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(71) Applicant *(for all designated States except US)*: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SPINDLER, Stephen, R.** [US/US]; 6184 Omega Street, Riverside, CA 92506 (US). **DHAHBI, Joseph, M.** [US/US]; 828 Grape Street, Riverside, CA 92507 (US).

(74) Agents: **LOCKYER, Jean, M.** et al.; Two Embarcadero Center, 8th Floor, San Francisco, CA 94111 (US).

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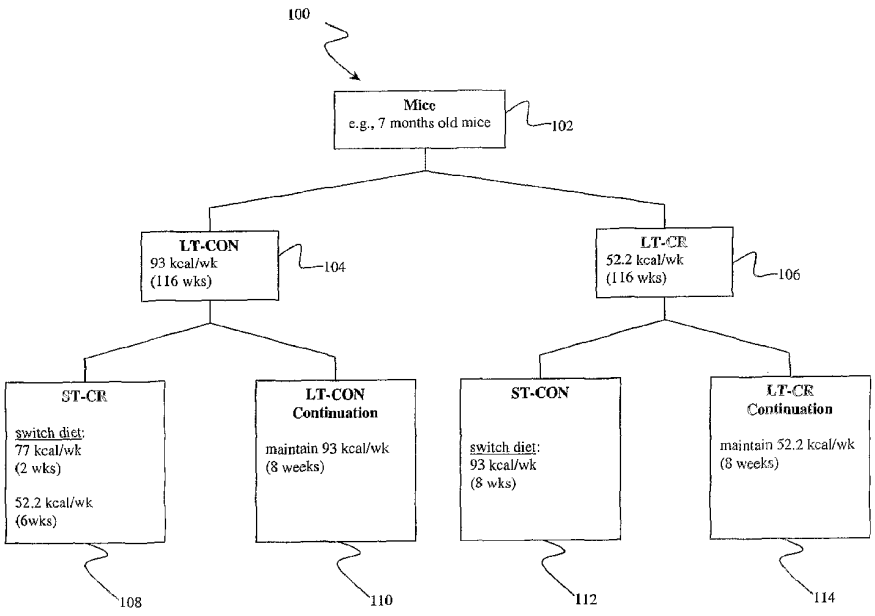
(54) Title: METHODS OF EVALUATING CALORIC RESTRICTION AND IDENTIFYING CALORIC RESTRICTION MIMETICS



(57) Abstract: The invention provides methods of identifying biomarkers of calorie restriction and of examining the dynamics of calorie restriction. In addition, the invention provides methods of selecting mimetics of calorie restriction.

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METHODS OF EVALUATING CALORIC RESTRICTION AND IDENTIFYING CALORIC RESTRICTION MIMETICS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Serial No. 10/387,743, filed March 12, 2003; U.S. Serial No. 10/387786, filed March 12, 2003; and U.S. Serial No. 10/622,160, filed July 16, 2003, each of which applications is incorporated by reference herein.

BACKGROUND OF THE INVENTION

[0002] A major goal of pharmaceutical research has been to discover ways to reduce morbidity and delay mortality. Several decades ago it was discovered that a decrease in caloric intake, termed caloric restriction, can significantly and persistently extend healthy life in animals; see for example, Weindruch, et al., *The Retardation of Aging and Disease by Dietary Restriction*, (Charles C. Thomas, Springfield, Illinois) 1988. CR remains the only reliable intervention capable of consistently extending lifespan and reducing the incidence and severity of many age-related diseases, including cancer, diabetes, and cardiovascular disease. Additionally, physiological biomarkers linked to lifespan extension in rodents (e.g., mice, shrews, and squirrels), other mammals (e.g., rabbits) and monkeys that have been subjected to CR have been shown to be associated with extended lifespan in humans; see for examples, Weyer, et al., *Energy Metabolism after Two Years of Energy Restriction: the Biosphere Two Experiment*, Am. J. Clin. Nutr. 72, 946-953, 2000, and Roth, et al., *Biomarkers of Caloric Restriction may Predict Longevity in Humans*, Science 297, 811, 2002. A study by Walford et al. indicated that healthy nonobese humans on CR diet programs show physiologic, hematologic, hormonal, and biochemical changes resembling those of rodents and monkeys on such CR diets. See Walford, et al., *Calorie Restriction in Biosphere Two: Automations in Physiologic, Hematologic, Hormonal and Biochemical Parameters in Humans Restricted for a Two-Year Period*, J. Gerontol.: Biol. Sci. 57A, 211-224, 2002. These preliminary findings suggest that the anti-aging effects of CR may be universal among all species. The molecular and genetic processes that lead to lifespan extension in animals may extend lifespan in humans.

[0003] It has been known that CR affects gene expression. Understanding what kind of genes or what groups of genes CR affects will be advantageous in the field of genomic

medicine. The understanding of the dynamics of the changes in gene expression in response to CR has been a daunting task. There is currently no method that allows the understanding of the relatedness of genes and how certain genes are affected by similar CR treatments. Understanding of the dynamics of the changes in gene expression in response to CR is important and can lead to more understanding of the behavior, structure, and function of genes. Understanding the behavior, structure, and function of genes also enables grouping of genes that behave similarly and discovering ways to regulate genes as a group. Motif discovery involves taking co-regulated genes and deducing the signal transduction systems that are affected by CR and these systems can be targets for interventions (e.g., drug therapies).

[0004] Most CR studies have led to the widespread idea that CR acts incrementally to prevent the age-related accumulation of deleterious or harmful changes in biological macromolecules and in gene expression. This idea caused many investigators to undervalue the effects of CR on the genes that do not change in expression with age. Additionally, the detailed dynamics or kinetics of the transition of the CR phenotype remain unclear in these studies. Understanding the dynamics of CR enables effective use of CR to perform various treatments, for instance, extending longevity or delaying the onset of age-related diseases. Understanding the dynamics of CR thus also enables the discovery of CR mimetics that can be used efficiently to treat diseases of animals and humans.

[0005] Furthermore, historically, the only accepted assay for evaluating compounds for their effects on aging and the development of age-related diseases has been lifespan studies. However, this method has distinct limitations. Even a "short-lived" mammal like a mouse lives 40 months. Use of a shorter-lived, enfeebled rodent strain introduces confounds into the study. A cohort of at least 60 rodents is required to have the statistical power to reliably detect a 10% change in longevity. Thus, a large-scale CR mimetic screening is impractical using this standard. For more than 25 years, scientists have been searching for biomarkers that would make it possible to detect the development of age-related diseases and the underlying rate of aging over short periods of time. For the most part, these efforts have not met with success. Thus, there is a need for new methods of identity interventions that mimic CR. This invention addresses this need.

BRIEF SUMMARY OF THE INVENTION

[0006] In certain embodiments, the invention provides methods of evaluating the dynamics of caloric restriction and methods of identifying biomarkers of calorie restriction. Furthermore, even though CR brings many benefits to animals and humans, it is not likely that many will avail themselves of a CR lifestyle. The identification and development of CR mimetic compounds or drugs are thus desirable. The invention therefore also provides methods of identifying mimetics of CR and methods of prolonging lifespan by administering such mimetics.

[0007] Certain exemplary embodiments of the present invention allow screening and/or evaluation of at least one compound that mimics or reproduces the effects or some of the effects induced by CR in mammals, for example, mice. In one embodiment, the effectiveness of several compounds (e.g., Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones as well as combinations thereof) are identified and evaluated as CR mimetics because they reproduce at least some of the effects induced by CR. The effects induced by CR and each of the compounds, alone, or in combination, in organs (e.g., livers, hearts, and brains) of mice are evaluated. In one embodiment, gene-expression profiles of mice subjected to CR and mice subjected to the administration of the compounds are evaluated and compared. In other embodiments, a compound or compounds are screened for their ability to inhibit or retard the aging process in mammals.

[0008] In one embodiment, the invention provides a method of analyzing genes comprises administering a first type of a CR dietary program for a first period of time for a first sample; administering a second dietary program for the first sample after the first period of time; and, administering a control diet to a second sample. The gene expression effects or other effects between the first sample and the second sample are analyzed.

[0009] In another embodiment, a method for identifying targets for interventions comprises comparing gene expression levels or protein activity levels in a sample exposed to a first type of CR and to a second type of CR. Genes that appear to have similarity in the responses of both the first and the second types of CR are identified.

[0010] In another aspect, the invention provides methods of identifying interventions that mimic caloric restriction. Thus, in another embodiment, the invention provides a method for identifying a compound that potentially reduces collagen accumulation in myocardium comprising obtaining control data from administering a CR dietary program to one group and

administering a dosage of a compound to another group. At least one collagen measurement resulting from the CR dietary program is compared to at least one collagen measurement resulting from administering a dosage of the compound. The compound is identified to be potentially effective in reducing collagen accumulation based at least in part on the comparison between the collagen measurement resulting from the CR dietary program and the collagen measurement resulting from administering the compound.

[0011] In another embodiment, a method for identifying a compound that potentially reduces collagen accumulation in myocardium and blood vessels comprises obtaining control data from an administering of a CR dietary program to a first mammalian group. The CR dietary program includes at least one of a long-term CR (LT-CR) dietary program and a short-term CR (ST-CR) dietary program. The method also comprises administering an effective dosage of a compound to a second mammalian group. At least one of collagen gene expression or collagen accumulation between the first mammalian group and the second mammalian group are compared. The compound is chosen to be potentially effective in reducing collagen accumulation based at least in part on comparing the collagen gene expression or collagen accumulation between the first mammalian group and the second mammalian group.

[0012] A method of fractionating genetic information into groups is also disclosed. Control data from an administering of a long-term control (LT-CON) dietary program is obtained. A first sample group is subjected to a LT-CON dietary program for a first predetermined period after which, the first sample group is switched from the LT-CON dietary program to a ST-CR dietary program for a second predetermined period. A second sample group is subjected to a LT-CR dietary program for the first predetermined period after which, the second sample group is divided to a third sample group that is switched to a short-term control (ST-CON) dietary program and a fourth sample group that is maintained on the same LT-CR dietary program for the second predetermined period. The effects among the first sample group, the third sample group, and the fourth sample group are compared to the control data and to each other.

[0013] As noted above, the invention provides a method for identifying a compound that mimics at least some of the effects induced by a CR program. In one embodiment, the method comprises administering a CR diet program to a first group of mammals for a predetermined amount of time and administering a dosage of at least one compound to a

second group of mammals for a term which is less than or equal to the predetermined amount of time. The method further comprises assessing changes in gene expression levels, levels of nucleic acids, proteins, or protein activity levels and determining whether the agent mimics the effects induced by the CR diet program.

[0014] Another embodiment describes a method of reproducing at least one effect in mammals that have been subjected to long-term caloric restriction (LT-CR). The method comprises administering a LT-CR diet program to a first group of mammals for a first duration of time and administering at least one compound to a second group of mammals for a second duration of time. The second duration of time is substantially shorter than the first duration of time. The first group of mammals and the second group of mammals are similar, for example, both are groups of mice. Control data from adminsitration of a control diet program is obtained. Effects of the LT-CR diet program and the compound are determined by comparing data obtained from the first group of mammals and the second group of mammals to the control data. Effects between the LT-CR diet program and the compound are compared to determine whether the compound reproduces at least one effect caused by LT-CR.

[0015] Another embodiment describes a method of identifying a compound that reproduces effects of CR. The method comprises administering an effective dosage of a compound to a first group of mammals for a duration of time; administering a CR diet program to a second group of mammals; and obtaining control data from administering a control diet program. The first group of mammals and the second group of mammals are similar, for example, both are groups of mice. The method further comprises analyzing changes in gene expression levels, levels of nucleic acids, protein, or protein activity levels, in each of the first group of mammals and the second group of mammals. The compound is identified as one that reproduces changes induced by CR when the compound produces analyzed changes in the first group of mammals wherein at least about 1% or one or more changes of the analyzed changes are a subset of the changes induced by CR. In one embodiment, the changes in gene expression levels, levels of nucleic acids, protein, or protein activity levels, in each of the first group of mammals and the second group of mammals are compared to the control data to identify and compare the changes.

[0016] Another embodiment describes a method for searching for a compound. The method comprises administering a ST-CR diet program to a first group of mammals for a

predetermined amount of time and administering a dosage of at least one compound to a second group of mammals, for a term which is less than or equal to the predetermined amount of time. The method further comprises assessing changes in gene expression levels, levels of nucleic acids, proteins, or protein activity levels and determining the compound's ST-CR mimetics effects.

[0017] Another embodiment describes a method of extending longevity (or increasing maximum life span) for a mammal that is otherwise healthy. The method comprises administering an effective dosage of at least one of Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones (or combinations thereof) to the mammal for an effective amount of time.

[0018] Another embodiment disclosed a method of reproducing effects of CR comprising administering an effective dosage of at least one of Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones to a mammal for an effective amount of time.

[0019] In other embodiments, the biological age or metabolic state of an organism (e.g., a mammal) may be assessed by determining the gene expression level of one or more of the genes listed in Tables 5-9, Table 2, or Tables 14-16.

[0020] In some embodiments, methods of evaluating the initial effect of CR on longevity and gene expression are disclosed. The results obtained from these embodiments indicate that the effects of CR on lifespan are induced rapidly after a shift from the normal diet to the restricted diet (e.g., CR diet program). They also indicate that the gene expression effects are rapidly induced in a stepwise manner. In addition the gene expression effects of CR are rapidly reversible. The results from these embodiments have major implications for fully understanding CR and CR dynamics.

[0021] In another aspect, the invention provides a method of evaluating the dynamics of CR. In one exemplary embodiment the method comprises obtaining control data from administering a long-term control diet program. Next, each of several mammalian sample groups is subjected to a CR diet program for a different amount of time relative to other sample groups. The effects of CR between each of the several mammalian sample groups and the control data are compared to each other. Additionally, the effects of CR for different amounts of time are analyzed.

[0022] In another exemplary embodiment the method comprises dividing a mammalian sample group into a first sample group and second sample group. The first sample group is

subjected to a long-term control diet program (e.g., a normal, non CR diet) for a first predetermined period. The second sample group is subjected to a long-term caloric restriction diet program for a second predetermined period. After the first predetermined period, portions of the first sample group are switched to a caloric restriction diet program for different amounts of time. After the second predetermined period, at least a portion of the second sample group is switched to a control diet program for a third predetermined period and the remaining portion of the second sample group is maintained on the long-term caloric restriction diet program. Effects of CR among members of the first sample group and the second sample group are compared to one another.

[0023] In another exemplary embodiment, a method of reversing some effects of CR is disclosed. The method comprises administering a control diet program to a mammalian sample group that has been subjected to a long-term caloric restriction diet program, wherein the control diet program includes higher caloric intake for the mammalian sample group than the caloric intake for a long-term caloric restriction diet program.

[0024] In another exemplary embodiment, a method of extending longevity in an old mammal is disclosed. The method comprises administering a caloric restriction diet program to the old mammal. In one case the old mammal is an old mouse. The old mouse may be more than 18 months old. In another case, the old mammal is a human of about more than 50 years old. Additionally, administering the CR diet program includes shifting the old mammal to the CR diet program in stages, with at least one stage including a gradual decrease in the number of calories in the diet program.

[0025] In another exemplary embodiment, a method of identifying an intervention for use in old subjects is disclosed. The method comprises administering a control diet program (e.g., a diet with a normal amount of calories) to individuals in the first sample group. After start of old age, at least one candidate intervention is administered to the individuals in the first sample group. The effects of the candidate intervention are compared to the effects from a CR or control or another diet program administered to the second sample group. Normally, a single candidate intervention may be administered to individuals in the first sample group in order to avoid interactions between interventions. However, it is also desirable to perform alternative methods in which a group of two or more candidate interventions is administered concurrently to individuals in another first sample group to observe the effects from the group of interventions.

[0026] In another exemplary embodiment, a method of identifying an intervention and performing at least one biochemical measurement after exposing a biological sample to the intervention is disclosed. The biochemical measurement is designed to show whether the intervention mimics substantially or at least some of the effects of CR. The intervention is then withdrawn from the biological sample. At least one further biochemical measurement is performed after withdrawing the intervention. At least one further biochemical measurement is made to determine whether withdrawing the intervention mimics substantially or at least some of the effects of withdrawing CR. Normally, a single candidate intervention may be administered to a biological sample in order to avoid interactions between interventions. However, it is also desirable to perform alternative methods in which a group of two or more candidate interventions is administered concurrently to another biological sample to observe the effects from the group of interventions.

[0027] These and other features and advantages of embodiments of the present invention will be more readily apparent from a detailed description of the embodiments, set forth below, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] Figure 1 illustrates an exemplary dietary regimen scheme that various groups of samples are subjected to.

[0029] Figures 2A-2B illustrate how genes are categorized into clusters based on various caloric restriction dietary regimens.

[0030] Figure 3 illustrates exemplary results of real time RT-PCR (reverse transcriptase-PCR) data validating microarray data to confirm gene changes; (PCR is Polymerase Chain Reaction).

[0031] Figure 4 illustrates an exemplary dietary regimen scheme that various test groups are subjected to.

[0032] Figure 5 illustrates an analysis of gene expression changes in mouse liver following 8 weeks of treatment with various compounds according to some embodiments. Analysis of gene expression changes in mouse liver following 8-weeks of treatment with the indicated glucoregulatory compound. The extent to which a given drug reproduces CR-specific gene expression profiles is represented by the size of the indicated area of the charts. The number

of genes in each group is indicated by the colored "slices" of the chart. The number of genes in each category is indicated within each slice. The number of total genes altered by each drug or combination of drugs is given in parentheses. The gene numbers are from Tables 5-10. The percentages of the total genes in each group are given in Table 4.

[0033] Figure 6 illustrates a Venn diagram analysis. The analysis shows the overlap between the effects of LT-CR, ST-CR and of each of the drugs used. The numbers in parentheses indicate genes which a given dug induced to change expression in a direction opposite to that produced by LT-CR. The gene numbers are from Tables 5-10.

[0034] Figure 7 illustrates an exemplary embodiment in which various diet programs (for different periods of time) are administered to old mammals such as old mice;

[0035] Figure 8 illustrates the effect of CR on longevity of mice that are subjected to CR at an old age.

[0036] Figure 9 illustrates diet programs that are administered to mice in accordance with some embodiments of the present invention.

[0037] Figures 10A-10B illustrate the dynamics of changes in expression of genes whose expression is affected by CR. 10A) For 54 Affymetrix unique identifiers, early changes in expression initiated after 2, 4 or 8 weeks are sustained through the subsequent time points. For 35 Affymetrix unique identifiers, changes in expression require more than 8 weeks of CR treatment (LT-CR). 10B) For the remaining 34 Affymetrix unique identifiers, there is no consistent pattern after changes in gene expression are initiated after 2, 4 or 8 weeks. The changes in gene expression are not maintained in the same direction through the subsequent time points. An 8-week switch of LT-CR to the control diet segregated the 123 Affymetrix unique identifiers into more clusters (CON8).

[0038] Figures 11A-11E illustrate a result using real-time reverse transcriptase PCR (real time RT-PCR) to validate the changes in gene expression of the genes affected by CR; PCR is Polymerase Chain Reaction.

[0039] Figure 12 illustrates a method of identifying an intervention in accordance with some embodiments of the present invention.

[0040] Figure 13 illustrates a method of identifying an intervention for use in mammalian subjects of old age.

[0041] Figure 14 illustrates an exemplary method of determining whether a CR effect is reversible.

[0042] Figure 15 illustrates an exemplary method of determining whether the effects of a CR mimetic are reversible.

Description of the Tables

[0043] Table 1 illustrates exemplary primer sequences for real time RT-PCR that can be used for some embodiments of the present invention.

[0044] Table 2 illustrates some effects of LT-CR, ST-CR and ST-CON dietary regimens.

[0045] Table 3 illustrates 8 various treatments (with exemplary dosage of the compounds) that can be administered to a test group such as mice.

[0046] Table 4 illustrates percentage of compound-specific or drug-specific effects and overlap between the effects of CR and those of each of the treatments used.

[0047] Table 5 illustrates effects of Metformin and CR on hepatic gene expression.

[0048] Table 6 illustrates effects of Glipizide and CR on hepatic gene expression.

[0049] Table 7 illustrates effects of Glipizide and Metformin and CR on hepatic gene expression.

[0050] Table 8 illustrates effects of Rosiglitazone and CR on hepatic gene expression.

[0051] Table 9 illustrates effects of Soy Isoflavones and CR on hepatic gene expression.

[0052] Table 10 illustrates genes with gene expression that are altered in the opposite direction by LT-CR and the compounds/drugs being tested.

[0053] Table 11 illustrates the percentage of CR effects reproduced by different compounds.

[0054] Table 12 illustrates dietary compositions of the control diet program and the CR diet program. Values are g ingredient/100 g of diet for these formulations. Mice on the control diet were fed 93 kcal per week fo the control diet (AIN-93M). Mice on the CR diets were fed 77 kcal per week of the CR diet or 52 kcal per week 0f the CR diet (40% calorie restricted AIN-93M).

[0055] Table 13 illustrates exemplary primary sequences for real time RT-PCR that can be used for some embodiments of the present invention.

[0056] Table 14 illustrates genes whose expression is affected by long-term CR.

[0057] Table 15 illustrates genes that display consistent changes in expression in response to CR administered for varying time points (e.g., a two-week CR, a four-week CR, an eight-week CR, and a long-term CR), such expression level changes being either consistently higher or lower than the control group, across all time points of CR.

[0058] Table 16 illustrates genes whose expression is affected by short-term CR and long-term CR in different directions.

DETAILED DESCRIPTION OF THE INVENTION

[0059] In the following description, for purposes of explanation, numerous specific details are set forth in order to provide a thorough understanding of exemplary embodiments of the present invention. It will be evident, however, to one skilled in the art, that these embodiments may be practiced without these specific details. In other instances, specific structures and methods have not been described so as to not obscure the present invention. The following description and drawings are illustrative of the invention and are not to be construed as limiting the invention.

[0060] Throughout the discussion, the following terminologies are used. A control (CON) diet program or regimen refers to a normal feeding program having a normal number of calories (e.g., 93 kcal per week for a mouse test subject). A CR diet program refers to a dietary regimen with a reduced amount of calories (e.g., 77 kcal per week or 52 kcal per week for a mouse test subject). It is to be appreciated that the number of calories per week can be modified to adjust to what is considered normal for a particular test subject. A long-term caloric restriction (LT-CR) diet program refers to a reduced dietary regimen for a long duration of time, e.g., for more than eight weeks in the case of mice, or between about several months to about 36 months, or to about the end of life in some cases. A short-term caloric restriction (ST-CR) diet program refers to a reduced dietary regimen for a short duration of time, e.g., for about eight weeks or less than eight weeks, e.g., six weeks, four weeks, two weeks, two days, or one day, in the case of mice. In certain situations, a diet program may be an ST-CR diet program which runs until about the end of life, when the ST-CR diet program is begun after a control diet program (e.g., a control diet program was administered to one or

more animals in a test group for a long duration and the diet program for these animals was switched to a ST-CR diet program for the rest of the animals' lives). It is to be appreciated that the number of weeks or months that constitutes a short or long duration of time for a diet program or regimen can vary depending on experimental designs, test groups, mammalian species, etc.

[0061] A ST-CR group refers to a test group or a sample group that is subjected to a ST-CR diet program. A ST-CR group may further be divided into several sub ST-CR groups, for example a CR2 group, a CR4 group, and a CR8 group. A CR2 group refers to a ST-CR group that is subjected to the ST-CR diet program for a two-week duration. A CR4 group refers to a ST-CR group that is subjected to the ST-CR diet program for a four-week duration. A CR8 group refers to a ST-CR group that is subjected to the ST-CR diet program for an eight-week duration.

[0062] A short-term control (a ST-CON) group refers to a test group or a sample group that is subjected to a control diet program for a short duration of time relative to another diet program for a longer duration of time. A CON8 group refers to a test group or a sample group that is subjected to a control diet program for a duration of 8 weeks. Similarly, CON4, CON6, etc. refer to the length of time (in weeks) that an animal is subjected to a control diet.

[0063] A LT-CR group refers to a test group or a sample group that is subjected to a LT-CR diet program. A long-term control (LT-CON) group refers to a test group or a sample group that is subjected to a control diet program for a long duration of time.

[0064] CR mimetic compounds or drugs are compounds capable of mimicking at least some of the anti-aging, anti-disease effects, and other beneficial effects of CR without a substantial reduction in dietary calorie intake or without reducing the subject's weight below a normal weight.

[0065] A drug group refers to a test group or a sample group that is subjected to a regimen for a duration of time (e.g., a predetermined period of time). The regimen includes an administration of at least one intervention or a candidate intervention. An intervention can be a compound or a pharmaceutical agent (e.g., drug) that can be a potential CR mimetic. A candidate intervention can be a compound or a pharmaceutical agent (e.g., drug) that can be a potential CR mimetic or it may be a group of compounds or pharmaceutical agents. The drug group can also be divided into several sub-drug groups, for example, a 2Wk-drug, a 4Wk-drug, and an 8Wk-drug, which represent different periods of exposure to an intervention (2

weeks, 4 weeks, and 8 weeks, respectively, in this example). A drug-withdrawn group refers to a test group or sample group that is subjected to withdrawal of the intervention that is administered to one of the groups as described above. The withdrawal of the intervention may be for a predetermined amount of time.

[0066] Moreover, a long-term drug group refers to a test group or a sample group that is subjected to a dietary regimen that includes administration of at least one compound, test compound or a pharmaceutical agent for a long duration of time, wherein the compound can be a CR mimetic candidate or a potential CR mimetic candidate. A short-term drug group refers to a test group or a sample group that is subjected to a dietary regimen that includes administration of at least one compound, test compound, or a pharmaceutical agent for a short duration of time, wherein the compound can be a CR mimetic candidate or a potential CR mimetic candidate. A short-term drug withdrawn group refers to a test group or a sample group that is subjected to a withdrawal of the compound that is administered to the group as described in either the long-term drug group or the short-term drug group where the withdrawal is for a short term.

[0067] Exemplary embodiments are described with reference to specific configurations and techniques. In some aspects of the inventions, exemplary embodiments pertain to methods of analyzing effects induced by CR or CR mimetics and in some embodiments at different stages of CR treatment or CR mimetic treatment. Other embodiments relate to methods of screening for CR mimetics and reproducing the effects induced by CR.

[0068] The effects of CR and CR mimetics can be assessed using a variety of assays. Such assays include at least one of the changes in gene expression levels (e.g., mRNA levels), changes in protein levels, changes in protein activity levels, changes in carbohydrate or lipid levels, changes in nucleic acid levels, changes in rate of protein or nucleic acid synthesis, changes in protein or nucleic acid stability, changes in protein or nucleic acid accumulation levels, changes in protein or nucleic acid degradation rate, and changes in protein or nucleic acid structures or function. The effects also include extending the longevity or life span of mammals (e.g., extending the longevity of mice). The following discussion focuses on several exemplary methods of identifying and categorizing genes that are expressed, not expressed, or otherwise altered (e.g., negatively or positively regulated) as induced by CR or a CR mimetic. The following discussion also focuses on extending the longevity of old

mammals, for example, old mice, by subjecting the old mammals to a CR diet program in at least one stage.

[0069] A CR mimetic refers to a compound, a test compound, an agent, a pharmaceutical agent, or the like, that reproduces at least some effects induced by CR. It is to be appreciated by one skilled in the art that the exemplary methods are not limited to analyzing gene expressions that are affected by CR or CR mimetics but may include changes in physiological biomarkers such as changes in protein levels, protein activity, nucleic acid levels, carbohydrate levels, lipid levels, the rate of protein or nucleic acid synthesis, protein or nucleic acid stability, protein or nucleic acid accumulation levels, protein or nucleic acid degradation rate, protein or nucleic acid structures or functions, and the like.

[0070] Currently, CR that is started early, either early in life, or middle age, represents the best-established paradigm of retardation of aging in mammals. See for example, Weindruch, et al., *The Retardation of Aging and Disease by Dietary Restriction,* (C.C. Thomas, Springfield, IL, 1988). The effects of CR on age-related parameters are broad. CR increases maximum lifespan, reduces and delays the onset of age-related disease, reduces and delays spontaneous and induced carcinogenesis, suppresses autoimmunity associated with aging, and reduces the incidence of several age-induced diseases (Weindruch, *supra* 1988).

[0071] Even though CR brings many beneficial effects to animals and humans, it is not likely that many will avail themselves of a CR lifestyle. As is known, it is difficult for any animal or human to maintain a diet program. Additionally, many believe that CR only acts incrementally or progressively to bring benefits to mammals such as extending lifespan and reducing and delaying the onset of age-related diseases. Such belief has not encouraged the use of CR to treat old mammals. Thus, there is a need to identify the dynamics of CR to determine whether CR can act rapidly such that CR can be beneficial to old mammals and not just young or middle-age mammals. There is also a need to identify, evaluate, and/or develop an intervention that is capable of mimicking some of the effects of CR, especially the beneficial effects, without the reduction of dietary calorie intake as required by CR diet programs. There is also a need to identify an intervention that can be administered to a mammal and that can rapidly reproduce the beneficial effects of CR. Identifying such interventions will enable treatments for mammals at almost all stages of life.

[0072] CR or CR mimetics may affect some genes in similar ways. Understanding the dynamics of the changes in gene expression in response to CR or CR mimetics is important

since it may allow for more understanding of the behavior, structure and function of genes in a particular group. Furthermore, understanding the behavior, structure, and function of genes, how they interact within a group, and how they respond to CR and CR mimetics will enable the discovery of ways to regulate genes as a group. Thus, there is a need to identify the dynamics of changes of gene expression in groups of genes and to identify relatedness of genes to one another based on singular CR or CR mimetic treatments. When the dynamics of the changes in gene expression for groups of genes are better understood, it becomes easier and more efficient to regulate genes as a group or groups using fewer compounds and mechanisms.

[0073] In one embodiment, a mammalian sample group is chosen. The sample group can be any mammal. Often, rodents such as laboratory mice are employed. The mice are divided into groups, each of which will undergo a different treatment. For example, one or more groups of mice is subjected to a CR dietary program (reduced number of calories in the diet) generating one or more CR groups. Another group of mice can be a control group, which is subjected to a control (normal number of calories) dietary program generating a control group.

[0074] The CR group can, for example, be then divided into sub-groups, *e.g.,* two subgroups, one of which is switched to the control dietary program while the other is maintained on the same CR dietary program. The control group is also be divided into sub-groups, *e.g.,* two sub-groups one of which is switched to a CR dietary program while the other is maintained on the same control dietary program. Under these switching of dietary regimens, genes that are similarly affected by a certain CR regimen individually and as a group can also be determined. As will be apparent below, switching the dietary regimen affects certain genes or groups of genes in the same way. This allows for the discovery of regulatory factors and signal transduction pathways that control gene expression. In another embodiment, a compound (or a CR mimetic) can also be administered to a group of mice in similar manner, for example, switching a control diet group to a test compound group. From the results, it can be determined whether the compound can reproduce or mimic at least some effects that are caused by CR.

[0075] Other sample groups, *e.g.,* mice, can be used for testing interventions such as pharmaceutical compounds or agents, to determine whether such intervention reproduce the effects (oar at least some of the effects) of CR. The effects caused by the different

interventions are compared to the control group and/or to each other. Comparing the effects of CR and the various compounds on the mice will allow determination or identification of CR mimetic compounds.

[0076] It will be recognized that the various embodiments described herein can be used with non-mammal organisms such as insects, nematodes, yeast, bacteria, and other organisms. In some situations, techniques may be performed in these non-mammal organisms and then candidate drugs, discovered in those organisms, can be tested in mammals (e.g., humans).

[0077] It is also to be noted that control data can be obtained from a prior study, the results of which are recorded, as opposed to a control treated concurrently with a test group. For example, data can be obtained from a control group of mice subjected to a control diet program and the data recorded, or the data may be obtained from control animals treated with a control diet when the test animals are subjected to treatment. Thus, the control data may be obtained from an administering of a control diet program which was previously performed.

[0078] This control data may be obtained once and stored for recall in later screening studies for comparison against the results in the later screening studies. Similarly, gene expression levels from LT-CR or ST-CR (or other types of measurements such as changes in protein levels, changes in protein activity levels, changes in carbohydrate or lipid levels, changes in nucleic acid levels, changes in rate of protein or nucleic acid synthesis, changes in protein or nucleic acid stability, changes in protein or nucleic acid accumulation levels, changes in protein or nucleic acid degradation rate, and changes in protein or nucleic acid structure or function) may be evaluated and recorded once for recall in later screening studies for comparison against the results in the later screening studies. Of course, it is typically desirable to have the prior stored studies have a similar (if not identical) set of genes (or other parameters such as proteins) relative to the genes (or other parameters) in the later screening studies in order to perform a comparison against a similar set of genes or other parameters.

[0079] An "expression pattern", as used herein, refers to changes in a biomarker. An "expression pattern" can be determined by measuring levels of mRNA, levels of protein, changes in protein activity levels, changes in protein activity, changes in protein modification, e.g., phosphorylation, changes in carbohydrate or lipid levels, changes in rate of protein or nucleic acid synthesis, changes in protein or nucleic acid stability, changes in protein or nucleic acid accumulation levels, changes in protein or nucleic acid degradation

rate, and changes in protein or nucleic acid structure or function, and the like. Such changes can be measured using methodology known in the art. Typically, the parameters to be measured are determined using cells, typically a tissue or organ, obtained from the test and control samples.

[0080] For example, isolated organs or tissues can be used to perform many different types of analysis that allow for determination of effects of each of the different treatments. Some embodiments focus on the determination of changes in gene expression levels. It is to be noted that the exemplary methods discussed are not limited only to analyzing genes expressions that are affected by CR or CR mimetics but are also to include changes in physiological biomarker expression patterns as set forth above.

EXAMPLES

[0081] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

[0082] Figure 1 illustrates an exemplary scheme 100 of the various dietary regimens for mammalian samples. In one embodiment, the mammalian samples are mice. Male mice of the long-lived F1 hybrid strain B6C3F1 were fed and maintained as described in Dhahbi, et., al., *Caloric intake alters the efficiency of catalase mRNA translation in the liver of old female mice*, J.Gerontol.A Biol.Sci.Med.Sci.; 53: B180-B185, 198, which is hereby incorporated by reference. Briefly, the mice were purchased from Jackson Laboratories (Bar Harbor, ME 04609). For the first seven months, mice were fed rodent diet No. 5001 (TMI Nutritional International LLC, Brentwood, MO 63044). At seven months, all mice were individually housed. The seven-month old mice are indicated as mice group 102 as shown in Figure 1. The mice from the group 102 were randomly assigned to one of two groups, a LT-CON group 104 and a LT-CR group 106. Each mouse in the LT-CON group 104 was subjected to a LT-CON dietary program with feeding of 93 kcal per week of a semi-purified control diet in 1 gm pellets (AIN-93M, Diet No. F05312, BIO-SERV, Frenchtown, NJ, 08825). A complete list of diet ingredients can be found on the Harland Teklad website http://www.teklad.com/custom/index.htm. Each mouse in the LT-CR group 106 was subjected to a LT-CR dietary program with feeding of 52.2 kcal per week of a semi-purified CR diet (AIN-93M 40% Restricted, Diet No. F05314, BIO-SERV).

[0083] In one embodiment, after 29 months of age (116 weeks), the mice from both the LT-CON group 104 and the LT-CR group 106 were subjected to a crossover (or switching) experiment in which LT-CR and LT-CON mice were switched to the opposite dietary regimen for 2 months (8 weeks). In one embodiment, half of the mice from the LT-CON group 104 were switched to a ST-CR dietary program for 8 weeks generating a ST-CR group 108. The other half of the mice from the LT-CON group 104 continued with the LT-CON dietary program for 8 weeks generating a LT-CON continuation group 110. Note that there is no change in the dietary regimen for the mice that are not switched to the ST-CR dietary program. Hence, for clarity of discussion, the group of mice that is maintained on the LT-CON dietary program is referred to as a LT-CON continuation group. Thus, a LT-CON continuation group may simply refer to a group of mice that is subjected to a LT-CON dietary program. Additionally, half of the mice from the LT-CR group 106 were switched to a short-term ST-CON dietary program for 8 weeks generating a ST-CON group 112. The other half of the mice from the LT-CR group 106 continued with the LT-CR dietary program for 8 weeks generating a LT-CR continuation group 114. There is no change in the dietary regimen for the mice that are not switched to the ST-CON dietary program. The group of mice that are continued with the LT-CR dietary program is thus referred to as a LT-CR continuation group, which simply refers to a group of mice that is subjected to a LT-CR dietary program.

[0084] In one embodiment, the mice from the ST-CR group 108 were mice from the LT-CON group 104 that were switched from a 93 kcal per week diet to a 77 kcal per week diet for 2 weeks, followed by a 52.2 kcal per week diet for 6 weeks. The mice from the ST-CON group 112 were the mice from the group LT-CR 106 that were switched to a control dietary program for 8 weeks in which the mice were switched from a 52.2 kcal per week diet to a 93 kcal per week diet. Thus, in one embodiment, the switching of the groups of mice to different dietary programs generates 4 sample groups, LT-CON continuation group 110, LT-CR continuation group 114, ST-CON group 112, and ST-CR group 108. In one embodiment, each group includes 4 mice.

[0085] All mice were killed at 124-weeks of age (31 months). Mice from all groups were fasted for 48 hours before killing. Mice were killed by cervical dislocation, and hearts rapidly excised, rinsed in PBS to remove blood, and flash frozen in liquid nitrogen. No signs of pathology were detected in any of the animals used. All animal use protocols were approved by an institutional animal use committee.

[0086] It is also to be noted that control data can be obtained from a prior study, the results of which are recorded as opposed to a control group of mice subjected to a control diet program concurrently with the test groups of mice as illustrated in Figure 1. Thus, the control data may be obtained from an administering of a control diet program which was previously performed. This control data may be obtained once and stored for recall in later screening studies for comparison against the results in the later screening studies. Similarly, gene expression levels from LT-CR or ST-CR (or other types of measurements such as protein levels, nucleic acid levels, carbohydrate levels, lipid levels) may be evaluated and recorded once for recall in later screening studies for comparison against the results in the later screening studies. Of course, it is typically desirable to have the prior stored studies have a similar (if not identical) set of genes (or other parameters such as proteins) relative to the genes (or other parameters) in the later screening studies in order to perform a comparison against a similar set of genes or other parameters.

[0087] The effects caused to each of the four groups of mice (LT-CON continuation group 110, LT-CR continuation group 114, ST-CON group 112, and ST-CR group 108) were compared to each other. In one embodiment, the effects were used to determine the effects of CR on gene expression caused by each of the different dietary programs. In one embodiment, the effects of LT-CR on gene expression were determined by comparing the results between the LT-CON continuation group 110 and the LT-CR continuation group 114. The effects of ST-CR were determined by comparing the results between the LT-CON continuation group 110 and the ST-CR group 108. The effects of ST-CON were determined by comparing the results between the LT-CON continuation group 110 and the ST-CON group 112.

[0088] In other embodiments, a test compound (or test compounds) that is a CR mimetic candidate or a potential CR mimetic can be administered to the a group of mice. For example, in addition to, or instead of, switching some of the LT-CON group 104 to the ST-CR dietary program (e.g., to generate the ST-CR group 108), some of the mice from the LT-CON group 103 can be switched to a dietary program that includes the test compound. The effects of this test compound can then be determined by comparing the results between the LT-CON group and the test compound group in the same way that the results for the ST-CR is obtained by comparing the results between the ST-CR group 108 and the LT-CON continuation group 110. Similarly, a group of mice can be subjected to a dietary program that includes the test compound for the same duration as the LT-CR dietary program generating

for example, a long-term drug group. After this duration, some of the mice from this group are subjected to a control dietary regimen without the test compound generating a short-term drug withdrawal group. One effect that can be determined from comparing the long-term drug group and the short-term drug withdrawal group may include determining whether the effects of the test compound are reversible by a control dietary regimen or by withdrawing the test compound.

[0089] In one embodiment, specific mRNA levels from the hearts of mice from all of the various test groups were measured. It is to be appreciated that measuring specific mRNA levels is only one exemplary method of identifying the effects caused by various dietary regimens or test compounds. Other methods such as those conventionally used for measuring specific protein activity levels, specific protein level changes, specific carbohydrate level changes, specific lipid level changes, and specific nucleic acid levels can be used. Other heart RNA was isolated from frozen tissue fragments by homogenization in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) as described by the suppliers. mRNA levels were measured using the Affymetrix U74v2A high-density oligonucleotide arrays according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, cDNA was prepared from total RNA from each animal using Superscript Choice System with a primer containing oligo(dT) and the T7 RNA polymerase promoter sequence. Biotinylated cRNA was synthesized from purified cDNA using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem). cRNA was purified using RNeasy mini columns (Qiagen, Chatsworth, CA). An equal amount of cRNA from each animal was separately hybridized to U74v2A high-density oligonucleotide arrays. The arrays were hybridized for 16 hours at 45 °C. After hybridization, arrays were washed, stained with streptavidin-phycoerythrin, and scanned using a Hewlett-Packard GeneArray Scanner. In one embodiment, image analysis and data quantification were performed using the Affymetrix GeneChip™ analysis suite v5.0.

[0090] In embodiments where the Affymetrix GeneChip™ analysis suite are used, the U74vA array contains targets for more than 12,422 mouse genes and expressed sequence tags (ESTs). Each gene or EST is represented on the array by 20 perfectly matched (PM) oligonucleotides and 20 mismatched (MM) control probes that contain a single central-base mismatch. All arrays were scaled to a target intensity of 2500. The signal intensities of PM and MM were used to calculate a discrimination score, R, which is equal to (PM - MM) / (PM + MM). A detection algorithm utilized R to generate a detection p-value and assign a

Present, Marginal or Absent call using Wilcoxon's signed rank test. Details of this method can be found in Wilcoxon F. *Individual Comparisons by Ranking Methods*, Biometrics 1, 80-83, 1945, and Affymetrix, *I. New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays*, Technical Notes 1, Part No. 701097 Rev. 1, 2001. Only genes that were "present" in at least 2 out of 4 arrays per experimental group were considered for further analysis. In addition, genes with signal intensity lower than the median array signal intensity in any of the 16 arrays were eliminated from the analysis. These selection criteria reduced the raw data from 12,422 genes to only 3456 genes which were considered for further analysis.

[0091] In one embodiment, to identify differentially expressed genes between any two groups, each of the 4 samples in one group was compared with each of the 4 samples in the other group, resulting in 16 pairwise comparisons. These data were analyzed statistically using a method based on Wilcoxon's signed rank test. Difference values (PM-MM) between any two groups of arrays were used to generate a one-sided p-value for each set of probes. Default boundaries between significant and not significant p-values were used. (See Affymetrix, I. *New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays*, mentioned above, for more details). In one embodiment, genes are considered to have changed expression if the number of increase or decrease calls was 8 or more of the 16 pairwise comparisons, and an average fold change, derived from all 16 possible pairwise comparisons, was 1.5-fold or greater. Empirically, these criteria for identifying gene expression changes can be reliably verified by methods such as Western blot, Northern blot, dot blot, primary extension, activity assays, real time PCR, and real time RT-PCR (reverse transcriptase PCR). Gene names were obtained from the Jackson Laboratory Mouse Genome Informatics database as of August 1, 2002.

[0092] In one embodiment, the effects caused by LT-CR, ST-CR, and ST-CON dietary regimens are listed in Table 2. These effects are illustrated in terms of fold changes. The numbers in the LT-CR column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the LT-CR and LT-CON groups (n = 4). The numbers in the ST-CR column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the ST-CR and LT-CON groups (n = 4). The numbers in the ST-CON column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the ST-CON and LT-CON groups (n = 4). Where there is no

change in gene expression, an "NC" is denoted. In one embodiment, the ratios of the fold changes are determined to illustrate the effects on gene expression. For each ratio, the numerator is the level of expression of each gene from the LT-CR, ST-CR, or ST-CON group, and the denominator is the level of expression of that gene in the LT-CON group. For example, the fold changes in gene expression caused by LT-CR is the ratio of the level of expression of each gene in the LT-CR group divided by the level of expression of that gene in the LT-CON group. The fold changes in gene expression caused by ST-CR is the ratio of the level of expression of each gene in the ST-CR group divided by the level of expression of that gene in the LT-CON group. The fold changes in gene expression caused by ST-CON is the ratio of the level of expression of each gene in the ST-CON group divided by the level of expression of that gene in the LT-CON group.

[0093] As mentioned above, gene expressions can be validated by real time RT-PCR. In one embodiment, the expression of a total of 9 genes randomly chosen from among the genes which changed expression was examined by real time RT-PCR using total cardiac RNA purified from the mice used in the microarray studies. Total RNA was treated with DNase I (Ambion Inc., Austin, TX) and used to synthesize cDNA in a 20 µl total volume reaction. Briefly, 2 µg of total RNA were incubated with 250 ng random primer (Promega, Madison, WI) for 5 min at 75°C, and then on ice for 5 min. 2 µl of 0.1 M DTT, 4 µl of 5 X buffer, 4 µl of 2.5 mM dNTP, 100 U (units) reverse transcriptase (Invitrogen, Carlsbad, CA), and 16.5 U RNase inhibitor (Promega) were added and incubated for 2 hr at 37°C. The reaction was stopped by boiling for 2 min at 100°C. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA. All samples were reverse-transcribed at the same time and the resulting cDNA was diluted 1:4 in water and stored at -80°C.

[0094] Relative quantification with real-time, two-step real time RT-PCR was performed with Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Primers were designed using Netaffx analysis center and verified against the public databases to confirm unique amplification products (http://www.affymetrix.com/analysis/index.affx and http://www.ncbi.nlm.nih.gov), (Table 1). Primers for transcription factor S-II were amplified in parallel with the genes of interest. Transcription factor S-II was used as a reference gene because its mRNA levels are unaffected by a CR diet. For each gene, single real time RT-PCR was performed with each individual mRNA sample obtained from mice from each of the sample groups, for example,

the LT-CON continuation group 110 (n = 4), the LT-CR continuation group 114 (n = 4), the ST-CON group 112 (n=4) and the ST-CR group 108 (n=4). Briefly, real time RT-PCR was carried out in 25 µl volumes containing 2 µl of diluted cDNA, 1X SYBR Green PCR Master Mix, 0.5 mM of each forward and reverse primers, and 0.5 unit uracil N-glycosylase. The reactions were incubated for 2 min at 50°C to allow degradation of contaminating cDNA by uracil N-glycosylase, and 15 min at 95°C to activate HotStarTaq DNA polymerase. Target amplification reactions were cycled 40 times with denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C with 30 sec. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis.

[0095] The heart tissue from each mouse from each of the test groups including the LT-CON continuation group 110, the LT-CR continuation group 114, the ST-CON group 112, and the ST-CR group 108 was isolated for determination of effects of each of the different treatments. For example, profiles such as gene expression levels, nucleic acid levels, protein levels, protein activity levels, carbohydrate levels, and lipid levels, to name a few, can be analyzed for the hearts isolated from mice from the various groups. The methods for such analysis are well known in the art. Some embodiments of the present invention focus on the determination of changes in gene expression levels. It is to be noted that such determination is not the only method that can be used to analyze the effects of CR, LT-CR, ST-CR, switching of the CR dietary programs, and mimetic compounds.

[0096] In one embodiment, microarray assessment of the relative levels of mRNA of 12,422 genes and ESTs revealed that 47 genes in the heart changed expression with a LT-CR dietary program as illustrated in Figure 2A. These differentially expressed genes are further grouped into categories by their putative functions as illustrated in Table 2. LT-CR and ST-CR affected the expression of genes whose products are components of extracellular matrix and cytoskeleton, intermediary metabolism, immune and stress responses and signal transduction.

[0097] Expression of a subset of the genes listed in Table 2 was also measured using real time RT-PCR. In Figure 3, 9 randomly chosen genes (with gene names AB005450, Z68618, Y08027, X58251, X52046, X04653, U47737, D16497, and X00496) were monitored by quantitative PCR. As illustrated in Figure 3, PCR confirmed the changes found by microarray for each of the 9 chosen genes. As can be seen from this figure, the fold changes

are in the same direction and are substantially similar in the amount of the fold changes. The results in Figure 3 indicate that the analytical methods used here reliably identified genes that change expression.

[0098] In another aspect, the invention provides methods of evaluating the dynamics of changes in gene expression in CR. In one embodiment, to elucidate the dynamics of the changes in gene expression in response to caloric intake, LT-CR and LT-CON mice were subjected to an 8-week switch to an opposite diet. For instance, as previously mentioned, some mice from the LT-CR group were switched from the LT-CR dietary program to the ST-CON dietary program (Figure 1). Additionally, some mice from the LT-CON group were switched from the LT-CON dietary program to the ST-CR dietary program (Figure 1). This switching or crossover feeding further distinguished the 47 genes whose expression was altered by LT-CR. In one embodiment, the switched feeding fractionates or categorizes the 47 genes into 4 subgroups (discussed below) according to their response to changes in caloric intake as illustrated in Figure 2A. The differences in the dynamics of changes in mRNA levels suggest that CR involves multiple complex molecular mechanisms in its effects on gene expression. Moreover, when these 47 genes were sorted according to the mode of regulation (positive or negative), the 4 subgroups were further separated into 7 gene clusters as illustrated in Figure 2B. Genes assemble into clusters most likely because of similarities in the molecular mechanisms of their regulation. For example, several genes may have a common regulatory factor (e.g., enhancer sequences) or a common signal transduction pathway, and these common features are revealed through the gene clusters identified as a result of switching the diet programs. Thus, this switching allows for motif discovery.

[0099] Figures 2A-2B illustrate the effects of switched or crossover feeding on gene expression in heart tissue which was the source of the RNA in one exemplary embodiment. LT-CR altered the expression of 47 genes. The genomic effects of an 8-week switch of LT-CR and LT-CON mice to opposite diets further distinguished these 47 genes into 4 subgroups (Figure 2A). A subgroup of 35 genes for which expression is altered by LT-CR but unaffected by either of the dietary regimen switches to the opposite diet, ST-CON or ST-CR dietary regimen. A subgroup of 8 genes for which ST-CR reproduced the gene expression changes induced by LT-CR. A subgroup of 1 gene for which ST-CON did not reverse the gene expression changes induced by LT-CR. Finally, a subgroup of 3 genes for which ST-CR reproduced but ST-CON did not reverse the gene expression changes induced by LT-CR.

[0100] The 47 genes were further sorted according to the direction of the changes in gene expression across the different experimental conditions. This sorting further segregated the 4 subgroups of genes into 7 gene clusters with similar patterns of expression (Figure 2B). Cluster 1 (2 genes) illustrates that the increase in mRNA levels by LT-CR was reproduced by ST-CR but was not reversed by ST-CON treatment. Cluster 2 (1 gene) illustrates that the increase in mRNA levels by LT-CR was neither reproduced by ST-CR nor reversed by ST-CON treatment. Cluster 3 (1 gene) illustrates that the increase in mRNA levels by LT-CR was reproduced by ST-CR and was reversed by ST-CON treatment. Cluster 4 (21 genes) illustrates that the increase in mRNA levels by LT-CR was not reproduced by ST-CR but was reversed by ST-CON treatment. Cluster 5 (14 genes) illustrates that the decrease in mRNA levels by LT-CR was not reproduced by ST-CR but was reversed by ST-CON treatment. Cluster 6 (7 genes) illustrates that the decrease in mRNA levels by LT-CR was reproduced by ST-CR and was reversed by ST-CON treatment. Cluster 7 (1 gene) illustrates that the decrease in mRNA levels by LT-CR was reproduced by ST-CR but was not reversed by ST-CON treatment.

[0101] These genes, it is believed, congregated into clusters because of similarities in their expression profiles. Genes in the same cluster are thought to be regulated by similar mechanisms and thus, the regulatory sequences such as 5' upstream regions of the genes can be analyzed to identify shared cis-regulatory elements. DNA sequence motifs specific to expression clusters constitute the primary hypothesis for the cis-regulatory elements though which co-regulation of the genes within a cluster is achieved. Algorithms such as AlignACE have been used to identify known and novel motifs based on gene expression data from microarray experiments. Thus, promoter comparison between genes within clusters and genes of different clusters can identify potential binding sites for known or novel factors that might control gene expression during CR.

[0102] The exemplary methods discussed allow for ways to categorize genes. As apparent from Figures 2A-2B, genes are fractionated into clusters (or groups) as certain genes are similarly affected by a particular CR dietary regimen. Genes in the same cluster are likely to be transcriptionally co-regulated and their promoter regions can be analyzed for the presence of shared sequence motifs. Motif discovery begins by identifying genes that are co-regulated under different conditions by CR. Genes which respond in the same way to given physiological conditions are grouped together. For example, as illustrated in Figure 2B, genes which are responsive to ST-CR and LT-CR form 2 clusters (3, 8); genes which are

responsive to LT-CR only form 2 clusters (22, 14); and ST-CON further subdivides genes into 7 clusters (2, 1, 1, 21, 14, 7, 1). The expression of different genes can be stimulated or inhibited by the same regulatory factors and signal transduction systems.

[0103] The most parsimonious explanation for the co-behavior of each of these clusters of genes is that they are co-regulated by the same signal transduction pathway. Gene regulation in eukaryotes mainly involves transcription factors binding to short DNA sequence motifs located upstream of the coding region of genes. Thus, the upstream sequences of a set of co-regulated genes can be analyzed for shared cis-regulatory motifs (short DNA sequences). These known or unknown DNA sequence motifs (regulatory motifs) common to gene clusters are putative binding sites for transcription factors. Algorithms such as AlignACE have been used to identify known and novel sequence motifs based on gene expression data from microarray experiments. Thus, promoter comparison within clusters and genes can identify potential binding sites for known or novel transcription factors that might control gene expression during CR. Knowledge of the identity of the transcription factors bound by the putative regulatory motifs will suggest which signal transduction systems may be responsible for the regulation of the genes by CR. The signal transduction systems responsible for gene regulation by many transcription factors are known. The signal transduction systems responsible for regulation of the activity of other transcription factors, including novel transcription factors which may be identified, may be determined experimentally. Drugs which alter the activity of identified, known signal transduction systems may be possible candidate CR mimetics. In other cases, potential CR mimetics which alter the activity of the identified signal transduction systems may be identified experimentally by monitoring some feature of the activity of the signal transduction system. This feature might be, for example, the phosphorylation or other modification of the structure or activity of a protein or changes in the activity of a specific gene. In this way, motif discovery may aid in the discovery or development of pharmaceuticals capable of mimicking the life- and health-span extending effects of CR.

[0104] Table 2 illustrates that LT-CR affects genes in the extracellular matrix (ECM) and cytoskeleton. LT-CR decreased the expression of several collagen encoding genes (e.g., procollagen genes U03419, X58251, and X52046) . In the myocardium, a collagen matrix maintains the heart architecture, elasticity of the ventricles and vessels and the myocyte-capillary relationship. Previous studies in humans and rats show an increase in myocardial collagen associated with aging. See for example, Gazoti et. al., *Age related changes of the*

collagen network of the human heart, Mech.Ageing Dev., 122: 1049-58, 2001 and Eghbali et. al., *Collagen accumulation in heart ventricles as a function of growth and aging*, Cardiovasc.Res., 23: 723-9, 1989. This increase of the myocardial collagen may contribute to the age-related decrease in ventricular and cardiovascular elasticity. Possible mechanisms for collagen accumulation include loss of myocytes which is a characteristic of the aging heart and age-related increase in systolic blood pressure. It has been shown through microarray studies of cardiomyopathies that increased expression of collagen and several other extracellular matrix proteins leads to fibrosis and impaired contractile function. Extracellular matrix, cytoskeleton, and their modification play important roles in cardiovascular functioning.

[0105] As shown in Table 2, mice subjected to LT-CR showed decreased expression of collagen genes (e.g., U03419, X58251, and X52046). Additionally, mice subjected to ST-CR also showed decreased expression of collagen genes (e.g., U03419, X58251, X52046, and M15832). In contrast, mice under a control feeding program showed increased expression of collagen genes (e.g., U03419, X58251, and X52046) relative to mice in a CR dietary regimen. The decreased expression of extracellular matrix genes in CR (LT-CR or ST-CR) mice suggests less fibrosis and more elasticity in the myocardium of CR mice as opposed to the control mice. These effects may be part of the anti-aging strategy of CR to delay the age-associated decline in cardiovascular hemodynamics. The results indicate that mice subjected to CR may have extended longevity or delayed onset of age-related ventricular diseases since the expression of collagen genes are decreased as a result of CR.

[0106] Table 2 also illustrates that CR alters the expression of other extracellular matrix genes. For example, CR increased the expression of tissue inhibitor of metalloproteinase 3 gene which is a physiological inhibitor of matrix-degrading endopeptidases. Matrix remodeling results from a shift in the balance between metalloproteinases and their inhibitors. Disruption of this balance has been implicated in pathological states including cardiovascular diseases where tissue inhibitor of metalloproteinase activity was decreased. Thus, the results indicate that CR may delay the onset of cardiovascular diseases through decreasing tissue inhibitor of metalloproteinase activity. Additionally, CR decreased the expression of cysteine rich protein b1 gene. The product of this gene associates with extracellular matrix and binds directly to integrins to support cell adhesion and induces cell migration. Cysteine rich protein b1 expression is associated with the cardiovascular system during embryonic development. Later in life, its expression has been linked to angiogenesis and tumor growth.

[0107] Additionally, CR decreased the expression of microtubule-associated protein tau which promotes microtubule assembly and regulates cytoskeletal-membrane interactions. Tau is associated with Alzheimer's disease and was thought to be a neuron-specific protein. Tau is also expressed in the heart and other tissues. Even though the role of tau in cardiac microtubule assembly has not been shown yet, increased microtubule density is linked to contractile dysfunction in cardiac hypertrophy. Additionally, CR increased the expression of transgelin which plays a role in cytoskeleton organization and regulates smooth muscle cell morphology. Its expression is elevated in models of endothelial injury where transgelin is thought to mediate the conversion of myofibroblasts into smooth muscle cells. Moreover, transgelin is in human atherosclerotic plaque. These positive CR effects on the expression of EMC, cytoskeletal, signal transducer, and metabolism genes may be involved in retardation of cardiovascular diseases such as atherogenesis and hypertension.

[0108] Table 2 further illustrates that CR increased the expression of stearoyl-CoA desaturase gene, which is a rate-limiting enzyme in the synthesis of unsaturated fatty acids. The balance between saturated and monounsaturated fatty acids directly influences the membrane fluidity and its physical properties, and alterations in the ratio of these fatty acids have been implicated in many pathologies including vascular and heart diseases. Changes in lipid composition and decreased membrane fluidity occur with aging in several tissues. Thus, CR enhances membrane fluidity by increasing the desaturase gene expression.

[0109] Table 2 also illustrates that CR increases the expression of cytosolic acyl-CoA thioesterase 1 which controls levels of acyl-CoA/free fatty acids in the cytosol by hydrolysis of acyl-CoAs. While in tissues such as liver and kidneys thioesterases regulate gene transcription via nuclear receptors, cardiac thioesterases seem to be involved in the release of arachidonic acid (AA) from cellular phospholipids. AA can be metabolized to various cardioactive compounds, including prostanoids, leukotrienes, and epoxyeicosatrienioic acids. These metabolites and AA itself modulate a variety of systems in cardiomyocytes, including ion channels, gap junctions, and protein kinase C activity. More interestingly, the effects of AA on cardiac contractility combine a positive effect at low AA concentrations and a negative effect at high AA concentrations. The relative activation of the positive and negative pathways determines the nature of the final response. The effects of CR on cardiac cytosolic acyl-CoA thioesterase gene expression may be a fine tuning of these opposed pathways to result in an improved heart function.

[0110] Table 2 also illustrates that CR alters the expression of other metabolic genes. The expression of ADP-ribosyltransferase 3 gene, which is involved in posttranslational processing of nascent proteins, was increased by CR. The functional effects of the ADP-ribosyltransferase 3 gene differ depending on the tissue. In the skeletal muscle, the ADP-ribosyltransferase 3 gene ribosylates integrin to affect cell-cell and cell-matrix interactions. The role of ADP-ribosyltransferase 3 in cardiac muscle has not yet been determined. CR also increased the expression of the carbonic anhydrase 14 gene, which is most abundant in the kidney and heart. Carbonic anhydrase participates in various physiological processes including acid-base balance and ion transport. In the heart, acid-base homeostasis is important because of the pH sensitivity of myocardial contractility. Moreover, the failing myocardium is characterized by reduced carbonic anhydrase activity. The results here also indicate that CR delays progression toward cardiovascular diseases.

[0111] Table 2 further illustrates that CR alters the expression of several growth factor genes. CR decreased the expression of epithelial membrane protein 1 gene which has been implicated in tumorigenesis. CR increased the expression of p53 regulated PA26 nuclear protein gene which is a regulator of cellular growth and plays a role in tumor suppression. CR decreased the expression of the interferon induced transmembrane protein 3-like gene. It has been suggested that interferon-inducible transmembrane proteins transduce the antiproliferative activity of interferon. The implications of these opposed effects of CR on growth in the heart are unclear. In addition, beyond birth, cardiac growth occurs by hypertrophy rather than hyperplasia and primary tumors of the heart are rare.

[0112] Table 2 further illustrates that CR decreases the expression of several signal transducers relevant to cardiovascular diseases. CR decreases the expression of G protein-coupled receptor kinase 5 which is one of the two major G protein-coupled receptor kinases expressed in the heart. Increased expression and activity of these kinases have been shown to play an important role in the development of cardiac hypertrophy and congestive heart failure. Myocardial levels of G protein-coupled receptor kinase 5 mRNA and protein content are increased in experimental congestive heart failure. In addition, transgenic over expression of G protein-coupled receptor kinase 5 in mice leads to a significant decrease in myocardial performance. These results suggest that the CR-related decreased expression of this gene may improve and maintain healthy myocardial functioning. CR also decreased the expression of three other genes implicated in cardiovascular diseases, Ribosomal protein S6 kinase, 90kD, polypeptide and stromal cell derived factor 1 and natriuretic peptide precursor

type B. Ribosomal protein S6 kinase has been found to be activated in failing myocardium. Stromal cell derived factor 1 expression is induced in a permanent coronary artery occlusion model of myocardial infarction in rat. Ventricular expression of natriuretic peptide type B is increased in animal models of congestive heart failure. Increased production of this cardiac hormone is a marker of left ventricular dysfunction and has prognostic significance in patients with congestive heart failure. Since higher expression levels of natriuretic peptide type B are considered a protective response against myocardial damage, the lower expression levels in CR animals may reflect a healthier myocardium and thus, a more efficient cardiac function.

[0113] Table 2 further illustrates that CR affects genes associated with immune response and inflammation. Expression of genes related to inflammation, such as complement component 1, q subcomponent, c polypeptide and histocompatibility 2, k region locus 2 were decreased in CR mice. Cardiomyocytes and endothelial cells express MHC (major histocompatibility complex) class I and II antigens in and around inflammatory regions in the heart. Both MHC class II genes and the early genes of the classical complement system are expressed at low levels in resting macrophages and up-regulated by activation of macrophages. Decreased expression of such genes suggests that CR may ameliorate inflammation in CR mice.

[0114] Table 2 further illustrates that CR affects genes associated with stress response and xenobiotic metabolism. CR increased the expression of cytochrome P450 enzyme 2e1. This enzyme is expressed most highly in the liver where it metabolizes a broad spectrum of drugs and endogenous substances. However, it is also expressed in the heart. It is still not known if cytochrome P450 enzymes contribute significantly to drug and xenobiotic metabolism in the heart. CR also increased the expression of thioether S-methyltransferase which plays a role in the detoxification and solubilization of endogenous and exogenous sulfur- and selenium-containing compounds. Even though the physiological role of cytochrome P450 enzymes and thioether S-methyltransferase in the heart is still unclear, the increase of their expression by CR suggests they may play a role in protecting the heart against xenobiotics. However, the cytochrome P450 system was shown to modulate cardiomyocyte contraction in cell culture through metabolism of arachidonic acid. This suggests that cytochrome P450 enzymes, in the heart, may be involved in intracellular signal transduction.

[0115] Figure 4 provides an additional illustration of an exemplary scheme 100 of the various dietary regimens or programs and compound administration programs for mammalian samples. In one embodiment, the mammalian samples are mice. One-month-old male mice of the long-lived strain C57Bl6 x C3H F1 were purchased from Harlan (Indianapolis, IN). Mice were housed in groups of four per cage and fed a non-purified diet, PMI Nutrition International Product # 5001 (Purina Mills, Richmond, IN). In one embodiment, at five months of age, the mice were individually housed. In one embodiment, at five months, the mice are subjected to various diet or treatment programs. As illustrated in Figure 4, the five-month old mice as shown in box 102 were randomly assigned to one of two groups, a control (CON) group 104, and a long-term CR (LT-CR) group 106. In one embodiment, each mouse in the CON group 104 was fed 93 kcal per week of the purified control diet (AIN-93M, Diet No. F05312, BIO-SERV). In one embodiment, each mouse in the LT-CR group 106 was fed 52.2 kcal per week of a purified CR diet (AIN-93M 40% Restricted, Diet No. F05314, BIO-SERV). In one embodiment, each mouse in the LT-CR mice 106 consumed approximately 40% fewer calories than each mouse in the CON group 104. The CR diet was enriched in protein, vitamins, and minerals so that the CR mice consumed approximately the same amount of these nutrients per gram body weight as the control mice. Mice had free access to acidified tap water. No signs of pathology were detected in any of the animals used. All animal use protocols were approved by an institutional animal use committee.

[0116] In one embodiment, at 20 months of age, mice in the LT-CR group 106 continued to be fed with the CR diet for another two months (eight weeks). The mice in the CON group 104 were divided into various groups subjected to various test compounds and in one embodiment, the test compounds are gluco-regulatory compounds. In one embodiment, the mice in the CON group 104 were randomly assigned to seven experimental groups, a CON group 108, a short-term CR (ST-CR) group 110, a Metformin group 112, a Glipizide group 114, a Rosiglitazone group 116, a Metformin-Glipizide combination group 118, and a Soy Isoflavone group 120. Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones are some of the test compounds that can be used. Metformin, Glipizide, and Rosiglitazone are examples of glucoregulatory compounds. Each mouse in the CON group 108 continued to be fed 93 kcal per week of control diet alone for eight weeks. Each mouse in the ST-CR group 110 was fed 77 kcal per week of CR diet for two weeks, followed by 52.2 kcal per week of CR diet for six weeks. The mice in the other five groups were fed the control diet containing one drug or a combination of two drugs for a total of eight weeks. The drug or compound

administration can be shorter than eight weeks, for example, between about 1 day to about 8 weeks. In one embodiment, each mouse in the Metformin group 112 was fed the 93 kcal per week control diet plus 2100 mg of Metformin in 1 kg of the control diet; each mouse in the Glipizide group 114 was fed the 93 kcal per week control diet plus 1050 mg of Glipizide in 1 kg of the control diet; each mouse in the Rosiglitazone group 116 was fed the 93 kcal per week control diet plus 80 mg of Rosiglitazone in 1 kg of the control diet; each mouse in the Metformin-Glipizide combination group 118 was fed the 93 kcal per week control diet plus 1050 mg of Metformin and 525 mg of Glipizide in 1 kg of the control diet; and, each mouse in the Soy Isoflavone group 120 was fed with the 93 kcal per week control diet having 0.25% (by weight) Soy Isoflavones in the control diet.

[0117] The amounts of the drugs or the compounds such as Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones, to be administered to the mice can vary depending on the types of compounds and/or their concentrations. In one embodiment, dosages for Metformin may be approximately between 0.2 mg and 2.0 gm of Metformin per kg body weight per day. Dosages for Glipizide may be approximately between 1.05×10^{-3} mg and 105 mg of Glipizide per kg body weight per day. Dosages for Rosiglitazone may be approximately between 8.0×10^{-4} mg and 8.0 mg of Rosiglitazone per kg body weight per day. The dosages for the combination of Metformin and Glipizide may be approximately between 0.1 mg and 1.0 gm per kg body weight per day of Metformin plus approximately between 0 mg and 52.5 mg of Glipizide per kg body weight per day. The dosages for Soy Isoflavones may be approximately between 0.025-2.5% of daily diet (by weight) of Soy Isoflavones in the control diet.

[0118] Metformin was obtained from Sigma, St. Louis, MO; Glipizide was also obtained from Sigma; Rosiglitazone (known as Avandia), was obtained from SmithKline Beecham; and Soy Isoflavone extract was NOVASOY 400, obtained from Life Extension Foundation. These compounds were mixed with the powered control diet and cold-pressed into one-gram pellets by the diet supplier (BIO-SERV).

[0119] Mice were killed at 22 months of age. They were fasted for 48 hours and killed by cervical dislocation. The organs were removed rapidly, placed in plastic screw-cap tubes, and flash frozen in liquid nitrogen. The tissues were stored in liquid nitrogen.

[0120] In one embodiment, mice in the LT-CR group 106 are subjected to the CR diet for a duration of time that is longer or substantially longer than mice in the ST-CR group 110, for

example, 5 weeks to 40 months longer. Similarly, mice in the LT-CR group 106 are subjected to the CR diet for a duration of time that is longer or substantially longer (e.g., 5 weeks to 40 months longer) than mice in the drug groups, such as the Metformin group 112, the Glipizide group 114, the Rosiglitazone group 116, the Metformin-Glipizide combination group 118, and the Soy Isoflavone group 120. In some embodiments, mice in the LT-CR group 106 are subjected to the CR diet to about the end of their life.

[0121] It is to be noted that other compounds can be chosen in addition to or in place of the compounds (e.g., Metformin, Glipizide, and Rosiglitazone) listed above. In some embodiments, glucoregulatory compounds such as Metformin, Glipizide, and Rosiglitazone, alone and in combination, were tested. Glucoregulatory agents are chosen because CR produces a marked reduction in blood insulin levels (~50%), lowers blood glucose levels (~15%) and enhances insulin sensitivity in tissues. These same effects are often produced by glucoregulatory pharmaceuticals. Compounds known to lower circulating glucose and insulin levels are promising candidate CR mimetics. Thus, other test compounds that are glucoregulatory agents can be used in the embodiments of the present invention without deviating from the scope of the disclosure. In addition, small molecule cancer chemopreventatives (e.g., Soy Isoflavones) can also be used in addition to the test compounds listed in Figure 1 to screen for a CR mimetic compound(s).

[0122] As previously indicated, control data can be obtained from a prior study, the results of which are recorded as opposed to a control group of mice subjected to a control diet program concurrently with the test groups of mice as illustrated in Figure 4. Thus, the control data may be obtained from an administering of a control diet program which was previously performed. This control data may be obtained once and stored for recall in later screening studies for comparison against the results in the later screening studies. Similarly, gene expression levels from LT-CR or ST-CR (or other types of measurements such as changes in protein levels, changes in protein activity levels, changes in carbohydrate or lipid levels, changes in nucleic acid levels, changes in rate of protein or nucleic acid synthesis, changes in protein or nucleic acid stability, changes in protein or nucleic acid accumulation levels, changes in protein or nucleic acid degradation rate, and changes in protein or nucleic acid structure or function) may be evaluated and recorded once for recall in later screening studies for comparison against the results in the later screening studies. Of course, it is typically desirable to have the prior stored studies have a similar (if not identical) set of genes (or other

parameters such as proteins) relative to the genes (or other parameters) in the later screening studies in order to perform a comparison against a similar set of genes or other parameters.

[0123] Additionally, a compound can be evaluated or determined to see whether it will reproduce the effects of CR or mimic CR by being fed to the mice in a scheme similar to that illustrated in Figure 4.

[0124] In one embodiment, mRNA levels of specific genes or nucleic acid sequences in the different groups of the mice were measured in various organs of the mice. In one embodiment, total liver RNA was isolated from frozen tissue fragments by Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) homogenization in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) as described by the supplier. mRNA levels were measured using the Affymetrix U74v2A high-density oligonucleotide arrays according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, cDNA was prepared from total RNA from each animal's organ using Superscript Choice System with a primer containing oligo(dT) and the T7 RNA polymerase promoter sequence. Biotinylated cRNA was synthesized from purified cDNA using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem). cRNA was purified using RNeasy mini columns (Qiagen, Chatsworth, CA). An equal amount of cRNA from each animal was separately hybridized to U74v2A high-density oligonucleotide arrays. The arrays were hybridized for 16 hours at 45 °C. After hybridization, arrays were washed, stained with streptavidin-phycoerythrin, and scanned using a Hewlett-Packard GeneArray Scanner. Image analysis and data quantification were performed using the Affymetrix GeneChip™ analysis suite v5.0.

[0125] In one embodiment, image analysis and data quantification were performed using Affymetrix Microarray Suite 5.0. The U74vA array contains targets for more than 12,422 mouse genes and expressed sequence tags (ESTs). Each gene or EST is represented on the array by 20 perfectly matched (PM) oligonucleotides and 20 mismatched (MM) control probes that contain a single central-base mismatch. All arrays were scaled to a target intensity of 2500. The signal intensities of PM and MM were used to calculate a discrimination score, R, which is equal to (PM - MM) / (PM + MM). A detection algorithm utilizes R to generate a detection p-value and assign a Present, Marginal or Absent call using Wilcoxon's signed rank test. Details of this method can be found in Wilcoxon F. *Individual Comparisons by Ranking Methods*, Biometrics 1, 80-83, 1945 and Affymetrix, *I. New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays*,

Technical Notes 1, Part No. 701097 Rev. 1, 2001. Only genes that were "present" in at least 75% of all arrays in an experimental group were considered for further analysis. In addition, genes with signal intensity lower than the median array signal intensity in any of all the arrays were eliminated from the analysis. These selection criteria reduced the raw data from 12,422 genes to 3505 genes that were considered for further analysis. The use of these microarrays allows for rapid gene expression profiling between the groups of test subjects allowing for rapid screening of possible compounds which may reproduce some effects of CR and may also extend maximum life span.

[0126] In one embodiment, a study included eight experimental groups as illustrated in Table 3. In one embodiment, the control group was compared to each of the seven treatment groups to determine the specific effects of each treatment on gene expression. It is to be appreciated that the control group can also be compared to each of the seven treatment groups to determine the specific effects of each treatment on nucleic acid levels, protein activity levels, and protein levels. The results from the LT-CR and ST-CR groups were compared to results from each of the treatments of the five test compounds. In one embodiment, these comparisons were used to characterize gene expression profiles common to drug treatments and CR.

[0127] To identify differentially expressed genes between any treatment and the control group, each of the four samples in the control group was compared with each of the four samples in the treatment group, resulting in sixteen pairwise comparisons. These data were analyzed statistically using a method based on Wilcoxon's signed rank test. Difference values (PM-MM) between any two groups of arrays were used to generate a one-sided p-value for each set of probes. Default boundaries between significant and not significant p-values were used (See Affymetrix, I. *New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays*, mentioned above, for more details). Genes are considered to have changed expression if the number of increase or decrease calls is 50% or higher in the pairwise comparisons, and an average fold change, derived from all possible pairwise comparisons, is 1.5-fold or greater. Empirically, we found that these criteria identified gene expression changes which were reliably verified by Northern blots, details can further be found in Cao, et. al., *Genomic profiling of short- and long-term caloric restriction in the liver of aging mice*, Proc. Natl. Acad. Sci. U.S.A. 98, 10630-10635 (2001). The gene expression changes can also be verified by methods such as Western blot, dot blot, primary extension, activity assays, real time PCR, and real time RT-PCR (reverse transcriptase PCR).

[0128] Gene names were obtained from the Jackson Laboratory Mouse Genome Infomatics database as of December 1, 2002.

[0129] In one embodiment, the effects caused by LT-CR and ST-CR dietary regimens and Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones and combinations thereof are listed in Tables 5-10. These effects are illustrated in terms of gene expression fold changes for various genes. In Table 5, the numbers in the Metformin column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the Metformin and the control (CON) groups (n = 4). The numbers in the LT-CR column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the LT-CR and the CON groups (n = 4). The numbers in the ST-CR column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the ST-CR and the CON groups (n = 4). Where there is no change in gene expression, an "NC" is denoted. Table 6 is similar to Table 5 except it applies to Glipizide. Thus, numbers in the Glipizide column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the Glipizide and the CON groups (n = 4). Table 7 is similar to Table 5 except it applies to the Glipizide and Metformin (GM) combination. Thus, numbers in the GM column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the GM combination and the CON groups (n = 4). Table 8 is similar to Table 5 except it applies to Rosiglitazone. Thus, numbers in the Rosiglitazone column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the Rosiglitazone and the CON groups (n = 4). Table 9 is similar to Table 5 except it applies to Soy Isoflavones. Thus, numbers in the Soy Isoflavone column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from the Soy Isoflavone and the CON groups (n = 4).

[0130] In one embodiment, the fold changes are determined to illustrate the effects on gene expression. If the level of expression of a gene in the treatment groups is equal to or greater than the level of expression in the CON group, the fold change in expression is calculated as a ratio in which the numerator is the level of expression of a gene after one of LT-CR, ST-CR, Metformin, Glipizide, a combination of Metformin and Glipizide, Rosiglitazone, or Soy Isoflavone treatment, and the denominator is the level of expression of the gene in the CON group. For example, the fold change in the expression of a gene in the LT-CR group is the

ratio of the expression level of that gene in LT-CR mice to the level of expression of that gene in the CON group; the fold change in the expression of a gene caused by ST-CR is the ratio of the expression of the gene in the ST-CR group to the level of expression of that gene in the CON group; and the fold change in the expression of a gene in the Metformin, Glipizide, a Glipizide Metformin combination, Rosiglitazone, or Soy Isoflavone groups, is the ratio of the expression of a gene in one of the Metformin, Glipizide, a Glipizide Metformin combination, Rosiglitazone, or Soy Isoflavone groups, to the expression level of that gene in the CON group. If the level of expression of a gene in the treatment groups is less than the level of expression in the CON group, the fold change in expression is calculated as the negative inverse of the ratio. Thus, the level of expression of the gene in the CON group is the numerator and the level of expression of that gene in the treatment group is the denominator and a minus sign is used to indicate a decrease in fold change.

[0131] In one embodiment, the ability of several glucoregulatory pharmaceuticals (e.g., Metformin, Glipizide, and Rosiglitazone), and other compounds such as Soy Isoflavones to produce CR-specific gene expression profiles in the liver of mice was assessed using the Affymetrix microarrays. The compounds were fed to mice using the mentioned scheme illustrated in Figure 4.

[0132] Figure 5 illustrates that in one embodiment, administering the drugs to mice for eight weeks significantly changed the expression of 63 genes for Metformin, 46 for Glipizide, 46 for a combination of Metformin and Glipizide, 44 for Rosiglitazone, and 3 for Soy Isoflavones. Of the 63 genes with changed expression caused by Metformin: 4 genes with changed expression have identical changes as those caused by ST-CR; 17 genes with changed expression have identical changes as those caused by LT-CR and ST-CR; 15 genes with changed expression have identical changes as those caused by LT-CR; 3 genes with changed expression have the opposite direction of change compared to those caused by LT-CR and ST-CR; and 24 genes with changed expression that are just due to the administration of Metformin alone.

[0133] Still with Figure 5, of the 46 genes with changed expression caused by Glipizide: 0 genes with changed expression have identical changes as those caused by ST-CR; 7 genes with changed expression have identical changes as those caused by LT-CR and ST-CR; 7 genes with changed expression have identical changes as those caused by LT-CR; 6 genes with changed expression have the opposite direction of change compared to those caused by

LT-CR and ST-CR; and 26 genes with changed expression that are just due to the administration of Glipizide alone.

[0134] Still with Figure 5, of the 44 genes with changed expression caused by Rosiglitazone: 5 genes with changed expression have identical changes as those caused by ST-CR; 12 genes with changed expression have identical changes as those caused by LT-CR and ST-CR; 4 genes with changed expression have identical changes as those caused by LT-CR; 5 genes with changed expression have the opposite direction of change compared to those caused by LT-CR and ST-CR; and 18 genes with changed expression that are just due to the administration of Rosiglitazone alone.

[0135] Still with Figure 5, of the 46 genes with changed expression caused by the Metformin and Glipizide combination: 2 genes with changed expression have identical changes as those caused by ST-CR; 6 genes with changed expression have identical changes as those caused by LT-CR and ST-CR; 8 genes with changed expression have identical changes as those caused by LT-CR; 5 genes with changed expression have the opposite direction of change compared to those caused by LT-CR and ST-CR; and 25 genes with changed expression that are just due to the administration of Metformin and Glipizide combination alone.

[0136] Figure 5 further illustrates that of the 3 genes that changed expression caused by the administration of Soy Isoflavones, 1 of them is identical to LT-CR, 1 of them is identical to LT-CR and ST-CR, and 1 is due to the administration of Soy Isoflavones alone.

[0137] Table 4 summarizes in percentages the extent to which a compound or compound combination reproduces CR-specific gene expression profiles in the results illustrated in Figure 5. For Metformin, 57% (36 genes) of the induced changes in expression were a subset of the changes induced by either LT- or ST-CR. The other values were 48% (21 genes) for Rosiglitazone, 35% (16 genes) for the combination of Metformin and Glipizide, 30% (14 genes) for Glipizide, and 67% (2 gene) for Soy Isoflavones. These percentages clearly indicate that the glucoregulatory pharmaceuticals substantially reproduce CR-specific gene expression profiles.

[0138] Additionally, of the 63 genes altered by Metformin, 51% (32 genes) were changed similarly by LT-CR and 33% (21 genes) by ST-CR (Figure 5; Table 4). A total of 57% (36 genes) of the Metformin-induced gene expression changes were reproduced with either LT- or ST-CR. Twenty seven percent of the genes whose expression was affected by

Metformin were altered by both LT-CR and ST-CR (17 genes). Metformin produced 24 changes in the expression of genes which were not affected by LT- or ST-CR (38% of the changes). Here, we term these effects drug specific changes to distinguish them from the effects in common with CR. Finally, there were 3 genes which Metformin induced to change expression in a direction opposite to that produced by LT-CR (Figure 5).

[0139] Additionally, of the 44 genes altered by Rosiglitazone, 36% (16 genes) were changed similarly by LT-CR and 39% (17 genes) by ST-CR (Figure 5; Table 4). A total of 48% (21 genes) of the Rosiglitazone -induced gene expression changes were reproduced with either LT- or ST-CR. Twenty seven percent of the genes whose expression was affected by Rosiglitazone were altered by both LT-CR and ST-CR (12 genes). Rosiglitazone produced 18 changes in the expression of genes which were not affected by LT- or ST-CR (41% of the changes). Finally, there were 5 genes which Rosiglitazone induced to change expression in a direction opposite to that produced by LT-CR (Figure 5).

[0140] Additionally, of the 46 genes altered by Glipizide, 30% (14 genes) were changed similarly by LT-CR and 15% (7 genes) by ST-CR (Figure 5; Table 4). Fifteen percent of the genes whose expression was affected by Glipizide were altered by both LT-CR and ST-CR (7 genes). Glipizide produced 26 changes in the expression of genes which were not affected by LT- or ST-CR (56% of the changes). Finally, there were 6 genes which Glipizide induced to change expression in a direction opposite to that produced by LT-CR (Figure 5).

[0141] Additionally, of the 46 genes altered by the Glipizide-Metformin combination, 30% (14 genes) were changed similarly by LT-CR and 17% (8 genes) by ST-CR (Figure 5; Table 4). A total of 35% (16 genes) of the Glipizide-Metformin -induced gene expression changes were reproduced with either LT- or ST-CR. Thirteen percent of the genes whose expression was affected by Glipizide-Metformin were altered by both LT-CR and ST-CR (6 genes). Glipizide-Metformin produced 25 changes in the expression of genes which were not affected by LT- or ST-CR (54% of the changes). Finally, there were 5 genes which Glipizide-Metformin induced to change expression in a direction opposite to that produced by LT-CR (Figure 5).

[0142] Additionally, of the 3 genes altered by Soy Isoflavones, 67% (1 gene) was changed similarly by LT-CR and 1 gene which Soy Isoflavones induced to change expression that was not observed in LT-CR or ST-CR (Figure 5).

[0143] As illustrated further in Table 5, the genes that changed expression with Metformin and CR are associated with stress and chaperone proteins, metabolism, signal transduction, and the cytoskeleton. Table 5 indicates the changes in various gene expressions that are caused by Metformin as well as LT-CR and ST-CR. These results indicate that Metformin can be used as a compound that reproduces the effects (or at least some of the effects) of CR including delaying aging and delaying onset of aging related diseases. For example, the expression of glucose 6-phosphatase was induced with Metformin and LT-CR. This is a key enzyme in gluconeogenesis. These results are consistent with other microarray and conventional studies which show that CR increases the enzymatic capacity of the liver for gluconeogenesis and the disposal of the byproducts of extrahepatic protein catabolism for energy production. See for example, Dhahbi, et. al., *Caloric restriction alters the feeding response of key metabolic enzyme genes*, Mech. Ageing Dev. 122, 35-50, 2001, and Dhahbi, et al., *Calories and aging alter gene expression for gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes*, Am. J. Physiol. 277, E352-E360, 1999. This CR effect, which is reproduced with Metformin, is consistent with theories of aging, such as the oxidative stress theory, which postulates that the accumulation of damaged proteins contributes to the rate of aging. CR prevents or retards the development of age-related diseases, and extends average and maximum life span in otherwise healthy rodents as well as variety of other species. Metformin, being able to reproduce the key effects to the gene expression mentioned above and as illustrated in Table 5, is expected to be able to, like CR, prevent or retard the development of age-related diseases, and extend average and maximum life span in otherwise healthy rodents as well as variety of other species such as fish, dogs, monkeys, and other mammals including humans.

[0144] Furthermore, analysis of genes for which expression is different between the control diet group (e.g., CON group 108) and the CR diet groups (e.g., ST-CR group 110 and LT-CR group 122) can demonstrate that specific genes are preferentially expressed during CR, LT-CR, or ST-CR. The same kind of analysis performed for gene expression that is caused by the test compounds can also be performed. The results which indicate that genes which change expression during treatments with the test compounds, such as Metformin and that are the same genes which change expression during CR, indicate that such compounds can be a CR mimetic compound that reproduces at least some of the effects of CR such as preventing or retarding the development of age-related diseases and extending average and maximum life span in otherwise healthy rodents as well as variety of other species (e.g., humans).

[0145] Expression of the molecular chaperone, glucose regulated protein 58 kDa, was decreased with Metformin, and LT- and ST-CR. Studies with microarray analysis have indicated that CR negatively regulates the expression of nearly all endoplasmic reticulum chaperones. Reduced chaperone expression is proapoptotic and anti-neoplastic; elevated chaperone levels tip the balance away from apoptosis and toward cell survival. Thus, there is an inverse correlation between chaperone protein expression and the survival of pre-cancerous cells. Lowering chaperone proteins will tend to reduce cancer incidence. Compounds such as Metformin that reduce chaperone protein expression will tend to reduce the incidence of cancer.

[0146] Additionally, chaperone induction has emerged as a new anti-apoptotic mechanism in some cells and tissues. Elevated chaperone levels during tumorigenesis allow cells to survive carcinogenesis and tumor formation. Induced GRP78, GRP94 and GRP170 are essential for the survival, growth and immuno-resistance of transformed cells. Tumorigenesis-associated chaperone induction confers drug resistance to the tumors. Chaperone induction allows precancer cells to survive the DNA damage and mutations which result in transformation, proliferation and onset of carcinogenesis. Metformin reduces chaperone levels in liver and this will tend to reduce the incidence of cancer.

[0147] Tables 6-9 illustrate the changes in gene expression caused by Glipizide, a Metformin & Glipizide combination, Rosiglitazone and Soy Isoflavones as well as by LT-CR and ST-CR. These tables include the genes that changed expression with the drug and CR as well as genes that changed expression with the drug only.

[0148] Table 10 includes genes whose expression is altered in the opposite direction by LT-CR and the compounds administered to mice.

[0149] As can be seen from the results, Rosiglitazone (Table 8) and Glipizide (Table 6) can also be CR mimetics to reproduce the effects (or at least some of the effects) of CR, LT-CR, and/or ST-CR. On the other hand, Soy Isoflavones produce only three changes in gene expression. One change was identical to LT-CR and ST-CR, and one change was identical to LT-CR (Table 9). Soy Isoflavones are putative chemopreventatives. Thus, Soy Isoflavones did not give a strong positive outcome in this assay as did Glipizide, Metformin, a Metformin and Glipizide combination, and Rosiglitazone.

[0150] It is to be appreciated that not all effects of CR are desirable. For example, CR suppresses immunity, reduces libido, reduces fertility, and suppresses adrenal and gonadal

steroid production. Thus, not all, or indeed, not many of the effects induced by CR need to be reproduced by a test compound such as Metformin in order for the test compound to be recognized as a drug that reproduces beneficial effects of CR.

[0151] Various embodiments of the present invention were used to screen several test compounds, e.g., glucoregulatory pharmaceuticals such as Metformin, Glipizide, and Rosiglitazone and Soy Isoflavone extract for their ability to mimic or reproduce the effects of ST-CR and/or LT-CR on gene expression. The glucoregulatory pharmaceuticals, and the combination of two of these pharmaceuticals produced a significant number of changes in hepatic gene expression that are identical to those produced by LT- and/or ST-CR. These findings suggest that these compounds are promising candidate CR-mimetics. Soy Isoflavones did not produce a strongly positive gene-expression signature. These results suggest that microarray profiling is a rapid method of screening drugs for the anti-aging and anti-disease properties. It is expected that Metformin, Glipizide, and Rosiglitazone (and analogous compounds) may be administered at effective dosages, to mammals including humans, to reproduce at least some of the effects of CR. Furthermore, Metformin, Glipizide, and Rosiglitazone(and analogous compounds) may be administered to mammals, including humans and mice, to increase the maximum life span of an otherwise healthy mammal. The analogous compounds include derivatives (e.g., salt derivatives) and other chemically similar structures. The effective dosages for Metformin may be approximately between 0.2 mg and 2.0 gm of Metformin per kg body weight per day. The effective dosages for Glipizide may be approximately between 1.05×10^{-3} mg and 105 mg of Glipizide per kg body weight per day. The effective dosages for Rosiglitazone may be approximately between 8.0×10^{-4} mg and 8 mg of Rosiglitazone per kg body weight per day. The effective dosages for the combination of Metformin and Glipizide may be approximately between 0.1 mg and 1.0 gm per kg body weight per day of Metformin plus approximately between 0 mg and 52.5 mg of Glipizide per kg body weight per day.

[0152] In one embodiment, the gene expression profiles induced by the different compounds or drugs are compared to the gene expression profiles induced by LT- and ST-CR to identify the common changes in gene expression and to determine the extent to which the drugs reproduce CR specific effects. The extent to which each of the tested compound (e.g., Metformin, Glipizide, Rosiglitazone, and Soy Isoflavones) reproduced the effects of CR on gene expression was determined. Figure 6 illustrates a Venn diagram analysis of the overlap between the effects of LT-CR, ST-CR, and of each of the compounds or drugs administered

to the test groups as shown in Figure 4. The numbers in parentheses indicate genes which a given drug induced to change expression in a direction opposite to that produced by LT-CR. The gene numbers are from Tables 5-10. As illustrated in Table 11 and Figure 6, Metformin reproduced 11.3% (32 out of 283 genes) of the effects of LT-CR on gene expression. Metformin reproduced 39.6% (21 out of 53 genes) of the effects of ST-CR on gene expression. Glipizide reproduced 5.0% (14 out of 279 genes) of the effects of LT-CR on gene expression. Glipizide reproduced 13.5% (7 out of 52 genes) of the effects of ST-CR on gene expression. The combination of Metformin and Glipizide reproduced 5.0% (14 out of 280 genes) of the effects of LT-CR on gene expression. The combination of Metformin and Glipizide reproduced 15.1% (8 out of 51 genes) of the effects of ST-CR on gene expression. Rosiglitazone reproduced 5.7% (16 out of 280 genes) of the effects of LT-CR on gene expression. Rosiglitazone reproduced 32.1% (17 out of 48 genes) of the effects of ST-CR on gene expression. Soy Isoflavones reproduced 0.7% (2 out of 285 genes) of the effects of LT-CR on gene expression. Soy Isoflavones reproduced 0% (1 out of 53 genes) of the effects of ST-CR on gene expression. These percentages clearly indicate that Metformin, Glipizide, and Rosiglitazone share several common effects on hepatic gene expression with CR. As can be seen, Metformin is more effective in reproducing some of the effects of CR than Glipizide, Rosiglitazone, and a Glipizide-Metformin combination. Soy Isoflavones are not effective in reproducing effects of CR as were the other tested compounds.

[0153] The various methods described herein may be used to search for (e.g., screen) drug candidates (e.g., an intervention), which can reproduce at least some of the effects of CR (e.g., either ST-CR or LT-CR) in mammals, including humans. Further, these methods may be used to search for (e.g., screen) drug candidates (e.g., an intervention), which can extend the maximum life span of an organism, including a human.

[0154] It can be expected that agents, identified in the embodiments described above, will extend lifespan, delay aging related diseases, and increase the age of onset and reduce the incidence of age-related diseases. Agents which reproduce the LT-CR or ST-CR signature (e.g., a similar pattern of gene expression changes) in microarray assays or other assays are likely to act as authentic CR mimetics and to extend maximum lifespan and improve health generally by delaying the onset and reducing the incidence of age related diseases.

[0155] In one embodiment of the invention, the maximum lifespan of old mammals can be extended by treating the old mammals with a CR diet program. The old mammals can be gradually subjected to the CR diet program in stages, such as at least one stage. Treating the old mammals with the CR diet program in at least one stage should be done incrementally rather than suddenly (all at once). Thus, there is no sudden reduction in the number of calories in the mammals' diets. Figure 7 illustrates an exemplary embodiment of treating old mammals 101, such as mice, with a CR diet program. As will be seen from below, treating old mammals with CR in stages can extend the maximum lifespan of the old mammals and bring other benefits of CR to the old mammals. In one embodiment, the old mammals 101 were divided into several groups, each of which underwent a CR diet program for a different amount of time. In one embodiment, the old mammals 101 were divided into a CR2 group 103, a CR4 group 105, a CR8 group 107, and a CON group 109.

[0156] In one embodiment, the old mammals 101 are male mice of the long-lived F1 hybrid strain B6C3F1. The old mammals 101 may be about 18 months old in the case of these mice. The mice were purchased from Harland (Indianapolis, IN). Each mouse from the CR2 group 103 was fed a 77 kcal per week CR diet for one week followed by a 52 kcal per week CR diet for another week. Each mouse in the CR4 group 105 was fed a 77 kcal per week CR diet for two weeks followed by 52 kcal per week CR diet for another two weeks. Each mouse in the CR8 group 107 was fed a 77 kcal per week CR diet for two weeks followed by a 52 kcal per week CR diet for six weeks. Each mouse in the CON group 109 was fed a 93 kcal per week control diet for eight weeks.

[0157] In one embodiment, the diet that was fed to each of the mice includes a semi-purified control diet in 1 gm pellets with a Control No. AIN-93M, Diet No. 505312, from BIO-SERV of Frenchtown, NJ, 08825. As illustrated in Figure 7, for a particular group such as the CR2 group 103, CR4 group 105, and CR8 group 107, each mouse in these groups was subjected to a reduced diet program that ultimately resulted in a CR diet program that consisted of 52 kcal per week of a CR diet. It can be seen from Figure 7 that the reduction was carried out in stages; for example, in the CR2 group 103, each mouse was subjected to the reduced diet by first going through the 77 kcal per week CR diet for one week and then finally to the 52 kcal per week CR diet for another week. The gradual reduction of calories in the diet, in stages, prevents the mice in each group from experiencing a sudden drop in caloric intake that may lead to death.

[0158] In addition, Table 12 illustrates the difference in dietary composition between the CR diet program and the control diet program. The control diet program consist of about 14 gm/100 gm diet casein, about 0.2 gm/100 gm diet L-cysteine, about 46.6 gm/100 gm diet cornstarch, about 15.5 gm/100 gm diet dextrinized cornstarch, about 10 gm/100 gm diet sucrose, about 4 gm/100 gm diet corn oil (Mazola), about 5 gm/100 gm diet cellulose, about 3.5 gm/100 gm diet mineral mix (AIN-76), about 0.3 gm/100 gm diet choline bitartrate, and about 1 gm/100 gm diet vitamin mix. The CR diet consist of about 23.3 gm/100 gm diet casein, about 0.3 gm/100 gm diet cysteine, about 29.5 gm/100 gm diet cornstarch, about 15.5 gm/100 gm diet dextrinized cornstarch, about 10 gm/100 gm diet sucrose, about 6.7 gm/100 gm diet corn oil, about 6.8 gm/100 gm diet cellulose, about 5.8 gm/100 gm diet mineral mix, about 0.4 gm/100 gm diet choline bitartrate, and about 1.7 gm/100 gm diet vitamin mix. Note that the 40% CR diet composition listed in Table 12 is for both the 52 kcal per week CR diet and the 77 kcal per week CR diet. The dietary composition for the diet in the reduction stage, where the diet includes a 77 kcal per week diet program, can be adjusted accordingly from the CR diet to obtain a 77 kcal per week diet. The CR diet was used for both 52 and 77 kcal per week CR diets.

[0159] In one embodiment, the effects of the CR diet program on the old mammals 101 are determined by comparing the results obtained from the CR diet program of the CR2 group 103, CR4 group 105, and CR8 group 107 to the results from the CON group 109. In one embodiment, the results include analyses of longevity of the mice in each of the groups CR2 group 103, CR4 group 105, and CR8 group 107, which were compared to the longevity of the mice in the CON group 109.

[0160] In some embodiments, parametric survival analyses were performed on the mice survival data. We assumed the data followed a Weibull distribution and the observed data were used to estimate the survival function. A change point regression analysis was also performed on the survival data to find the break points in the mortality data.

[0161] Unlike the conventional belief that CR acts progressively or incrementally, some embodiments of the present invention illustrate that CR rapidly affects the mammals that are subjected to CR, even at a later stage of their lives. For example, as illustrated in Figure 7, a CR diet program was administered to the old mammals 101 (e.g., mice) for various lengths of time. In one embodiment, the rapid effects of CR in old mice and their similarity to the effects of LT-CR indicate to us that CR may have robust effects on life span even when

initiated late in life. In one embodiment, a CR diet program (such as a long term CR diet program) was initiated in old mice (e.g., 19-month old mice) using the method shown in Figure 7, just prior to the onset of accelerated mortality.

[0162] Figure 8 illustrates that in one embodiment, the longevity of the old mice subjected to the CR diet program was compared to the longevity of the mice subjected to the control diet program. Figure 8 illustrates that after the initiation of the CR diet program at 19 months of age, the mean time to death increased from 11.8 ± 0.7 (SE) months in the control mice to 16.8 ± 1.2 months (SE) in the CR mice (P = 0.004), which is a 42% increase. These results indicate that CR initiated late in life is as effective at extending the remaining lifespan as CR initiated early in life. Late-life CR increased the mean and maximum lifespan by approximately 5 months. In Figure 8, the open circles represent the results obtained from a CR group (e.g. a LT CR group) and the filled circles represent the results obtained from the CON group 109. These groups can be summarized as the results of the CR diet administered to old mammals.

[0163] Conventional methods in the art have led to the conclusion that shifting mice at an advanced age to the CR diet program increased rather than decreased mortality. See for example, Forster, et. al., *Genotype and age influence the effect of caloric intake on mortality in mice*, FASEB J., (2003). The conventional methods shifted old mice that have been on a control diet program abruptly to the CR diet without progressively reducing the caloric intake over time (in stages). Rapid introduction of a CR diet program in old rodents results in elevated mortality. Furthermore, the average weight of the CR mice in these studies was too low, especially the DBA/2 mice, which did not show any effects of CR, suggesting that the CR diet programs used, imposed a state of overt starvation on these mice.

[0164] In one embodiment, a regression analysis revealed that the decrease in the mortality rate of the mice subjected to a CR diet program began within 2 to 3 months of initiating the CR diet program. A break point in the survival curve of the CR and control mice occurred at approximately 21.5 months of age as illustrated in Figure 8. CR thus decreased the mortality rate by 3.1-fold between 21.5 and 31 months of age ($p<0.001$). Thereafter, the mortality rate of the CR mice approximated that of the control mice, but the lifespan was extended by about 5 months ($p<0.001$). These results indicate that CR very rapidly decelerated the underlying rate of aging, even though it was initiated late in life. Additionally, the CR and control mice died primarily of large tumors, mainly adenomas and carcinomas of the liver and lung (data

not shown). Thus, late-life CR appeared to very rapidly delay and/or to decrease the onset and the progression of tumors.

[0165] Referring back to Figure 7, in one embodiment, the results from the CR2 group 103, CR4 group 105, and the CR8 group 107 can be compared to LT-CR and control groups (e.g. LT-CON) to determine the shortest duration of time that the old mammals 101 need to be subjected to a CR diet program to obtain the benefits of CR. In some cases, a short duration such as a two-week duration as in the CR2 group 103 is sufficient to cause a positive effect on the gene expression profiles of the old mammals. In other cases, a longer duration is required, for example, a four-week duration (CR4 group 105) or an eight-week duration (CR8 group 107).

[0166] As in other embodiments discussed above and the those to be discussed below, control data can be obtained from a prior study, the results of which are recorded, as opposed to subjecting a control group of mice to a control diet program concurrently with the test groups of mice as illustrated in Figure 7.

[0167] Figure 9 illustrates an exemplary method 100 of subjecting a group of mammals to various dietary regimens. In one embodiment, the mammalian samples are mice. Male mice of the long-lived F1 hybrid strain B6C3F1 were purchased from Harland Laboratories, Indianapolis. For the first six months the mice were fed Rodent Diet No. 5001 (TMI Nutritional International LLC, Brentwood, MO, 63044). At six months, all mice were individually housed. The 6-month old mice are indicated as mice group 102 as shown in Figure 9. The mice in the group 102 were randomly assigned to two groups, an LT-CON group 104 and an LT-CR group 106. Each mouse in the LT-CON group 104 was subjected to a control diet program with a feeding of 93 kcal per week of a semi-purified control diet in 1gm pellets for a long duration of time (e.g., 20 months in one group of mice). A complete list of diet ingredients or composition can be found in Table 12. Each mouse in the LT-CR group 106 was subjected to an CR diet program with a feeding of 52 kcal per week of the semi-purified diet for a long duration of time (e.g., 14 months in one case of mice). A complete list of the diet ingredients or composition can be found in Table 12.

[0168] In one embodiment, after 20 months of age, the mice from both the LT-CON group 104 and the LT-CR group 106 were subjected to a cross-over (or switching) experiment in which the mice in the LT-CR and the LT-CON groups were switched to opposite dietary regimens. The LT-CON group 104 was sub-divided into four groups, a CR2 group 108, a

CR4 group 110, a CR8 group 112, and an LT-CON continuation group 114. Each mouse in the CR2 group 108 was subjected to a 77 kcal per week CR diet for 1 week followed by a 52 kcal per week CR diet for another 1 week. Each mouse in the CR4 group 110 was subjected to a 77 kcal per week CR diet for 2 weeks followed by a 52 kcal per week for another 2 weeks. Each mouse in the CR8 group 112 was subjected to a 77 kcal per week CR diet for 2 weeks followed by a 52 kcal per week CR diet for 6 weeks. Each mouse in the LT-CON continuation group 114 was maintained on the 93 kcal per week control diet for 8 weeks. Note that the LT-CON continuation group 114 simply refers to a group of mice that is subjected to the control diet for the additional amount of time such as 8 weeks. In one embodiment, the LT-CR group 106 was subdivided into two groups, a CON8 group 116 and an LT-CR continuation group 118. Each mouse in the CON8 group 116 was a LT-CR mouse subjected to a 93 kcal per week control diet for 8 weeks. Each mouse in the LT-CR continuation group 118 was maintained on the 52 kcal per week CR diet for 8 weeks. The LT-CR continuation group 118 simply refers to a group of mice that is subjected to a CR diet program for the additional amount of time such as 8 weeks.

[0169] In one embodiment, the results obtained for all of the test groups can be compared to each other (or to the control data previously recorded) to determine the effects of various CR diet programs and at various durations of time. The test groups can be evaluated using a biochemical measurement such as gene expression level.

[0170] In one embodiment, total liver RNA was isolated from frozen tissue fragments by homogenization in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, as described by the supplier) with an Ultra-Turrax (IKA Works, Inc. Wilmington, NC). mRNA levels were measured using Affymetrix M11K sets A and B high-density oligonucleotide arrays according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA). Briefly, cDNA was prepared from total RNA from each animal using Superscript Choice System with a primer containing oligo(dT) and the T7 RNA polymerase promoter sequence. Biotinylated cRNA was synthesized from purified cDNA using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem). cRNA was purified using RNeasy mini columns (Qiagen, Chatsworth, CA). An equal amount of cRNA from each animal was separately hybridized to MU11 sets A and B high-density oligonucleotide arrays. The arrays were hybridized for 16 h at 45 °C. After hybridization, arrays were processed as described above.

[0171] In embodiments where the Affymetrix GeneChip™ analysis suite is used, each of the MU11K sets A and B comprises targets for more than 12,000 mouse Affymetrix unique identifiers. Each Affymetrix unique identifier is represented on the array by 20 perfectly matched (PM) oligonucleotides and 20 mismatched (MM) control probes that contain a single central-base mismatch. All arrays were scaled to a target intensity of 2500. The signal intensities of PM and MM were used to calculate a discrimination score, R, which is equal to (PM - MM) / (PM + MM). A detection algorithm that utilized R was used to generate a detection p-value and assign a Present, Marginal or Absent call using Wilcoxon's signed rank test. A detailed description of this method can be found in Affymetrix, I, *New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays. Technical Notes 1*, Part No. 701097 Rev.1 (2001), and Wilcoxon F., *Individual Comparisons by Ranking Methods*, Biometrics, 1:80-83 (1945). Only Affymetrix unique identifiers that were "present" in at least 75% of the arrays per experimental group were considered for further analysis. In addition, Affymetrix unique identifiers with signal intensities lower than the median array signal intensity in less than 75% of the arrays per experimental group were eliminated. These selection criteria reduced the raw data from 12,422 Affymetrix unique identifiers to only 2194 Affymetrix unique identifiers, which were considered for further analysis.

[0172] In one embodiment, to identify differentially expressed Affymetrix unique identifiers between any two groups, each of the samples (n) in one group was compared with each of the samples (p) in the other group, resulting in nxp pairwise comparisons. In one embodiment, n is equal to 3 or 4 and p is equal to 3 or 4. In one embodiment, the effects of LT-CR on gene expression were determined by comparing the results between the LT-CON continuation group and the LT-CR continuation group. In another embodiment, the effects of 2 weeks, 4 weeks, and 8 weeks of CR on gene expression were determined by comparing the results between the LT-CON continuation group 114 and the CR2 group 108, CR4 group 110, and CR8 group 112. The effects on gene expression produced by 8 weeks of control feeding were determined by comparing the results between the LT- CON group 114 and the CON8 group 116. In another embodiment, the effects of 2 weeks, 4 weeks, and 8 weeks of CR on gene expression were determined by comparing the results between the LT-CON continuation group 506 and the CR2 group 508, CR4 group 510, and CR8 group 512. The effects on gene expression of 2 weeks, 4 weeks, and 8 weeks of treatment with a candidate intervention will be determined by comparing the results between the LT-CON continuation group 506 and the 2Wk-drug group 514, 4Wk-drug group 516, and 8Wk-drug group 518. In

yet another embodiment, the effects of 2 weeks, 4 weeks, and 8 weeks of CR on gene expression in old mammals were determined by comparing the results between the CON group 109 and the CR2 group 103, CR4 group 105, and CR8 group 107.

[0173] The data were analyzed statistically using a method based on Wilcoxon's signed rank test. Difference values (PM-MM) between any two groups of arrays were used to generate a one-sided p-value for each set of probes. Default boundaries between significant and not significant p-values were used. See Affymetrix, I. *New Statistical Algorithms for Monitoring Gene Expression on GeneChip Probe Arrays*, mentioned above, for more details. The Affymetrix unique identifiers (known genes or ESTs) are considered to have changed expression if the number of increase or decrease calls was at least 75% of the pairwise comparisons. An average fold change, derived from all possible pairwise comparisons, of 1.5-fold or greater was considered significant. Empirically, these criteria for identifying gene expression changes can be reliably verified by methods such as Western blot, Northern blot, dot blot, primary extension, activity assays, real time PCR, and real time reverse transcriptase PCR (RT-PCR).

[0174] The results of the data are illustrated in Tables 14-16 and Figures 10A-10B. Gene names were obtained from the Jackson Laboratory Mouse Genome Informatics database as of August 1, 2002. Gene names were obtained from the LocusLink and Affymetrix databases as of January 23, 2003.

[0175] Tables 14-16 list some of the gene expression effects caused by the LT-CR diet program, CR diet program for 2 weeks, CR diet program for 4 weeks, CR diet program for 8 weeks, and the control diet program administered to mice that have been subjected to the LT-CR diet program and switched to the control diet program (e.g., CON 8 group 116) according to some embodiments. These gene expression effects are illustrated in terms of fold changes. In each of these tables, the Category/Gene column represents the category of the genes and the names of the genes and the Genebank column represents the Genebank identification number of the corresponding genes. In one embodiment, the numbers in the LT-CR column represent the average fold change in specific mRNA derived from all possible pairwise comparison (e.g., 16 possible pairwise comparisons) among individual mice from the LT-CR continuation group and the LT-CON continuation group 114 (e.g., number (n) of mice in each of these two groups is 4). The numbers in the CR2 column represent the average fold change in specific mRNA derived from all possible pairwise comparison (e.g., 16 possible

pairwise comparisons) among individual mice from the CR2 group 108 and the LT-CON continuation group 114 (e.g., n = 4). The numbers in the CR4 column represent the average fold change in specific mRNA derived from all possible pairwise comparison (e.g., 16 possible pairwise comparisons) among individual mice from the CR4 group 110 and the LT-CON continuation group 114 (e.g., n = 4). The numbers in the CR8 column represent the average fold change in specific mRNA derived from all possible pairwise comparison (e.g., 16 possible pairwise comparisons) among individual mice from the CR8 group 112 and the LT-CON continuation group 114 (e.g., n = 4). The numbers in the CON8 column represent the average fold change in specific mRNA derived from all possible pairwise comparison (e.g., 16 possible pairwise comparisons) among individual mice from the CON8 group 116 and the LT-CON continuation group 114 (e.g., n = 4). Where there is no change in gene expression, an "NC" is denoted.

[0176] In one embodiment, the fold changes for each of the genes listed in Tables 14-16 are expressed in ratios. For each ratio, the numerator is the level of expression of each gene from the particular LT-CR, CR2, CR4, CR8, or CON8 group, and the denominator is the level of expression of that gene in the LT-CON continuation group. For example, the fold changes in gene expression caused by an LT-CR diet program is the ratio of the level of expression of each gene in the LT-CR continuation group divided by the level of expression of that gene in the LT-CON continuation group. The fold change in gene expression caused by a CR diet program for 2 weeks is the ratio of the level of expression of each gene in the CR2 group divided by the level of expression of that gene in the LT-CON continuation group. The fold change in gene expression caused by a CR diet program for 4 weeks is the ratio of the level of expression of each gene in the CR4 group divided by the level of expression of that gene in the LT-CON continuation group. The fold changes in gene expression caused by a CR diet program for 8 weeks is the ratio of the level of expression of each gene in the CR8 group divided by the level of expression of that gene in the LT-CON continuation group. The fold changes in gene expression caused by an 8-week switch to a control diet program after a LT-CR diet program is the ratio of the level of expression of each gene in the CON8 group divided by the level of expression of that gene in the LT-CON continuation group.

[0177] Table 14 lists genes which required more than 8 weeks of a CR diet program to change expression. Table 15 lists genes which responded early to a CR diet program and sustained their initial CR-induced expression levels at all subsequent time points for example, across 2 weeks, 4 weeks, 8 weeks, and longer than 8 weeks of a CR diet program; the genes

in Table 15 may be referred to as "stables." Table 16 lists genes which responded early to a CR diet program then returned to control levels briefly, before assuming their LT-CR expression level; the genes in Table 16 may be referred to as "oscillators."

[0178] Validation of the relative gene expression levels was performed using real time RT-PCR. In one embodiment, the expression of a total of 9 genes randomly chosen from among the genes that have changed expression was examined by real time RT-PCR using total liver RNA purified from the mice used in the microarray studies. Total RNA was treated with DNase I (Ambion Inc., Austin, TX) and used to synthesize cDNA in a 20 µl total volume reaction. Briefly, 2 µg of total RNA were incubated with 250 ng random primer (Promega, Madison, WI) for 5 min at 75°C, and then on ice for 5 min. 2 µl of 0.1 M DTT, 4 µl of 5 X buffer, 4 µl of 2.5 mM dNTP, 100 U (units) reverse transcriptase (Invitrogen, Carlsbad, CA), and 16.5 U RNase inhibitor (Promega) were added and incubated for 2 hr at 37°C. The reaction was stopped by boiling at 100°C for 2 min. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA. All samples were reverse-transcribed at the same time and the resulting cDNA was diluted 1:4 in water and stored at -80°C.

[0179] Relative quantification with real-time, two-step real time RT-PCR was performed with a Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) and using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Primers were designed using Netaffx analysis center and verified against the public databases to confirm unique amplification products (http://www.affymetrix.com/analysis/index.affx and http://www.ncbi.nlm.nih.gov), (see Table 13). Primers were chosen for transcription elongation factor A (S-II) 1 to amplify S-II in parallel with the gene of interest. S-II mRNA level is unaffected by a CR diet program. For each of the 9 genes in Table 13, real time RT-PCR was performed with each individual mRNA sample obtained from each mouse from each of the sample groups, for example, the LT-CON continuation group 114 (n = 4), the LT-CR continuation group 118 (n = 4), the CON8 group 116 (n=4), the CR2 group 108 (n=4), the CR4 group 110 (n=4), and the CR8 group 112 (n=4). Briefly, real time RT-PCR was carried out in a 25 µl volume containing 2 µl of diluted cDNA, 1X SYBR Green PCR Master Mix, 0.5 mM of each forward and reverse primers, and 0.5 unit uracil N-glycosylase. The reactions were incubated for 2 min at 50°C to allow degradation of contaminating cDNA by uracil N-glycosylase, and 15 min at 95°C to activate HotStarTaq DNA polymerase. Target amplification reactions were cycled 40 times

with denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis.

[0180] In one embodiment of the invention, the kinetics of the early effects by CR on gene expression are determined to gain insight into the mechanism of the rapid deceleration of aging and the reduction in the incidence of age-related pathology and diseases that result from shifting from a normal diet program to a CR diet program. In this embodiment, Affymetrix microarrays containing probes for approximately 12,000 Affymetrix unique identifiers were used to interrogate RNA samples purified from the old mice that were shifted from the life-long control feeding (e.g., the LT-CON group 104) to a CR diet program for 2, 4, and 8 weeks (e.g., the CR2 group 108, the CR4 group 110, and the CR8 group 112, respectively). In one embodiment, the gene expression profiles of the mice from these CR groups were compared to the gene expression profiles of the mice from the LT-CON group. In addition, the gene expression profiles of the mice from these CR groups were also compared to the gene expression profiles of the mice subjected to a LT-CR diet program (e.g., the LT-CR continuation group 118). Additionally, the gene expression profiles of mice that are shifted from a LT-CR diet program to a control diet program for a short duration of time (e.g., 8 weeks) are also determined by comparing the gene expression profiles of the mice from the CON8 group 116 to the mice from the LT-CON continuation group 114.

[0181] In one embodiment, of the approximately 12,000 Affymetrix unique identifiers interrogated, reliable signals for 2194 identifiers were obtained after data reduction. Figures 10A-10B and Tables 14-16 indicate that LT-CR diet programs altered the expression of 123 the Affymetrix unique identifiers (1% of the interrogated Affymetrix unique identifiers, 6% of the reporting Affymetrix unique identifiers). Figures 10A-10B and Tables 14-16 further indicate the effects of 2 to 8 weeks of CR diet program on the genes whose expression levels are monitored by these Affymetrix unique identifiers.

[0182] In Figures 10A-10B, the various durations of CR and control diets are represented on the x-axis with the indicators CR2, CR4, CR8, LT-CR, and CON8. CR2, CR4, and CR8 indicate the gene expression results for the mice that were subjected to a CR diet program for 2 weeks, 4 weeks, and 8 weeks, respectively (e.g., the CR2 group 108, the CR4 group 110, the CR8 group 112, respectively, of Figure 9). LT-CR indicates the gene expression results for the mice that were subjected to a CR diet program for a long duration of time, e.g., 22

months (e.g., the LT-CR continuation group 118, Figure 9). CON8 indicates the gene expression results for the mice that were subjected to a shift to the control diet program (e.g., for 8 weeks) after being subjected to a CR for a predetermined duration of time (e.g., 20 months) (e.g., the CON8 group 116, Figure 9). The results in Figures 10A-10B demonstrate that following the onset of the CR diet program, there is a rapid and progressive shift toward the gene expression profile associated with the LT-CR diet program.

[0183] In one embodiment, the responding genes are divided into three temporal classes termed early responders, middle responders, and late responders. The early responders are those genes that changed expression between 2 to 4 weeks of the CR diet program. The middle responders are those genes that changed expression between 4 and 8 weeks of the CR diet program. The late responders are those genes that required more than 8 weeks of the CR diet program to respond (e.g., the genes that changed expression in the LT-CR diet program but did not change expression in the CR2, CR4, and CR8 groups). Among the early and middle responders, some genes sustained their CR-induced expression levels at all subsequent time points. For example, as illustrated in Figure 10A, clusters of genes remained in the increased or decreased levels from CR2, to CR4, to CR8, and to LT-CR. These genes may be referred to as stables. Among the early responders, some genes returned to control levels briefly, before assuming their LT-CR expression levels (Figure 10B). These genes may be referred to as oscillators.

[0184] As illustrated in Figures 10A-10B, 71 of the 123 Affymetrix unique identifiers (58%) were early responders, and these were nearly evenly divided between stables and oscillators (37 stables and 34 oscillators). 77 of the Affymetrix unique identifiers (14%) were middle responders (all stables), and 35 Affymetrix unique identifiers (28%) were late responders. These results indicate that the majority of the genes responded early to the effects of CR, and that the stables somewhat outnumber the oscillators.

[0185] Quantitative change in the activity of specific genes can control the rate of aging and/or age-related diseases. For example, quantitative change in the activity of specific genes can decelerate the rate of aging and/or age-related diseases. CR diet programs can alter the expression of genes that affect or decelerate the rate of aging or age-related diseases. Insight into the mechanism or the dynamics of the changes of the genes enables a more complete understanding of the relationship and effects of a CR diet program or a CR mimetic and the observed deceleration of aging and reduction in incidence of age-related pathology and

diseases.. At least some embodiments of the present invention indicate that the deceleration of aging and/or beneficial effects on age-related diseases caused by a CR diet program or a CR mimetic is rapid.

[0186] The early, middle, and late CR responsive genes are likely regulated by different signal transduction pathways. Combinatorial interactions among the components of the pathways may induce or repress genes at each time point. In one embodiment, the pathways involved are further analyzed using motif discovery.

[0187] Switching sample groups to different diet programs according to some of the embodiments discussed above, (e.g., Figure 9) allows for motif discovery. For instance, the switching or crossover feeding distinguished some genes whose expression was altered by LT-CR but not by CR2, CR4, or CR8. Thus, the switching of diet programs allows for motif discovery and allows for genes to be categorized.

[0188] As is apparent from Figures 10A-10B, genes are fractionated into clusters as certain genes are similarly affected by a particular dietary regimen. Genes in the same cluster are likely to be transcriptionally co-regulated and their promoter regions can be analyzed for the presence of shared sequence motifs. Motif discovery begins by identifying genes that are co-regulated under different conditions by CR. Genes which respond in the same way to given physiological conditions are grouped together. For example, as illustrated in Figure 10A, genes which are responsive to CR2 and LT-CR form 2 clusters (14, 17); genes which are responsive to CR4 and LT-CR form 2 clusters (1, 5); and genes which are responsive to CR8 and LT-CR form 2 clusters (7, 10). Also as illustrated in Figure 10A, genes which are only responsive to LT-CR form 2 clusters (14, 21). Switching the mice to an 8-week control diet program following a LT-CR diet program further subdivides genes into 12 clusters (3, 2, 11, 1, 5, 14, 21, 10, 4, 15, 1, 2). The results from Figures 10A-10B indicate that the expression of different genes can be stimulated or inhibited by the same regulatory factors and signal transduction systems.

[0189] In one embodiment, the effects of the transition from a CR diet program to a control diet program are determined. Using some of the embodiments discussed above (for example, the embodiments discussed with reference to Figure 9), it was determined that many, if not most, of the gene expression levels of the mice that were switched to a control diet after a period of being subjected to a CR diet program returned to the control expression levels (Figures 10A-10B). The control expression levels are the gene expression levels of the genes

from mice that are subjected only to a control diet program. These embodiments thus provide methods to directly study the transition of the CR diet program to the control diet program. Switching the mice from the CR diet program (e.g., the LT-CR group 106) to the control diet program (e.g., the CON8 group 116) revealed that many genes that were affected by CR returned to the control expression levels after the switch. In one embodiment, 110 of the 123 (90%) Affymetrix unique identifiers that were affected by the LT-CR diet program returned to control expression levels. Figures 10A-10B indicate that all of the late responsive genes were shifted from their LT-CR expression levels to control expression levels (see for example, the cluster with 14 genes and the cluster with 21 genes at the LT-CR mark which were shifted to the control expression levels at the CON8 mark in Figure 10A). The results in Figures 10A-10B indicate that many of the effects of CR are reversible. These results suggest that even though the late responder genes required more than 8 weeks of the CR diet program to change expression, they were rapidly responsive to changes in caloric intake. These results further indicate that most genes respond rapidly to changes in caloric intake. These results also indicate that a method may be used, as shown in Figure 15 and as discussed further herein, to test a candidate CR mimetic (e.g., a candidate intervention) to determine if the effects of the candidate CR mimetic are reversible.

[0190] In one embodiment, switching to the control diet program for 8 weeks after a CR diet program provides a method of fractionating genes that are responsive to CR into defined clusters amenable to further study.

[0191] In one embodiment, the genes that changed expression due to various CR diet programs at various time points were clustered into functional classes including (1) carbohydrate, fat, and protein metabolism; (2) growth factor and signal transduction; (3) cytoprotective stress-responses, oxidative and reductive xenobiotic metabolism, and chaperones; and (4) immune response and inflammation. Tables 14-16 include the gene expression results of the genes belonging to these classes and how the gene expression of these genes is affected by a CR diet program at 2, 4, and 8 weeks, by an LT-CR diet program, and by a switch to a control diet program after a CR diet program.

[0192] In one embodiment, the genes in the carbohydrate, fat, and protein metabolism class that are altered by a CR diet program are listed in Tables 14-16. Of the 26 metabolic genes discussed below, 23 were early or middle responders. Thus, the initial phases of the metabolic transition from the control to the CR state occur essentially completely during the

first 8 weeks of the CR diet program. Some oscillators return to control expression levels before reaching their LT-CR expression levels. Consistent with their rapid shift in response to the CR diet program, 23 of the 26 genes reverted to control expression levels after only 8 weeks of control diet program.

[0193] As illustrated in Table 15, CR induced the expression of three urea cycle enzymes, arginase 1, argininosuccinate lyase, and argininosuccinate synthetase 1. Nitrogen derived from amino acid catabolism in the periphery is disposed of from the liver via the urea cycle. Thus, CR enhances the disposal of nitrogen in the liver. CR also increased the expression of cathepsin L (Table 14), phenylalanine hydroxylase (Table 16), homogentisate 1, 2-dioxygenase (Table 14), ornithine aminotransferase (Table 16) and histidine ammonia lyase (Table 14). These genes are involved in amino acid degradation to provide substrates for gluconeogenesis. Consistent with these effects, CR induced expression of phosphoenolpyruvate carboxykinase 1 (Table 16) and glucose-6-phosphatase (Table 16), which are the key gating enzymes of gluconeogenesis. These results indicate that CR enhances the enzymatic capacity of the degradation of amino acids for energy production. Because the weights of the animals are approximately at steady state, CR apparently enhances the turnover and resynthesis of whole body protein. It is to be noted that such effects are observed not only during fasting, but also in the hours following feeding.

[0194] Continuing with the metabolism class, CR positively affected the function of lipid metabolism. CR decreased the expression of acetyl-CoA acetyltransferase 1 (Table 15), fatty acid Coenzyme A ligase, long chain 2 (Table 15), 2,4-dienoyl-CoA reductase mitochondrial (Table 15), liver fatty acid binding protein 1 (Table 15), and hepatic lipase (Table 16). The decrease in the expression of these genes should reduce the enzymatic capacity for lipid biosynthesis and metabolism. The decrease in the expression of these genes may account for the decrease in serum triglycerides observed in rodents that were subjected to a CR diet program.

[0195] Still continuing with the carbohydrate, fat, and protein metabolism class, CR also increased the expression of apolipoprotein B-100 (Table 15), which is a major component of low density lipoprotein and very low density lipoproteins. The increased expression of this gene also enhances its role in the distribution of hepatic lipid to other tissues for use as fuel. Additionally, CR also decreased the expression of hydroxysteroid 17-beta dehydrogenase 5 (Table 16) and hydroxysteroid 17-beta dehydrogenase 2 (Table 14), which are enzymes

responsible for the biological inactivation of testosterone. The decreased expression of these genes may help in maintaining or controlling the level of testosterone in aging mammals. For instance, the decreased expression of these genes may account for the higher testosterone levels seen in old rodents that were subjected to a CR diet program compared to old rodents that were not subjected to a CR diet program.

[0196] Still continuing with the carbohydrate, fat, and protein metabolism class, CR also decreased the expression of the mRNA for hydroxyprostaglandin dehydrogenase 15 (Table 16). This gene catalyzes the initial step in the inactivation of circulating prostaglandins, including prostaglandin E(2). The inactivation of the circulating prostaglandins may be a compensatory response to a reduced and/or age-associated systemic inflammation in animals that were subjected to a CR diet program.

[0197] Continuing with the carbohydrate, fat, and protein metabolism class, CR beneficially affected methylation activity. CR decreased the expression of thioether S-methyltransferase (Table 16), which catalyzes the transfer of the methyl group from S-adenosylmethionine to sulfur, selenium, or tellurium compounds. CR increased the expression of S-adenosylhomocysteine hydrolase (Table 16), which hydrolyzes the S-adenosylhomocysteine (SAH) formed after donation of the methyl group of S-adenosylmethionine (SAM) to a methyl acceptor. CR also increased the expression of glycine N-methyltransferase (Table 16), which catalyzes the methylation of glycine by S-adenosylmethionine to form N-methylglycine (sarcosine) and SAH. Glycine N-methyltransferase and S-adenosylhomocysteine hydrolased together can control the SAM to SAH ratio. Increased SAH leads to decreased transmethylation of phospholipids, proteins, small molecules, DNA and RNA. Decreased methylation is generally associated with enhancement of transcriptional activity and differentiation.

[0198] In one embodiment, the genes in the class of signal transducers and growth factors that were affected by a CR diet program are listed in Tables 14-16. As illustrated, CR altered the expression of genes associated with cell growth and proliferation. In one embodiment, CR decreased the expression of lymphocyte antigen 6 complex, locus E (Table 14), Ras homolog gene family, member U (Table 16), and inhibitor of DNA binding 2 gene (Table 14).

[0199] CR also decreased the expression of two genes associated with angiogenesis, Eph receptor B4 (Table 15) and ectonucleotide pyrophosphatase/phosphodiesterase 2 (Table 14).

Moreover, CR induced the expression of phosphatase and tensin homolog gene (Table 15), which has a tumor suppression activity. Thus, CR appears to enhance anti-proliferative growth control.

[0200] CR also decreased the expression of transthyretin (Table 15), and thyroid hormone receptor alpha (Table 15), which are the major thyroid hormone carrier proteins in rodents. The decreased expression of these genes leads to reduced thyroid hormone signals in animals and humans that are subjected to a CR diet program. The reduction of thyroid hormone signal in turn reduces a diverse set of energy utilization-related processes, including the metabolism of lipids, carbohydrates, and proteins, and oxygen consumption.

[0201] The results above indicate that CR extends the longevity and delays the onset of age-related diseases in mammals. Furthermore, these results indicate that CR is also effective in treating old mammals (as well as younger mammals) and that CR acts rapidly to bring the benefits of CR to the mammals.

[0202] In one embodiment, the results in Tables 14-16 also indicate that CR altered the expression of chaperone proteins. Most proteins require interactions with molecular chaperones for their biosynthesis, maturation, processing, transport, secretion, and degradation. It has been found that the mRNA and protein levels of most endoplasmic-reticulum chaperones increase with age. CR decreases the caloric intake in the liver and other tissues thus decreasing the mRNA and protein levels of most endoplasmic reticulum chaperones. The linkage between caloric intake and chaperone expression may match protein folding, assembly, and processing capacity to the level of insulin stimulated protein biosynthetic activity. Elevated chaperone expression also decreases apoptotic responsiveness to genotoxic stress. Chaperones repress apoptosis through both the endoplasmic stress and the mitochondrial apoptosis signaling pathways. The anti-cancer benefits of CR may result from the fact that CR reduces endoplasmic reticulum chaperone levels and enhances apoptosis in liver and other cell types. In contrast, in non-dividing cells, such as neurons, CR appears to induce chaperone expression, thereby enhancing cell survival.

[0203] In one embodiment, the results in Tables 14-16 also indicate that CR altered the expression of genes in the xenobiotic metabolism class. CR differentially regulated the expression of a number of phase I and II enzyme genes. For example, CR enhanced the expression of N-sulfotransferase (Table 15), flavin-containing monooxygenase 5 (Table 16), several cytochrome P450 isozymes and glutathione S-transferase, mu2 (Table 15). Examples

of some of the cytochrome P450 isozymes that are enhanced by CR include cytochrome P450, 3a16 (Table 16), cytochrome P450, steroid inducible 3a11 (Table 16), cytochrome P450, steroid inducible 3a13 (Table 16), cytochrome P450, 2b13, phenobarbitol inducible, type c (Table 15), cytochrome P450, 2b13, phenobarbitol inducible, type a (Table 15), and cytochrome P450 oxidoreductase (Table 15). The increased expression of these genes may enhance drug metabolization and detoxification functions of the liver. It is to be noted that many of these enzymes also can enhance toxicity and carcinogenicity of some substrates. Thus, the effects that CR will have on xenobiotic metabolism are dependent on the xenobiotic environment. The physiological impact of the CR on the decreased expression of cytochrome p450, 1a2 (Table 14), cytochrome p450, 2f2 (Table 14), cytochrome p450, 2j5 (Table 16) and cytochrome p450, 7b1 (Table 16), and glutathione S-transferase, pi 2 (Table 14) is difficult to predict. For example, CR is reported to induce Cyp2e1, which leads to 2.5-fold greater bioactivation thioacetamide, a potent hepatotoxin and carcinogen. However, CR also increased resistance to thioacetamide hepatotoxicity, perhaps by enhancing the rate of liver apoptosis and regeneration. Thus, through differential gene regulation CR may strike a balance between toxin and carcinogen activation and deactivation, and cellular growth and apoptosis.

[0204] In one embodiment, to verify or validate that the genes responded as indicated in Figures 10A-10B the expression of 9 randomly chosen genes was monitored by a quantitative PCR (e.g., real time RT-PCR) as illustrated in Figures 11A-11E. In every case, quantitative PCR confirmed the changes found by microarray gene expression profiling at each of the CR time-points.

[0205] Figures 11A-11E illustrate the results of validating the 9 randomly chosen genes (with gene names V00835, U51805, AF026073, M27796, M16358, U00445, X51942, U70139, and U44389) using the real time RT-PCR. Real time RT-PCR confirmed the changes found by microarray gene expression profiling for each of the 9 chosen genes. As can be seen from this figure, the fold changes are in the same direction and are substantially similar in the amount of the fold changes.

[0206] Figure 11A illustrates validation of some of the genes that change with LT-CR (see genes V00835, U51805, AF026073, M27796, and M16358). The open bars represent the microarray data and the solid bars represent the real time RT-PCR data. The real time RT-PCR data represent the fold changes in the specific mRNA derived from comparing the

results between the mice from the LT-CR continuation group and mice from the LT-CON continuation group measured using real time RT-PCR. The microarray data represent the average fold changes in the specific mRNA derived from all possible pairwise comparisons among individual mice from the LT-CR continuation group and the LT-CON continuation group.

[0207] Figures 11B-11E illustrate some of the genes that have changes in expression that fluctuates across the various time points. The triangles represent the microarray data and the squares represent the real time RT-PCR data. Figure 11B compares the microarray data and the real time RT-PCR data for the gene U00445. Figure 11C compares the microarray data and the real time RT-PCR data for the gene X51942. Figure 11D compares the microarray data and the real time RT-PCR data for the gene U70139. Figure 11E compares the microarray data and the real time RT-PCR data for the gene U44389.

[0208] The results in Figures 11A-11E indicate that the use of the microarray analytical methods that are used for some of the embodiments of the present invention reliably identified genes that change expression. The validation of the microarray analytical methods also insures that the complexity of the response of the genes did not arise simply from the stringency of the selection criterion. As can be seen from these figures, in no case did the assignment increase, or decrease in fold change in the gene expression levels arise from close calls in the selection criterion.

[0209] As shown in Figures 11A-11B, the fold changes in the 9 randomly chosen genes confirmed the data obtained using the microarray methods. Thus, the expression patterns shown in Figures 10A-10B represent the true kinetics of the response to CR in old mice.

[0210] Using some of the techniques previously described, a candidate intervention can be discovered and analyzed. Figure 12 illustrates that in one embodiment, a candidate intervention that is a CR mimetic candidate or a potential CR mimetic can be administered to a group of mammals for different lengths of time. This figure illustrates that in one embodiment, mammalian samples 502 (e.g., mice) are subjected to an LT-CON diet program generating an LT-CON group 504. Each member of the mammalian samples 502 is fed a 93-kcal per week control diet for a predetermined duration of time, e.g., 20 months, to generate the LT-CON group 504. In one embodiment, the 93-kcal per week control diet is a normal diet program in the embodiments where the mammalian samples are mice. The normal

number of calories may change accordingly depending on the type of the mammalians samples.

[0211] After the predetermined duration of time, the members in the LT-CON group 504 are divided into several groups, which include an LT-CON continuation group 506, a CR2 group 508, a CR4 group 510, a CR8 group 512, a 2Wk drug group 514, a 4Wk drug group 516, and an 8Wk drug group 518. Each of the members in the CR2 group 508 is subjected to a CR diet program that reduces the number of calories from 93 kcal per week to 77 kcal per week for 1 week followed by a 52 kcal per week for another 1 week. Each of the members in the CR4 group 510 is subjected to a CR diet program that reduces the number of calories from 93 kcal per week to 77 kcal per week for 2 weeks followed by a 52 kcal per week for another 2 weeks. Each of the members in the CR8 group 508 is subjected to a CR diet program that reduces the number of calories from 93 kcal per week to 77 kcal per week for 2 weeks followed by a 52 kcal per week for 6 weeks.

[0212] Each of the members in the 2Wk drug group 514 is subjected to an administration of the candidate intervention for a specified duration of time. Each member of the 2Wk-drug group 514 is subjected to the administration of the candidate intervention for 2 weeks. Similarly, each member of the 4Wk-drug group 516 is subjected to an administration of the candidate intervention for a duration of 4 weeks. Each member of the 8Wk-drug group 518 is subjected to an administration of the candidate intervention for a duration of 8 weeks. The number of calories in the control diets fed to these groups is maintained at the normal level, e.g., 93 kcal per week for the mammalian species used in this case. The dosage of the intervention can be an effective dosage or a testing dosage. For instance, a candidate intervention can be Metformin, which may be administered in the diet of the members of the drug groups with a dosage of approximately between 0.2 mg and 2.0 gm of Metformin per kg body weight per day. In one embodiment, the 2100 mg of Metformin are added to 1 kg of the control diet. It is to be appreciated that Metformin is not the only candidate intervention. Examples of other possible candidate interventions include glucose regulatory agents such as Glipizide, and Rosiglitazone as well as countless others which may be screened as possible CR mimetics or other types of candidate intervention which may reproduce or mimic at least some of the benefits of CR.

[0213] The results of biochemical measurements (e.g., gene expression levels) from the LT-CON continuation group 506, CR2 group 508, CR4 group 510, CR8 group 512, 2Wk-

drug group 514, 4Wk-drug group 516, and 8Wk-drug group 518 are compared to each other. In one embodiment, the results include the changes in gene expression profiles and/or life extension for each of the groups tested. The gene expression profiles for the mice in these test groups can be determined using the methods described above. In one embodiment, the effects of the CR2 group 508, CR4 group 510, and CR8 group 512 are obtained by comparing the results from each of the CR2 group 508, CR4 group 510, and CR8 group 512 to the results of the LT-CON group 504. The effects of the 2Wk-drug group 514, 4Wk-drug group 516, and 8Wk-drug group 518 are obtained by comparing the results from each of the 2Wk-drug group 514, 4Wk-drug group 516, and 8Wk-drug group 518 to the results of the LT-CON group 504. The results from each of the 2Wk-drug group 514, 4Wk-drug group 516, and 8Wk-drug group 518 can also be compared to the results from the CR2 group 508, CR4 group 510, and CR8 group 512.

[0214] Administering the candidate intervention to the mammalian samples for different durations of time allows for the determination of the dynamics of the candidate intervention in reproducing the effects or some of the effects of CR. Additionally, using this approach, it can be determined whether the candidate intervention can act rapidly to bring some of the CR beneficial effects to the mammalian samples (and thus mimic at least some of the effects of CR). For example, when the gene expression profiles for mice from a particular drug group (e.g., 2Wk-drug, 4Wk-drug, or 8Wk-drug group) substantially correlate with the gene expression profiles for mice from a particular CR group (CR2, CR4, CR8, or LT-CR group), the candidate is identified as a CR mimetic that reproduces at least some of the effects of CR or at least some of the effects of CR administered for a particular duration of time.

[0215] Figure 13 illustrates that, in another embodiment, a candidate intervention is administered to individuals in a mammalian group. This embodiment is particularly helpful to determine whether the candidate intervention can be used to bring the beneficial effects of CR to old mammals. The mammalian group can be a human group, a rodent group, or any other animal group. The candidate intervention can be an intervention identified using the methods previously described. In Figure 13, a control diet program is administered to individuals in the mammalian group (box 402). After the start of old age for the mammalian group (e.g., 20 months if the mammalian group is a mouse group), the candidate intervention is administered to some of the individuals in the mammalian group (box 404). The remaining individuals of the mammalian group are maintained on the control diet program (box 406). The results (e.g., gene expression profiles or longevity) between the individuals subjected to

the candidate intervention and the individuals maintained on the control diet program can be compared to each other to determine whether a candidate intervention brings benefits to the mammals tested. Additionally, the results of the candidate intervention can be compared to a pre-recorded data of CR testing to determine whether the candidate intervention can reproduce at least some of the effects of CR and be effective in treating the mammals at an older age.

[0216] In one embodiment, the candidate intervention is added concurrently with the control diet program. Thus, the candidate intervention can be mixed or added into the control diet or administered in addition to the diet. The control diet includes a normal number of calories for the particular mammals, for instance, when the mammals are mice, the number of calories of control food to be fed to each mouse may be about 93 kcal/week.

[0217] In one embodiment, to determine the effects of the candidate intervention, the gene expression profiles obtained from the mammals that were subjected to the candidate intervention are compared to the gene expression profiles obtained from the mammals that were subjected to the control diet program without the candidate intervention and to mammals that were subjected to a CR diet program. In one embodiment, a plurality of gene expression levels from the mammal subjected to the candidate intervention is compared the same type of plurality of gene expression levels from the mammals subjected to the control diet program and to mammals that were subjected to a CR diet program. The extent to which the effects (e.g., gene expression levels) of the candidate intervention match or correlate with the effects of CR will determine the likelihood that the candidate intervention is a CR mimetic. The higher the match or correlation in effects then the more likely that the candidate intervention is a CR mimetic and may be capable of reproducing at least some of the benefits of CR. The extent to which the dynamics of the effects (e.g., early responders versus late responders, etc.,) of the candidate intervention match the dynamics of the effects of CR will also determine the likelihood that the candidate intervention is a CR mimetic and thus may produce some of the benefits of CR.

[0218] In another embodiment, another group of mammals (same type of mammals) is subjected to a CR diet program (e.g., a ST-CR or a LT-CR diet program) (not shown in Figure 13). This group of mammals may consist of old mammals, young mammals, or middle-age mammals. The results from the mammal group that was subjected to the candidate intervention can be compared to the results from the mammal group that was

subjected to the CR diet program. When the gene expression levels of the mammal group that was subjected to the candidate intervention matches or substantially correlates with the corresponding gene expression levels of the mammal group that was subjected to the CR diet program, the candidate intervention can be identified as a CR mimetic or an intervention that deserves further screening. In one embodiment, the gene expression levels substantially correlate when the gene expression levels have the same direction of expression or changes and about the same magnitude of expression or changes.

[0219] The embodiments below take advantage of the finding that CR is rapidly reversible as discussed in several embodiments above. Figure 14 illustrates that in one embodiment, an LT-CR diet program is administered to individuals in a mammalian group, (box 302). The mammalian group can be a human group, a rodent group, or any other animal test group. After a predetermined amount of time, e.g., 20 months in the case of mice, some individuals of the mammalian group are switched to an ST-CON diet program for a short amount of time, e.g., 2 months in the case of mice. Also after this predetermined amount of time, the remaining individuals of the mammalian group are maintained on the LT-CR for the same short amount of time, e.g., 2 months in the case of mice.

[0220] The results from this embodiment indicate that CR is reversible. This embodiment can also be used to analyze whether a candidate intervention is reversible as CR is. An example of this method of testing the reversibility of a candidate intervention is shown in Figure 15. For example, a candidate intervention can be administered to another group of mammals (same type of mammals) for a long (or short) duration of time (as in box 302). Thus, instead of being subjected to an LT-CR as indicated in box 302, this other group of mammals is subjected to the administration of the intervention. Following that administration, the intervention is withdrawn, and the effects of the withdrawal of the intervention are compared to the effects of the withdrawal of CR. For instance, when the individuals are switched to the control diet, the reversible effects that are caused by CR should be reversed.

[0221] In one embodiment, at least one biochemical measurement (e.g., gene expression level measurement) is performed after the drug groups were exposed to the candidate intervention. The biochemical measurement is designed to show whether the candidate intervention substantially mimics or mimics at least some of the effects of CR (e.g., gene expression levels of genes known to change due to CR are measured after the candidate

intervention). In one embodiment, the gene expression levels of the mammals from the particular drug group (e.g., 2Wk-drug group, 4Wk-drug group, and 8Wk-drug group) are compared to the corresponding gene expression levels of the mammals from the particular CR group (CR2 group, CR4 group, or CR8group). In another embodiment, the gene expression levels for the mammals from the particular drug group are compared to the corresponding gene expression levels for the mammals from a LT-CR group (e.g., the LT-CR continuation group 118 of Figure 9).

[0222] Continuing with Figure 15, in one embodiment, the candidate intervention is withdrawn from a test group. In this embodiment, the administration of the candidate intervention to a mammalian group, (e.g., as in the 8Wk-drug group 518), is withdrawn from this group. In one embodiment, the 8Wk-drug group 518 is converted to a drug-withdrawal group and is subjected to only a control diet for a duration of time, e.g., 1-2 weeks. Similarly, a CR group can also be withdrawn from the CR diet program. In one embodiment, the CR8 group 512 is withdrawn from the CR diet program for the same duration of time (e.g., 1-2 weeks). The effects of withdrawing the candidate intervention can be compared to the effects of withdrawing the CR diet program to determine whether the intervention substantially mimics or mimics at least some of the effects of withdrawing the CR diet program. This embodiment enables one to determine whether the effects produced by a candidate intervention are substantially the same as the effects produced by the CR diet program.

[0223] In another embodiment, the CR diet program may be administered to a group for more than 8 weeks. Thus, for the CR8 group 512, instead of being subjected to the CR diet program for 8 weeks, the mammalian sample group may be subjected to an LT-CR diet program, a CR diet program with a duration longer than 8 weeks, e.g., 20 weeks in a case of mice. Similarly, the candidate intervention may be administered to a drug group for more than 8 weeks. Thus, for the 8Wk-drug group, instead of being subjected to an administration of the candidate intervention for only 8 weeks, the mammalian sample group is subjected to the administration of the candidate intervention for longer 8 weeks, e.g., 20 weeks in a case of mice. Following the long duration, these groups may be switched to a control diet program. The results of the switch can be determined to see if the effects of the exposure and the withdrawal of the candidate intervention are similar to the effects of the exposure to and then withdrawal of the CR diet program. Normally, in one exemplary embodiment, these effects are measured by comparing gene expression levels, of genes known to change due to the introduction and/or withdrawal of CR, of members of a CR group (exposed to CR and

then withdrawn from CR) to gene expression levels (for the same genes) of members of a drug group (exposed to a candidate intervention and then withdrawn from the candidate intervention).

[0224] All publications, patent applications, and accession numbers cited in this specification are herein incorporated by reference as if each individual publication, patent application, or accession number were specifically and individually indicated to be incorporated by reference.

[0225] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Table 1. Primer sequences for real-time RT-PCR.

GenBank	Gene	PCR Primer Sequences	Product Size (bp)
X00496	Ia-associated invariant chain	CTGGGTCAAGTCACCCTGTGAAGAC CGATGAAACAGACACCAGTCTCAAG	156
X58251	Procollagen, type I, alpha 2	CCAACAAGCATGTCTGGTTAGGAGA TGTTCTGAGAAGCACGGTTGGCTAG	138
U47737	Lymphocyte antigen 6 complex, locus E	CCCTGGTATCATTGTACCCACCTTG GATGGGACTCAACTGCATCGGGTAG	108
X04653	Lymphocyte antigen 6 complex, locus A	TGCTGGGTAGGTAGGTGCTCTAATC GATACATGTGGGAACATTGCAGGAC	196
X52046	Procollagen, type III, alpha 1	AGAAGTCTCTGAAGCTGATGGGATC GCCTTGCGTGTTTGATATTCAAAGA	148
Y08027	ADP-ribosyltransferase 3	AATTGTATCGCGAACGCAGAATATA AAGGTTGTTCCTACCAGAGTCTTCA	96
AB005450	Carbonic anhydrase 14	TCTGAGCCCCTTGTACAGAACTACA GACCCAGCATCTCTCCTGTGGTATA	112
Z68618	Transgelin	TCTTAGCCCTGACAGCTCTGAGGTG ACTTCTCCCTGCTTACTCCAGGATG	179
D16497	Natriuretic peptide precursor type B	AGCTCTTGAAGGACCAAGGCCTCAC TATCTTGTGCCCAAAGCAGCTTGAG	137
M18209	Transcription elongation factor A (SII), 2	CCAGCTGAAATGTAGGCTGTAGCAA ACAGGAGTCTGAACACAGGCAGAAG	199

Table 2. Effects of LT-CR and switch to opposite dietary regimens on heart gene expression

Gene / Protein	GenBank	LT-CR[1, 4]	ST-CR[2, 4]	ST-CON[3, 4]
ECM and Cytoskeleton				
Actin, alpha 1, skeletal muscle	M12347	NC	-1.7	1.5
Connective tissue growth factor	M70642	NC	-1.9	NC
Microtubule-associated protein tau	M18775	-1.5	NC	NC
Procollagen, type I alpha 1	U03419	-1.6	-1.8	NC
Procollagen, type I, alpha 2	X58251	-1.5	-1.5	NC
Procollagen, type III, alpha 1	X52046	-1.5	-1.8	NC
Procollagen, type IV, alpha 1	M15832	NC	-1.5	NC
Tissue inhibitor of metalloproteinase 3	U26437	1.6	NC	NC
Transgelin	Z68618	1.6	NC	NC
Metabolism				
ADP-ribosyltransferase 3	Y08027	1.7	NC	NC
Apolipoprotein B editing complex 2	AW124988	1.5	NC	NC
Carbonic anhydrase 14	AB005450	1.6	NC	NC
Carboxylesterase 3	AW226939	-1.5	NC	NC
CCAAT/enhancer binding protein (C/EBP), delta	X61800	NC	-1.6	NC
Cysteine dioxygenase 1, cytosolic	AI854020	2.3	NC	NC
Cytosolic acyl-CoA thioesterase 1	Y14004	1.5	NC	NC
Iduronidase, alpha-L-	L34111	1.8	NC	NC
Stearoyl-Coenzyme A desaturase 1	M21285	5.4	2.4	2.6
Sulfotransferase family 1A, phenol-preferring, member 1	L02331	1.8	NC	NC
Suppressor of K+ transport defect 3	U09874	-1.6	NC	NC
Signal Transducers, Growth Factors				
A disintegrin and metalloproteinase domain 19 (meltrin beta)	AA726223	NC	-1.7	NC
Cyclin-dependent kinase inhibitor 1A (P21)	AW048937	1.8	NC	NC
Cysteine rich protein b1	M32490	-1.6	-1.5	NC
Down syndrome critical region homolog 1	AI846152	-1.5	-1.6	NC
Epithelial membrane protein 1	X98471	-1.7	NC	NC
G protein-coupled receptor kinase 5	AI639925	-1.5	NC	NC
Interferon induced transmembrane protein 3-like	AW125390	-1.5	NC	NC
Natriuretic peptide precursor type B	D16497	-1.9	-2.3	-1.5
Nuclear factor I/X	AA002843	2.1	NC	NC
p53 regulated PA26 nuclear protein	AI843106	1.5	NC	NC
Profilin 2	AW122536	-1.5	NC	NC
Ribosomal protein S6 kinase, 90kD, polypeptide	AJ131021	-1.5	NC	NC
Stromal cell derived factor 1	AV139913	-1.6	NC	NC
Immune Response and Inflammation				
B-cell translocation gene 3	D83745	1.5	NC	NC
Complement component 1, q subcomponent, c polypeptide	X66295	-1.7	NC	NC
Cytotoxic T lymphocyte-associated protein 2 alpha	X15591	-1.7	NC	NC
Histocompatibility 2, K region locus 2	M27134	-1.6	NC	NC
Ia-associated invariant chain	X00496	-2.1	-1.6	NC
Ig kappa chain V-region	M18237	1.6	NC	1.9
Interferon activated gene 205	M74123	-1.5	NC	NC
Lymphocyte antigen 6 complex, locus A	X04653	-1.7	NC	NC
Lymphocyte antigen 6 complex, locus E	U47737	-1.8	-1.5	NC
Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	M33960	NC	-1.5	NC
Stress Response and Xenobiotic Metabolism				
Cytochrome P450, 2e1, ethanol inducible	X01026	1.9	NC	NC
Homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1	AI846938	1.6	NC	NC
Thioether S-methyltransferase	M88694	1.5	1.5	NC
Miscellaneous				

Catechol-O-methyltransferase	AF076156	NC	-1.5	NC
H19 fetal liver mRNA	X58196	1.5	NC	NC
RNA binding motif protein 3	AB016424	NC	1.6	NC
Zinc finger protein 145	AI553024	2.9	1.5	1.5
EST	AI596360	1.6	NC	NC
EST	AV376312	1.9	NC	NC
EST	AI847069	1.5	NC	NC
EST	AA833425	1.6	NC	NC
EST	AI851695	1.6	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and LT-CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and LT-CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CON and LT-CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 3. Experimental Groups.

Group	Drug or diet
1	Metformin (2100)
2	Glipizide (1050)
3	Metformin (1050) & Glipizide (525)
4	Rosiglitazone (80)
5	Soy (.25%)
6	Long-term calorie restriction
7	Short-term calorie restriction (8 weeks)
8	Control

Notes: Numbers in parentheses indicate the amount of each compound in mg/kilogram of the control diet, unless otherwise indicated.

Table 4. Percentage of drug-specific effects and overlap between the effects of CR and those of each of the drugs used.

	Metformin	Glipizide	Metformin & Glipizide	Rosiglitazone	Soy
LT- or ST-CR	57%	30%	35%	48%	67%
LT-CR	51%	30%	30%	36%	67%
ST-CR	33%	15%	17%	39%	33%
LT- and ST-CR	27%	15%	13%	27%	33%
Drug-specific	38%	57%	54%	41%	33%

Table 5. Effects of Metformin and CR on hepatic gene expression.

Gene / Protein	GenBank	Metformin[1]	LT-CR[2, 4]	ST-CR[3, 4]
Changes in gene expression induced by Metformin and reproduced with either LT- or ST-CR				
Stress and chaperone proteins				
Cytochrome P450, 2b13, phenobarbitol inducible, type c	M60358	3.4	2.7	1.8
Cytochrome P450, 4a12	Y10221	-3.1	-3.2	-2.8
ATP-binding cassette, sub-family G (WHITE), member 2	AF103875	-1.5	-1.6	NC
Metallothionein 2	K02236	-1.9	-4.4	NC
Glucose regulated protein, 58 kDa	M73329	-1.5	-1.6	-1.5
Heat shock 70kD protein 5 (glucose-regulated protein, 78 kD)	AJ002387	-1.5	-1.8	-1.5
Metabolism				
Farnesyl pyrophosphate synthase	AI846851	3.1	1.5	1.7
Farnesyl pyrophosphate synthase (Second time)	AW045533	3.7	1.5	1.5
Fatty acid synthase	X13135	2.4	NC	1.6
ATP-binding cassette, sub-family A (ABC1), member 1	AI845514	-1.5	-1.5	NC
Glucose-6-phosphatase, catalytic	U00445	1.6	2.8	NC
Aquaporin 1	L02914	1.6	1.5	NC
Arylsulfatase A	X73230	-1.7	-2.4	-2.2
Arylsulfatase A (second time)	AF109906	1.8	4.6	NC
Cytoskeleton				
keratin complex 1, acidic, gene 18	M22832	-1.7	-1.7	-1.5
Keratin complex 2, basic, gene 8	X15662	-1.5	-2.2	-1.7
Actin, gamma, cytoplasmic	M21495	-1.5	-3.2	-2.1
Actin, beta, cytoplasmic	M12481	-1.6	-1.5	NC
Vinculin	AI462105	-1.5	-1.6	NC
Signal Transduction				
Ectonucleotide pyrophosphatase/phosphodiesterase 2	AW122933	-1.5	-2.9	-1.5
Dual specificity phosphatase 1	X61940	1.5	1.7	NC
Suppressor of cytokine signaling 2	U88327	1.6	1.9	1.7
Interferon gamma induced GTPase	U53219	-1.7	-3.1	-1.7
Interferon-g induced GTPase	AJ007972	-1.5	-2.7	-1.7
Interferon-inducible GTPase	AA914345	-1.7	-2.9	-1.5
Interferon-inducible GTPase (second copy)	AJ007971	-1.6	-2.7	-1.6
Pre B-cell leukemia transcription factor 1	AW124932	1.8	NC	1.5
Regulator of G-protein signaling 16	U94828	2.0	NC	1.6
Activating transcription factor 3	U19118	-1.9	-1.8	-1.5
Cholinergic receptor, nicotinic, beta polypeptide 3	AI842969	-1.5	-1.7	NC
Miscellaneous				
Complement component 9	X05475	-1.5	-2.1	NC
Hermansky-Pudlak syndrome 1 homolog (human)	AI551087	-1.6	-1.5	NC
Major urinary protein 1	AI255271	-1.6	NC	-1.5
EST	C79248	-1.6	-1.7	NC

EST	AI787317	-1.6	-1.7	NC
EST	AA690218	1.5	2.6	NC

Metformin-specific changes in gene expression

Energy metabolism				
Pyruvate kinase liver and red blood cell	D63764	1.8	NC	NC
Glucokinase	L41631	1.6	NC	NC
Diaphorase 1 (NADH)(cytochrome b-5 reductase)	AW122731	1.5	NC	NC
Guanidinoacetate methyltransferase	AF010499	1.5	NC	NC
NAD(P) dependent steroid dehydrogenase-like	AW106745	1.9	NC	NC
Phospholipid transfer protein	U28960	1.8	NC	NC
Thyroid hormone responsive SPOT14 homolog (Rattus)	X95279	2.4	NC	NC
Trans-golgi network protein 2	AA614914	-1.5	NC	NC
Glutathione S-transferase, alpha 2 (Yc2)	J03958	-1.5	NC	NC
NAD(P) dependent steroid dehydrogenase-like	AL021127	2.0	NC	NC
Transketolase	U05809	1.5	NC	NC
Signal transduction				
Programmed cell death 4	D86344	-1.6	NC	NC
Protein phosphatase 1, catalytic subunit, beta isoform	M27073	-1.5	NC	NC
Diazepam binding inhibitor	X61431	1.7	NC	NC
Enolase 1, alpha non-neuron	AI841389	1.5	NC	NC
Miscellaneous				
Ia-associated invariant chain	X00496	1.5	NC	NC
Murinoglobulin 1	M65736	-1.5	NC	NC
Zinc finger protein 265	AI835041	-1.6	NC	NC
EST	AI853364	1.7	NC	NC
EST	AI852741	-1.5	NC	NC
EST	AV291989	-1.5	NC	NC
EST	AA733664	-1.5	NC	NC
EST	AW212131	-1.5	NC	NC
EST	AW124226	-1.6	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from Metformin and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 6. Effects of Glipizide and CR on hepatic gene expression.

Gene / Protein	GenBank	Glipizide[1]	LT-CR[2, 4]	ST-CR[3, 4]
Changes in gene expression induced by Glipizide and reproduced with either LT- or ST-CR				
Stress and chaperone proteins				
Heat shock protein, 105 kDa	L40406	1.7	2.3	NC
Cytochrome P450, 4a12	Y10221	-1.9	3.2	-2.8
ATP-binding cassette, sub-family G (WHITE), member 2	AF103875	-1.5	-1.6	NC
Metabolism				
Vanin 1	AJ132098	-1.5	-1.6	-1.5
Ectonucleotide pyrophosphatase/phosphodiesterase 2	AW122933	-1.6	-2.9	-1.5
Retinoic acid early transcript gamma	D64162	-1.5	-3.1	NC
Hydroxysteroid dehydrogenase-6, delta<5>-3-beta	AF031170	-1.6	-1.5	NC
Signal Transduction				
Suppressor of cytokine signaling 2	U88327	2.0	1.9	1.7
Complement component 2 (within H-2S)	AF109906	1.8	4.6	NC
Activating transcription factor 3	U19118	-2.0	-1.8	-1.5
Cytoskeleton				
Actin, gamma, cytoplasmic	M21495	-1.7	-3.2	-2.1
Miscellaneous				
Lectin, galactose binding, soluble 1	X15986	-1.7	-2.6	-1.8
EST	AA959954	-1.5	-2.2	NC
EST	AI266885	-1.7	-1.6	NC
Glipizide-specific changes in gene expression				
Stress and chaperone proteins				
Cytochrome P450, 1a2, aromatic compound inducible	X04283	1.6	NC	NC
Cytochrome P450, 4a10	AB018421	-1.7	NC	NC
Cytochrome P450, 4a14	Y11638	-1.5	NC	NC
DnaJ (Hsp40) homolog, subfamily C, member 3	U28423	1.6	NC	NC
Metabolism				
Stearoyl-Coenzyme A desaturase 1	M21285	-1.8	NC	NC
Hydroxysteroid dehydrogenase-3, delta<5>-3-beta	M77015	-1.5	NC	NC
Thyroid hormone responsive SPOT14 homolog (Rattus)	X95279	-1.7	NC	NC
Glutathione S-transferase, alpha 2 (Yc2)	J03958	-1.6	NC	NC
Cathepsin C	U74683	1.5	NC	NC
DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)	AI225445	-1.5	NC	NC
Signal transduction				
Activating transcription factor 5	AB012276	1.5	NC	NC
Hepcidin antimicrobial peptide	AI255961	1.5	NC	NC
Angiogenin	U22516	1.5	NC	NC
Butyrylcholinesterase	M99492	-1.5	NC	NC
Wee 1 homolog (S. pombe)	D30743	-1.5	NC	NC

Miscellaneous				
Staphylococcal nuclease domain containing 1	AB021491	1.5	NC	NC
Pre-B-cell colony-enhancing factor	AI852144	-1.5	NC	NC
Complement component 1, q subcomponent, alpha polypeptide	X58861	1.5	NC	NC
EST	AA612450	-1.5	NC	NC
EST	AA959954	-1.5	NC	NC
EST	AI850090	-1.5	NC	NC
EST	AI852184	1.6	NC	NC
EST	AW047688	-1.5	NC	NC
EST	AW060549	-1.6	NC	NC
EST	AW122942	1.5	NC	NC
EST	AW212131	-1.5	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from Glipizide and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 7. Effects of Glipizide & Metformin (GM) and CR on hepatic gene expression.

Gene / Protein	GenBank	GM[1]	LT-CR[2, 4]	ST-CR[3, 4]
Changes in gene expression induced by GM and reproduced with either LT- or ST-CR				
Stress and chaperone proteins				
Heat shock protein, 105 kDa	L40406	1.7	2.3	NC
DnaJ (Hsp40) homolog, subfamily B, member 1	AB028272	1.5	1.6	NC
Cytochrome P450, 4a12	Y10221	-1.5	3.2	-2.8
Metabolism				
Farnesyl pyrophosphate synthase	AI846851	1.5	1.5	1.7
Farnesyl pyrophosphate synthase (Second time)	AW045533	1.8	1.5	1.5
Retinoic acid early transcript gamma	D64162	-1.6	-3.1	NC
Sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase; GM3 synthase)	Y15003	1.6	2.5	NC
Signal Transduction				
Suppressor of cytokine signaling 2	U88327	2.7	1.9	1.7
Complement component 2 (within H-2S)	AF109906	2.0	4.6	NC
Regulator of G-protein signaling 16	AV349152	1.5	NC	1.6
Regulator of G-protein signaling 16	U94828	1.7	NC	1.6
Angiopoietin-like 4	AA797604	1.6	1.8	NC
Insulin-like growth factor binding protein 1	X81579	1.5	2.4	NC
Cytoskeleton				
Actin, gamma, cytoplasmic	M21495	-1.7	-3.2	-2.1
Miscellaneous				
Lectin, galactose binding, soluble 1	X15986	-1.6	-2.6	-1.8
EST	AI266885	-1.5	-1.6	NC
GM-specific changes in gene expression				
Stress and chaperone proteins				
Cytochrome P450, 2b10, phenobarbitol inducible, type b	M21856	-1.6	NC	NC
DnaJ (Hsp40) homolog, subfamily C, member 3	U28423	1.6	NC	NC
Serum amyloid P-component	M23552	1.5	NC	NC
Metabolism				
3'-phosphoadenosine 5'-phosphosulfate synthase 2	AF052453	-1.5	NC	NC
Glutathione S-transferase, alpha 2 (Yc2)	J03958	-2.0	NC	NC
Phospholipid transfer protein	U28960	-1.5	NC	NC
Stearoyl-Coenzyme A desaturase 1	M21285	-1.9	NC	NC
Thyroid hormone responsive SPOT14 homolog (Rattus)	X95279	-1.6	NC	NC
Cytochrome c oxidase, subunit VIc	AV071102	-1.6	NC	NC
DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)	AI225445	-1.5	NC	NC
Signal transduction				
Angiogenin	U22516	1.6	NC	NC
Bcl2-associated athanogene 3	AI643420	1.6	NC	NC

Prolactin receptor	D10214	1.5	NC	NC
Transducin-like enhancer of split 1, homolog of Drosophila E(spl)	U61362	1.5	NC	NC
Deoxyribonuclease II alpha	AW120896	1.5	NC	NC
cAMP-regulated guanine nucleotide exchange factor II	AF115480	1.5	NC	NC
Wee 1 homolog (S. pombe)	D30743	-1.6	NC	NC
Cytoskeleton				
Reelin	U24703	-1.6	NC	NC
Miscellaneous				
Butyrylcholinesterase	M99492	-1.5	NC	NC
Lysophospholipase 1	AA840463	-1.5	NC	NC
Leucine-rich alpha-2-glycoprotein	AW230891	1.5	NC	NC
Dynein, cytoplasmic, light chain 1	AF020185	1.5	NC	NC
EST	C79676	-1.5	NC	NC
EST	AI842968	-1.6	NC	NC
EST	AW124226	-1.7	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from GM and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 8. Effects of Rosiglitazone and CR on hepatic gene expression.

Gene / Protein	GenBank	Rosiglitazone [1]	LT-CR[2, 4]	ST-CR[3, 4]
Changes in gene expression induced by Rosiglitazone and reproduced with either LT- or ST-CR				
Stress and chaperone proteins				
Cytochrome P450, 2f2	M77497	-1.6	-1.5	-1.5
Cytochrome P450, 2b13, phenobarbitol inducible, type c	M60358	1.9	2.7	1.8
Cytochrome P450, 4a12	Y10221	-2.9	3.2	-2.8
Cytochrome P450, 7a1	L23754	-1.7	-1.7	NC
Metabolism				
Ectonucleotide Pyrophosphatase/phosphodiesterase 2	AW122933	-1.8	-2.9	-1.5
Apolipoprotein A-IV	M64248	-3.4	NC	-1.8
Signal Transduction				
Activating transcription factor 3	U19118	-1.5	-1.8	-1.5
Cytokine inducible SH2-containing protein 2	U88327	1.7	1.9	1.7
Inhibitor of DNA binding 3	M60523	-1.7	NC	-1.5
Regulator of G-protein signaling 16	AV349152	1.6	NC	1.6
Regulator of G-protein signaling 16	U94828	1.8	NC	1.6
Cytoskeleton				
Actin, gamma, cytoplasmic	M21495	-1.8	-3.2	-2.1
Keratin complex 1, acidic, gene 18	M22832	-1.6	-1.7	-1.5
Keratin complex 2, basic, gene 8	X15662	-1.7	-2.2	-1.7
Tubulin, beta 2	M28739	-1.5	NC	-1.5
Miscellaneous				
Lectin, galactose binding, soluble 1	X15986	-1.8	-2.6	-1.8
Arylsulfatase A	X73230	-1.6	-2.4	-2.2
Macrophage expressed gene 1	L20315	-1.6	-2.4	-1.9
Quiescin Q6	AW04575	1.6	1.6	NC
EST	AI530403	1.5	1.7	NC
EST	AI266885	-2.0	-1.6	NC
Rosiglitazone-specific changes in gene expression				
Stress and chaperone proteins				
Cytochrome P450, 8b1, sterol 12 alpha-hydrolase	AF090317	-1.5	NC	NC
Metabolism				
Glutathione S-transferase, alpha 2 (Yc2)	J03958	-1.7	NC	NC
Flavin containing monooxygenase 5	U90535	-1.5	NC	NC
Thyroid hormone responsive SPOT14 homolog (Rattus)	X95279	-1.5	NC	NC
Amine N-sulfotransferase	AF026073	-1.5	NC	NC
DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)	AI225445	-1.6	NC	NC
Cathepsin C	U74683	1.7	NC	NC
Cathepsin C (second time)	AI842667	1.7	NC	NC
Signal transduction				
G0/G1 switch gene 2	X95280	1.5	NC	NC
Cytoskeleton				
Inter-alpha trypsin inhibitor, heavy chain 3	X70393	1.5	NC	NC
Miscellaneous				

Orphan nuclear receptor; Rev-ErbA-alpha protein	AI834950	1.5	NC	NC
RAD51-like 1 (S. cerevisiae)	U92068	1.5	NC	NC
Pre-B-cell colony-enhancing factor	AI852144	-1.5	NC	NC
Hemoglobin, beta adult minor chain	V00722	1.5	NC	NC
Quiescin Q6	AW123556	1.7	NC	NC
EST	AA619207	-1.7	NC	NC
EST	AA959954	-1.5	NC	NC
EST	AW060549	-1.7	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from Rosiglitazone and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 9. Effects of Soy Isoflavone and CR on hepatic gene expression.

Gene / Protein	GenBank	Soy Isoflavone[1]	LT-CR[2, 4]	ST-CR[3, 4]
Changes in gene expression induced by Soy and reproduced with either LT- or ST-CR				
Immunoglobulin kappa chain variable 28 (V28)	M18237	-1.8	-2.0	1.5
EST	M80423	-2.1	-2.0	NC
Soy-specific changes in gene expression				
EST	V00817	-1.5	NC	NC

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from Soy and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[3] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from ST-CR and CON groups (n = 4).

[4] "NC" indicates no change in gene expression.

Table 10. Genes whose expression is altered in the opposite direction by LT-CR and the drugs used.

Gene / Protein	GenBank	LT-CR[1]	DRUG[2]
Metformin			
Cytochrome P450, 7a1	L23754	-1.7	1.8
Sterol-C4-methyl oxidase-like	AI848668	-2.4	2.4
EST	AI844396	-1.6	1.9
Glipizide			
Splicing factor 3b, subunit 1, 155 kDa	AI844532	-1.5	1.5
EST	AJ011864	1.6	-1.7
Arginine-rich, mutated in early stage tumors	AW122364	-1.7	1.5
Neuropilin	D50086	-1.6	1.5
Calcium binding protein, intestinal	Y00884	-1.5	1.9
Phosphatase and tensin homolog	U92437	-1.5	1.6
Glipizide & Metformin			
Calcium binding protein, intestinal	Y00884	-1.5	1.7
Metallothionein 1	V00835	-4.1	1.6
Splicing factor 3b, subunit 1, 155 kDa	AI844532	-1.5	1.5
Carbon catabolite repression 4 homolog (S. cerevisiae)	AW047630	-1.5	1.5
Serum amyloid A 1	M13521	-1.5	2.1
Rosiglitazone			
metallothionein 2	K02236	-4.4	1.6
insulin-like growth factor binding protein 1	X81579	2.4	-1.6
metallothionein 1	V00835	-4.1	1.7
calcium binding protein, intestinal	Y00884	-1.5	1.6
Phosphatase and tensin homolog	U92437	-1.5	1.5

[1] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from LT-CR and CON groups (n = 4).

[2] The numbers in this column represent the average fold change in specific mRNA derived from all 16 possible pairwise comparisons among individual mice from a drug and CON groups (n = 4).

Table 11. Percentage of CR effects reproduced by the different drug treatments.

	LT-CR	ST-CR
Metformin	11.3%	39.6%
Glipizide	5.0%	13.5%
Metformin & Glipizide	5.0%	15.1%
Rosiglitazone	5.7%	32.1%
Soy	0.7%	0%

TABLE 12. Control and CR diet composition

Ingredient	Control	CR
Casein	14.0	23.3
L-Cysteine	0.2	0.3
Corn starch	46.6	29.5
Dextrinized cornstarch	15.5	15.5
Sucrose	10.0	10.0
Corn oil (Mazola)	4.0	6.7
Cellulose	5.0	6.8
Mineral mix, AIN-76	3.5	5.8
Choline bitartrate	0.3	0.4
Vitamin mix	1.0	1.7

TABLE 13. Primer sequences for qPCR.

Gene Name	GenBank Accession No.	Primer sequences (5'-3') (Forward and Reverse Primers)	PCR Product size (bp)
Metallothionein 1	V00835	CTCCTGCGCCTGCAAGAACTG ACACAGCCCTGGGCACATTTG	96
Arginase 1, liver	U51805	AAAGGAAAGTTCCCAGATGTACCAGG TATAGTGTTCCCCAGGGTCTACGTCTC	109
Amine N-sulfotransferase	AF026073	CTCTTGGTCTTGAAATGTACAGATATCAGG GGCCACTGAGACTATTTCAGACAGG	192
Carbonic anhydrase 3	M27796	CCTTCAAGTAAGGCTCTGAGCTTGC GTGAAATTCATGCTTCTGGGTGAGA	183
Major urinary protein 4	M16358	TTGACTTAACCAAAACCAATCGCTG TGTGAGACAGGATGGAAAGCAGATC	165
Glucose-6-phosphatase, catalytic	U00445	TGTGCTTGCATTCCTGTATGGTAGTG AACAGTTGCCTACCAGACACAGCAG	161
Phenylalanine hydroxylase	X51942	GTCTGTTCATTTTTACCTCTCAGGTAAGC AGTTCTCAGAGCCATAATGAATGAATGTAG	156
Carbon catabolite repression 4 homolog (S. cerevisiae)	U70139	TTGCTGATCGAACAGGATGTACACTA AGAAGTCAAAGGCATAGCAAACAGG	111
Transcription elongation factor A (SII), 2	U44389	TCTCCATTACTCGTAAAAGCTCCATAACC GATACAGGAAGGTAGGTTTCATCGTATGG	143
	M18209	CCAGCTGAAATGTAGGCTGTAGCAA ACAGGAGTCTGAACACAGGCAGAAG	199

Table 14. Genes changed by Long-Term CR

Category/Gene	Genebank	CR2	CR4	CR8	LT-CR	CON8
ECM/Cytoskeleton						
Clathrin, heavy polypeptide (Hc)	AA139495	NC	NC	NC	-1.5	NC
Metabolism						
ATP synthase gamma chain, mitochondrial	AA114811	NC	NC	NC	1.5	NC
Cathepsin L	AA096813	NC	NC	NC	2.2	NC
Histidine ammonia lyase	L07645	NC	NC	NC	1.8	NC
Homogentisate 1, 2-dioxygenase	U58988	NC	NC	NC	1.5	NC
Amylase 1, salivary (also called liver alpha-amylase)	V00719	NC	NC	NC	1.5	NC
Tryptophan 2,3-dioxygenase	U24493	NC	NC	NC	1.5	NC
Sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase)	W36875	NC	NC	NC	1.5	NC
Aldehyde dehydrogenase family 1, subfamily A1	M74570	NC	NC	NC	-1.8	NC
Alpha-1-antiproteinase precursor (alpha-1-antitrypsin)	W34969	NC	NC	NC	-1.5	NC
Hydroxysteroid 17-beta dehydrogenase 2	X95685	NC	NC	NC	-1.5	NC
T-complex protein 1, related sequence 1 or acetyl-Coenzyme A C-acetyltransferase 2 (Provisional)	W81884	NC	NC	NC	-1.5	NC
Signal Transducers, Growth Factors						
mVL30-1 retroelement	C77421	NC	NC	NC	1.6	NC
Annexin A7	C78610	NC	NC	NC	-1.5	NC
Ectonucleotide pyrophosphatase/phosphodiesterase 2	AA059550	NC	NC	NC	-1.6	NC
Inhibitor of DNA binding 2	M69293	NC	NC	NC	-1.5	NC
Lymphocyte antigen 6 complex, locus E	U04268	NC	NC	NC	-1.7	NC
Major urinary protein 1	M16355	NC	NC	NC	-2.6	NC
Immune Response, Inflammation						
Complement component 4 binding protein	M17122	NC	NC	NC	-1.8	NC
Stress Response, Xenobiotic Metabolism and Chaperones						
Hydroxyacyl-Coenzyme A dehydrogenase, type II	U96116	NC	NC	NC	2.0	NC
Peroxiredoxin 3	M28723	NC	NC	NC	1.5	NC
Cytochrome P450, 1a2, aromatic compound inducible	X00479	NC	NC	NC	-2.2	NC
Cytochrome P450, 2f2	M77497	NC	NC	NC	-1.8	NC
Glutathione S-transferase, pi 2	D30687	NC	NC	NC	-1.7	NC
Proteasome (prosome, macropain) subunit, alpha type 2	X70303	NC	NC	NC	-1.5	NC

Proteasome (prosome, macropain) subunit, alpha type 4	AA008321	NC	NC	NC	-1.6	NC
Miscellaneous						
Hemopexin	U89889	NC	NC	NC	1.5	NC
L1 repeat, Tf subfamily, member 14	D84391	NC	NC	NC	1.6	NC
T10	X74504	NC	NC	NC	1.5	NC
EST	AA238331	NC	NC	NC	-1.9	NC
EST	AA213083	NC	NC	NC	-1.7	NC
EST	AA690887	NC	NC	NC	-1.6	NC
EST	AA048018	NC	NC	NC	-1.6	NC
EST	C76068	NC	NC	NC	1.6	NC
EST	C77864	NC	NC	NC	-1.8	NC

Table 15. Genes which changes in expression remained in the same direction across all time points.

Category/Gene	Genebank	CR2	CR4	CR8	LT-CR	CON8
Metabolism						
Apolipoprotein B-100 precursor	AA120586	2.9	3.5	1.5	1.7	1.8
Arginase 1, liver	U51805	3.0	2.3	1.5	2.3	NC
Argininosuccinate lyase	AA237297	2.3	1.5	1.7	2.6	NC
Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	AA117646	1.5	1.5	1.8	1.6	NC
N-sulfotransferase	AF026073	5.0	3.4	1.6	1.8	NC
Fatty acid binding protein 1, liver	AA591003	-4.3	-3.7	-1.7	-1.9	NC
Fatty acid binding protein 1, liver (same gene)	AA087320	-6.8	-6.8	-1.7	-1.8	NC
Carbonic anhydrase 3	M27796	-8.2	-7.5	-1.8	-5.5	NC
2,4-dienoyl-CoA reductase, mitochondrial	AA521793	-2.4	-1.9	-1.6	-1.5	NC
Acetyl-Coenzyme A acetyltransferase 1 (acetoacetyl Coenzyme A thiolase)	AA710204	-2.0	-2.5	-1.5	-1.5	NC
Fatty acid Coenzyme A ligase, long chain 2	U15977	NC	-3.4	-2.0	-1.7	NC
Transglutaminase 2, C polypeptide	M55154	NC	-2.4	-1.5	-1.5	NC
Argininosuccinate synthetase 1	M31690	NC	NC	2.0	2.3	NC
Argininosuccinate synthetase 1 (same gene)	M31690	NC	NC	2.1	2.7	1.5
Carbonyl reductase 1	U31966	NC	NC	1.7	1.7	NC
Glycine N-methyltransferase	W14826	NC	NC	1.8	2.0	NC
S-adenosylhomocysteine hydrolase	L32836	NC	NC	1.5	1.9	NC
Signal Transducers, Growth Factors						
Tumor differentially expressed 1	L29441	2.4	1.8	1.5	1.8	NC
Phosphatase and tensin homolog	U92437	2.5	1.9	1.5	1.6	NC
Poly A binding protein, cytoplasmic 1	AA106783	3.8	2.4	1.6	1.7	NC
Major urinary protein 4	M16358	-3.5	-2.5	-1.6	-2.6	NC
Major urinary protein 4 (same gene)	M16358	-4.0	-2.5	-1.5	-2.7	NC
Thyroid hormone receptor alpha	W13191	-3.6	-2.1	-1.5	-2.1	-1.6
Transthyretin	D89076	-2.3	-2.2	-1.5	-1.8	NC
Major urinary protein 5	M16360	-2.4	-1.5	-1.7	-2.2	NC
MORF-related gene X	AA529583	NC	NC	-1.6	-1.7	NC
Eph receptor B4	Z49085	NC	NC	-1.5	-1.5	NC
X-box binding protein 1	AF027963	NC	NC	-1.5	-1.8	NC
X-box binding protein 1 (same gene)	AA016424	NC	NC	-1.5	-1.6	NC
Immune Response, Inflammation						
Orosomucoid 1	M27008	3.3	1.5	2.0	2.0	1.5
Complement component 9	X05475	NC	NC	-1.5	-2.0	NC
Interferon-inducible GTPase	AA415898	NC	NC	-1.5	-1.9	NC

Isocitrate dehydrogenase 2 (NADP+), mitochondrial	U51167	NC	NC	1.5	1.5	NC
Stress Response, Xenobiotic Metabolism and Chaperones						
Cytochrome P450, 2b13, phenobarbitol inducible, type c	M60358	1.6	1.8	1.8	2.0	NC
P450 (cytochrome) oxidoreductase	D17571	2.8	1.7	1.5	2.1	1.6
Cytochrome C oxidase subunit VIIb	AA521794	4.3	3.4	1.5	1.6	NC
Metallothionein 1	V00835	4.3	2.9	1.6	2.3	NC
Glutathione S-transferase, mu 2	J04696	1.6	1.8	2.4	1.9	NC
Tumor rejection antigen gp 96 (94 KD glucose-regulated protein)	W55140	-3.1	-2.6	-1.7	-1.5	NC
Cytochrome P450, 2b9, phenobarbitol inducible, type a	M21855	NC	1.6	2.5	2.3	NC
Glucose regulated protein, 58kDa	M73329	NC	-3.0	-1.7	-1.7	NC
Heat shock 70kD protein 5 (glucose-regulated protein, 78kD)	D78645	NC	NC	-1.9	-2.1	-1.5
Heat shock 70kD protein 5 (glucose-regulated protein, 78kD)	D78645	NC	-2.5	-1.9	-2.0	-1.5
DnaJ (Hsp40) homolog, subfamily B, member 11	AA204094	NC	NC	-1.5	-1.5	NC
Calreticulin	X56603	NC	NC	-1.7	-1.7	NC
Calnexin	AA163552	NC	NC	-1.6	-1.7	NC
Miscellaneous						
Arginine-rich, mutated in early stage tumors	AA408789	-3.0	-1.8	-1.9	-2.2	-1.7
ATP-dependent protease LA2	AA120387	-2.1	-1.5	-1.6	-1.7	NC
EST	AA217076	-4.4	-3.2	-1.7	-1.5	NC
EST	AA537958	-1.9	-1.9	-1.5	-1.6	NC
EST	AA711625	-2.1	-1.8	-1.6	-2.2	NC
EST	AA120109	NC	-2.8	-1.6	-2.2	NC
EST	C76068	NC	NC	1.7	2.1	1.5

Table 16. Genes Changed by ST- and LT-CR (not same direction)

Category/Gene	Genebank	CR2	CR4	CR8	LT-CR	CON8
Metabolism						
Aminolevulinate, delta-, dehydratase	X13752	-2.7	-2.3	NC	-1.6	NC
Cathepsin L	X06086	3.6	1.6	NC	2.3	NC
Cathepsin L (same gene)	X06086	2.3	NC	NC	2.3	NC
Glucose-6-phosphatase, catalytic	U00445	2.7	NC	1.5	2.3	1.8
Hydroxyprostaglandin dehydrogenase 15 (NAD)	U44389	-2.1	-1.8	NC	-1.7	NC
Hydroxysteroid 17-beta dehydrogenase 5	D45850	-1.6	-1.7	NC	-1.8	NC
Lipase, hepatic	X58426	-2.8	-2.4	NC	-1.5	NC
Ornithine aminotransferase	X64837	1.6	NC	1.5	2.0	1.5
Phenylalanine hydroxylase	X51942	6.0	2.5	NC	1.6	NC
Phosphoenolpyruvate carboxykinase 1, cytosolic	AA110781	1.6	-2.3	NC	1.5	NC
S-adenosylhomocysteine hydrolase	AA237376	2.1	2.0	NC	1.6	NC
S-adenosylhomocysteine hydrolase	L32836	1.5	NC	1.5	2.0	NC
Stearoyl-Coenzyme A desaturase 1	M21285	3.3	NC	6.9	7.9	4.2
Stearoyl-Coenzyme A desaturase 1	AA137436	2.1	NC	6.0	6.6	3.8
Thioether S-methyltransferase	M88694	-2.6	-2.8	NC	-1.6	NC
Signal Transducers, Growth Factors						
Serine (or cysteine) proteinase inhibitor, clade A, member 6 (corticosteroid binding globulin precursor))	X70533	-2.9	-6.4	NC	-2.2	NC
Gap junction membrane channel protein beta 2	M81445	-2.6	-2.9	NC	-1.6	NC
Ras homolog gene family, member U	AA240968	-1.9	-2.4	NC	-1.6	NC
Tumor differentially expressed 1	L29441	4.2	NC	1.5	1.9	NC
Immune Response, Inflammation						
Carbon catabolite repression 4 homolog (S. cerevisiae)	U70139	2.2	NC	NC	-1.5	NC
FK506 binding protein 5 (51 kDa)	U36220	2.3	1.6	NC	1.5	NC
Mannose-binding protein A	U09010	-2.3	-1.8	NC	-1.7	NC
Stress Response, Xenobiotic Metabolism and Chaperones						
Aldo-keto reductase family 1, member 13	AA592828	NC	-1.8	NC	-1.5	NC
Cytochrome P450, 2j5	U62294	-1.5	-1.8	NC	-1.7	NC
Cytochrome P450, 3a16	D26137	NC	-1.9	NC	1.7	NC
Cytochrome P450, 7b1	U36993	-1.5	-1.9	NC	-2.3	NC
Cytochrome P450, steroid inducible 3a11	X60452	NC	-3.2	NC	1.9	1.5
Cytochrome P450, steroid inducible 3a13	X63023	4.1	NC	1.6	2.1	NC
Esterase 31	L11333	-2.4	-2.2	NC	-2.2	NC
Flavin containing monooxygenase 5	U90535	2.1	NC	1.5	1.5	NC

Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	AA254963	NC	-1.8	NC	-1.6	NC
Solute carrier family 22 (organic cation transporter), member 1	U38652	-2.6	-2.2	NC	-1.6	NC
Miscellaneous						
EST	AA097626	5.8	2.0	NC	1.7	NC
Hepcidin antimicrobial peptide	W12913	-1.5	-1.7	NC	-2.4	NC

WHAT IS CLAIMED IS:

1. A method of identifying a marker of caloric restriction, the method comprising:

determining an expression pattern of one or more biomarkers in a biological sample obtained from a first mammal subjected to a caloric restricted diet program for a first period of time;

determining the expression pattern of one or more biomarkers in a biological sample obtained from a second mammal subjected to a caloric restricted diet program for a second period of time, wherein the second period of time is different from the first;

comparing the expression pattern of the one or more biomarkers in the biological sample from the first mammal to the expression pattern in the biological sample from the second mammal;

comparing the expression pattern of the one ore more biomarkers in the biological samples from the first and second mammals to the expression pattern in a biological sample from a control mammal administered a control dietary program, thereby identifying a marker of caloric restriction.

2. The method of claim 1, wherein the first mammal is subjected to long term caloric restriction.

3. The method of claim 1, wherein the first mammal is subjected to short caloric restriction.

4. The method of claim 3, wherein the second animal is subjected to long term caloric restriction for a period of time.

5. The method of claim 4, wherein the second animal is switched to a control diet following the period of time the animal is subjected to the long term caloric restriction.

6. The method of claim 1, wherein the expression patterns in the biological samples are determined by detecting a change in mRNA level.

7. The method of claim 6, wherein the change in mRNA level is determined using a microarray.

8. The method of claim 1, wherein the expression patterns in the biological samples are determined by detecting a change in the level or activity of the protein encoded by the gene.

9. The method of claim 8, wherein the expression pattern is determined is heart tissue.

10. The method of claim 1, further comprising determining the expression pattern of the one or more biomarkers in a biological sample obtained from a third mammal subjected to a caloric restricted diet program for a third period of time, wherein the third period of time is different from the first and the second periods of time; and comparing the expression pattern to the expression patterns in the first mammal, second mammal and control mammal.

11. The method of claim 1, wherein the first or second mammal that is subjected to caloric restriction is switched to a control diet following the period of time of caloric restriction.

12. The method of claim 1, wherein the mammal is selected from the group consisting of rodents, monkeys, and humans.

13. The method of claim 12, wherein the mammal is a rodent.

14. The method of claim 13, wherein the rodent is a mouse.

15. A method of identifying an intervention that reduces collagen accumulation in at least one of heart or blood vessels, the method comprising:

treating a mammalian sample with a candidate intervention;

measuring collagen gene expression or collagen accumulation in the mammalian sample; and

comparing the changes in collagen gene expression or collagen accumulation to that of a mammalian sample administered a caloric restricted diet; and identifying a decreases in collagen gene expression or collagen accumulation that mimics a decrease present in the mammalian sample administered a caloric restricted diet, thereby identifying an intervention that reduces collagen accumulation in at least one of heart or blood vessels.

16. The method of claim 17, wherein the mammalian sample is a mouse.

17. A method of identifying an intervention that mimics caloric restriction, the method comprising:

treating a sample with a candidate intervention;

and identifying a change in expression pattern of a biomarker identified in accordance with claim 1, wherein the change in expression pattern correlates with the change observed in caloric restriction, thereby identifying an intervention that mimics caloric restriction.

18. The method of claim 17, wherein the sample is a mammal.

19. The method of claim 18, wherein the mammal is a mouse

20. The method of claim 17, wherein the sample is a mammalian cell sample.

21. The method of claim 17, wherein the biomarker is a protein encoded by a gene set forth in Table 2, 5, 6, 7, 8. 9, 10, 14, 15, or 16.

22. The method of claim 17, wherein the expression pattern is determined by measuring the level of mRNA encoding the biomarker.

23. A method of identifying an intervention that mimics caloric restriction, the method comprising:

treating a sample with a candidate intervention;

comparing the expression pattern of protein encoded by a gene set forth in Table 2, 5, 6, 7, 8. 9, 10, 14, 15, or 16; and comparing the change in expression pattern to that observed in an animal that is treated with caloric restriction, thereby identifying an intervention that mimics caloric restriction.

24. The method of claim 23, wherein the caloric restriction is short term caloric restriction.

25. A method of analyzing genes comprising:

administering a long term control (LT-CON) dietary program to a LT-CON group and a long term caloric restriction (LT-CR) dietary program to a LT-CR group for a

first predetermined period, said LT-CON group and said LT-CR group comprised of similar mammalian samples;

after said first predetermined period, dividing said LT-CON group to a ST-CR group and a LT-CON continuation group, and switching said ST-CR group to a short-term caloric restriction (ST-CR) dietary program while maintaining said LT-CON continuation group on said LT-CON dietary program for a second predetermined period;

after said first predetermined period, dividing said LT-CR group to a ST-CON group and a LT-CR continuation group, and switching said ST-CON group to a short-term control (ST-CON) dietary program while maintaining said LT-CR continuation group on said LT-CR dietary program for said second predetermined period; and

comparing gene expression effects among said ST-CR group, said LT-CON continuation group, said ST-CON group, and said LT-CR continuation group.

26. A method of identifying at least one regulatory nucleic acid sequence motif for a group of genes comprising:

administering a LT-CON dietary program to a LT-CON group and a LT-CR dietary program to a LT-CR group for a first predetermined period, said LT-CON group and said LT-CR group comprised of similar mammalian samples;

after said first predetermined period, dividing said LT-CON group to a ST-CR group and a LT-CON continuation group, and switching said ST-CR group to a ST-CR dietary program while maintaining said LT-CON continuation group on said LT-CON dietary program for a second predetermined period;

after said first predetermined period, dividing said LT-CR group to a ST-CON group and a LT-CR continuation group, and switching said ST-CON group to a ST-CON dietary program while maintaining said LT-CR continuation group on said LT-CR dietary program for said second predetermined period;

comparing gene expression effects among said ST-CR group, said LT-CON continuation group, said ST-CON group, and said LT-CR continuation group; and

identifying genes that exhibit similar behaviors for each of said ST-CR group, said LT-CON continuation group, said ST-CON group, and said LT-CR continuation group to identify genes affected by said switchings.

27. A method of identifying a compound that potentially reduces collagen accumulation in at least one of heart and blood vessels comprising:

obtaining control data from an administering of a CR dietary program to one sample group;

administering a dosage of a compound to another sample group;

comparing at least one of collagen measurement resulting from said CR dietary program to at least one collagen measurement resulting from said administering a dosage of a compound; and

identifying said compound to be potentially effective in reducing collagen accumulation based at least in part on said comparing.

28. A method of reproducing at least one effect in mammals that have been subjected to long-term caloric restriction (LT-CR) comprising:

administering a LT-CR diet program to a first group of mammals for a first duration of time;

administering at least one compound to a second group of mammals for a second duration of time wherein said second duration of time is substantially shorter than said first duration of time, said first group of mammals and said second group of mammals being similar;

obtaining control data from an administering of a control diet program;

determining effects of said LT-CR diet program and said at least one compound by comparing data obtained for said first group of mammals and said second group of mammals to said control data; and

comparing effects between said LT-CR diet program and said at least one compound to determine whether said at least one compound reproduces at least one effect caused by said LT-CR.

29. A method of identifying a compound that reproduces effects of a CR comprising:

administering an effective dosage of a test compound to a first mammal for a duration of time;

administering a CR diet program to a second mammal, said first mammal and said second mammal being similar;

analyzing changes in gene expression levels, levels of nucleic acids, protein, or protein activity levels, in each of said first mammal and said second mammal; and

identifying said test compound as one that reproduces changes induced by said CR when said test compound produces analyzed changes in said first mammal wherein at least about 1% or one or more gene changes of said analyzed changes are a subset of said changes induced by said CR.

30. A method for searching for a compound comprising:

administering a ST-CR diet program to a first group of mammals for a predetermined amount of time;

administering a dosage of at least one compound, for a term which is less than or equal to said predetermined amount of time, to a second group of mammals;

assessing changes in gene expression levels, levels of nucleic acids, proteins, or protein activity levels; and

determining whether said at least one compound mimics at least some effects induced by said ST-CR diet program.

31. A method of evaluating the dynamics of caloric restriction (CR) comprising:

obtaining control data from an administering of a long-term control (LT-CON) diet program;

subjecting each of several mammalian sample groups to a CR diet program wherein each of said several mammalian sample groups is subjected to said CR diet program for a different amount of time; and

comparing effects of said CR diet program between each of said several mammalian sample groups and said control data and comparing effects among members of said several mammalian sample groups.

32. A method of evaluating dynamics of CR comprising:

dividing a mammalian sample group into a first sample group and a second sample group;

subjecting said first sample group to a LT-CON diet program for a first predetermined period and said second sample group to a long-term caloric restriction (LT-CR) diet program for a second predetermined period;

after said first predetermined period, switching portions of said first sample group to a CR diet program for a third predetermined period;

after said second predetermined period, switching at least a portion of said second sample group to a control diet program for said third predetermined period and maintaining the other portion of said second sample group on said LT-CR diet program; and

comparing the effects of CR among members of said first sample group and said second sample group.

33. A method of reversing effects of CR comprising:

administering a control diet program to a mammalian sample group that has been subjected to a LT-CR diet program wherein said control diet program includes higher calorie allowance for said mammalian sample group than the calories for said LT-CR diet program.

34. A method of identifying an intervention for use in old age subjects comprising:

administering a control diet program to individuals in a first sample group;

administering, after a start of old age, at least one candidate intervention to said individuals in said first sample group; and

comparing effects of said candidate intervention to effects from a calorically restricted diet program on a second sample group.

35. A method of identifying an intervention comprising:

exposing a biological sample to at least one intervention;

performing at least one biochemical measurement after exposing said biological sample to said intervention, said biochemical measurement being designed to show whether said intervention mimics at least some of the effects of CR (caloric restriction);

withdrawing said intervention from said biological sample; and

performing at least one further biochemical measurement after withdrawing said intervention, said at least one further biochemical measurement being designed to show whether said withdrawing mimics at least some of the effects of withdrawing CR.

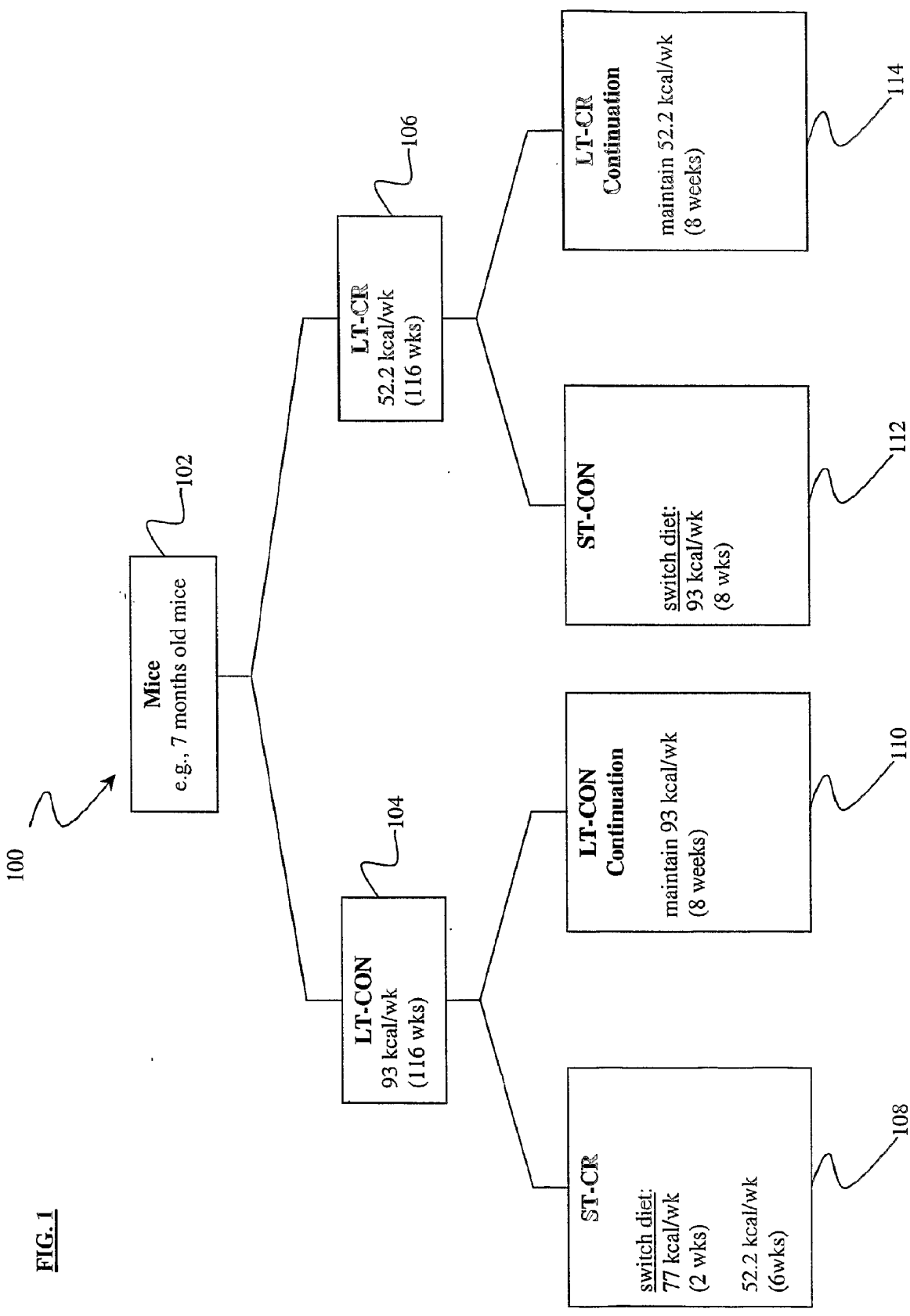

100
Mice
e.g., 7 months old mice
102
LT-CR
52.2 kcal/wk
(116 wks)
106
LT-CON
93 kcal/wk
(116 wks)
104
LT-CR
Continuation
maintain 52.2 kcal/wk
(8 weeks)
114
ST-CON
switch diet:
93 kcal/wk
(8 wks)
112
LT-CON
Continuation
maintain 93 kcal/wk
(8 weeks)
110
ST-CR
switch diet:
77 kcal/wk
(2 wks)
52.2 kcal/wk
(6wks)
108

FIG. 1

FIG. 2A

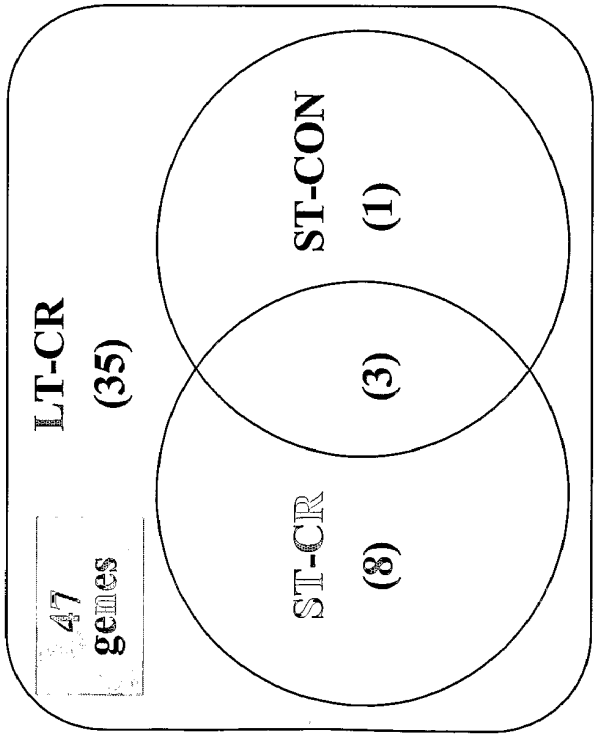
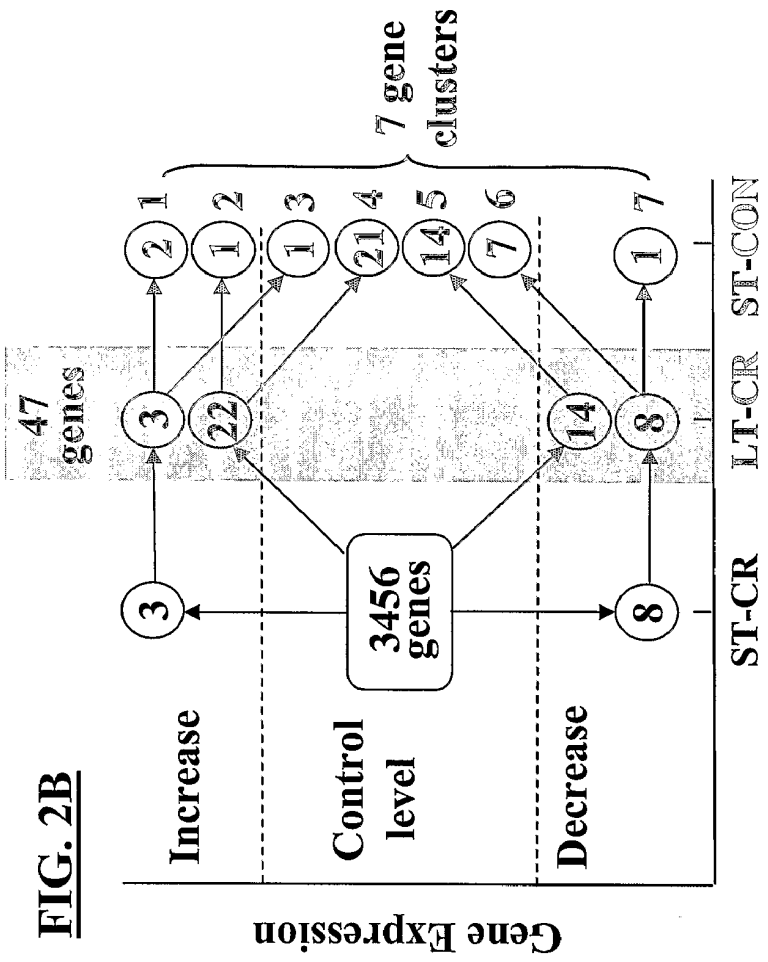

47 genes
LT-CR (35)
ST-CR (8)
(3)
ST-CON (1)

FIG. 2B


7 gene clusters
47 genes
3456 genes
Increase
Control level
Decrease
Gene Expression
ST-CR
LT-CR
ST-CON

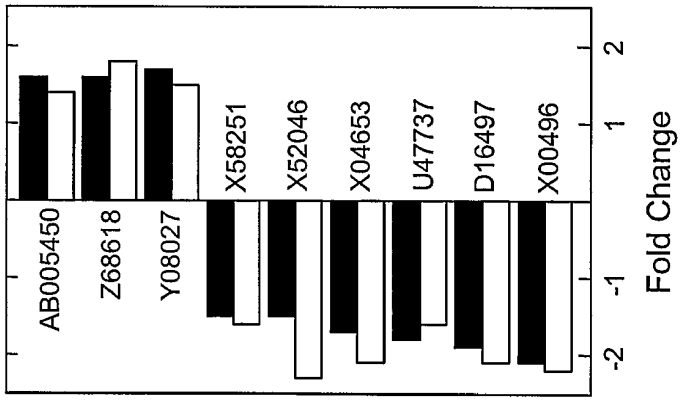

AB005450
Z68618
Y08027
X58251
X52046
X04653
U47737
D16497
X00496
-2
-1
1
2
Fold Change

FIG. 3

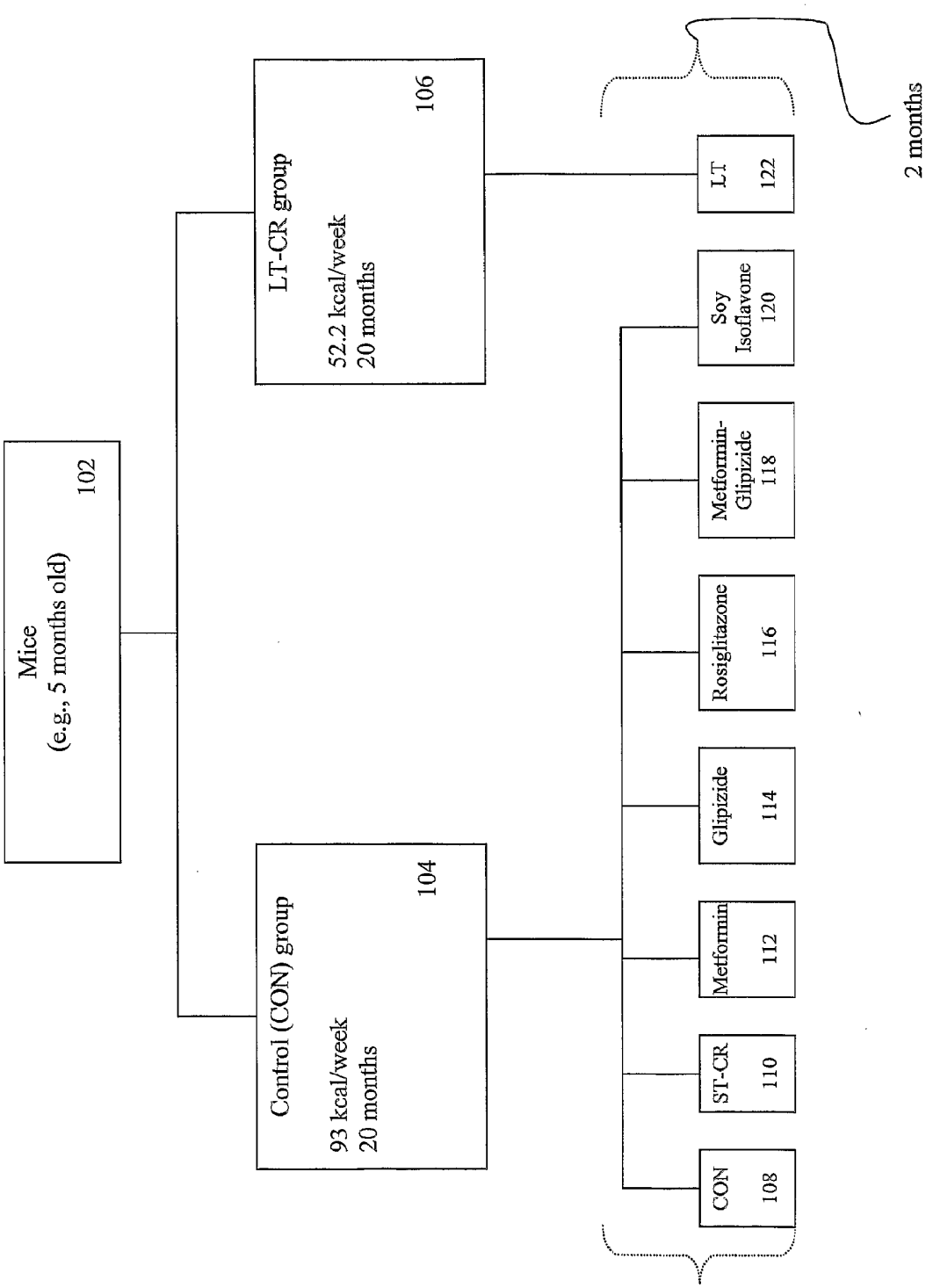

Mice
(e.g., 5 months old)
102
Control (CON) group
93 kcal/week
20 months
104
LT-CR group
52.2 kcal/week
20 months
106
CON
108
ST-CR
110
Metformin
112
Glipizide
114
Rosiglitazone
116
Metformin-Glipizide
118
Soy Isoflavone
120
LT
122
2 months

FIG 4

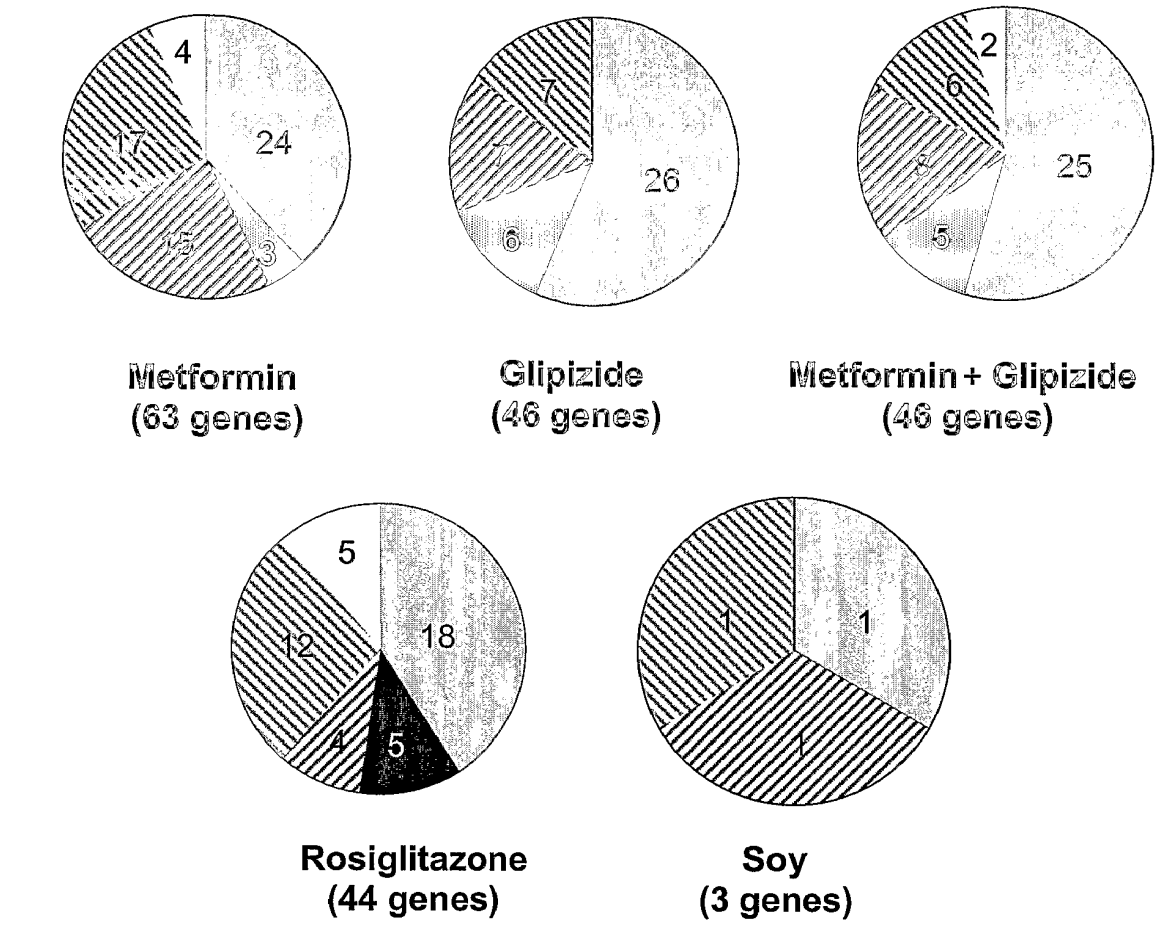

4
24
17
5
3
Metformin
(63 genes)
7
26
6
Glipizide
(46 genes)
2
6
25
5
Metformin + Glipizide
(46 genes)
5
18
12
4
5
Rosiglitazone
(44 genes)
1
1
1
Soy
(3 genes)
Identical to ST-CR
Identical to LT & ST-CR
Identical to LT-CR
Opposite Direction to LT-CR & CT-CR
Drug Only

FIG. 5

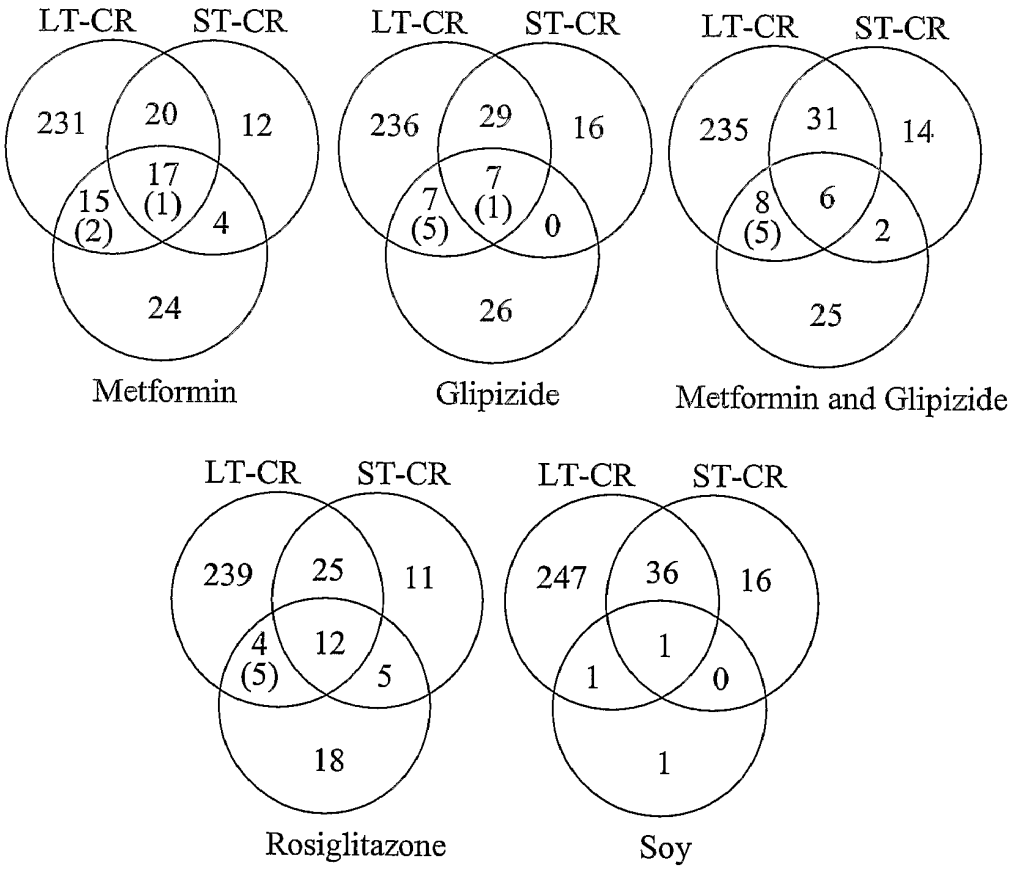

LT-CR
ST-CR
231
20
12
17
15
(1)
(2)
4
24
Metformin
LT-CR
ST-CR
236
29
16
7
7
(1)
(5)
0
26
Glipizide
LT-CR
ST-CR
235
31
14
8
6
(5)
2
25
Metformin and Glipizide
LT-CR
ST-CR
239
25
11
4
12
(5)
5
18
Rosiglitazone
LT-CR
ST-CR
247
36
16
1
1
1
0
1
Soy

Figure 6

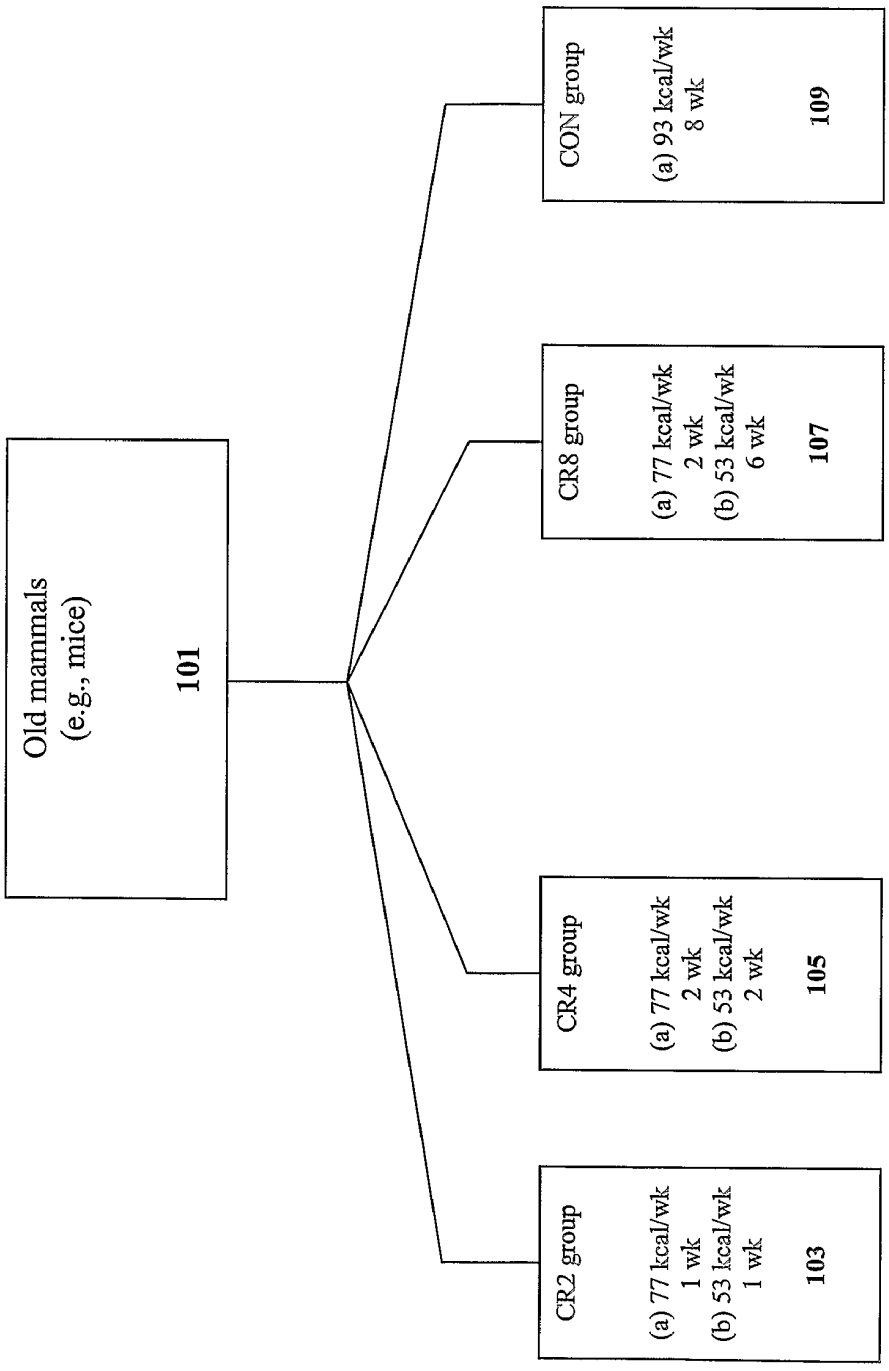

Old mammals
(e.g., mice)
101
CR2 group
(a) 77 kcal/wk
1 wk
(b) 53 kcal/wk
1 wk
103
CR4 group
(a) 77 kcal/wk
2 wk
(b) 53 kcal/wk
2 wk
105
CR8 group
(a) 77 kcal/wk
2 wk
(b) 53 kcal/wk
6 wk
107
CON group
(a) 93 kcal/wk
8 wk
109

FIGURE 7

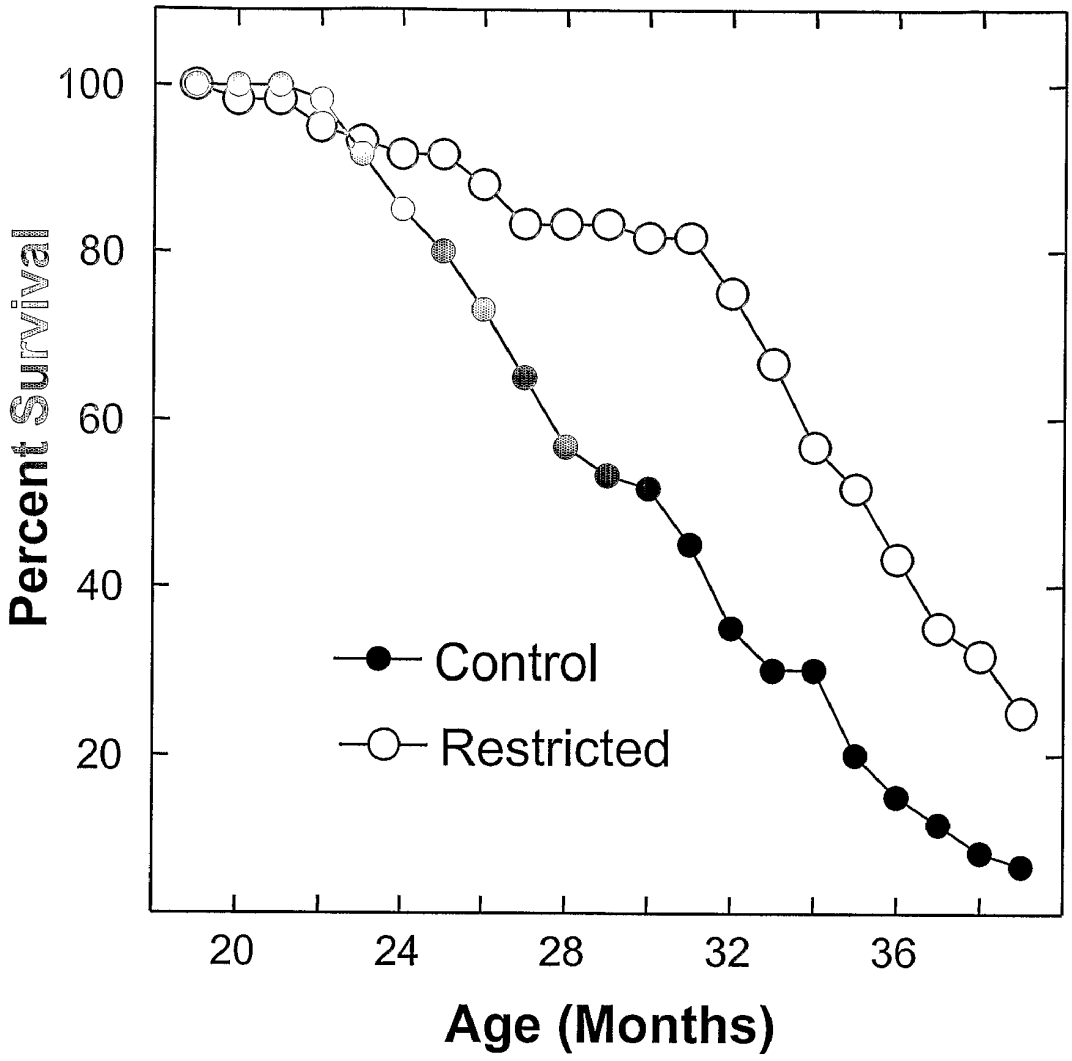


FIGURE 8 Longevity of mice subjected to caloric restriction from 19 months of age. The CR group is represented by the open circles, while the control group by the filled circles.

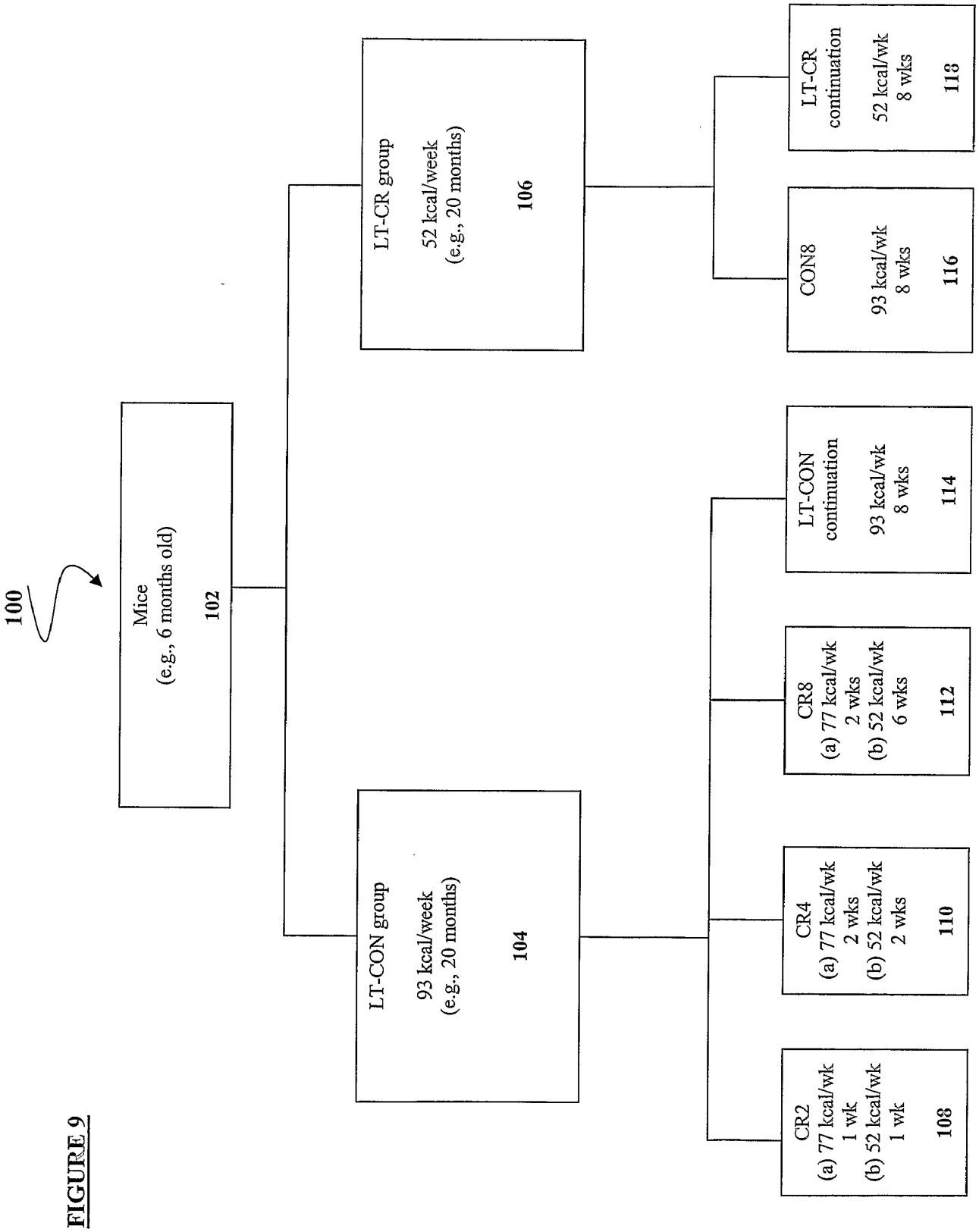
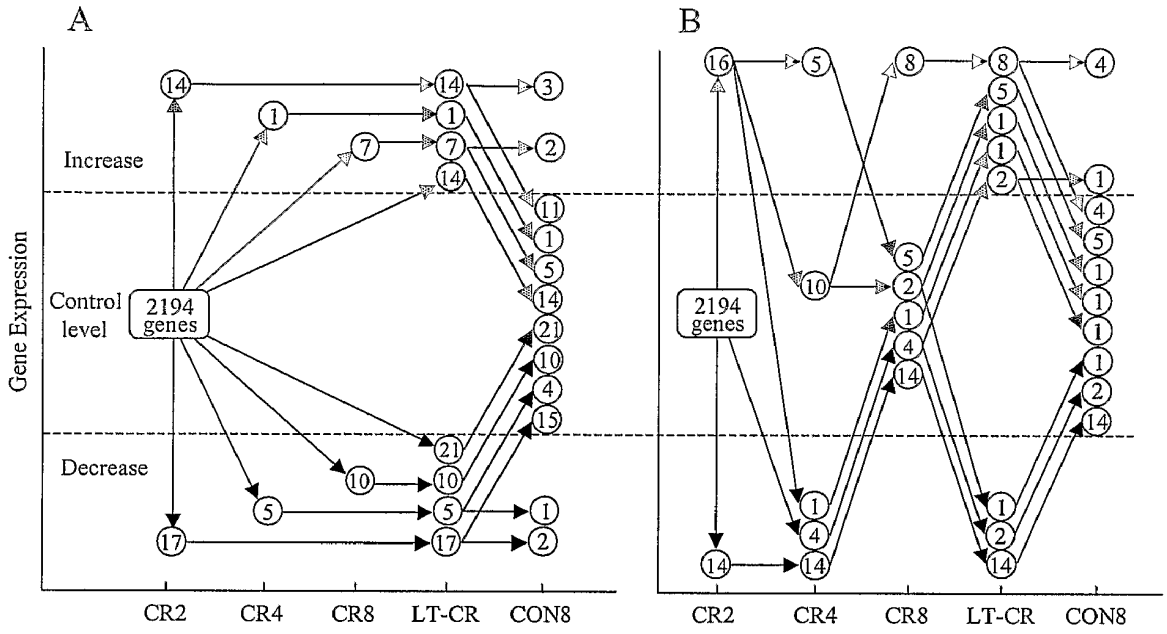
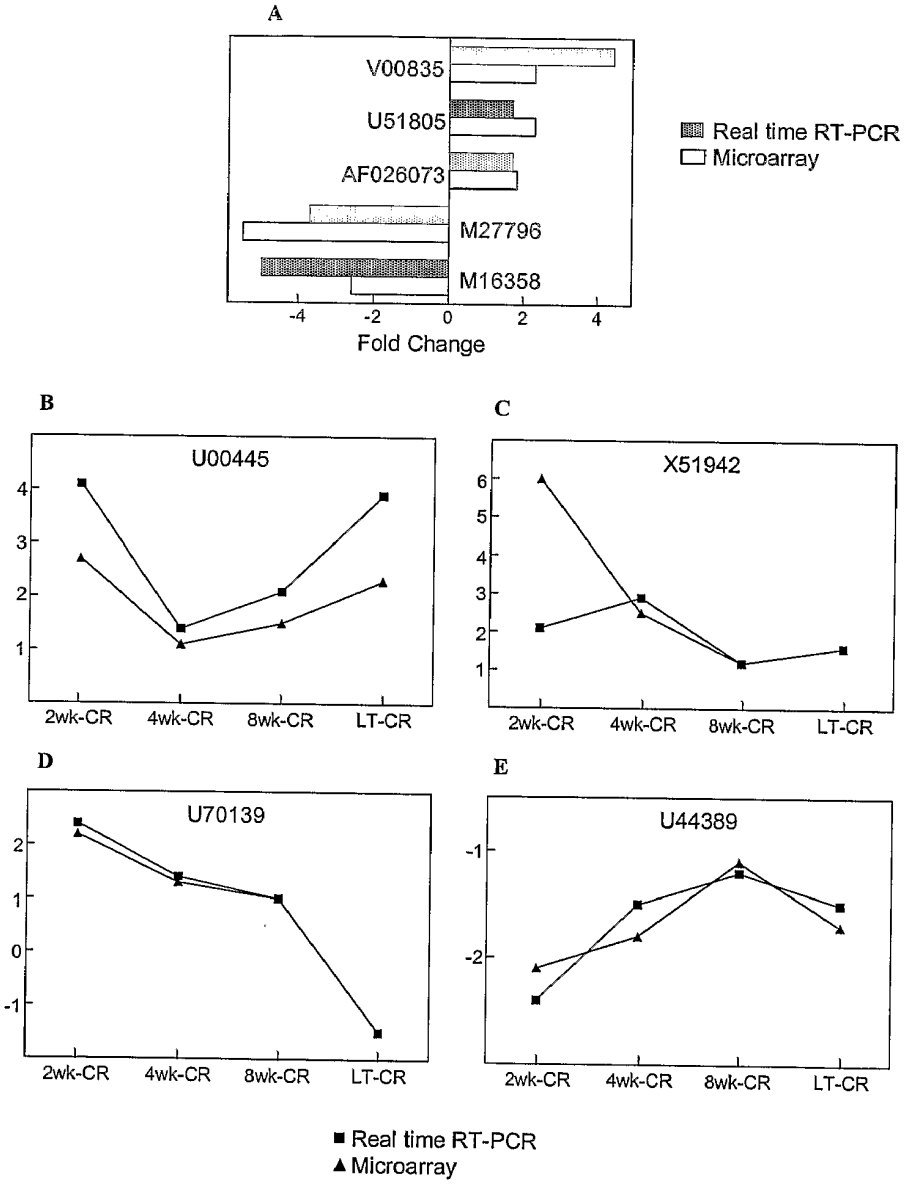

100
Mice
(e.g., 6 months old)
102
LT-CON group
93 kcal/week
(e.g., 20 months)
104
LT-CR group
52 kcal/week
(e.g., 20 months)
106
CR2
(a) 77 kcal/wk
1 wk
(b) 52 kcal/wk
1 wk
108
CR4
(a) 77 kcal/wk
2 wks
(b) 52 kcal/wk
2 wks
110
CR8
(a) 77 kcal/wk
2 wks
(b) 52 kcal/wk
6 wks
112
LT-CON
continuation
93 kcal/wk
8 wks
114
CON8
93 kcal/wk
8 wks
116
LT-CR
continuation
52 kcal/wk
8 wks
118

FIGURE 9



FIGURES 10A-10B Dynamics of the early changes in hepatic expression of genes whose expression is affected by LT-CR.

FIGURES 11A-11E



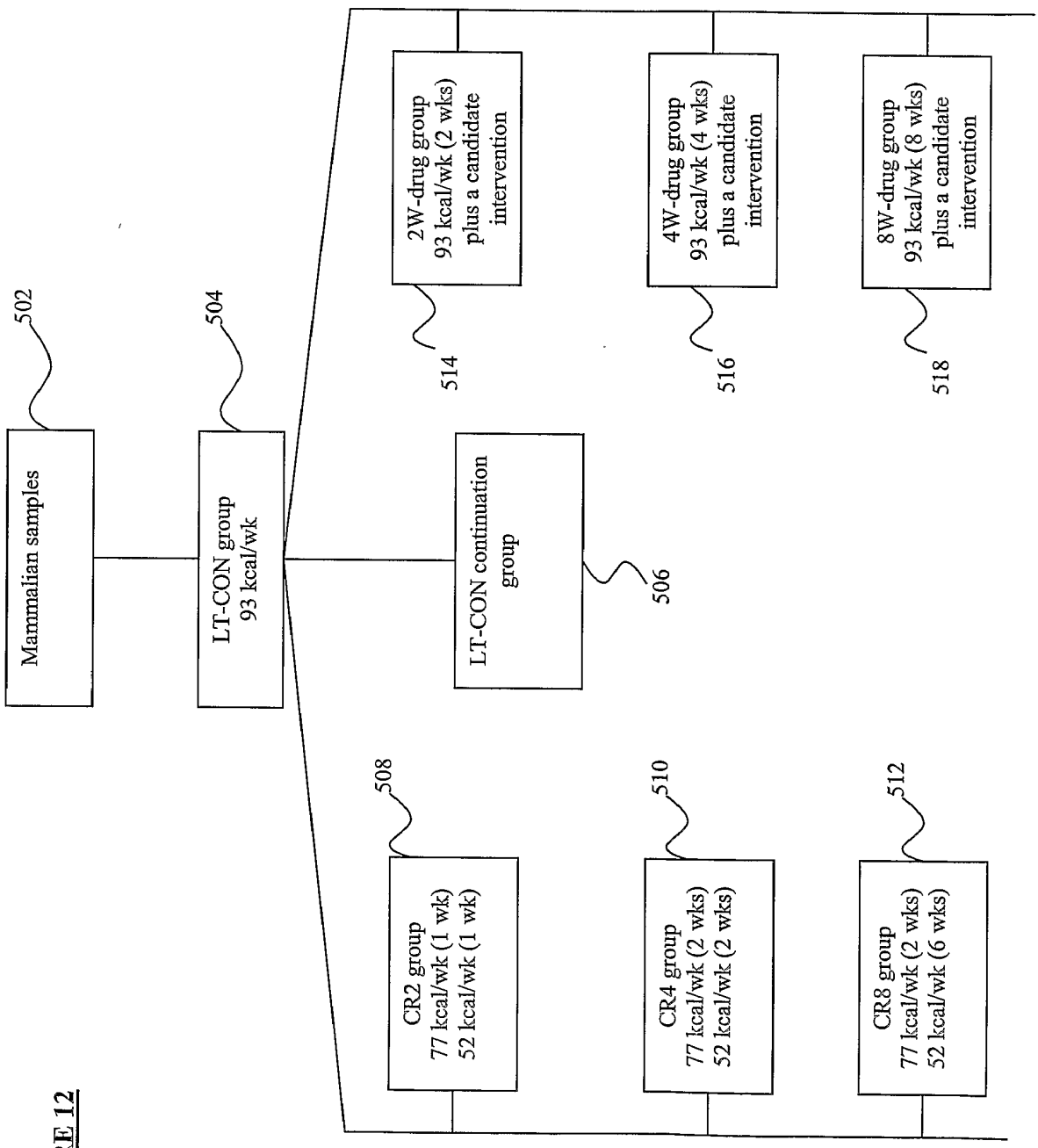

Mammalian samples
502
LT-CON group
93 kcal/wk
504
LT-CON continuation group
506
2W-drug group
93 kcal/wk (2 wks)
plus a candidate intervention
514
4W-drug group
93 kcal/wk (4 wks)
plus a candidate intervention
516
8W-drug group
93 kcal/wk (8 wks)
plus a candidate intervention
518
CR2 group
77 kcal/wk (1 wk)
52 kcal/wk (1 wk)
508
CR4 group
77 kcal/wk (2 wks)
52 kcal/wk (2 wks)
510
CR8 group
77 kcal/wk (2 wks)
52 kcal/wk (6 wks)
512

FIGURE 12

FIGURE 13

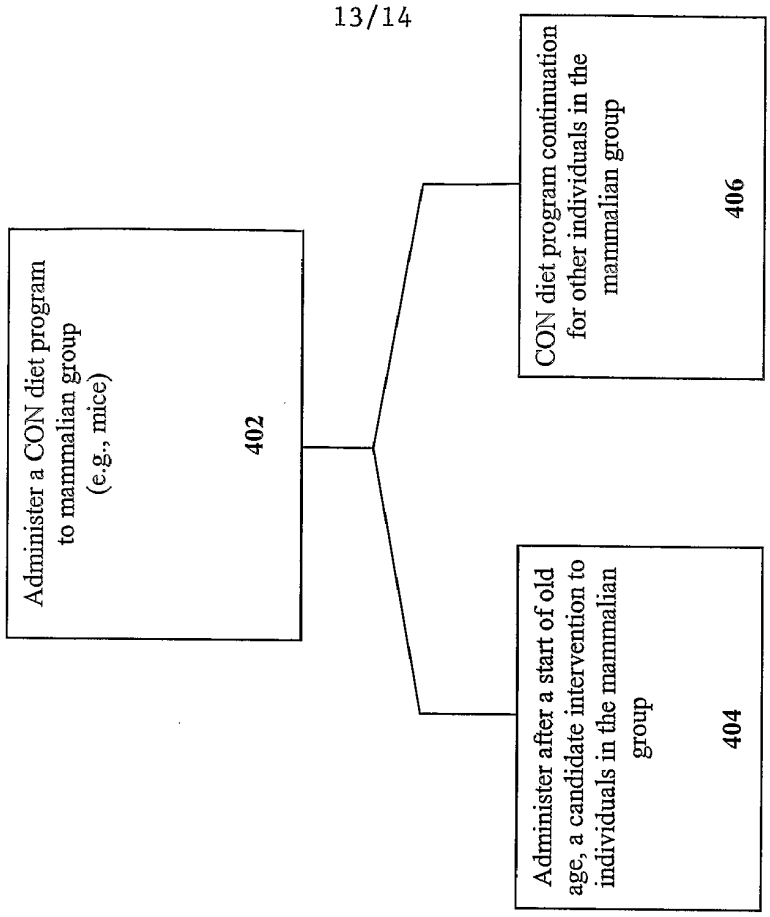


FIGURE 14

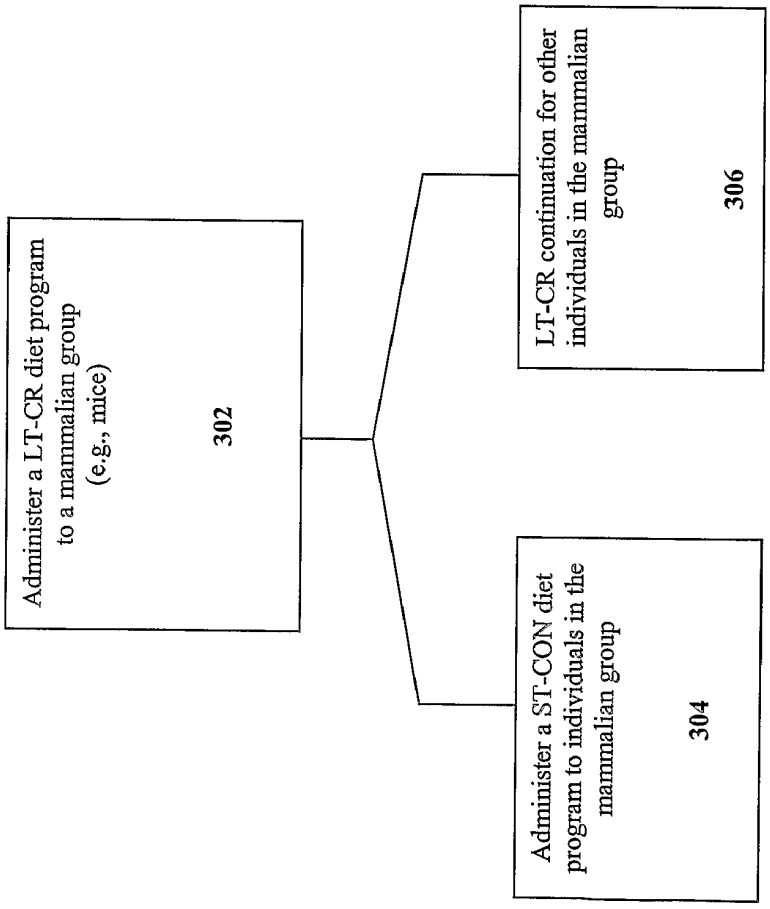
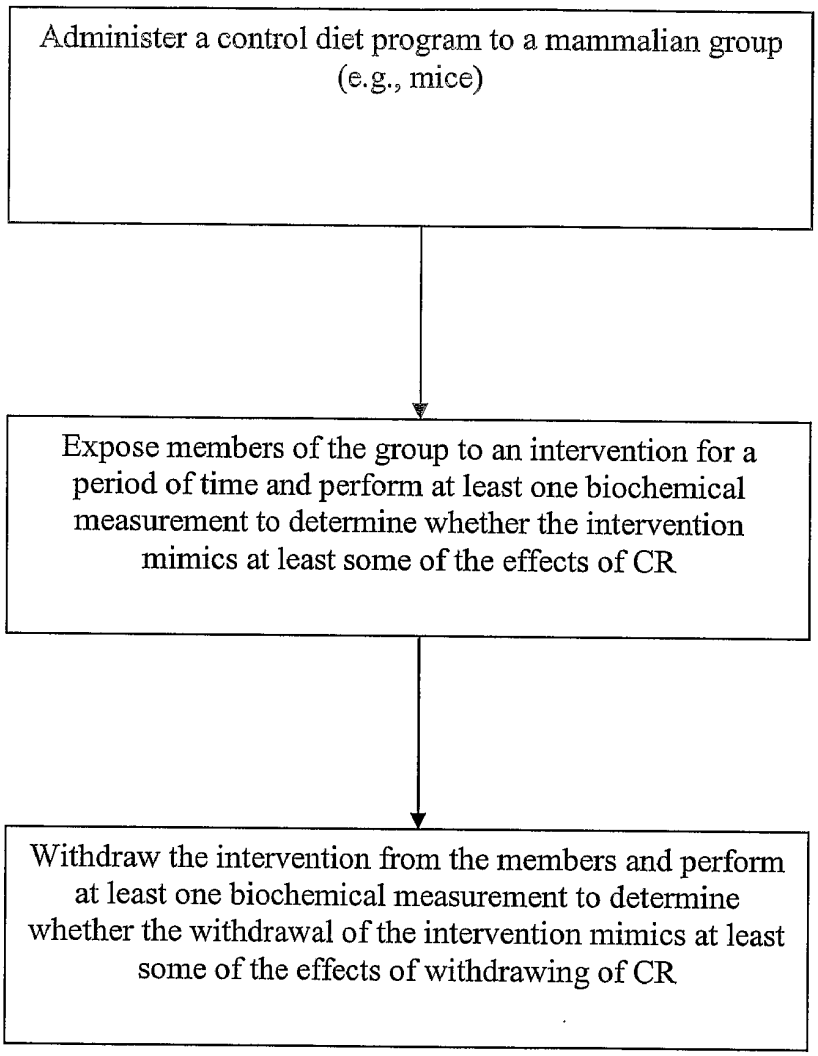


FIGURE 15


Administer a control diet program to a mammalian group (e.g., mice)
Expose members of the group to an intervention for a period of time and perform at least one biochemical measurement to determine whether the intervention mimics at least some of the effects of CR
Withdraw the intervention from the members and perform at least one biochemical measurement to determine whether the withdrawal of the intervention mimics at least some of the effects of withdrawing of CR

专利名称(译)	评估热量限制和识别热量限制模拟物的方法		
公开(公告)号	EP1601949A4	公开(公告)日	2007-07-18
申请号	EP2004720476	申请日	2004-03-12
[标]申请(专利权)人(译)	加利福尼亚大学董事会		
申请(专利权)人(译)	加利福尼亚大学董事会		
当前申请(专利权)人(译)	加利福尼亚大学董事会		
[标]发明人	SPINDLER STEPHEN R DHAHBI JOSEPH M		
发明人	SPINDLER, STEPHEN, R. DHAHBI, JOSEPH, M.		
IPC分类号	C12Q1/68 A61K49/00 C12Q1/00 G01N G01N1/00 G01N33/50 G01N33/53		
CPC分类号	G01N33/5308		
优先权	10/387743 2003-03-12 US 10/387786 2003-03-12 US 10/622160 2003-07-16 US		
其他公开文献	EP1601949A2		
外部链接	Espacenet		

摘要(译)

本发明提供了鉴定卡路里限制的生物标志物和检查卡路里限制的动态的方法。此外，本发明提供了选择卡路里限制模拟物的方法。