Animal Models of Diabetic Complications Consortium
(R01)

Annual Report
(2005)

Animal Models of Diabetic Complications Consortium

Title of Project – R01 HL 069364 Atherosclerosis in Insulin Resistant Hyperlipidemic Pigs

UPDATE REPORT
(September 1, 2001 – February 24, 2006)

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Co-P.I. Timothy C. Nichols, M.D.

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Part A:

Principal Investigator's Summary

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1. Program Accomplishments:

The overall goal of our grant is to produce pigs that have an atherogenic risk factor profile resembling patients with insulin resistance (IR) and hyperlipidemia that will develop coronary, abdominal aortic, and carotid atherosclerosis. Our main strategy is to utilize two strains of pigs available at the University of North Carolina. One strain has traits that predispose it to insulin resistance (IR) and the other has familial hypercholesterolemia (FH). Control strains with normal lipids (NL) and normal insulin sensitivity (IS) are also available. Our breeding program utilizes animals from these groups to develop two new phenotypes: one with more severe IR and one with FH plus IR. As we originally estimated, at least 4 generations of animals have been produced and successfully phenotyped during the first 4 years of this project. All offspring that survived to puberty (~9 months old in pigs) underwent characterization of their degree of insulin resistance and abnormalities in lipid metabolism.

Our major achievement to date has been to produce pigs with one of the following four phenotypes: NL/IS, and NL/IR and downsized (DS-) FH/IS and FH/IR pigs. The NL pigs develop hypercholesterolemia when fed a high fat diet. The DS-FH pigs exhibit hypercholesterolemia while being fed low fat pig chow along with elevated triglycerides and depressed HDL cholesterol. Our most important preliminary finding is that pigs with the IR trait develop more severe and importantly more diffuse coronary and aortic atherosclerosis. We have now entered nine NL/IS and nine NL/IR pigs into a yearlong study to confirm these preliminary findings. The number of pigs was chosen based on statistical power analyses from our preliminary findings. We anticipate completing the yearlong study in Jan 2007. We have not been successful in creating an IR strain in the regular sized FH pigs, possibly due to the fact that these pigs have been selected for leanness. To address this issue, we are actively breeding the downsized FH pigs for a yearlong study to begin in Feb 2007. The downsized FH pigs have two advantages: (1) they are ~40% smaller than the NL pigs and (2) the cost of low fat pig chow is significantly less expensive than high fat pig chow. They also have more fat back than the regular size FH pigs. The goal of our proposed studies is to validate the usefulness of these pigs to investigators who are attempting to identify genes that predispose to the development of insulin resistance and to determine the pathophysiological factors that link insulin resistance and atherosclerosis in coronary and carotid arteries and the abdominal aorta. Accordingly, our long-range goal is to exploit new insights into the mechanism(s) by which IR alters atherogenesis and thereby develop and test novel treatments for the growing epidemic of type II diabetes in children and adults and the associated cardiovascular disease.

2. Collaboration within your group: Describe interrelationship between projects

Not applicable.

3.

Collaboration with other AMDCC groups:

Tissues from the pigs with the four phenotypes are being distributed to the investigators listed on the table.

INVESTIGATOR	INSTITUTION	SAMPLE	GOAL
Dale Able Don McClean	Utah	Myocardium	Biochemistry and EM mitochondrial analyses
Tim Kern	Case Western	Eyes	Retinal vessel analyses
Firousz Daneshgari	Cleveland Clinic	Bladder	Physiological analyses
Eva Feldman	Univ of Michigan	Neurological tissues (skin biopsies, sciatic nerve, CNS)	Biochemical and microscopic analyses
Eva Feldman	Univ of Michigan	EDTA plasma	Screen for ROS

4. Pertinent non-AMDCC Collaboration:

Charles Jeannette	UNC	Urine and kidneys	Urine protein, glomeruli & other vessel analyses

5. Address previous EAC comments:

Please include both the EAC comment and your response

- The CV working group is strongly encouraged to publish a position paper regarding validation criteria and strain differences/susceptibility.

Our group was asked to write a peer reviewed paper for the *ILAR Journal* on using pigs as models of type 2 diabetes mellitus with emphasis on cardiovascular disease including validation criteria and strain differences. The manuscript has now been accepted after revision as requested by peer review. Publication is anticipated in June 2006.

1. Bellinger DA, Merricks EP, Nichols TC. Swine models of type 2 diabetes mellitus: Insulin resistance, glucose tolerance, and cardiovascular complications. ILAR J, accepted, 51 pages.

Animal Models of Diabetic Complications Consortium
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Part B:

Update by Project Leaders

Project 1: “R01 HL 069364 Atherosclerosis in Insulin Resistant Hyperlipidemic Pigs”

Responsible Investigators: David Clemmons and Timothy C. Nichols

1. Rationale and Relevance:

This grant was written in response to RFA HL-01-010 entitled "Non-mouse models of diabetes complications in cardiovascular and microvascular diseases." The RFA stated: "The purpose of this solicitation is to support efforts to develop non-mouse animal models of diabetic complications. The animal models are expected to mimic vascular diseases in patients with type 1 or type 2 diabetes mellitus with an emphasis on, but not limited to, cardiovascular disorders of coronary heart disease, stroke, peripheral arterial disease, cardiomyopathy, and congestive heart failure. Improved animal models of microvascular complications are also needed. The goal of this initiative is to obtain these non-mouse animal models, through the use of selective breeding, dietary manipulation, or molecular genetic approaches. Applicants to this initiative are also expected to characterize and validate the models for use in various aspects of basic, developmental, or translational research including testing prevention, early detection, therapeutic, or diagnostic imaging strategies. Applicants should also propose plans to make these models available to other research investigators for studies to advance our understanding of the etiology, pathobiology, clinical progression, management and prevention of diabetic vascular diseases."

In response to the RFA, the major purpose of our grant is to produce pigs that have an atherogenic risk factor profile resembling patients with insulin resistance (IR) and hyperlipidemia that will develop coronary, abdominal aortic, and carotid atherosclerosis. We proposed to utilize two strains of pigs available at the University of North Carolina. One strain has traits that predispose it to insulin resistance (IR) and the other has familial hypercholesterolemia (FH). Control strains with normal lipids (NL) and normal insulin sensitivity (IS) are also available. Our breeding program utilized animals from these groups to develop two new phenotypes: one with more severe IR and one with FH plus IR. As we originally estimated, at least 4 generations of animals have been produced and successfully phenotyped during the first 4 years of this project. All offspring that survived to puberty (~9 months old in pigs) underwent characterization of their degree of insulin resistance and abnormalities in lipid metabolism. The end result has been to produce pigs with one of the following four phenotypes: downsized-FH/IS, downsized-FH/IR, NL/IS, and NL/IR. Pigs with all phenotypes are being entered into our proposed studies to validate their usefulness to investigators who are attempting to identify genes that predispose to the development of insulin resistance and to determine the pathophysiological factors that link insulin resistance and atherosclerosis in coronary and carotid arteries and the abdominal aorta. Accordingly, our long-range goal is to exploit new insights into the mechanism(s) by which IR alters atherogenesis and thereby develop and test novel treatments for the growing epidemic of type II diabetes in children and adults and the associated cardiovascular disease.[4,5]

2. Summary of Accomplishments:

Aim I. Develop two strains of pigs by selective breeding: Strain #1 will possess insulin resistance, strain #2 will be a cross between insulin resistant pigs and animals with familial hypercholesterolemia.

1. Identification of IR pigs by Oral Feeding and 3 point sampling. IS and IR pigs were initially identified based on insulin (RIA, ICN) and glucose (2300 STAT PLUS, YSI, Yellow Springs, Ohio) levels measured after an overnight fast and 1 and 2 hours after a normal meal (35 kcal/kg/day via once daily feeding), and insulin to glucose ratios. Our rationale for using this approach is based on reports that various strains of pigs have greater glucose tolerance after an oral glucose load than is found in humans.[6] Pigs are tested when they are postpubertal pigs (> 8 months) since humans rarely exhibit DM prior to puberty.

At the start of this grant in 09/01, we had 6 founder hyperinsulinemic pigs (average serum insulin 17.9 +

7.1 IU/ml). At present (01/06), we have produced four generations of pigs as we had projected and performed 241 three point tests on 178 post pubertal pigs. By selectively breeding pigs we have identified as hyperinsulinemic in the three point oral feeding test, we were able to obtain an ~37% increase in fasting hyperinsulinemic pigs (NL – 2.2 ± 7.1 n = 7, and FH 24.8 ± 9.2, n = 5). Of equal importance we have preserved pigs that have normal fasting and post prandial insulin levels and thus are insulin sensitive so that they are available for comparisons. These "normal insulinemic" or insulin sensitive pigs have mean fasting levels of 11.1 to 14.2 (**Table 1**). It is also worth noting that even among the 11 hyperinsulinemic animals, there is heterogeneity and several of these animals have an even more severe phenotype with mean two-hour postprandial insulin levels greater than 100 µU/ml. Thus our selective breeding strategy of mating hyperinsulinemic pairs may result in a further increase IR in the future.

At present, we do not know the inheritance pattern of hyperinsulinemia in our pigs. Based on our experience to date, we estimate conservatively that approximately 25% of a given litter from hyperinsulinemic parents will exhibit hyperinsulinemia as adults. Our current breeding strategy accounts for this level of phenotypic expression of hyperinsulinemia. Most (>80%) of the pigs born to parents with normal insulin levels also have normal insulin levels. The remaining offspring usually have hyperinsulinemia of intermediate severity

Table 1. Phenotype of Proven Breeder FH & NL & Ossabaw pigs with or without IR at UNC

		Cholesterol	Serum Insulin level (µU/ml)		
Pig Phenotype	**Gender + n**	**(mg/dl)**	**fasting**	**1hr**	**2hr**
1. FH/IS	3M/2F	572.5 ± 99.7	11.1 ± 2.9	15.9 ± 2.4	13.0 ± 2.4
2. FH/IR	3M/2F	494 ± 78.5	24.8 ± 9.2	74.5 ± 30.4	52.3 ± 24.4
3. NL/IS	2M/2F	119.5 ± 28.8	7.9 ± 2.6	15.1 ± 2.2	14.5 ± 4.4
4. NL/IR	5M/2F	109.7 ± 25.8	22.2 ± 8.2	80 ± 68.4	44.8 ± 30.2
5. Ossabaw*	2M/3F	65.6 ± 4.9	14.2 ± 2.6	75.7 ± 46.2	32.6 ± 15.3

*The Ossabaw pigs exhibit an NL/IR phenotype but are 75 to 100 kg vs. 200+ kg.

2. Measurement of insulin resistance (Si) by Bergman Frequently Sample Insulin Glucose Tolerance Test (FSIGT).

All pigs undergo formal Bergman testing upon entry into the yearlong study.[7] Of note, various strains of pigs have been reported to clear intravenous glucose loads more efficiently than humans and, therefore, absolute S_i values may not be directly comparable between the two species.[6] Hyperinsulinemic pigs have exhibited a mean S_i of 3.3 ± 0.45 (n = 5) and thus defined as IR as compared to pigs with normal insulin levels that exhibited a mean S_i of 4.6 ± 0.5 (n = 6) and are thus defined as IS. We have used this data to define Insulin Resistance as an S_i value of less than 4.0 by the Bergman Frequently Sampled Insulin Glucose Tolerance Test.[7]

3. Insulin Resistant Normolipidemic (NL) and Familial Hypercholesterolemic (FH) Pigs.

Two unique strains of pigs have been used in our studies. The first strain, normolipidemic pigs (NL) from the Chapel Hill colony, has increased backfat and by using our selective breeding we were able to obtain two subgroups: one with insulin resistance (NL/IR) and the second with insulin sensitivity (NL/IS). The normolipidemic pigs were made hypercholesterolemic by feeding a high fat diet thus allowing us to determine the effect of feeding this diet on insulin resistance and on atherosclerotic lesion development.

The second strain has familial hypercholesterolemia (FH) and exhibit a spontaneous hypercholesterolemia while ingesting low fat pig chow as well as a leaner body habitus. We have produced and entered 6 regular size FH/IS pigs into our yearlong study during which they had a stable S_i (see item 4 below). We have not produced sufficient numbers of regular size FH/IR pigs to complete this study. To address this issue, we are actively breeding these regular size FH pigs with mates that have produced IR pigs and we also acquired a third strain of pigs that had been derived from a cross between FH pigs and "pot-bellied" pigs that are termed "downsized FH pigs (DS-FH)." These DS-FH pigs are ~40% smaller but have larger amounts of backfat when compared to regular sized FH pigs. We screened 28 of these DS-FH pigs that were prepared by Dr. Rapacz in Wisconsin and identified 10 that were IR then transported them to Chapel Hill. These DS-FH pigs have been successfully bred and ~25% of the progeny also appear to be IR based on fasting, 1 and 2 hour post prandial hyperinsulinemia. A major goal for this year is to produce sufficient numbers of these DS-FH/IS and DS-FH/IR pigs to complete a year long study that will be similar in design to the studies with NL pigs. In addition to

providing an essential control for the effects of feeding a high fat diet, these FH pigs are less expensive to feed since they only receive low fat pig chow (~$1 vs $7/day) and the DS-FH strain is a more practical size for use by the scientific community.

4. Effect of High Fat Diet on Bergman S_i and Fasting Glucose Levels.

Six NL pigs who had S_i values that were <4.0 have been entered into the study. After one year four had a decrease in S_I; one has had a stable S_i; one had an increasing S_i. Except for that animal, 69G, the S_i varied inversely with weight and backfat once the pigs were fed the high fat diet. Conversely, S_I increases in pigs when they lose weight (5 to 10%). Thus we believe the Bergman methodology results in an accurate prediction of changes in the insulin sensitivity index for pigs that have been entered in this study. More importantly, the fluctuations in S_i that are noted on the high fat diet reinforce the need to repeat the S_i during the study period to accurately correlate insulin resistance with the development of atherosclerosis. In contrast as mentioned above, 6 regular size FH pigs that started the study with similar S_i values but who were placed on a low fat diet had no significant changes during the 12 month study: Baseline, 4.8 ± 0.6; 3 months, 4.5 ± 0.5; 6 months, 4.5 ± 6.2, and 12 months, 4.5 ± 0.5. A second unexpected finding is the emergence of fasting hyperglycemia that was noted in 3 of 6 in the NL pigs. In contrast, to date we have not detected hyperglycemia in any of the 6 FH pigs. These findings suggest that the NL animals reproduce to some extent the changes that occur in humans in response to feeding a high fat diet.

Summary of Aim 1 Accomplishments:

1. Our breeding program has shown that we can create pigs with progressive increases in IR phenotype by selective breeding of hyperinsulinemic pigs in the NL and downsize FH backgrounds.
2. Selective breeding of pigs with normal insulin levels produces progeny with normal insulin levels in both the NL and downsized FH backgrounds.
3. Proven breeders for all 4 phenotypes have been identified.
4. Ossabaw pigs exhibit significant hyperinsulinemia in a normolipid background and are small.
6. The Bergman FISGT appears to be a valid measure of insulin sensitivity and resistance in these pigs since it segregates IS and IR pigs and is sensitive to variables that are known to alter insulin resistance (e.g., changes in body weight).
6. Feeding the high fat diet to NL pigs has caused worsening of the S_i in some pigs.
7. During the past year, some NL/IR pigs have exhibited fasting hyperglycemia with blood sugars ranging from 115 to 165, suggesting that a diabetes mellitus phenotype is emerging in the IR background.

Aim II. To characterize the time course of lesion development over one year and relate these changes to changes in insulin sensitivity and lipoproteins in 4 groups of animals.

1. Effect of IR on the severity and diffuseness of coronary and aortic atherosclerosis. The studies in this specific aim will be the most important in the grant because they will validate the usefulness of the NL/IR and downsized FH/IR pigs and the feasibility of using these animal models to analyze the relationship between the presence of insulin resistance and the development of atherosclerosis. The primary objective is to determine if IR pigs have differences in the development of atherosclerotic lesions and changes in markers of atherosclerosis compared to IS animals.

Cardiovascular complications due to diabetes mellitus generally take years to develop in humans. Therefore, we designed our experiments to be of at least one-year duration in post pubertal pigs. These data, however, will be essential to determine if future mechanistic and intervention studies could be done in a shorter time frame and thus with less expense.

We have entered 9 NL/IR and 9 NL/IS animals into this year long study and we anticipate completing this study at the end of 01/07 (Aim II, Exp 1). Our most important finding to date is that the IR pigs develop more severe and diffuse atherosclerosis than IS pigs in the NL and DS-FH backgrounds. The number of pigs that have undergone necropsy is too few for formal statistical analyses but the trend for IR pigs to have more severe and diffuse atherosclerosis is present in all animals that have been studied to date. In addition, complicated lesions with hemorrhage in coronary atherosclerotic plaques have been noted in DS-FH/IR pigs (**Fig 2**). Similar pathology was found in streptozotocin treated pigs fed a high fat diet.[8]

2. Systemic Arterial Blood Pressure in Pigs - Effect of IR.

In a small cross section analysis of our herd including pigs of ages from 1 to 5 years old, our downsized FH/IR pigs had a higher systolic and diastolic blood pressure compared to IS controls when measured in the pigs tail by a validated method (Dynamap, **Table 2**).[2,3,9] This was an unexpected finding. The NL/IR and NL/IS animals had similar blood pressure while ingesting low fat chow. At present, the number of pigs is too small to determine if these differences are statistically significant. Also, we do not know if the blood pressure is different in our experimental pigs and whether or not it varies during the course of the 1-year study especially when the NL pigs will be eating a high fat diet. The severity and time course and progression of this difference in blood pressures in these animals needs to be completely characterized, that is whether the animals have higher blood pressure at baseline in the immediate postpubertal state or when in the course of the natural history of their disease they develop higher blood pressure needs to be determined. Likewise the rate of progression of severity of these blood pressure differences needs to be documented. Our analyses will correlate blood pressure with our primary endpoints of severity and diffuseness of atherosclerosis.

Table 2. Pig Tail Arterial Blood Pressure in Pigs by Phenotype

Phenotype	n	Systolic	Diastolic	MAP	Pulse
NL/IS	5	154.61 ± 30.72	99.37 ± 26.41	122.52 ± 27.78	90.48 ± 19.31
NL/IR	4	153.24 ± 22.22	104.57 ± 19.90	123.14 ± 20.57	98.75 ± 14.65
DS-FH/IS	4	146.83 ± 27.91	93.70 ± 21.85	112.79 ± 25.34	95.97 ± 25.33
DS-FH/IR	2	168.79 ± 23.56	115.05 ± 26.64	136.71 ± 29.58	91.86 ± 15.38

3. Validation of Measuring Pig Lipoproteins by Nuclear Magnetic Resonance (NMR).

The use of physicochemical methods to measure lipoprotein concentrations is time consuming and labor intensive, and, while the concentration of lipoproteins can be determined by these methods, the lipoprotein particle size and particle number cannot be determined. Increasing evidence suggests that such measurements are of particular importance in IR humans.[5,10-12] We collaborated with Jim Otvos, Ph.D., LipoScience, Research Triangle Park, NC, to develop validated NMR methods to measure pig lipoprotein particle sizes and concentrations, thus greatly facilitating our studies and broadening their relevance to IR humans. Results of the NMR determined HDL concentrations and sizes using pig plasma yielded a high degree of correlation with concentrations that were measured by traditional methods (**Fig 1)**. Comparable studies validated measurement of pig LDL and showed a high degree of correlation.

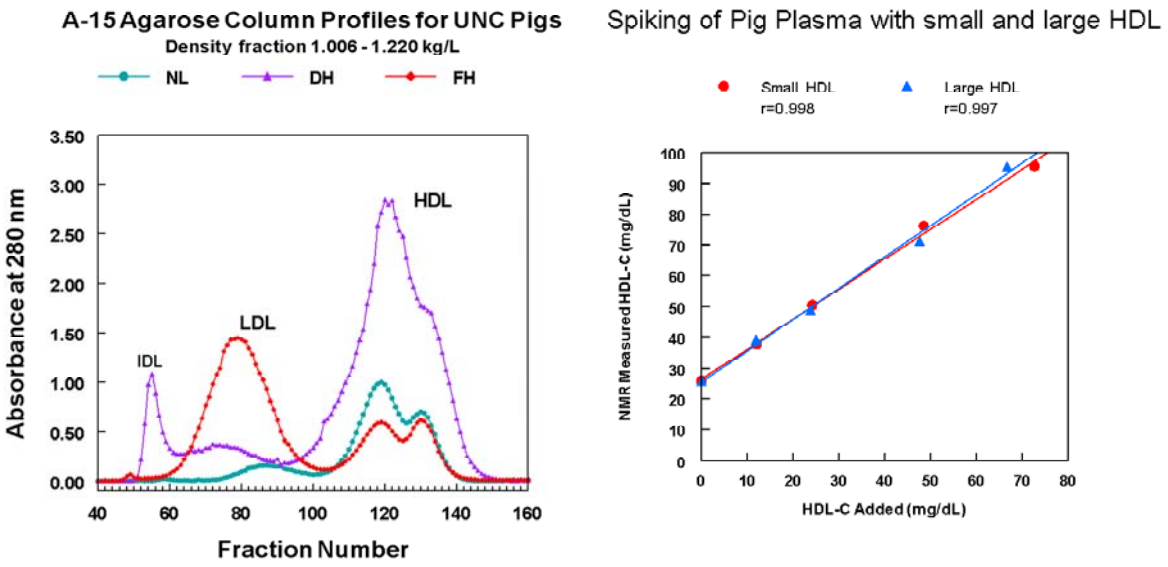

Fig 1. Validation of NMR Measurement of NL and FH Pig HDLs. Purified lipoprotein components were generated from NL/IS fed normal low fat pig chow or high fat diet, FH/IS and FH/IR pig plasmas using a combination of ultracentrifugation and agarose column chromatography (Left Panel). The components were then characterized concurrently by NMR and chemical analysis. Two separate models were developed to

adjust for subtle differences between human and pig NMR lipoprotein signals: one for NL and FH pigs fed low fat chow (pig-II model), and the other for NL pigs fed high fat diet (pig-high fat diet model). The pig-II model incorporates 7 VLDL, 10 LDL and 11 HDL subcomponents isolated from pigs whereas the pig-diet model consists of 7 VLDL, 8 LDL and 14 HDL subcomponents. To verify that these models correctly classify the quantity and size of HDL subclasses, a standard spiking or addition study was performed to optimize these computer algorithms. Two HDL subclasses isolated from pig plasma representing small HDL (average size 8.7 nm) and large HDL (average size 13.0 nm) were concentrated. Plasma samples from a NL pig were then spiked by addition of increasing amounts of the small and large HDL stock solutions and the NMR LipoProfile analyses were done in the usual manner. A high degree of correlation was obtained (Right Panel).

4. Femoral Artery Ultrasound - Measurement of Atherosclerosis Progression.

At present, we do not know if the increased severity and diffuseness of atherosclerosis described in the IR pigs below is due to a faster rate of disease progression or not. We had originally proposed to monitor atherogenesis in these pigs by intravascular ultrasound (IVUS). However, transcutaneous femoral artery ultrasound was chosen over IVUS for three reasons. First, it is easy to repeat frequently in the pig's pen without requiring vascular access in a sterile operating room. Second, although non-invasive ultrasound is known to have mechanical bioeffects and can cause some subtle, transient vascular cell deformation, it does not disrupt the artery as does the invasive IVUS procedure.[13] Third, the expected rate of change is 0.0147 mm/yr in humans without known arterial disease and higher in patients with vascular disease who have a degree of atherogenic stimulus that is comparable to our FH and IR pigs.[14] Our ultrasound is identical to that used in these studies and thus can detect this level of change. Of note, in humans the carotid artery is often assessed by ultrasound. We chose the femoral artery because the porcine carotid artery is more deeply located than in humans and thus requires a lower frequency transducer that achieves greater penetration but less resolution. The femoral artery in the pig is more superficial and thus can be imaged with a high-frequency transducer that produces superior resolution of intima-media thickness. Moreover, recent reports have suggested that data that are equivalent to changes in the carotids can be obtained from lower extremity vessels in normals and diabetics.[15] Thus transcutaneous ultrasound appears to be a reproducible and less invasive alternative to the originally proposed intravascular ultrasound (IVUS) for determining the rate of atherosclerosis progression between insulin resistant and insulin sensitive pigs (i.e., rate of change of femoral intima-media thickness as well as plaque number and volume and percent stenosis). From these data, we are determining if there is a differential rate of atherosclerotic lesion progression between IS and IR pigs during the 1-year study.

We have obtained data in three pigs over a 3 month interval showing progression of femoral atherosclerosis using transcutaneous ultrasound (**Fig 3**). Whether these changes can or cannot be reproducibly quantified and the extent to which they can be quantified as well as the precision of the quantification and the rate of change need to be determined for femoral arteries in at least 8-10 animals. These results need to be compared to control hypercholesterolemic animals that are insulin sensitive.

We are also beginning to measure arterial mechanical properties during atherogenesis by Acoustic Radiation Force Impulse (ARFI, publication 2). This information will allow us to compare changes in arterial mechanical properties between IS and IR pigs.

Summary of Aim 2. Accomplishments:

1. Atherosclerosis study is in progress with NL/IS (n=9) and NL/IR (n=9) pigs, the number our statistical power analyses support being sufficient to detect significantly different endpoints.
2. Preliminary results suggest that the IR trait exacerbates coronary artery and abdominal aortic atherogenesis in both the NL and downsized FH backgrounds.
3. DS-FH/IR pigs appear to have a higher blood pressure than DS-FH/IS pigs
4. Porcine NMR lipoprotein validation of measuring pig LDL and HDL particle size and

concentrations has been achieved.
5. Ultrasound measurements of femoral atherosclerosis will be used to determine the rate of disease progression.

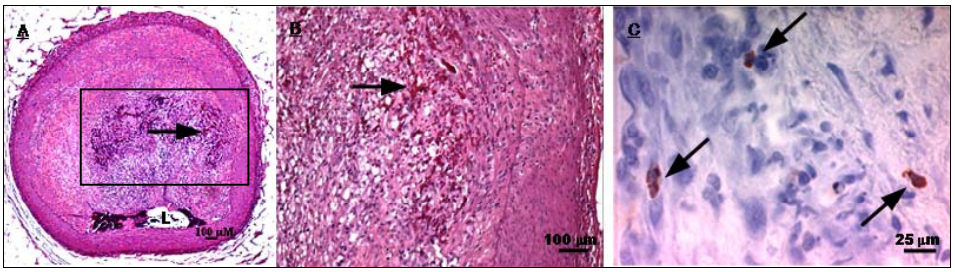

Fig. 2. Panel A. Hemorrhage into coronary atherosclerotic plaque of a 2-year FH/IR pig (total cholesterol 561 mg/dl; insulin: fasting, 4.6; 1 hr, 93.6; 2 hr, 27.1 µU/ml). Box indicates region of hemorrhage. Panel B shows higher magnification of region indicated by arrow from Panel A. (L = lumen.) Panel C. Macrophages in atherosclerotic plaque in FH pig coronary artery (monoclonal antibody MAC387, NeoMarkers, Fremont, CA).

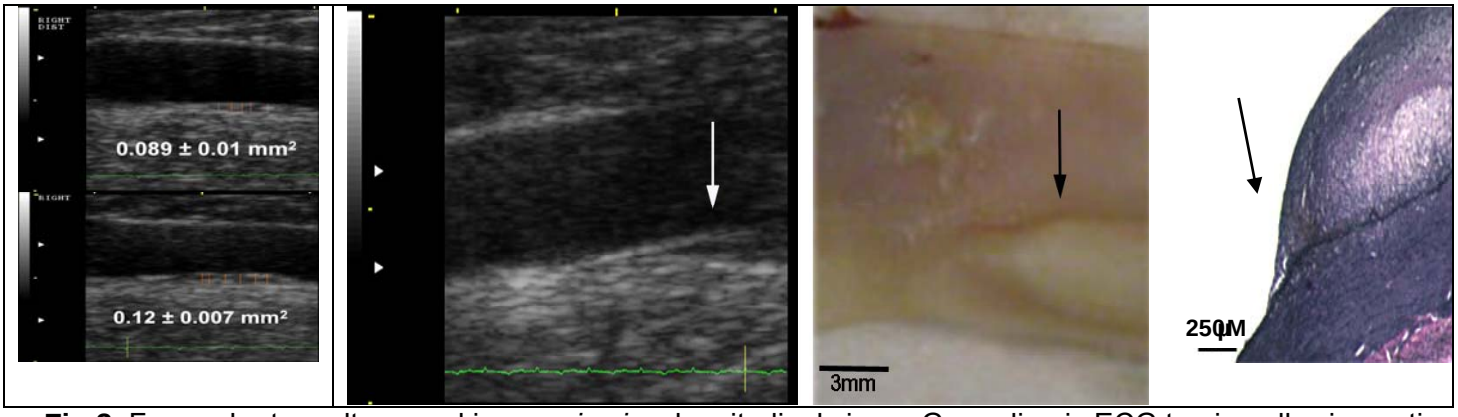

Fig 3. Femoral artery ultrasound images *in vivo,* longitudinal views. Green line is ECG tracing allowing gating to R wave. Left Panel: Comparison of top and bottom images shows progressive intimal thickening over 3 months in an FH/IS pig. The numbers listed are an average of 3 pigs over the same time interval. Second Panel: Atherosclerotic plaque from an FH/IS pig (total cholesterol 412 mg/dl; insulin: fasting, 11; 1 hr, 22; 2 hr, 13 µU/ml). Third Panel. En face image opened artery (arrow indicates raised plaque). Fourth Panel: Micrograph of plaque from section taken at arrow in panels B and C. (Verhoff van Gieson).

Completion of new pig housing unit

The additional accomplishment that cannot be over emphasized is the January 2005 completion of a new pig housing unit. This facility is now fully operational. In the original grant application, we requested support for this facility to maintain the number of pigs required for this study. The grant and UNC have generously provided the support that made this facility feasible.

3. Plans for the coming year:

During the remainder of Year 5, our emphasis will be on the following 4 items:

1. Continued evaluation of pigs entered into Aim II Exp 1 (NL/IS n = 9 vs NL/IR n = 9). We anticipate completing the live animal portion of this experiment in Jan 2007.
2. The Bergman frequently sampled insulin glucose tolerance test will be used to measure insulin sensitivity in experimental pigs during the year long study.
3. Monitor femoral artery intimal medial thickness with ultrasound. This approach should allow us to achieve our goal of documenting the extent and rate of development of atherosclerosis in both strains of IR pigs and comparing to IS (Insulin Sensitive) controls.
4. Monitor blood pressure in experimental pigs.

5. Determine LDL and HDL particle sizes and concentrations with LipoScience, Inc, Raleigh NC using the recently validated methodology.
6. Characterizion of biochemical changes that occur with disease markers in serum and plasma (e.g. CRP, IL-6, PAI-1, and fibrinogen), and/or lesions.
7. In addition, we will be breeding additional pigs for Aim II Exp 2 (DS-FH/IS vs DS-FH/IR). Selective breeding to obtain increased insulin resistance will continue. The breeders listed on **Table 1** have been and will be used to produce the remaining pigs needed to initiate Aim II Exp 2. We will continue to re-evaluate the breeding strategies with each data set to maintain the goal of producing pigs with an increased severity of IR (> 30 to 50 µU/ml fasting insulin or elevated mean fasting insulin concentrations ≥ 2.1 times greater than age and weight matched control animals and 1 and/or 2 hour postprandial insulin levels that are elevated ≥ 4-fold times that of controls).
8. Production of smaller pigs with FH and IR. Both the downsized FH pigs and the recently acquired Ossabaw strains that exhibit insulin resistance will be used to produce smaller pigs for current and future studies.

4. Most significant achievement:

Our most significant achievement is the significant amount of progress made towards achieving our 4 program goals.

Goal 1. To create NL (i.e. normolipidemic diet-inducible atherosclerosis) and FH pigs with and without IR. We have used a 3 point oral feeding test that measures fasting and post prandial insulin and glucose levels to identify hyperinsulinemic pigs. Following puberty (8 months of age), the hyperinsulinemia trait is often but not always expressed but the NL and FH traits are stable. Pigs with hyperinsulinemia have been shown to have insulin resistance on Bergman FSIGT. Selective breeding strategies implemented in Years 1 to 4 were designed to increase the severity of hyperinsulinemia in the NL and FH pigs. We had projected producing and phenotyping four generations during the first four years of the grant and we have done that. Our results suggest that breeding strategies consisting of two hyperinsulinemic parents, sib crosses, and backcrosses selecting for hyperinsulinemia have produced breeder pigs with an increased hyperinsulinemia while preserving the FH and NL phenotypes. Equally important, pigs with normal insulin levels in the FH and NL backgrounds have been preserved for producing essential control pigs. Breeding strategies remain in place to produce pigs with a more severe insulin resistance phenotype in sufficient.

Goal 2. Document the extent and rate of development of atherosclerosis in both strains of pigs and compare to IS controls. We have entered 9 NL/IS and 9 NL/IR pigs in the year long study and anticipate its completion in Jan 2007. We are actively breeding downsized FH pigs to have sufficient numbers to enter into the year long study starting Feb 2007 that will compare DS-FH/IS and DS-FH/IR pigs. Although the number of animals is too few for formal statistical analyses, the preliminary results suggest that the IR trait exacerbates the severity and diffuseness of coronary artery and abdominal aortic atherosclerosis in both the NL and downsized FH backgrounds. We are using transcutaneous femoral artery ultrasound to monitor the rate of atherosclerosis progression in all study pigs.

Goal 3. Characterize biochemical changes that occur with disease markers in serum, plasma, or lesions. We have established Bergman methodology for testing insulin sensitivity, S_i, in our pigs. To date, the S_i appears to be stable in the FH pigs, however some NL pigs are exhibiting worsening of the IR trait while ingesting the high fat diet. Lipoprotein analyses, serum and plasma markers and tissue markers will be analyzed as originally described with the addition of NMR analyses by

LipoScience, Inc., Research Triangle Park, NC. Blood pressure is also being monitored in all experimental pigs. Atherosclerotic and non-atherosclerotic tissue will be characterized as described in the grant.

Goal 4. Establish a colony of well-characterized animals for dissemination to the research community. We anticipate having sufficient breeders of pigs among the four phenotypes to achieve this long-range goal by the end of the granting period in Aug 2006. We emphasize that we will have breeders only and not experimental pigs for dissemination. The down-sized FH offer the possibility of reducing expenses and handling difficulties without loss of the inherited human-type FH phenotype. The even smaller Ossabaw pigs that have been recently transferred to UNC will also increase the feasibility of achieving this goal. These backgrounds have the greatest potential for providing a scientifically useful animal model insulin resistance. We are actively pursuing additional funding to preserve all of these germ lines.

Publications:

1. Bellinger DA, Merricks EP, Nichols TC. Swine models of type 2 diabetes mellitus: Insulin resistance, glucose tolerance, and cardiovascular complications. ILAR J, accepted, 51 pages.
2. Dumont, Behler R,Merricks EP, Nichols TC, Gallippi C. Toward Acoustic Radiation Force Imaging (ARFI) Classification of Atherosclerosis. Submitted, 25 pages.

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15. van der Meer IM, Iglesias del Sol A, Hak AE, Bots ML, Hofman A, Witteman JC. Risk factors for progression of atherosclerosis measured at multiple sites in the arterial tree: the Rotterdam Study. Stroke. 2003;34:2374-2379.

Action Items

1. Please email a copy of your final 2005 Annual Report to me no later than *February 24, 2006*.

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2. Tom Coffman will compile a "general response" to the previous EAC comments that will be included at the front of the Annual Report booklet.

Tom – please send me this document by *February 24, 2006* for inclusion into the booklet.

3. Please email updated "Feldman tables" for the 3 animal models that you are focusing on to Rick McIndoe by *February 10, 2006*.

Rick – please compile these tables into a single "master Feldman table" and send this to me by *February 24, 2006*.