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(54) Title: IDENTIFICATION OF 5-LIPOXYGENASE EFFECTS ON OBESITY AND INSULIN RESISTANCE

(57) Abstract: 5-LO deficiency is shown to exacerbate markers of the metabolic syndrome. The enzyme has significant effects on adiposity and metabolism as well as bone density and fertility.

IDENTIFICATION OF 5-LIPOXYGENASE EFFECTS ON OBESITY AND INSULIN RESISTANCE

INTRODUCTION

[01] This invention was made with Government support under contract P01 HL30568 awarded by the National Institutes of Health. The Government has certain rights in this invention.

[02] Insulin resistance occurs in 25% of non-diabetic, non-obese, apparently healthy individuals, and predisposes them to both diabetes and coronary artery disease. Diabetes mellitus is a major health problem in the United States affecting approximately 7% of the population. The most common form of diabetes mellitus is non-insulin-dependent diabetes mellitus (NIDDM or type II diabetes). Hyperglycemia in type II diabetes is the result of both resistance to insulin in muscle and other key insulin target tissues, and decreased beta cell insulin secretion. Longitudinal studies of individuals with a strong family history of diabetes indicate that the insulin resistance precedes the secretory abnormalities. Prior to developing diabetes these individuals compensate for their insulin resistance by secreting extra insulin. Diabetes results when the compensatory hyperinsulinemia fails. The secretory deficiency of pancreatic beta cells then plays a major role in the severity of the diabetes.

[03] Reaven (1988) Diabetes **37**:1595-607 were the first to have investigated insulin resistant, non-diabetic, healthy individuals from the general population who are non-obese. Strikingly, they observed that 25% of them have insulin resistance that is of a similar magnitude to that seen in type II diabetes patients. These individuals compensate by having insulin levels that are 3-4 times higher than normal. These elevated insulin levels are sufficient to maintain normoglycemia. Others have also confirmed that a large proportion of the non-diabetic population is insulin resistant. These insulin resistant, non-diabetic individuals have a much higher risk for developing type II diabetes than insulin sensitive subjects.

[04] However, even without developing hyperglycemia and diabetes, these insulin resistant individuals pay a significant price in terms of general health. Insulin resistance results in an increased risk for having elevated plasma triglycerides (TG), lower high density lipoproteins (HDL), and high blood pressure, a cluster of abnormalities that have been termed by different investigators as either Syndrome X, the insulin resistance syndrome, or the metabolic syndrome. It is believed that either the hyperinsulinemia, insulin resistance, or both play a direct role in causing these abnormalities. Data from ethnic, family, and longitudinal studies suggest that a major component of resistance is inherited.

[05] Many mechanisms may potentially contribute to insulin resistance. One major mechanism is the impairment of insulin receptor tyrosine kinase (IR-TK) activity, a key step

in insulin receptor signalling. Several inhibitors of IR-TK have been associated to insulin resistance. The identification and characterization of genetic sequences involved in insulin resistance is of great medical interest.

Publications.

[06] The sequence of human 5LO is reported by Dixon et al. (1988) Proc. Nat. Acad. Sci. 85: 416-42. Drazen *et al.* (1999) Nature Genetics 22:168-170 report a pharmacogenetic association between 5-LO promoter genotype and the response to anti-asthma treatment, which article is herein specifically incorporated by reference. In *et al.* (1997) J. Clin. Invest. **99**:1130-1137 describe naturally occurring mutations in the human 5-LO gene promoter.

[07] Lusis (2000) Nature **407**:233-41 reviews atherosclerosis. Mehrabian *et al.* (2001) Circ Res **89**:125-30 describes the positional mapping of a locus involved in susceptibility to atherosclerosis.

SUMMARY OF THE INVENTION

[08] The present invention provides methods and compositions for the treatment and diagnosis of diseases related to hyperglycemic conditions, including diabetes, insulin resistance, and the like. Alleles, including variations in the 5-lipoxygenase promoter region, are associated with disease susceptibility, and their detection is used in the diagnosis of a predisposition to these conditions.

[09] To determine whether arachidonate 5-lipoxygenase (5-LO/*Alox5*) is responsible for the metabolic QTL traits in the cross between B6 and DBA (BXD), we characterized *Alox5* deficient mice (*Alox5*$^{-/-}$) by expression profiling and metabolic phenotyping. Microarray analysis revealed that the relative transcript abundances for a significant proportion of genes differentially regulated in *Alox5*$^{-/-}$ mice are partially controlled by the *Alox5* locus in the F2 cross. Through clinical phenotyping, we show that QTL traits, including adiposity, leptin levels, lipids, and bone density, are altered in *Alox5*$^{-/-}$ mice, demonstrating that 5-LO and its associated pathway underlie these metabolic disturbances. Animals lacking 5LO are at an increased risk for obesity and insulin resistance. Methods for treatment of these altered metabolic conditions include agents that enhance or mimic the activity of 5LO.

BRIEF DESCRIPTION OF THE DRAWINGS

[10] Figure 1 is a graph depicting the correlation of 5-LO promoter genotypes with insulin levels in humans.

[11] Figure 2 is a graph depicting the correlation of 5-LO genotypes with insulin resistance in humans.

[12] Figure 3. Differences in QTL traits as a function genotype in the BXD cross. F2 mice homozygous for DBA alleles at the chromosome 6 locus have significantly increased total

fat mass, omental fat mass, leptin levels (expressed as a ratio of percent body fat), bone mineral density, and VLDL/LDL levels compared to mice homozygous for B6 alleles.

[13] Figure 4. Significant enrichment of the genes in the *Alox5*$^{-/-}$ liver expression signature with the genes in the BXD liver microarray data set whose expression values are linked to *Alox5*. Of the 20,107 genes with eQTL lod scores > 2 (point-wise significance = 0.01), 1,991 (9.9%) were within an 18cM window encompassing *Alox5*. Restricting attention to the 444 genes from the *Alox5*$^{-/-}$ signature, 104 (23.1%) genes have eQTLs with lod scores greater than 2.0 in the 18cM interval. The *Alox5*$^{-/-}$ liver gene expression signature is even more significantly enriched for genes in the BXD liver expression data set whose expression values are linked to *Alox5* and correlated with omental fat mass (shown in parentheses). Of the 20,107 genes with eQTLs with lod scores greater than 2.0, 1,177 (5.9%) are correlated with omental fat pad mass at the 0.05 significance level and map to the 18cM *Alox5* interval. Restricting attention to the 444 genes from the *Alox5*$^{-/-}$ signature, 87 genes (19.6%) have eQTL with lod scores greater than 2.0 that map to the interval and correlate with omental fat pad mass at the 0.05 significance level.

[14] Figure 5. Histograms of correlation coefficients computed between omental fat mass and the gene expression levels in 5 different gene sets. The Pearson correlation coefficient was computed between omental fat mass and every gene expression trait in the set of 23,574 genes on BXD microarray. After taking the absolute value of the correlation coefficients computed for each set, the histograms were plotted with the number of genes on the y-axis and the correlation coefficients on the x-axis. Highlighted in each plot is the percentage of genes in the given set that had statistically significant correlation coefficients at the 0.05 significance level. The different sets of genes represented for each panel are A) a simulated set of 23,574 gene expression traits; B) the set of 23,574 gene expression traits represented in the BXD data set; C) the set of 1,991 genes whose eQTLs give lod scores greater than 2 and map to the 18cM *Alox5* interval; D) the set of 444 genes in the *Alox5*$^{-/-}$ liver expression signature; and E) the set of 104 genes from the *Alox5*$^{-/-}$ liver expression signature whose eQTLs give lod scores greater than 2 in the BXD cross and map within the 18cM interval flanking *Alox5*.

[15] Figure 6. 5-LO deficiency increases body weight and measures of adiposity on chow and HFC diets. (A) Male and female *Alox5*$^{-/-}$ mice weigh significantly more than their wildtype counterparts on chow and HFC diets. (B and C) The increased body weight of female *Alox5*$^{-/-}$ mice is predominantly due to increased body fat, which is increased in all four fat depots. (D) Plasma leptin levels, expressed as a ratio of percent body fat, is disproportionately higher in *Alox5*$^{-/-}$ female mice compared to wildtype mice. Data are expressed as mean ± SE and there are 4-7 animals in each group.

[16] Figure 7. 5-LO deficiency increases plasma lipoprotein levels and bone density. (A) Total, HDL, and VLDL/LDL cholesterol is significantly increased in 4-7 month old female *Alox5*$^{-/-}$ mice (n = 4) compared to wildtype mice (n = 5). (B) Femoral bone density is significantly increased in *Alox5*$^{-/-}$ mice (n = 12) compared to wildtype mice (n = 10). Data are expressed as mean ± SE.

[17] Figure 8. *Alox5*$^{-/-}$ mice have delayed glucose clearance due to defective insulin secretion. An IPGTT were performed as described in Methods. Asterisks (* $P < .05$; ** $P < .005$) denote time points with significant differences between female *Alox5*$^{-/-}$ (n=10) and control mice (n=10). Data are expressed as mean ± SE.

[18] Figure 9. 5-LO is expressed in ovaries and adrenals and *Alox5*$^{-/-}$ mice exhibit adrenal lipid depletion. (A) Cryosections of ovaries and adrenals from wildtype B6 stain positive for 5-LO whereas *Alox5*$^{-/-}$ mice exhibit minimal staining (100X magnification). (B) Adrenal glands from mice maintained on either chow or HFC diets were cryosectioned and stained for cholesteryl esters using oil red O. The cortex normally stains intensely because of the presence of cholesteryl esters that serve as a pool of cholesterol for steroid hormone synthesis (40X magnification).

DETAILED DESCRIPTION OF THE EMBODIMENTS

[19] Methods and compositions for the diagnosis and treatment of altered metabolic conditions related to insulin/glucose dynamics, are described. The invention is based, in part, on the evaluation of the expression and role of 5-LO, which is both differentially expressed in disease models, and for which alleles predisposing to altered metabolic conditions are herein identified. This permits the definition of 5-LO and other leukotriene synthesis genes as part of a disease pathway and the identification of a target in the pathway that is useful both diagnostically, in drug screening, and therapeutically. Alleles of 5-LO that predispose to coronary artery disease (CAD) are also associated with altered insulin metabolism and may be indicative of a predisposition to diabetes.

[20] The leukotrienes constitute a group of arachidonic acid-derived compounds with biologic activities suggesting important roles in inflammation and immediate hypersensitivity. The enzyme 5-lipoxygenase (EC 1.13.11.34) catalyzes 2 reactions in the formation of leukotrienes. Matsumoto *et al.* (1988) Proc. Nat. Acad. Sci. **85**:26-30, herein incorporated by reference, isolated cDNA clones for human lung and placenta 5-lipoxygenase and deduced the complete amino acid sequence of the enzyme.

[21] Alleles of the human 5-LO gene have a promoter polymorphism, in which there is a variable number of tandem binding sites for the transcription factors Sp1/Egr-1 (Drazen *et al.* (1999) Nat Genet **22**:168-70; and In *et al.* (1997) J. Clin. Invest. 99:1130-1137, herein incorporated by reference), where each repeat has the sequence motif GGGCGG. The

common allele in the human population consists of five repeated binding sites and has been termed the "5", or "N" allele. Alleles with less than 5 repeats, usually 3 repeats or 4 repeats, may be referred to numerically as "3" or "4", or collectively as deleted, or "D" alleles. Alleles with expanded repeats greater than 5 in number, usually 6 or 7 repeats, may be referred to collectively as "E" expanded or "A" addition alleles. Four genotypic groups have been defined: homozygous 55 (indicating that both alleles consisted of five repeated binding sites); 33, 34, and 44 (one or two binding sites deleted); 35 and 45 (one allele deleted); and 56, 57, and 67 (one or both alleles expanded). A comparison between the genotypic groups revealed that individuals carrying deleted repeat alleles (genotypes 33, 34, or 44) had greatly increased incidence of coronary artery disease compared to individuals with either wild type alleles or larger numbers of repeats. The sequence is the human 5-LO gene is provided as SEQ ID NO:1, where the promoter region extends from nucleotides 1-1844, and the coding sequence starts at nucleotide 1845.

[22] Predisposing 5-LO allele can have one or more Sp1/Egr-1 binding sites deleted, usually at least one binding site deletion on each chromosome, relative to the common allele in the human population, which wild type allele consists of five repeated Sp1/Egr-1 binding sites. Typically such susceptible alleles will have not more than 4 Sp1/Egr-1 binding site repeats. Other predisposing alleles are those changes in the 5-LO DNA sequence that confer an increased susceptibility.

[23] In addition to 5-LO, other members of the metabolic pathway leading to the biosynthesis of leukotrienes may be involved in susceptibility to disease. This pathway involves several enzymes and consists of two main branches. Upon activation of the cell by calcium, arachidonic acid is released from the nuclear membrane by cytosolic phospholipase A2 (cPLA2). 5-lipoxygenase activating protein (FLAP) then presents the fatty acid to 5-LO, which subsequently catalyzes the rate-limiting step of LT synthesis by incorporating molecular oxygen into arachidonic acid and generating LTA4. LTA4 can then be converted to LTB4 via LTA4 hydrolase (LTA4H) or shunted into the cysteinyl leukotriene pathway and converted to LTC4 by LTC4 synthase (LTC4S), which is then converted to LTD4 and subsequently LTE4 by g-glutamyl transferase and LTD4 peptidase, respectively. LTB4 binds to cell surface receptors known as LTB4 receptor 1 (LTB4R1) or LTB4 receptor 2 (LTB4R2) and the cysteinyl leukotrienes (LTC4, LTD4, and LTE4) bind to their respective receptors, CysLTR1 and CysLTR2. As a result, these molecules stimulate proinflammatory signaling pathways in target cells. Thus, the identification of the entire LT synthesis pathway and all the genes involved in this metabolic process are considered as having effects on atherosclerosis development, measures of adiposity (i.e. fat mass and leptin levels), lipid (ie. cholesterol and triglyceride) levels, insulin/glucose metabolism, and bone density. Genetic variations in other genes of the pathway, including but not limited to,

cPLA2, FLAP, LTA4H, LTC4S, LTB4R1, LTB4R2, CysLTR1, and CysLTR2 can be associated with and causal in disease.

[24] In one aspect of the present invention, methods are provided for determining a predisposition to altered metabolic conditions involving insulin/glucose dynamics in an individual. The methods comprise an analysis of genomic DNA in an individual for an allele of the 5- lipoxygenase promoter, which confers an increased susceptibility. Individuals are screened by analyzing their genomic 5-LO gene sequence for the presence of a predisposing allele, as compared to a normal 5-LO sequence. The normal 5-LO sequence shall be understood to include sequence variants in non-coding regions that do not affect the level of expression of the gene, and coding region variants that do not change the amino acid sequence, e.g. "third position" changes. The methods also comprise the analysis of genomic DNA in an individual for other leukotriene synthesis genes, which can also confer increased risk.

[25] The effect of a sequence variation on 5-LO expression or function can be determined by analysis for segregation of the sequence variation with the disease phenotype, *e.g.* presence of glucose tolerance, insulin levels, *etc.* A predisposing mutation will segregate with incidence of the disease. As an alternative to kindred studies, biochemical studies are performed to determine whether a candidate sequence variation in the 5-LO coding region or control regions affects the quantity or function of the protein. Expression levels of a candidate variant allele are compared to expression levels of the normal allele by various methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed natural protein; insertion of the variant control element into a vector with a reporter gene such as β-galactosidase, chloramphenical acetyltransferase, etc. that provides for convenient quantitation; and the like.

[26] A number of methods are used for determining the presence of a predisposing mutation in an individual. Genomic DNA is isolated from the individual or individuals that are to be tested. DNA can be isolated from any nucleated cellular source such as blood, hair shafts, saliva, mucous, biopsy, feces, *etc.* Methods using PCR amplification can be performed on the DNA from a single cell, although it is convenient to use at least about 10^5 cells. Where large amounts of DNA are available, the genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis, or amplified by conventional techniques. Of particular interest is the use of the polymerase chain reaction (PCR) to amplify the DNA that lies between two specific primers. The use of the polymerase chain reaction is described in Saiki *et al.* (1985) Science **239**:487, and a review of current techniques may be found in McPherson *et al.* (2000) PCR (Basics: From Background to Bench) Springer Verlag; ISBN: 0387916008. A detectable label may be included in the amplification reaction. Suitable labels include fluorochromes,

e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'- dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, e.g. ^{32}P, ^{35}S, ^{3}H; *etc.* The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, *etc.* having a high affinity binding partner, *e.g.* avidin, specific antibodies, *etc.*, where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

[27] Primer pairs are selected from the 5-LO genomic sequence using conventional criteria for selection. The primers in a pair will hybridize to opposite strands, and will collectively flank the region of interest. The primers will hybridize to the complementary sequence under stringent conditions, and will generally be at least about 16 nt in length, and may be 20, 25 or 30 nucleotides in length. The primers will be selected to amplify the specific region of the 5-LO gene suspected of containing the predisposing mutation. Typically the length of the amplified fragment will be selected so as to allow discrimination between repeats of 2 to 8 units. Multiplex amplification may be performed in which several sets of primers are combined in the same reaction tube, in order to analyze multiple exons simultaneously. Each primer may be conjugated to a different label.

[28] A diagnostic screening method of particular interest detects the number of SP-1 repeats in the promoter region of the human 5-LO gene. The organization of the region comprises a repeat region of from about 2 to about 8 6 base pair repeats of the binding motif GGGCGG, flanked by unique sequences. Within the 5' and 3' flanking sequences, sequences are selected for amplification primers. The exact composition of the primer sequences are not critical to the invention, but they must hybridize to the flanking sequences under stringent conditions. Criteria for selection of amplification primers are as previously discussed. To maximize the resolution of size differences at the locus, it is preferable to chose a primer sequence that is close to the repeat sequence, such that the total amplification product is at least about 30, more usually at least about 50, preferably at least about 100 or 200 nucleotides in length, which will vary with the number of repeats that are present, to not more than about 500 nucleotides in length. The number of repeats has been found to be polymorphic, as previously described, thereby generating individual differences in the length of DNA that lies between the amplification primers.

[29] The primers are used to amplify the region of genomic DNA that contains the repeats. Conveniently, a detectable label will be included in the amplification reaction, as previously described. Multiplex amplification may be performed in which several sets of primers are

combined in the same reaction tube. This is particularly advantageous when limited amounts of sample DNA are available for analysis. Conveniently, each of the sets of primers is labeled with a different fluorochrome.

[30] After amplification, the products are size fractionated. Fractionation may be performed by gel electrophoresis, particularly denaturing acrylamide or agarose gels. A convenient system uses denaturing polyacrylamide gels in combination with an automated DNA sequencer, see Hunkapillar et al. (1991) Science 254:59-74. The automated sequencer is particularly useful with multiplex amplification or pooled products of separate PCR reactions. Capillary electrophoresis may also be used for fractionation. A review of capillary electrophoresis may be found in Landers, et al. (1993) BioTechniques 14:98-111. The size of the amplification product is proportional to the number of repeats (n) that are present at the locus specified by the primers. The size will be polymorphic in the population, and is therefore an allelic marker for that locus.

[31] The amplified or cloned fragment may be sequenced by dideoxy or other methods, and the length of the amplified region, or the sequence of bases, is compared to the normal 5-LO sequence. Alternatively, where the predisposing mutation creates or destroys a recognition site for a restriction endonuclease, the fragment is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel electrophoresis, particularly acrylamide or agarose gels. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, *etc.* Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices is used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilised on a microarray, may also be used as a means of detecting the presence of variant sequences.

[32] The presence of a predisposing mutation is indicative that an individual is at increased risk of developing altered metabolic conditions relating to diabetes. The diagnosis of a disease predisposition allows the affected individual to seek early treatment of potential lesions, and to avoid activities that increase risk.

[33] Hyperglycemia in type II diabetes is the result of both resistance to insulin in muscle and other key insulin target tissues, and decreased beta cell insulin secretion. Longitudinal studies of individuals with a strong family history of diabetes indicate that the insulin resistance precedes the secretory abnormalities. Prior to developing diabetes these individuals compensate for their insulin resistance by secreting extra insulin. Diabetes results when the compensatory hyperinsulinemia fails. The secretory deficiency of pancreatic beta cells then plays a major role in the severity of the diabetes.

[34] Insulin resistance is an essential feature of a great variety of clinical disorders in addition to diabetes, including coronary artery disease, hyperlipidemia, obesity and hypertension. Individuals with non-insulin dependent diabetes have insulin resistance in peripheral tissues. They have a subnormal glucose utilization in skeletal muscle, where glucose transport across the cell membrane of skeletal muscle is the rate limiting step in glucose metabolism. In adipose and muscle cells, insulin stimulates a rapid and dramatic increase in glucose uptake, primarily by promoting the redistribution of the GLUT4 glucose transporter from its intracellular storage site to the plasma membrane. Impaired glucose tolerance (IGT) is associated with a normal fasting blood glucose but an elevated postprandial blood sugar between 7.8 and 11 mmol/L (140 and 199 mg/dL). Some patients with IGT are hyperinsulinimic, and progress to NIDDM.

[35] However, even without developing hyperglycemia and diabetes, these insulin resistant individuals pay a significant price in terms of general health. Insulin resistance results in an increased risk for having elevated plasma triglycerides (TG), lower high density lipoproteins (HDL), and high blood pressure, a cluster of abnormalities that have been termed by different investigators as either Syndrome X, the insulin resistance syndrome, or the metabolic syndrome. It is believed that either the hyperinsulinemia, insulin resistance, or both play a direct role in causing these abnormalities. Data from ethnic, family, and longitudinal studies suggest that a major component of resistance is inherited.

[36] The response to insulin has been measured by a number of different methods, and insulin resistance has been quantified by a number of different indices. A variety of procedures have been developed to detect the presence of insulin resistance. Using any of these techniques, there is a wide range of insulin sensitivity in normal individuals, some of whose values overlap with similar values in people with diabetes. Therefore, one cannot distinguish between nondiabetic and diabetic individuals on the basis of measures of insulin resistance.

[37] The most widely accepted research method or 'gold standard' is the euglycemic insulin clamp technique. With this procedure, exogenous insulin is infused, so as to maintain a constant plasma insulin level above fasting, while glucose is fixed at a basal level by infusing glucose at varying rates. This glucose infusion is delivered via an indwelling catheter at a rate based on plasma glucose measurements every 5 min. When the plasma glucose level falls below basal, the glucose infusion rate is increased to return plasma glucose to basal levels and vice versa. The amount of glucose infused over time (M value) is an index of insulin action on glucose metabolism. The more glucose that has to be infused per unit time, then the more sensitive the patient is to insulin. Conversely, the insulin-resistant patient requires much less glucose to maintain basal plasma glucose levels. The effect of insulin on fuel metabolism can be assessed in the absence of the

confounding effects of hypoglycemic counterregulation, endogenous insulin secretion, or variable levels of hyperglycemia, and multiple insulin actions can be assessed by using isotopes, including regulation of glucose uptake and production, inhibition of lipolysis, and changes in protein metabolism.

[38] An alternative is the minimal model. With this procedure, glucose and insulin are sampled frequently from an indwelling catheter during an intravenous glucose tolerance test; the results are entered into a computer model, which generates a value that is an index of insulin sensitivity (called Si). The acute insulin release (AIR) in response to glucose is also determined by the test. This measure of insulin resistance correlates reasonably well with the euglycemic insulin clamp in nondiabetic subjects. Its accuracy deteriorates in diabetes because the immediate plasma insulin response to the glucose challenge is diminished. Therefore, additional maneuvers are needed to raise plasma insulin levels, such as giving tolbutamide or exogenous insulin in the course of the test.

[39] The most practical way of assessing insulin resistance is the homeostasis model assessment (HOMA), involving fasting insulin and glucose levels. This value is calculated as fasting plasma insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5 (Matthews et al. (1985) *Diabetologia*. 28:412-9). The steady-state basal plasma glucose and insulin concentrations are determined by their interaction in a feedback loop. A computer-solved model is been used to predict the homeostatic concentrations which arise from varying degrees beta-cell deficiency and insulin resistance. Comparison of a patient's fasting values with the model's predictions allows a quantitative assessment of the contributions of insulin resistance and deficient beta-cell function to the fasting hyperglycaemia. The estimate of insulin resistance obtained by homeostasis model assessment correlates with estimates obtained by use of the euglycaemic clamp, the fasting insulin concentration, and the hyperglycaemic clamp. The lower limit of the top quintile of HOMA distribution (i.e. 2.77) in nonobese subjects with no metabolic disorders has been chosen as the threshold for insulin resistance in some studies (Bonora *et al.* (1998) Diabetes **47**:1643-9). The results of this study documented that 1) in hypertriglyceridemia and a low HDL cholesterol state, insulin resistance is as common as in NIDDM, whereas it is less frequent in hypercholesterolemia, hyperuricemia, and hypertension; 2) the vast majority of subjects with multiple metabolic disorders are insulin resistant; 3) in isolated hypercholesterolemia, hyperuricemia, or hypertension, insulin resistance is not more frequent than can be expected by chance alone; and 4) in the general population, insulin resistance can be found even in the absence of any major metabolic disorders.

[40] The measurement of insulin concentration can be done in the overnight fasted condition, since in the postprandial state, glucose levels are changing rapidly and the variable levels of glucose confound the simultaneous measure of insulin levels as an index

of insulin action. There is a significant correlation between fasting insulin levels and insulin action as measured by the clamp technique. Very high plasma insulin values in the setting of normal glucose levels are very likely to reflect insulin resistance. As individuals develop diabetes, plasma glucose increases and plasma insulin decreases and so the plasma insulin level no longer reflects only insulin resistance because it becomes influenced by the appearance of a ß-cell defect and hyperglycemia.

[41] The genes in the 5-LO/leukotriene pathway have been found to be differentially expressed in an animal model for altered metabolic states relating to diabetes. "Differential expression" as used herein refers to both quantitative as well as qualitative differences in the genes' temporal and/or tissue expression patterns. Thus, a differentially expressed gene may have its expression diminished or inactivated in protective versus susceptible conditions. The 5-LO gene therefore finds use in screening for agents that modulate expression or activity, and which find use in treatment of diabetes and insulin resistant states. Drug candidates of interest include known 5-LO inhibitors, many of which are known in the art, for example zileuton, ABT-761 (see Drazen *et al., supra.*); 2,5-Diaryl tetrahydrofurans, 2,5-diaryl tetrahydrothiophenes, 2,4-diaryl tetrahydrofurans, 2,4-diaryl tetrahydrothiophenes, 1,3-diaryl cyclopentanes, 2,4-diaryl pyrrolidines, and 2,5-diaryl pyrrolidines as disclosed in U.S. Patent no. 6,294,574; compounds described in U.S. Patent no. 6,194,585, and the like.

[42] Screening assays identify compounds that modulate the expression or activity of 5-LO or other genes in the leukotriene pathway. A 5-LO inhibitor can, for example, act as the basis for amelioration of hyperglycemic disease. Such compounds may include, but are not limited to peptides, antibodies, or small organic or inorganic compounds. Methods for the identification of such compounds are described below.

[43] Cell- and animal-based systems can act as models for hyperglycemic disease and are useful in such drug screening. The animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions that are effective in treating hyperglycemic disease. In addition, such animal models may be used to determine the LD_{50} and the ED_{50} in animal subjects, and such data can be used to determine the *in vivo* efficacy of potential treatments. Animal-based model systems of disease may include, but are not limited to, non-recombinant and engineered transgenic animals. Additionally, animal models exhibiting hyperglycemic disease symptoms may be engineered by utilizing, for example, 5-LO gene sequences in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, target gene sequences may be introduced into, and knocked out or overexpressed in the genome of the animal of interest. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea

pigs, pigs, micro-pigs, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate cardiovascular disease animal models.

[44] Any technique known in the art may be used to introduce a target gene transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); *etc.*

[45] Specific cell types within the animals may be analyzed and assayed for cellular phenotypes characteristic of hyperglycemic disease. Further, such cellular phenotypes may include a particular cell type's fingerprint pattern of expression as compared to known fingerprint expression profiles of the particular cell type in animals exhibiting hyperglycemic disease symptoms. Cells that contain and express 5-LO and/or other leukotriene synthesis pathway genes can be utilized to identify compounds that exhibit anti-hyperglycemic disease activity.

[46] Cells of a cell type known to be involved in hyperglycemic disease may be transfected with sequences capable of increasing or decreasing the amount of 5-LO gene expression within the cell. For example, 5-LO gene sequences may be introduced into, and overexpressed in, the genome of the cell of interest, or, if endogenous target gene sequences are present, they may be either overexpressed or, alternatively disrupted in order to underexpress or inactivate target gene expression.

[47] Transfection of target gene sequence nucleic acid may be accomplished by utilizing standard techniques. Transfected cells can be evaluated for the presence of the recombinant 5-LO gene sequences, for expression and accumulation of 5-LO gene mRNA, and for the presence of recombinant 5-LO protein. Where a decrease in 5-LO gene expression is desired, standard techniques may be used to demonstrate whether a decrease in expression is achieved.

[48] *In vitro* systems may be designed to identify compounds capable of inhibiting 5-LO. Such compounds may include, but are not limited to, peptides made of D-and/or L-configuration amino acids, phosphopeptides, antibodies, and small organic or inorganic molecules. The principle of the assays used to identify compounds that inhibit 5-LO involves preparing a reaction mixture of 5-LO and a test compound under conditions and for a time sufficient to allow the two components to interact, and detecting the resulting change in the catalytic activity in the formation of leukotrienes. Alternatively, a simple binding assay can be used as an initial screening method. These assays can be conducted in a variety of

ways. For example, one method to conduct such an assay would involve anchoring 5-LO protein or a test substance onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In another embodiment of such a method, the assay tests the presence of products catalyzed by 5-LO.

[49] For example, a routine assay of 5-LO activity can be performed in a mixture containing 50 mM potassium phosphate buffer at pH 7.4, 2 mM $CaCl_2$, 2 mM ATP, 25 M arachidonic acid (0.1 Ci) and 5-LO enzyme (50-100 mg of protein) in a final volume of 200 ml. The reaction is carried out at 24° C. for 3 minutes. The mixture is extracted with 0.2 ml of an ice-cold mixture of ethyl ether:methanol: 0.2 M citric acid (30:4:1). The extract is subjected to thin-layer chromatography at –10° C. in a solvent system of petroleum ether:ethyl ether:acetic acid (15:85:0.1). The silica gel zones corresponding to authentic arachidonic acid and its metabolites are scraped into scintillation vials for counting. The enzyme activity is expressed in terms of the amount of arachidonic acid oxygenated for 3 minutes.

[50] In a binding assay, the reaction can be performed on a solid phase or in liquid phase. In a solid phase assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

[51] Alternatively, a binding reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for target gene product or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

[52] Cell-based systems such as those described above may be used to identify compounds that act to ameliorate disease symptoms. For example, such cell systems may be exposed to a test compound at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed cells. After exposure, the cells are examined to determine whether one or more of the disease cellular phenotypes has been altered to resemble a more normal or more wild type, non-disease phenotype.

[53] In addition, animal-based disease systems, such as those described, above may be used to identify compounds capable of ameliorating disease symptoms. Such animal

models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies, and interventions, which may be effective in treating disease. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to ameliorate disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with disease.

[54] With regard to intervention, any treatments that reverse any aspect of insulin resistance and other hyperglycemic conditions should be considered as candidates for human disease therapeutic intervention. Dosages of test agents may be determined by deriving dose-response curves.

[55] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[56] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[57] Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

[58] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

[59] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[60] The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[61] The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[62] In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[63] The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[64] The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific 5-LO nucleic acid reagent described herein, which may be conveniently used, e.g., in clinical settings, for prognosis of patients susceptible to disease.

[65] Before the subject invention is described further, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as variations of the particular embodiments may be made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended to be limiting. Instead, the scope of the present invention will be established by the appended claims.

[66] In this specification and the appended claims, the singular forms "a," "an" and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

[67] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[68] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[69] All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the subject components of the invention that are described in the publications, which components might be used in connection with the presently described invention.

EXPERIMENTAL

Example 1

Materials and Methods

[70] *Animal Husbandry.* Mice were purchased from the Jackson Laboratories, Bar Harbor, Maine, and housed 4 per cage at 25°C on a 12-hour light/dark cycle. They were maintained either on a chow diet or a high-fat, high-cholesterol diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid (diet No. 90221, Harlan-Teklad). The mice used in the experiments described below were of both sexes and between 4 to 6 months of age. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the UCLA Animal Research Committee.

[71] 5-LO$^{-/-}$ mice on a B6 background were generated as described previously. To generate double knockout animals, 5-LO $^{-/-}$ mice were first bred to LDLR $^{-/-}$ mice (also on a B6 background), and the F1 progeny were backcrossed to LDLR $^{-/-}$ mice to produce 5-LO $^{+/-}$ / LDLR $^{-/-}$ mice. These mice were then intercrossed to generate double knockout animals. Although a small number of 5-LO $^{-/-}$ / LDLR $^{-/-}$ mice were obtained, they did not produce offspring. Therefore, the experiments described herein were performed with 5-LO $^{+/-}$ /LDLR $^{-/-}$ mice. The segregation of the 5-LO$^{-/-}$ mutation was followed using PCR primers specific for the targeted allele (neo primer) (SEQ ID NO:2) 5'-ATCGCCTTCTTGACGAGTTC-3'; downstream primer for both +/+ and KO within intron 6 (SEQ ID NO:3) 5'-GCAGGAAGTGGCTACTGTGGA-3'; primer specific to +/+ 5'-(SEQ ID NO:4) TGCAACCCAGTACTCATCAAG-3'. PCR primers used for the LDLR +/+ allele were (SEQ ID NO:5) 5'-ACCCCAAGACGTGCTCCCAGGATGA-3' and (SEQ ID NO:6) 5'-CGCAGTGCTCCTCATCTGACTTGT-3' and for the mutant allele were (SEQ ID NO:7) 5'-AGGATCTCGTCGTGACCCATGGCGA-3' and (SEQ ID NO:8) 5'-GAGCGGCGATACCGTAAAGCACGAGG-3'.

[72] *Plasma Lipid and Insulin Measurements.* Mice were fasted overnight and bled retro-orbitally under isoflurane anesthesia. Enzymatic assays for plasma cholesterol levels were performed as described previously. Insulin levels were measured in duplicate by ELISA (Crystal Chemical IUSKRO20).

[73] *Northern Blot Analysis* Total RNA was isolated from bone marrow cells using Trizol reagent (Life Technologies Inc). The RNA (10 μg) was run on a 1% agarose formaldehyde gel, transferred to nylon membrane, and hybridized with a 700-bp mouse-specific probe

from the 3' end of the 5-LO cDNA. The blots were stripped and probed for GAPDH as an internal control. Levels of 5-LO mRNA were quantitated by phosphorimaging and are expressed as the ratio of 5-LO to GAPDH mRNA.

[74] *Western Blot Analysis.* Homogenates of bone marrow cells (80 μg protein) in SDS sample buffer were subjected to electrophoresis on NuPAGE 4% to 12% precast SDS polyacrylamide gradient gels (Novex) under reducing conditions as suggested by the manufacturer. Proteins were transferred to nitrocellulose membranes, incubated (1:3000 dilution) overnight with antibodies to 5-LO, LTA4 hydrolase, or LTB4 omega-hydroxylase (Cayman Chemical), and visualized by ECL detection (Amersham, Little Chalfont). Image-Quant software (Molecular Dynamics) was used for the quantification of bands, which were normalized to GAPDH.

[75] *Measurement of LTB4 Levels.* LTB4 levels were determined in duplicate using a commercially available ELIZA kit (Cayman Chemical). Assays were performed on bone marrow cells (25 μg protein) homogenized in 10 mmol/L Tris, pH 8.0.

[76] *Sequence Analysis of 5-LO cDNA.* cDNA was prepared from peritoneal macrophage RNA of CAST and B6 mice using an Superscript rtPCR kit (Gibco BRL). The PCR primers used for sequencing were as follows: (SEQ ID NO:9) 5-'ATGCCCTATGCCCTCCTACACTGTCAC-3'/ (SEQ ID NO:10) 5'-CCACTCCATCCATCTATACTG-3'; (SEQ ID NO:11) 5'-GCAGCACAGACGTAAAGAACTG-3'; (SEQ ID NO:12) 5'-GAGGAAGTCACTGGAACGCAC; (SEQ ID NO:13) 5'-CTACGGATTCAAAGTACGACTG-3'/ (SEQ ID NO:14) CAGGTACTCGGACAGCTTCTC-3'; (SEQ ID NO:15) 5'-GCTATCCAGTCGTTCACGATG-3' (SEQ ID NO:16) 5'-GCAGCACTTCGAGCTTGGAAG-3'. The products were purified and sequenced by Laragen, Inc. (Los Angeles). The results were analyzed by the use of programs available through NCBI.

[77] *Isolation of Bone Marrow Cells and Peritoneal Macrophages.* Bone marrow cells were flushed from mouse femurs with DMEM/5% fetal calf serum (FCS) and centrifuged at 1500 RPM for 15 minutes (3 repetitions of washing and centrifugation). Peritoneal macrophages were isolated after lavage with DMEM/5% FCS, as described for bone marrow cells.

[78] *Measurement of 5-LO by Immunohistochemistry.* Immunostaining was performed on aortic lesion cryostat sections from apolipoprotein E $^{-/-}$ (apoE $^{-/-}$) and LDLR $^{-/-}$ mice, as described below. Alternate sections were fixed with formaldehyde, washed with PBS, and incubated in blocking buffer, followed by either rabbit anti-human 5-LO (Cayman Chemical, Mich) or rat anti-mouse MOMA-2 (Accurate Chemical, NY) antiserum. The sections were then washed and incubated with biotinylated goat anti-rabbit IgG at a dilution of 1:200. After extensive washing, the macrophages and 5-LO protein were visualized by alkaline phosphatase using Vector Red as substrate. Appropriate control experiments, including

omission of primary antibody, were performed. Peritoneal monocyte/macrophages were harvested with 20 mL DMEM/5% FCS 3 days after 4% thioglycolate (DIFCO, MI) injection. The cells were centrifuged at 1500 rpm, washed 3 times with media, and cultured overnight on glass slides. The slides were stained with a 1:200 dilution of rabbit anti-human 5-LO and hematoxylin.

[79] *Statistical Analyses.* Differences in measured variables between groups of mice were determined by ANOVA (Statview version 5.0). Values are expressed as mean±SEM, and differences were considered statistically significant at P±0.05.

Results

[80] *CON6 Mice Have Reduced Expression of 5-LO.* Quantitative trait locus mapping of a cross between resistant CAST and susceptible B6 mice for atherosclerotic lesion development revealed a locus with a powerful effect on atherosclerosis on mouse chromosome 6. Subsequently, a congenic strain, CON6, containing the locus derived from CAST on the background of B6 was constructed. The congenic strain was almost entirely resistant to atherosclerosis, even when an LDL receptor–null mutation was introduced. These studies defined the critical region of the gene to between ~45 cM and 74 cM on mouse chromosome 6. To complement this approach, various candidate genes within the locus were tested. The 5-LO gene is located near the middle of the congenic region, at ~53 cM.

[81] *5-LO $^{+/-}$ Mice on an LDLR $^{-/-}$ Background Have Reduced Insulin Levels.* In previous studies of the CAST/B6 intercross, we observed a significant quantitative trait locus for insulin levels on chromosome 6 that was coincident with the locus for lesion formation. Moreover, the CON6 strain exhibited decreased insulin levels as compared with B6 mice. To examine whether 5-LO could also account, in part, for the linkage of insulin to this locus, we measured insulin levels in the 5-LO $^{+/-}$ /LDLR $^{-/-}$ mice. Analogous to the lesion results, heterozygosity for a 5-LO–null allele on an LDL $^{-/-}$ background decreased insulin levels 3-fold compared with 5-LO $^{+/+}$ /LDLR $^{-/-}$ controls (Figure 7). This suggests that variations of the 5-LO gene may also have a role in regulation of insulin levels associated with this locus.

EXAMPLE 2

[82] Based on the above mouse studies, the contribution of the 5LO gene to human disease was assessed. These results demonstrate that 5LO is also involved in susceptibility to diabetes in humans. The nomenclature for the 5-LO genotype is as discussed in Drazen et al. (1999), supra. The genotype is determined by the number of Sp1 repeats in the promoter region, where 3 and 4 repeats are referred to as a "D", or deleted allele. 5 repeats is the "N", or normal allele; and 6 repeats is an "A", or addition allele. The genotyping is performed essentially as set forth in Drazen *et al.*

[83] The deleted alleles also lead to significantly higher fasting insulin levels (figure 1) as well as insulin resistance (HOMA analysis; figure 2), both of which are known risk factors for diabetes. Taken together, these results strongly suggest that genetic variation in the 5LO gene contributes to CAD-related traits in the human population, with deleted alleles predisposing individuals to diabetes. We believe that identification of individuals who are carriers of deleted alleles would serve as a beneficial and powerful screening test in the general population or for those who are already at risk of developing diabetes.

[84] We have developed a method that can be used as a diagnostic DNA test to determine the form of the 5LO gene that an individual has. PCR is used to amplify a portion of the 5LO gene from an individual. Based on the size of this amplified fragment, it is possible to determine whether an individual carries the normal form (wildtype allele) or a variant version (deleted allele) of the 5LO gene. There are currently no genetic tests available for common forms of diabetes. Given the importance and prevalence of diabetes, we believe that this is the first such test that can be widely used to identify at-risk individuals in the population.

[85] It is evident that subject invention provides a convenient and effective way of determining whether a patient will be susceptible to hyperglycemic disease. The subject methods will provide a number of benefits, including preventive treatment and diet. As such, the subject invention represents a significant contribution to the art.

Example 3

[86] The use of inbred strains of mice to dissect the genetic complexity of common diseases offers a viable alternative to human studies since experimental parameters, such as environment, breeding scheme, and detailed phenotyping, can be controlled. In recent years, QTL mapping has led to the identification of numerous genetic loci for a variety of traits relevant to human diseases, including behavioral differences, lipid levels, obesity, and atherosclerosis. Although the use of QTL analysis to identify complex disease genes is still a long and laborious undertaking, important progress in the development of genomics and bioinformatics tools, such as the creation of whole genome congenic strains, microarray technologies, and the availability of the genomic sequence from several different strains, will accelerate this process.

[87] As an example of this approach, we reported QTLs for various traits related to cardiovascular and metabolic diseases in a BXD intercross. In particular, we identified a locus on mouse chromosome 6 with pleiotropic effects on adiposity, plasma lipoprotein levels, and bone density. *Alox5* is located directly under the linkage signals and is the rate-limiting enzyme in leukotriene (LT) biosynthesis, which are well known mediators of the acute inflammatory response, particularly that associated with asthma. Interestingly, we

mapped a locus for atherosclerosis and plasma insulin levels directly over *Alox5* in a separate cross between B6 and CAST/Ei (CAST). Subsequent studies demonstrated that mice congenic for the CAST allele of *Alox5* were markedly resistant to atherosclerosis and had significantly reduced expression of the enzyme, which could be attributed to amino acid substitutions in the c-terminus of 5-LO. We also reported that *Alox5*$^{-/-}$ mice on a hyperlipidemic background were resistant to aortic lesion formation and had reduced levels of plasma insulin, an observation supported by recent genetic studies in humans that have implicated the 5-LO pathway in atherosclerosis. Taken together, there is convincing evidence that *Alox5* leads to the metabolic traits that map to the chromosome 6 locus.

[88] In the present study, we evaluated *Alox5* to determine whether this gene could be the underlying cause for the QTLs identified between B6 and DBA. Using an integrative genomics approach involving sequence comparisons, microarray analysis, and the clinical characterization of mouse models, we demonstrate that the pleiotropic metabolic effects of the chromosome 6 locus in the BXD cross can be attributed to *Alox5*.

[89] *Examination of trait and genetic variation in the BXD cross.* In the all female cross, F2 animals homozygous for the DBA allele at the *Alox5* locus had significantly increased fat mass, leptin levels, bone density, and very low density/low density lipoprotein (VLDL/LDL) levels compared to mice homozygous for the B6 allele, demonstrating the underlying genetic basis for linkage to this locus (Figure 3). Given that precise positioning of QTLs in a moderately sized cross such as the BXD data set is known to be problematic, the linkage peaks do not directly implicate *Alox5*. However, since the joint lod score curve for the composite trait was centered directly over its physical location, *Alox5* was considered as an attractive positional candidate gene. We first compared the sequences of *Alox5* between B6 and DBA using the available public and private genome databases and our own sequencing of the *Alox5* cDNA. In total, there were 66 single nucleotide polymorphisms (SNPs) that were polymorphic between B6 and DBA in *Alox5*, including 63 intronic SNPs, 1 missense mutation, and 2 SNPs in the 3' UTR, as represented in the Celera Mouse Genome Database component of the Celera Discovery System. The single missense mutation in DBA is identical to a V646I substitution we previously identified in CAST, which based on our previous studies dramatically decreases 5-LO levels and activity. This suggests that DBA mice are similar to CAST (and *Alox5*$^{-/-}$ mice) in this phenotypic regard.

[90] In considering other potential candidate genes located underneath the QTL linkage signals, we also evaluated peroxisome proliferator activated receptor gamma (*Pparγ*). This transcription factor is involved in adipocyte differentiation, insulin sensitivity, and is the target of pharmaceutical agonists, such as rosiglitazone, that are used to treat type 2 diabetes. However, compared to the variation observed in *Alox5*, only two polymorphic SNPs were identified in *Pparγ* between B6 and DBA and both occurred in introns.

[91] *Significant overlap in the perturbed liver transcriptional networks of Alox5$^{-/-}$ and BXD F2 mice.* To further evaluate whether *Alox5* and/or *Pparγ* could account for the chromosome 6 QTLs, we compared the changes in liver transcriptional networks induced by perturbations of these genes in mouse models. Significant overlap between the set of genes whose expression vary in *Alox5*$^{-/-}$ or rosiglitazone-treated mice (compared to wildtype mice) and the set of genes whose expression is linked to the chromosome 6 locus in the BXD cross, would provide direct experimental support for that corresponding gene(s) to underlie the QTLs. As previously described, gene expression values from livers of the BXD animals were treated as quantitative traits in a standard QTL analysis. Figure 4 shows that, of the 23,574 genes on the BXD microarray, 20,107 genes had expression QTLs (eQTLs) with lod scores > 2 (point-wise significant at the 0.01 significance level). Nearly 10% (1,991) of these eQTLs mapped to an 18cM window encompassing *Alox5* (Table 1). Since roughly 1% (236 genes) would have been expected by chance, this region of the mouse genome is considered a hotspot for eQTL activity in this cross.

[92] To determine the overlap between these 1,991 genes and those comprising the expression signature of 5-LO deficiency, we profiled the livers of *Alox5*$^{-/-}$ mice. From this set of expression experiments, 444 genes were identified with significantly variable relative transcript abundances between *Alox5*$^{-/-}$ and control B6 mice (Table 2) and were also represented on the BXD microarray. Of these 444 genes, 104 (23.4%) had eQTLs directly over *Alox5* (Figure 4; Table 3). Since only 44 (~10%) genes in the *Alox5*$^{-/-}$ signature would be expected to give eQTLs over *Alox5* by chance, the almost 2.5-fold enrichment is highly significant with a P-value of 3.3×10^{-17}, as calculated by the Fisher Exact Test. Interestingly, deficiency of 5-LO alters the expression of other LT pathway genes, such as 5-LO activating protein, LTB_4 receptor 1, and LTB_4 omega hydroxylase, and the expression of metabolic genes, such as leptin receptor (LEPR) (see Table 2).

[93] We next determined whether the gene expression traits linked to the *Alox5* locus in the BXD cross were significantly correlated with the clinical QTL traits that also linked to this locus. Using a simulated set of 23,574 genes, only 5% are correlated with omental fat mass (Figure 5A). Overall, of the 23,574 genes on the BXD microarray, the expression levels of 28% were correlated with omental fat mass in the BXD mice at the 0.05 level (Figure 5B). By comparison, of the 1,991 genes with eQTLs over *Alox5*, 1,177 (59%) were correlated with omental fat mass (Figure 5C). When we examined the correlation of omental fat mass with the 444 genes in the *Alox5*$^{-/-}$ signature, 58% were correlated (Figure 2D). However, of the 104 genes that fell in the *Alox5*$^{-/-}$ signature set and were linked to the *Alox5* locus in the cross, 84% (87 genes) were significantly correlated with omental fat mass (Figure 5E; Table 4). We next re-compared the enrichment of genes in the *Alox5*$^{-/-}$ signature with those having eQTLs over *Alox5* in the BXD cross by further constraining the gene set to the 87

that were correlated with fat pad mass. Overall, only 1,177 (5.9%) of the genes in the BXD dataset met these criteria compared to 87 (19.6%) genes in the *Alox5*$^{-/-}$ signature (Figure 4). This nearly 4-fold enrichment is highly significant with a P-value of 7.3 X 10^{-24}, as determined by the Fisher Exact Test. Similar results hold for total fat mass, VLDL/LDL cholesterol, leptin levels, and measures of bone density.

[94] In contrast to the comparisons described above, there was no likewise statistically significant overlap between eQTLs over the chromosome 6 locus and the expression profile in liver tissue from mice treated with rosiglitazone. For example, employing the same type of analysis described above, there were 595 genes represented in the rosiglitazone signature (Table 5). However, only 56 of these genes had expression values linked to the chromosome 6 locus, which is not significantly different from the 46 expected by chance (Fisher Exact Test P-value = 0.68) (Table 6). These data suggest that *Pparγ* is unlikely to be the gene underlying the chromosome 6 QTLs in the BXD cross. Therefore, we focused our attention on characterizing *Alox5*$^{-/-}$ mice.

[95] *Characterization of Alox5*$^{-/-}$ *mice for QTL traits in the BXD cross.* To determine whether *Alox5*$^{-/-}$ mice exhibit the clinical QTL traits mapping to chromosome 6 in the BXD cross, we first measured body weight and composition. On both chow and high-fat, high-cholesterol (HFC) diets, *Alox5*$^{-/-}$ mice weighed 30% more than B6 controls, a difference that persisted over time (Fig 6A). Though similar trends were observed between males and females regardless of diet, the most significant differences were among females on the chow diet. To determine whether these observations were the result of behavioral differences, we measured daily food intake over a 6-day period. Food consumption was not different between *Alox5*$^{-/-}$ and wildtype mice (4.0 ± 0.27g/day vs. 4.3 ± 0.05 g/day, respectively) and the mice exhibited similar activity levels when observed during the light cycle. We next measured whole-body fat using nuclear magnetic resonance (NMR) and by the dissection and weighing of individual fat pads to determine whether increased adipose tissue explained the weight differences. The body fat content of *Alox5*$^{-/-}$ females on a chow diet was 32% compared to 16% for wild-type mice, as determined by NMR (Figure 6B). Notably, this difference was not isolated to one fat depot since all four depots exhibited increased mass (Figure 6C). Similar differences were also observed on the HFC diet (Figure 6C).

[96] Since *Alox5*$^{-/-}$ mice had increased body fat compared to wild-type mice, elevated leptin levels were expected. However, plasma leptin was disproportionately increased 3-8 fold in *Alox5*$^{-/-}$ females compared to controls even though adiposity was increased only 2-3-fold (Figure 6D). Male *Alox5*$^{-/-}$ mice also exhibited increased leptin levels (> 2-fold) but not to the same extent as females. Moreover, the dysregulation of leptin by 5-LO appears, in part, to be independent of adiposity since young *Alox5*$^{-/-}$ mice at 12 wks of age exhibited

strikingly elevated leptin levels compared to wildtype mice (860 pg/μl vs. 180 pg/μl) without significantly increased body weight.

[97] Given the QTLs for plasma lipids and bone density in the BXD cross, we also phenotyped *Alox5*$^{-/-}$ mice for these traits as well. On a chow diet, *Alox5*$^{-/-}$ mice had elevated total, HDL, and LDL/VLDL cholesterol levels compared to wildtype mice (Figure 7A). Similarly, both femoral bone mineral density (BMD) and content (BMC) were significantly higher in *Alox5*$^{-/-}$ mice compared to wildtype mice (Figure 7B). These results, and those described above, are consistent with the trends observed in the BXD cross, where F2 mice homozygous for the DBA allele at the *Alox5* locus had significantly increased body fat, leptin levels, lipid levels, and bone density compared to B6 homozygotes (Figure 7).

[98] *Alox5*$^{-/-}$ *mice exhibit altered glucose-stimulated insulin secretion.* In a previous cross between B6 and CAST, we observed a QTL for insulin levels over *Alox5*. Taken together with the observation that 5-LO deficiency increases adiposity, we next determined whether *Alox5*$^{-/-}$ mice exhibit other associated metabolic abnormalities by performing intraperitoneal glucose tolerance tests (IPGTTs). Fasting glucose and insulin levels were similar in *Alox5*$^{-/-}$ and wildtype mice, but plasma glucose levels were significantly elevated in *Alox5*$^{-/-}$ mice at all time points examined following glucose administration, which appeared to be due to reduced second phase insulin secretion (Figure 8). For example, by 15 min post-injection, the wild type mice had doubled the amount of plasma insulin as compared with *Alox5*$^{-/-}$ mice, which only became similar to control mice after 30 min. To assess whether the decreased insulin secretion capacity in *Alox5*$^{-/-}$ mice was mediated by an altered pancreatic response, we isolated β-cells from *Alox5*$^{-/-}$ and wildtype mice and measured insulin secretion *in vitro* after incubating in 4, 12, and 16mM glucose. However, there were no differences in intracellular insulin levels or its secretion by the β-cells in these experiments.

[99] *5-LO deficiency results in reduced fertility and adrenal lipid content.* Previous work has suggested that 5-LO is involved in endocrine metabolism, and, in separate crosses between mice heterozygous for the 5-LO null allele, we often obtain a decreased proportion of homozygous *Alox5*$^{-/-}$ mice, particularly on a hyperlipidemic background. To gain insight into these mechanisms, we examined 5-LO protein expression in ovarian and adrenal tissues from wildtype mice. Staining for 5-LO was abundant in both of these organs and specific since it was absent or dramatically reduced in *Alox5*$^{-/-}$ mice (Figure 9A). In addition, we previously reported that CAST mice, which have substantially reduced 5-LO expression, have marked depletion of adrenal lipids. To determine whether 5-LO was also involved in this phenotype, we examined adrenal glands from *Alox5*$^{-/-}$ mice on both chow and HFC diets and observed strikingly decreased lipid staining in the adrenal cortex compared to controls (Figure 9B). It is therefore possible that 5-LO deficiency contributes to reduced fertility by affecting the endocrine functions of ovaries and/or adrenals.

[100] Using a novel integrative genomics approach involving genotypic, gene expression, and clinical trait data in segregating mouse populations and genetically targeted mice, we demonstrate that 5-LO influences various metabolic parameters, such as adiposity, lipoprotein levels, glucose/insulin metabolism, and bone density. Several lines of evidence support these conclusions: first, QTLs for these traits map to *Alox5* in a cross between B6 and DBA; second, there is very significant enrichment and overlap between the liver gene expression signature of *Alox5*$^{-/-}$ mice with those genes that exhibit eQTLs over *Alox5* in the BXD cross; third, there was no comparative statistically significant enrichment in the expression signature obtained by the pharmacological manipulation of *Pparγ*, another positional candidate gene known to influence metabolic processes including adiposity, insulin resistance, and bone density; fourth, *Alox5*$^{-/-}$ mice exhibit the same differences in QTL traits as F2 mice homozygous for the DBA allele of *Alox5*, which substantially decreases 5-LO levels and activity. Taken together, these data provide compelling evidence that 5-LO and its role in inflammatory processes influence a variety of physiological pathways related to human metabolic disorders.

[101] Previous studies have utilized microarray approaches in mice and humans to identify gene expression patterns that redefine a complex disease trait, identify subtypes of a given disease, elucidate the complex genetic networks of causal and reactive expression changes, and/or enhance the ability to identify the key drivers of disease. In the present study, we highlight one important variation to this approach that led to the identification of a gene affecting multiple traits. We demonstrate that a multi-faceted approach to QTL mapping can directly lead to the causal gene by incorporating a comparison of the perturbed transcriptional network in segregating populations with that in single-gene perturbation experiments of positional candidate genes (i.e. knockout mice).

[102] The striking enrichment of eQTLs for genes comprising the *Alox5*$^{-/-}$ expression signature and those linking to the *Alox5* locus in the BXD data set provides direct experimental support that *Alox5* is the underlying gene. The most plausible explanation for this enrichment is that variation in *Alox5* in the BXD mice gives rise to similar transcriptional network perturbations as that associated with 5-LO deficiency. Since DBA has an amino acid substitution in *Alox5* that decreases enzyme levels and activity, the results of the liver microarray experiments in the *Alox5*$^{-/-}$ and BXD mice are entirely consistent.

[103] There is now convincing evidence that obesity is associated with a chronic, low-grade, state of inflammation, and recent studies suggest that macrophages may be important participants in this process. For example, two separate reports have shown that adipose tissue is characterized by macrophage infiltration, and it is plausible that such inflammation contributes to the development of type 2 diabetes and CAD. Since 5-LO is expressed

primarily in leukocytes, our results suggest that macrophages in fat tissue may also have effects on adipogenesis.

[104] It is noteworthy that although *Alox5*$^{-/-}$ mice have elevated leptin, they do not consume less food than their wild-type littermates. Given that LEPRs are present in other tissues, it is possible that 5-LO deficiency decreases their peripheral expression, which could, in turn, lead to elevated plasma leptin. This notion is supported by the decreased expression of LEPR we observed in the livers of *Alox5*$^{-/-}$ mice. Moreover, since leptin is also known to inhibit insulin secretion, the hyperleptinemia in *Alox5*$^{-/-}$ mice could further contribute to the altered glucose-stimulated insulin response. Interestingly, Mancuso and colleagues have also demonstrated that leptin can induce LT synthesis 2-4-fold in murine macrophages, which, by extension, could increase inflammation and atherogenesis in the artery wall.

[105] The effect of 5-LO deficiency on glucose clearance is of particular interest. Recent studies by Prasad and colleagues have shown that 12/15-LO, a related enzyme that also metabolizes arachidonic acid, influences beta cell function and insulin secretion *in vitro*. In our studies, delayed glucose clearance appears to result from abnormal second phase insulin secretion, which is in contrast to the loss of first phase insulin secretion that type 2 diabetic patients tend to exhibit. Thus, while 5-LO deficiency, in general, exacerbates markers of the metabolic syndrome, there may also be distinct physiologic differences with the human condition. Interestingly, altered insulin secretion was not observed with isolated beta cells perhaps because the *in vitro* experiments do not mimic the hyperleptinemic environment of the circulation *in vivo*.

[106] In agreement with several previously reported studies, we also observed significant effects of 5-LO on bone density. For example, LTB_4 and peptide LTs have been shown to stimulate osteoclast formation *in vitro* and *in vivo*. A locus influencing bone density was also identified on chromosome 6 near *Alox5*, which we also observed in our QTL study of the BXD cross. Furthermore, a separate positional cloning study in mice revealed that bone mass is controlled by common variations of the 12/15-LO gene. This latter study also demonstrated that deficiency of 12/15-LO increased bone density in mice, as did inhibitors of the enzyme. Our present results now provide further evidence that 5-LO, and, more broadly arachidonic acid metabolism, plays a role in bone growth and/or remodeling.

[107] In conclusion, our studies have revealed surprising pleiotropic effects associated with 5-LO deficiency, a key enzyme in the LT biosynthetic pathway. We show that the enzyme has significant effects on adiposity and metabolism as well as bone density and fertility. Inhibitors and antagonists of the 5-LO pathway are now widely used to treat asthma and several studies now imply that such therapeutic strategies may also be effective for the treatment of other conditions.

Methods

[108] *Animal Husbandry.* *Alox5*$^{-/-}$ mice on a B6 background were bred in house from known homozygous parental breeders, which were backcrossed to B6 for more that 10 generations. Control B6 mice were either bred in house or purchased from the Jackson Laboratories (Bar Harbor, Maine). All animals were housed 4 per cage at 25°C on a 10-hr dark/14-hr light cycle and maintained on either on a chow diet (Purina diet # 5015) or a HFC containing 15%fat, 1.25% cholesterol, and 0.5% cholic acid (Harlan-Teklad diet # 90221). The mice used in the experiments were of both sexes and age matched between 4-7 months of age. All procedures were in accordance with current the National Research Council, Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Animal Research Committee.

[109] *Treatment Of Mice With Rosiglitazone.* Male B6 mice (n=12-13, 9-11 weeks old) were treated daily with either rosiglitazone (100 mg/kg) or vehicle (0.25% methylcellulose) by oral gavage for 7 days. Animals were fed standard rodent chow for at least 1 week before study initiation and weighed daily during treatment. Mice were euthanized 6 hours after the last treatment and the livers were removed for RNA isolation and microarray analysis.

[110] *Probe Selection for Mouse Gene Expression Arrays.* The mouse microarray used for the BXD cross has been previously described. The mouse microarray used for the present studies is an updated version, containing 23,574 non-control oligonucleotide probes for mouse genes and 2,186 control oligos. Full-length mouse sequences were extracted from Unigene clusters, build 168 (Feb 2004), combined with RefSeq mouse sequences from Release 3 (Jan 2004) and RIKEN full-length sequences, version fantom1.01. We clustered this collection of full-length sequences and selected one representative sequence per cluster. To complete the array, we selected 3' ESTs from Unigene clusters that did not cluster with any full-length sequence from Unigene, RefSeq, or RIKEN. To select a probe for each gene sequence, we used a series of filtering steps, taking into account repeat sequences, binding energies, base composition, distance from the 3' end, sequence complexity, and potential cross-hybridization interactions. For each gene, every potential 60bp sequence was examined and the 60bp oligonucleotide that best satisfied the criteria was printed on the microarray. All microarrays used in this study were manufactured by Agilent Technologies, Inc., Palo Alto, CA.

[111] *Preparation of Labeled cDNA.* After euthanization, livers from *Alox5*$^{-/-}$, roziglitazone-treated B6 mice, and controls (housed under similar conditions) were removed, immediately flash-frozen in liquid nitrogen, and stored at –80C. Total RNA was purified from 25mg portions using an RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Liver cDNA was prepared in the same fashion as for the F2 mice in BXD cross, as described previously. Competitive hybridizations were performed by mixing

fluorescently labeled cRNA (5mg) from *Alox5*$^{-/-}$ mice and wildtype controls or from rosiglitazone-treated mice and B6 controls.

[112] *Analysis of Expression Data.* Array images were processed as previously described to obtain background noise, single channel intensity, and associated measurement error estimates. Expression changes between two samples were quantified as $\log_{10}$ (expression ratio) where the 'expression ratio' was taken to be the ratio between normalized, background-corrected intensity values for the two channels (red and green) for each spot on the array. An error model for the log ratio was applied as described by Mutch *et al.* to quantify the significance of expression changes between two samples.

[113] *Plasma Measurements.* Mice were fasted overnight and bled retro-orbitally 2-3 hrs into the light cycle under isoflurane anesthesia. Enzymatic assays for total cholesterol, HDL cholesterol, and triglycerides were performed as described previously. Glucose levels were determined using commercially available kits from Sigma (St. Louis, MO). Insulin levels were measured using a commercial ELISA kit from Crystal Chemical (IUSKR020) and leptin levels were analyzed using a murine leptin ELISA kit (R&D Systems, Minneapolis, MN). All measurements were performed in duplicate or triplicate according to the manufacturers' instructions.

[114] *Body Composition.* Whole body fat, fluids, and lean tissue mass of isoflurane anesthetized mice were determined using a Bruker Optics Minispec NMR analyzer (The Woodlands, TX) according to the manufacturer's recommendations. After euthanization, individual fat depots (retroperitoneal, epididymal, subcutaneous, and omental) were dissected out and weighed separately.

[115] *Food Intake. Alox5*$^{-/-}$ and wildtype B6 mice were individually caged in a minimum of bedding and fed 6g of chow per day. Every 24 hrs for 6 days, the remaining uneaten food was carefully removed and weighed to determine differences in food intake.

[116] *Intraperitoneal Glucose Tolerance Tests.* Animals were fasted overnight and 50μl of blood was drawn from the retro-orbital plexus under isoflurane anesthesia. A bolus of glucose (10% wt/vol in sterile H_2O) was injected into the peritoneal cavity (1mg/g body wt) and blood samples were drawn from the retro-orbital plexus at 5, 10, 15, 30, 60, and 120 minutes post injection. Glucose levels were measured immediately following each time point using the OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Inc, Johnson & Johnson). We have previously established the accuracy of glucose values using this monitor by measuring plasma glucose with the Sigma kit described above. Insulin levels were measured as described above.

[117] *Immunohistochemistry.* Adrenals and ovaries of both *Alox5*$^{-/-}$ and control mice were fixed in 10% neutral-buffered formalin. Immunostaining was performed on 4-μm sections cut from paraffin embedded tissues which were first dehydrated in graded alcohols and

embedded in paraffin. The sections were deparaffinized by baking the slides at 65° C for 30 min, then washed in xylene, and a series of graded alcohols. Tissues were permeabilized using a preheated vegetable steamer and 0.2M citrate buffer, pH 6.0 for 30 min. Endogenous peroxidase activities were blocked by 1% H_2O_2 in methanol for 15 min. The sections were blocked using 10% Normal Serum (Vector Laboratories, Inc., CA), and antigens were detected with affinity-purified polyclonal antibodies against 5-LO (Cayman Chemical, Ann Arbor, MI), which were used at 1:100 dilution and applied for 1.5 hrs. The tissue sections were washed and the primary antibodies were detected using biotin conjugated secondary antibodies (Vector Laboratories, Burlingame, CA), incubated in HRP-Aviden (Vector Laboratories, Burlingame, CA), detected by DAB (Vector Laboratories, Burlingame, CA) and lightly counterstained with hematoxylin. Each tissue was stained in duplicate. Adrenal glands from mice maintained on either chow or HFC diets were cryosectioned and stained for cholesteryl esters using oil red O. The cortex normally stains intensely because of the presence of cholesteryl esters that serve as a pool of cholesterol for steroid hormone synthesis.

[118] *Statistical Analyses.* QTL analysis of clinical and expression data in the BXD cross was performed as described previously. Genotypes in the BXD F2 animals at the *Alox5* locus were predicted by imputing the genotype probability distribution using markers flanking this locus and selecting the genotype that provided the largest contribution to the LOD score at the *Alox5* location for the omental fat mass trait. Differences in measured variables between F2 mice predicted to be homozygous for the DBA allele and F2 mice predicted to be homozygous for the B6 allele were determined using a standard t test (Statview v5.0). Differences in measured variables between *Alox5*$^{-/-}$ and control mice were determined by ANOVA (Statview v5.0). In all cases, values are expressed as mean ± SEM and differences were considered statistically significant at $P < 0.05$.

[119] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[120] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is 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.

WHAT IS CLAIMED IS:

1. A method for detecting a predisposition to diseases related to hyperglycemic conditions, the method comprising:

analyzing an individual for quantitative or qualitative change in phenotype of a 5-lipoxygenase pathway gene, specifically including 5-LO.

2. The method of Claim 1, wherein said quantitative or qualitative change is a genetic polymorphism in an allele sequence of that gene.

3. The method of Claim 2, wherein said allele comprises not more than 4 Sp1/Egr-1 binding sites in the 5-LO promoter region of either chromosome.

4. The method of Claim 3, wherein said analyzing the genomic sequence comprises the steps of:

amplifying a region of the 5-lipoxygenase promoter from isolated genomic DNA to provide an amplified fragment;

detecting the presence of a polymorphic sequence in said amplified fragment.

5. The method of Claim 4, wherein said detecting step comprises hybridization with a probe specific for the sequence of said polymorphism.

6. The method of Claim 1, wherein said disease related to altered metabolic conditions such as hyperglycemia and insulin resistance.

7. The method of Claim 1, wherein said disease related to hyperglycemic conditions is diabetes.

8. A method of screening for biologically active agents that affect susceptibility to hyperglycemic conditions, the method comprising:

combining a candidate biologically active agent with any one of:

(a) a 5-lipoxygenase polypeptide;

(b) a cell comprising a nucleic acid encoding a 5-lipoxygenase; or

(c) a non-human transgenic animal model for 5-lipoxygenase gene function comprising one of: (i) a knockout of a 5-lipoxygenase gene; (ii) an exogenous and stably transmitted 5-lipoxygenase gene; and determining the effect of said agent susceptibility to hyperglycemic conditions.

9. A method of treating hyperglycemic conditions, the method comprising: administering a patient an effective dose of a 5-lipoxygenase modulating agent.

FIGURE

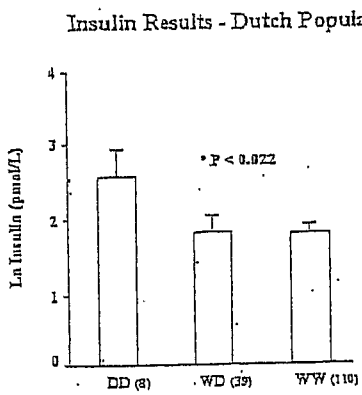
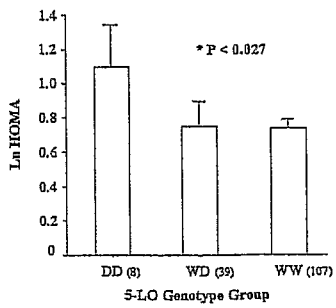
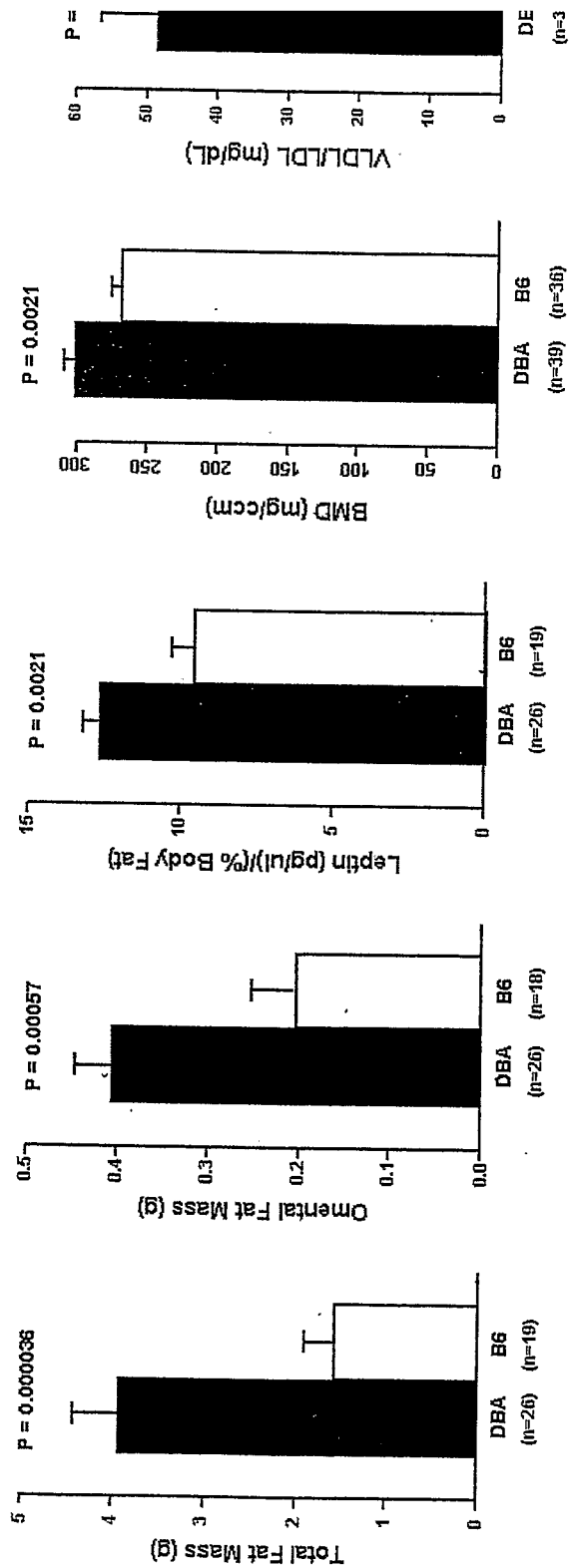


Figure 3. HOMA Results - Dutch Population





Online Figure 3

Figure 4

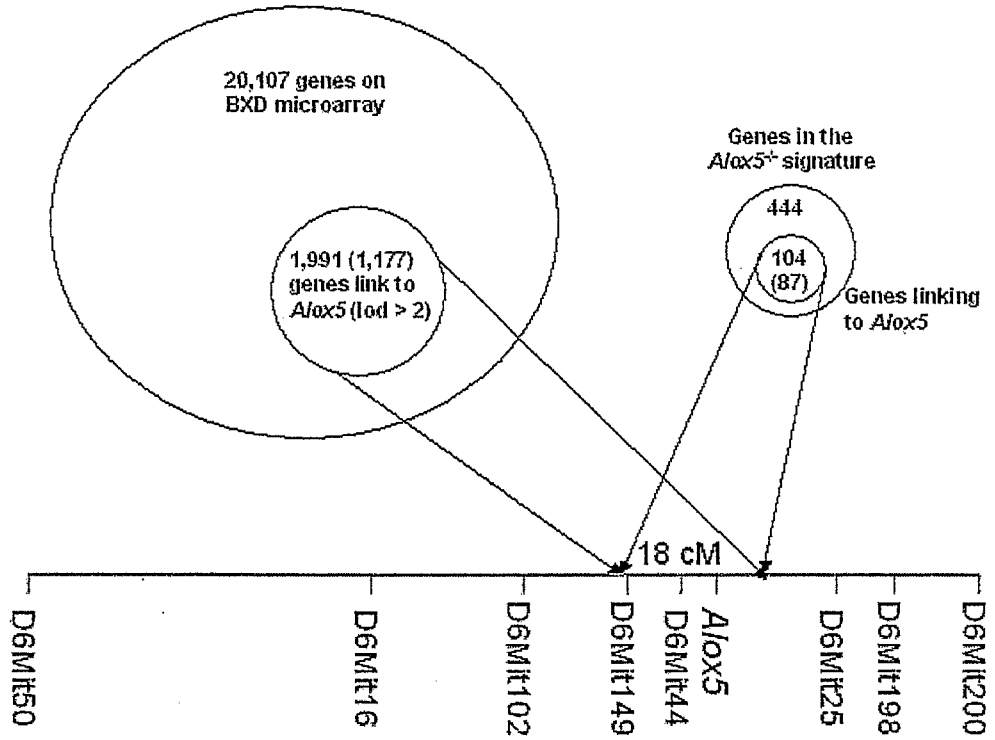
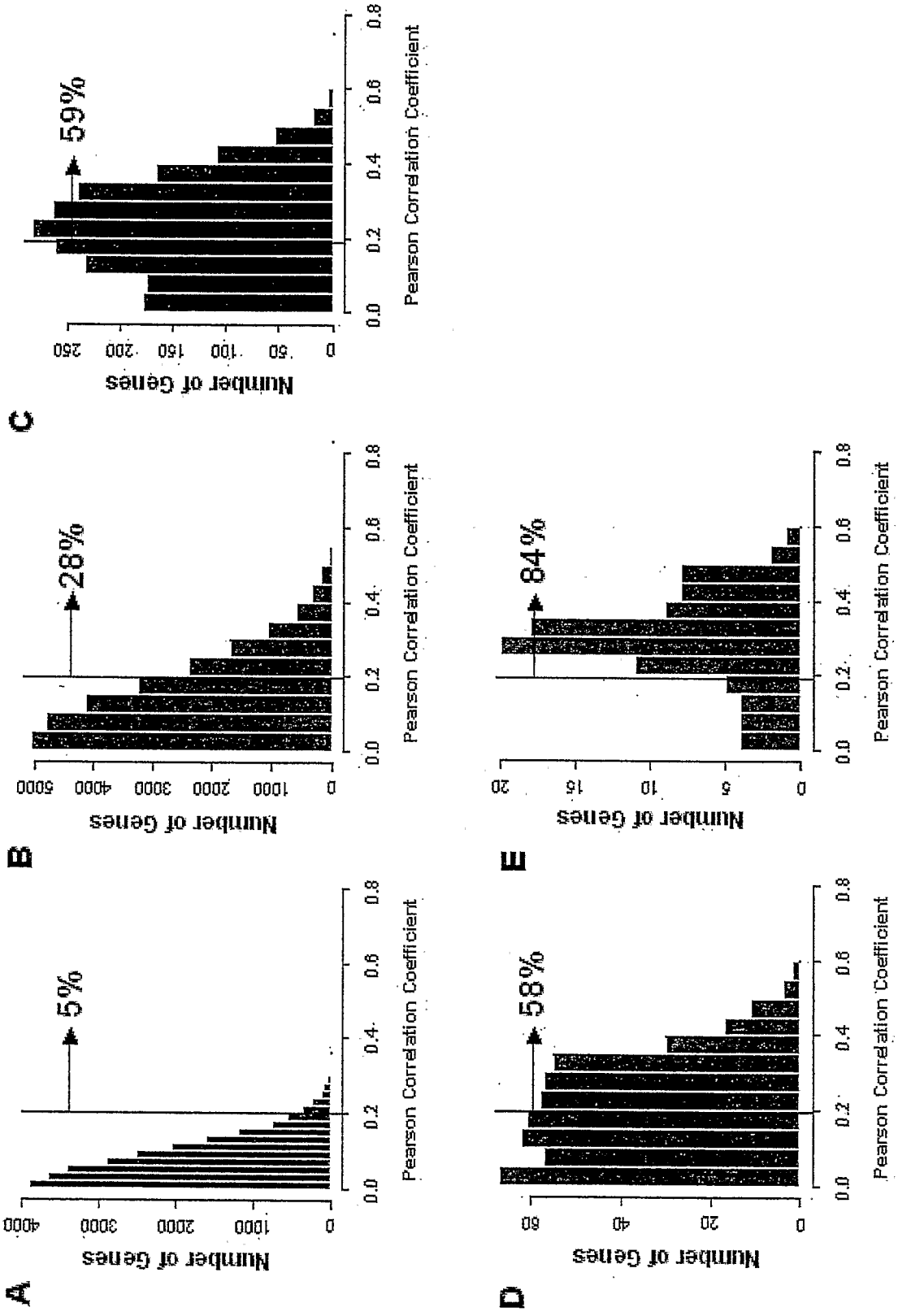

20,107 genes on BXD microarray
1,991 (1,177) genes link to Alox5 (lod > 2)
Genes in the Alox5-/- signature
444
104 (87)
Genes linking to Alox5
18 cM
D6Mit50
D6Mit16
D6Mit102
D6Mit149
D6Mit44
Alox5
D6Mit25
D6Mit198
D6Mit200

Figure 5


A
5%
Number of Genes
Pearson Correlation Coefficient
B
28%
Number of Genes
Pearson Correlation Coefficient
C
59%
Number of Genes
Pearson Correlation Coefficient
D
58%
Number of Genes
Pearson Correlation Coefficient
E
84%
Number of Genes
Pearson Correlation Coefficient

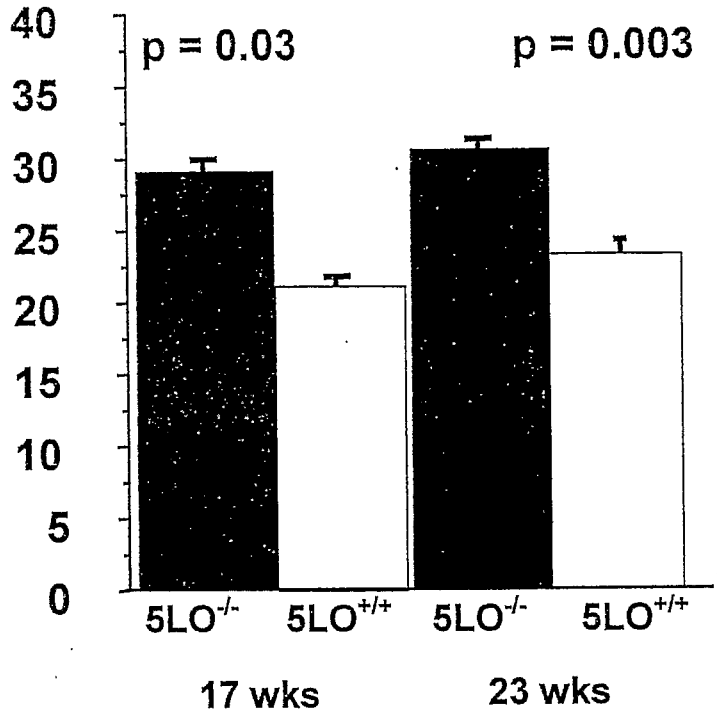
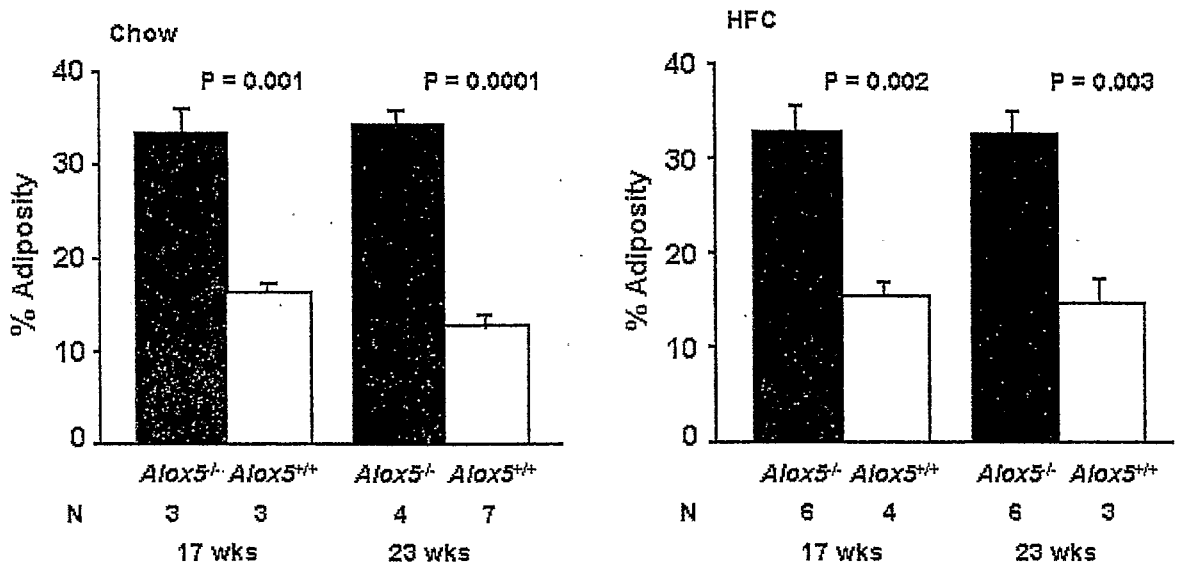

p = 0.03
p = 0.003
40
35
30
25
20
15
10
5
0
5LO-/-
5LO+/+
5LO-/-
5LO+/+
17 wks
23 wks

Figure 6B


Chow
HFC
% Adiposity
P = 0.001
P = 0.0001
P = 0.002
P = 0.003
Alox5-/- Alox5+/+ Alox5-/- Alox5+/+
N 3 3 4 7
N 6 4 6 3
17 wks
23 wks

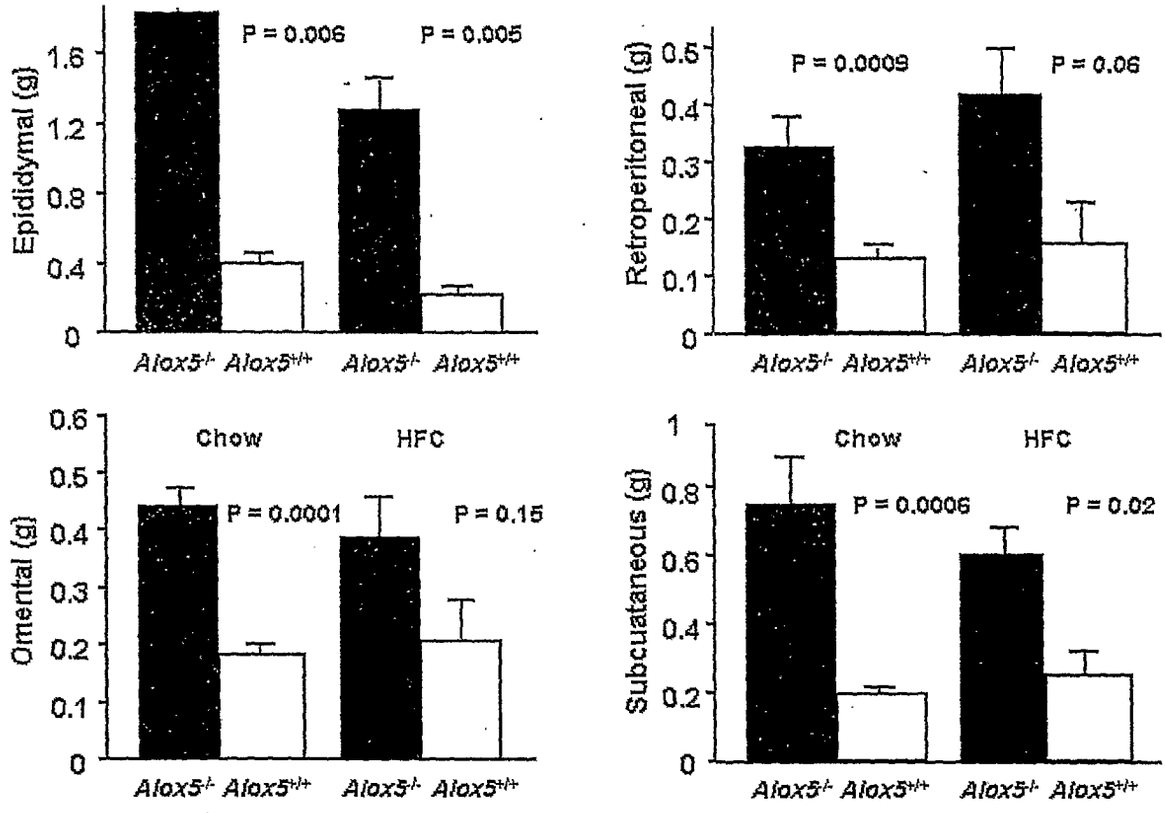
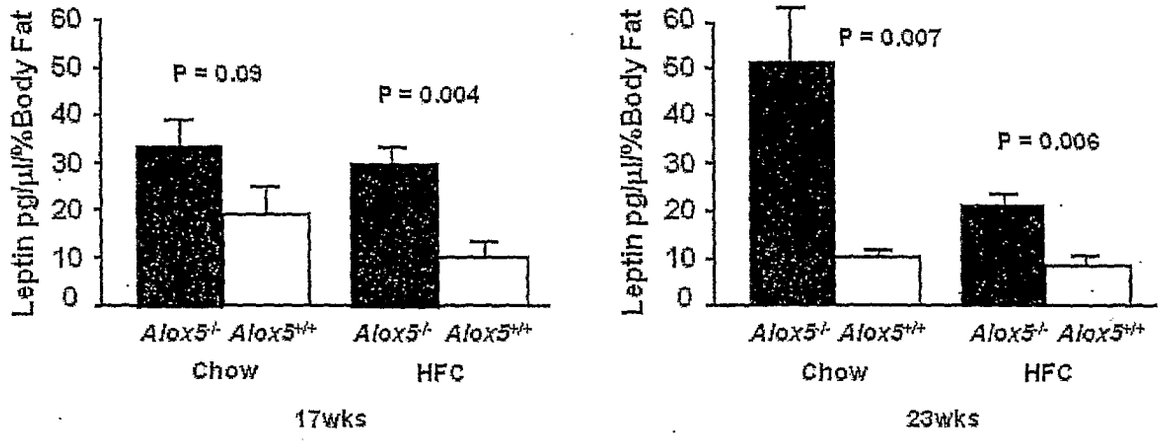

Epididymal (g)
P = 0.006
P = 0.005
Retroperitoneal (g)
P = 0.0009
P = 0.06
Omental (g)
Chow
HFC
P = 0.0001
P = 0.15
Subcuatateneous (g)
Chow
HFC
P = 0.0006
P = 0.02
Alox5-/- Alox5+/+ Alox5-/- Alox5+/+

Figure 6D


Leptin pg/µl/%Body Fat
P = 0.09
P = 0.004
P = 0.007
P = 0.006
Alox5-/- Alox5+/+ Alox5-/- Alox5+/+
Chow
HFC
17wks
23wks

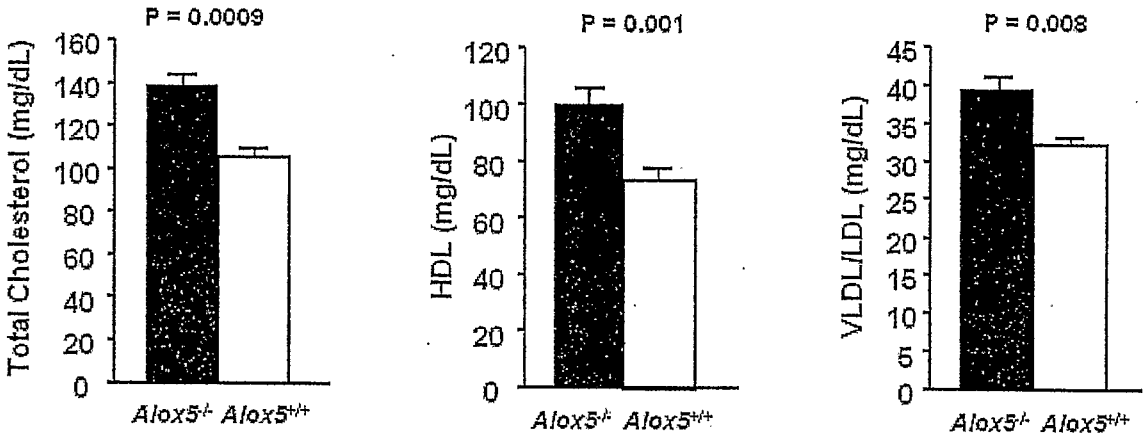
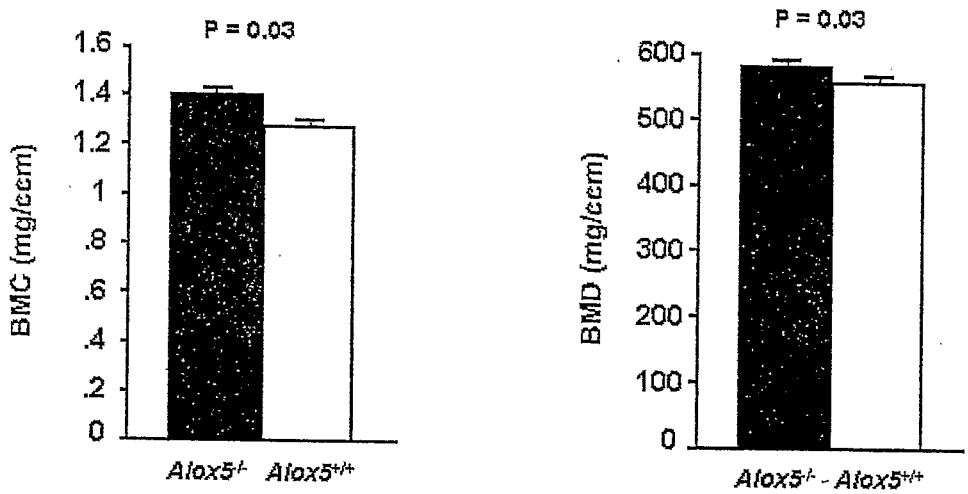
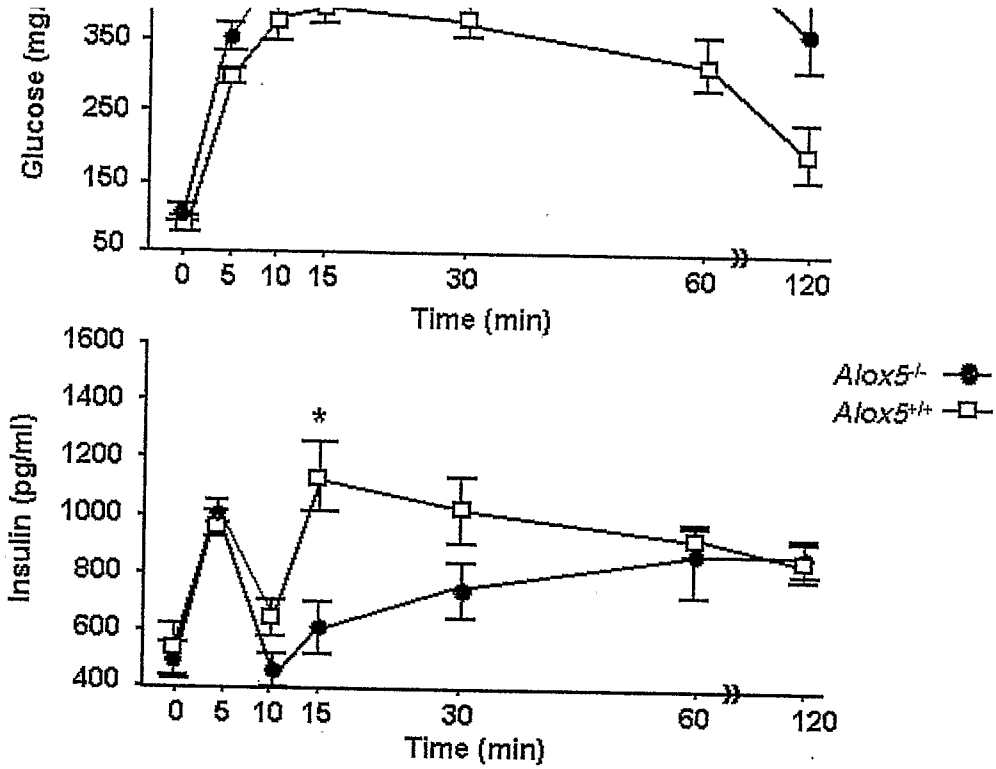

P = 0.0009
Total Cholesterol (mg/dL)
160
140
120
100
80
60
40
20
0
Alox5-/- Alox5+/+
P = 0.001
HDL (mg/dL)
120
100
80
60
40
20
0
Alox5-/- Alox5+/+
P = 0.008
VLDL/LDL (mg/dL)
45
40
35
30
25
20
15
10
5
0
Alox5-/- Alox5+/+

Figure 7B


P = 0.03
BMC (mg/ccm)
1.6
1.4
1.2
1
.8
.6
.4
.2
0
Alox5-/- Alox5+/+
P = 0.03
BMD (mg/ccm)
600
500
400
300
200
100
0
Alox5-/- Alox5+/+


Glucose (mg
350
250
150
50
0 5 10 15 30 60 120
Time (min)
1600
1400
1200
1000
800
600
400
Insulin (pg/ml)
*
0 5 10 15 30 60 120
Time (min)
Alox5-/-
Alox5+/+

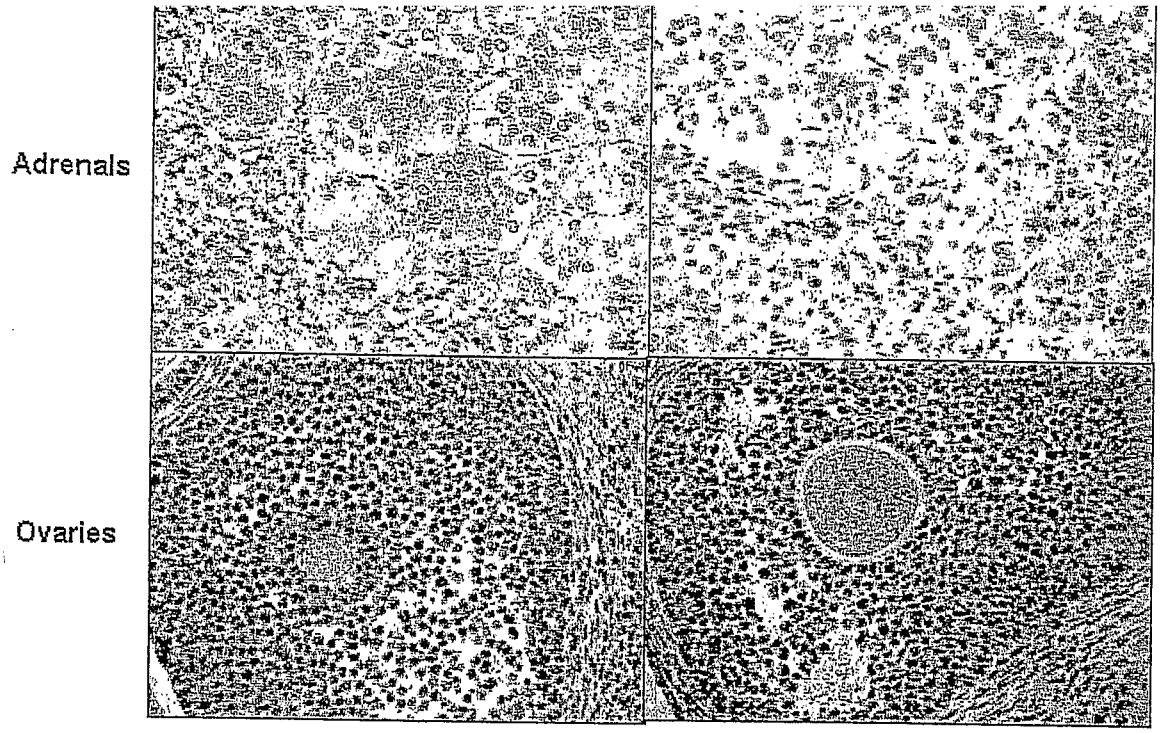
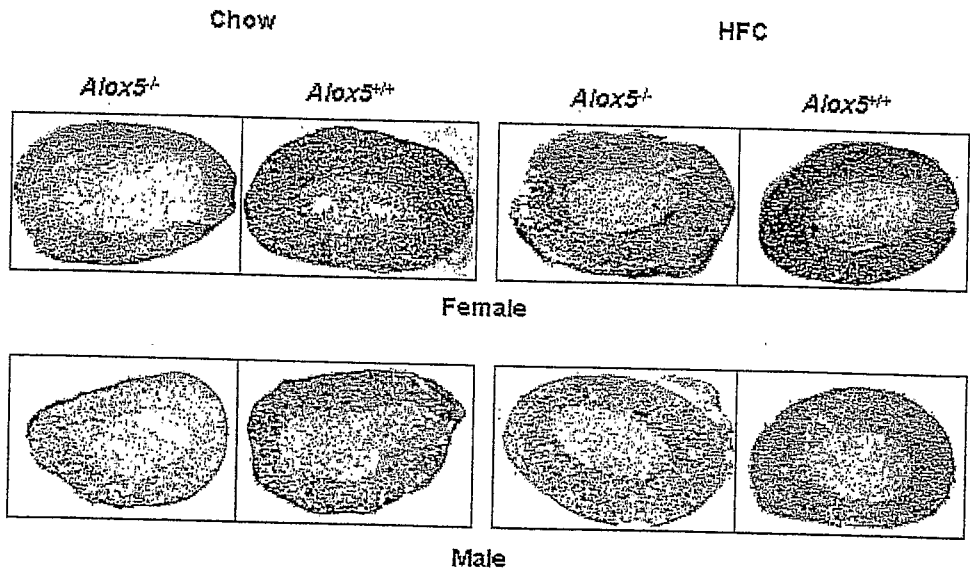

Adrenals
Ovaries

Figure 9B


Chow
HFC
Alox5-/-
Alox5+/+
Alox5-/-
Alox5+/+
Female
Male

SEQLIST

SEQ ID NO:1

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SEQ ID NO:1

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