AMDCC Annual Report (2011)

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Project Title: Atherosclerosis /other complications in hyperlipidemic BKS diabetic mouse
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Abstract: Diabetes, and the complications of diabetes, are the result of complex genetic and environmental interactions. In the present cycle of the AMDCC, we have sought to identify realistic animal models of the complications of diabetes by characterizing naturally occurring variations among inbred strains of mice that impact diabetes, insulin resistance and atherosclerosis. Among the most promising models is the classic BKS.db mouse. BKS.db consists of a combination of alleles derived from inbred strain DBA on the background of strain C57BL/6, along with a null mutation of the leptin recepter gene db. During the present AMDCC cycle we identified all of the regions of BKS that are derived from DBA using very high density SNP mapping. These regions are scattered throughout the genome, and it is clear that multiple DBA alleles are required for the development of diabetes and its complications. We then identified two strong candidates for the DBA alleles required for diabetes: 5-lipoxygenase (5LO) and lipin, both of which are dramatically reduced in activity in BKS as compared to C57BL/6. In this application we will extend the investigations of 5LO, lipin and other genes predisposing to diabetes and its complications in BKS.db. We will also introduce the apolipoprotein E (Apo E) null mutation onto BKS to produce a model for diabetes - induced atherosclerosis. To facilitate the genetic dissection of this model we have produced a whole genome set of congenic strains in which regions of DBA have been introgressed on the background of C57BL/6. Our analyses will incorporate state of the art physiologic and bioinformatic approaches and will integrate an expression quantitative trait locus (eQTL) database that we have developed over the past several years.

1. Program Accomplishments:

For the past several years, our hypothesis has been that susceptibility to diabetes and its complications are genetically complex and result from the interaction of multiple biochemical and metabolic pathways. And, while the specific gene variations that lead to pathology vary from individual to individual and between humans and animal models, the common pathologies are founded on relatively few shared overlapping pathways that are driven by the net impact of a multitude of individual genetic variations.

Progress: Mapping diabetes susceptibility in the BXD F2 db/db cross

As we have described previously, we have constructed a cross between strains DBA and B6 on the background of the *db* mutation in order to examine susceptibility to obesity-induced diabetes. Mice were studied at 5 weeks of age, prior to anticipated islet depletion (n= 221) and at 12 weeks of age when C57BLKS *db/db* mice exhibit reduced insulin production (n= 218).

In the past year we have extended the analysis of the cross as follows.

F2 animals were genotyped for a set of single nucleotide polymorphisms, about 2800 of which are informative between B6 and DBA, resulting an average marker spacing of about 0.5 cM. Using the SNP genotype data and phenotype data collected for all F2 mice we carried out QTL mapping for diabetes related traits.

In 5-week animals, a QTL for plasma glucose (LOD 4.0) was observed on distal chromosome 5, coinciding with the second highest peak for insulin QTL (Figure 1a and b). And, for 12 week animals there is a secondary glucose QTL (LOD 3) on central chromosome 2 that coincides with a QTL for insulin (LOD 3.3) seen in both 5- and 12-week animals (Figure 1a & b).

Interestingly, there were also co-incident LOD peaks for glucose, insulin and plasma triglycerides present on distal chromosome 4 and central chromosome 7 (Figure 1a, b, and c). For triglycerides (Figure 1c) and insulin (Figure 1b) these peaks are prominent but do not achieve genome-wide significance. For glucose (Figure 1a) the peaks are discernable but not prominent. However, the co-incidence of peaks for all three traits suggested the presence of genetic variation at these loci that contributes to the biological modulation of each phenotype. To improve the statistical power to identify significant QTL associated with plasma insulin glucose and triglyceride levels, we created a combined trait, the glucose-insulin-triglyceride eigenvector using the 'moduleEigengenes' function in the WGCNA package. The LOD-plot mapping data for this eigenvector are shown in Figure 1d. The QTL on Chr.7 for the combined trait is strong (LOD 5.8, $p<0.05$). While the LOD-score for Chr.4 peak is increased over the QTL peaks for the individual traits, it fails to reach the cutoff for genome-wide significance and remains suggestive. However, the eigenvector approach significantly improved our ability to map these key diabetes-associated traits that individually failed to achieve significance.

Legend for Figure 1: Quantitative trait loci (QTL) for plasma insulin (uIU/g) (a), plasma glucose (mg/dl) (b) and plasma triglycerides (mg/dl) (c) of F2 mice. The QTL for the eigenvector for combined traits of insulin, glucose and triglycerides (d) has a strong peak on chromosome 7 and a suggestive peak on Chr.4. QTL are shown for 5-week cohort (gray) and 12-week cohort (blue) with gender as an interactive covariate. For (d) the LOD scores for genome-wide significance as determined by n=1000 permutations of the genotype data are indicated by horizontal lines with blue for p=0.05 and black for p=0.1.

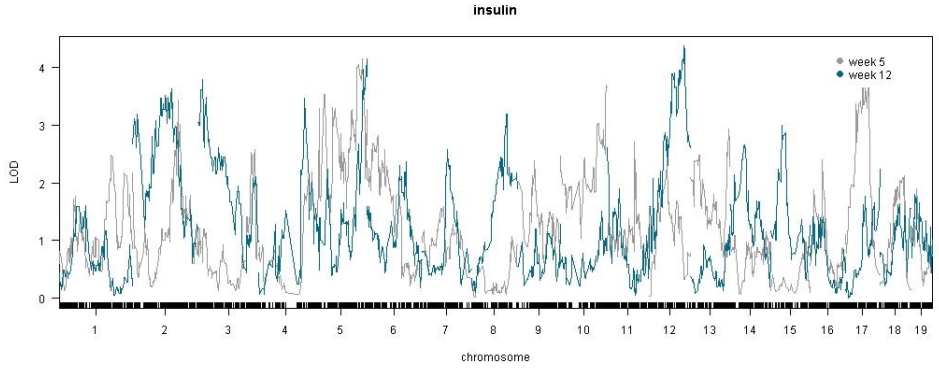


Figure 1A Insulin

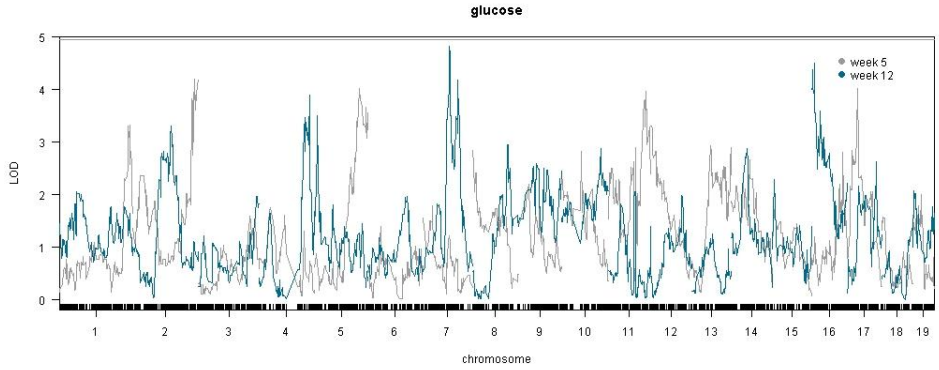


Figure 1B Glucose

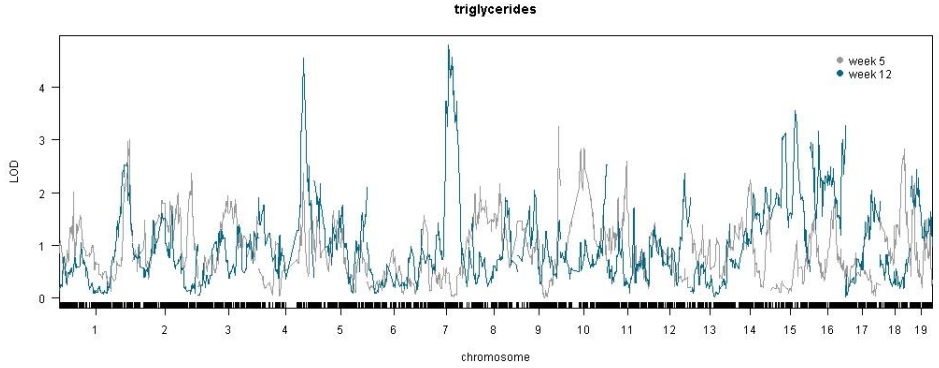


Figure 1C Triglycerides

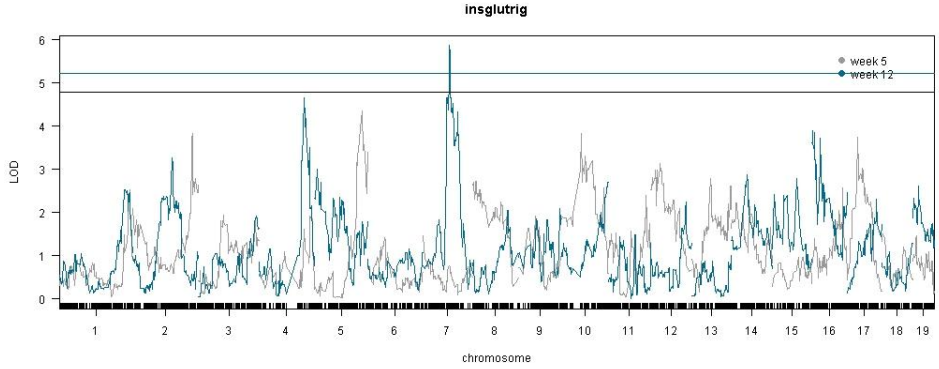


Figure 1D Eigenvector

Because we and others have previously observed a correlation between diabetes susceptibility and hepatic lipid accumulation, we carried out comprehensive gene expression analysis in liver samples from the F2 mice for network analysis and correlation mapping of gene expression and clinical traits. Altogether, expression arrays were performed on total RNA isolated from livers of 221 F2 mice at 5 weeks of age and 218 F2 mice at 12 weeks of age.

One use of this expression data was to identify candidate genes for the diabetes-associated QTLs above. For instance, the peak marker for the chromosome 4 eigenvector QTL occurred at 127Mb. To identify possible genetic variants that might underlie this QTL we looked for cis-eQTLs in the liver expression profiles that coincided with the eigenvector QTL. For this, we selected genes with significant co-localizing eQTL (LOD >4.2) that also lie within 10 Mb of the eigenvector QTL. Significantly, the gene list for the chromosome 4 eigenvector QTL includes the zinc finger domain transcription factor Zfp69 that has previously been associated with the obesity-induced diabetes locus Nidd/SJL.

We carried out a similar search for cis-eQTLs that co-localize with the chromosome 7 eigenvector QTL (See supplemental table ST2b). While there are many strong cis-eQTLs in the region, a literature search for the corresponding genes failed to reveal any previous association with diabetes. Nor are any type 2 diabetes-related QTL identified for this region (See review by Clee and Attie *Endocr Rev* 28: 48-83, 2007). This suggests that the Chr.7 QTL represents a novel diabetes locus that contributes to plasma insulin, glucose and triglyceride levels in the obese mouse.

Gene expression data were further analyzed using weighted gene co-expression network analysis (WGCNA) to identify clusters of genes showing highly correlated expression profiles. Of the 23,000 genes on the arrays, about 15,000 had sufficient signal quality to be used for WGCNA.

Correlation of the each gene module in male F2 *db/db* mice at 12 weeks of age with diabetes-related phenotypes, including plasma glucose, insulin and triglycerides, and bodyweight, liver fat and size of fat depots, is shown in Figure 2a. The individual co-expression modules are identified by an arbitrary color on the left of the figure and the specific clinical traits are identified across the bottom of the figure. Positive correlation between a trait and a gene-module is shown in red, and negative correlation is shown in green, with stronger correlations indicated by increased color intensity.

A few of the modules show strong correlation across numerous relevant phenotypes. For instance the greenyellow module in 12-week males shows strong positive correlation with, plasma cholesterol and with various measures of adiposity as well as with fat in the liver. Weaker positive correlation was seen with plasma insulin. The same module showed weak negative correlation with plasma glucose and triglycerides (Figure 2b). These correlations suggest that genes in this module contribute to, or are driven by, the diabetic state.

Figure 2a

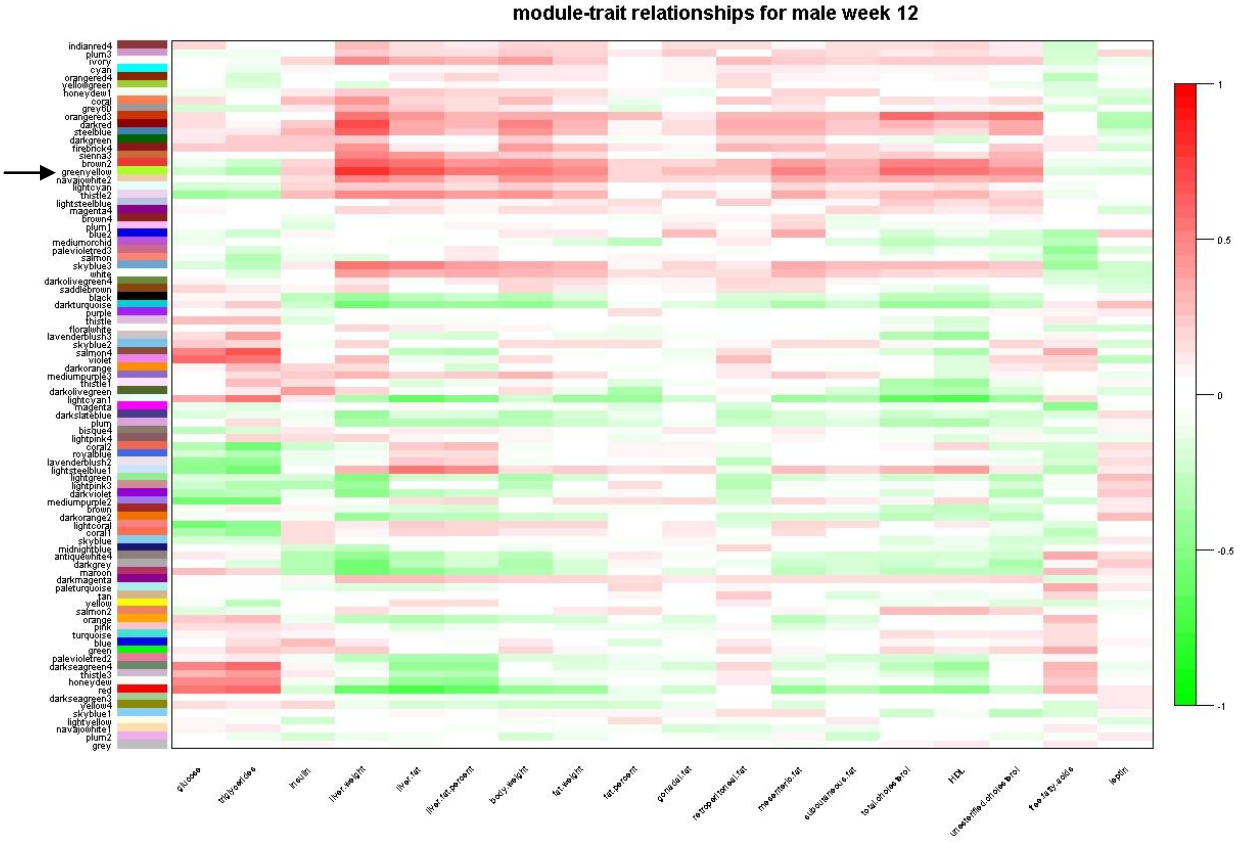
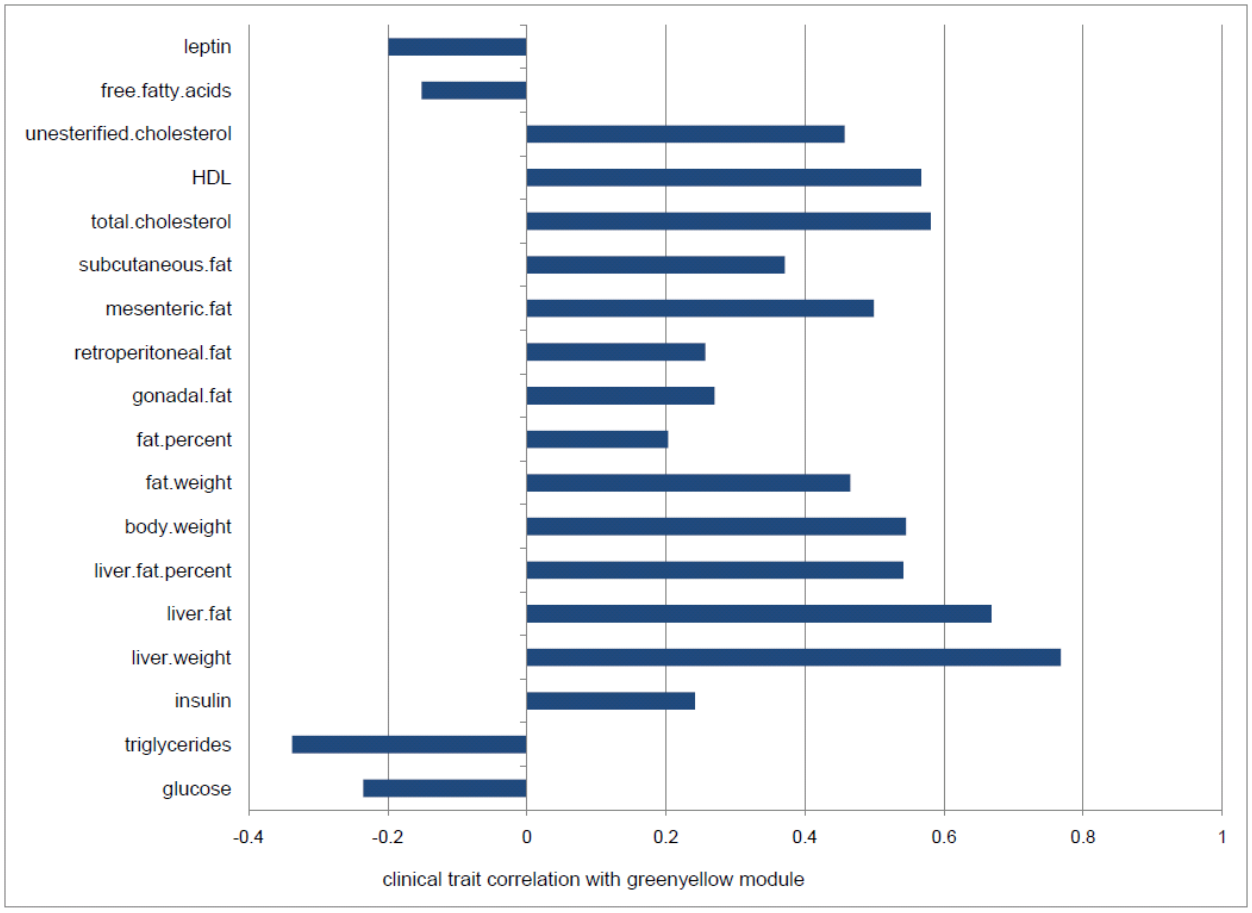


Figure 2b
Correlation of diabetes-related traits in 12-week F2 males with 'greenyellow' coexpression module in liver. The statistical threshold significance for modules correlated with traits was R>0.4 (p<1e-6). The greenyellow module was positively correlated with unesterified cholesterol, HDL, total cholesterol, mesenteric fat, fat weight, body weight, liver fat percent, liver fat and liver weight.



To better characterize these common pathways, we carried out functional annotation clustering analysis using the DAVID platform. Consistent with its correlation to adiposity in the liver and other diabetes-related traits, the greenyellow module for 12-week male liver shows enrichment for lipid biosynthetic processes, glucose catabolism and steroid biosynthesis and metabolism

In order to identify genetic loci associated with the diabetes- and obesity-related greenyellow module for 12-week male liver, we carried out mapping analysis for the eigenvector of expression values for module-genes. Figure 3a shows the resulting LOD plot. For this module, there is a single strong QTL on distal chromosome 12 that appears to coincide with QTL peaks for bodyweight (Fig.3b) and liver fat (Fig 3c).

Figure 3a: Mapping of the eigenvector for genes in the green yellow module for 12-week male F2 animals. Genome-wide significance was determined by measuring the maximum lod score achieved by an equal number of randomly selected genes. The horizontal line represents the LOD-score exceeded by less than 5% of 1000 such randomly selected gene sets. ($p<0.05$).

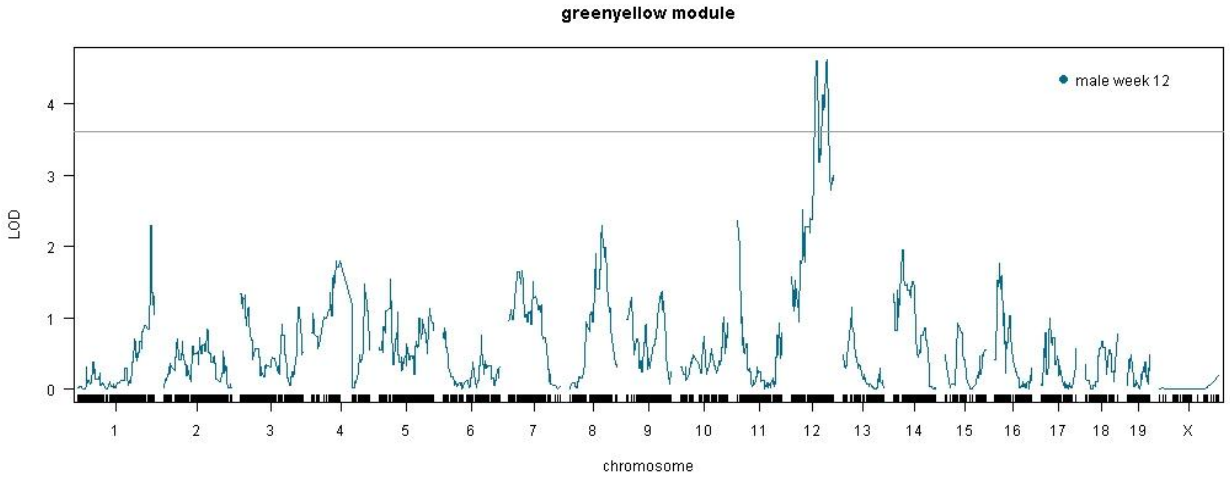
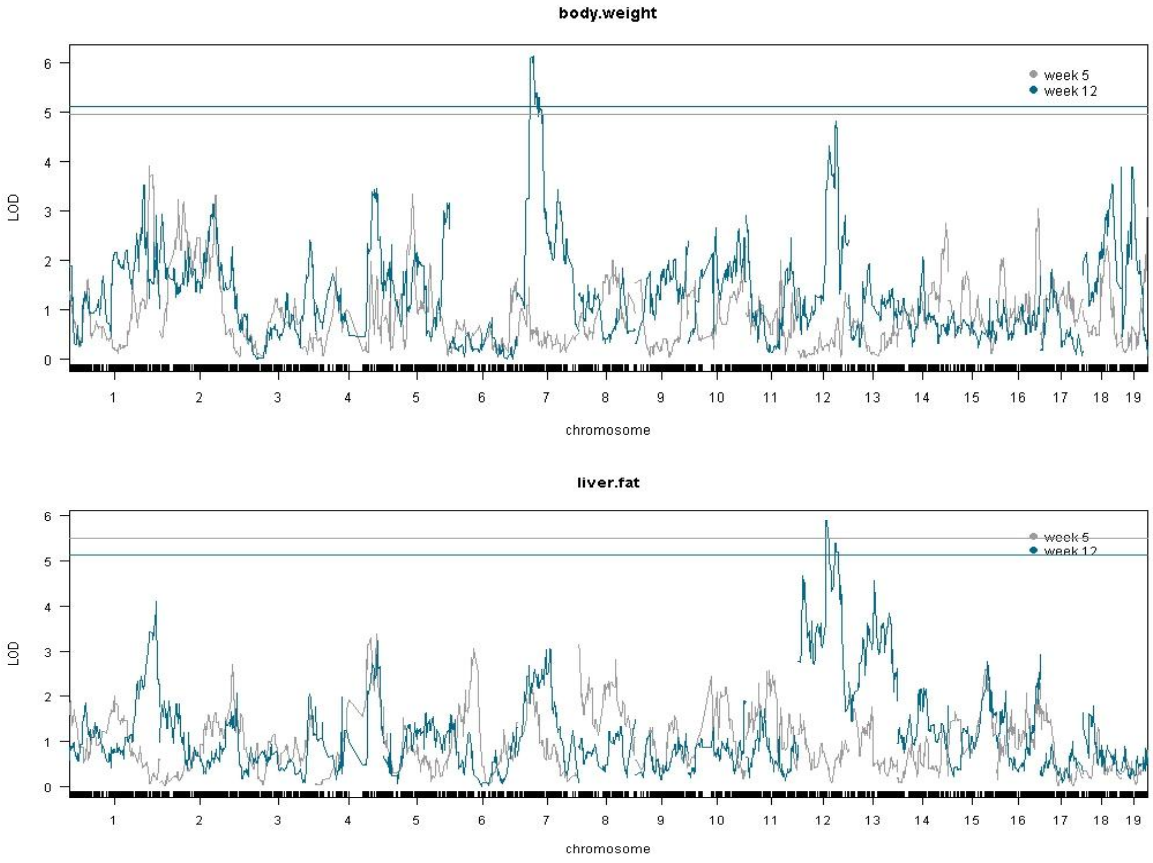


Figure 3b and 3c: Bodyweight and liver fat QTL

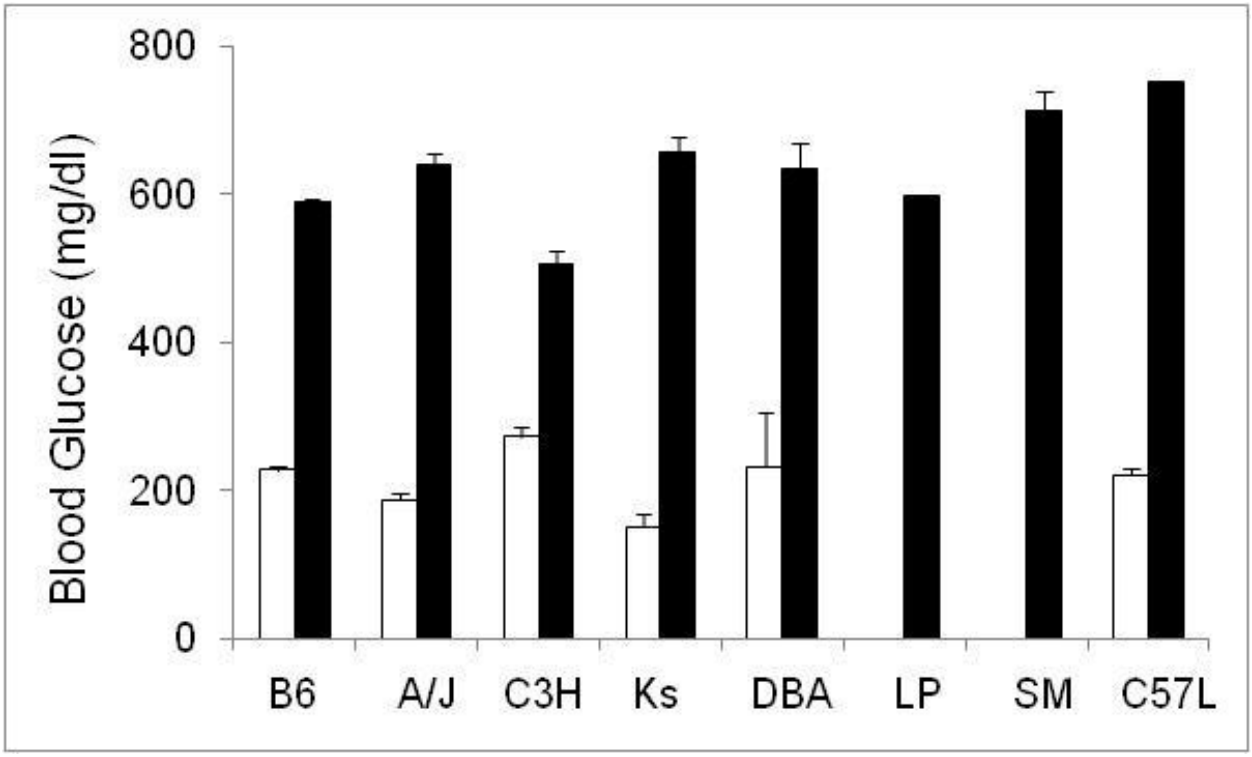


Thus, this locus appears to contain genetic variation that regulates genes in the greenyellow module and likely modulates diabetes-related traits that also map to this locus.

Progress: Mapping diabetes complication susceptibility in F1 Akita mice.

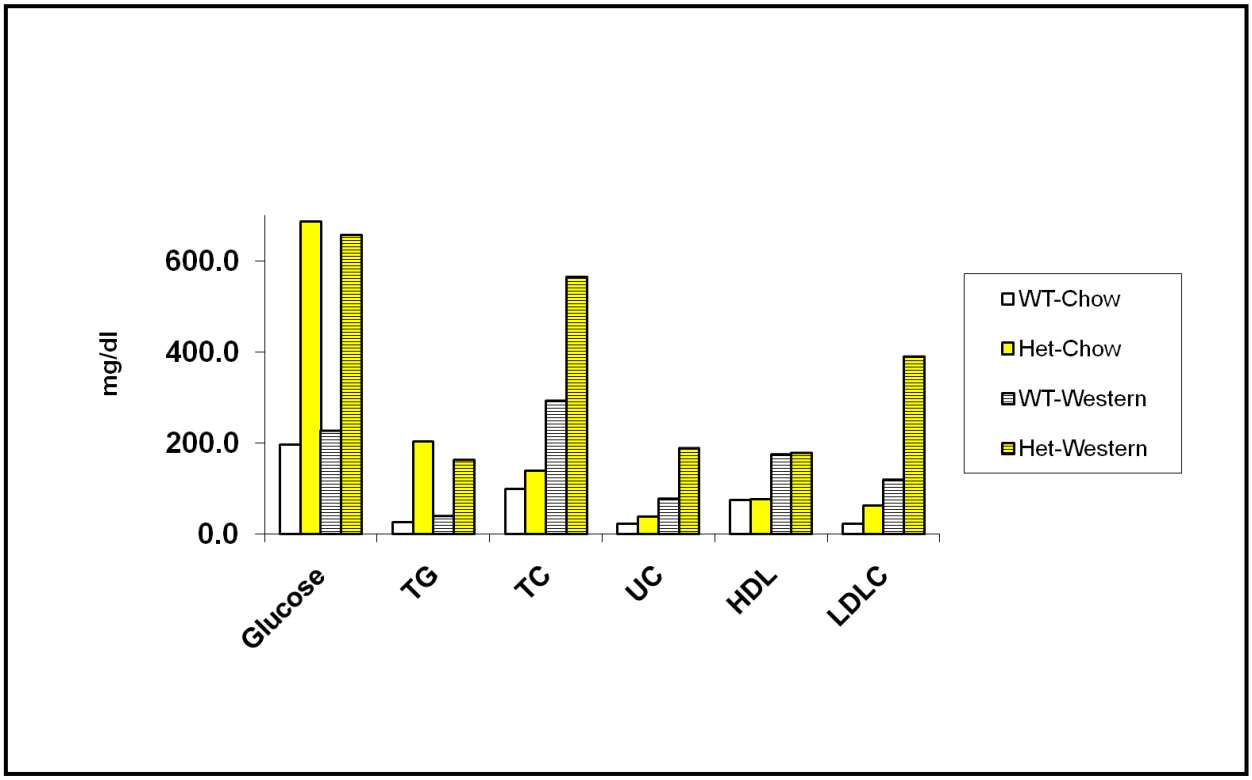
Rationale: Several inbred mouse strains carrying the Akita mutation have been developed at The Jackson Laboratory including C57BL/6, DBA/2 and FVB. Male mice carrying a single copy of the mutation experience apoptosis of beta cells in the pancreatic islets and as a result develop a type 1 diabetic phenotype. While the affected mice are relatively uniform in hyperglycemia, they differ widely in the development complication severity. The most tested phenotype is nephropathy and, among the three common Akita strains, DBA/2 is markedly more affected in terms of ACR and kidney histology. We have been carrying out pilot studies to determine the feasibility of using this system to map genetic loci that determine susceptibility to diabetic complications.

The strategy is to carry out genome wide association to map loci for complication severity in a panel of mouse strains each carrying the Akita mutation to cause diabetes. The diabetic mice are created by mating common inbred strains to a strain carrying the Akita mutation and then measuring complication phenotypes in F1 males inheriting the mutation and also in normo-glycemic littermates.
Preliminary studies have shown that such F1 males are relatively uniform in levels of glycemia among strains as was seen in purebred strains carrying the Akita mutation.

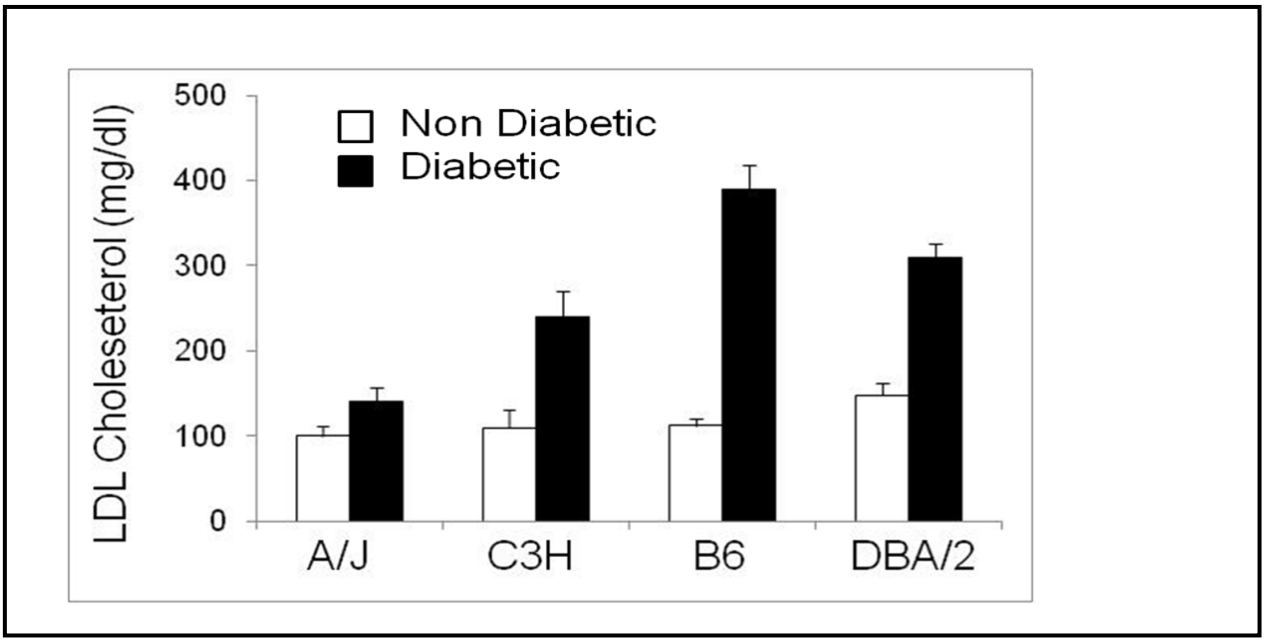


Black bars represent glucose levels in F1 Akita mice vs non-diabetic F1 littermates.
Similarly, glycated hemoglobin levels are relatively uniform across F1 Akita strains.

Because we wanted to assess diabetic atherosclerosis, we also measured the effect of 8 weeks western diet on plasma lipids of C57BL/6 mice carrying the Akita mutation.

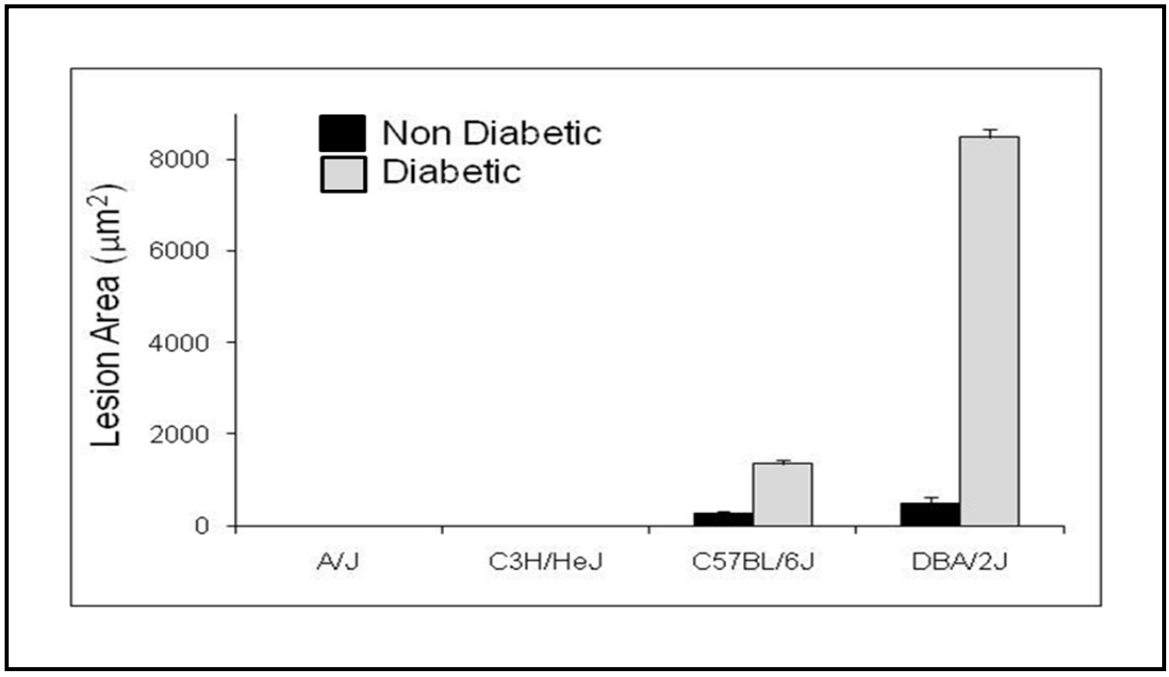


Plasma glucose and triglycerides were dramatically increased by the Akita mutation independent of diet. Conversely HDL cholesterol was increased substantially by Western diet while the Akita mutation had no impact. However, total cholesterol, unesterified cholesterol and LDL cholesterol were increased synergistically by diabetes and diet.



Again, while plasma glucose among F1 males receiving western diet for 8 weeks was relatively uniform across strains (not shown), LDL cholesterol varied widely, depending on genetic background (above).

Moreover, western diet led to small but significant atherosclerotic lesions at levels that were dependent on genetic background and not simply related to glucose or cholesterol levels (see below).



For other diabetic complications, we also saw significant effects of genetic background on complication severity. This includes nephropathy (ACR, blood creatinine and mesangial expansion), neuropathy (Hargreaves test and IENF density) and cardiomyopathy (expression of ANP and BNP, and diastolic function by echocardiography). Generally, the functional and molecular screens for complication severity (ACR, Hargreaves, ANP expression levels) were more sensitive and quantitatively varying across strains than were histological assessments.

The clear impact of genetic background on variations in complication severity make these strong candidates for mapping by the GWAS approach. Moreover, the GWAS approach in mice has been demonstrated to give much higher resolution than traditional genetic crosses and has the additional advantage that replicate animals for each strain may be created to meet the need for statistically significant differences among strains and to simultaneously monitor phenotypes that are incompatible in individual mice or which require collections of tissues at different stages of the animal's life.

Plans for the year and completion of the project:

1) A paper examining systems genetics of diabetes susceptibility with a focus on gene expression in liver is under submission.
2) We will now focus on the genetics of gene expression in pancreatic islets in the BXD F2 db/db cross. Depending on results, this analysis may be combined with 3) below.
3) We have sent data from our BXD F2 db/db cross to be combined with data from Alan Attie's cross of C57BL/6 X BTBR F2 ob/ob. The objective is to increase the power to detect diabetes susceptibility QTL and tissue interactions.
4) Preliminary data for the project to map loci underlying differences in susceptibility to diabetic complications using GWAS in F1 Akita mice is strong and promising. However the full mapping project is ambitious and will require substantial additional funding to complete. We will submit the developing preliminary data as part of our application.
5) The genetic basis of hepatic steatosis and its relationship to diabetes susceptibility is poorly understood. We will begin the genetic mapping of liver fat content and its relationship to diet and insulin sensitivity using the GWAS approach. This is work that can be carried out as an adjunct to other funded research but will have important implications for our understanding of diabetes and its complications.

2. Collaborations:

With other AMDCC PIs: We have been working with Eva Feldman to establish quantitative histological measures of neuropathy in our panel of Akita mice and, she has kindly quantified intra-epidermal nerve fiber (IENF) density in several of our F1 strains. While some animals show clearly reduced nerve-fiber density associated with diabetes, the strength of the response in the few strains we have examined to date has not been sufficiently robust to appear in all the diabetic animals. As with nephropathy (see collaboration with MMPC's below) and with cardiomyopathy, the histological measures for diabetic neuropathy have proven to be less sensitive than functional measures. Input from other AMDCC PIs has been very valuable in the design of pilot studies to examine complication-phenotypes in Akita F1 animals. This includes, in particular, Dale Abel, Chip Brosius and Firouz Daneshgari for cardiomyopathy, nephropathy and uropathy, respectively.

With the MMPCs: We have a strong ongoing collaboration with Renee LeBoeuf and Charlie Alpers at the Seattle MMPC. This has included both analysis of tissues from the BXD F2 db/db cross as well as preliminary data for the examination of complication phenotypes in Akita F1 mice.

With other non-AMDCC PIs: A number of collaborative efforts have evolved at UCLA for measurement of diabetic complications in our Akita F1 panel. This includes Kristina Bostrom for assessment of diabetic vascular calcification and BMP signaling as well as Nigel Maidment and Wendy Walwyn for functional tests of neuropathy (Hargreaves and von Frey tests), Yibin Wang for tests of heart function (echo-cardiography) and W. Robb McClellan for molecular screens of cardiomyopathy.

3. Address previous EAC comments:

- Dr. Davis' group continues to characterize the F2 cross of the C57BLKS and C57BL/6 mice. A noted inverse relationship between glucose and liver fat was noted. As mentioned by Dr. Davis, interpretation of these findings is hampered somewhat by the severe phenotype at this age (e.g. anorexia) so it is difficult to relate them to the human situation. One interpretation that appears to be supported by the data is that liver fat is related to the relative level of insulin.

  RESPONSE: As discussed above, the genetics of diabetes susceptibility in the BXD F2 db/db cross has proven to be extremely complex and, we agree that anorexia, particularly in males at 12 weeks contributes to difficulties in interpretation. However, in females and in males at five weeks anorexia is not major feature but the genetic complexity remains. There does not appear to be a simple relationship between liver

fat and insulin levels.

- They report a QTL for plasma insulin levels on distal Ch5 but a weaker QTL in a similar location for glucose at 5 weeks does not correlate at 12 weeks of age. This may be the result of differences in insulin sensitivity between individual mice. One suggestion would be to look at some other quantitative trait associated with insulin sensitivity such as previously mentioned clamp studies or area under the curve of insulin and/or glucose tolerance tests.

  RESPONSE: We agree that individual differences in insulin sensitivity are of fundamental interest in these mapping studies. And, the suggested measures, clamp studies or area under the curve of insulin and/or glucose tolerance tests, are certainly the gold standard for determination of differences in insulin sensitivity between groups of mice. However, in the context of an F2 cross, there are reasons to approach these particular measures with caution. First, the observed variability, even among genetically identical animals, can be high and will tend to obscure the subtle gradation in insulin sensitivity that is expected to occur across the F2 population. And, since every F2 animal is genetically unique, each clamp or glucose tolerance test would have n=1, a situation that would be fairly discomforting to those who routinely carry out such studies. Still, there are other equally variable endpoints, such as atherosclerotic lesion area, that have been pursued in F2 studies although, in this case, the variability in lesion area is presumed to be almost entirely biological. By contrast, GTT's and particularly clamp studies are very subject to variations in surgical and other handling by the investigator. Our choice was to monitor as many diabetes-related phenotypes as possible, but carefully selecting those screens that could be could be reasonably completed without distorting results of other measures in the panel. On this basis, we chose not to include GTT or clamp studies. And while we agree that clamp and GTT studies would yield valuable information, these would require producing a separate set of F2 animals.

- Gene expression analysis is being used to identify highly correlated gene modules in islet samples and similar analysis will be completed for adipose, intestine, and liver. These novel subnetworks will then be used in gene set enrichment analysis to aid in the identification specific genes/pathways that are associated with diabetes. The constructed gene networks should be compared to some that were already published to determine whether these are universally applicable. Cross tissue analysis will also be interesting.

  RESPONSE: We agree and have established a collaborative effort to pursue these issues with Alan Attie who carried out a similar cross with C57BL/6 X BTBR F2 ob/ob mice. Our idea is compare networks in the two crosses both within tissues and between tissues. Moreover, by combining the expression data from the two crosses, we hope to increase the power to detect diabetes susceptibility loci. Analysis of the combined data is

underway.

- P&F project continues with phenotyping done at the UW MMPC. Are there thoughts on how the QTLs will be identified? For example, for the kidney histology, will a general score be used? The other kidney phenotypes (BUN, etc.) and atherosclerosis are somewhat more obvious.

  RESPONSE: We have been looking at quantitative measures of nephropathy (for instance, percent mesangial matrix/glomerular tuft area) for use in QTL analysis. These may prove satisfactory but, experience to date suggests these morphological features may be less satisfactory because they become apparent mostly in the severely affected mice. Other indicators such as BUN and ACR are more sensitive indicators of early disease that are more widely variable across the entire F2 population.

- The website is set-up to display microarray data. Has all of the data from your P&F and primary award been uploaded to the site?

  RESPONSE: We have uploaded the major phenotypic data. We have the micro-array data tables ready to upload and are awaiting some corrections to the AMDCC template to complete this process. We will continue to work with Rick McIndoe to ensure that this happens.

- The same issues identified last year are operant this year. Dr. Davis continues to generate data, identifying QTLs for insulin and body fat and generating tissue transcriptomes, which have been analyzed by weighted gene co-expression network analysis (WGCNA) to identify sets of genes whose expression is highly correlated. The progress report does not include conclusions from experiments to date (e.g. do any of the expression QTLs map to the phenotypic QTLs) or discuss the work to be accomplished in the upcoming year. While the approach was viewed with enthusiasm, we are concerned that the robust amounts of data and analysis have not resulted in more specific conclusions/hypotheses. Nor has the data been interpreted in light of human genetic data about glucose levels, insulin function or BMI.

  RESPONSE: A summary of results and interpretation is given in the progress report, above.

- The goal of this project is to identify susceptibility genes and novel gene pathways in diabetic mice. Dr. Davis is uniquely positioned to perform the proposed goals. The PI has made excellent progress during the last year, and has begun to examine the F2 animals. There has been extensive collaboration with Drs. Renee LeBoeuf and Charlie Alpers to examine nephropathy in these animals. The PI has also measured atherosclerosis in the F2 animals and found that lesion area was correlated with plasma glucose levels, not necessarily hyperlipidemia, which is an important finding.

RESPONSE: The preliminary screen of atherosclerosis in a subset of the F2 animals showed lesion area that was correlated with glucose levels and not with hyperlipidemia. Unfortunately, lesions, when present, were very small and, when the whole F2 set was examined, very few animals had lesions at all, thus thwarting our effort to map the underlying loci. The Akita F1 mice show much more promise in this regard. For these pilot animals we supplied a western diet and observed more substantial lesions, particularly in the DBA F1. Again, these early lesions show no simple relationship to plasma lipids and, given the relatively uniform glycemia, suggests independent determinants of lesion initiation. While we do not yet know what fraction of F1 strains will show robust initial lesions, the presence of significant differences among parental strains for the major RI strain sets strongly supports that we will be able to map QTL for this diabetes driven atherosclerotic phenotype.

- The response to the prior EAC comments seems adequate at best; our prior questions about TG synthesis genes were not answered in the report, nor were the 5-LO questions. Finally, the prioritization questions were only vaguely addressed, with no real answer provided. In any case, the PI has made progress and continues to be on track for completion of the project goals. .

  RESPONSE: The 5LO-experments were terminated because of breeding difficulties. The analysis of TG-synthesis genes is further addressed in the progress report above. More specific prioritization of objectives for completion of the studies also listed.

- Below is a list of your AMDCC publications from the website. Should any publications be added or subtracted? Has all of the relevant data from these publications been uploaded to the website? Please work with Dr. Rick McIndoe to ensure that the website and database are up-to-date and complete.

  RESPONSE: As described above, we are working with Rick McIndoe to upload all relevant data. The following publications should be added:

1. Bhasin KK, Van Nas A, Martin LJ, Davis RC, Devaskar SU, Lusis AJ. Maternal low-protein diet or hypercholesterolemia reduces circulating essential amino acids and leads to intrauterine growth restriction. Diabetes. 2009;58(3):559-66. PMCID: 19073773.
2. Kopf D, Cheng LS, Blandau P, Hsueh W, Raffel LJ, Buchanan TA, et al. Association of insulin sensitivity and glucose tolerance with the c.825C>T variant of the G protein beta-3 subunit gene. J Diabetes Complications. 2008;22(3):205-9. PMCID: 18413224.
3. Davis RC, Castellani LW, Hosseini M, Ben-Zeev O, Mao HZ, Weinstein MM, et al. Early hepatic insulin resistance precedes the onset of diabetes in obese C57BLKS-db/db mice. Diabetes. 2010;59(7):1616-25. PMCID: 20393148.
4. Davis RC, Van Nas A, Castellani LW, Zhao Y, Zhou Z, Wen PZ, et al. Systems Genetics of Susceptibility to Obesity-Induced Diabetes in Mice. Submitted 2011.