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The association of protein intake (amount and type) with ovarian antral follicle counts among infertile women: results from the EARTH prospective study cohort

Irene SOUTER[1,A,*], **Yu-Han CHIU**[A,2], **Maria BATSIS**[1,3,a], **Myriam C. AFEICHE**[3], **Paige L. WILLIAMS**[4,5], **Russ HAUSER**[1,3,5], and **Jorge E. CHAVARRO**[2,5,6] **for the EARTH Study Team**

[1]Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, Harvard Medical School, Massachusetts General Hospital Fertility Center, Yawkey 10-A, 55 Fruit Street, Boston, MA, USA, 02114

[2]Department of Nutrition, Harvard T. H. Chan School of Public Health, Boston, MA, United States, 02115

[3]Department of Environmental Health, Harvard T. H. Chan School of Public Health Boston, MA, USA 02115

[4]Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, MA, 02115

[5]Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, MA, 02115

[6]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States, 02115

Abstract

[*]**Corresponding Author**: Irene Souter, MD, Massachusetts General Hospital Fertility Center, Yawkey 10-A, 55 Fruit Street, Boston, MA, USA, 02114, isouter@partners.org, Tel: + 1 617 726 4962.

[a]Present Address: Maria Batsis, University of Texas Southwestern, Children's Medical Center, Department of Pediatrics, 1935 Medical District Drive, Dallas, TX 75235

[A]**Disclaimer:** The authors consider that the first two authors should be regarded as joint first authors.

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DISCLOSURE OF INTERESTS

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CONTRIBUTION TO AUTHORSHIP

IS: substantially contributed to the conception, and design of the study, the acquisition, analysis and interpretation of the data, and wrote the manuscript.
YHC: substantially contributed to the design of the study, analysis and interpretation of the data, and the drafting of the manuscript.
MB: substantially contributed to the acquisition of the data.
MCA: substantially contributed to the analysis and interpretation of the data.
PW: substantially contributed to the analysis and interpretation of the data.
RH: substantially contributed to the conception, and design of the study, the acquisition, analysis and interpretation of the data.
JEC: substantially contributed to the conception, and design of the study, the acquisition, analysis and interpretation of the data.
All authors critically revised the manuscript for important intellectual content, read and approved the final manuscript.

ETHICAL APPROVAL

The study was approved by the Institutional Review Boards of the Massachusetts General Hospital and the Harvard T. H. Chan School of Public Health (IRB #: 1999P008167, original approval: 12/13/1996, most recent renewal: 03/01/2016), and informed consent was obtained from all participants prior to study enrollment.

Objective—To evaluate the association between protein intake (amount and type) and antral follicle count (AFC).

Design—Prospective cohort.

Setting—Academic fertility center.

Population—265 women undergoing fertility treatments at an academic fertility center and participating in an ongoing study on environment and reproductive health.

Methods—We measured AFC in ultrasonographic evaluation among women undergoing infertility treatments. Women completed a previously validated semi-quantitative food frequency questionnaire. We used Poisson regression to evaluate the relation between protein intake and AFC while adjusting for age, body mass index, race, smoking status, and total energy intake.

Main Outcome Measures—Antral follicle count.

Results—Among 265 women (mean age: 35.0±3.9 years, 85% Caucasian), total protein intake (% energy) was unrelated to AFC. When protein from different food sources was considered separately, we found a negative association between dairy protein intake and AFC. The mean AFC was 14.4% (3.9%–23.7%) lower for women in the highest quintile of dairy protein intake than for women in the bottom quintile after adjusting for potential confounders (p-trend=0.04). This association was stronger among women who had never smoked (p-trend=0.002) but was not observed among previous smokers (p-trend=0.36). There were no associations between protein intake from either non-dairy animal or vegetable sources and AFC.

Conclusion—Higher dairy protein intake (≥ 5.24% of energy) was associated with lower antral follicle counts among women presenting for infertility treatment. These findings should be further investigated in prospective studies designed to also clarify the biology underlying the observed associations.

Keywords

antral follicle count; dairy intake; protein intake; ovarian reserve; ovary; female infertility

INTRODUCTION

Infertility affects 15.5% of couples seeking conception[1] and bears significant financial and psychosocial repercussions for both the individuals involved and society in general.[2–4] Despite a well-established notion that both nutrition and modifiable lifestyle factors impact female[5–9] and male reproductive potential,[10–12] research in this field is not extensive leaving couples planning pregnancy with few evidence-based resources to guide preconception diet advice.

Even though diminished ovarian reserve is one of the major causes of female infertility, the process leading to reproductive senescence is currently poorly understood. In light of emerging population trends towards delayed pregnancy,[13] the identification of reversible factors (including diet) that affect the individual rates of reproductive decline might be of significant clinical value. Diets restricting the use of certain types of protein are gaining popularity, mainly due to increasing health and environmental awareness and compassion for

animals. However, their effects on reproductive health and ovarian aging remain unknown. Animal studies suggest a possible adverse effect of a low-protein diet on conception rates, ovarian follicular numbers and ovarian reserve in adulthood,[14–18] effects potentially mediated by accelerated accumulation of oxidative stress, altered ovarian telomere length and mitochondrial DNA copy number. Human data are lacking.

AFC and anti-mullerian hormone (AMH) are markers known to predict ovarian primordial follicle numbers better than basal follicle stimulating hormone (FSH) levels.[19] AFC, in particular, appears to be even more sensitive than AMH in predicting both ovarian primordial follicle numbers[19] and response to medication in in-vitro fertilization (IVF) cycles[20] and has been found to be independently associated with age at natural menopause.[21]

Study Objective

The objective of the present analysis was to examine the relation between protein intake (both amount and source of dietary protein) and AFC (as a measure of ovarian reserve) in a group of women attending an infertility clinic.

METHODS

Study Population

Participants were women enrolled in the EARTH (Environment and Reproductive Health) study, an ongoing prospective cohort started in 2004 aimed at evaluating the effects of various environmental factors on reproductive health.[22–24] Couples presenting to the Massachusetts General Hospital (MGH) for infertility treatments were invited to participate. Women between 18 and 45 years using their own gametes for intrauterine insemination or IVF were eligible to enroll. At enrollment, all participants underwent an anthropometric evaluation and completed a nurse-administered general health questionnaire where data on demographics, lifestyle, medical and reproductive history was collected. In 2007, a previously validated[25] semi-quantitative food frequency questionnaire (FFQ) was introduced to assess participant's dietary habits. Of 326 women who prospectively completed diet questionnaires and underwent ultrasound assessment, we excluded women with a prior oophorectomy (n=4), incomplete or missing AFC data (n=25), as well as women whose ultrasound for AFC determination was performed more than one year after FFQ completion (n=32), leaving 265 women for the present analysis. Included participants did not differ significantly from those excluded in age, body mass index (BMI), smoking status or race/ethnicity but did so in the prevalence of female factor infertility ($p<0.01$).

Diet assessment

All study participants completed a previously validated FFQ, thus providing information on how often, on average, they consumed specified amounts of each food, beverage and supplement included in the questionnaire during the year preceding their enrollment in the study. For each food, the questionnaire offered nine possible responses, ranging from never or less than once a month to six or more times per day. Nutrient content of each item was obtained from the nutrient database of the US Department of Agriculture[26] (USDA) and

supplemented with data from food manufacturers. Nutrient intakes were estimated by summing the contribution of all relevant food items and were expressed as daily intakes. Total protein intake, as well as protein intake from different food sources (dairy foods, animal foods, vegetables) was estimated and expressed as the percentage of energy consumed. In a validation study, the correlation between FFQ-assessed protein intake and protein intake assessed with prospectively collected diet records representing one year of diet was 0.44.[27] Among the major food sources of protein, recall was better for dairy foods (skim milk r=0.88) and worse for vegetables (beans r= 0.34).[28]

Ultrasonographic Determination of Antral Follicle Counts

All women participating in the study underwent a standard infertility work-up which included the ultrasonographic determination of the AFC for ovarian reserve evaluation, either on the 3rd day of an unstimulated menstrual cycle or on the 3rd day of a progesterone withdrawal bleed (at which time a serum FSH level was measured as well). All transvaginal ultrasounds were performed by one of the MGH reproductive endocrinology and infertility physicians. No fertility medications were used in the cycle preceding the ultrasonographic determination of the AFC.

Statistical Analysis

We divided women into quintiles of protein intake (total, vegetable, and animal (dairy and non-dairy)). We first summarized participant characteristics by quintiles of total protein intake and tested for differences across quintiles using Kruskal-Wallis test for continuous variables and Chi-square test for categorical variables. Poisson regression models were used to examine the relation between protein intake and log(AFC), while adjusting for potential confounders. We compared AFC of women in increasing quintiles of protein intake in relation to those of women in the lowest quintile (reference). Population marginal means were utilized to present marginal population averages adjusted for the covariates in the model, and results were exponentiated to express them in the original count scale. Tests for linear trend were performed using the median values of protein intake in each quintile as a continuous variable. Protein intake was adjusted for total energy intake using the nutrient density method. Specifically, terms for fat intake (% of calories) and total energy intake were added to the models to allow the protein intake parameters to represent the isocaloric substitution of carbohydrates with the same amount of energy from protein. In addition, we estimated the effect of substituting a type of protein for another by including energy contribution from all protein types as continuous variable in the same model. The effect of substituting one type of protein for another was estimated using linear combinations of the regression coefficients; the 95% confidence interval of a substitution was estimated based on the variance of each regression coefficient and their covariance. Multivariable-adjusted models included additional terms for age, BMI, race, and smoking status. We evaluated whether the association between protein intake and AFC was modified by BMI (≥ 25 kg/m^2 and < 25 kg/m^2), age (≥ 35 and < 35 years) and smoking (current/former and never smokers) by introducing cross-product terms to the final multivariate models. All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Two-sided p values ≤ 0.05 were considered statistically significant.

RESULTS

The study population was primarily Caucasian (85%) with a mean (±SD) age of 35.0 (±3.9) years. The majority of women (65%) were of normal weight defined as a BMI <25 kg/m^2 (median (25th, 75th): 23.3 (21.2, 26.2) kg/m^2), while 25%, and 10% of them were either overweight or obese, respectively. Most of the women had never smoked (72%). Mean (±SD) alcohol and caffeine intake were 7.7 (±8.9) g/day and 120 (±115) mg/day, respectively. Median intakes of protein, fat and carbohydrates (% energy) were 17, 33, and 50% energy, respectively (US mean protein intake: 14.6% energy).[29] The most common infertility diagnosis was idiopathic (46%), followed by male factor (28%), ovulatory dysfunction (9%) and diminished ovarian reserve (7%). 38% of participants reported at least one prior pregnancy. Overall, 37% of participants had an AFC ≥ 15 whereas 6.4% had an AFC ≤ 5 and were thus expected to be either high or poor responders, respectively.

There were no appreciable differences in age, BMI, smoking status, reproductive history or intakes of caffeine, alcohol, total energy or total fat across quintiles of total protein intake (Table 1). As expected, carbohydrate intake decreased with increasing intake of protein (p-trend: <.0001).

Total protein intake was unrelated to AFC in age-adjusted and multivariable-adjusted analyses (Table 2). While AFC was significantly lower among women in the second and third quintile of total protein intake when compared to women in the bottom quintile, there was not a clear pattern across quintiles of intake. Similarly, when this relation was examined separately for protein coming either from vegetable or animal sources, we noted no association between vegetable protein intake and AFC (Table 2) and while AFC was significantly lower among women in the 2nd and 5th quintile compared to the 1st, no significant trend was noted across quintiles of animal protein intake (Table 2).

We then further divided animal protein intake into protein coming from dairy and protein coming from other animal sources. Women in the highest quintile of dairy protein intake (≥5.24% of energy, or ≥2.3 cups of milk/day) had 14.4% (3.9%–23.7%, p=0.009) lower AFC than women in the lowest quintile after adjusting for potential confounders (Table 2). In addition, we estimated the effect of substituting 2% energy of dairy protein (around 1 cup of milk) with vegetable protein, and calculated that the AFC would be 5% higher (0.3%, 10%, p=0.04).

Last, we evaluated whether the relation between protein intake and AFC was modified by smoking, BMI and age. Smoking modified the association between dairy protein intake and AFC (p-interaction=0.003, fig. 1A–1B). Consumption of dairy protein was inversely associated with AFC among the 190 (72%) women who had never smoked (p-trend=0.002, fig. 1A), but not among the 75 (28%) women who had a history of ever smoking (p-trend=0.36, fig. 1B). There was no evidence of significant heterogeneity in the relation of dairy protein intake and AFC by BMI (p-interaction=0.12) or age (p-interaction=0.58).

DISCUSSION

In light of the increasing popularity of diets that limit certain protein sources and the growing trend towards delayed pregnancy, the identification of dietary factors that might affect reproductive aging can help guide preconception dietary counseling.

Main Findings

We evaluated the association between protein intake and AFC in a prospective cohort of reproductive age women attending an infertility clinic and found that overall greater consumption of dairy protein (≥5.24% energy, or ≥2.3 cups of milk/day) was associated with lower AFC. This association was stronger among women that never smoked. Neither vegetable nor animal protein intake from non-dairy sources were related to AFC in any of the analyses.

Studies investigating the effects of various dietary components on reproductive targets are emerging, however data on a potential effect of diet on ovarian reserve are scarce. To the best of our knowledge our study is the first to explore the possible association of dietary protein consumption with human ovarian reserve.

Strengths and Limitations

Our study has the following strengths: i) all data were derived from one large, fertility center treating a diverse population, ii) all AFC determinations were performed by infertility specialists only, following the same protocol thus minimizing “between operators” variability, and iii) the use of a previously validated FFQ with adequate validity and reproducibility for epidemiological study use.[25,30] One of the biggest challenges when evaluating the effects of a particular dietary component on either a reproductive target or on disease risk in general, is the correlation among dietary nutrients (i.e.: low-fat dairy consumers might have an overall “healthier” diet, while high-fat food consumption might be associated with a sedentary and overall “less healthy” lifestyle). In order to address this, our study and each individual sub-analysis within it controlled not only for total energy intake and alcohol/tobacco consumption but for all other dietary factors that might have correlated with the dietary component under consideration.

A potential limitation of our study is the lack of AMH levels to correlate with the AFC findings, mainly because the assay was not commercially available during most of the study period and the test was neither required nor covered by insurance. However, the AFC is considered a robust and reliable ovarian reserve predictor, slightly more sensitive than AMH in predicting ovarian primordial follicle number[19] and IVF response.[20] Finally, the results should be interpreted with caution because the findings may not be generalizable to a spontaneously conceiving population, and the consumption of dairy may be reflective of other unknown dietary or lifestyle factors that might be affecting ovarian reserve.

Interpretation

While it is not possible in the present study to identify the underlying mechanism linking higher dairy protein intake to lower AFC, dairy products are a diverse food group in terms of

factors that could potentially influence the ovarian reserve and there are several hypotheses that could explain the findings and include the following: 1) the presence in dairy products of measurable amounts of steroid hormones and growth factors that might have physiological and other effects in humans,[31–33] and 2) the contamination of milk products by pesticides and endocrine disrupting chemicals[34–35] that may negatively impact folliculogenesis and oocyte competence.

Regarding the former, studies suggest that commercial milk (derived from both pregnant and non-pregnant animals) contains large amounts of estrogens, progesterone and other placental hormones that are eventually released into the human food chain,[36] with dairy intake accounting for 60–80% of the estrogens consumed.[37] Dairy estrogens overcome processing, appear in raw whole cow's[38–39] and commercial milk products,[40] are found in substantially higher concentrations with increasing amounts of milk fat, with no apparent difference between organic and conventional dairy products,[39] and once inside the human body get converted to estrone and estradiol.[41] Following absorption, bovine steroids may alter reproductive outcomes. Human studies documented associations between dairy consumption and both plasma steroid hormone concentrations[42] and secretion of gonadotropins.[36] It is therefore possible, that absorbed bovine steroids may target either the hypothalamic-pituitary-gonadal axis or directly the oocyte and the local, intra-ovarian/intra-follicular supporting environment, through mechanisms involving altered gene expression and modified neuroendocrine signaling.

Serum levels of growth factors are also altered by dietary protein intake. Increased intake of i) dairy products, ii) animal vs. vegetable and iii) milk vs. meat protein, was associated with higher serum levels of insulin-like growth factor-I (IGF-I).[43–50] It is possible that the higher circulating IGF-I levels (resulting either from the diet itself or from stimulation of endogenous production[44]) adversely impact the ovary and its reserve (IGF-I regulates granulosa cell steroidogenesis and apoptosis during follicular development thus playing an essential role in reproduction).[51] IGF-I is also known to influence fertility at multiple other levels within the reproductive system, including effects on the hypothalamic–pituitary-gonadal axis[52–55] and the gonads.[56–60]

It is also possible that the observed association between dairy protein and AFC is mediated by the presence of environmental contaminants in the dairy. Dairy consumption has been associated with higher serum concentrations of certain organochlorine pesticides in both adult and pediatric populations[61–65] and with higher bisphenol-A (BPA) concentrations among lactating mothers,[66] while phthalates[67] and bisphenol analogues have been detected in dairy products.[35] In our previous study on this same cohort, higher urinary BPA concentrations were associated with lower AFC.[68]

Lastly, it is also possible that the differences in association with AFC of different sources of protein may simply reflect different degrees of measurement error. A validation study of the questionnaire used in this study found that the validity of recall for dairy foods was substantially higher than that for major sources of vegetable protein.[28] This alone could lead to a situation where we would identify an association with dairy protein only when in reality protein intake in general was inversely related to AFC.

Whether the observed association between dairy consumption and lower AFC results from an effect of steroid hormones, growth factors, environmental contaminants or other factors (i.e.: timing and duration of exposure) remains to be determined and is well beyond the study's scope. Finally, a 14% reduction in AFC might not be clinically significant at the individual level but can be of public health importance at the population level (across infertile women of reproductive age).

The fact that smoking modified the observed association between dairy protein intake and AFC was not entirely unexpected since smoking has been linked to both indicators of increased ovarian age and adverse reproductive outcomes.[69–72] The fact that the observed effect reached significance only among women who were never smokers potentially suggests that smoking's negative effect on ovarian reserve is a lot stronger and "masks" that of certain dietary habits.

CONCLUSION

We evaluated the relation between protein intake and ovarian reserve in a population of women presenting for infertility treatment and found an association between increased dairy intake (≥5% energy) and lower AFC. Given the lack of data on this topic, and the fact that multiple environmental and genetic factors, as well as complex neuroendocrine interactions, may alter the fate of the non-regenerating oocyte pool, it is imperative that these findings are reproduced in prospective studies designed to also clarify the biology underlying the observed associations. The latter might be crucial given that consumption of another species' milk by humans is an evolutionary novel dietary behavior that has the potential to alter reproductive parameters and may have long-term adverse health effects.

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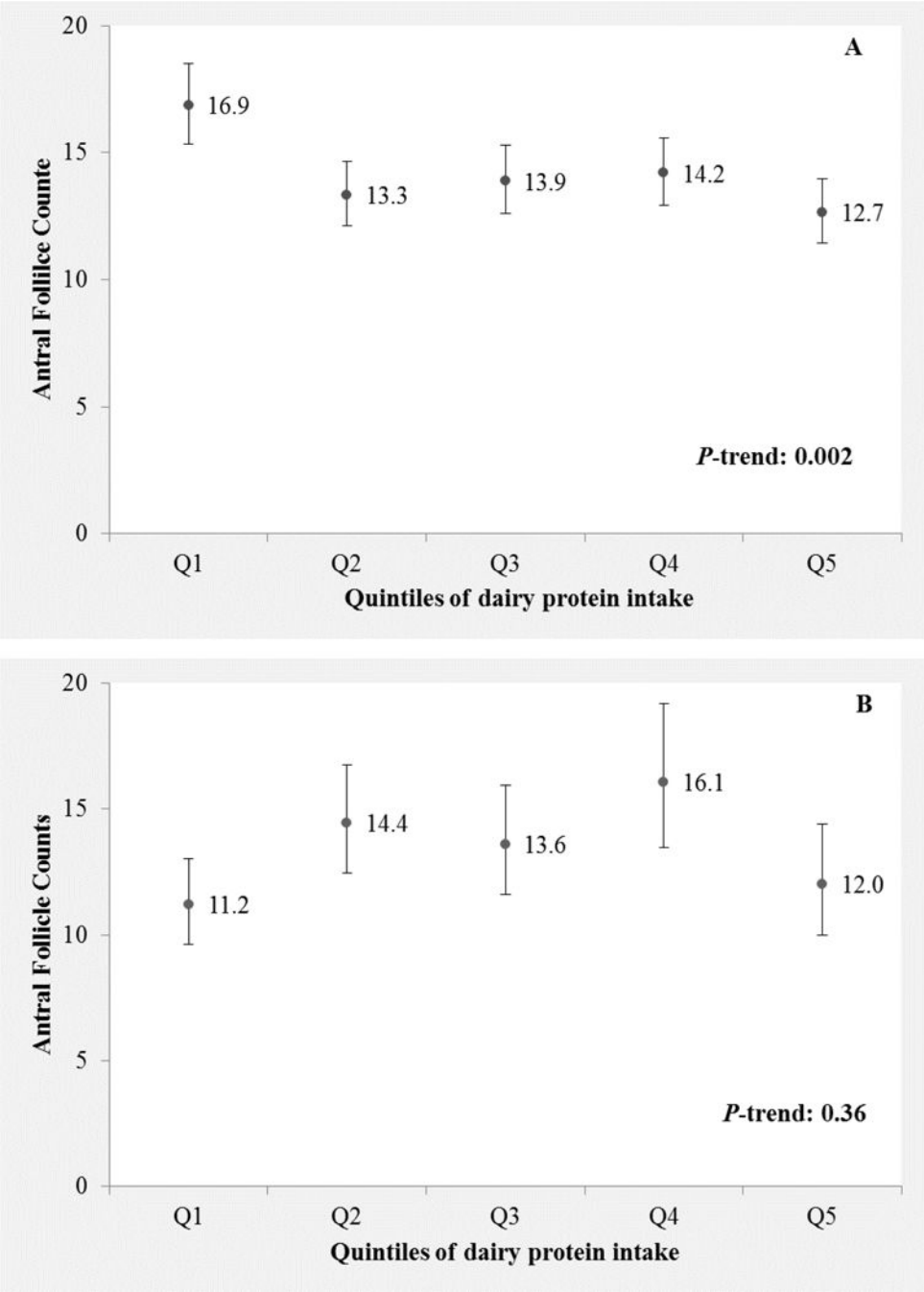

Figure 1.

Antral follicle count (AFC) according to quintiles of dairy protein intake among never (Fig. 1A) and ever smokers (Fig. 1B). Values are adjusted antral follicle counts with 95% confidence intervals. Results are adjusted for total energy intake, age, BMI, smoking status (ever vs. never), race (white vs non-white), vegetable protein intake and non-dairy protein intake, with each of these covariates at their mean levels. Tests for trend were conducted across quintiles using the median intake in each quintile as a continuous variable.

Table 1

Demographic characteristics of 265 EARTH study women according to quintile of PI.

	Total Protein Intake					
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**P-value[1]**
N	53	53	53	53	53	
Median, % energy	13.6	15.3	16.4	17.9	20.9	
Range, % energy	10.35,14.44	14.48,15.96	15.98,17.02	17.03,18.73	18.76,26.14	
	Mean ± SD or N (%)					
Demographics						
Age, years	34.8±4.1	35.2±3.7	35.6±4.4	34.3±3.7	35.0±3.7	0.53
BMI, kg/m^2	23.9±3.9	24.0±3.8	23.2±3.4	25.3±6.2	25.3±5.0	0.26
Ever smokers, N (%)	14 (26)	17 (32)	8 (15)	20 (38)	16 (30)	0.11
M-V Exercise, hrs/week[2]	3.5±3.7	3.4±3.8	4.0±4.7	3.8±4.1	4.9±6.1	0.96
White	45 (85)	46 (87)	44 (83)	45 (85)	46 (87)	0.98
Diet						
Alcohol, g/d	6.5±7.3	9.1±9.3	7.6±10.4	9.4±10.6	6.0±5.8	0.21
Caffeine, g/d	116±100	119±88	112±122	133±115	120±144	0.48
Total fat, % energy	32.4±6.6	32.6±5.9	32.3±7.1	32.6±5.7	33.5±5.6	0.55
Total carbohydrate, % energy	54.5±7.4	51.0±6.4	50.4±7.2	48.0±6.9	44.6±6.3	<.0001
Total energy intake, kcal/d	1840±586	1904±627	1777±632	1778±577	1627±564	0.15
Reproductive history, N (%)						
Prior pregnancy	26 (49)	19 (36)	19 (36)	18 (34)	22 (42)	0.51
Day 3 FSH, IU/ml	7.2±2.0	7.5±3.2	7.8±2.8	7.4±1.8	7.3±2.5	0.82
Infertility diagnosis, N (%)						0.16
Male factor	11 (21)	14 (26)	14 (26)	22 (42)	13 (25)	
Diminished ovarian reserve	3 (6)	4 (8)	7 (13)	2 (4)	2 (4)	
Ovulatory	8 (15)	7 (13)	3 (6)	1 (2)	4 (8)	
Endometriosis, uterine, tubal	3 (6)	5 (9)	4 (8)	6 (11)	9 (17)	
Unexplained	28 (53)	23 (43)	25 (47)	22 (42)	25(47)	

[1] Kruskal–Wallis & x^2-test: for continuous and categorical variables, respectively;

[2] Moderate-Vigorous Exercise.

Table 2

Association between antral follicle counts (AFC) and protein intake in 265 women.

Adjusted Mean Antral Follical Count (95% Confidence Intevals) by Protein Intake						
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**P- trend[1]**
Subject, N	53	53	53	53	53	
Total protein intake (% energy)						
Range:	10.35,14.44	14.48,15.96	15.98,17.02	17.03,18.73	18.76,26.14	
MDLl[2]	14.8 (13.8, 15.8)	13.0 (12.0, 14.0) *	13.2 (12.2, 14.2) *	13.8 (12.8, 14.8)	14.1 (13.2, 15.2)	0.90
MDL2[3]	14.7(13.7, 15.8)	13.0 (12.0, 14.0) *	13.1 (12.1, 14.1) *	13.8 (12.9, 14.9)	14.2 (13.2, 15.3)	0.97
Vegetable protein intake (% energy)						
Range:	2.14,5.00	5.05,5.83	5.84,6.60	6.62,7.51	7.52,15.16	
MDL1[4]	13.8 (12.7, 14.9)	13.4 (12.5, 14.5)	14.0 (13.0, 15.1)	13.8 (12.8, 14.8)	13.8 (12.8, 15.0)	0.84
MDL2[5]	14.1 (13.0, 15.4)	13.7 (12.6, 14.9)	14.3 (13.2, 15.5)	14.1 (13.1, 15.3)	14.1 (13.0, 15.4)	0.86
Animal protein intake (% energy)						
Range:	0.16,7.84	7.86,9.48	9.49,10.89	10.89,12.98	12.99,22.01	
MDL1[6]	14.9 (13.7, 16.1)	12.9 (12.0, 13.9) *	13.5 (12.5, 14.5)	14.5 (13.5, 15.6)	13.1 (12.1, 14.1) *	0.27
MDL2[7]	14.9 (13.7, 16.2)	12.8 (11.9, 13.8) *	13.4 (12.4, 14.4)	14.5 (13.5, 15.6)	13.1 (12.1, 14.2) *	0.31
Dairy protein intake (% energy)						
Range:	0.00,2.31	2.33,3.20	3.21,4.12	4.12,5.24	5.24,9.27	
MDL1[8]	14.5 (13.5, 15.7)	13.4 (12.5, 14.5)	13.7 (12.7, 14.7)	14.8 (13.7, 15.9)	12.4 (11.5, 13.5) *	0.04
MDL2[9]	14.8 (13.7, 16.0)	13.7 (12.6, 14.8)	13.9 (12.8, 15.1)	14.9 (13.8, 16.2)	12.6 (11.6, 13.8) *	0.04
Non-dairy animal protein intake (% energy)						
Range:	0.03,4.12	4.15,5.57	5.58,7.13	7.15,8.98	9.01,21.04	
MDL1[10]	13.7 (12.7, 14.9)	13.4 (12.4, 14.4)	13.8 (12.8, 14.8)	13.7 (12.7, 14.7)	14.2 (13.2, 15.3)	0.48
MDL2[11]	14.0 (12.8, 15.3)	13.5 (12.5, 14.7)	14.0 (13.0, 15.2)	14.0 (12.9, 15.2)	14.6 (13.4, 15.8)	0.42

[1] Estimated using median intake in each quartile as a continuous variable;

[2] **Model (MDL) 1**: adjusted for total energy intake (TEI), age, and total fat intake (TFI);

[3] **Model 2:** adjusted for TEI, TFI, age, BMI, smoking status (ever vs. never), race (white vs non-white);

[4] **Model 1:** adjusted for TEI, TFI, age, and animal protein intake (PI);

[5] **Model 2:** adjusted for TEI, TFI, age, BMI, smoking status (ever vs. never), race (white vs non-white), and animal PI;

[6] **Model 1:** adjusted for TEI, TFI, and vegetable PI;

[7] **Model 2:** adjusted for TEI, TFI, age, BMI, smoking status (ever vs. never), race (white vs non-white), and vegetable PI;

[8] **Model 1:** adjusted for TEI, TFI, age, non-dairy PI, and vegetable PI;

[9] **Model 2:** adjusted for TEI, TFI, age, BMI, smoking status (ever vs. never), race (white vs. non-white), non-dairy PI and vegetable PI;

[10] **Model 1:** adjusted for TEI, TFI, age, dairy PI and vegetable PI;

[11] **Model 2:** adjusted for TEI, TFI, age, BMI, smoking status (ever vs. never), race (white vs. non-white), dairy PI and vegetable PI;

* P-value for trend <0.05 compared to women in the lowest quartile of PI.