Animal Models of DIABETES COMPLICATIONS CONSORTIUM

Organization – University of North Carolina at Chapel Hill

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

UPDATE REPORT
(September 1, 2001 – January 1, 2004)

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A. 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 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 3 generations of animals have been produced and successfully phenotyped during the first 3 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: FH/IS, FH/IR, NL/IS, and NL/IR. The FH/IR pig resembles the human metabolic syndrome exhibiting IR in combination with both elevated triglycerides and LDL cholesterol, and depressed HDL cholesterol. 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.[1,2]

B. Summary of Accomplishments

We have summarized our accomplishments in each Aim and the objectives of each Aim.

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.

Objective 1. Our first objective was to prepare two strains of pigs, one that possesses traits that predispose them to develop IR, and another that has familial hypercholesterolemia (FH). IR is defined as decreased biological response to normal concentration of serum insulin that overtime leads to

compensatory hyperinsulinemia.[2] A total of 129 post pubertal normal lipid (NL) and FH pigs over at least 3 new generations have been screened for IR (total screening tests = 179). Pigs are defined as normal or insulin sensitive (IS) if they exhibit mean fasting serum insulin levels of 9.5 ± 5.5 μU/ml and 1 and 2 hour postprandial levels between 10 and 20 μU/ml. Pigs are defined as having IR if they exhibit an elevated mean fasting insulin concentrations ≥ 2.1 times greater than age and weight matched control animals, and one or two hour postprandial insulin levels elevated ≥ 4-fold times that of controls. We now have produced 12 breeders with the IR phenotype (see Objective 2 and Table 1).

FH pigs exhibit elevated levels of triglycerides, LDL-cholesterol, apolipoproteins (apo) B, CIII and E and reduced levels of HDL-cholesterol, apoA1 and A4.[3,4] The FH pigs have been bred to expand the germline (see also Objective 2).

Objective 2. Our second objective was to utilize animals from the IR and FH groups to develop two new phenotypes, one with more severe IR and one with combined FH/IR. Our animal husbandry accounted for both the IR and FH status to successfully propagate the existing pig phenotypes, FH/IS and NL/IS, while both developing the FH/IR and NL/IR phenotypes as well as achieving an increased level of IR. At the beginning of this grant, six pigs were identified that had a mean fasting insulin of 17.9 ± 7.1 μU/ml and a two hour postprandial mean insulin of 75 ± 18.3 μU/ml. These pigs were defined as having an IR founder phenotype. We tested whether or not we could increase the level of IR by breeding sib pairs, backcrossing to parents selecting for IR, and utilizing these founder IR pigs whenever possible. This approach has provided the 12 breeder pigs with an increased severity of IR when compared to our founder pigs (Table 1). Specifically, the 6 FH and 6 NL breeder pigs classified as IR have mean fasting insulins of 24.8 and 22.2 μU/ml, respectively, and their mean one and two hour postprandial insulin levels range from 44 to 80 μU/ml, a significant increase with eating ($p<0.01$). In contrast, the mean fasting insulin levels in the 4 FH and 4 NL breeder pigs classified IS are 11.1 and 7.9 μU/ml respectively, all of whom had mean one and two hour postprandial insulin levels less than 16 μU/ml (Table 1). Again, this represents a significant increase with eating ($p <0.05$). Moreover, when the mean fasting insulin levels are compared between the IS and IR groups, the difference is also significant ($p < 0.05$), and when the postprandial insulin levels are compared, the difference is highly significant ($p<0.001$). Most importantly, breeders for all four phenotypes have now been produced. Most are proven. Pigs with combined FH/IR exhibit a phenotype that strongly mirrors the metabolic syndrome: IR, elevated triglycerides and LDL, and depressed HDL and some exhibit increased total body fat (Table 1).[1,2]

Transmission of the FH gene is documented by persistent hypercholesterolemia and is inherited in an autosomal codominant manner (see values on Table 1). Transmission of the IS and IR phenotypes is defined as post pubertal pigs exhibiting either (1) fasting insulin < ~10 μU/ml and 1 and 2 hour postprandial levels between 10 and 20 μU/ml or (2) mean fasting insulin concentrations ≥ 2.1 times greater than age and weight matched control animals and one or two hour postprandial insulin levels elevated ≥ 4-fold times that of controls, respectively. The inheritance pattern of the IS and IR traits in either the FH or NL pigs is unknown at present. Careful breeding records and DNA samples are being archived on all pigs that are phenotyped but identification of the molecular basis for inheriting the IR phenotype is beyond the scope of this grant.

Objective 3. With encouragement from the AMDCC Advisory Committee, a third objective was added that utilized down-sized pigs to produce smaller animals with insulin resistance and familial hypercholesterolemia. The added objective has been approached in two ways. First, 29 down-sized FH pigs from the Rapacz colony in Wisconsin were screened and 5 were identified as having

combined FH/IR. These five have been moved to Chapel Hill, completed quarantine, and are being bred to produce pigs for this grant. This down-sized FH strain is in a "pot-bellied" background and weighs around 250 lbs as an adult. This size has proven to be too large for reliable intravascular ultrasound (IVUS) due to the limitations of the existing animal fluoroscopy at UNC. We are dealing with this limitation by producing even smaller pigs and upgrading the fluoroscopy. To produce an even smaller pig, we have recently acquired a new strain of Ossabaw pigs that weigh between 100 and 150 lbs as adults.[5] These pigs were not available when this grant was written due to their limited availability and herd health issues. This strain is descended from Spanish pigs either shipwrecked or purposefully left on Ossabaw Island, Georgia, in the 16th-17th century, where they have lived for the past 450 - 500 years in genetic isolation. Both hyperinsulinemia and increased percent body fat have been reported in this strain and we have confirmed that our Ossabaw pigs also exhibit IR (see Table 1. item 5. Ossabaw).[6-8] We will breed these Ossabaw pigs with the downsized FH pigs with IR to produce a smaller pig with combined FH/IR that would a more suitable size for monitoring atherogenesis by IVUS. In addition, an upgraded animal fluoroscopy suite is scheduled to be installed in the Division of Laboratory Animal Medicine at UNC during the second or third quarter of 2004.

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.

The studies in this specific aim will be the most important in the grant because they will validate the usefulness of the FH/IR and NL/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.

In **Exp 1**, the degree of these abnormalities will be determined in the NL/IR pigs that are being fed a high fat diet to induce hypercholesterolemia (Table 5. Aim II Exp 1. NL/IS vs. NL/IR pigs). These animals will be fed the high fat diet for one year. Serum markers that predict the risk of lesion development will be analyzed at 3, 6, and 12 months to determine how these markers change in response to the presence of insulin resistance and hypercholesterolemia (Table 2). These changes will be compared to NL/IS pigs that are being fed the identical high fat diet. Insulin sensitivity, triglycerides, total cholesterol HDL-C, LDL-C, small dense LDL and HDL_2-C will be determined at the initiation of study and will be repeated at 1, 3, 6 and 12 months (Tables 2 and 3 and Fig 1). Several serum markers of atherosclerotic risk that have been shown to be increased in patients with insulin resistance such as C-reactive protein, interleukin-6 and P-selectin will be determined. After one year, both groups of animals will be sacrificed and the degree of atherosclerosis determined by morphometric and histological analysis. The lesions themselves and surrounding normal arterial wall will be analyzed with several different probes to determine the abundance of inflammatory mediators, extracellular matrix proteins, growth related molecules, parameters of insulin sensitivity and lipoprotein alterations (Table 4). The results should provide future investigators with a compendium of data regarding the development of anatomical and biochemical changes that occur in these animals and a parallel comparison between changes in insulin sensitivity and markers of disease activity.

In **Exp 2**, FH/IR pigs will be used in order to compare the changes in serum markers and in atherosclerotic lesions that develop over one year in FH pigs that are not being fed a special diet but have comparable levels of hypercholesterolemia and IR (Table 6. Aim II Exp 2 FH/IS vs. FH/IR). FH animals fed normal pig chow achieve cholesterol levels that are very similar to those that are induced in our control, non-FH animals on a high fat diet. Therefore utilizing these FH pigs will eliminate the need to feed a high fat diet. Also, these results should provide control data that will allow us to

determine whether in the presence of hypercholesterolemia, insulin resistance adds an additional risk that is independent of the diet. This is important for two reasons. One is the theoretical aspect that there may be interaction between diet and insulin resistance that leads to a more severe set of secondary abnormalities in lipoprotein subfractions such as small dense LDL and HDL_2-C. These animals will allow us to determine the extent of atherosclerosis that occurs in insulin resistant animals fed a diet high in fiber and low in fat. We anticipate that markers, such as C-reactive protein, may correlate with the additional atherosclerotic risk conferred by insulin resistance whereas, cytokines such as, interleukin-6, will probably increase as a function of the underlying inflammatory state that develops in lesions, whether or not insulin resistance is present. Because all of the data in humans are correlative, the establishment of this animal model in which potential causal relationships can be demonstrated will provide a new means of making more accurate predictions. These results will be compared to results obtained using control animals that exhibit an FH/IS phenotype. Our rationale is that many of the same traits that predict the development of atherosclerosis in insulin resistant humans, such as fasting hypertriglyceridemia, elevations in apolipoprotein B-100, and in inflammation markers such as C-reactive protein, will be present in the FH and IR pigs.

Exp 1 and 2 are initiated in pigs that are 9 months of age and have completed puberty. There are two reasons for this choice. First, type II diabetes most commonly appears after puberty in humans. Second, both the FH and IR traits appear to be most fully expressed after puberty.

Our goal for the IVUS is to determine whether the presence of IR correlates with a more rapid onset of atherosclerosis and a more severely affected phenotype at any given time point and to correlate differences in changes in marker proteins with morphometric differences. We will be able to determine if specific marker proteins are altered differently in IR animals as compared to IS animals. This model system allows us to compare differences, both in the ontogeny of the changes in serum markers and changes in morphometry that occur over time in related but not identical subpopulations.

Analysis of tissue markers at the time of euthanasia in all pigs will yield additional information regarding the role of IR in the pathogenesis of atherosclerosis. For example, changes over time in components of the extracellular matrix in atherosclerotic lesions can be correlated with IR and various serum and tissue markers (Table 4). Another rationale for analyzing tissue markers at the time of euthanasia is that they may yield additional information regarding the pathogenesis of lesions in the IR versus the IS state. We should be able to determine whether components of extracellular matrix in lesions or whether smooth muscle cell growth factors such as IGF-I or PDGF are overexpressed preferentially in lesions from IR animals as compared to IS animals and the degree to which these changes correlate with the severity of atherosclerosis. For example, some markers may correlate better with PCNA labeling, an index of smooth muscle cell proliferation and some may correlate better with markers of differentiation such as caveolin-1. These data will provide extremely valuable documentation of the morphometric and time-dependent changes in serum markers that occur in these animals as they develop progressive atherosclerosis. Similarly, the experimental results will identify markers that correlate most strongly with degree of atherosclerosis at each time point analyzed. These data will be of great help for designing future studies

Two of a planned 4 NL/IS pigs have been placed on the atherogenic diet for Aim II Exp 1 (Table 5, Fig 1). The remaining two NL/IS pigs will be placed on the study in early 2004. In addition, 4 FH/IS pigs have been started in Aim II Exp 2 (Table 6, Fig 1). All of these animals have successfully undergone Bergman testing with a frequently sampled intravenous glucose tolerance test at 3-month intervals. Preliminary results suggest that insulin sensitivity remains stable in the NL/IS pigs on the high fat diet and in the FH/IS pigs on normal low fat pig chow.

C. Plans for the coming year

Plans for the remainder of Year 3 and beginning of Year 4 - During the remainder of Year 3, our emphasis will be on (1) continuing evaluation of pigs entered into Exp 1 and 2 of Aim II and breeding additional pigs for these studies, (2) selective breeding to obtain increased insulin resistance, (3) completion of the new pig housing unit, and (4) production of smaller pigs with FH and IR. The NL/IS pigs started Exp 1 in 9/03 and will complete the experiment in 9/04. The FH/IS pigs started in 4/03 will complete sampling in 4/04. The breeders listed on Table 1 will be used to produce the remaining pigs needed to complete Exp 1 and 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 as proposed (> 30 to 50 mU/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).

We are approaching the recently identified constraints on IVUS in two ways. First, during the next year, an upgraded animal fluoroscopy suite sufficient to guide IVUS on pigs that are 250 lbs or less is scheduled to be constructed. Second, we will continue to characterize our newly acquired strain of Ossabaw pigs and breed them with the down-sized FH pigs to create a more suitably sized model both for monitoring atherogenesis by IVUS as well as for dissemination to the scientific community.

Currently, we plan to breed four sibling pairs and to perform two backcrosses of progeny to parents to produce offspring with more severe IR. Six to eight is the current maximum number of adult pigs we can maintain on the atherogenic diet in our facility due to space constraints. If we identify additional pigs with the desirable phenotypes among those currently being screened, they will be entered in the study as space allows. Additional space will become available in the new pig housing unit by September 2004 that will have capacity for 19-25 adults (i.e., >200 lbs). This space will allow us to maintain up to 20 experimental pigs concurrently.

Revised timetable for new pig housing unit - The UNC Medical School Planning Office retained Woolpert Engineering Inc who has completed an engineering study that identified no unexpected problems with installing the new pig-housing unit at our facility, the Francis Owen Blood Research Laboratory (FOBRL). Zoning approval from the Town of Carrboro was obtained in 11/03. Facilities Services at UNC issued a work order for the new hog unit to be purchased from Hog Slats, Inc, Newton Grove, NC in 11/03. The bidding process for site preparation was first performed on 12/30/03, and repeated in Jan 2004. The site contractor was chosen from these Jan 2004 bidders. UNC Facilities Services has issued a work order and ground breaking is anticipated in 3/04. This timetable will allow Hog Slats potentially to begin construction in 4/04 with completion in 120 days allowing occupancy of the new hog unit by late summer 2004. This unit is essential for housing the breeder and experimental pigs required for this grant.

Revised Timetable for Experiments.

Grant Year	1		2		3		4		5	
Calendar Year	9/01	9/02	9/03		9/04		9/05		9/06	
Aim I: Breeding IR, FH, and DS-FH pigs for all Aims	----------									
Aim II: Effect IR on atherosclerosis										
Aim II- Exp 1 NL/IR vs. NL/IS				4----------						
Projected Completion of New Hog Unit					*					
						8----------				
							8----------			
Aim II- Exp 2 FH/IR vs. FH/IS						4----------				
							8----------			
							8----------			
Aim 3: Effect of IGF on IR and atherosclerosis (Deleted, Funding Denied)										
Aim 4: Effect of alphaVbeta3 inhibition on IR & atherosclerosis (Deleted, Funding Denied)										
Breeding, processing samples & data analysis	----------									

KEY TO PHENOTYPE OF PIGS:
1. NL/IR: Normolipidemic Chapel Hill pigs that are insulin resistance
2. NL/IS: Normolipidemic Chapel Hill pigs that are insulin sensitive
3. FH/IR: Familial hypercholesterolemic pigs that are insulin resistance
4. FH/IS: Familial hypercholesterolemic pigs that are insulin sensitive.

D. Most significant achievement (September 1, 2001 – January 1, 2004).

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

Goal 1. To Create NL (i.e. Chapel Hill pigs with diet-inducible atherosclerosis) and FH pigs with and without IR. Selective breeding strategies implemented in Years 1 to 3 were designed to increase the severity of IR in the NL and FH pigs. Pigs have a 4-month gestation and achieve puberty at 9 months of age. Following puberty, the IR trait should be fully expressed and the FH trait is stable. We had projected producing and phenotyping three generations during the first three years of the grant and we have done that. Our results suggest that breeding strategies consisting of two IR parents, sib crosses, and backcrosses selecting for IR have produced breeder pigs with an increased IR severity while preserving the FH and NL phenotypes. Equally important, the IS trait in the FH and NL backgrounds has been preserved for producing essential control pigs. Breeding strategies remain in place to produce pigs with a more severe insulin resistance phenotype in sufficient numbers for the experiments in Aim II (see Table 1).

Goal 2. Document the extent and rate of development of atherosclerosis in both strains of pigs and compare to IS controls. Both of the proposed atherosclerosis studies have been initiated (Tables 5 and 6). Additional pigs will be entered into the study when they are documented to have the desired phenotype.

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 experimental pigs. Lipoprotein analyses, serum and plasma markers and tissue markers will be analyzed as originally described (Tables 2 – 4).

Goal 4. Establish a colony of well-characterized animals for dissemination to the research community. This long-range goal appears to be within reach by the end of the granting period. The down-sized FH and even smaller Ossabaw pigs have been transferred to UNC and offer the possibility of reducing expenses and handling difficulties without loss of the inherited human-type FH phenotype (Table 1). This background has the greatest potential for providing a scientifically useful animal model of the human metabolic syndrome.

Table 1. Serum cholesterol, fasting and 1 and 2 hour postprandial insulin levels in breeder pig stock at the Francis Owen Blood Research Laboratory for producing familial hypercholesterolemic and normal lipid pigs either with or without insulin resistance

1. Familial Hypercholesterolemia with normal insulin sensitivity (FH/IS)

		cholesterol	Serum Insulin level (µU/ml)		
Pig ID	Gender	(mg/dl)	fasting	1hr	2hr
56D	F	588	10.8	14.3	14
39D	F	680	11	15	9.8
49F	F	612	9.6	20.9	16.3
52F	M	410	12.8	13.3	12
Average ± SD		572.5 ± 99.7	11.1 ± 2.9	15.9 ± 2.4	13.0 ± 2.4

2. Familial Hypercholesterolemia (FH) with insulin resistance (FH/IR)

		cholesterol	Serum Insulin level (µU/ml)		
Pig ID	Gender	(mg/dl)	fasting	1hr	2hr
31	F	545	21.7	86.7	41.8
34	F	588	23.3	72.9	40.7
38	F	442	33.8	53.4	91.4
9028	M	369	11.8	118.9	58.7
45F	M	526	33.5	40.8	28.7
Average ± SD		494 ± 78.5	24.8 ± 9.2	74.5 ± 30.4	52.3 ± 24.4

3. Normal lipids – with normal insulin sensitivity (NL/IS)

		cholesterol	Serum Insulin level (µU/ml)		
Pig ID	Gender	(mg/dl)	fasting	1hr	2hr
76G	F	144	5.5	15.7	8.6
78G	F	112	6.5	17.5	16.1
69G	M	140	11.4	12.1	14.2
02C	M	82	8	14.9	19
Average ± SD		119.5 ±28.8	7.9 ± 2.6	15.1 ± 2.2	14.5 ± 4.4

4. Normal lipids - with insulin resistance IR (NL/IR)

		cholesterol	Serum Insulin level (µU/ml)		
Pig ID	Gender	(mg/dl)	fasting	1hr	2 hr
05F	F	127	13	42	21.1
06F	F	117	18.5	59.3	24.9
70F	F	76	31.8	32.9	78.7
20F	F	144	17.7	65.9	22.4
30B	F	82	30	200	77
14F	M	128	30.7	77.1	88.4
67F	M	94	17.6	135.4	36.1
Average ± SD		109.7 ± 25.8	22.2 ± 8.2	80 ± 68.4	44.8 ± 30.2

5. Ossabaw Pigs -

		cholesterol	Serum Insulin level (µU/ml)		
Pig ID	Gender	(mg/dl)	fasting	1hr	2 hr
1-1	M	65	11	104	22.6
1-2	F	62	17.9	58.4	11.5
1-3	F	69	15.3	22.5	47
1-4	M	60	12.9	140	36.8
52	F	72	15	53.8	45.3
Average ± SD		65.6 ± 4.9	14.2 ± 2.6	75.7 ± 46.2	32.6 ± 15.3

Table 2. Sampling schedule for Aim 2 Exp 1 and 2 (months)

Time (months)	0	1	2	3	4	5	6	7	8	9	10	11	12
1. Pig ID, age, gender, phenotype													
2. DNA sample	x												x
3. Weight and % body fat	x			x			x						x
4. Total chol, HDL, LDL, triglycerides	x	x	x	x	x	x	x	x	x	x	x	x	x
5. Bergman insulin sensitivity (S_i)	x	x		x			x						x
6. Abdominal IVUS				x			x						x
7. Plasma and serum markers (Table 3)	x	x		x			x						x
8. Coronary and carotid arteries, aorta													x
9. Tissue markers (Table 4)													x

Table 3. Characterization of inflammatory serum markers	**D. Serum extracellular matrix & ECM related proteins**: Vitronectin, Thrombospondin, Osteopontin, Matrix metalloproteinases –2 and -9
A. Serum markers of inflammation: TNF-α, IL-1β, IL-6, MCP-1, CRP, P-selectin, E-selectin	**E. Serum lipoproteins**: Total cholesterol, triglycerides, HDL-C, LDL-C and Apolipoprotein B-100, Small dense LDL and HDL_2-C
B. Serum markers of IGF-1 & insulin actions: IGF-1, IGFBP-5, and insulin	
C. Serum markers of insulin sensitivity: Bergman, IGFBP-1, Free IGF-1, PAI-1	

Table 4. Characterization of tissues & tissue markers	4. Extracellular matrix and ECM related proteins: Vitronectin, Thrombospondin, Osteopontin, Matrix metalloproteinase 2 and 9, and integrins $\alpha V\beta 3$, $\alpha 5\beta 1$, and $\alpha 2\beta 3$
A. Body weight and percent body fat.	5. Tissue lipoproteins: ApoB-100 and oxidized LDL
B. Biochemical and immunohistochemical detection and mRNA analyses of tissue markers of inflammation, cell growth, insulin sensitivity, extracellular matrix, and lipoproteins	**C. Coronary and carotid arteries and aortic atherosclerosis morphometry.** Computer-assisted morphometry.[9-11]
1. Inflammation: IL-1β, IL-6, E-selectin, P-selectin, monocyte chemoattractant protein-1, CRP	**D. Smooth muscle cell markers:** caveolin-1, SMC actin, PCNA labeling index
2. Growth regulatory proteins: IGF-1, IGFBP-5, PDGF, IGF-1 receptor phosphorylated forms	
3. Insulin sensitivity and action: IGFBP-1 and PEPCK (Hepatic mRNA), PI-3 kinase, phosphorylated form, Insulin receptor, phosphorylated form, Insulin receptor substrate-(PO4 by IKK-2). Liver, skeletal muscle, and fat will be collected.	

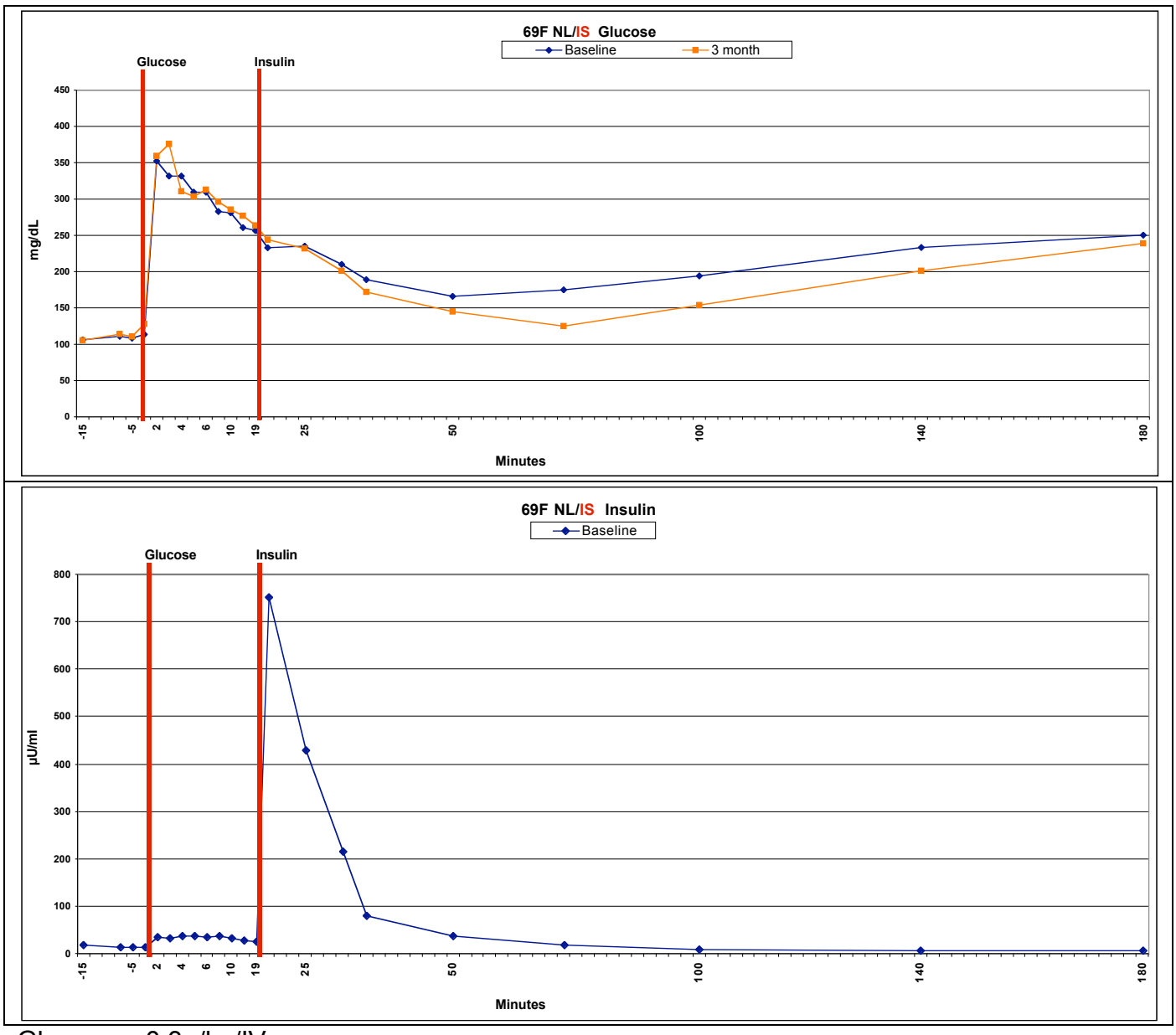
Table 5. Preliminary Results for Aim II Exp 1. NL/IS vs. NL/IR pigs

Animal Model/Background Strain Phenotype	Swine (Sus scrofa)/Poland China mixed normolipidemic /insulin Sensitive
Protocol (describe age at start and sacrifice, diet, etc)	2-2.5 yrs at start of study. High fat, high cholesterol (1%) diet, 12 months with monthly sampling. Bergman Insulin Sensitivity testing at 4 pts
Primary Screening	
Insulin Resistance	Yes No x
Type 2 Diabetes mellitus	Yes No x
Type 1 Diabetes mellitus	Yes No x
Fasting Glucose (mg/dl)	73.5 ± 2.1
Fasting Insulin (μU/ml)	11.5 ± 0.9
1 Hour Insulin (μU/ml)	18.0 ± 3.0
2 Hour Insulin (μU/ml)	24.1 ± 1.8
Aortic atherosclerosis % surface area with raised lesions intimal area (mm^2) intimal area as a % medial area	pending
Coronary artery atherosclerosis intimal area (mm^2) % luminal narrowing by intima intimal area as a % medial area	pending
Carotid artery atherosclerosis % surface area with raised lesions intimal area (mm^2) intimal area as a % medial area	pending
Lesion complexity ()	pending
Secondary Screening	monthly serum & plasma
Total Serum Cholesterol (mg/dl)	111 ± 41 baseline, increased to 327 on diet
Serum Triglycerides (mg/dl)	38 ± 5.6
Serum HDL-C (mg/dl)	pending
Body Weight (g)	446 ± 45 lbs
Body Fat (%)	pending
Blood Pressure (mmHg)	NA
Evidence of inflammation in the lesions (also see Table 4) 1. IL-1β, IL-6 2. MCP-1, CRP, P-selectin, E-selectin	pending
Serum markers of inflammation (also see Table 3) 1. TNF-α, IL-1β, IL-6 2. MCP-1, CRP, P-selectin, E-selectin	pending
Presence of Calcification	pending
Gene Expression (include specific genes of interest) Growth regulatory proteins: IGF-1, IGFBP-5, PDGF, IGF-1 receptor phosphorylated forms Insulin sensitivity and action: IGFBP-1 and PEPCK (Hepatic mRNA), PI-3 kinase, phosphorylated form, receptor, phosphorylated form, Insulin receptor substrate-(PO4) Extracellular matrix and ECM related proteins: Vitronectin, Thrombspondin, Osteopontin, Matrix metalloproteinase 2 and 9	pending
Other comments	study in progress, n=2 at present
Collaborators	

Table 6. Preliminary Results for Aim II Exp 2. FH/IS vs. FH/IR pigs

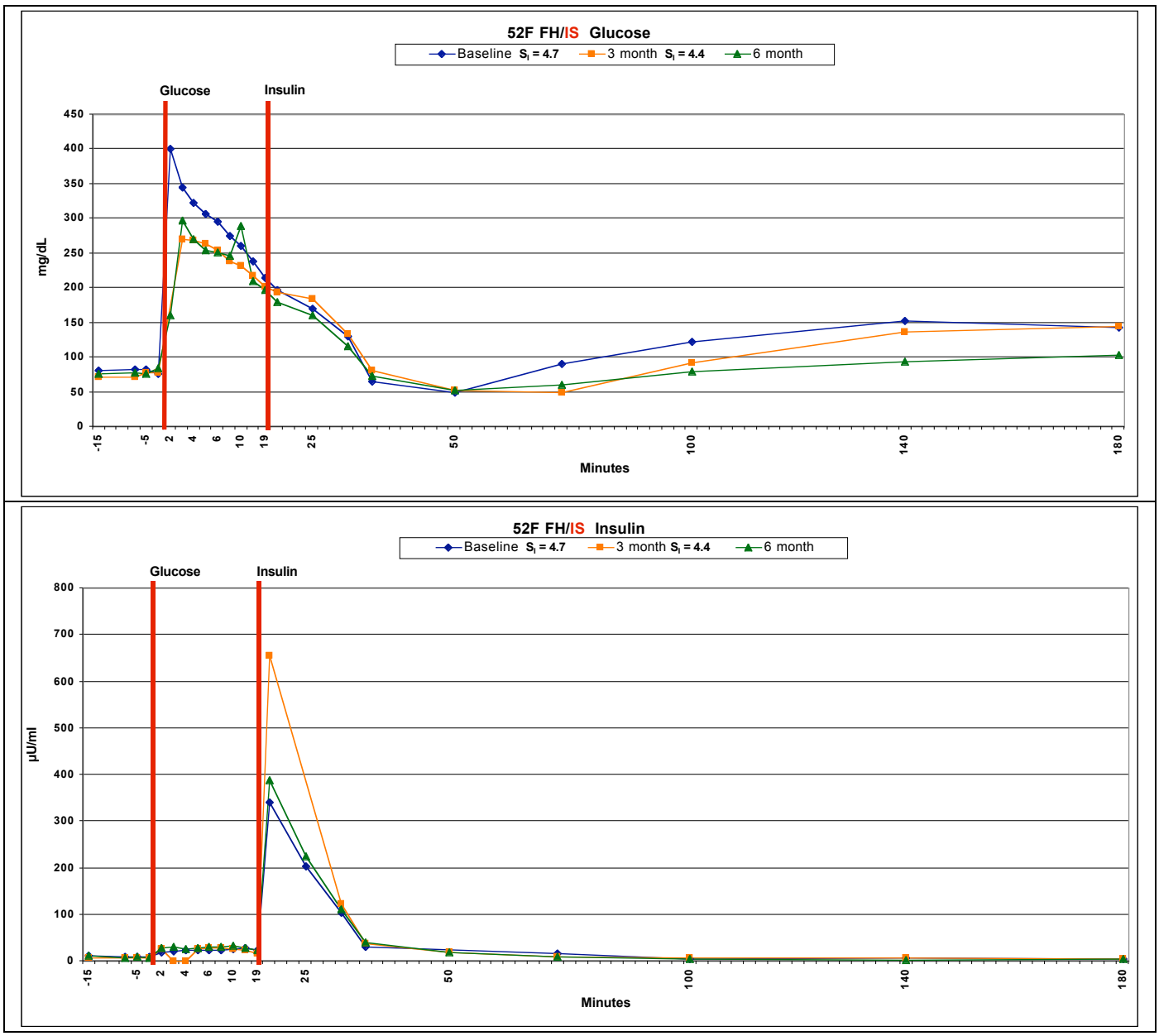
Animal Model/Background Strain Phenotype	Swine (Sus scrofa)/mixed breed Familial Hypercholesterolemia/Insulin Sensitive
Protocol (describe age at start and sacrifice, diet, etc)	1.5-2 years age at start. Basic swine maintenance chow, 12 months with monthly sampling. Bergman Insulin Sensitivity testing at 4 pts
Primary Screening	
Insulin Resistance	Yes No x
Type 2 Diabetes mellitus	Yes No x
Type 1 Diabetes mellitus	Yes No x
Fasting Glucose (mg/dl)	71.5 ± 3.8
Fasting Insulin (μU/ml)	11.0 ± 1.9
1 Hour Insulin (μU/ml)	19.0 ± 9.1
2 Hour Insulin (μU/ml)	12.1 ± 2.4
Aortic atherosclerosis % surface area with raised lesions intimal area (mm^2) intimal area as a % medial area	pending
Coronary artery atherosclerosis intimal area (mm^2) % luminal narrowing by intima intimal area as a % medial area	pending
Carotid artery atherosclerosis % surface area with raised lesions intimal area (mm^2) intimal area as a % medial area	pending
Lesion complexity ()	pending
Secondary Screening	monthly serum & plasma
Total Serum Cholesterol (mg/dl)	456.8 ± 89.4
Serum Triglycerides (mg/dl)	56.5 ± 26.9
Serum HDL-C (mg/dl)	pending
Body Weight (g)	417.3 ± 52.3 lbs
Body Fat (%)	pending
Blood Pressure (mmHg)	NA
Evidence of inflammation in the lesions (also see Table 4) 1. IL-1β, IL-6 2. MCP-1, CRP, P-selectin, E-selectin	pending
Serum markers of inflammation (also see Table 3) 1. TNF-α, IL-1β, IL-6 2. MCP-1, CRP, P-selectin, E-selectin	pending
Presence of Calcification	pending
Gene Expression (include specific genes of interest) Growth regulatory proteins: IGF-1, IGFBP-5, PDGF, IGF-1 receptor phosphorylated forms Insulin sensitivity and action: IGFBP-1 and PEPCK (Hepatic mRNA), PI-3 kinase, phosphorylated form, receptor, phosphorylated form, Insulin receptor substrate-(PO4) Extracellular matrix and ECM related proteins: Vitronectin, Thrombspondin, Osteopontin, Matrix metalloproteinase 2 and 9	pending
Other comments	study in progress, n=4 at present
Collaborators	

Figure 1 A. Aim 2 Exp 1 NL/IS vs. NL/IR



Glucose = 0.3g/kg/IV
Insulin = 0.03U/kg/IV

Figure 1 B. Aim 2 Exp 2 FH/IS vs. FH/IR pigs



Glucose = 0.3g/kg/IV
Insulin = 0.03U/kg/IV

Figure 1. Bergman Assay for insulin sensitivity (S_i) in Aim 2 Exp 1 NL/IS vs. NL/IR pigs (Fig 1A) and Aim 2 Exp 2 FH/IS vs. FH/IR pigs (Fig 1B). All pigs were studied after an overnight fast. The food intake of the animals to be tested had been monitored for 3 days prior to the fast to insure that carbohydrate intake was adequate (~35 kcal/kg/day). Two intravenous catheters were placed. Blood samples were obtained at - 15, - 10, - 5, and - 1 minutes and then a bolus of glucose (0.3 gm/kg/IV) was given as a 50% solution over 1 min and then samples were drawn at 2, 3, 4, 5, 6, 8, 10, 14, and 19 minutes. At 20 minutes, an insulin bolus (0.03U/kg/IV) was injected and blood sampling continued at 22, 25, 30, 40, 50, 70, 100, 140, and 180 minutes post-glucose infusion. Glucose and insulin concentrations were measured for all time points. Glucose was measured by an automated analyzer (YFI 2300 STAT Plus, YFI Inc, Ohio). Insulin was measured by a solid phase RIA (ICN, lower unit of

detection is 2 μU/ml). The data are analyzed by the Bergman method to calculate insulin sensitivity index (S_I).[12] Preliminary results suggest that the S_I is not significantly changed at the early time points in the NL/IS pigs fed a high fat diet and is stable over time in the FH/IS pigs fed normal pig chow. The high fat diet contains 1% cholesterol (~10 grams/day), 20% beef tallow, 0.75% cholate by weight. The normal pig chow contains 3.58% fat consisting of corn oil and lard, and provides 60 to 65 mg cholesterol total per day (June 2003 analysis, eurofins, Woodson-Tenent Laboratories Division, Goldston NC).[3,11]

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