Abstract: The present invention relates to methods for the diagnosis of functional disorders in humans. A method of the invention, in certain embodiments, comprises the detection of one or more polymorphisms in mitochondrial DNA of a human. The current invention further provides kits for use in a method of the invention.
DIAGNOSTIC METHODS AND KITS FOR FUNCTIONAL DISORDERS

1.0 FIELD OF THE INVENTION

The invention relates to methods and kits for the diagnosis of functional disorders.

2.0 BACKGROUND

Functional disorders are various conditions that affect the well-being of a human. Examples of functional disorders are chronic fatigue syndrome (CFS), migraine, irritable bowel syndrome (IBS), depression, fibromyalgia, and complex regional pain syndrome. Symptoms of functional disorders commonly relate to nervous function (especially function of the autonomic nervous system), muscle function and/or gastrointestinal function.

Functional disorders are believed to be common, affecting the well-being and quality of life of many and thereby also having a significant economical impact. In order to alleviate the impact of functional disorders, it is necessary to diagnose those disorders and to take remedial measures. A challenge in the diagnosis of functional disorders is that a physiological or anatomical cause for functional disorders cannot be identified. Moreover, the symptoms observed in patients suffering from functional disorders are also seen in patients not suffering from functional disorders. Rather, symptoms associated with functional disorders may result from other afflictions, which may be less severe or more severe, and which may be of a physiological nature and/or a psychological nature.

Functional disorders were found to track patterns of mitochondrial lineage. Mitochondria have their own DNA in the form of a plasmid of 16569 nucleotides that is passed on through maternal inheritance. Symptoms of functional disorders are typically found in family members who are direct descendants from a maternal donor with those symptoms.

Methods and kits (reagents) to diagnose functional disorders would be highly desirable. The current invention provides such methods and kits.
3.0 SUMMARY OF THE INVENTION

The current invention relates to methods for the diagnosis of functional disorders. A method of the current invention, in certain embodiments, comprises the detection of a polymorphism in mitochondrial DNA of a human. In certain embodiments, a polymorphism detected with a method of the invention is an indicator that the carrier of the polymorphism is more likely to suffer from functional disorders than an individual who is not a carrier of the polymorphism.

In certain embodiments, a method of the invention detects a polymorphism at one, two, three, four, five or more nucleotides of the human mitochondrial genome. In certain embodiments, a method of the invention detects a polymorphism at one, two, three, four, five or more nucleotides numbers 239, 2259, 3010, 4727, 4745, 7337, 9380, 13326, 13680, 14831, 14872, and/or 16519, of the human mitochondrial genome. In certain embodiments, a method of the invention detects a defined polymorphism at one, two, three, four, five or more nucleotides by detecting the presence of a defined nucleotide. In certain preferred embodiments, a method of the invention detects the presence or absence of one, two, three, four, five or more polymorphisms of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and/or 16519C (each being the number of the nucleotide in the human mitochondrial genome followed by the nucleotide that is detected if the defined polymorphism is present).

In certain other embodiments, the absence of one or more polymorphisms of the current invention is an indicator that the individual who is not a carrier of such one or more polymorphisms is more likely not to suffer from functional disorders than is an individual who carries one or more of these polymorphisms.

In certain embodiments, a method of the invention detects the absence of a polymorphism at one, two, three, four, five or more nucleotides of the human mitochondrial genome. In certain embodiments, a method of the invention detects the absence of a polymorphism at one, two, three, four, five or more of nucleotides numbers 239, 2259, 3010, 4727, 4745, 7337, 9380, 13326, 13680, 14831, 14872, and/or 16519, of the human mitochondrial genome. In certain preferred embodiments, a method of the invention detects the absence of one, two, three, four, five or more of the defined polymorphisms of 239C,

In certain other embodiments, the invention provides kits for the detection of the presence or absence of a polymorphism for the diagnosis of functional disorders.

4.0 DETAILED DESCRIPTION OF THE INVENTION

The current invention is related to methods and kits for the diagnosis of functional disorders. In certain embodiments, the current invention relates to methods comprising the detection of one or more polymorphism and the determination of a predisposition and/or affliction with functional disorders.

Particular methods and/or compositions described herein are provided for purposes of illustration and not to limit the invention in any way. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims. Where a range of values is provided, each intervening value between the upper and lower limits of that range, to the tenth of the unit of the lower limit, is also specifically disclosed, unless the context clearly dictates otherwise.

Unless expressly defined otherwise, each and every technical, scientific and other term used herein has the same meaning and the same breadth as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. 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 described. All publications mentioned herein are incorporated herein by reference for any purpose and to disclose and describe the methods and/or materials in connection with which the publications are cited.

4.1 METHODS TO DIAGNOSE FUNCTIONAL DISORDERS.

The current invention relates to methods that are useful for the diagnosis of functional disorders. A method of the current invention, in certain embodiments, comprises the detection of a polymorphism in mitochondrial DNA of a human. In certain embodiments, a method of the invention comprises detecting one, two, three, four, five, or more
polymorphisms in the mitochondrial DNA of a human. Background on human mitochondrial DNA is also described in U.S. Patent Application Nos. 20040029133; 20050026167; 20050123913; 20060078881; 20060147925; 20070190534; and in U.S. Patent Nos. 5,670,320; 6,605,433; 6,759,196, all of which are incorporated herein by reference for all purposes, and a method of the current invention may be applied to a mitochondrial DNA described in any of these references.

In certain embodiments, a polymorphism of the invention is an indicator that the carrier of the polymorphism (referred to herein as "carrier") is more likely to suffer from functional disorders than an individual who is not a carrier of the polymorphism (referred to herein as "non-carrier"). In certain embodiments, the presence of one, two, three, four, five, or more polymorphisms of the invention in a human is an indicator that the carrier of the polymorphisms is more likely to suffer from functional disorders than an individual who is not a carrier of the polymorphisms. In certain embodiments, a carrier of a polymorphism of the invention is at least 2x (two times) more likely to suffer from functional disorders than a non-carrier, or at least 3x, or at least 4x, or at least 5x, or at least 6x, or at least 7x, or at least 8x, or at least 9x, or at least 10x more likely. In certain embodiments, a carrier of one, two, three, four, five, or more polymorphisms of the invention is at least 2x (two times) more likely to suffer from functional disorders than a non-carrier of those polymorphism(s), or at least 3x, or at least 4x, or at least 5x, or at least 6x, or at least 7x, or at least 8x, or at least 9x, or at least 10x more likely.

In certain embodiments, the presence of one, two, three, four, five, or more polymorphisms of the invention in a human is an indicator that the carrier of the polymorphisms is more than 50 percent likely to suffer from functional disorders, or more than 60 percent, more than 70 percent, more than 80 percent, more than 90 percent, more than 95 percent, or that the carrier is 100 percent likely to suffer from functional disorders.

In certain other embodiments, the absence of one, two, three, four, five, or more polymorphisms of the current invention is an indicator that the individual who is not a carrier of such one or more polymorphisms is more likely not to suffer from functional disorders than to suffer from functional disorders. In certain other embodiments, the absence of one, two, three, four, five, or more polymorphisms of the current invention is an indicator that the individual who is not a carrier of such one or more polymorphisms is at least 2x (two times) less likely to suffer from functional disorders than a carrier of such one or more
polymorphisms, or at least 3x, or at least 4x, or at least 5x, or at least 6x, or at least 7x, or at least 8x, or at least 9x, or at least 10x less likely.

In certain embodiments, the absence of one, two, three, four, five, or more polymorphisms of the invention in a human is an indicator that the non-carrier of the polymorphisms is more than 50 percent likely not to suffer from functional disorders, or more than 60 percent, more than 70 percent, more than 80 percent, more than 90 percent, more than 95 percent, or that the carrier is 100 percent likely not to suffer from functional disorders.

4.2 FUNCTIONAL DISORDERS

The current invention relates to methods to identify a predisposition for and/or an affliction with functional disorders in a human. Functional disorders are disorders that exhibit no demonstrable structural pathology while a patient suffering from functional disorders exhibits deficient nerve and/or muscle function when compared to a healthy person.

Symptoms observed in functional disorders include gastrointestinal dysmotility, gas, pain, migraine, cyclic vomiting, chronic fatigue, limb pain, constipation, diarrhea, sleep apnea, frequent urination, and/or gastroesophageal reflux. Examples of functional disorders include chronic fatigue syndrome (CFS), migraine, irritable bowel syndrome (IBS), depression, fibromyalgia, complex regional pain syndrome, nonspecific abdominal pain, chronic temporal mandibular joint pain, myalgic encephalitis, chronic pelvic pain, chronic pain syndromes, interstitial urethritis, post-traumatic stress syndrome, gulf war syndrome, and functional tinnitus.

4.3 POLYMORPHISMS IN MITOCHONDRIAL DNA

In certain embodiments, a method of the invention detects a polymorphism at one, two, three, four, five or more nucleotides of the human mitochondrial genome. In certain embodiments, a method of the invention detects a polymorphism at one, two, three, four, five or more of nucleotides numbers 239, 2259, 3010, 4727, 4745, 7337, 9380, 13326, 13680, 14831, 14872, and/or 16519, of the human mitochondrial genome. In certain embodiments, a method of the invention detects a polymorphism at one, two, three, four, five or more of nucleotides by detecting the presence of a defined nucleotide. In certain preferred embodiments, a method of the invention detects the presence or absence of one, two, three, four, five or more defined polymorphisms of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and/or 16519C (each being the number of the nucleotide in the human mitochondrial genome followed by the nucleotide that
is detected if the defined polymorphism is present). In certain preferred embodiments, a method of the invention detects the presence or absence of one or two defined polymorphisms of 3010A, 3010G, 16519T, and/or 16519C.

In certain embodiments, a method of the invention detects the absence of a polymorphism at one, two, three, four, five or more nucleotides of the human mitochondrial genome. In certain embodiments, a method of the invention detects the absence of a polymorphism at one, two, three, four, five or more of nucleotides numbers 239, 2259, 3010, 4727, 4745, 7337, 9380, 13326, 13680, 14831, 14872, and/or 16519, of the human mitochondrial genome. In certain preferred embodiments, a method of the invention detects the absence of one, two, three, four, five or more polymorphisms by assaying for the absence of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and/or 16519C.

4.4 PATIENTS

A method of the invention, in certain embodiments, may be used on an individual who has never experienced symptoms associated with functional disorders and on an individual who has experienced such symptoms. In certain embodiments, a method of the invention may be used on an individual who has experienced symptoms associated with functional disorders on more than one occasion (i.e., who has a history of functional symptomatology). In certain other embodiments, a method of the invention may be used on an adult (at least 18 years old), a child, a male, and/or a female patient. In certain embodiments, a method of the invention may be used on an individual who has been diagnosed with one or more functional disorders, for example, chronic fatigue syndrome (CFS), migraine, irritable bowel syndrome (IBS), depression, fibromyalgia, complex regional pain syndrome, nonspecific abdominal pain, chronic temporal mandibular joint pain, myalgic encephalitis, chronic pelvic pain, chronic pain syndromes, interstitial urethritis, post-traumatic stress syndrome, gulf war syndrome, and/or functional tinnitus.

In certain embodiments, a polymorphism of the invention is detected in mitochondrial DNA of any haplogroup, sub-haplogroup and/or any haplotype. As used in the art and herein, a haplotype is a particular combination of genetic markers, many haplogroups are divided into two or more sub-haplogroups, and haplogroups and sub-haplogroups are groups of haplotypes in association with one another. Examples of known haplogroups are A, B, C, D, E, F, G, H,
HV, I, J, K, L1, L2, L3, M, N, P, Q, R, T, U, V, W, X, Y, and/or Z. Background on haplogroups and haplotypes of mitochondrial DNA is also described in U.S. Patent Application Nos. 20040029133; 20050123913; and in U.S. Patent Nos. 5,670,320; 6,759,196, all of which are incorporated herein by reference for all purposes.

4.5 DETECTION OF POLYMORPHISMS

A polymorphism of invention may be detected using any method known in the art. For example, any method capable of determining the sequence of a polynucleotide or oligonucleotide may be used to identify the nucleotide at the site of the nucleotide number of the polymorphism that is the subject of analysis. A method useful for the detection of a polymorphism of the invention, in certain embodiments, may sequence mitochondrial DNA, or a DNA or RNA copy thereof, at and/or around the site of the polymorphism. In certain other embodiments, a method useful for the detection of a polymorphism of the invention may detect a sequence at and/or around the site of a polymorphism, for example, through restriction enzyme digestion. In certain other embodiments, a method useful for the detection of a polymorphism of the invention may examine properties of a DNA or RNA oligonucleotide or polynucleotide, for example, migration properties on a polyacrylamide gel. In certain other embodiments, a method useful for the detection of a polymorphism of the invention may examine the amplification (in other words, multiplication) of a polynucleotide or oligonucleotide sequence through the polymerase chain reaction or another technique involving an amplification of a polynucleotide or oligonucleotide sequence.

In certain embodiments, a polymorphism of the invention is detected by analyzing mitochondrial DNA from a sample obtained from a human. Examples of such a sample include blood, skin, saliva, cerebrospinal fluid, muscle tissue, nerve tissue, placenta, and any other type of organ, tissue and/or cell. Mitochondrial DNA for analysis of the presence of a polymorphism of the invention may also be obtained, for example, by growing cells in culture and by obtaining mitochondrial DNA from such cells.

Examples of methods known in the art that are useful for detecting a polymorphism in mitochondrial DNA include, but are not limited to, any direct sequencing methodology such as cyclosequencing, as well as any indirect sequencing method, of which examples include fluorescence in-situ hybridization (FISH), Southern blot analysis, single stranded conformation analysis (SSCP), denaturing gradient gel electrophoresis, denaturing high
pressure liquid chromatography (DHPLC), RNAase protection assays, allele-specific oligonucleotides (ASO), dot blot analysis, PCR-SSCP, allele-specific PCR, cleavase fragment length polymorphism (CFLP), temperature modulation heteroduplex chromatography (TMHC), sandwich hybridization methods.

A polymorphism of the invention may also be detected, for example, by differential hybridization techniques using allele-specific oligonucleotides. For example, a polymorphism may be detected on the basis of the higher thermal stability of the perfectly matched probes as compared to the mismatched probes.

In certain embodiments, a hybridization reaction in any of the methods for detecting a polymorphism of the invention may be carried out in any format, for example, a filter-based format, Southern blots, slot blots, "reverse" dot blots, solution hybridization, solid support hybridization, solid support based sandwich hybridization, bead-based hybridization, array-based hybridization, chip-based hybridization, silicon chip-based hybridization, and microtiter well-based hybridization formats.

In certain embodiments, a hybridization reaction in any of the methods for detecting a polymorphism of the invention may be carried out using a solid support of any kind, for example, supports comprising organic and/or inorganic polymers, porous supports, small beads, natural and/or synthetic supports, for example, nitrocellulose, nylon, glass, quartz, diazotized membranes (paper or nylon), silicones, polyformaldehyde, cellulose, and cellulose acetate. Or, for example, plastics such as polyethylene, polypropylene, polystyrene, and the like can be used as solid supports in certain embodiments. Or, for example, paper, ceramics, metals, metalloids, semiconductive materials, cermets or the like can be used as solid supports in certain embodiments. In certain other embodiments, substances that form gels can be used, for example, proteins (e.g., gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides. In certain other embodiments, a hybridization reaction in any of the methods for detecting a polymorphism of the invention may be carried out in the liquid state, such as denaturing high pressure liquid chromatography (DHPLC). In certain other embodiments, electronic chips may be employed to obtain direct or indirect sequence information.

In certain embodiments, an amplification-based assay is used to detect a polymorphism. In such amplification-based assays, mitochondrial polynucleotide sequences act as a template in an amplification reaction (e.g., PCR). In certain other embodiments, a
suitable amplification method may be a ligase chain reaction (LCR), a transcription amplification, or a self-sustained sequence replication.

Background on detecting a polymorphism, including methods and reagents used in those methods, are also described in U.S. Patent Application Nos. 20070254289; 20070277251; 20080015112; and in U.S. Patent Nos. 5,976,798; 6,087,107; 6,268,142; 6,284,466; 6,322,978; 6,355,425; 6,379,671; 6,403,307; 6,485,908; 6,492,115; 6,551,780; 6,689,561; 6,713,258; 6,746,839; 7,033,753; 7,105,299; 7,169,561; 7,273,699; 7,316,903; 7,332,277, all of which are incorporated herein by reference for all purposes, and methods, reagents, equipment and anything else useful for detecting a polymorphism described in these references may be used to detect a polymorphism of the current invention.

4.6 KITS

The current invention also provides kits that are useful for some or all steps of a method of the invention. In certain embodiments, a kit of the invention is useful for some or all steps in the detection of the presence of a polymorphism of the invention. In certain other embodiments, a kit of the invention is useful for some or all steps for in detection of the absence of a polymorphism of the invention.

A kit of the current invention in certain embodiments comprises reagents useful in detecting a polymorphism of the invention. In certain embodiments, a kit of the invention comprises instructions for using the kit in detecting a polymorphism of the invention. In certain embodiments, a kit of the invention comprises instructions for determining whether a person is likely to suffer from functional disorders.

A kit of the current invention in certain embodiments comprises an oligonucleotide complementary to mitochondrial DNA 5’ to a site of a polymorphism (nucleotide number where the polymorphism is located) of the invention, or 3’ to a site of a polymorphism of the invention. In certain embodiments, a kit of the invention comprises an oligonucleotide complementary to mitochondrial DNA around the site of a polymorphism of the invention, for example, so that the last, or second to last, or third to last nucleotide at the 3’ end of the oligonucleotide is at the site of the polymorphism. An oligonucleotide in a kit of the invention may be 4 to 50 nucleotides in length, or 6 to 40 nucleotides, or 8 to 30 nucleotides, or 10 to 24 nucleotides, or 12 to 20 nucleotides.
In certain embodiments, a kit of the invention comprises two oligonucleotides complementary to mitochondrial DNA, where one oligonucleotide is complementary 5' to a site of a polymorphism of the invention and the other oligonucleotide is complementary 3' to the site of the polymorphism. In certain embodiments, a kit of the invention comprises two oligonucleotides complementary to mitochondrial DNA at two sites that are separated by 0 to 2000 nucleotides, or 0 to 1000 nucleotides, or 0 to 500 nucleotides, or 0 to 200 nucleotides, or 50 to 400 nucleotides. In certain embodiments, a kit of the invention comprises two oligonucleotides that are complementary to sequences that are in the same strand of mitochondrial DNA or to sequences that are in opposite strands of mitochondrial DNA. In certain other embodiments, a kit of the invention comprises an array of oligonucleotides, for example, an array of oligonucleotides on a solid support.

In certain other embodiments, an oligonucleotide in a kit of the invention is degenerate (and therefore capable of specifically to more than one sequence). In certain other embodiments, an oligonucleotide of a kit of the invention is labeled (for example, to facilitate detection of the oligonucleotide or a compound or complex comprising the oligonucleotide). An oligonucleotide of a kit of the invention may be labeled, in certain embodiments, with a radiolabel, an enzyme, a fluorescent compound, streptavidin, avidin, biotin, a magnetic moiety, a metal-binding moiety, an antigen, an antibody, and/or an antibody fragment.

In certain other embodiments, a kit of the invention comprises an enzyme capable of catalyzing a step in a diagnostic method for which the kit is useful, for example, a kit may comprise a polymerase, a DNA polymerase, an RNA polymerase, a heat stable polymerase, a PCR polymerase, a ligase, a kinase, a restriction enzyme. In certain embodiments, a kit of the invention comprises a buffer, a buffer useful for an enzyme in the kit, a nucleotide, a triphosphate nucleotide (for example, ATP, CTP, GTP and/or TTP), a tube, an Eppendorf tube, a filter, a filter-paper, or any other component or reagent useful in a method of the current invention.

In certain other embodiments, a kit of the invention comprises instructions for using the kit in the diagnosis of functional disorders.

Background on components, reagents and instructions useful for a kit of the invention is also described in U.S. Patent Application Nos. 20070254289; 20070277251; 200800151 12; and in U.S. Patent Nos. 5,976,798; 6,087,107; 6,268,142; 6,284,466; 6,322,978; 6,355,425; 6,379,671; 6,403,307; 6,485,908; 6,492,115; 6,551,780; 6,689,561; 6,713,258; 6,746,839;
EXAMPLES

Example 1: Two Mitochondrial DNA Polymorphisms Are Associated With Migraine Headache and Cyclic Vomiting Syndrome

1.1 Abstract

Mitochondrial dysfunction is a hypothesized component in the multi-factorial pathogenesis of migraine without aura (MoA "common migraine") and its variant, cyclic vomiting syndrome (CVS). In this study, the entire mitochondrial genome was sequenced in 20 haplogroup-H CVS patients, a subject group studied because of greater genotypic and phenotypic homogeneity. Sequences were compared against haplogroup-H controls. Polymorphisms of interest were tested in 10 additional CVS subjects and in 112 haplogroup-H adults with MoA. The 165 19C>T polymorphism was found to be highly disease associated: 21/30 CVS subjects (70%, odds ratio = 6.2) and 58/12 migraineurs (52%, odds ratio = 3.6) versus 63/231 controls (27%). A second polymorphism, 3010G>A was found to be highly disease associated in those subjects with 16519T: 6/21 CVS subjects (29%, odds ratio 17) and 15/58 migraineurs (26%, odds ratio 15) versus 1/63 controls (1.6%). Our data suggest that these polymorphisms constitute a substantial proportion of the genetic factor in migraine pathogenesis, and strengthen the hypothesis that there is a component of mitochondrial dysfunction in migraine.

1.2 Introduction

Migraine is a very common condition, affecting approximately 15% of adults (1, 2), with high economic costs, especially in terms of lost time from employment and as a frequent cause of health care utilization. Although comprised of many variants, the majority of migraineurs have migraine without aura (MoA, previously known as "common migraine"). Like most other common disorders, the etiology of migraine and its variants is multi-factorial, with many known genetic components, including genes for calcium and sodium channels,
dopamine and insulin receptors, Na+/K+ ATPase pump subunits, and components of mitochondrial energy metabolism (3, 4).

A mitochondrial component to migraine has been postulated. This is supported by the findings in migraine sufferers of lactic acidosis (5, 6), mitochondrial accumulations and cytochrome-c-oxidase negative fibers in skeletal muscle (5), decreased respiratory chain complex activities (5, 7), and reduced in vivo brain phosphocreatine to inorganic phosphate ratio by 31P magnetic resonance spectroscopy (5). In addition, co-enzyme Q10 and riboflavin, a component and a precursor of a component of the mitochondrial respiratory chain, have shown efficacy in migraine prophylaxis in double-blind, placebo-controlled clinical trials (8, 9). The mitochondrial dysfunction hypothesis of migraine was recently reviewed (10).

Mitochondria are cytoplasmic organelles that produce the bulk of the ATP for cellular energy needs. Mitochondrial proteins are encoded on both the nuclear DNA (chromosomes) as well as the 16-kilobase mitochondrial DNA (mtDNA). Thus, sequence variants (polymorphisms) that adversely affect energy metabolism and predispose towards migraine pathogenesis theoretically could be on either or both of those genomes. The cytoplasmic-located mtDNA generally is derived solely from the ova without recombination, and individuals related through women carry an identical mtDNA sequence in the absence of a recent mutation.

Pilot studies have suggested a preferential maternal bias in migraine inheritance (11, 12), suggesting the presence of disease-predisposing mtDNA sequence variants. In support of this, about 20 different mtDNA sequence variants have been associated or possibly associated with migraine (10), especially 3243A>G (13). Most of those sequence variants are located in the "coding regions" that comprise 94% of the mtDNA and that code for subunits of the respiratory chain or the transfer and ribosomal RNA molecules needed to translate those subunits. In one study (14), migraine and its variant cyclic vomiting syndrome were found to be associated with any sequence variation in a 150 base-pair area of the control region believed to regulate mtDNA replication. Furthermore, an entire mtDNA haplogroup (U) was found to predispose towards migraine with occipital stroke (15).

However, migraine is a heterogeneous phenotype in which diagnostic criteria exist but are not always definitive. As a model disorder in which to study migraine genetics, we chose cyclic vomiting syndrome (CVS), a condition that's presence or absence is almost always clear by expert application of diagnostic criteria (16). CVS is a disabling condition characterized by recurrent, distinct episodes of nausea, vomiting and lethargy separated by
asymptomatic intervals (16-18). Most sufferers encounter recurrent identical episodes that often result in frequent school or work absences and multiple hospitalizations for dehydration. CVS is not rare; it was reported in nearly 2% of Western Australian (19) and Scottish (20) school children. CVS is widely believed to be a "migraine-like condition" secondary to a strong family history of migraine headache, as well as frequent prodromic symptoms, headache, abdominal pain, evolution to migraine headache, and a positive response to "anti-migraine" medications in CVS patients (21, 22). mtDNA sequence variation is hypothesized to be a substantial risk factor in the pathogenesis of CVS due to the preferential maternal inheritance of functional disorders, including MoA, and the presence of an energy-depleted pattern on urine organic acid measurements in most cases (17). In addition, lactic acidosis, reduced electron transport chain activities and/or heteroplasmic mtDNA sequence variants have been reported in selected cases (23-25). Most mtDNA sequence variants in CVS patients have been reported in the 1 kb mtDNA control region (14, 26), although 3243A>G and large rearrangements have been reported in the 15.6 kb comprising the coding regions (26-30).

In this study, the entire mtDNA was sequenced in 20 individuals with CVS. Any potential disease-associated sequence variants were assayed in an additional 10 CVS cases and in 112 adults with MoA. In order to minimize background mtDNA sequence variability (noise), all patient and control subjects belonged to mtDNA haplogroup H.

1.3 Subjects and methods

1.3.1 Subjects

The CVS subjects of whom the entire mtDNA was sequenced included 20 individuals from an earlier study (17) who were ascertained randomly throughout North America based upon postal codes from the database of the Cyclic Vomiting Syndrome Association (CVSA). Additional CVS subjects who were only assayed for selected mtDNA polymorphisms included six subjects recruited by the above means, and four subjects recruited as part of another earlier study on CVS with co-morbid neuromuscular disease (24). All subjects were unrelated, met the research definition for CVS (16) as determined by telephone interview, and belong to mtDNA haplogroup H.

The adult migraineur group consisted of 77 hospital-based patients from a headache clinic near Frankfurt and 35 outpatients recruited from an outpatient clinic in Munich, both
from Germany. All subjects were unrelated, met the International Headache Society definition of migraine without aura (31) as determined by a mailed-in questionnaire (32) and belong to mtDNA haplogroup H. A telephone interview was performed in cases with diagnostic uncertainty.

The control group consisted of 195 full-mtDNA sequences from published sources, 213 additional almost-complete mtDNA sequences (missing the control region), and 36 healthy Caucasian children ascertained in Los Angeles who were genotyped for the polymorphisms of interest (Table 1). Only haplogroup H sequences from individuals ascertained as part of a population or control study from Europe or North America were included. To reduce potential bias, we excluded samples ascertained due to any illness or symptoms, from self-selected groups (commercial heritage testing), and from islands with small founding and/or geographically-isolated populations (Iceland and Sardinia).

Informed consent was obtained from each subject or parent, except for de-identified control subjects, and all aspects of the study was approved by the Childrens Hospital Los Angeles Institutional Review Board.

<table>
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<tr>
<th>Subject Population</th>
<th>Number of Subjects</th>
<th>Area Sequenced</th>
<th>Number with 16519T</th>
<th>Number with 3010A</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>American and British</td>
<td>213</td>
<td>Coding regions only</td>
<td>N/A</td>
<td>69</td>
<td>33</td>
</tr>
<tr>
<td>European-American</td>
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<td>24</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
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<td>16519 and 3010 only</td>
<td>11</td>
<td>14</td>
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<td>31</td>
<td>Entire mtDNA</td>
<td>9</td>
<td>14</td>
<td>37</td>
</tr>
</tbody>
</table>

The present study

1.3.2 Methods

DNA was isolated from blood by standard methods or from saliva using a commercially-available kit (Oragene, DNA Genotek Inc., Ottawa, ON, Canada). Haplogroup H was defined in the conventional manner as the presence of a C at position 7028, as listed in published sequence databanks, by cyclosequencing or by PCR/restriction fragment length polymorphism (RFLP; 16519: \textit{Haelll} F GGATGACCCCCCTCAGATA (SEQ ID NO:1), R
CTTATTTAAGGGGAACGTG (SEQ ID NO:2); 3010: Bed F CATGCTAAGACTTCACCA (SEQ ID NO:3), R TCGTTGAACAAACGAACC (SEQ ID NO:4)).

The entire mtDNA was amplified using 28 overlapping primer sets (26, 14; and additional sets available upon request). Cyclosequencing was performed by SeqWright (Houston, TX), Agencourt (Beverly, MA), or Eton (San Diego, CA). Individual sequences were aligned and compared on Sequencher® software (Gene Codes Corp., Ann Arbor, MI) versus our reference sequence. Our reference consists of the most common nucleotide among haplogroup H individuals in our control group for each nucleotide position throughout the mtDNA, and is termed the haplogroup H reference sequence (HhRS). The HhRS is identical to the revised Cambridge Reference Sequence (rCRS) [MITOMAP] (38), which corresponds to the actual sequence of one individual of sub-haplogroup H2, with the exception of 9 changes correcting for rare and uncommon polymorphisms in the rCRS (see Table 2 legend).

Statistics were performed by WinSTAT Statistics for Windows, Kalmia Co. Inc., Cambridge, MA, and by custom-made software.

<table>
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<th>Subject Number</th>
<th>mtDNA Sequences Changes Relatives to the Haplologroup H Reference Sequence (HhRS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>302.1ins, 302.2ins, 989T&gt;A, 3010G&gt;A, 6272A&gt;G, 14869G&gt;A</td>
</tr>
<tr>
<td>5</td>
<td>152T&gt;C, 1438G&gt;A, 8598T&gt;C, 16129G&gt;A, 16311T&gt;C, 16519C&gt;T</td>
</tr>
<tr>
<td>7</td>
<td>980T&gt;C, 3010G&gt;A, 8020G&gt;A, 15394T&gt;C, 16519C&gt;T</td>
</tr>
<tr>
<td>9</td>
<td>477T&gt;C, 3010G&gt;A, 9091A&gt;G, 15734G&gt;A</td>
</tr>
<tr>
<td>11</td>
<td>456C&gt;T, 4336T&gt;C, 10325G&gt;A, 11719G&gt;A, 15833C&gt;T, 16304T&gt;C, 16519C&gt;T</td>
</tr>
<tr>
<td>12</td>
<td>3010G&gt;A, 16519C&gt;T</td>
</tr>
</tbody>
</table>
The HhRS is identical to the revised Cambridge Reference Sequence (rCRS) (38), with the exception of 7 rare polymorphisms (263A>G, 310G>A, 750A>G, 1438A>G, 4769A>G, 8860A>G, 15326A>G) and two variants (309OT and 16519T>C).

1.4 Results

All sequence variants relative to the HhRS in our 20 fully-sequenced CVS subjects are listed in Table 2. Excluding insertions in the ultra-variable 302-315 region, 20 different mtDNA variants were identified in two or more CVS subjects (Table 3), all of which are single-nucleotide polymorphisms (SNPs) listed on MITOMAP (38). Most prominently, one of the SNPs, 165 19OT, was found to be highly associated with CVS versus controls, and another, 3010G>A, was found to be highly associated with CVS in subjects with 165 19OT versus in the controls with 165 19OT (Table 4).

Table 3 mtDNA Single-Nucleotide Polymorphisms (SNPs) Found in 10% or More of the 20 Fully-Sequenced CVS or 195 Fully-Sequenced Control Subjects

<table>
<thead>
<tr>
<th>SNP</th>
<th>mtDNA Gene</th>
<th>Number with this SNP among 20 CVS Subjects</th>
<th>Number with this SNP among 195 Controls</th>
<th>P</th>
<th>CVS Subject Numbers With This SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>73A&gt;G</td>
<td>MT-CR</td>
<td>2</td>
<td>26</td>
<td>NS</td>
<td>15, 18</td>
</tr>
<tr>
<td>152T&gt;C</td>
<td>MT-CR</td>
<td>3</td>
<td>39</td>
<td>NS</td>
<td>2, 5, 10</td>
</tr>
<tr>
<td>239T&gt;C</td>
<td>MT-CR</td>
<td>2</td>
<td>2</td>
<td>NS</td>
<td>13, 17</td>
</tr>
<tr>
<td>456C&gt;T</td>
<td>MT-CR</td>
<td>2</td>
<td>23</td>
<td>NS</td>
<td>11, 14</td>
</tr>
<tr>
<td>477T&gt;C</td>
<td>MT-CR</td>
<td>2</td>
<td>14</td>
<td>NS</td>
<td>9, 16</td>
</tr>
<tr>
<td>1438G&gt;A</td>
<td>MT-RNR1</td>
<td>2</td>
<td>7</td>
<td>NS</td>
<td>5, 8</td>
</tr>
<tr>
<td>2259C&gt;T</td>
<td>MT-RNR2</td>
<td>3</td>
<td>7</td>
<td>NS</td>
<td>2, 4, 10</td>
</tr>
<tr>
<td>3010G&gt;A</td>
<td>MT-RNR2</td>
<td>7</td>
<td>61</td>
<td>NS</td>
<td>1, 3, 7, 9, 12, 16, 18</td>
</tr>
</tbody>
</table>
Referring to the subject numbers in Table 2.

Amino acid change of Ala to Thr, all other SNPs do not change the amino acid sequence.

In addition, in the 302-315 region, 60% of CVS subjects had 8 or 9 Cs (versus the more-common 7 Cs) prior to the T, versus 41% of controls (P = 0.08).

Table 4: Prevalence of 16519T and 3010A in Cyclic Vomiting Syndrome and Migraine without Aura

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Cyclic Vomiting Syndrome</th>
<th>Odds Ratio (95% C.I.)</th>
<th>P vs. Control</th>
<th>Migraine Without Aura</th>
<th>Odds Ratio (95% C.I.)</th>
<th>P vs. Control</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>16519T</td>
<td>21/30(^1) 70%</td>
<td>6.2(^2) (2.7-14)</td>
<td>2 x 10(^{-6})</td>
<td>58/112 52%</td>
<td>3.6(^2) (2.2-5.9)</td>
<td>8 x 10(^{-6})</td>
<td>63/231 27%</td>
</tr>
<tr>
<td>3010A</td>
<td>9/30 30%</td>
<td>N/A</td>
<td>NS</td>
<td>37/112 33%</td>
<td>N/A</td>
<td>NS</td>
<td>143/444 32%</td>
</tr>
<tr>
<td>3010A patients with 16519T</td>
<td>6/21 29%</td>
<td>17(^2) (2.0-156)</td>
<td>8 x 10(^{-4})</td>
<td>15/58 26%</td>
<td>15(^3) (1.9-117)</td>
<td>8 x 10(^{-5})</td>
<td>1/63 1.6%</td>
</tr>
</tbody>
</table>

C.I. = confidence interval.

1. One subject had 16519C/T heteroplasmy (both variants present) as confirmed by both sequencing and RFLP. The genotype assigned was the dominant species, T.

2. Assumes a population prevalence of 2% for CVS (19, 20) and 15% for migraine (1, 2).

3. The odds ratio would be higher, but to avoid dividing by zero we assumed that no controls suffer from migraine.
Excluding 16519C>T and 3010G>A, there was a mean of 5.2 mtDNA SNPs per individual among the fully-sequenced 20 CVS subjects and 4.9 mtDNA SNPs per individual among the 195 fully-sequenced controls (P = NS).

The 165 19C>T polymorphism was found to be highly associated with MoA (Table 4). In addition, among the subset with 165 19T, the 3010G>A polymorphism was found to be highly associated with MoA.

1.5 Discussion

Complex multifactorial conditions, usually influenced by multiple genetic and environmental factors, are the cause of the vast majority of human disease. These conditions are becoming better understood as (nuclear) genetic polymorphisms that confer an increased risk toward disease pathogenesis are rapidly being identified. Although the mitochondrial genome is small, at high copy number it constitutes up to one third of the total cellular mass of DNA and has a very-high polymorphic density (39). Thus, mtDNA sequence variation is likely to affect an individual's risk towards the development of some multifactorial conditions in a manner analogous to nuclear DNA polymorphisms (40). Migraine (including MoA and CVS) may follow such a hypothesis since a genetic component in its pathogenesis, preferential maternal inheritance and mitochondrial dysfunction are all well established. Furthermore, in converse, migraine is very common in patients with maternally inherited mitochondrial dysfunction (41). Establishing disease-associated mtDNA sequence variant(s) in migraine, or in any other condition, indicates that energy metabolism is a factor in disease pathogenesis, since the 37 mtDNA genes are exclusively involved in oxidative phosphorylation (39). In addition, disease-associated mtDNA sequence variation constitutes an important justification for the use of mitochondrial-directed therapies, some of which have demonstrated efficacy by double-blind clinical trials in migraine, and by retrospective studies and anecdotal observation in CVS (25, 42).

In the present study, we demonstrate that the common mtDNA polymorphisms 16519T and 3010A are highly associated with the most common form of migraine and the migraine-variant cyclic vomiting syndrome. Given the lack of significant disability in most MoA cases, we assume that our control group, ascertained for forensic or evolutionary studies, contains about the same proportion of migraineurs as does the general population. With this assumption, the 165 19T polymorphism alone was found to have an odds ratio of 3.6 in MoA and 6.2 for CVS (Table 4). This corresponds to predicted prevalence rates of 28%
and 11% for migraine in individuals with 16519T and 16519C, respectively. Even more striking is the combined effects of the two polymorphisms, which are very rarely seen together in control populations. Although the 3010 polymorphism alone does not appear to confer risk to developing migraine, on a 16519T background, the 3010A polymorphism has an odds ratio of 15 for MoA and 17 for CVS. Thus, the data predict that migraine is present in 74% of individuals with the combined 16519T/3010A genotype, and that these mtDNA polymorphisms are likely acting synergistically.

In addition, our data suggest that there may be other mtDNA polymorphic modifiers on a 16519T background, but the numbers are too small for significance in most cases (Table 3). In one case that did reach statistical significance, the 4727A>G polymorphism was found in 2/20 CVS cases versus in 1/195 fully-sequenced controls, respectively. Furthermore, three CVS subjects with 16519T but without 3010A>G, share six other polymorphisms (2259C>T, 4745A>G, 7337G>A, 13326T>C, 13680C>T and 14872C>T) in common, a combination not present in any of the 195 fully-sequenced controls (P = 7x10^{-4}). Those three subjects are not closely related as their mtDNA sequences vary at other polymorphisms. Additional CVS samples were studied following the completion of the present manuscript (see Table 8 below).

The overall genetic component to migraine pathogenesis is moderate, with first-degree relatives being at about a two-fold increased risk for being affected themselves (43). Thus, 16519T and 3010A likely constitute a substantial proportion of the overall genetic factor in MoA. As we have full mtDNA sequences in 20 of our CVS subjects, in CVS the data do not support that these variants are in linkage disequilibrium with other deleterious mtDNA sequences (Tables 2 and 3). More likely, it is the 16519T and 3010A nucleotides themselves that affect energy metabolism in a manner that predisposes towards the development of MoA and CVS. Furthermore, excluding 16519T and 3010A, all other mtDNA SNPs were found at essentially the same frequency in our CVS subjects and controls. While we certainly cannot state that one or more of these other polymorphisms among our CVS subjects may be disease-predisposing or modifying, it does not appear that any of them are singularly important in a sizable proportion of CVS patients, at least not in haplogroup H.

The 16519T polymorphism is located in the 1-kb non-coding mtDNA control region (often referred to as the "D-loop"), not far from the origin of heavy-strand replication and putative membrane-attachment site (38). This is a very atypical mtDNA nucleotide. In terms of nomenclature, 16519T is one of nine uncommon or rare single nucleotide polymorphisms
that are present in the single individual whose mtDNA was first sequenced and thus comprises the established reference sequence (rCRS) (38). Thus, while 16519C is the ancestral (present in chimpanzees) and the most common nucleotide among humans in general, 16519T is the rCRS nucleotide. While located in an area of the control region with relatively low sequence heterogeneity, 16519T is considered to have one of the highest mutation rates of any mtDNA position and has arisen multiple times in human evolution, including among Americans of West Eurasian (44), East Asian (45) African (46), and Hispanic/Native American (47) origins. Interestingly, 16519T was recently shown to be associated with diabetes and a poorer prognosis in individuals with pancreatic cancer (48). A physiological effect of this polymorphism is also suggested by its complex effects on human exercise physiology (49).

The 3010A polymorphism is located in the 16S-ribosomal RNA gene, whereas it rebuilds a base pair in a stem of the ribosomal A-site (38). Bacterial mutations in this stem confer resistance to certain antibiotics such as chloramphenicol. This nucleotide demonstrates evolutionary conservation in primates, although A is the nucleotide in mice and frogs. 3010A has appeared in human evolution at least 15 times on 10 different mtDNA haplogroups, and may be under positive selection in humans. It defines the sub-haplogroups/clusters of Hl, Jl, U3 (West Eurasian), D4, C (East Asian) and L2a (African) (38). J1 and D4 are overrepresented in centenarians (50), but 3010A by itself has not previously found to be statistically associated with human disease.

The mtDNA haplogroups denote sets of ancient matrilineal ancestry tens of thousands of years old. The West Eurasian haplogroup H is well suited for genetic association studies due to the relative lack of intra-group sequence variability and high prevalence. Among our fully-sequenced control group of 195 European-derived individuals with haplogroup H, the mean number of nucleotide changes throughout the entire mtDNA genome relative to the HhRS is only about 8 nucleotides (data to be published separately), versus several times this number for unrelated individuals of mixed haplogroups. Thus, limiting our study to subjects with haplogroup H substantially decreases background sequence variability and correspondingly increases statistical power. It is also practical as haplogroup H is the most common among European-derived populations, including prevalence rates of about 45% in the native population of Germanic countries, and in about 33% of North Americans of apparent-European ancestry (40). Thus, we chose to sacrifice a larger number of subjects for substantially greater genotypic homogeneity by limiting this study to subjects of haplogroup
Unlike some haplogroups, the 16519 and 3010 nucleotide positions are quite heterogeneous among individuals within haplogroup H. For example, had we chosen to study haplogroups J or T, whereas 3010A and 16519C are nearly universal, respectively, we would have missed the association with either polymorphism. Of course, this reasoning states that we may have missed migraine-associated polymorphisms that are not heterogeneous among haplogroup H individuals, and research in individuals with other haplogroups is needed.

One potential problem with our study is the lack of a German control group. However, the polymorphic frequency of 16519T appears to be highly homogeneous among continental European-derived haplogroup H populations, including 27% from the USA, 28% from Italy, and 29% from Finland (Table 1). Also considering that the migraineurs were ascertained from two large cities in the center of Europe, Frankfurt and Munich, it is quite unlikely that our results are due to a local increased prevalence of 16519T. Our results are also not likely due to systematic errors in our procedures, as most of our subjects’ genotypes were confirmed by both cyclosequencing and RPLP. Furthermore, in our own control group, 31% have 16519T, although the total is limited to 36 haplogroup H subjects. The polymorphic frequency for 3010A is also highly similar among the two larger control groups, 33% in Americans and 32% in mixed Brits/Americans (Table 1). The proportion does vary in our smaller groups, 22% in Italians and 45% in Finns, but these are non-significant differences (P = 0.18) likely due to the small numbers of subjects in those groups (Table 1).

Furthermore, since 3010A is present in only 1/63 controls from diverse locations with 16519T, it is highly unlikely that our findings of 3010A in 6/21 CVS and 15/58 MoA subjects with 16519T is an artifact of local polymorphic differences.

We conclude that the common mtDNA polymorphisms 16519T and 3010A are strongly associated with migraine and its variant cyclic vomiting syndrome among individuals with the common West Eurasian haplogroup H, and likely are disease-predisposing. Although only haplogroup H was studied, 16519T and 3010A are found in individuals with a multitude of haplogroups and within all major races. In fact, among Americans of European origin, 16519T is actually slightly more common among non-haplogroup H individuals (34). The effect of these polymorphisms may or may not be dependent upon the background haplogroup, and further study is needed. Our data strengthen the hypothesis that there is a component of mitochondrial dysfunction in migraine and provide additional rationale towards the use of mitochondrial-directed therapies in this condition.
1.6 References


34. Coble MD, Just RS, O'Callaghan JE, Letmanyi IH, Peterson CT, Irwin JA, Parsons TJ. Single nucleotide polymorphisms over the entire mtDNA genome that increase the power of forensic testing in Caucasians. Int J Legal Med 2004; 118:137-46.


Example 2: Mitochondrial DNA Polymorphisms and Predisposition Towards Depression and Its Co-Morbidities

2.1 Abstract

Data are presented to demonstrate an association between specific mtDNA sequence variants (termed SNPs, pronounced "snips" or single nucleotide polymorphisms) and functional disorders, as well as with functional symptoms in patients with major depressive disorder (MDD) and chronic fatigue syndrome (CFS). We found that the 165 19T mtDNA
SNP is found statistically more frequently in patients with CFS than in individuals found in the general population. Previously (Example 1), we demonstrated that 16519T, and 3010A on a 16519T background, are found statistically more frequently in patients with migraine and cyclic vomiting syndrome (CVS). Thus, together the data suggest that these mtDNA SNPs increase the chance that an individual will develop these functional conditions.

Furthermore, these SNPs significantly modify the chances that patients diagnosed with functional disorders will report a wide range of specific functional symptoms. Among patients with CFS, 3010A statistically increases the chances of reporting headache, muscle pain, muscle weakness, tingling or numbness, fainting or dizziness, and sleep disturbances. Among patients with MDD, 3010A appears to increase the chance of developing migraine. However, in MDD the opposite SNP, 3010G, quadruples the chance that the individual will suffer from a bowel disorder, and appears to increase the risk several fold for learning disabilities as well.

2.2 Methods

The presence of 165 19T versus 165 19C and of 3010A versus 3010G was determined by RFLP or cyclosequencing in the following groups of subjects:

1) DNA samples from 295 American MDD subjects from the GenRED (Genetics of Recurrent Early-onset Depression) study were kindly supplied by Dr. Douglas Levinson (Stanford University) and Dr. James Knowles (University of Southern California). The GenRED study comprises 1100 European-American patients with MDD of onset before age 31, all of whom have a first-degree relative with MDD or bipolar 2 of onset before age 41. Among the MDD samples, 112 were of haplogroup H and were further tested. Clinical data on co-morbid functional symptomatology was correlated with the 16519 and 3010 genotypes (Table 5).

2) DNA samples from 162 individuals that meet the 1994 Centers of Disease Control diagnostic criteria for CFS were kindly supplied by Dr. Jonathan Kerr at St. George's University of London. Among the CFS samples, 58 were of haplogroup H and were further tested. Clinical data was available from between 46 to 49 of the subjects (data on all clinical manifestations were not available on all subjects), almost all from London, and was correlated with the 165 19 and 3010 genotypes (Table 6).

3) DNA samples from 20 subjects with adult-onset cyclic vomiting syndrome (CVS) were ascertained from the clinic of Dr. R. McCallum, Kansas University in Kansas City. Among the CVS samples, 8 were of haplogroup H and were further tested. The data was
combined with that from the 5 adult-onset haplogroup H CVS subjects ascertained from throughout North America from the database of the Cyclic Vomiting Syndrome Association (CVSA). Data was compared against data obtained from the remainder of the CVSA sample (pediatric-onset CVS) and with controls ascertained from the United States, United Kingdom, Italy and Finland.

2.3 Results

2.3.1 Among patients with MDD, 3010A and 3010G are associated with different co-morbid functional symptoms.

At the present time, 27% of the full data set was analyzed and there is a trend with 3010A being associated with migraine among MDD patients (Table 5), as expected from our previous data. In contrast, the opposite nucleotide of the same SNP, 3010G, is associated with GI disorders among the MDD patients (Table 5). The 3010G+16519C genotype is present in 69% of MDD subjects with and 35% of subjects without any GI disorder (odds ratio = 3.92, 95% C.I. = 1.3-11.7, P = 5x10^4). A trend was noted suggesting that learning disabilities may also be associated with 3010G (Table 5).

Table 5. Pilot GenRED-Derived Data Comparing mtPNA SNPs to Selected Phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Migraine</th>
<th>COm</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3010A</td>
<td>13/31</td>
<td>41.9%</td>
<td>6/31</td>
<td>19.4%</td>
<td>P = 0.012</td>
<td>1/31</td>
<td>3.2%</td>
<td>P = 0.098</td>
</tr>
<tr>
<td>3010G</td>
<td>23/80</td>
<td>28.8%</td>
<td>36/80</td>
<td>45%</td>
<td>P = 0.012</td>
<td>11/80</td>
<td>13.8%</td>
<td>P = 0.098</td>
</tr>
<tr>
<td>16519T</td>
<td>9/28</td>
<td>32.1%</td>
<td>7/28</td>
<td>25%</td>
<td>P = 0.011</td>
<td>2/28</td>
<td>7.1%</td>
<td>P = 0.37</td>
</tr>
<tr>
<td>16519C</td>
<td>27/83</td>
<td>32.5%</td>
<td>35/83</td>
<td>42.2%</td>
<td></td>
<td>10/83</td>
<td>12.5%</td>
<td></td>
</tr>
</tbody>
</table>

*Colitis, enteritis, or other gastrointestinal disorders

2.3.2 The 16519T mtDNA SNP is associated with CFS, while among patients with CFS, 3010A is associated with several co-morbid functional symptoms.

A pilot study in English subjects found 16519T in 22 of 58 (38%) individuals with chronic fatigue syndrome (CFS) versus in 27% of controls, yielding an odds ratio of 2.0 (95% C.I = 1.1-3.7). DNA control samples from London blood donors revealed a proportion of 16519T of 26%, which is essentially the same as the 27% figure obtained from our multinational (USA, UK, Italy and Finland) control group.
In contrast, 3010A is highly associated with an increase in co-morbid functional symptoms among the subjects with CFS (Table 6).

Table 6. **3010A is highly associated with a wide variety of functional symptoms** among patients with CFS.

"[0]" = the absence of symptoms, while increasingly higher levels of somatic symptomatology are represented by higher integer numbers. "[1+]" = any score 1 or higher.

\( \chi^2 \) = chi square

Data on polymorphisms at 3010:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>7/21</td>
<td>14/21</td>
<td>17/25</td>
<td>8/25</td>
</tr>
<tr>
<td>Exertional Malaise</td>
<td>6/21</td>
<td>15/21</td>
<td>14/28</td>
<td>14/28</td>
</tr>
<tr>
<td>Muscle Weakness</td>
<td>7/21</td>
<td>14/21</td>
<td>18/28</td>
<td>10/28</td>
</tr>
<tr>
<td>Sore Throat</td>
<td>13/21</td>
<td>8/21</td>
<td>11/24</td>
<td>13/24</td>
</tr>
<tr>
<td>Cognitive Deficit</td>
<td>5/21</td>
<td>16/21</td>
<td>13/21</td>
<td>5/21</td>
</tr>
</tbody>
</table>

\( \chi^2 P = 0.019 \)

\( \chi^2 P = 0.13; \) Student's T: A 4.5+-2.4, G 3.5+-2.2, \( P = 0.12 \) (NS)

\( \chi^2 P = 0.011 \)

\( \chi^2 P = 0.032 \)

\( \chi^2 P = 0.0034 \)

\( \chi^2 P = \text{NS} \)
χ² P = NS

Muscle Pain 3010A: [0] = 2/21 = 9.5%, [3+] = 13/21 = 62%
χ² P = 0.001

χ² P = NS

Sleep Problems 3010A: 0 = 1/22 = 5%, 1-3 = 2/22, [4+] = 19/22 = 86%
3010A: [0-3] = 3/22 = 14%
3010G: [0-3] = 14/27 = 45%
χ² ([0-3] vs. [4+]) P = 0.0052

χ² ([0] vs. [K]) P = 0.10 (NS)

Numbness/Tingling 3010A: [0] = 9/21 = 43%, [2+] = 7/21 = 33%
Numbness/Tingling 3010G: [0] = 18/24 = 75%, [2+] = 2/24 = 8.3%
Fisher exact P = 0.026

The chronic fatigue data is very encouraging, and provides substantial support to our data demonstrating that 3010A is associated with a higher degree of somatic/functional symptomatology across the board, with the possible exception of bowel symptoms.

2.3.3 Data From Adult-Onset Versus Pediatric-Onset CVS Patients.
Analysis of data from adult-onset (18 years +) versus pediatric-onset of vomiting episodes in North American subjects with cyclic vomiting syndrome is presented below.
Table 7. Adult versus pediatric onset in CVS, pilot statistics
The 3010G+16519C genotype was found in:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult-onset CVS</td>
<td>10/13</td>
<td>77%</td>
</tr>
<tr>
<td>Child-onset CVS</td>
<td>2/17</td>
<td>11%</td>
</tr>
<tr>
<td>Controls</td>
<td>95/231</td>
<td>41%</td>
</tr>
</tbody>
</table>

P adult-onset CVS vs. child-onset CVS  P = 3.1 x 10^-5
P adult-onset CVS vs. controls        P = 0.011
P child-onset CVS vs. controls        P = 0.017

2.3.4 Data From SNPs other than 16519 and 3010.

Although 16519 and 3010 comprise a substantial proportion of the inherited risk to develop functional disease and functional symptoms, they do not comprise all such risk. The data demonstrate that additional functional-disease associated mtDNA SNPs likely exist, some of which have been identified. These other SNPs were found together in sets, in both patients with functional disorders and in controls (Tables 3 and 8), which does not allow at present the determination of which one, or ones, in said sets are pathogenic and which one, or ones, are neutral markers. The 2259T, 4745G, 7337A, 13326C, 13680T, 14831A and 14872T cluster was found in CVS subject numbers 2, 4 and 10, and the 239C, 4727G and 9380A cluster was found in CVS subject numbers 13 and 17 (Table 3). Among 16519T haplogroup H subjects, further data analysis revealed that the 13326C SNP was found in 4/30 CVS and in 3/22 CFS patients versus in 1/52 controls (P = 0.03). The sample size of our pilot data is underpowered such that few of the other SNPs in the clusters achieved statistical significance, but a trend suggesting that these clusters are associated with CVS is apparent in the data shown in Table 3.

2.4 Discussion

This analysis revealed that mtDNA SNPs 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C are associated with functional disease, especially the SNPs at 3010 and 16519. The data further showed that 3010 is associated with co-morbid functional symptoms among subjects with a diagnosis of a functional disorder. Identifying mtDNA sequence variants that predispose towards depression and co-morbid functional symptomatology will help identify subgroups of patients where mitochondrial dysfunction is a particularly important pathogenic mechanism. These individuals may respond differently to medications, and may benefit from
mitochondrial-targeted treatments, including frequent feedings, co-enzyme Q10, riboflavin and antioxidants. Our data and clinical experience suggest that most patients with functional symptoms do indeed respond favorably to these treatments.

These studies identify 16519T at an increased prevalence versus controls in functional disorders like pediatric-onset CVS in North Americans ascertained from the CVSA, migraine headache in Germans seen in headache clinics, and chronic fatigue syndrome in English patients.

3010A was identified at an increased prevalence versus controls in subjects with 16519T in functional disorders like pediatric-onset CVS in North Americans ascertained from the CVSA and in migraine headache in Germans seen in headache clinics. 3010A was identified at an increased prevalence in English patients with chronic fatigue syndrome among the subset of patients reporting the following co-morbid functional symptoms: headache, muscle pain, muscle weakness, numbness or tingling, fainting or dizziness and/or sleep problems. A trend was found for 3010A being more common in American adults with recurrent early-onset major depressive disorder among those reporting any kind of headache.

3010G was identified at an increased prevalence in patients with the following gut dysmotility-related phenomenon: in American adults with recurrent early-onset major depressive disorder among those reporting any gastrointestinal disorder versus among those reporting no GI disorders, and in adult-onset CVS in North Americans versus in controls. A trend was found for 3010G being more common in American adults with recurrent early-onset major depressive disorder among those reporting learning disabilities.

These studies show that 16519T predisposes towards the development of multiple functional disorders; that on a 16519T background, 3010A predisposes towards the development of multiple functional disorders; that among patients diagnosed with functional conditions, 3010A predisposes towards multiple manifestations of functional symptomatology; and that 3010G predisposes individuals towards gastrointestinal-related functional symptomatology.

The results and conclusions are further summarized in Table 8 below.
Table 8. Summary of Patient Groups, Polymorphisms, and Analyses when finding a Polymorphism in a Patient Group as indicated.

<table>
<thead>
<tr>
<th>Patient Group:</th>
<th>Polymorphism:</th>
<th>Analysis:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplogroup H children</td>
<td>16519T</td>
<td>6-fold more likely to have CVS</td>
</tr>
<tr>
<td>Haplogroup H adults</td>
<td>16519T</td>
<td>2 and 4-fold more likely to have CFS or migraine, respectively</td>
</tr>
<tr>
<td>Haplogroup H children</td>
<td>16519T+3010A</td>
<td>17-fold more likely to have CVS</td>
</tr>
<tr>
<td>Haplogroup H adults</td>
<td>16519T+3010A</td>
<td>15-fold more likely to have migraine</td>
</tr>
<tr>
<td>Haplogroup H adults</td>
<td>16519C+3010G</td>
<td>A few fold more likely to have CVS</td>
</tr>
<tr>
<td>Haplogroup H adults diagnosed with chronic fatigue syndrome</td>
<td>3010A</td>
<td>3 to 7-fold more likely to have headache, fainting or dizziness, muscle pain, muscle weakness, numbness or tingling, or sleep problems</td>
</tr>
<tr>
<td>Haplogroup H adults diagnosed with depression</td>
<td>3010G</td>
<td>4-fold more likely to have any bowel disorder; possibly more likely to have learning disabilities</td>
</tr>
<tr>
<td>Haplogroup H adults diagnosed with depression</td>
<td>3010A</td>
<td>Possibly more likely to have headaches</td>
</tr>
<tr>
<td>Haplogroup H individuals</td>
<td>Any of the group: 2259T, 4745G, 7337A, 13326C, 13680T, 14831A or 14872T</td>
<td>More likely to have child-onset CVS, CFS or migraine</td>
</tr>
<tr>
<td>Haplogroup H individuals</td>
<td>Any of the group: 239C, 4727G or 9380A</td>
<td>More likely to have child-onset CVS, CFS or migraine</td>
</tr>
</tbody>
</table>

2.5 References


The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety for any purpose.
WHAT IS CLAIMED IS:

1. A method to diagnose a functional disorder or functional symptomatology in a human comprising:
   determining the presence of a polymorphism in mitochondrial DNA of the human, wherein the polymorphism is selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C; and
   identifying the human as being at an increased risk to suffer from a functional disorder when compared to a human without said polymorphism.

2. The method according to claim 1, said method comprising determining the presence of at least two of said polymorphisms selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C.

3. The method according to claim 1, said method comprising determining the presence of at least three of said polymorphisms selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C.

4. The method according to claim 1, said method comprising determining the presence of at least four of said polymorphisms selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C.

5. The method according to claim 1, said method comprising determining the presence of at least five of said polymorphisms selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C.

6. The method according to claim 1, said method comprising determining the presence of a polymorphisms selected from the group consisting of 3010A, 3010G, 16519T, and 16519C.
7. The method according to claim 6, said method comprising determining the presence of at least two of polymorphisms selected from the group consisting of 3010A, 3010G, 16519T, and l6519C.

8. The method according to claim 6, said method comprising determining the presence of at least three of said polymorphisms selected from the group consisting of 3010A, 3010G, 16519T, and l6519C.

9. The method according to claim 6, said method comprising determining the presence of four of said polymorphisms selected from the group consisting of 3010A, 3010G, 16519T, and l6519C.

10. The method according to claim 1, wherein said functional disorder is selected from the group consisting of chronic fatigue syndrome (CFS), migraine, irritable bowel syndrome (IBS), depression, fibromyalgia, complex regional pain syndrome, nonspecific abdominal pain, chronic temporal mandibular joint pain, myalgic encephalitis, chronic pelvic pain, chronic pain syndromes, interstitial urethritis, post-traumatic stress syndrome, gulf war syndrome, and functional tinnitus.

11. The method according to claim 1, wherein said functional symptomatology is selected from the group consisting of gastrointestinal dysmotility, gas, pain, migraine, cyclic vomiting, chronic fatigue, limb pain, constipation, diarrhea, sleep apnea, frequent urination, and gastroesophageal reflux.

12. The method according to claim 6, wherein said functional disorder is selected from the group consisting of chronic fatigue syndrome (CFS), migraine, irritable bowel syndrome (IBS), depression, fibromyalgia, complex regional pain syndrome, nonspecific abdominal pain, chronic temporal mandibular joint pain, myalgic encephalitis, chronic pelvic pain, chronic pain syndromes, interstitial urethritis, post-traumatic stress syndrome, gulf war syndrome, and functional tinnitus.

13. The method according to claim 6, wherein said functional symptomatology is selected from the group consisting of gastrointestinal dysmotility, gas, pain, migraine, cyclic vomiting, chronic fatigue, limb pain, constipation, diarrhea, sleep apnea, frequent urination, and gastroesophageal reflux.

14. A kit for diagnosing a functional disorder in a human comprising two oligonucleotides capable of hybridizing to human mitochondrial DNA 5' and 3' to a
nucleotide in the DNA, wherein the nucleotide is selected from the group consisting of 239, 2259, 3010, 4727, 4745, 7337, 9380, 13326, 13680, 14831, 14872, and/or 16519; and wherein said oligonucleotides are separated by 0 to 200 nucleotides; and further comprising instructions for using the kit to diagnose functional disorders.

15. A kit for diagnosing a functional disorder in a human comprising means for detecting the presence of a polymorphism in mitochondrial DNA of the human, wherein the polymorphism is selected from the group consisting of 239C, 2259T, 3010A, 3010G, 4727G, 4745G, 7337A, 9380A, 13326C, 13680T, 14831A, 14872T, 16519T, and 16519C; and further comprising instructions for using the kit to diagnose functional disorders.
**INTERNATIONAL SEARCH REPORT**

**A** CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68 (2008.04)

USPC - 435/6

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

**B** FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC - 435/6

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

USPC - 435/91 2 (text search)

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


**C** DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2004/0029133 A1 (HERRNSTADT) 12 Feb 2004 (12 02 2004),Abstract, Table 1, 5, para [0020], [0053], [0070]</td>
<td>1, 2, 14, 15 3-13</td>
</tr>
<tr>
<td>Y</td>
<td>Lertzet al A new disease-related mutation for mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS) syndrome affects the ND4 subunit of the respiratory complex</td>
<td>3-13</td>
</tr>
<tr>
<td>A</td>
<td>Uusimma et al Molecular epidemiology of childhood mitochondrial encephalopathies in a Finnish population sequence analysis of entire mtDNA of 17 children reveals heteroplasmic mutations in tRNAGlu, tRNAArg, and tRNALeu(UUR) genes</td>
<td>1-15</td>
</tr>
<tr>
<td>A</td>
<td>Wang et al Mitochondrial DNA control region sequence variation in migraine headache and cyclic vomiting syndrome Am J Medical Genetics 2004, 131A 50-58</td>
<td>1-15</td>
</tr>
<tr>
<td>A</td>
<td>Boles et al A high predisposition to depression and anxiety in mothers and other maternal relatives of children with presumed maternally inherited mitochondrial disorders Am J of Medical Genetics 2005, 137B 20-24</td>
<td>1-15</td>
</tr>
</tbody>
</table>

**D** Further documents are listed in the continuation of Box C

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"Z" document member of the same patent family

**Date of the actual completion of the international search**

14 July 2008 (14 07 2008)

**Date of mailing of the international search report**

19 AUG 2008

**Name and mailing address of the ISA/US**

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

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Lee W Young

PCT/US 08/03994

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