Title: POLYMORPHISMS IN A KLOTHO GENE

Abstract: Methods and means are described for diagnosing a disease or a predisposition to disease by genotyping a klotho gene from an individual and identifying the presence of one or more risk polymorphisms. These risk polymorphisms are associated either directly or indirectly with predisposition to a range of chronic diseases including osteoporosis, skin disorders, age-related disease, lung dysfunction and metabolic syndrome. Methods of treatment, kits for diagnostic purposes and an isolated klotho gene including all or some of a range of polymorphisms are also described as well as methods of isolating molecules that modulate a klotho gene.
POLYMORPHISMS IN A KLOTHO GENE

FIELD OF THE INVENTION

The present invention relates to a method for determining polymorphism(s) in a gene, as well as means for using such a method in therapeutic applications.

In particular, the present invention relates to a method for the diagnosis of a disease or a predisposition to a disease by screening for the presence of polymorphism(s) in a gene.

More in particular, the present invention relates to the diagnosis of a disease or a predisposition to all or a sub-set of disease states, including age-related disease states such as metabolic syndrome, obesity, skin disorders, osteoporosis and disorders of lung function.

The present invention further relates to directed treatment of such disease states.

In addition, the present invention relates to a kit for diagnosis for susceptibility or predisposition to a number of diseases, including age-related disease, and to components for inclusion in said kit.

BACKGROUND TO THE INVENTION

The incidence of chronic disease is a significant drain on resources in both developing and developed nations. By way of example, diseases of the lungs - such as asthma and emphysema - and of the cardiovascular system - such as atherosclerosis - are of particular relevance to public health. Chronic age related disease may also manifest itself in a multiplicity of metabolic disorders - such as obesity and high blood pressure. Consequently, the costs for both national and international public health programmes attempting to deal with the consequences of these diseases are substantial. It would therefore be desirable to provide a means for screening individuals to identify those who are predisposed to disease and especially to identify to which chronic diseases they are susceptible.
At present, treatment of chronic diseases can be effective in slowing the progression of the disease, but only after the disease has been diagnosed. However, such treatments are seldom capable of reversing the effects of the disease once it has set in. Prophylactic treatment of the general population is expensive and a significant drawback is that such treatments are not targeted to the needs of the individual and may be either redundant or even counterproductive. In some cases adverse side effects may be experienced from prolonged exposure to inappropriate prophylactic treatments.

By way of example, prophylactic treatments, such as hormone replacement therapy (HRT), are established therapies for diseases like osteoporosis. These treatments have proven successful in halting further decline in bone mineral density (BMD) which is a characteristic seen in women suffering from the disease. However, HRT is generally not able to bring about a reversal of osteoporosis. That is to say, it is not capable of inducing an increase in BMD of sufferers.

Accordingly, it would be of particular advantage to be able to identify with increased accuracy those individuals having a predisposition or increased susceptibility to diseases such as osteoporosis. Suitable therapy could then be put into place before the effects of a disease like osteoporosis sets in.

A relatively new gene, termed klotho, has been identified that is involved in the suppression of several ageing phenotypes (Kuro-o et al (1997) Nature, 390:45-51). These investigators have shown that mice possessing a defect in klotho gene expression exhibit a number of conditions that may be associated with ageing. This defect was deemed to have been caused by a severe hypomorphic mutation in the 5' upstream region. As a result, this defect may result in only a slight transcription of the klotho gene and hence only a slight suppression of ageing phenotypes. Accordingly, this type of defect, which may result in only a slight transcription of the klotho gene, may have limited relevance to the clinician.

The human klotho gene has also been isolated (Matsumura et al. (1998) Biochem. Biophys. Res. Comm. 242: 626-630). The isolated human klotho cDNA encodes a protein of 1, 012 amino acids and shows about 86% identity to the mouse klotho protein (Kuro-o et al ibid). An association has also been described between a CA-microsatellite repeat located downstream of the final exon (that is, in the non-coding 3' region) of the human klotho gene, and bone loss in
post menopausal women (Ogata *et al.* 1998 Abstract T302 ASBMR-IBMS Second Joint Meeting). These results have suggested that the *klotho* gene may play a role in the progression of senile osteoporosis in post menopausal women.

However, it would be more desirable to provide a means of diagnosing individuals predisposed to chronic diseases, such as osteoporosis, much younger in life before the effects of the disease become apparent. It would therefore be an advantage to provide further means for diagnosis of predisposition to reduced BMD, osteopaenia and osteoporosis, in order to enable diagnosis of these conditions in younger as well as older individuals.

The present invention seeks to provide additional means for diagnosis of disease and/or predisposition to disease.

The present invention also seeks to provide diagnosis and/or treatment of disease that solves or at least ameliorates the disadvantages associated with the prior art.

SUMMARY ASPECTS

The present invention relates to methods for *inter alia* identifying and/or diagnosing the presence or absence of one or more risk polymorphisms within a *klotho* gene sequence. In particular, these methods relate to screens to determine the presence or absence of a risk polymorphism such as single nucleotide polymorphism (SNP). The methods of the present invention may also be used to determine the relative position of multiple risk polymorphisms within a *klotho* gene sequence in order to provide a set of risk polymorphisms or a haplotype for a *klotho* gene in an individual. The identified risk polymorphisms may be used to diagnose a disease and/or predisposition to disease by correlating the identified polymorphisms with inherited genetic factors and/or phenotypic traits. The identified polymorphisms in a *klotho* gene may be used as targets for the discovery of agents (such as modulators) which may be effectively used to prevent or delay or treat a disease or a predisposition to a disease associated with these genetic variations.
DETAILED ASPECTS OF THE INVENTION

According to a first aspect of the present invention, there is provided a method of diagnosis for a disease or a predisposition to a disease associated with a risk polymorphism in a klotho gene wherein the risk polymorphism is an SNP risk polymorphism; and wherein the method comprises: (i) genotyping a klotho gene; and (ii) determining whether the genotype comprises a risk genotype.

In this embodiment, typically the klotho gene is taken from an individual or is in a sample taken from an individual.

According to a second aspect of the present invention, there is provided a method of diagnosis for a disease or a predisposition to a disease associated with a risk polymorphism in a klotho gene from an individual; wherein the risk polymorphism is located in a region of the klotho gene selected from the group consisting of a 5' region of the klotho gene, an exon of the klotho gene and an intron of the klotho gene; wherein the method comprises: (i) genotyping a klotho gene; and (ii) determining whether the genotype comprises a risk genotype.

In this embodiment, typically the individual is a human.

Preferably, in this embodiment, the risk polymorphism is located in a 5' region of a klotho gene.

According to a third aspect of the present invention there is provided a method of preventing and/or treating a disease or a predisposition to a disease associated with a risk polymorphism in a klotho gene wherein the risk polymorphism is an SNP risk polymorphism; wherein the method comprises: (i) genotyping a klotho gene; (ii) determining the presence of a risk genotype; and (iii) applying a treatment in order to prevent, delay, reduce or treat the disease or the predisposition to the disease if said klotho gene comprises said risk genotype.

According to a fourth aspect of the present invention there is provided a method of preventing and/or treating a disease or a predisposition to a disease associated with a risk polymorphism in a klotho gene from an individual; wherein the risk polymorphism is located in a region of the klotho gene selected from the group consisting of a 5' region, an exon of the klotho gene and an
intron of the klotho gene: wherein the method comprises: (i) genotyping a klotho gene; (ii) determining the presence of a risk genotype; and (iii) applying a treatment in order to prevent, delay, reduce or treat the disease if said klotho gene comprises said risk genotype.

According to a fifth aspect of the present invention there is provided a method for identifying an SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene; wherein the method comprises: (i) genotyping for said SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene; and (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

According to a sixth aspect of the present invention there is provided a method of identifying a risk polymorphism in a klotho gene that is associated with a risk polymorphism in a klotho gene from an individual; wherein the risk polymorphism is located in a region of the klotho gene selected from the group consisting of a 5' region of the klotho gene, an exon of the klotho gene and an intron of the klotho gene; wherein the method comprises: (i) genotyping for said SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene; and (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

According to a seventh aspect of the present invention there is provided a isolated klotho gene comprising at least one SNP risk polymorphism is located within a klotho gene at a position selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.

According to an eighth aspect of the present invention there is provided a kit for diagnosis of a disease or a predisposition to disease, wherein the kit comprises: (i) means for genotyping a klotho gene; and (ii) reference means for determining whether the genotype comprises a risk genotype.
Other aspects of the present invention are presented in the accompanying claims and in the following description and drawings. These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section are not necessarily limited to that particular section heading.

5

PREFERABLE ASPECTS

For some embodiments, preferably the risk polymorphism is located in an exon of the klotho gene.

10

For some embodiments, preferably the risk polymorphism is located in an intron of the klotho gene.

Preferably the treatment is or comprises hormone replacement therapy (HRT).

15

Preferably the treatment is or comprises gene therapy.

In a preferred embodiment of the present invention, the risk polymorphism is an SNP risk polymorphism.

20

In a preferred embodiment of the present invention, the SNP risk polymorphism is located in a 5' region of a human klotho gene.

In a preferred embodiment of the present invention, the SNP risk polymorphism is located in an exon of a human klotho gene.

25

In an alternative preferred embodiment of the present invention, the SNP risk polymorphism is located in an intron of a human klotho gene.

Preferably the polymorphism has a one risk polymorphism association.

30

For some applications, preferably the polymorphism has a two risk polymorphism association.
Preferably the risk polymorphism(s) is/are selected from SNPs of the present invention are located within said klotho gene at the following positions: nucleotide 1122 (5' region); nucleotide 1337 (5' region); nucleotide 1686 (5' region); nucleotide 2406; nucleotide 12707 (exon 2); nucleotide 12753; nucleotide 19489 (exon 4); nucleotide 19969 (exon 4); and nucleotide 20445.

Preferably the SNP risk polymorphism is located in a region selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.

Preferably PCR techniques are used to genotype a nucleic acids comprising a klotho gene or part thereof from an individual.

Preferably the screening is carried out using PCR primers.

Preferably the genotyping (which may be a screening method) is carried out using allelic specific primers.

Preferably the results of genotyping of klotho polymorphisms may be used to identify patients most likely to suffer from adverse reactions to certain therapies.

Preferably the invention provides for diagnosis of predisposition to reduced BMD and osteoporosis.

ADVANTAGES

The present invention is advantageous in that it facilitates the genotyping of klotho gene polymorphisms which:

(i) provides for a more accurate diagnosis of predisposition to disease states and conditions.

Thus, by genotyping a single gene, such as a klotho gene, an individual may be identified as being predisposed to some or all of a number of chronic diseases such as age related diseases.
(ii) allows for the identification of individuals who are predisposed to a range of chronic diseases or who have an increased risk of contracting such diseases. A suitable therapy may then be put in place to prevent or treat or delay the onset of these diseases.

(iii) helps to identify patients most likely to respond positively to treatment with certain classes of therapies or particular therapeutics.

(iv) allows for the selection of optimal clinical trial patient samples thereby reducing the size of a trial and/or decreasing the time of the clinical trial.

Other advantages are discussed and are made apparent in the following commentary.

**KLOTHO GENE**

Wherever appropriate, the term “klotho gene” may be used interchangeably with the gene coding for same - otherwise expressed as being a nucleotide sequence of interest (NOI) – and/or any biologically active fragment(s) thereof and/or the expression product thereof – otherwise expressed as EP and/or any biologically active fragment(s) thereof.

The term “NOI” includes DNA, RNA and single and double stranded sequences. It also refers to sequences which are prepared by synthetic means. For some applications, the NOI is in an isolated and/or purified form.

The murine klotho gene and the human klotho gene have been isolated (Kuro-o et al ibid). A severe hypomorphic mutation, thought to be located in the 5' upstream region, has been identified in the murine klotho gene and has been shown to be associated with certain ageing phenotypes. In addition, a microsatellite repeat, associated with the progression of senile osteoporosis, has been identified downstream of the final exon in the human klotho gene (Ogata et al ibid). No polymorphisms have been located in regions such as the 5' region, exonic or intronic regions of the human klotho gene. Moreover, no single nucleotide polymorphisms (SNPs) have been identified in either the murine or human klotho genes.
The present invention is based on the surprising finding that risk polymorphisms exist in this gene.

Hence, the present invention provides methods for identifying and/or diagnosing polymorphisms in a *klotho* gene which are associated with phenotypic traits present in certain age related disease states. Such polymorphisms may be used to identify agents capable of modulating the onset of diseases associated with the phenotypic traits of such genetic variations.

The *klotho* gene of the present invention is different to the wild type sequence. Here, the term “wild type” refers to the published wild type nucleic acid sequence for the human *klotho* gene – i.e. the human *klotho* gene published by Matsumura *et al.* (1998) Biochem. Biophys. Res. Comm. 242: 626-630. This sequence is contained in Genbank records, accession numbers AB009666 and AB009667.

Hence, some embodiments of the present invention are based on methods for identifying *klotho* genes other than the wild type *klotho* gene. The genes which are to be identified include polymorphic or variant or allelic *klotho* genes with risk polymorphisms. It is to be understood that these variant *klotho* genes may be identified by reference to either the wild type *klotho* gene or another reference/control sequence.

**ISOLATED KLOTHO GENE**

The isolated *klotho* gene of the present invention may be introduced into a vector and expressed under *in vitro*, and/or *in vivo* and/or *ex vivo* conditions. In this way, the expression product may be used in applications which include but are not limited to gene therapy, identification of potential pharmaceutical targets in high throughput screening (HTS) assays and forensic analysis.

Preferably the isolated *klotho* gene of the present invention comprises at least one SNP selected from the group consisting a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at
nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445

Preferably the isolated *klotho* gene of the present invention is introduced into a vector and expressed under *in vitro*, and/or *in vivo* and/or *ex vivo* conditions.

**POLYMORPHISM**

As used herein, the term “polymorphism” refers to the coexistence of multiple forms of a sequence.

The term “multiple” refers to two or more genetically determined alternative sequences or alleles in a population.

The term “allele” refers to a variant form of a gene occurring at a same locus or to different sequence variants found at given markers.

The term “marker” refers to a specific site in a gene which exhibits sequence variations between individuals.

The term “sequence variations” includes but is not limited to single or multiple base changes including insertions, deletions or substitutions or a variable number of sequence repeats. As used herein, the terms “sequence variant” and “allele” are used interchangeably with the term “polymorphism”.

The risk polymorphisms of the present invention may be located in a region of a *klotho* gene. Such a region is termed a polymorphic region.

As used herein, the term “polymorphic region” is the locus or locations at which divergence occurs.
TYPES OF POLYMORPHISMS

Several different types of polymorphisms have been reported. Polymorphic markers include restriction fragment length polymorphisms (RFLPs), variable number of tandem repeats (VNTR's), single nucleotide polymorphisms (SNPs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. A polymorphic locus may be as small as one base pair. A one base pair change may occur in a codon.

As used herein, the term “codon” means a sequence of three adjacent nucleotides (a trinucleotide sequence) that may designate an amino acid or a start/stop site for translation.

RFLPs

A restriction fragment length polymorphism (RFLP) means a variation in DNA sequence that alters the length of a restriction fragment as described in Botstein et al., Am. J. Hum. Genet. 32, 314-331 (1980). The RFLP may create or delete a restriction site, thus changing the length of the restriction fragment. RFLPs have been widely used in human and animal genetic analyses (see WO 90/13668; WO90/11369; Donis-Keller, Cell 51, 319-337 (1987); Lander et al Genetics 121, 85-99 (1989)). When a heritable trait can be linked to a particular RFLP, the presence of the RFLP in an individual can be used to predict the likelihood that the individual will also exhibit the trait.

TANDEM REPEAT POLYMORPHISMS

Other polymorphisms take the form of short tandem repeats (STRs) that include tandem di-, tri- and tetranucleotide repeated motifs. These tandem repeats are also referred to as variable number tandem repeat (VNTR) polymorphisms. VNTRs have been used in identity and paternity analysis (U.S. Pat. No. 5,075,217; Armour et al., FEBS Lett. 307, 113-115 (1992); Horn et al. WO 91/14003; Jeffreys, EP 370,719), and in a large number of genetic mapping studies.
SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs).

Other polymorphisms take the form of single nucleotide variations between individuals of the same species. Such single nucleotide variations may arise due to substitution of one nucleotide for another at the polymorphic site or from a deletion of a nucleotide or an insertion of a nucleotide relative to a referenced allele. These single nucleotide variations are referred to herein as single nucleotide polymorphism (SNPs). Such SNPs are far more frequent than RFLPs, STRs and VNTRs. Some SNPs may occur in protein-coding sequences, in which case, one of the polymorphic forms may give rise to the expression of a defective or other variant protein and, potentially, a genetic disease. Other SNPs may occur in noncoding regions. Some of these polymorphisms may also result in defective protein expression (e.g., as a result of defective splicing). Other SNPs may have no phenotypic effects.

SNPs may be used in the same manner as RFLPs, and VNTRs but offer several advantages. SNPs occur with greater frequency and are spaced more uniformly throughout the genome than other forms of polymorphism. The greater frequency and uniformity of SNPs means that there is a greater probability that such a polymorphism will be found in close proximity to a genetic locus of interest than would be the case for other polymorphisms. Also, the different forms of characterized SNPs are often easier to distinguish that other types of polymorphism (e.g., by use of assays employing allele-specific hybridization probes or primers).

RISK ASSOCIATIONS

As used herein, the term “one risk polymorphism association” means that a risk polymorphism associated with a disease state would indicate that an individual was in a risk category for that disease state. By way of example, identification of a risk polymorphism in a klotho gene associated with lung dysfunction - would indicate that an individual was at risk of lung disease (one risk polymorphism association).

As used herein, the term “two risk polymorphism association” means that a risk polymorphism associated with a first disease state (such as reduced BMD) and a second disease state (such as
reduced BMD and lung dysfunction) would indicate that an individual was in a very high risk category for BMD and therefore osteoporosis (two risk polymorphism association).

In another embodiment of the present invention, there is provided a means for diagnosis of disease or predisposition to disease by identifying at least two risk polymorphisms in a klotho gene. In this embodiment of the invention, a combination of data from at least two identified polymorphisms allows for a precise diagnosis of which disease states to which an individual is susceptible.

In one embodiment, the present invention provides for a method of diagnosing a disease or a predisposition disease by genotyping a klotho gene. By genotyping the klotho gene, the methods of the present invention enable either direct diagnosis of a disease or a diagnosis of a predisposition to certain disease conditions.

RISK POLYMORPHISM

As used herein, the term “risk polymorphism” means a polymorphism which is associated with a disease or a predisposition to a disease or an increased susceptibility to disease.

GENOTYPE

As used herein, the term “genotype” means a klotho gene which has been screened for the presence of at least one risk polymorphism at a specific genetic locus. Otherwise expressed, the screened klotho gene could be called a “genotyped klotho gene”.

RISK GENOTYPE

As used herein, the term “risk genotype” refers to a klotho gene which comprises at least one risk polymorphism which is associated with at least one disease phenotype or phenotypic trait.
PHENOTYPE

As used herein, the term "phenotype" means any detectable trait that is the result of one or more genes. A polymorphisms may contribute to the phenotype of an individual in different ways. Some polymorphisms may occur within a protein coding sequence (such as an exon) and contribute to phenotype by affecting protein structure. Other polymorphisms may occur in non-coding regions (such as a promoter region or an intron) but may exert phenotypic effects indirectly via influence on replication, transcription, and translation. A single polymorphism may affect more than one phenotypic trait. Likewise, a single phenotypic trait may be affected by polymorphisms in different genes. Further, some polymorphisms predispose an individual to a distinct mutation that is causally related to a certain phenotype or phenotypic trait.

PHENOTYPIC TRAITS

The polymorphisms of the invention may contribute to the phenotype of an individual in different ways. Some polymorphisms may occur within a protein coding sequence (such as an exon) and contribute to phenotype by affecting protein structure. Other polymorphisms may occur in non-coding regions (such as a promoter region or an intron) but may exert phenotypic effects indirectly via influence on replication, transcription, and translation. A single polymorphism may affect more than one phenotypic trait. Likewise, a single phenotypic trait may be affected by polymorphisms in different genes. Further, some polymorphisms predispose an individual to a distinct mutation that is causally related to a certain phenotype.

CORRELATION OF POLYMORPHISMS WITH PHENOTYPIC TRAITS

As an example of the present invention in use (described in more detail below) common SNP risk polymorphisms in the human klotho gene were identified in a cohort of 1,435 twin pairs and their association with multiple risk traits were assessed.

In another example of the present invention, the genotype of four SNP risk polymorphisms in the human klotho gene may be used individually or in combination in the form of a haplotype for the diagnosis of a number of age related disease states.
As used herein, the term “haplotype” means a collection or a set of polymorphic sites in a particular gene sequence, such as a klotho gene sequence that are inherited as a group (that is, are in linkage disequilibrium (LD)). The identification of a disease associated haplotype (such as a set of risk polymorphism in LD) facilitates the determination of which of the risk polymorphisms are causative rather than merely linked to a disease causing allele. This information may then be used to design rational treatment approaches. Moreover, the determination of disease associated alleles in LD is advantageous as it allows the diagnostic testing of more than one risk polymorphism which helps to eliminate the possibility of false positive testing.

By way of example, the present invention (described in more detail below) provides a method for the diagnosis of predisposition to osteoporosis using the genotype of up to three SNP risk polymorphisms in the human klotho gene, either individually or in combination in the form of a haplotype.

In a further example, the present invention (described in more detail below) provides a method for the diagnosis of predisposition to disorders of lung function using the genotype of three SNP risk polymorphisms in the human klotho gene, either individually or in combination in the form of a haplotype.

In yet a further example, the present invention (described in more detail below) provides a method for the diagnosis of predisposition to age related skin disorders using the genotype of two SNP risk polymorphisms in the human klotho gene, either individually or in combination in the form of a haplotype.

Another example of the invention in use, described in more detail below, provides for a method for the diagnosis of predisposition to metabolic syndrome using the genotype of three SNP risk polymorphisms in the human klotho gene, either individually or in combination in the form of a haplotype.

OTHER RISK FACTORS

Optionally, the assessment of an individual’s risk factor is calculated by reference also to other known genetic or physiological or dietary or other indications. The invention in this way
provides further information on which measurement of an individual's risk of disease or predisposition can be based.

GENOTYPING

As used herein, the term "genotyping" means determining whether a klotho gene includes at least one risk polymorphism. The term "genotyping" is synonymous with terms such as "genetic testing", "genetic screening", "determining or identifying an allele or polymorphism", "molecular diagnostics" or any other similar phrase.

Any method capable of distinguishing nucleotide differences in the appropriate sample DNA sequences may also be used. In fact, a number of known different methods are suitable for use in genotyping (that is, determining the genotype) for a klotho gene of the present invention. These methods include but are not limited to direct sequencing, PCR-RFLP, ARMS-PCR, Taqman™, Molecular beacons, hybridization to oligonucleotides on DNA chips and arrays, single nucleotide primer extension and oligo ligation assays.

GENOTYPE SCREENING

In one embodiment, the present invention provides a method for genotype screening of a nucleic acid comprising a klotho gene from an individual. The methods for genotype screening of a nucleic acid comprising a klotho gene from an individual may require amplification of a nucleic acids from a target sample from that individual.

TARGET SAMPLE

The target samples of the present invention may be any target nucleic acid comprising a klotho gene from an individual being analyzed. For assay of such nucleic acids, virtually any biological sample (other than pure red blood cells) is suitable. For example, convenient target samples include but are not limited to whole blood, leukocytes, semen, saliva, tears, urine, fecal material, sweat, buccal, skin and hair. For assay of cDNA or mRNA, the target sample is typically obtained from a cell or organ in which the target nucleic acid is expressed.
GENOTYPING SNPS

A number of different methods are suitable for use in determining the genotype for an SNP. These methods include but are not limited to direct sequencing, PCR-RFLP, ARMS-PCR, Taqman™, Molecular beacons, hybridization to oligonucleotides on DNA chips and arrays, single nucleotide primer extension and oligo ligation assays. Any method capable of distinguishing single nucleotide differences in the appropriate DNA sequences may also be used.

AMPLIFICATION

As used herein, the term “amplification means nucleic acid replication involving template specificity. The template specificity relates to a “target sample” or “target sequence” specificity. The target sequences are “targets” in the sense that they are sought to be sorted out from other nucleic acids. Consequently, amplification techniques have been designed primarily for sorting this out. Examples of amplification methods include but are not limited to polymerase chain reaction (PCR), polymerase chain reaction of specific alleles (PASA), ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication and nucleic acid based sequence amplification (NASBA).

TAQMAN™

Suitable means for determining genotype may be based on the Taqman™ technique. The Taqman™ technique is disclosed in the following US patents 4,683,202; 4,683,195 and 4,965,188. The use of uracil N-glycosylase which is included in Taqman™ allelic discrimination assays is disclosed in US patent 5,035,996.

PCR

PCR techniques are well known in the art (see for example, EP-A-0200362 and EP-A-0201184 and US patent Nos 4 683 195 and 4 683 202). The process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. With PCR, it is possible to amplify a single copy of a specific
target sequence in, for example, genomic DNA to a level detectable by several different methodologies (such as hybridisation with a labelled probe, incorporation of biotinylated primers followed by avidin-enzyme conjugate detection and incorporation of $^{32}$P labelled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified sequence). Alternatively, it is possible to amplify different polymorphic sites (markers) with primers that are differentially labelled and thus can each be detected. One means of analysing multiple markers involves labelling each marker with a different fluorescent probe. The PCR products are then analysed on a fluorescence based automated sequencer. In addition to genomic DNA, any oligonucleotide sequence may be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. By way of example, PCR can also be used to identify primers for amplifying suitable sections of a klotho gene in or from a human.

**PRIMERS**

The present invention also provides a series of useful primers.

As used herein, the term “primer” refers to a single-stranded oligonucleotide capable of acting as a point of initiation of template-directed DNA synthesis under appropriate conditions (i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as, DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template.

The term “primer site” refers to the area of the target DNA to which a primer hybridizes.

The term “primer pair” means a set of primers including a 5' upstream primer that hybridizes with the 5' end of the DNA sequence to be amplified and a 3' downstream primer that hybridizes with the complement of the 3' end of the sequence to be amplified.
The primers of the present invention may be DNA or RNA, and single-or double-stranded. Alternatively, the primers may be naturally occurring or synthetic, but are typically prepared by synthetic means.

5 PRIMER HYBRIDISATION CONDITIONS

As used herein, the term “hybridisation” refers to the pairing of complementary nucleic acids. Hybridisation and the strength of hybridisation (ie the strength of association between the nucleic acids) is impacted by such factors as the degree of complementarity between nucleic acids, stringency of conditions involved, the melting temperature (Tm) of the formed hybrid and the G:C ratio within the nucleic acids.

As used herein, the term “stringency” is used in reference to the conditions of temperature, ionic strength and the presence of other compounds such as organic solvents under which the nucleic acid hybridisations are conducted.

Hybridizations are typically performed under stringent conditions, for example, at a salt concentration of no more than 1M and a temperature of at least 25°C. For example, conditions of 5X SSPE (750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C. are suitable for allele-specific primer hybridizations.

ALLELE SPECIFIC PRIMERS

An allele-specific primer hybridizes to a site on target DNA overlapping a polymorphism and only primes amplification of an allelic form to which the primer exhibits perfect complementarity (See Gibbs, Nucleic Acid Res. 17, 2427-2448 (1989)). This primer may be used in conjunction with a second primer which hybridizes at a distal site. Amplification proceeds from the two primers leading to a detectable product signifying the particular allelic form is present. A control may be performed with a second pair of primers, one of which shows a single base mismatch at the polymorphic site and the other of which exhibits perfect complementarity to a distal site. The single-base mismatch prevents amplification and no detectable product is formed. The method works best when the mismatch is included in the 3’-most position of the oligonucleotide aligned with the polymorphism because this position is
most destabilizing to elongation from the primer (see, for example WO 93/22456). Hybridisation probes capable of specific hybridisation to detect a single base mismatch may be designed according to methods known in the art and described in Maniatis et al Molecular Cloning: A Laboratory Manual, 2nd Ed (1989) Cold Spring Harbour.

(i) PCR ALLELE SPECIFIC PRIMERS

Preferably the screening is carried out using PCR allele specific primers designed to amplify portions of the human *klotho* gene that include either nucleotide 1686, nucleotide 12707, nucleotide 19489 or nucleotide 19969.

Examples of such PCR primers have the following nucleotide sequences.

For the amplification of a DNA sequence containing nucleotide 1686:

5’ AAATCTACTTTGTCTTCTCG 3’
5’ AAAGGCACCTGTTTCTCCC 3’

For the amplification of a DNA sequence containing nucleotide 12707:

5’ TGAATCTGAGAAAAAGTTCATC 3’
5’ CATTATGTTAGATAATCTTAGAG 3’

For the amplification of a DNA sequence containing nucleotide 19489:

5’ AGTGGAATATTGTCTTCCCTC 3’
5’ CCATAGGCTGCCACAGGG 3’

For the amplification of a DNA sequence containing nucleotide 19969:

5’ CACCATCCTGCAGTACTATC 3’
5’ TTTGGTTCAAGCCAGTCCCTC 3’
In one embodiment, preferably the PCR primers are designed to amplify portions of the human klotho gene that includes nucleotide 1686.

In one embodiment, preferably the PCR primers are designed to amplify portions of the human klotho gene that includes nucleotide 12707.

In one embodiment, preferably the PCR primers are designed to amplify portions of the human klotho gene that includes nucleotide 19489.

In one embodiment, preferably the PCR primers are designed to amplify portions of the human klotho gene that includes nucleotide 19969.

Other primers are shown in Table 4.

DETECTION OF POLYMORPHISMS IN AMPLIFIED TARGET SEQUENCES

The amplified nucleic acid sequences may be detected using procedures including but not limited to allele-specific probes, tiling arrays, direct sequencing, denaturing gradient gel electrophoresis and single-strand conformation polymorphism (SCCP) analysis.

ALLELE-SPECIFIC PROBES

Allele-specific probes can be designed that hybridize to a segment of target DNA from one individual but do not hybridize to the corresponding segment from another individual due to the presence of different polymorphic forms in the respective segments from the two individuals.

As used herein, the term “probe” refers to an oligonucleotide (ie a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of hybridising to another oligonucleotide sequence of interest. Probes are useful in the detection, identification and isolation of particular gene sequences. The hybridization probes of the present invention are typically oligonucleotides capable of binding in a base-specific manner to a complementary strand of nucleic acid.
The probes of the present invention may be labelled with any "reporter molecule" so that it is detectable in any detection system, including but not limited to enzyme (for example, ELISA, as well as enzyme based histochemical assays), fluorescent, radioactive and luminescent systems. The target sequence of interest (that is, the sequence to be detected) may also be labelled with a reporter molecule. The present invention is not limited to any particular detection system or label.

The hybridization conditions chosen for the probes of the present invention are sufficiently stringent that there is a significant difference in hybridization intensity between alleles, and preferably an essentially binary response, whereby a probe hybridizes to only one of the alleles. The typical hybridisation conditions are stringent conditions as set out above for the allele specific primers of the present invention so that a one base pair mismatch may be determined.

TILING ARRAYS

The polymorphisms of the present invention may also be identified by hybridization to nucleic acid arrays, some example of which are described in WO 95/11995. The term "tiling" generally means the synthesis of a defined set of oligonucleotide probes that is made up of a sequence complementary to the sequence to be analysed (the "target sequence"), as well as preselected variations of that sequence. The variations usually include substitution at one or more base positions with one or more nucleotides.

DIRECT SEQUENCING

The direct analysis of the sequence of polymorphisms of the present invention may be accomplished using either the dideoxy chain termination method or the Maxam Gilbert method (see Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2nd Ed., CSHP, New York 1989) or using, for example, Standard ABI sequencing technology using Big Dye Terminator cycle sequencing chemistry analyzed on an ABI Prism 377 DNA sequencer.
DENATURING GRADIENT GEL ELECTROPHORESIS

Amplification products of the present invention, which are generated using PCR, may also be analyzed by the use of denaturing gradient gel electrophoresis. Different alleles may be identified based on the different sequence-dependent melting properties and electrophoretic migration of DNA in solution. Erlich, ed., PCR Technology, Principles and Applications for DNA Amplification. (W.H. Freeman and Co, New York, 1992), Chapter 7.

SINGLE-STRAND CONFORMATION POLYMORPHISM (SCCP) ANALYSIS

Alleles of target sequences of the present invention may also be differentiated using single-strand conformation polymorphism (SCCP) analysis, which identifies base differences by alteration in electrophoretic migration of single stranded PCR products, as described in Orita et al., Proc. Nat. Acad. Sci. 86. 2766-2770(1989). Amplified PCR products can be generated as described above, and heated or otherwise denatured, to form single stranded amplification products. Single-stranded nucleic acids may refold or form secondary structures which are partially dependent on the base sequence. The different electrophoretic mobilities of single-stranded amplification products may be related to base-sequence difference between alleles of target sequences.

IDENTIFYING DIFFERENCES BETWEEN TEST AND CONTROL SEQUENCES

These detection procedures for amplified nucleic acid sequences may be used to identify difference of one or more points of variation between a reference and test nucleic acid sequence or to compare different polymorphic forms of the klotho gene from two or more individuals.

REFERENCE NUCLEIC ACID SEQUENCES

As used herein the term “reference nucleic acid sequence” means a control nucleic acid sequence such as a control DNA sequence representing one or more individuals homozygous for each of the alleles being tested in that assay. By way of example, control DNA sequences may include but are not limited to: (i) a genomic DNA from homozygous individuals; (ii) a PCR product containing a relevant SNP amplified from homozygous individuals; or (iii) a DNA sequence
containing a relevant SNP that has been cloned into a plasmid or other suitable vector. The control sample may also be an allelic ladder comprising a plurality of alleles from known set of alleles. There may be a plurality of control samples, each containing different alleles or sets of alleles. Other reference/control samples typically include diagrammatic representations, written representations, templates or any other means suitable for identifying the presence of a polymorphism in a PCR product or other fragment of nucleic acid. The terms “reference nucleic acid sequence”, reference samples and control samples are used interchangeable throughout the text.

In one embodiment, an isolated klotho gene comprising at least one SNP selected from the group consisting a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445 may also be used as a reference fragment for application in, for example, a kit of the invention. The reference sequence may be a wild type klotho gene sequence. Alternatively, the reference sequence may be a consensus wild type klotho gene sequence.

As used herein, the term “consensus” refers to an idealised sequence of nucleotides, or their constituent bases, that represent the nucleotides most likely to occur at each position in the sequence.

5' REGION

As used herein, the term “5' region” means a region which is 5' to a first exon of a structural gene such as a klotho gene. The term “5' region” includes but is not limited to regions such as a 5' non-coding region and putative promoter regions or regions comprising promoter elements.

PROMOTER

As used herein, the term “promoter” refers to a segment of DNA that contains the start signals for RNA polymerase and hence promotes transcription at the start of a structural gene. It also comprises the binding site of transcription factors that regulate gene expression. The
promoter DNA segment is typically located in a region 5′ to a structural gene. That is, the promoter DNA segment is typically located it is located in a 5′ region.

EXON

As used herein, the term “exon” means any segment of an interrupted gene that is represented in the mature RNA product. By way of example, an exon may be a region within a gene that codes for a polypeptide chain or domain. Typically, a mature protein is composed of several domains coded by different exons within a single gene.

_INTRON_

As used herein, the term “intron” refers to a segment of an interrupted gene that is not represented in the mature RNA product. Introns are part of the primary nuclear transcript but are spliced out to produce mRNA, which is then transported to the cytoplasm.

LINKED

As used herein, the term “linked” describes the tendency of genes, alleles, loci or genetic markers to be inherited together (that is, the alleles are co-inherited or co-segregate with each other) as a result of their location on the same chromosome. Linkage between genes, alleles, loci or genetic markers may be measured by percent recombination between the two genes, alleles, loci or genetic markers.

In a further embodiment, the present invention provides for diagnoses of disease and/or predisposition to disease by combining information from a plurality of risk polymorphisms which are either themselves linked to certain disease states or are in linkage disequilibrium (LD) with other risk polymorphisms.

LINKAGE DISEQUILIBRIUM (LD)

As used herein, the term “linkage disequilibrium (LD)” refers to the co-inheritance of two alleles at greater frequencies than would be expected from the separate frequencies of occurrence of each
allele in a given control populations. In other words, LD refers to the tendency of specific alleles to occur together more frequently than would be expected by chance. The cause of disequilibrium is often unclear. It may be due to certain allele combinations or to a recent admixture of genetically heterogeneous populations. In addition, in the case of markers that are very tightly linked to a disease gene, an association of an allele (or a group of alleles) with the disease gene is expected if the disease mutation occurred in the recent past so that sufficient time has not elapsed for equilibrium to be achieved through recombination events in that small chromosomal region.

LINKAGE EQUILIBRIUM (LE)

Alleles at a given loci are in linkage equilibrium (LE) if they occur at expected frequencies. The expected frequency of occurrence of two alleles that are inherited independently is the frequency of the first allele multiplied by the frequency of the second allele. In other words, alleles are in linkage equilibrium if the frequency of any particular set of alleles is the product of their individual population frequencies.

VECTORS

As it is well known in the art, a vector is a biological tool that allows or facilitates the transfer of an entity from one environment to another. Examples of vectors used in recombinant DNA techniques include but are not limited to plasmids, chromosomes, artificial chromosomes or viruses.

The term "vector" includes expression vectors and/or transformation vectors.

The term "expression vector" means a construct capable of in vivo or in vitro/ex vivo expression.

The term "transformation vector" means a construct capable of being transferred from one species to another.
VECTOR TRANSFER

The vectors comprising nucleotide sequences (NOIs) of the present invention may be introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation, electroporation and biolistic transformation.

As used herein, the term “transfection” refers to a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

HOST CELLS

A wide variety of host cells can be employed for expression of the NOIs of the present invention, both prokaryotic and eukaryotic. Suitable host cells include bacteria such as E. coli, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, e.g., mouse, CHO, human and monkey cell lines and derivatives thereof. Preferred host cells are able to process the NOI expression products (EPs) to produce an appropriate mature polypeptide. Processing includes but is not limited to glycosylation, ubiquitination, disulfide bond formation and general post-translational modification.

EXPRESSION VECTOR

Preferably, the nucleotide sequence of interest (NOI) which is inserted into a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The expression product (EP) produced by a host recombinant cell may be secreted or may be contained
intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing the NOI can be designed with signal sequences which direct secretion of the NOI coding sequences through a particular prokaryotic or eukaryotic cell membrane.

REGULATION OF EXPRESSION IN VITRO/ VIVO/EX VIVO

The present invention also encompasses gene therapy whereby the NOI is regulated in vitro/in vivo/ex vivo. For example, expression regulation may be accomplished by administering compounds that bind to NOI or control regions associated with the NOI, or its corresponding RNA transcript to modify the rate of transcription or translation.

CONTROL SEQUENCES

Control sequences that may be operably linked to sequences encoding the NOI include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell and/or target cell in which the expression vector is designed to be used. The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

OPERABLY LINKED

The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The NOIs of the present invention can be expressed in an expression vector in which a variant gene is operably linked to a native promoter or other promoter. Usually, the promoter is an eukaryotic promoter for expression in a mammalian cell. The transcription regulation sequences typically include a heterologous promoter and optionally an enhancer which is recognized by the host. The selection of an appropriate promoter, for example trp, lac, phage
promoters, glycolytic enzyme promoters and tRNA promoters, depends on the host selected. Commercially available expression vectors may also be used. Vectors may also include but are not limited to host-recognized replication systems, amplifiable genes, selectable markers, host sequences useful for insertion into the host genome.

**EP ISOLATION**

The expression product (EP) may be isolated by conventional means of protein biochemistry and purification to obtain a substantially pure product, i.e., 80, 95 or 99% free of cell component contaminants, as described in Jacoby, Methods in Enzymology Volume 104, Academic Press, New York (1984); Scopes, Protein Purification, Principles and Practice, 2nd Edition, Springer-Verlag, New York (1987); and Deutscher (ed), Guide to Protein Purification, Methods in Enzymology, Vol. 182 (1990). If the EP is secreted, it can be isolated from the supernatant in which the host cell is grown. If not secreted, the EP can be isolated from a lysate of the host cells.

**TRANSGENIC ANIMALS**

The invention further provides transgenic nonhuman animals capable of expressing the NOI of the present invention and/or having one or more of the NOIs inactivated and/or removed. Expression of an NOI is usually achieved by operably linking the NOI to a promoter and optionally an enhancer, and microinjecting the construct into a zygote. See Hogan et al., "Manipulating the Mouse Embryo, A Laboratory Manual," Cold Spring Harbor Laboratory. Inactivation of NOIs can be achieved by forming a transgene in which a cloned NOI is inactivated by insertion of a positive selection marker. See Capecchi, Science 244, 1288-1292 (1989). The transgene is then introduced into an embryonic stem cell, where it undergoes homologous recombination with an endogenous variant gene. Mice and other rodents are preferred animals. Such animals provide useful drug screening systems.

**BIOLOGICALLY ACTIVE FRAGMENTS**

In addition to substantially full-length EPs (such as a polypeptide) expressed by NOIs of the present invention, the EPs of the present invention may include biologically active fragments,
or analogs thereof, including organic molecules which simulate the interactions of the peptides. Biologically active fragments include any portion of the full-length polypeptide which confer a biological function on the EP, including ligand binding, and antibody binding. Ligand binding includes binding by nucleic acids, proteins or polypeptides, small biologically active molecules, or large cellular structures.

ANTIBODIES

Polyclonal and/or monoclonal antibodies that specifically bind to NOIs of the present invention but not to corresponding wild type gene products are also provided. Antibodies can be made by injecting mice or other animals with the NOI EP or synthetic peptide fragments thereof. Monoclonal antibodies may be screened as is described, for example, in Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, New York (1988); Goding, Monoclonal antibodies, Principles and Practice (2d ed.) Academic Press, New York (1986). Monoclonal antibodies may be tested for specific immunoreactivity with an NOI EP and lack of immunoreactivity to the corresponding wild type gene product. These antibodies are useful in diagnostic assays for detection of the variant form, or as an active ingredient in a pharmaceutical composition.

SCREENS

The NOIs of the present invention and/or a cell line that expresses the NOIs of the present invention may be used to screen for agents capable of affecting the expression of the sequences and/or the biological activity of the EPs thereof.

As used herein, the term “agent” may include but is not limited to a chemical compound, a mixture of chemical compounds, peptides, organic or inorganic molecules a biological macromolecule, or an extract made from biological materials such as bacteria, fungi, or animal (particularly mammalian) cells or tissues.

In one embodiment, the screens of the present invention may identify agonists and/or antagonists of the expression product of the present invention.
In another embodiment, the NOIs of the present invention may be used in a variety of drug screening techniques. By way of example, the NOI or EP thereof to be employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The abolition of binding specificity/biological activity or the formation of binding complexes between the NOI and/or EP thereof and the agent being tested may be measured.

Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity for the NOI of the present invention and is based upon the method described in detail in WO 84/03564.

It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

The invention further provides for a method of identifying a compound to prevent and/or delay and/or reduce and/or treat a disease and/or a predisposition to a disease comprising the steps of: (a) administering a compound to an animal tissue; and (b) determining whether said compound modulates an NOI of the present invention.

In an example of the invention in use a range of compounds are administered to animal cells in tissue culture (in vitro). In this way, thousands of potential small molecule modulators of klotho gene expression and function can be screened. Modulation of the klotho gene can either be determined by assessing whether the candidate compound affects levels of expression of a klotho gene, or whether klotho gene expression product (EP) function is affected. The invention also provides for administration of candidate compounds, that may modulate a klotho gene, to animal tissues in vivo, i.e. administration of compounds to live animals and then assessing their effects by routine methods such as histopathological analysis of tissues.

DIAGNOSIS OF DISEASE

The methods of diagnosis of predisposition to all these disease states involve determining whether an individual possesses the published wild-type sequence or the polymorphism at one or more of the polymorphic sites. In this respect, the genotype of the individual is compared with
the phenotype of the individual. As already used herein, the term “phenotype” means any detectable trait of an individual that is the result of one or more genes.

PREDISPOSITION TO DISEASE

As used herein, the term “predisposition to a disease” means that certain risk polymorphisms are shown to be associated with a given disease state. They are thus over represented in individuals with disease as compared with healthy individuals and indicate that these individuals may be at higher risk for developing disease or may develop a more severe form or particular subset of a disease type.

DISEASE STATES

The disease states which may be associated with risk polymorphisms in the klotho gene include, but are not limited to: metabolic syndrome; obesity; skin disorders; skin atrophy; a reduction in bone mineral density (BMD); osteopaenia; osteoporosis; an age-related disease; a cancer; a cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction and/or a contributory component disease component thereof.

In one embodiment of the invention, the predisposition of an individual to osteoporosis may be assessed by determining whether that individual contains a risk polymorphism of a klotho gene.

TREATMENT

Thus, the therapeutic regime of the present invention may be tailored to the needs of the individual being treated and exposure to adverse side effects minimised. The present invention can therefore be utilised to identify which individuals would be most likely to benefit from, for example, gene therapies. Gene therapy techniques include but are not limited to techniques which replace a faulty gene, such as a klotho gene and/or which downregulate expression of the gene and/or function of the gene product.

In another embodiment, the present invention provides for a method of treating age related disease in an individual, comprising the steps of: (a) genotyping a klotho gene; (b)
determining whether said genotype is a risk genotype; and (c) if said genotype is a risk genotype, applying a treatment to said human in order to prevent premature onset of said age related disease.

COMBINATION THERAPY

The methods of diagnosis of the present invention may be suitable for use in combination with a range of treatments for those suffering or predisposed to disease conditions such as osteoporosis. These treatments include hormone replacement therapy and/or the administration of biphosphonates.

BISPHOSPHONATES TREATMENT

Another suitable treatment (for osteoporosis) is use of biphosphonates. Two specific treatments involve using xanthine oxidase inhibitors or substituted benzodiazepines and are described in US-A-5436258 and US-A-5441964, the contents of which are incorporated herein by reference. Still further treatments will be known to a person of skill in the art. Potential treatments are described, for example, in JP-A-09030977, WO-A-97/06254, JP-A-09025293, WO-A-97/04799, WO-A-97/03060 and JP-A-09012592. Currently authorised treatments for osteoporosis include the use of oestrogens, with and without progestogen, the use of selective oestrogen receptor modulators, the use of anabolic steroids such as nandrolone, the use of the biphosphonates alendronate and disodium etidronate, the use of salcatonin and administration of calcium supplements.

HORMONE REPLACEMENT THERAPY

Hormone replacement therapy (HRT) is an example of a prophylactic therapy that can be used to pre-empt onset of a disease (such as osteoporosis) and can be implemented before symptoms become apparent with the intention of delaying or even preventing the disease. However, is believed that the use of HRT carries with it a concomitant increased risk of breast cancer. The invention offers the advantage that the increased risk of breast cancer associated with HRT can be accepted only by those women who are known to have a likelihood of predisposition to osteoporosis.
OSTEOPOROSIS AND REDUCED BMD

Osteoporosis is a common condition characterised by reduced bone mass, microarchitectural deterioration of bone tissue and increased fracture risk. The risk of osteoporotic fracture is related to bone mineral density (BMD), which in turn is under genetic control.

In one embodiment, the present invention provides for a method of treating disease in a human, comprising the steps of (a) genotyping a klotho gene in said human; (b) determining whether said genotype comprises a risk genotype; and (c) if said genotype comprises a risk genotype, applying a treatment to said human in order to prevent, delay or reduce said disease.

ADMINISTRATION OF OSTEOPOROSIS TREATMENT

In pharmaceutical treatment of osteoporosis, all routes of administration are suitable and include but are not limited to oral administration, injection intravenously, intraperitoneally, intramuscularly and subcutaneously, intranasal administration and topical administration. Typical dosages and durations of treatment are as described in clinician's textbooks such as British National Formulary, incorporated herein by reference. Currently, none of the osteoporosis medications that have been approved by the Food and Drug Administration (FDA) for postmenopausal women has been approved for men. Testosterone replacement therapy may be prescribed for a man with a low testosterone level. Calcitonin is a medication that slows or stops bone loss and may relieve the pain of fractures in some patients. Calcitonin is approved by the FDA for the treatment of osteoporosis in postmenopausal women. While its effect in men has not been studied, evidence suggests that it may work the same in men as in women. Calcitonin is available as an injection and as a nasal spray. Its use is described in US-A-5440012.

Bisphosphonates are a class of drugs that have been shown to help preserve and increase bone density by slowing or stopping bone loss. The FDA has approved the bisphosphonate known as alendronate for the treatment of postmenopausal osteoporosis in women; it is currently being studied for treatment of osteoporosis in men. There are other bisphosphonates under development - and in fact etidronate has been approved, though only outside the USA. Sodium fluoride has recently been recommended for approval by an FDA committee. Parathyroid
hormone, calcitriol, and others are investigational drugs. It will be some time before research findings are available on these preparations. Decrease in bone mineral density can also be slowed by taken calcium supplements, and some suggested levels are 1,000 mg of calcium a day for women on oestrogen replacement therapy and 1,500 mg of calcium daily for women not receiving oestrogen therapy.

MODULATORS

Examples of modulators of a klotho gene of the present invention include but are not limited to compounds and substances that affect the expression of the gene as well as the activity and/or amount of the expressed gene product. Typical modulators suitable for use in the invention include klotho agonists and/or antagonists - either of a klotho gene or of the klotho gene product; large and small molecular weight inhibitors of klotho gene expression or function; inducers and suppressors of a klotho gene; antisense sequences - including antisense oligonucleotides to a klotho gene or transcript; antibodies; klotho gene product binding proteins - including dominant negative versions of the klotho gene product that may be involved in oligomerisation; and klotho gene product kinases; or fragments, variants and derivatives thereof.

An example of a modulator according to the invention is an antisense oligonucleotide that binds to and prevents or reduces transcription of klotho mRNA. This modulator may be used to reduce the activity and/or amount of a klotho gene EP.

As used herein, the term “antisense” is used to refer to a nucleic acid strand that is complementary to the “sense” strand. The designation (-) is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (or positive) strand. An antisense nucleic acid to the klotho NOIs of the present invention may be produced by any method including the synthesis of the klotho NOIs in a reverse orientation to a promoter which permits the synthesis of the coding strand. This transcribed strand may combine with, for example, a natural mRNA produced by a cell to form a duplex. These duplexes may then block either the further transcription of the mRNA and/or its translation.
Another suitable modulator is the EP itself, which can be administered by injection and is used to increase the activity and/or amount of the *klotho* EP.

A further embodiment of the present invention provides for a method of preventing and/or treating disease comprising administering a modulator of a *klotho* NOI.

**ADMINISTRATION OF MODULATORS**

The modulators of the *klotho* gene of the present invention may be suitable for a number of disease states and conditions. Such modulators may be suitable for either prophylactic administration or after a disease has been diagnosed. The route of administration is suitably chosen according to the disease or condition to be treated, however, typical routes of administration of the modulator of the present invention include but are not limited to oral, rectal, intravenous, parenteral, intramuscular and sub-cutaneous routes. The invention also provides for *klotho* modulators to be administered either as DNA or RNA and thus as a form of gene therapy, or as proteins. The modulators may be delivered into cells directly by means including but not limited to liposomes, viral vectors and coated particles (gene gun).

The modulators of the invention may be suitable for treatment of a range of diseases and conditions. In some individuals a combination of diseases may be present or predicted wherein in others only one is diagnosed. Diseases and conditions for which modulators of the *klotho* may be suitably used for treatment typically include diseases such as metabolic syndrome; diabetes; obesity; skin disorders; skin atrophy; reduction in bone mineral density; osteopaenia; osteoporosis; age-related disease; cancer; cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction. Syndromes such as age-related disease and metabolic syndrome are known clinically to manifest as a wide range of physiological conditions and therefore include other conditions, some of which are described in more detail in the examples and figures below.

The present invention also provides for a kit for diagnosis of or predisposition to disease, said kit typically comprising: (a) means for determining the genotype of a *klotho* gene in a human; and (b) reference means for identifying the presence of a risk polymorphism.
KITS

Typically, the kit of the present invention contains all of the necessary components to determine the presence/absence of a polymorphism of the present invention in an individual. These components include, but are not limited to, PCR primers, PCR enzymes, restriction enzymes, a DNA purification means, a DNA sampling means and any other component useful for determining a polymorphic difference between the wildtype klotho gene and an allelic klotho variant of the present invention. By way of example, the kits may comprise at least one allele-specific oligonucleotide primer and/or allele-specific oligonucleotide probe. Alternatively, the kits contain one or more pairs of allele-specific oligonucleotides capable of hybridizing to different forms of a polymorphism. In some kits, the allele-specific oligonucleotides may be immobilized to a substrate. By way of example, the same substrate can comprise allele-specific oligonucleotide probes for detecting at least the SNP risk polymorphisms such as those discussed above. Optional additional components of the kit may include, for example, means used to label (for example, an avidin enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for reverse transcription, PCR, or hybridization reactions. The control/reference sample may comprise a wild type klotho gene or may contain an allele known to be associated with an age-related disease. Alternatively, the reference/control sample may comprise actual PCR products produced by amplification of relevant disease related alleles or may contain genomic or cloned DNA from an individual with a known set of particular disease related alleles. Usually, the kit also contains instructions for carrying out the methods. The kit may also contain a modulator capable of overcoming the risk polymorphism. The kits may be used for detection and measurement of the risk polymorphism, such as SNPs, in biological fluids and tissues, and for localization of an SNP in tissues. The kits may also be used in simultaneously or sequentially with a modulator.

SUMMARY

In summation, methods and means are described for diagnosing a disease or a predisposition to disease by genotyping a klotho gene from an individual and identifying the presence of one or more risk polymorphisms. These risk polymorphisms are associated either directly or indirectly with predisposition to a range of chronic diseases including osteoporosis, skin disorders, age-
related disease, lung dysfunction and metabolic syndrome. Methods of treatment, kits for
diagnostic purposes and an isolated klotho gene including all or some of a range of
polymorphisms are also described as well as methods of isolating molecules that modulate a
klotho gene.

For some aspects, the present invention relates to a method of diagnosis for a disease or a
predisposition to a disease associated with a risk polymorphism in a klotho gene wherein the
risk polymorphism is an SNP risk polymorphism; and wherein the method comprises: (i)
genotyping a klotho gene; and (ii) determining whether the genotype comprises a risk genotype.

The present invention also relates to a method of diagnosis for a disease or a predisposition to a
disease associated with a risk polymorphism in a klotho gene from an individual; wherein the
risk polymorphism is located in a region of the klotho gene selected from the group consisting
of a 5' region of the klotho gene, an exon of the klotho gene and an intron of the klotho gene;
wherein the method comprises: (i) genotyping a klotho gene; and (ii) determining whether the
genotype comprises a risk genotype.

The present invention also relates to a method of preventing and/or treating a disease or a
predisposition to a disease associated with a risk polymorphism in a klotho gene wherein the
risk polymorphism is an SNP risk polymorphism; wherein the method comprises: (i)
genotyping a klotho gene; (ii) determining the presence of a risk genotype; and (iii) applying a
treatment in order to prevent, delay, reduce or treat the disease or the predisposition to the
disease if said klotho gene comprises said risk genotype.

The present invention also relates to a method of preventing and/or treating a disease or a
predisposition to a disease associated with a risk polymorphism in a klotho gene from an
individual; wherein the risk polymorphism is located in a region of the klotho gene selected
from the group consisting of a 5' region, an exon of the klotho gene and an intron of the klotho
gene; wherein the method comprises: (i) genotyping a klotho gene; (ii) determining the presence
of a risk genotype; and (iii) applying a treatment in order to prevent, delay, reduce or treat the
disease if said klotho gene comprises said risk genotype.
The present invention also relates to a method for identifying an SNP risk polymorphism associated with a known risk SNP risk polymorphism in a klotho gene; wherein the method comprises: (i) genotyping for said SNP risk polymorphism associated with an SNP risk polymorphism in a klotho gene; and (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

The present invention also relates to a method of identifying a risk polymorphism in a klotho gene that is associated with a known risk polymorphism in a klotho gene from an individual; wherein the risk polymorphism is located in a region of the klotho gene selected from the group consisting of a 5' region of the klotho gene, an exon of the klotho gene and an intron of the klotho gene; wherein the method comprises: (i) genotyping for said SNP risk polymorphism associated with an SNP risk polymorphism in a klotho gene; and (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

The present invention also relates to an isolated klotho gene comprising at least one SNP risk polymorphism is located within a klotho gene at a position selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.

The present invention also relates to a kit for diagnosis of a disease or a predisposition to disease, wherein the kit comprises: (i) means for genotyping a klotho gene; and (ii) reference means for determining whether the genotype comprises a risk genotype.

Other aspects of the present invention are presented below by way of numbered paragraphs, which include:

1. A method of diagnosing disease or predisposition to disease comprising genotyping a klotho gene.
2. A method according to paragraph 1, wherein said method comprises determining whether said *klotho* gene includes at least one risk polymorphism, wherein presence of said risk polymorphism is diagnostic of disease or predisposition to disease.

3. A method according to paragraph 2, wherein said method comprises determining whether said *klotho* gene includes at least two risk polymorphisms.

4. A method according to paragraph 2 or 3, wherein said risk polymorphism is located in a region of a *klotho* gene selected from the group consisting of a 5' non-coding region, a promoter element, an exon and an intron.

5. A method according to any of paragraph 2 to 4, wherein said risk polymorphism comprises a single nucleotide polymorphism (SNP).

6. A method according to paragraph 5, wherein said risk polymorphism comprises a SNP located within said *klotho* gene at a position selected from the group consisting of nucleotide 1122; nucleotide 1337; nucleotide 1686; nucleotide 2406; nucleotide 12707; nucleotide 12753; nucleotide 19489; nucleotide 19969; and nucleotide 20445.

7. A method according to paragraph 6, wherein said polymorphism comprises at least one SNP selected from the group consisting of a cytosine at nucleotide position 1122; a deleted adenine at position 1337; a guanine at position 1686; a guanine at position 2406; a cytosine at position 12707; an adenine at position 12753; a cytosine at position 19489; a thymine at position 19969; and a thymine at position 20445.

8. A method according to any previous paragraph, wherein said disease comprises at least one of the conditions selected from the group consisting of metabolic syndrome; obesity; skin disorders; skin atrophy; reduction in bone mineral density; osteopaenia; osteoporosis; age-related disease; cancer; cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction.
9. A method of treating disease, comprising: (a) genotyping a klotho gene; (b) determining whether said genotype comprises a risk genotype; and (c) if said genotype comprises a risk genotype, administering prophylactic treatment in order to prevent, delay or reduce said disease.

10. A method according to paragraph 9, wherein said risk polymorphism is located in a region of a klotho gene selected from the group consisting of a 5' non-coding region, a promoter element, an exon and an intron.

11. A method according to paragraph 9 or 10, wherein said risk polymorphism comprises a SNP.

12. A method according to paragraph 11, wherein said risk polymorphism comprises at least one SNP located at a position selected from the group consisting of nucleotide 1122; nucleotide 1337; nucleotide 1686; nucleotide 2406; nucleotide 12707; nucleotide 12753; nucleotide 19489; nucleotide 19969; and nucleotide 20445.

13. A method according to paragraph 12, wherein said risk polymorphism comprises at least one SNP selected from the group consisting of a cytosine at nucleotide position 1122; a deleted adenine at position 1337; a guanine at position 1686; a guanine at position 2406; a cytosine at position 12707; an adenine at position 12753; a cytosine at position 19489; a thymine at position 19969; and a thymine at position 20445.

14. A method according to any of paragraph 9 to 13, wherein said disease comprises at least one of the conditions selected from the group consisting of metabolic syndrome; obesity; skin disorders; skin atrophy; reduction in bone mineral density; osteopaenia; osteoporosis; age-related disease; cancer; cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction.

15. A method according to any of paragraph 9 to 14, wherein said prophylactic treatment comprises hormone replacement therapy (HRT).
16. A method according to any of paragraph 9 to 15 wherein said prophylactic treatment comprises gene therapy.

17. A method of treating age related disease in a human, comprising: (a) genotyping a klotho gene in said human; (b) determining whether said genotype is a risk genotype; and (c) if said genotype is a risk genotype, administering prophylactic treatment to said human for said age related disease.

18. A kit for diagnosis of disease or predisposition to disease, comprising: (a) means for determining the genotype of a klotho gene; and (b) reference means for identifying presence of a risk polymorphism.

19. An oligonucleotide primer comprising the sequence:
   5’ AAATCTACTTTGTCTTCTCG 3’

20. An oligonucleotide primer comprising the sequence:
   5’ AAAGGCACCTGTCTTCTCCC 3’

21. An oligonucleotide primer comprising the sequence:
   5’ TGAATCTGAGAAAAAGTTCATC 3’

22. An oligonucleotide primer comprising the sequence:
   5’ CATTTATGTTAGATAATCTTAGAG 3’

23. An oligonucleotide primer comprising the sequence:
   5’ AGTGGGAATATTGTCTTCTC 3’

24. An oligonucleotide primer comprising the sequence:
   5’ CCATAGGCTGCCACAGGG 3’

25. An oligonucleotide primer comprising the sequence:
   5’ CACCATCCTGCAGTACTATC 3’
26. An oligonucleotide primer comprising the sequence:

\[ 5' \text{T}TTGGTTCA\text{GCCAGTCCTC} 3' \]

27. An isolated klotho gene comprising at least one SNP selected from the group consisting of:
a cytosine at nucleotide position 1122; a deleted adenine at position 1337; a guanine at position 1686; a guanine at position 2406; a cytosine at position 12707; an adenine at position 12753; a cytosine at position 19489; a thymine at position 19969; and a thymine at position 20445.

28. A method of identification of a polymorphism that is correlated with a known polymorphism in a klotho gene comprising:
   - (a) locating a polymorphism and correlating it with the known klotho gene polymorphism;
   - (b) determining whether the new polymorphism is linked to disease or any contributory component thereof.

29. A method according to paragraph 28, comprising determining whether the new polymorphism is linked to a condition selected from the group consisting of metabolic syndrome; obesity; skin disorders; skin atrophy; reduction in bone mineral density; osteopaenia; osteoporosis; age-related disease; cancer; cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction, or a contributory component thereof.


31. A method according to paragraph 30, wherein said disease comprises at least one of the conditions selected from the group consisting of metabolic syndrome; diabetes; obesity; skin disorders; skin atrophy; reduction in bone mineral density; osteopaenia; osteoporosis; age-related disease; cancer; cardiovascular disease; blood pressure disorders; emphysema and lung dysfunction.

32. A method according to paragraph 30 or 31, wherein said modulator comprises a molecule selected from the group consisting of klotho agonists and/or antagonists; small molecular weight inhibitors of klotho gene expression or function; inducers and suppressors of a klotho gene; antisense sequences; antibodies; klotho gene product binding proteins; and klotho gene product kinases; or fragments, variants and derivatives thereof.
33. A method of identifying a compound for treatment of disease, comprising: (a) administration of a compound to animal tissue; and (b) determining whether said compound modulates a klotho gene.

34. A method according to paragraph 33, wherein said animal tissue comprises animal cells in tissue culture.
EXAMPLES

The invention will now be further described only by way of example in which reference is made to the following Figures:

FIGURES

Figure 1 which shows a diagram; and

Figure 2 which shows a diagram.

In more detail:

Fig 1 shows a schematic representation of the human klotho gene with the SNP risk polymorphisms of the present invention and their correlation to disease states indicated underneath. The solid coloured bar represents an association detected between an SNP and phenotype at a significance level of 0.05 or less.

Fig 2 shows a schematic representation of the human klotho gene with the SNP risk polymorphisms of the present invention and their correlation to disease states indicated underneath. A grey bar represents an association detected between an SNP and phenotype at a significance level of between 0.050 and 0.095.

Example 1

The DNA sequence of the human klotho gene has been published by Matsumura et al. (1998) Biochem. Biophys. Res. Comm. 242: 626-630. This sequence which is contained in Genbank records, accession numbers AB009666 and AB009667 is referred to as the wild type sequence. PCR primers were designed to amplify exons 1 to 5 of the human Klotho gene and 2 Kb of sequence 5’ to exon 1, containing the putative promoter regions of the gene. These PCR products were screened to identify common polymorphisms. SNP detection was performed by Standard ABI sequencing technology using Big Dye Terminator cycle sequencing chemistry analyzed on an ABI Prism 377 DNA sequencer. For each PCR fragment, the sequence from 16 unrelated
Caucasian individuals was compared with the consensus “wild type” sequence, to identify any sequence differences. Both forward and reverse strands were sequenced for each fragment and a SNP was only confirmed if it was present on both strands.

9 SNPs identified in the human klotho gene are included in the Table 1, below.

An estimate of allele frequencies in the Caucasian population was made on a sample of 192 unrelated individuals. The frequencies of the rarer alleles are given in Table 2, below. The allele frequencies of all SNPs were in Hardy-Weinberg equilibrium.

For those SNPs with a rare allele frequency of greater than 0.1, pair-wise linkage disequilibrium (LD) values have been estimated (as shown in Table 3). LD estimates indicate the presence of strong background LD across the gene. The presence of strong background LD means that associations between predisposition to disease and a particular genotype or combination of genotypes is representative of the genetic make up of the klotho gene as a whole. Associations with a SNP or combination of SNPs may be a direct result of the effect of the SNP being screened on the function of the klotho gene. Alternatively, the SNP may be in LD with the causative polymorphism.

The following Examples describe the use of four of the nine SNPs identified for diagnosis of disease and/or predisposition to disease. However, because of the strong background LD across the gene, the other five SNPs mentioned herein are also suitable for use in a similar manner for diagnosis of predisposition to disease, either alone or in combination with other SNPs described above.

The four major SNPs referred to in the following Examples are as follows:
<table>
<thead>
<tr>
<th>Accession no. and Nucleotide position</th>
<th>Nucleotide change</th>
<th>Location</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB009666 Nucleotide 1686</td>
<td>Adenine to Guanine</td>
<td>5’ region</td>
<td>None</td>
</tr>
<tr>
<td>AB009667 Nucleotide 12707</td>
<td>Guanine to Cytosine</td>
<td>Codon 370</td>
<td>Cysteine to Serine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 19489</td>
<td>Thymine to Cytosine</td>
<td>Codon 589</td>
<td>Histidine to Histidine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 19969</td>
<td>Cytosine to Thymine</td>
<td>Codon 749</td>
<td>Alanine to Alanine</td>
</tr>
</tbody>
</table>

This analysis is based on a total of 2870 female twin subjects comprising 1025 dizygotic (DZ) pairs and 410 monozygotic (MZ) twin pairs. 1086 women (37.8%) were pre-menopausal and 1135 women (39.5%) were well into their menopause.

Each polymorphism was tested for association with disease related phenotypes using a mean effect model.

A summary of the associations between the four SNPs in the human klotho gene and phenotypes relating to disease states discussed below are included in Figs 1 and 2.

**Example 2**

**Genotyping of SNPs in the human klotho gene**

A known technique that is suitable for genotyping the SNPs mentioned above is Taqman™. In this case two allele specific probes were included in the PCR reaction along with PCR primers. Allelic discrimination was achieved by hybridization of the appropriate probe to the polymorphic site followed by subsequent cleaving of the probe by the 5’ nuclease activity of Taq polymerase during PCR extension of the primers. Primers and probes thus obtained are suitable for screening nucleotide 1686, nucleotide 12707, nucleotide 19489 and nucleotide 19969.
Table 4 (see below) includes examples of primers and probes that are suitable for use in genotyping the klotho gene SNP risk polymorphisms using the Taqman™ technique.

Example 3

Diagnosis of predisposition to osteoporosis based on genotyping of the human klotho gene

Individuals were screened to determine whether they possessed the published wild-type sequence or one or more polymorphisms at nucleotide 12707 (Genbank accession number AB009667), nucleotide 19489 (Genbank accession number AB009667) and nucleotide 19969 (Genbank accession number AB009667). Screening was carried out using the Taqman™ assays described in Example 2.

The homozygous T/T genotype at nucleotide 19489 (Genbank accession number AB009666) was found to be associated with forearm and spine BMD in postmenopausal women. Similarly, the homozygous T/T genotype at nucleotide 19489 (Genbank accession number AB009666) and the homozygous G/G genotype at nucleotide 12707 (Genbank accession number AB009667) were associated decreased VOS in postmenopausal women. In addition, in premenopausal women, the C/C homozygous genotype at nucleotide 19969 (Genbank accession number AB009667) was associated with lower spine BMD and the heterozygous G/C genotype at nucleotide 12707 (Genbank accession number AB009667).

Example 4

Diagnosis of predisposition to disorders of lung function based on genotyping of the human klotho gene

Individuals were screened to determine whether they possessed the published wild-type sequence or the polymorphism at nucleotide 12707 (Genbank accession number AB009667), nucleotide 19489 (Genbank accession number AB009667) and nucleotide 19969 (Genbank accession number AB009667). Screening was carried out using the Taqman™ assays described in Example 2.

The heterozygous G/C genotype at nucleotide 12707 (Genbank accession number AB009666) was associated with lower forced expiratory volume (FEV) and forced expiratory volume/
forced vital capacity ratio (FEV/FVC) in pre-menopausal women. Similarly, the homozygous C/C genotype at nucleotide 19489 (Genbank accession number AB009666) was associated lower FEV pre-menopausal women. In postmenopausal women, the C/C homozygous genotype at nucleotide 19969 (Genbank accession number AB009667) was associated with lower FVC.

Example 5
Diagnosis of predisposition to age related skin disorders based on genotyping of the human klotho gene

Individuals were screened to determine whether they possessed the published wild-type sequence or the polymorphism at nucleotide 12707 (Genbank accession number AB009667 and nucleotide 19969 (Genbank accession number AB009667). Screening was carried out using the Taqman™ assays described in Example 2.

In pre-menopausal women high mole count was associated with the homozygous C/C genotype at nucleotide 19969 (Genbank accession number AB009667). In postmenopausal women the C/T heterozygous genotype at nucleotide 19969 (Genbank accession number AB009667) and the homozygous C/C genotype at nucleotide 12707 (Genbank accession number AB009667) were associated with increased mole count.

Example 6
Diagnosis of predisposition to metabolic syndrome based on genotyping of the human klotho gene

Individuals were screened to determine whether they possessed the published wild-type sequence or the polymorphism at nucleotide 1686 (Genbank accession number AB009666), nucleotide 12707 (Genbank accession number AB009667) and nucleotide 19489 (Genbank accession number AB009667). Screening was carried out using the Taqman™ assays described above.

The homozygous A/A genotype at nucleotide 1686 (Genbank accession number AB009666) was associated with lower serum triglyceride levels in pre-menopausal women. Similarly, the homozygous C/C genotype at nucleotide 12707 (Genbank accession number AB009667) was
also associated with lower serum triglyceride levels in pre-menopausal women. In addition, the C/T heterozygous genotype at nucleotide 19489 (Genbank accession number AB009667) was associated with increased total fat mass and increased weight.

Methods and means for diagnosis and treatment of a number of diseases are thus provided.
Table 1: Position and nature of SNPs identified in the human *klotho* gene

<table>
<thead>
<tr>
<th>Accession no. and Nucleotide position</th>
<th>Position</th>
<th>Nucleotide change</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB009666 Nucleotide 1122</td>
<td>5’ region</td>
<td>Guanine to Cytosine</td>
<td>None</td>
</tr>
<tr>
<td>AB009666 Nucleotide 1337</td>
<td>5’ region</td>
<td>Adenine deletion</td>
<td>None</td>
</tr>
<tr>
<td>AB009666 Nucleotide 1686</td>
<td>5’ region</td>
<td>Adenine to Guanine</td>
<td>None</td>
</tr>
<tr>
<td>AB009666 Nucleotide 2406</td>
<td>Codon 109</td>
<td>Thymine to Guanine</td>
<td>Leucine to Arginine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 12707</td>
<td>Codon 370</td>
<td>Guanine to Cytosine</td>
<td>Cysteine to Serine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 12753</td>
<td>Codon 385</td>
<td>Guanine to Adenine</td>
<td>Lysine to Lysine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 19489</td>
<td>Codon 589</td>
<td>Thymine to Cytosine</td>
<td>Histidine to Histidine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 19969</td>
<td>Codon 749</td>
<td>Cytosine to Thymine</td>
<td>Alanine to Alanine</td>
</tr>
<tr>
<td>AB009667 Nucleotide 20445</td>
<td>Intron 4</td>
<td>Adenine to Thymine</td>
<td>Non coding</td>
</tr>
</tbody>
</table>
Table 2: Estimate of frequency of rarer alleles in 192 unrelated individuals.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Rare Allele</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB009666</td>
<td>C</td>
<td>0.0026</td>
</tr>
<tr>
<td>Nucleotide 1122</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009666</td>
<td>A deletion</td>
<td>0.212</td>
</tr>
<tr>
<td>Nucleotide 1337</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009666</td>
<td>A</td>
<td>0.196</td>
</tr>
<tr>
<td>Nucleotide 1686</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009666</td>
<td>G</td>
<td>0.0078</td>
</tr>
<tr>
<td>Nucleotide 2406</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667</td>
<td>C</td>
<td>0.154</td>
</tr>
<tr>
<td>Nucleotide 12707</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667</td>
<td>A</td>
<td>0.170</td>
</tr>
<tr>
<td>Nucleotide 12753</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667</td>
<td>T</td>
<td>0.411</td>
</tr>
<tr>
<td>Nucleotide 19489</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667</td>
<td>T</td>
<td>0.132</td>
</tr>
<tr>
<td>Nucleotide 19969</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667</td>
<td>T</td>
<td>0.121</td>
</tr>
<tr>
<td>Nucleotide 20445</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Linkage disequilibrium (LD) estimates

<table>
<thead>
<tr>
<th></th>
<th>AB009666 Nucleotide 1337</th>
<th>AB009666 Nucleotide 1686</th>
<th>AB009667 Nucleotide 12707</th>
<th>AB009667 Nucleotide 12753</th>
<th>AB009667 Nucleotide 19489</th>
<th>AB009667 Nucleotide 19969</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB009666 Nucleotide 1337</td>
<td>-</td>
<td>0.999 (P&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009666 Nucleotide 1686</td>
<td>0.999 (P=0.016)</td>
<td>0.659 (P=0.015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667 Nucleotide 12707</td>
<td>-0.630 (P=0.021)</td>
<td>0.679 (P=0.014)</td>
<td>1.00 (P=0.00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667 Nucleotide 12753</td>
<td>-0.625 (P=0.049)</td>
<td>0.563 (P&lt;0.001)</td>
<td>0.999 (P&lt;0.0001)</td>
<td>0.999 (P&lt;0.0001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB009667 Nucleotide 19489</td>
<td>0.104 (P=0.449)</td>
<td>0.563 (P&lt;0.001)</td>
<td>0.999 (P=0.005)</td>
<td>0.999 (P=0.007)</td>
<td>0.999 (P&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>AB009667 Nucleotide 19969</td>
<td>0.620 (P&lt;0.001)</td>
<td>0.526 (P=0.109)</td>
<td>0.999 (P=0.005)</td>
<td>0.999 (P=0.007)</td>
<td>0.999 (P=0.007)</td>
<td>0.982 (P=0.0001)</td>
</tr>
<tr>
<td>AB009667 Nucleotide 20445</td>
<td>0.670 (P=0.09)</td>
<td>0.563 (P&lt;0.005)</td>
<td>0.999 (P=0.007)</td>
<td>0.999 (P=0.007)</td>
<td>0.999 (P=0.0001)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. PCR amplification primers and TaqMan™ probes used to type each allele of the four SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Forward primer sequence (5' to 3')</th>
<th>Reverse primer sequence (5' to 3')</th>
<th>Probes sequence (5' to 3')</th>
<th>Reporter label</th>
</tr>
</thead>
<tbody>
<tr>
<td>1686</td>
<td>TAGGGCCCCGGCAGGAT</td>
<td>CCTGGAGCGGCTCCTGTC</td>
<td>G specific CCCCCAAGTCGGGAAAGTTGGTC</td>
<td>TET</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A specific CCCCCAAGTCGGGAAAGTTGGTC</td>
<td></td>
<td>FAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C specific TTTGCTCTTGCCTTTGGACCCACCT</td>
<td></td>
<td>FAM</td>
</tr>
<tr>
<td>12707</td>
<td>GTTCAATCAAAGGAACCT</td>
<td>GGTCAATCCAGGAAAGCA</td>
<td>G specific TTTGCTCTTGCCTTTGGACCCACCT</td>
<td>TET</td>
</tr>
<tr>
<td></td>
<td>GCTGACTT</td>
<td>GTTG</td>
<td></td>
<td>FAM</td>
</tr>
<tr>
<td>19489</td>
<td>CTGCCATCCAGCCCCA</td>
<td>GGGCCAGTCCAGGGA</td>
<td>C specific TTACTCCAGGAAATGCAAGTTACACATTIT</td>
<td>TET</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T specific TTACTCCAGGAAATGCAAGTTACACATTIT</td>
<td>FAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T specific TTACTCCAGGAAATGCAAGTTACACATTIT</td>
<td>FAM</td>
</tr>
<tr>
<td>19969</td>
<td>CCTGCCCTTCTCCCCAA</td>
<td>AATCTCCAGGCGCAGAAA</td>
<td>C specific CCAAAACTCTCTCGCCACACCTCTT</td>
<td>TET</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>TGG</td>
<td>T specific CCAAAACTCTCTCGCCACACCTCTT</td>
<td>FAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FAM</td>
</tr>
</tbody>
</table>
All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be covered by the present invention.
CLAIMS

1. A method of diagnosis for a disease or a predisposition to a disease associated with a risk polymorphism in a *klotho* gene wherein the risk polymorphism is an SNP risk polymorphism; wherein the method comprises:
   (i) genotyping a *klotho* gene; and
   (ii) determining whether the genotype comprises a risk genotype.

2. A method of diagnosis for a disease or a predisposition to a disease associated with a risk polymorphism in a *klotho* gene in or from an individual; wherein the risk polymorphism is located in a region of the *klotho* gene selected from the group consisting of a 5' region of the *klotho* gene, an exon of the *klotho* gene and an intron of the *klotho* gene; wherein the method comprises:
   (i) genotyping a *klotho* gene; and
   (ii) determining whether the genotype comprises a risk genotype.

3. A method of preventing and/or treating a disease or a predisposition to a disease associated with a risk polymorphism in a *klotho* gene wherein the risk polymorphism is an SNP risk polymorphism; wherein the method comprises:
   (i) genotyping a *klotho* gene;
   (ii) determining the presence of a risk genotype in the *klotho* gene; and
   (iii) applying a treatment in order to prevent, delay, reduce or treat the disease or the predisposition to the disease if said *klotho* gene comprises said risk genotype.

4. A method of preventing and/or treating a disease or a predisposition to a disease associated with a risk polymorphism in a *klotho* gene from an individual; wherein the risk polymorphism is located in a region of the *klotho* gene selected from the group consisting of a 5' region, an exon of the *klotho* gene and an intron of the *klotho* gene; wherein the method comprises:
   (i) genotyping a *klotho* gene;
   (ii) determining the presence of a risk genotype in the *klotho* gene; and
   (iii) applying a treatment in order to prevent, delay, reduce or treat the disease or the predisposition to the disease if said *klotho* gene comprises said risk genotype.
5. A method according to claim 3 or claim 4 wherein said treatment is for an age related disease.

6. A method according to any one of claims 3 to 5 wherein said treatment is or comprises hormone replacement therapy (HRT).

7. A method for identifying an SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene; wherein the method comprises:
   (i) genotyping for said SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene; and
   (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

8. A method of identifying a risk polymorphism in a klotho gene that is associated with a known risk polymorphism in a klotho gene from an individual; wherein the risk polymorphism is located in a region of the klotho gene selected from the group consisting of a 5' region of the klotho gene, an exon of the klotho gene and an intron of the klotho gene; wherein the method comprises:
   (i) genotyping for said SNP risk polymorphism associated with a risk SNP risk polymorphism in a klotho gene a klotho gene; and
   (ii) determining whether the genotype is linked to a risk genotype or is in linkage disequilibrium (LD) with a risk genotype.

9. A method according to any one of the preceding claims wherein the risk polymorphism comprises at least two risk polymorphisms.

10. A method according to any one of the preceding claims wherein the risk polymorphism comprises a haplotype.

11. A method according to any one of the preceding claims wherein the risk polymorphism comprises at least one SNP risk polymorphism located within the klotho gene at a position selected from the group consisting of: nucleotide 1122; nucleotide
1337; nucleotide 1686; nucleotide 2406; nucleotide 12707; nucleotide 12753; nucleotide 19489; nucleotide 19969; and nucleotide 20445.

12. A method according to any one of the preceding claims wherein the polymorphism comprises at least one SNP risk polymorphism located within a klotho gene at a position selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.

13. A method according to any one of the preceding claims, wherein the disease comprises an age related disease and/or a disease associated with one or more of: a metabolic syndrome; obesity; a skin disorder; a skin atrophy; a reduction in bone mineral density (BMD); osteopaenia; osteoporosis; a cancer; a cardiovascular disease; a blood pressure disorder; emphysema; lung dysfunction; or a contributory component thereof.

14. An isolated klotho gene comprising at least one SNP risk polymorphism that is located within a klotho gene at a position selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.

15. A construct comprising the klotho gene according to claim 14.

16. A vector comprising the klotho gene according to claim 14.

17. A plasmid comprising the klotho gene according to claim 14.
18. A host cell comprising the \textit{klotho} gene according to claim 14.

19. A process for preparing an isolated \textit{klotho} protein comprising expressing a nucleotide sequence according to claim 14 such as when present in the entity of any one of claims 15-18 and optionally isolating and purifying the protein.

20. A \textit{klotho} protein produced by the process according to claim 19.

21. An assay method for identifying an agent capable of modulating a \textit{klotho} gene or the expression product thereof wherein the assay method comprises:
   (i) contacting the agent with the \textit{klotho} gene as defined in any one of claim 1 to 12 or as defined in any one of claims 14 to 19 or the expression product thereof;
   (ii) determining whether the agent modulates the \textit{klotho} gene or the expression product thereof.

22. An assay method according to claim 21 wherein the assay is to screen for an agent useful in the prevention and/or treatment of an age related disease.

23. A process comprising the steps of:
   (i) performing the assay according to claim 21 or 22;
   (ii) identifying one or more agents capable of modulating a \textit{klotho} gene or expression product thereof; and
   (iii) preparing a quantity of one or more of the identified agents.

24. A process comprising the steps of:
   (i) performing the assay according to claim 21 or claim 22;
   (ii) identifying one or more agents capable of modulating a \textit{klotho} gene or the expression product thereof; and
   (iii) preparing a pharmaceutical composition comprising one or more of the identified agents.

25. A process comprising the steps of:
   (i) performing the assay according to claim 21 or claim 22;
(ii) identifying one or more agents capable of modulating a \textit{klotho} gene or the expression product thereof;

(iii) modifying one or more of the identified agents; and

(iv) preparing a pharmaceutical composition comprising one or more of the modified agents.

26. An agent identified or modified by the process according to any one of claims 23 to 25.

27. A pharmaceutical composition comprising an agent according to claim 26 and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant or any combination thereof.

28. A method of preventing and/or treating disease associated with a \textit{klotho} gene comprising administering an agent according to claim 26 or a pharmaceutical according to claim 27 wherein said agent or said pharmaceutical is capable of modulating a \textit{klotho} gene or expression product thereof to cause a beneficial therapeutic effect.

29. A method according to claim 28 wherein the disease is that defined in claim 13.

30. Use of an agent according to claim 26 in the preparation of a pharmaceutical composition for the treatment of an age related disease associated with a \textit{klotho} gene.

31. A kit for diagnosis of a disease or a predisposition to disease; wherein the kit comprises:

(i) means for genotyping a \textit{klotho} gene; and

(ii) reference means for determining whether the genotype comprises a risk genotype.

32. A kit according to claim 31 wherein the kit additionally comprises an agent according to claim 26 or a pharmaceutical according to claim 27; wherein said agent or said pharmaceutical is capable of modulating and/or preventing and/or treating an age related disease.
33. A nucleotide sequence comprising any one of:

5' AAATCTACTTTGTCTTCCTCG 3'
5' AAAGGCACCTGTTTCTCCC 3'
5' TGAATCTGAGAAAAAGTTTCATC 3'
5' CATTTATGTTAGATAATCTTAGAG 3'
5' AGTGGAAATATTTGTCTTCCTC 3'
5' CCATAGGCTGCCACAGGG 3'
5' CACCATCCTGCAGTACTATC 3'
5' TTTGGGTCAGCCAGTCCCTC 3'

34. A method according to any one of claims 1-13 wherein the method comprises amplifying a risk polymorphism in a klotho gene using at least one of the nucleotide sequences according to claim 33.

35. A method according to claim 34 wherein the risk polymorphism is an SNP risk polymorphism.

36. A risk polymorphism for use in the diagnosis of a disease or a predisposition to a disease; wherein the risk polymorphism is an SNP risk polymorphism; and wherein the SNP risk polymorphism comprises at least one SNP located within a klotho gene at a position selected from the group consisting of: a cytosine at nucleotide position 1122; a deleted adenine at nucleotide position 1337; a guanine at nucleotide position 1686; a guanine at nucleotide position 2406; a cytosine at nucleotide position 12707; an adenine at nucleotide position 12753; a cytosine at nucleotide position 19489; a thymine at nucleotide position 19969; and a thymine at nucleotide position 20445.
Figure 1: Diagrammatic Representation of Significant (p < 0.05) Phenotype Associations with the KLOTHO gene

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>1686 (998)</th>
<th>cd370 (999)</th>
<th>cd589 (1001)</th>
<th>cd749 (1002)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic Syndrome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Osteoporosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Spine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Hip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Forearm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lung Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung FEV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung FVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV/FVC Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2: Diagrammatic Representation of Trends (p > 0.05, <0.095) in Phenotype Associations with the KLOTHO gene

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>1686 (998)</th>
<th>cd370 (999)</th>
<th>cd589 (1001)</th>
<th>cd749 (1002)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic Syndrome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP Systolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Hip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Forearm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hip Spacing (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>