            | 0.4                                                | 225                                                      | 0.3                                                          | 465                                               | 0.3                                                   |
| 202718_at       | 179                                            | 3.5                                                | 570                                                      | 2.2                                                          | 319                                               | 2.2                                                   |
| 209185_s_at     | 2166                                           | 2.0                                                | 2161                                                     | 1.8                                                          | 2136                                              | 2.3                                                   |
| 210095_s_at     | 553                                            | 3.0                                                | 390                                                      | 1.8                                                          | 373                                               | 2.6                                                   |
| 211355_x_at     | 111                                            | 2.5                                                | 58                                                       | 3.8                                                          | 76                                                | 2.0                                                   |
| 211959_at       | 2918                                           | 0.4                                                | 3881                                                     | 0.3                                                          | 3430                                              | 0.4                                                   |

TABLE 8B

IGFBP and IGF signalling-related gene expression changes in the three types of muscle

| <u>Probe id</u> | <u>Description</u>                                  |
|-----------------|-----------------------------------------------------|
| 1555997_s_at    | insulin-like growth factor binding protein 5        |
| 202718_at       | insulin-like growth factor binding protein 2, 36kDa |
| 209185_s_at     | insulin receptor substrate 2                        |
| 210095_s_at     | insulin-like growth factor binding protein 3        |
| 211355_x_at     | leptin receptor                                     |
| 211959_at       | insulin-like growth factor binding protein 5        |

[166] *Discussion.* The underlying mechanism for the difference in inter-statin and inter-individual tendency of developing statin-induced myopathy is very likely to be multifactorial. The major contributing factors may include: (1) Intrinsic properties of the statin in terms of its lipophilicity and its affinity to hepatic and muscular transporters. (2) Individual genetic variations in the relevant pathway genes modulating the statin effect. Such pathway genes may include the hepatic and muscular transporter genes, the cholesterol and fatty acid synthesis pathway genes, PDK/PDC glucose and fatty acid oxidation regulation pathway genes, the small GTPase Rho/Ras pathway genes, and the forkhead transcription factors, *etc.* (3) Individual non-genetic variations such as co-medication with fibrates or other types of drug or food that may affect the PK and PD of statin.

[167] In terms of the transporters, inter-individually difference may exist affecting the affinity and/or the activity of transporters, such as OATP-C and OATP-B. These two factors combined may contribute significantly to the difference in intra-muscular concentration of a statin in different individuals. Other factors, such as combination treatment with fibrate class of drugs or heavy exercise may simply change the equilibrium of the pathway of the invention to make the muscle cells more susceptible for developing myopathy at certain times. This provides the foundation for a powerful diagnostic tool for individual susceptibility for statin-induced myopathy and an important tool for differentiating the current statins on the market.

[168] A combination of fibrates and statins can lead more often to rhabdomyolysis. Synergistic induction of PDK4 by fibrates and statins have been reported by Motojima K & Seto K, *Biol. Pharm. Bull.* 26(7):954-8 (2003). The time course of PDK4 induction by fibrate is rapid and transient, whereas the effect of fibrate to reduce the serum triglycerides and fatty acids are chronic. So, for the combination treatment with fibrates and statins, the duration of the conditions to induce acute rhabdomyolysis may be limited to the early stage. Therefore, it may be possible to avoid fibrate and statin-induced rhabdomyolysis by improving the time schedule of medication and the doses of the drugs.

#### EQUIVALENTS

[169] The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present invention is to be limited only by the terms of the appended claims along with the full scope of equivalents to which such claims are entitled.

## CLAIMS

We claim:

1. The use of statin in the manufacture of a medicament for the treatment of a cardiovascular disorder in a selected patient population, wherein the patient population is selected on the basis of the gene expression of biomarkers of statin-induced muscle toxicity by the patients following administration of statin to the patients.
2. The use of claim 1, wherein the biomarker genes are selected from the group consisting of genes involved in oxidation, apoptosis, ubiquitin-dependent protein catabolism and proteolysis.
3. The use of claim 1, wherein the biomarker genes are selected from the group consisting of genes involved the regulation of glycolysis and fatty acid oxidation.
4. The use of claim 3, wherein the biomarker genes are selected from the group consisting of PDK4, 6-phosphofructo-2-kinase, and acetyl-Coenzyme A carboxylase beta.
5. The use of claim 1, wherein the biomarker gene is PDK4.
6. The use of claim 1, wherein the statin is selected from the group consisting of pitavastatin, atorvastatin, rosuvastatin, fluvastatin, lovastatin, simvastatin and pravastatin.
7. The use of claim 1, wherein the gene expression pattern is obtained in a skeletal muscle sample from the patient.
8. The use of claim 7, wherein the skeletal muscle sample from the patient is selected from the group consisting of soleus muscle, gastrocnemius muscle and extensor digitorum lateralis (EDL) muscle.

9. A method for treating cardiovascular disease in a subject, comprising the steps of:
  - (a) administering a statin to the subject;
  - (b) determining the gene expression pattern of the subject; and
  - (c) either:
    - (i) continuing with the statin therapy if the gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates a lack of muscle toxicity, or
    - (ii) stopping or reducing the statin therapy if the gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates muscle toxicity.
10. A method for diagnosing a propensity for muscle toxicity in a subject, comprising the steps of:
  - (a) administering a statin to the subject;
  - (b) determining the gene expression pattern of the subject; and
  - (c) determining whether the gene expression of biomarkers of statin-induced muscle toxicity by the subject indicates a propensity for muscle toxicity.
11. The method of claim 10, further comprising the step of differentiating among more than one statin for the propensity for muscle toxicity in the individual.
12. The method of claim 11, wherein the statin is selected from the group consisting of pitavastatin, atorvastatin, rosuvastatin, fluvastatin, lovastatin, simvastatin and pravastatin.

13. A method for diagnosing a propensity for muscle toxicity in a subject, comprising the steps of:
  - (a) determining for the two copies of a transporter gene in the subject, the polymorphic identity of a transporter genes; and
  - (b) identifying, based upon the polymorphic identity of a transporter genes, whether the subject has a propensity for statin-induced muscle toxicity.
14. The method of claim 13, wherein the transporter gene is selected from the group consisting of OATP-C and OATP-B.
15. A method for treating cardiovascular disease with reduced fibrate and statin-induced rhabdomyolysis, by improving the time schedule of medication and the doses of the drugs.

1/5

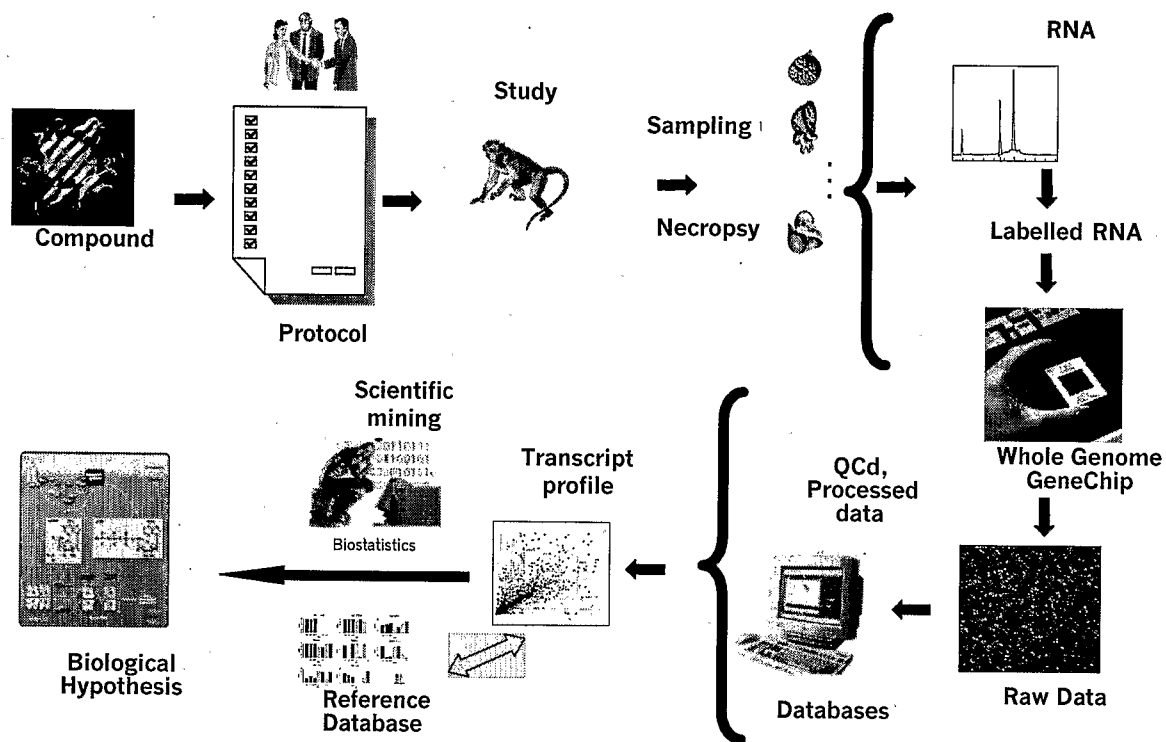


**FIG. 1**  
**THE WORKFLOW OF VELOCE GENOMICS STUDIES**

2/5

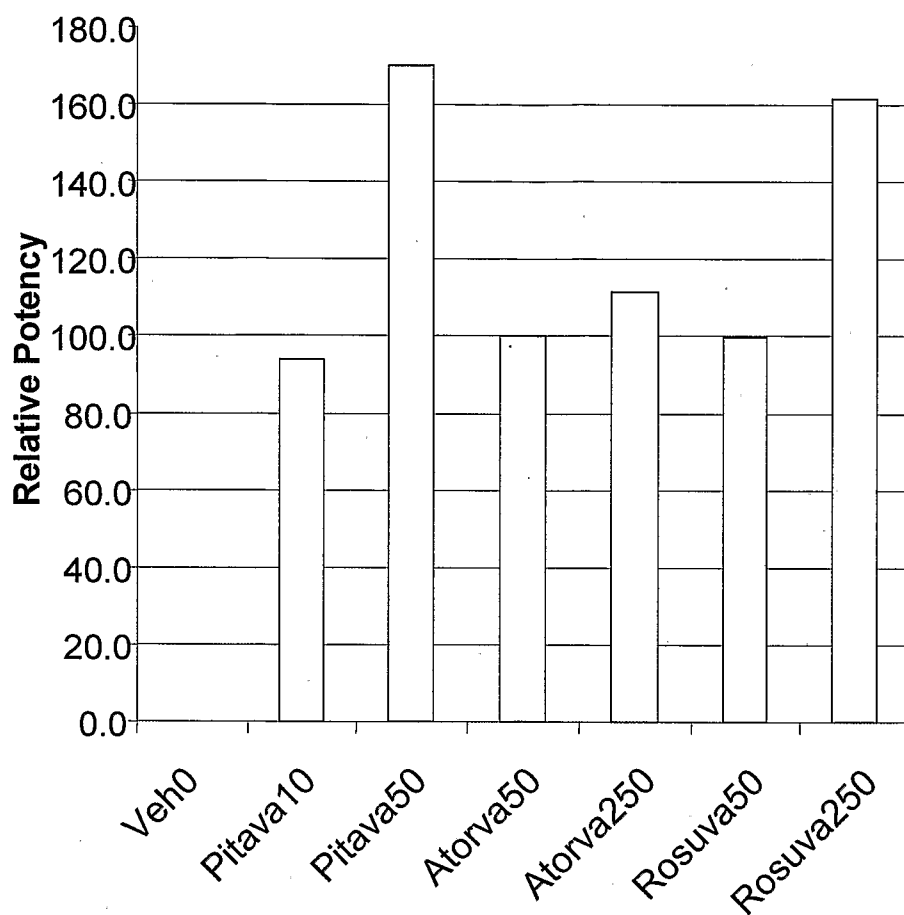


**FIG. 2**  
**RELATIVE POTENCY RANKING OF STATINS BASED**  
**ON PATHWAY GENES**

3/5

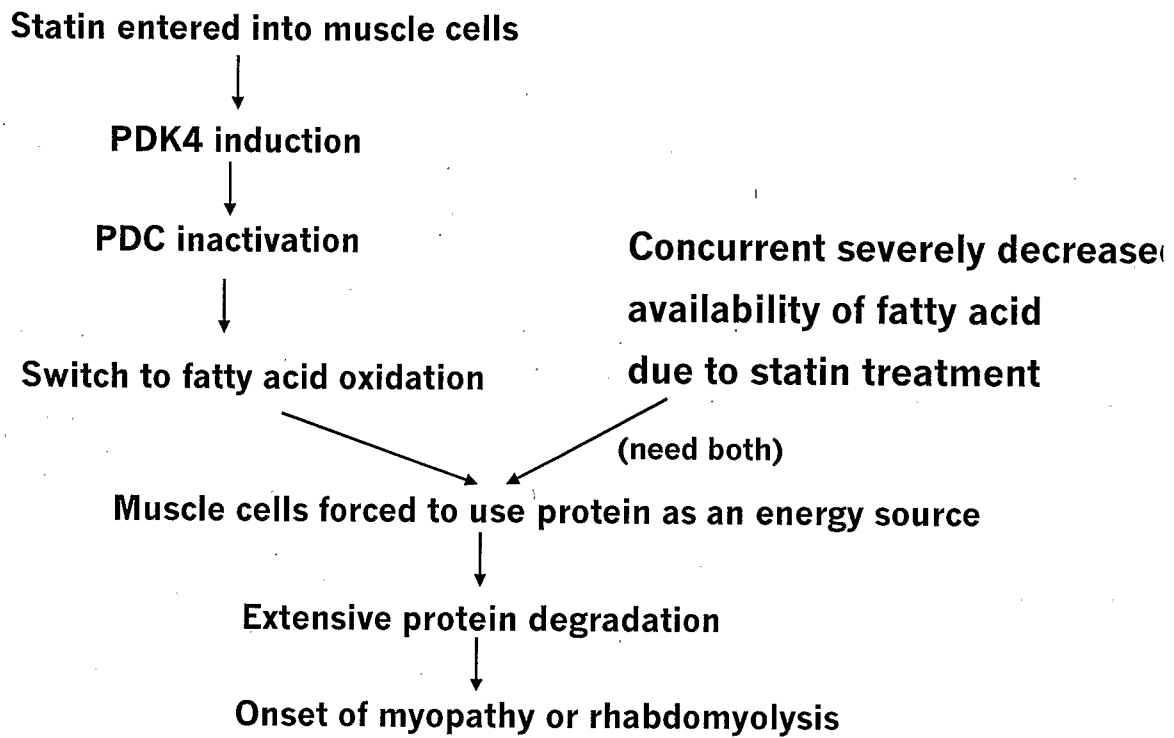


**FIG. 3**  
**A METABOLIC SWITCHING HYPOTHESIS —**  
**THE FIRST PART OF THE FULL HYPOTHESIS ON**  
**STATIN INDUCED MYOPATHY**

4/5

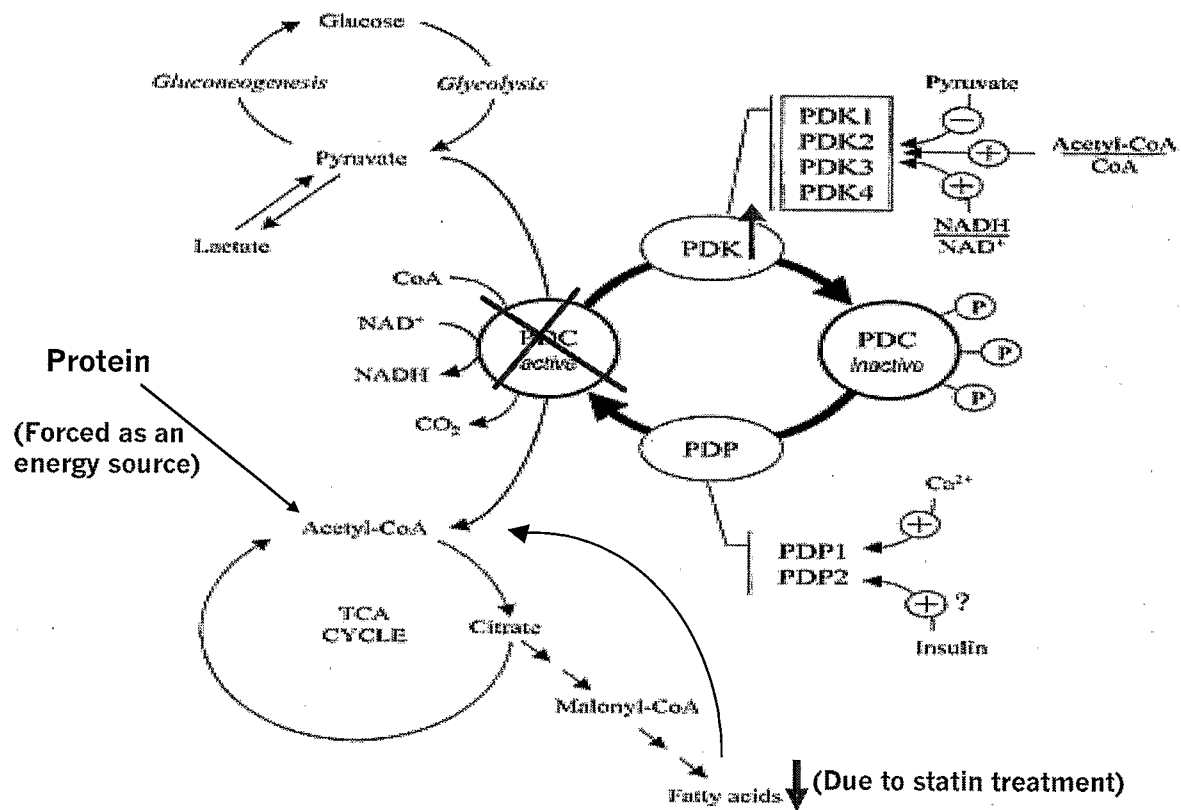


**FIG. 4**  
**THE DETAILED PATHWAY AND IMPACT OF PDK4**  
**INDUCTION**

5/5

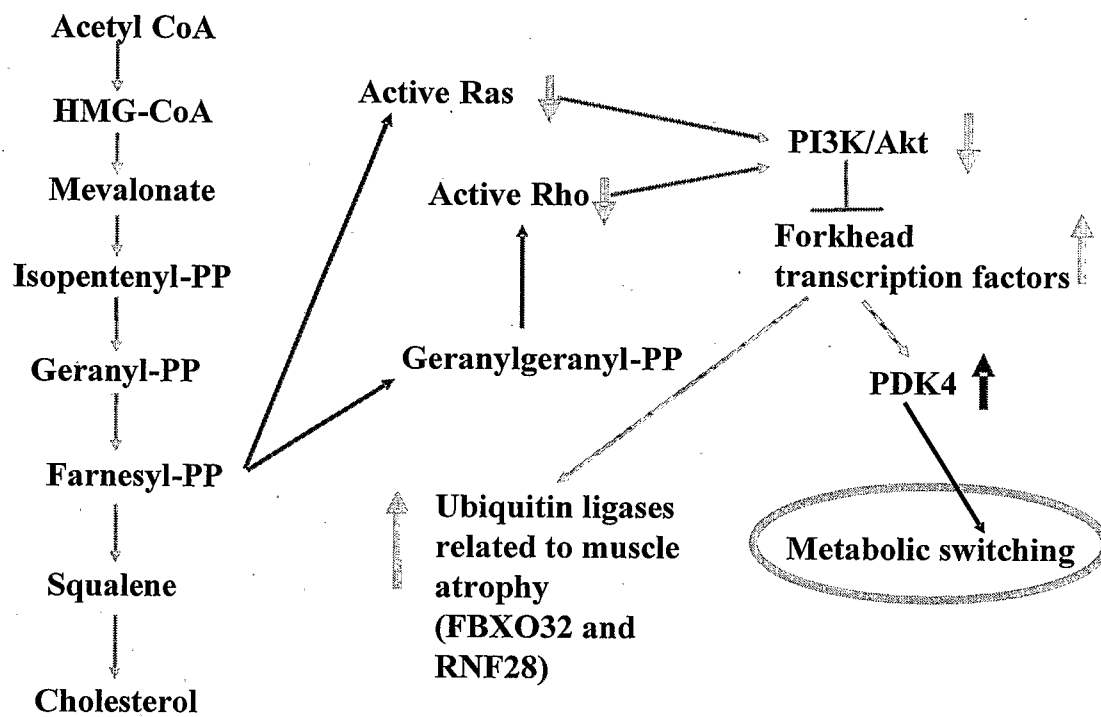


**FIG. 5**  
**THE FULL HYPOTHESIS ON STATIN-INDUCED MYOPATHY**