Anti-MUC1 Antibodies and Ovarian Cancer Risk: Prospective Data from the Nurses' Health Studies

Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2010 June ; 19(6): 1595–1601. doi:10.1158/1055-9965.EPI-10-0068.

Anti-MUC1 antibodies and ovarian cancer risk: prospective data from the Nurses' Health Studies

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Abstract

Background—Surface-epithelial glycoprotein, MUC1, becomes over-expressed and hypoglycosylated in adenocarcinomas; similar changes occur during non-malignant inflammatory events. Antibodies developed against tumor-like MUC1 in response to such events could be one way through which ovarian cancer risk factors operate.

Methods—We evaluated the association between anti-MUC1 antibodies and risk of ovarian cancer in a prospective nested case-control study in the Nurses' Health Studies. We used an enzyme-linked immunosorbent assay to measure plasma anti-MUC1 antibodies in 117 ovarian cancer cases collected at least 3 years prior to diagnosis and 339 matched controls.

Results—In controls, younger women (*p*-trend=0.03), those with a tubal ligation (*p*=0.03), and with fewer ovulatory cycles (*p*-trend=0.04) had higher antibody levels. In cases, women with late-stage disease (p=0.04) and those whose specimen was >11 years remote from diagnosis (p=0.01) had higher antibody levels. Overall, increasing anti-MUC1 antibody levels were associated with a non-significant trend for lower risk for ovarian cancer; but there was highly significant heterogeneity by age (*p*-heterogeneity=0.005). In women <64 years, the antibody level in quartiles 2–4 *vs.* 1 were associated with reduced risk, (RR=0.53; 95% CI (0.31, 0.93); *p*-trend=0.03), while in women ≥64 years, the corresponding RR was 2.11 (95% CI (0.73, 6.04); *p*-trend=0.05).

Conclusion—Anti-MUC1 antibodies evaluated several years prior to diagnosis may be associated with lower risk of subsequent ovarian cancer in women less than 64 years old at assessment.

Impact—Key elements of an "immune model" to explain ovarian cancer risk factors are confirmed and should be evaluated in larger prospective studies.

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Keywords

ovarian neoplasms; MUC1; Antibodies; Tubal Ligation; Ovulation

Introduction

Human mucin family member, MUC1, is a heavily glycosylated protein expressed in low levels by many normal epithelial cells, while a poorly glycosylated form is over-expressed by many adenocarcinomas, including breast and ovarian cancers (1). Circulating anti-MUC1 antibodies have been identified in patients at the time of cancer diagnosis and generally predict better survival (2–4). Antibodies also have been found in healthy individuals, especially in women who are pregnant or lactating (4–7), leading to the hypothesis that natural immunity against MUC1 might develop and account for the long-term protective effects of pregnancy and breastfeeding on breast cancer risk (8). In addition, several other factors that reduce risk of ovarian cancer, including oral contraceptive use, tubal ligation, and mastitis during breastfeeding, have been associated with higher antibody levels (9). These findings led to the hypothesis that immunity developed against MUC1 could account, at least in part, for the long-term protective effect of certain reproductive and hormonal factors on ovarian cancer risk (9).

Since ovarian tumors can express MUC1 and lead to antibody development, it is important to evaluate antibody levels well before diagnosis in a prospective study design. Thus, we measured anti-MUC1 antibodies in plasma samples collected from Nurses' Health Study (NHS) participants in a nested case-control study of ovarian cancer, where cases provided blood samples at least three years prior to a diagnosis, to evaluate the relationship between circulating anti-MUC1 antibodies and risk of ovarian cancer.

Material and Methods

Study Population

The Nurses' Health Study (NHS) was established in 1976 among 121,700 U.S. female registered nurses, aged 30 to 55 years at entry, and the Nurses' Health Study II (NHSII) in 1989 among 116,430 U.S. female registered nurses aged 25 and 42 years at enrollment (10, 11). Information on potential risk factors for ovarian cancer, including reproductive history, was obtained through baseline and follow-up questionnaires sent every two years. Heparinized blood specimens were provided by 32,826 NHS participants between 1989 and 1990 (12) and by 29,611 NHSII participants between 1996 and 1999 (13). Samples were shipped via overnight courier to the laboratory, where they were separated into plasma, RBC, and WBC components, and stored in liquid nitrogen freezers. Follow-up of NHS and NHSII blood cohorts was 98% in 2004 and 2003, respectively. NHS and NHSII protocols have been reviewed and approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital.

Incident cases of epithelial ovarian cancer diagnosed at least 3 years after blood collection but before June 1, 2004 (NHS) or June 1, 2003 (NHSII) were identified. A total of 117 cases (111 from NHS, 6 from NHSII) were confirmed by medical record review and matched to 339 controls (approximately 3 per case) on age (±1 year), menopausal status at blood collection and diagnosis, postmenopausal hormone use at blood collection, and month (± 1 month), time of the day (± 2 hours), and fasting status of the blood draw. All controls had intact ovaries at the time of case diagnosis. Except for non-melanoma skin cancer, cases and controls had no history of cancer prior to blood collection.

Laboratory Assays

Anti-MUC1 antibody assays were conducted at the Department of Immunology, University of Pittsburgh. Antibodies were measured against a synthetic 100-mer MUC1 peptide corresponding to five tandem repeats of the MUC1 polypeptide core repeat region (9,14). Briefly, MUC1-coated Immulon wells (Dynax, Chantilly, VA) and peptide-negative plates were incubated overnight and washed three times with PBS before addition of 100 μl of 2.5% bovine serum albumin in PBS. Serially diluted plasma (1:40 to 1:80 in PBS) was added to MUC1-coated plates and incubated at room temperature. Plates were washed 5 times with 100 μl PBS and 0.1% Tween 20 detergent. Alkaline phosphatase-labeled goat anti-human polyvalent IgM, IgG, IgA (50 μl) (Sigma-Aldrich, St. Louis, MO) diluted 1:1,000 was added before plates were again washed 5 times with PBS-Tween. Alkaline phosphatase substrate pNPP (100 μl) (Sigma-Aldrich) was added. Plates were incubated before the stop solution (0.5 mol/L NaOH) was added. We used the MRX Revelation plate reader (Thermo Labsystems, Chantilly, VA) to read absorbance values at 405 to 410 nm, which were subtracted from absorbance values obtained from antigen-negative plates to account for nonspecific binding.

Laboratory personnel were blinded to case/control status of the samples. Blood specimens from cases and their matched controls were assayed on the same plate, and each plate included two to eight quality control samples. The within-plate coefficient of variation (CV) was 9%, showing excellent reproducibility, and the between-plate CV was 23% for absorbance values at the 1:40 dilution and 9 and 24% at the 1:80 dilution. The Spearman rank correlation between the two dilutions was high (ρ=0.93, p <0.001); readings at the 1:40 dilution were used in this analysis. Cases and their matched controls were assayed together and on the same plate.

Statistical Analysis

The distribution of anti-MUC1 antibody levels among subjects was skewed right; therefore, values were natural-log transformed for all analyses. No antibody values were identified as outliers (15). Generalized linear models were used to compare mean anti-MUC1 antibody levels across categories of factors possibly associated with antibodies, adjusting for age and assay plate, among controls and characteristics of the cancer among cases. The estimated number of ovulatory cycles for each woman was calculated by subtracting age at menarche, one year for each term pregnancy, duration of oral contraceptive use and breastfeeding from age at menopause (or age if premenopausal) and multiplying by 12, as previously described (16).

We used conditional logistic regression to estimate relative risks (RR) and 95% confidence intervals (95% CI) comparing quartiles (based on the control distribution) of anti-MUC1 antibody levels. To examine whether the association between antibody levels and ovarian cancer risk differed according to categories of age at blood collection, we compared the likelihood of models with and without an interaction term between quartiles of antibody levels weighted by median antibody levels (modeled continuously) and age (in years). All p-values were two-sided. Analyses were conducted on SAS 9.1 version (SAS Institute, Cary, NC).

Results

The average age at blood collection was 57 years for both cases and controls and the average age at ovarian cancer diagnosis for cases was 65 years (Table 1). Compared to controls, women who developed ovarian cancer were more likely to be nulliparous, have a family history of ovarian cancer, and have relatively shorter durations of OC use.

Among controls, women in the top quartile of age at blood collection (*i.e.* ≥64 years) had significantly lower levels of antibodies compared to younger women (p=0.03) (Table 2).

Antibody levels also decreased with increasing number of estimated ovulatory cycles (*p*-trend=0.04), but were higher among controls with a history of tubal ligation (*p*=0.03). Antibody levels were slightly, but not significantly, lower among controls whose age at menopause was ≥ 51 years or had regularly used talc in genital hygiene.

Among cases, women whose age at blood collection was ≥64 had a non-significantly higher level of antibodies than those whose age at collection was younger (p=0.28) (Table 3). No significant differences in antibodies were observed between invasive or borderline tumors or by histology. Women who went on to develop late versus early stage disease tended to have higher antibody levels (p=0.04) and women who became cases more than 11 years after their blood was drawn had higher antibody levels than those diagnosed earlier in the follow-up period (p=0.01). As might be expected, only 5 (17%) of the 29 women with the shortest interval to diagnosis (and lowest anti-MUC1 antibody levels) were diagnosed after age 65 compared to 20 (67%) of the 30 women with longest interval to diagnosis (and highest antibody levels) (p<0.0001). None of the factors found to affect antibody levels in controls were significant in the cases (data not shown).

Among all women, those with antibody levels in the top quartile had a non-significant 28% lower risk of developing ovarian cancer compared to those in the lowest quartile (95% CI (0.39, 1.31)); there was no significant trend across quartiles (*p*-trend=0.30) (Table 4). However, there was a statistically significant difference in the association by age at blood draw (*p*-interaction=0.005). Since the RRs in quartiles two through four were similar, we collapsed these quartiles to increase power in the analysis stratified by age. Among those <64 at blood collection (n=90 cases and 257 controls), the RR for ovarian cancer comparing the quartiles 2–4 versus 1 was 0.53 (95% CI (0.31, 0.93); *p*-trend=0.03). The comparable RR in women ≥64 years at blood draw (n=27 cases and 82 controls) was 2.11 (95% CI (0.73, 6.04); *p*-trend=0.05). Neither adjusting for known risk factors of ovarian cancer (e.g. oral contraceptive use, parity, and tubal ligation) nor excluding NHSII participants (6 cases and 8 controls) altered the results (data not shown).

Discussion

In this first-of-kind study, we prospectively evaluated the relationship between circulating anti-MUC1 antibody levels and risk of ovarian cancer. We observed a statistically significant interaction by age for the association between anti-MUC-1 antibodies and risk of ovarian cancer, with an inverse association among women <64 years at assessment (i.e., the controls had higher antibody levels on average than the cases) and a positive association in those ≥64 years (i.e., the controls had lower antibody levels on average than the cases). A decline of anti-MUC1 antibodies in healthy individuals with increasing age has been observed previously (4,9). This may be due to a mechanism called immunosenescence, which reflects a declining immune response toward many common antigens with increasing age and time since antigen presentation, such as after immunization (17,18). Many of the exposures that we have postulated could lead to protective antibodies occur during the reproductive years and their effect could wane with an increasing time interval from those events. This interpretation would be compatible with data from published epidemiologic studies that the protective effect of OC use (19,20) and possibly with breast feeding (21) and tubal ligation (22), attenuates over time. In addition, the drop in antibody levels among controls matches age-specific increases in risk for ovarian cancer in the population; i.e. about a twofold change between women <64 compared to those ≥64, (27 vs. 56 cases per 100,000 in NHS data).

The events associated with antibody levels among controls found in our study include known risk factors for ovarian cancer. Notably, women who had a tubal ligation had about 20% higher antibody levels compared to those who had not; this is similar to a prior finding among 705

healthy women (9). Explanations for the protective effect of tubal ligation on ovarian cancer risk have focused on blockage of potentially harmful contaminants from reaching the ovaries; however, our results suggest the injury associated with tubal ligation may trigger the release of MUC1 into the circulation and development of protective anti-MUC1 antibodies.

Increasing number of ovulatory cycles, which has been linked with higher risk for ovarian cancer, was associated with lower antibody levels in this study and in one prior study examining this association (23). The ability of uninterrupted ovulations to lower anti-MUC1 antibody levels may be due to an absence of reproductive events, like OC use and breastfeeding that may have otherwise elicited antibody production. In addition, MUC1 has higher expression in secretory endometrium (24) and circulating levels are highest during the luteal phase (25). Thus, it is possible that repeated cyclic variation of MUC1 in blood over many years could lead to an immune tolerance that ultimately lowers anti-MUC1 antibody levels.

A prior study of 705 women without ovarian cancer noted associations between anti-MUC1 antibody levels and other factors, including OC use, hysterectomy, breastfeeding, bone fracture, and talc use (9). Although we did not observe statistically significant associations for these exposures in this study (possibly due to the smaller number of controls), the direction of these relationships were similar to the prior study. For example, talc use was associated with lower anti-MUC1 antibody levels in both studies, fitting with its role as a factor that has been shown to increase risk for ovarian cancer (26–28). Like ovulatory cycles, regular talc use may represent an exposure that can trigger a chronic inflammatory response, lead to MUC1 antigenemia, and result in MUC1-specific immune tolerance. Clearly, additional research is necessary to fully understand how “acute” events translate into protection and “chronic” events into increased risk and how these may relate to difference in the association by age. We believe this will require an understanding of the effects of the events and age on cellular immune reactions that may be most critical for cancer elimination or escape.

We suggested that the decline in anti-MUC1 antibodies in controls reflected immunosenescene. Clearly, the higher antibody levels observed among cases who were ≥age 64 at blood draw (Table 3) requires a different interpretation. First, we point out that the level of antibodies in the oldest age group was about the same as the younger controls. This may indicate that there is a subset of women who do not have the expected decline in antibody levels as they age, perhaps due to chronic antigen challenge provided by an ongoing chronic inflammatory condition or a developing cancer. In addition, we note that cases who were ≥age 64 at blood draw had an age at diagnosis of 75 and overlapped with the group in Table 3 who had the greatest lag between age at blood draw and cancer diagnosis and highest antibody levels. Unfortunately, our data cannot clarify whether the higher antibody levels in older cases represent a persistence of high antibodies that may delay onset of disease or new appearance of antibodies reflecting a chronic inflammatory response. Sorting the possibilities out will require larger studies with serial measurements of anti-MUC1 antibodies levels across the important mid-60’s age period. Besides anti-MUC1 antibodies, it may be necessary to measure free MUC1 antigen and immune complexes involving the antigen and antibodies. These have been described and could interfere with assays for either free antigen or free antibody (30). A comprehensive study might also establish whether the appearance (or persistence) of high levels of anti-MUC1 antibodies in later life might represent an early detection signal many years in advance of ovarian cancer.

In the current study, we focused our attention on MUC1 because of the wealth of data that exists on its expression in normal tissues versus cancer, inflammation, and other pathological conditions (1,31). We measured antibodies against the unglycosylated protein core of MUC1, commonly found in tumors. However, there are other aberrantly glycosylated MUC1 epitopes closely associated with tumor MUC1 that might be even more immunogenic eliciting additional

protective immunity (32,33). It also is possible that additional ovarian cancer antigens can be presented to the immune system through other non-malignant events and lead to the creation of antibodies and cellular immunity that would also be associated with a reduced risk for ovarian cancer. MUC1 thus represents one of many targets of immunosurveillance that collectively can elicit protective immunity and lower cancer risk. Development of new high throughput technologies may allow identification of antibodies that are present in circulation before ovarian cancer diagnosis and the exposures or events that are associated with increased antibody levels and define serologic signatures of cancer risk reduction. Further, understanding how antibodies against MUC1 or other antigens are formed during exposures or events that predate ovarian cancer development may suggest ways to duplicate or amplify these protective effects through vaccination.

A limitation of this study is that we were unable to measure MUC1-specific memory T cells due to the lack of archived viable white cells. However, T-cell activation is indirectly measured through the presence of various isotypes of anti-MUC1 antibodies, all of which require antigen-specific helper T cells for their generation. We also lacked information on certain exposures that previously have been associated with anti-MUC1 antibody levels, such as mastitis, mumps parotitis, and endometriosis. The relatively small number of cases in this study clearly limited power to examine the effect of antibodies by histologic type of ovarian cancer or by other potential effect modifiers; and the relatively high between-plate CVs, often seen for immune-based assays like that used here, also contributed to a lack of precision.

Although ours is the first prospective study investigating anti-MUC1 antibodies in relation to ovarian cancer, one study examined humoral immune responses to MUC1 among BRCA carrriers and related it to subsequent breast cancer, another MUC1-expressing cancer (34). Women who carried a mutation had significantly lower anti-MUC1 Ig antibodies compared to controls; and carriers who went on to develop breast cancer had lower (although not significantly so) antibody levels than carriers not found to have cancer at prophylactic mastectomy. Thus, this study fits with our observations in suggesting that anti-MUC1 antibodies correlate with a known risk factor for the cancer and risk for developing disease.

In conclusion, our prospective study provides important evidence that circulating anti-MUC1 antibody, either as an effector mechanism or as a marker of a more comprehensive immune response, may be associated with a lower risk of ovarian cancer in women <64 years and offers an explanation for the mechanism of action of several factors known to alter risk of ovarian cancer. These data further suggest a new paradigm that immune surveillance may play an important role in ovarian carcinogenesis. Larger studies are necessary to clarify the role of MUC1 immunity in ovarian cancer, particularly in relation to the potential differential association by age at antibody assessment. Ultimately this avenue of research could lead to new methods for ovarian cancer prevention or early detection.

Acknowledgments

We thank the participants in the Nurses' Health Studies for their continued participation and dedication.

This research was supported by the National Institutes of Health [grant numbers CA105009 and CA123170 to DWC, CA49449 and CA67262 to SHE and P01 CA73743 to OJF].

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Table 1

Characteristics of cases and controls at time of blood collection.

	Controls	Cases
Sample size	*339*	*117*
Age (mean, SD), years*	57 (7)	57 (7)
Age at diagnosis (mean, SD), years	n/a	65 (8)
Postmenopausal at blood collection (%)*	61	62
Postmenopausal at time of diagnosis (%)*,†	87	88
Use of postmenopausal hormones within three months of blood collection (%)*,‡	45	47
Height (mean, SD), inches	65 (4)	65 (3)
BMI (mean, SD), kg/m^2	25 (5)	25 (5)
Age at menarche (mean, SD), years	13 (1)	13 (1)
Parous (%)	96	91
Number of live births (mean, SD)§	3 (1)	3 (2)
Age at first birth (mean, SD), years§	25 (3)	25 (4)
Ever breastfed (%)§	67	61
Duration of oral contraceptive use (mean, SD), months§§	55 (47)	44 (40)
Tubal ligation (%)	20	18
Hysterectomy (%)	21	26
Age at natural menopause (mean, SD), years	51 (3)	51 (3)
Number of lifetime ovulations (mean, SD)	379 (67)	389 (64)
Weekly use of perineal talcum powder (%)	22	26
Family history of breast cancer (%)	9	9
Family history of ovarian cancer (%)	3	9

*Cases and controls were matched on these factors.

†For controls, menopausal status at the time of case's ovarian cancer diagnosis.

‡Among postmenopausal women at blood collection.

§Among parous women.

§§Among women who ever used OC for at least 3 months.

Table 2

Association between ovarian cancer risk factors and anti-MUC1 antibodies among 339 controls from the Nurses' Health Studies.

	N (%)	Geometric mean*	*p-value*†
Anti-MUC1 Antibody		0.57	
Age at blood draw (quartiles)			
<51 years	73 (22)	0.60	
51–56 years	98 (29)	0.60	
57–63 years	86 (25)	0.61	
64+ years	82 (24)	0.48	*0.03*
BMI			
<30 kg/m^2	195 (58)	0.59	
30+ kg/m^2	143 (42)	0.55	0.23
Height			
<65 inches	162 (48)	0.58	
65+ inches	177 (52)	0.57	0.87
Age at menarche			
< 13 years	151 (45)	0.58	
13+ years	188 (55)	0.57	0.93
Use of Oral Contraceptive			
Never	183 (54)	0.55	
Ever	156 (46)	0.61	0.13
Tubal ligation			
No	271 (80)	0.55	
Yes	68 (20)	0.66	*0.03*
Ever used IUD			
No	317 (94)	0.58	
Yes	22 (6)	0.54	0.62
Hysterectomy			
No	267 (79)	0.57	
Yes	72 (21)	0.59	0.67
Age at first birth (tertiles)§			
<23 years	88 (27)	0.59	
23–24 years	94 (29)	0.56	
25+ years	143 (44)	0.56	0.49
Number of live births§			
0	14 (4)	0.66	
1 or 2	105 (31)	0.54	
3 or 4	165 (49)	0.59	
5 or more	55 (16)	0.59	0.70
Breastfeeding§			
Never	105 (33)	0.54	

	N (%)	Geometric mean*	*p-value*†
Ever	218 (67)	0.59	0.16
Age at menopause			
<51 years	61 (30)	0.62	
51+ years	144 (70)	0.53	0.10
Current PMH use§§			
No	113 (55)	0.55	
Yes	93 (45)	0.56	0.80
Number of ovulatory months (quartiles)¶			
<339	71 (24)	0.63	
339–389	73 (25)	0.61	
390–426	73 (25)	0.55	
427+	73 (25)	0.51	*0.04*
Bone fractures/osteoporosis**			
No	285 (89)	0.56	
Yes	36 (11)	0.60	0.58
Talc use (in 1982)**			
Less than weekly	250 (78)	0.59	
At least weekly	71 (22)	0.51	0.09

*Adjusted for age at blood collection in years (continuous) and by assay plate (indicators).

†P-trend from linear regression modeling categorical variables linearly, adjusting for age (continuous) and assay plate (indicators).

§Among parous women.

¶Among women with known menopause status.

**Available in NHS only.

Table 3

Association between ovarian cancer characteristics and anti-MUC1 antibodies among 117 cases from the Nurses' Health Studies.

	N (%)	Geometric mean*	*p-value*†
Anti-MUC1 Antibody		0.54	
Age at blood draw (quartiles)			
<51 years	25 (21)	0.52	
51–56 years	34 (29)	0.50	
57–63 years	31 (27)	0.52	
64+ years	27 (23)	0.63	0.28
Invasiveness			
Borderline	12 (11)	0.49	
Invasive	99 (89)	0.53	0.65
Histologic type			
Serous borderline	6 (6)	0.54	0.89
Serous invasive	63 (66)	0.52	Ref.
Mucinous	12 (13)	0.43	0.33
Endometrioid	11 (11)	0.52	0.96
Clear cell	4 (4)	0.54	0.88
Stage			
Early (stages I–II)	40 (35)	0.45	
Late (stages III–IV)	75 (65)	0.59	*0.04*
Time between blood draw and diagnosis			
36–77 months	29 (25)	0.38	
78–102 months	29 (25)	0.59	
103–135 months	29 (25)	0.53	
136+ months	30 (25)	0.70	*0.01*

*Adjusted for age at blood collection in years (continuous) and by assay plate (indicators).

†P-trend from linear regression modeling dichotomous and ordinal variables linearly or as indicators for histologic type, adjusting for age (continuous) and assay plate (indicators).

Table 4

Relative risk of ovarian cancer by quartiles of anti-MUC1 antibodies (optical density readings at 1:40) in the Nurses' Health Studies

	Quartiles of anti-MUC1 antibodies *					
	Q_1	Q_2	Q_3	Q_4	*P*-trend†	Q_1 vs. $Q_{2,3,4}$
All women						
Cases, N(%)	36(31)	28(24)	27(23)	26(22)		81(69)
Controls, N(%)	84(25)	86(25)	84(25)	85(25)		255(75)
RR (95% CI) ‡	1.00	0.73 (0.40, 1.34)	0.74 (0.41, 1.35)	0.72 (0.39, 1.31)	*0.30*	0.73 (0.45, 1.18)
Age < 64 §						
Cases, N(%)	29(32)	23(26)	20(22)	18(20)		61(68)
Controls, N(%)	52(20)	68(26)	63 (25)	74(29)		205(80)
RR (95% CI) ‡	1.00	0.60 (0.30, 1.19)	0.58 (0.29, 1.15)	0.45 (0.23, 0.91)	*0.03*	0.53 (0.31, 0.93)
Age ≥ 64 §						
Cases, N(%)	7(26)	5(19)	7(26)	8(30)		20(74)
Controls, N(%)	32(39)	18(22)	21(26)	11(13)		50(61)
RR (95% CI) ‡	1.00	1.36 (0.35, 5.23)	1.83 (0.50, 6.73)	3.95 (1.05, 14.9)	*0.05*	2.11 (0.73, 6.04)

* Median anti-MUC1 antibody levels in quartiles 1,2,3 and 4 were 0.29, 0.47, 0.68, and 1.16, respectively (among all women regardless of age).

† Wald statistic P-values modeling antibody quartiles weighted by their median antibody values linearly.

‡ Conditional logistic regression stratified on matching factors (age, menopause status, date and fasting status at blood collection, and menopause status at time of cases' diagnosis).

§ Likelihood ratio test P-value for interaction between antibodies (weighted quartiles modeled linearly) and age (in years) was 0.005.