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A Joint Effect of New Western Diet and Retinoid X Receptor Alpha Prostate-Specific Knockout with Development of High Grade Prostatic Intraepithelial Neoplasia in Mice - A Preliminary Study

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Abstract

Background—The “New Western-style Diet” (NWD) characterized by high in fat and low in fiber, vitamin D, calcium and methyl donors - are considered as a risk factor for prostate cancer. Previous studies have shown that premalignant lesions of human prostate have decreased expression of the Retinoid X Receptor alpha (RXR α). This study was to determine the effect of diet in RXR α knockout mice in developing high grade prostate intraepithelial neoplasia (mPIN).

Methods—Male mice (N = 54) with or without the RXR α prostate null mutation were fed either NWD or AIN-76A control diet for 10 months; prostates were harvested at 11 months of age and examined for prostate mPIN.

Results—mPIN was seen in 79% of RXR α prostate null mice fed NWD (n=19), 30.8% RXR α prostate null mice fed AIN-76A (n=13), 42.9% RXR α wild type mice fed NWD (n=14), and 12.5% RXR α wild type mice fed AIN-76A (n=8). Unconditional Logistic analysis showed a significant joint effect of NWD and RXR α status in developing mPIN 26.3 (95% CI: 2.5-280), but interaction was not significant owing to the small sample size 1.6 (0.09-27.7, p=0.7441).

Conclusion—This study provides preliminary data to support a joint RXR α -diet effect in prostate carcinogenesis.

Keywords

prostate cancer; Retinoid X Receptor α ; PIN; New Western Diet; Prostate-specific gene knockout

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INTRODUCTION

With the success of the human genome project the opportunity exists to precisely study genetic, environmental, exogenous and endogenous risk factors contributing to the malignant phenotype. This is important for potentially pre screening individuals at risk (particularly family members) and designing chemoprevention strategies. However, this is a very complex process because environmental risk factors are variable and difficult to quantify during one's life time. Populations with a large difference in disease incidence provide a unique human model to investigate the multiple parameters contributing to both gene and environment factors. Prostate cancer (PC) is the most commonly diagnosed cancer in Western and European countries [1] whereas the incidence of prostate cancer in Asian countries is substantially lower [2]. When Asians migrate to the Western world the incidence of prostate cancer approaches that of the indigenous population. Dietary patterns may increase risk for prostate cancer supported by epidemiological studies examining the risks of a "Western" style diet characterized by high intakes of high-fat foods, and low intakes of nutrient-rich food such as vegetables and whole grains [3]. While epidemiological evidence has suggested that such high fat/low nutrient diets may play a role in the development of prostatic malignancy [4], the direct experiment evidence for this is lacking.

Phenotypic protein expression studies in human confirm that RXR α is markedly decreased not only in prostate adenocarcinoma but also in precancerous high grade PIN [5]. A conditional RXR α knockout system that disrupts the RXR α gene in the prostatic epithelium [6] showed that prostate-specific RXR α -deficient mice developed multifocal hyperplasia at 4 months of age and had increased incidence of mouse high grade PIN (mPIN) among animals 10-15 months of age [7]. These studies demonstrated that loss of RXR α function results in preneoplastic lesions in the prostate which may promote PC development. This study aims to examine the combined effect of the New Western diet (NWD), a diet high in fat and low in fiber, vitamin D, calcium and methyl donors, and prostatic specific RXR α -deficiency in the development of mPIN.

MATERIALS AND METHODS

Mice and tissue preparation

Prostatic epithelium-specific Cre recombinase PB-Cre4 male and floxed RXR α female breeding mice were provided by Dr. P. Roy-Burman and mice with RXR α -deficient prostates were bred as previously described [5,7]. Offspring mice were genotyped by RT-PCR analysis to confirm the homozygous prostate-specific RXR α knockout as reported previously [7]. Mice were housed in the animal facility of the UCLA Center for Human Nutrition, which is accredited by the American Association for Accreditation of Laboratory Animal Care. From birth, all mice were fed a standard AIN-76A diet. At 5 weeks of age, mice were randomized into four groups: (i) wild type (WT) mice fed AIN-76A (n=8); (ii) WT mice fed NWD (n=14); (iii) RXR α null mice fed AIN-76A (n=13); (iv) RXR α null mice fed NWD (n=19). After feeding diets for an additional 42 weeks, mice were sacrificed at approximately 11 months of age. At necropsy the prostate was removed, dissected, and weighed. Prostates were fixed in 10% formalin, embedded in paraffin, cut to 4 μ m tissue

sections, and stained with H&E for histological examination. The study was approved by UCLA Chancellor's Animal Research Committee (ARC).

Nutritional components of the diets

The nutritional compositions of the diets are shown in Table 1. AIN-76A is a mouse diet developed based on the standards of American Institute of Nutrition [8]. The NWD was developed based on nutrient densities by Newmark 2001 in which calcium, vitamin D, fat and phosphorus content are based on nutrient-density levels consumed by Western populations [9]. Compared to AIN-76A, the NWD has higher fat content, reduced calcium, vitamin D and fiber; it also has reduced levels of methyl donor nutrients (folic acid, methionine and choline) approximating the nutrient-density levels commonly consumed by Western populations [9]. The other dietary components (fiber, folic acid, methionine, cysteine and choline) have essentially equal nutrient-density level requirements in both humans and rodent diets [9]. The NWD modulated the levels of these components based on their potential to contribute to colon cancer in Western populations.

Pathological analysis of prostate

HE stained slides were reviewed by a board certified pathologist (Dr. Jianyu Rao) who specializes in genitourinary tract pathology and the diagnosis was further confirmed by another urological pathologist (Dr. Jiaoti Huang) who was not involved in the study. The PIN and cancer lesions were further verified by the two pathologists together by double scoping. Pathological review was performed in a blinded fashion without the knowledge of RXR α genotype and treatment status. The change of the epithelial tissue was described as normal, mouse high grade PIN (mPIN), or adenocarcinoma, using criteria as previously defined [10]. Immunohistochemical staining for p63 and Cytokeratin 5/6 (both antibodies from Abcam, Cambridge, MA, USA) was performed using standard immunohistochemical protocol of an automatic stainer (Dako North America, Inc. Carpinteria, CA). The positive control used was a clinically obtained cervical biopsy sample. The negative control used was omitting the primary antibody.

Statistical Analysis

Descriptive analyses were used to describe characteristics using frequency and percentage for both RXR α genotypes (normal=0, Null=1) and diet (AIN76A=0, NWD=1). Initially, Student t-test was used to compare means of two groups for continuous variables. Fisher's Exact Test (two-tails) was used to determine whether mPIN progression from normal was associated with diet or RXR α genotypes.

In order to test hypotheses that NWD diet as well as RXR α null type are positively associated with the risk of the development of mPIN (mPIN=1, normal=0), crude and adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) for RXR α and diet were estimated using unconditional logistic regression models. Stratified analyses by RXR α or diet were performed and OR for each stratum-specific ORs are reported in Table 2.

As shown in Table 2, the data were further analyzed by using a 2 x 4 table stratified by both main variables into four groups: RXR α normal and AIN-76A (baseline), RXR α null and

AIN-76A (group 1), RXR α normal and NWD (group2), and RXR α null and NWD (group 3). Three dummy variables were created corresponding to three groups and included into the unconditional logistic regression model. The OR comparing group 3 with the baseline group is the OR for combined effect of both RXR α null genotype and new western diet (NWD).

In order to assess departure from multiplicative interaction, an interaction term was created using the two main variables with codes as described above. The likelihood ratio test with a p-value for heterogeneity was estimated by comparing the goodness of fit of a full logistic regression model including two main effect variables (RXR α and diet) with a full model with an interaction term (RXR α , diet, and a product term of both main variables). All statistical analyses were performed using SAS v.9.1 (SAS Institute, Cary, NC).

RESULTS

At the time of sacrifice, the average body weight of mice fed the NWD (43.3 ± 5 g) was significantly higher than the average body weight of mice fed the control AIN-76A diet (36.5 ± 4.6 g, $P < 0.05$ by Student t-test). The body weight of WT mice fed AIN-76A (35.3 ± 6.9 g) and RXR α prostate null mice fed AIN 76A (37.2 ± 2.3 g) did not differ significantly and there was also no significant difference between WT and RXR α prostate null mice fed the NWD (43.0 ± 3.9 g and 43.6 ± 5.6 g, respectively, $P > 0.05$ by Student t-test), indicating that the effect of the RXR α conditional mutation did not have an effect on overall gain in body weight.

Prostates from all groups were evaluated histologically as either normal (no areas of high grade PIN or cancer); mPIN (areas with high grade PIN); or cancer (areas with adenocarcinoma), using criteria for mPIN defined by Shappell et al [10]. The typical phenotypes of the mouse prostates are shown in Figure 1. Figure 1A to C shows the low magnification view of normal lateral glands, anterior prostate and ventral prostate, respectively. Figure 1D to F depict representative high magnification images of mPIN with either tufting or cribriform atypical epithelial proliferations with nuclear hyperchromasia and prominent nucleoli and occasional mitosis. Foci of invasive adenocarcinoma were seen in two of the 19 RXR α -knockout mice fed with NWD, but not other groups (Figure 1G to I for one mice and 1J to L for another mice). In both cases the tumor showed poorly formed acinar structures infiltrating the stromal tissue. There was diffuse background of mPIN in the surrounding glands. The tumor area characteristically showed loss of basal cell markers (negative p63 in tumor area but present in the adjacent mPIN area (Figure 1D)). CK5/6 stain showed the same results as that of the p63 staining with loss of basal cells in areas of suspicious for invasion and results are not presented.

The number of mice with normal, mPIN or PC is summarized in Figure 2 and Table 2. Prostates from wild-type mice fed the AIN-76A control diet were predominantly normal (87.5%), with a small group of mPIN (12.5%) and no PC was observed. Wild-type mice fed the NWD had 42.9% mPIN and no PC. In the RXR α null mice fed AIN-76A, mPIN was found in 30.8% and no PC was found. The RXR α deficient prostates from mice fed the NWD produced 15 mPIN (79.0%) and among these with mPIN, 2 PC were found (10.5%) in 19 mice.

As shown in Table 2, the adjusted odds ratios (ORs) for mPIN were 7.3 (95% CI: 1.90-28.2) for NWD vs. AIN-76A, adjusting for RXR α genotype and 4.4 (95% CI: 1.2-16.2) for RXR α null vs. normal, when adjusting for diet. When stratified by RXR α status, the crude ORs for mPIN were 8.4 (95% CI: 1.7-42.4) for NWD vs. AIN-76A among RXR α null type and 5.3 (95% CI: 0.5-54.9) among RXR α normal group.

The joint OR for mPIN was 26.3 (95% CI: 2.5-280) when RXR α null with NWD group compared with the group of RXR α normal with control diet (AIN-76A). The large confidence interval of the joint odds ratio may be explained by small sample size. Thus, this result needs to be interpreted with caution. Departure from multiplicative interaction was assessed by using likelihood ratio test with p-value for heterogeneity of 0.74813 and the OR for interaction was 1.6 (0.09-27.7, p=0.7441), probably due to small sample size.

DISCUSSION

Our study provides preliminary experimental evidence for the combined effect of gene (RXR α deficiency)-environment (Western-style diet) in prostate cancer carcinogenesis. This study observed slightly higher background incidence of mPIN even in the WT-AIN-76A control diet group (12.5%) than what has been reported in other studies [11]. This may be due to the advanced age (11-13 months) of the mice at the time of sampling in this study. However, even with advancing age, WT-AIN-76A diet group did not result in increased prostate cancer. Huang [7] found that incidence of low or high-grade PIN in RXR α null mice 10-15 months of age was 62 and 17% respectively, which was similar to our study. In addition, we observed about 12% more mPIN in WT-NWD group mice than in the RXR α null-AIN-76A group. Overall we found that RXR α knockout mice fed with a high fat, low nutrient diet developed significantly more precancerous lesions (mPIN) than control mice with normal RXR α and AIN-76A diet (79% versus 12.5%, respectively) (P=0.006 by two-tailed Fisher's exact test). However, with the relatively small sample size studied one should be cautious with the findings, as reflected by rather large confidence intervals of the OR and an insignificant departure of multiplicative interaction.

The etiological factors leading to human PC are complex. The relationship between diet and PC risk remains unclear. Nutritional factors that may influence the disease include total energy intake, dietary fat, cooked meat, micronutrients and vitamins (carotenoids, retinoids, vitamins C, D and E), fruit and vegetable intake, minerals (calcium, selenium), and phytoestrogens (isoflavonoids, flavonoids, lignans) [12]. While some of these factors may act as protective factors and others may increase risk, overall the associations of these individual factors with prostate cancer development are rather weak and/or at times inconsistent from studies to studies. The complexity of identifying dietary risk factors for prostate cancer is highlighted by the findings of the Health Professionals Follow-Up Study (HPFS). This large prospective cohort study has been able to confirm a moderate lowered risk of consuming tomato products (presumably from lycopene) [13] and has also been able to isolate the contributor of increased risk from dietary fat as due to red meat, saturated and monounsaturated fat, and alpha-linolenic acid [14].

The New Western Diet contains increased dietary fat, with reduced calcium, vitamin D, folate, choline, methionine, B12, and fiber, to simulate human Western-style diets based on nutrient density in the diet and has been shown to induce adenomas and carcinoma in the colon of C57Bl/6 mice without carcinogenic exposure or targeted mutations [9]. A similar high-fat low-nutrient Western-style diet induces hyperproliferation of the mouse prostate after feeding for 16 weeks [11]. High-fat diets are associated with increased risk for aggressive prostate cancer [15] and several prospective cohort studies found that total energy intake is a risk factor for PC development and progression [16,17]. In our study, mice fed the NWD were exposed to a much higher fat diet and were significantly heavier than mice fed the control diet. High fat intake, or high energy, alone could have contributed to the increased and advanced mPIN found in our study. Dietary fat studies are difficult to control. Unless nutrients in the chow are balanced, animals fed ad libitum control their total caloric intake and will eat less of a high fat diet which may reduce intakes of other nutrients in the chow. The totality of the experimental data suggest that dietary fat *per se* does not affect the development or progressions of prostate cancer in rodent models but that fat in combination with as yet unknown concomitant dietary changes may enhance carcinogenesis [18]. The NWD has reduced levels of folate and other methyl donor constituents. Studies of dietary intake and blood levels of folate, methionine have generally found no associations with risk of prostate cancer although there is some evidence that high dietary intake of vitamin B12 are associated with increased risk. Recent studies suggest that supplemental dietary folate is associated with increased risk. High serum folate presumed to be from folic acid fortification in the diet is associated with increased cancer cell proliferation [19]. Folic acid supplementation was associated with increased risk of prostate cancer [20]. In contrast, dietary folate deficiency can block prostate cancer progression in the TRAMP model [21]. The effect of reduced folate in the NWD is unknown, but future dietary studies must account for the potential effects of folate intake.

While NWD may provide a useful simulating agent that can be used to study the effect of human Western-style diet with PC risk, the downside of that is one can not determine the relationship of individual nutrient (e.g., fat, folate, Vitamin B12, etc) within the mixture with prostate cancer. Individually these nutritional factors may all contribute to the risk of prostate cancer development, yet when combined one may argue that such association could be altered somehow. This is also a limitation of the study.

While Western-style diet may be a risk factor for PC, not all men consuming a Western-style diet develop PC. Genetic changes play an important role in PC carcinogenesis. Mice with the conditional disruption of the RXR α gene display increased proliferation and induction of PIN [7], whereas mice lacking both RXR β and RXR γ are normal in terms of prostate morphology [22]. Decreased RXR α would result in deficient RAR/RXR α heterodimers and RXR/RXR α homodimers. Thus, in the model in which RXR functions as a transcriptionally active partner [23], this could result in functional cellular retinoid deficiency. Moreover, RXR α can mediate multiple signaling pathways in the prostate by dimerizing with other nuclear receptors, such as the peroxisomal proliferator-activated receptor- γ and vitamin D receptor, the ligands of which have been shown to inhibit prostatic cell growth [24,25]. Vitamin D is emerging as an important dietary factor that affects the

incidence and progression of many malignancies including prostate cancer and the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)(2)D(3)], inhibits the growth and stimulates the differentiation of prostate cancer cells [26]. It may well be that RXR α and vitamin D act together in prostate cancer carcinogenesis, although the exact mechanisms remain to be elucidated. At the moment we do not have data to address the question of increasing vitamin D intake in the RXR α prostate null and the New Western Diet. A further study such as increasing vitamin D intake in the context of RXR α deficiency may be proposed to examine the interplay of vitamin D level, western diet, and RXR α in prostate cancer carcinogenesis.

Also to be determined are the exact mechanisms for the down regulation of RXR α in premalignant PIN and cancer. In human prostate, a highly heterogeneous pattern of RXR α protein expression, with some areas of low or no staining, was reported in 13 adenocarcinoma specimens by Zhong et al [27]. Mao et al [5] found RXR α protein expression was absent in basal and luminal secretory epithelial cells in 52% of human PIN specimens and that the basal cell expression of RXR α in PIN was significantly reduced compared to expression in basal cells of normal glands. That the loss of RXR α signal in PIN was a result of reduced RXR α expression in basal cells and not due to loss of basal cells was confirmed by positive Cytokeratin 903 (basal cell marker in human prostate tissue) stain in PIN. Loss of RXR α protein may indicate a premalignant alteration to basal cells along the pathway to neoplasia. Diet can influence the control of gene expression that might impact cancer development [28]. Dietary polyphenols have been shown to have cancer inhibition activities by reducing DNA hypermethylation properties [29, 30]. Green tea decreased CpG methylation in the promoter region of RXR α gene and increased RXR α protein levels in intestinal tumors in the azoxymethane-Apc Min/+ mouse [31]. While our study presented data to show the combined effect between RXR α and NWD in the development of mPIN in a mouse model, the direct link of such a combined effect for the initiation of human prostate cancer remains to be further determined. Additional studies are necessary to further explore the relationship between loss of RXR α expression and diet in prostate carcinogenesis. Prostate tissues from the study have been retained for additional studies which will include examination of markers for cellular proliferation and markers for prostate neoplasia, and larger sized animal experiments will be performed to validate the findings from this preliminary study.

In summary, both the NWD and RXR α prostate-specific deficiency showed increased risk of developing mPIN. However, there is a possible synergistic effect for developing mPIN when both genetic and dietary factors are present. Additional studies are needed to further explore the relationship between loss of RXR α expression and diet in prostate carcinogenesis.

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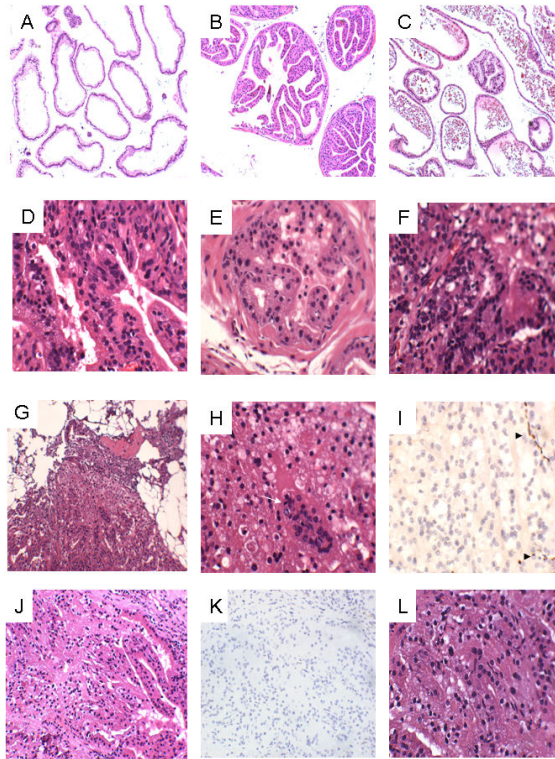


Figure 1.

Representative H&E and IHC images. A. Normal lateral glands (100x); B. Normal anterior prostate (100x); C. Ventral prostate (100x); D. Diffuse mPIN in ventral prostate (100x); E. mPIN in anterior prostate (400x) F. Diffuse mPIN in lateral prostate, elsewhere areas of invasive cancer are seen (400x); G to I. The first invasive cancer at low (100x, G), high (400x, H-note mitotic figure indicated by white arrow) magnification and IHC for p63 (400x, I). Note the presence of basal cells in adjacent mPIN areas but absence in tumor areas (I, solid arrowhead); J to L, The second invasive cancer at low (100x, J), IHC for p63 (100x, K) and high (400x, L) magnifications.

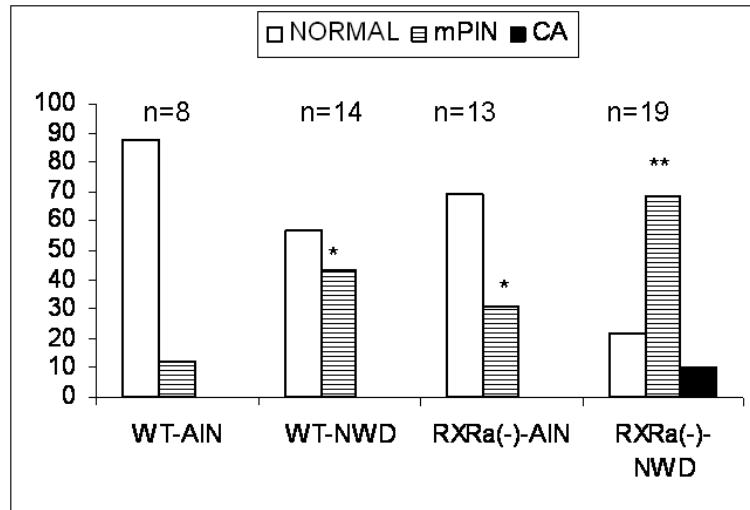


Figure 2. Percentage of prostate phenotypes according to genotype and diet. * $P < 0.05$, ** $P < 0.01$ comparing to WD-AIN by Chi-square test.

Table 1

Diet composition

Ingredients in % (wt) or amount (wt)	AIN-76A	NWD
Fat (corn oil), %	5	20
Calcium, mg/g	5	0.5
Vitamin D, IU/g	1	0.11
Phosphorus (PO ₄), mg/g	4	3.6
Fiber (cellulose), %	5	2
Folic acid, µg/g	2	0.2
DL- Methionine, %	0.3	-
L-Cysteine, %	-	0.3
Choline bitartrate, %	0.2	0.12
kcal/g (approximate)	3.6	4.5

Table 2

Diet, RXR α genotypes and mouse high grade PIN (mPIN)

Factors	mPIN (N, %)	Normal (N,%)	Crude OR, 95%CI	Adjusted OR [*] , 95%CI
Diet				
<i>AIN-76A</i>	5 (23.8)	16 (76.2)	1.0	1.0
<i>NWD</i>	21 (63.6)	12 (36.4)	5.6 (1.6-19.2)	7.3 (1.9-28.2)
p-value			0.0060	0.0038
RXR α				
<i>Normal</i>	7 (31.8)	15 (68.2)	1.0	1.0
<i>Null</i>	19 (59.4)	13 (40.6)	3.1 (1.0-9.8)	4.4 (1.2-16.2)
p-value			0.0500	0.0263
Joint OR^{**}				
<i>RXRα Diet</i>			<i>OR</i>	<i>p-values</i>
<i>Normal AIN-76A</i>	1 (12.5)	7 (87.5)	1.0	
<i>Null AIN-76A</i>	4 (30.8)	9 (69.2)	3.1 (0.3-34.4)	0.4964
<i>Normal NWD</i>	6 (42.9)	8 (57.1)	5.3 (0.5-54.9)	0.7862
<i>Null NWD</i>	15 (79.0)	4 (21.0)	25.3 (2.5-280)	0.0011
Interaction ^{***}			1.6 (0.09-27.7)	0.7441
Stratified Analysis by RXR α				
<i>Diet (RXRα Normal)</i>				
<i>AIN-76A</i>	1 (12.5)	7 (87.5)	1.0	
<i>NWD</i>	6 (42.9)	8 (57.1)	5.3 (0.5-54.9)	
p-value			0.1930	
<i>Diet (RXRα Null)</i>				
<i>AIN-76A</i>	4 (30.8)	9 (69.2)	1.0	
<i>NWD</i>	15 (79.0)	4 (21.0)	8.4 (1.7-42.4)	
p-value			0.0110	
Stratified Analysis by Diet				
<i>RXRα (AIN-76A diet)</i>				
<i>Normal</i>	1 (12.5)	7 (87.5)	1.0	
<i>Null</i>	4 (30.8)	9 (69.2)	3.1 (0.3-34)	
p-value			0.6070	
<i>RXRα (NWD diet)</i>				
<i>Normal</i>	6 (42.9)	8 (57.1)	1.0	
<i>Null</i>	15 (79.0)	4 (21.0)	5.0 (1.1-23.1)	
p-value			0.0660	

* Adjusted ORs: ORs were estimated when both variables (diet and RXR α) were included in the same logistic regression model.

** Joint OR was estimated when RXR α Null and NWD compared with RXR α Normal and AIN-76A diet

*** Multiplicative interaction OR was estimated when both variables (diet and RXR α) and a product of both variables were all included in the same logistic regression model.