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Oxidative stress and endometriosis

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BACKGROUND: Little is known about the aetiology of endometriosis; however, in the presence of oxidative stress, reactive oxygen species might increase growth and adhesion of endometrial cells in the peritoneal cavity, leading to endometriosis and infertility. Within a study investigating persistent organic compounds and endometriosis, the authors evaluated the association between oxidative stress and endometriosis. METHODS: Women aged 18–40 years who were undergoing laparoscopy were contacted to participate in the study ($n = 100$); 84 were eligible and agreed to be interviewed; 78 provided blood specimens. Four markers of oxidative stress and antioxidant status were measured in serum for 61 women. Multiple imputation of missing data was used to generate values for the missing oxidative stress data. RESULTS: Thirty-two women had visually confirmed endometriosis at laparoscopy while 52 did not, including 22 undergoing tubal ligation and 30 with idiopathic infertility. There was a weak association between thiobarbituric acid-reactive substances (nmol/ml) and endometriosis, after adjusting for age, body mass index, current smoking, hormone use in the past 12 months, gravidity, serum vitamin E, serum estradiol, and total serum lipids ($\beta = 1.18$; 95% CI −0.04, 2.39). CONCLUSIONS: These results suggest that oxidative stress might play a role in the development and progression of endometriosis, which should be evaluated in larger studies.

Key words: endometriosis/infertility/oxidative stress/thiobarbituric acid-reactive substances

Introduction

Endometriosis is a common gynaecological disorder characterized by the growth of endometrial glands and stroma outside the uterus. Approximately 10% of women of reproductive age in the USA are diagnosed with endometriosis (Wheeler, 1989) with lesions present in 20–50% of women undergoing laparoscopy for infertility (Matorras *et al.*, 1995). Little is known about the aetiology of endometriosis; however, it has been suggested that reactive oxygen species (ROS) or free radicals may increase growth and adhesion of endometrial cells in the peritoneal cavity, promoting endometriosis and infertility (Portz *et al.*, 1991; Murphy *et al.*, 1998).

Under normal conditions, antioxidants such as vitamin E, vitamin C and β-carotene counteract the effect of free radicals. When an imbalance exists and there is an excess of free radicals, oxidative stress arises, resulting in damage to circulating lipoproteins, proteins, carbohydrates and nucleotides (Slater, 1984). Oxidative stress has been associated with numerous adverse health effects including atherosclerosis (Schisterman *et al.*, 2001), pre-eclampsia (Madazli *et al.*, 2002), and male and female infertility (Agarwal *et al.*, 2003). Two studies have found a positive association between oxidative stress and endometriosis (Shanti *et al.*, 1999; Szczepanska *et al.*, 2003), whereas others have not found an association (Arumugam and Dip, 1995a; Ho *et al.*, 1997; Wang *et al.*, 1997). These studies have differed greatly in many regards including selection of the control population, eligibility criteria, markers of oxidative stress and antioxidant status, and the biological medium in which oxidative stress was measured, making it difficult to come to a definitive conclusion about this association.

Given the limited data and complex environment of the peritoneal cavity, it is unclear when and why oxidative stress may occur in relation to endometriosis; however, there are several different hypotheses. It has been suggested that the presence of iron (Arumugam and Yip, 1995b), macrophages (Murphy *et al.*, 1998), and/or environmental contaminants such as polychlorinated biphenyls (PCB) (Donnez *et al.*, 2002; Van Langendonckt *et al.*, 2002) in the peritoneal fluid may induce oxidative stress leading to tissue growth and endometriosis. In addition, circulating levels of oxidative stress due to other causes may further induce endometriosis. To evaluate this hypothesis, the investigators assessed the association between serum levels of oxidative stress and endometriosis among women undergoing laparoscopy within a study investigating the association between persistent organic compounds and endometriosis (Buck Louis *et al.*, 2005).

Materials and methods

Study population

Women who were aged 18–40 years and undergoing laparoscopy at one of two participating university-affiliated hospitals were identified for recruitment between April 1999 and January 2000. Among 100 women recruited for the study, 84 (84%) were eligible and consented to participation in the study.

Women were interviewed by a trained research assistant/phlebotomist to obtain information on sociodemographic factors, reproductive and medical history, lifestyle characteristics, and limited diet information. At the time of the interview, 78 (94%) women donated ~20 ml of blood. Specimens were placed on ice and transported immediately to the Toxicology Research Center at the University of Buffalo after the interview. Specimens were collected for the purpose of measuring PCB levels in serum and the remaining sample was used for measuring markers of oxidative stress and antioxidant status for the current study.

In order to minimize detection bias, laparoscopic surgeons were blinded on exposure status and instructed to do a complete examination for endometriosis during surgery regardless of the surgery purpose (i.e. tubal ligation, infertility, pelvic pain). Surgeons completed standardized operative reports designed for the study, which noted the presence or absence of endometriosis, the stage of endometriosis according to the revised American Fertility Society (AFS) criteria, and other gynaecological pathology. Thirty-two women had visually confirmed endometriosis at laparoscopy and 52 did not have endometriosis. Indication for laparoscopy among endometriosis cases included infertility, pelvic pain, pelvic mass, and tubal ligation. Among the 52 without disease, 22 had surgery for tubal ligation and 30 for idiopathic infertility. Institutional Review Board approval was given for the conduct of the study.

Laboratory methods

In 2003, markers of oxidative stress and antioxidant status were measured in serum for 61 women. Blood samples could not be analysed for 17 participants, as insufficient sample was available.

All chemicals, including high-performance liquid chromatography (HPLC) grade solvents, were purchased from Sigma Chemical Co. (USA). The concentration of haemoglobin from erythrocytes was measured using the cyanmethaemoglobin method (Fairbanks and Klee, 1987). Serum samples previously stored at −80°C for ~4 years were thawed at 4°C, assayed in duplicate and quantified from appropriate standard curves as described below. Instrumentation included a Cobas Mira or FARA 2 automated liquid-handling chemistry system (Roche Diagnostic Systems, Inc., USA), an RF 5000 spectrofluorometer, a UV-160 spectrometer and a LC-10A HPLC system (Shimadzu Corp., Japan). We selected four biomarkers of oxidative stress and antioxidant status from 40 that are commonly used (Dotan *et al.*, 2004) and are believed to measure the main targets in the biochemical pathways involved in oxidative stress. These include: (i) thiobarbituric acid-reacting substances (TBARS), which measure primarily malondialdehyde derived from lipid peroxidation, as well as other breakdown products from oxidatively modified proteins, carbohydrates and nucleic acids (Guichardant *et al.*, 2004); (ii) 8-F_2-isoprostane for a stable end-product of oxidized lipids derived from arachidonic acid (Fam and Morrow, 2003); (iii) fat-soluble antioxidants (vitamin A, vitamin E, β-carotene and lycopene) reflecting micronutrient antioxidant protection in serum; and (iv) paraoxonase (PON1) activity. PON1 is a 44 kDa glycoprotein synthesized in the liver and carried exclusively in the circulation by high-density lipoprotein (HDL) and is the component of HDL responsible for the ability of HDL to protect cellular membranes and lipoproteins from oxidative modification (Mackness *et al.*, 2000). PON1 has the ability to hydrolyse and detoxify oxidatively modified lipids (Aviram *et al.*, 2000) and its activity is a marker of antioxidant protection against lipid peroxidation (Watson *et al.*, 1995).

TBARS were measured on 100 μl samples using a commercial kit (OXI-TEK, cat. No. 0801192) purchased from ZeptoMetrix Corp. (USA). This assay is adapted from the Yagi (1982) procedure and is an indirect screening test of total oxidative stress (Armstrong and Browne, 1994). After incubation with thiobarbituric acid at 95°C for 30 min, samples were measured by fluorometer with excitation set at 530 nm and emission at 550 nm. Sensitivity was set high with a slit width of 5 nm. Concentration is expressed in nmol/ml of malondialdehyde equivalents. Total (free and esterified) 8-F_2-isoprostane was measured using a commercial kit (cat. No. 516351) purchased from Cayman Chemical Co. (USA). Samples of 250 μl were treated with potassium hydroxide, extracted with ethanol and purified through an affinity column (cat. No. 416358 Cayman Chemical Co.), eluted and analysed by enzyme immunoassay technology. Concentration is expressed in pg/ml. Fat-soluble antioxidants were measured by HPLC. Samples of 300 μl were mixed with equal volumes of ethyl alcohol containing internal standard (α-tocopherol acetate) and extracted twice with 2 ml of hexane, the upper organic phase removed, evaporated to dryness under nitrogen, reconstituted in 300 μl of mobile phase containing 60% acetonitrile–25% methanol and 15% ethylene chloride, sonicated and 60 μl injected onto a Supelco C 18 column with Supelcoguard pre-column and the vitamins separated as previously described (Browne and Armstrong, 1998). Absorbance data were obtained from a photodiode array spectrometer set to simultaneously record at 292 nm for α-tocopherol, 326 nm for retinol and 452 nm for carotenoids and then quantified with Shimadzu Spectrum Max Plot Class-VP software. Concentration is expressed in μg/ml. Verification of accuracy was obtained from the National Institute of Standards and Technology Micronutrients Measurement Quality Assurance Program (USA). Paraoxonase activity was measured as an indicator of protection against lipid peroxidation. The organophosphate activity closely parallels the antioxidant activity of PON and the hydrolysis of several organophosphate compounds is indicative of PON activity. We performed an enzyme kinetic assay measuring the rate of formation of *p*-nitrophenol using 1 mmol/l paraoxon as the substrate in 50 mmol/l glycine buffer, pH 10.5, containing 1.0 mmol/l $CaCl_2$. Serum samples were diluted 1:20 in 25 mmol/l triethanolamine (TEA) hydrochloride containing 1.0 mmol/l $CaCl_2$ and the reaction initiated by addition of 20 μl diluted sample to 360 μl working paraoxon reagent. The rate of *p*-nitrophenol was measured at 405 nm over 200 s. with a 25 second lag time. One unit (IU) of paraoxonase activity is defined as 1 μmol/l of *p*-nitrophenol formed per minute. Activity is expressed as IU/l based on the molar absorptivity of *p*-nitrophenol (molar extinction coefficient = 18, 290).

Statistical analysis

Multiple imputation of missing data was performed to impute missing values for the 17 subjects with missing oxidative stress data. Subjects with and without oxidative stress data did not differ substantially and it was assumed all data were missing at random. The expectation maximization (EM) algorithm was used to impute 20 complete datasets using the following predictive variables (% missing): case status, age, body mass index (BMI) (1%), total lipids (5%), current smoking status, current vitamin use, hormone use in the past 12 months, race, education, gravidity, TBARS (27%), 8-F_2-isoprostane (29%), paraoxonase activity (27%), vitamin A (31%), vitamin E (31%), β-carotene (31%), lycopene (31%), and estradiol

(27%). Twenty imputations provide 98.52% efficiency when data are missing for 30% of participants (Yuan, 2000). Each dataset was analysed using Student's *t*-test to compare mean levels of markers of oxidative stress and antioxidant status between women with and without endometriosis. Multivariate logistic regression was used to adjust for potential confounders including age, body mass index, current smoking, hormone use in the past 12 months, gravidity, serum estradiol, and total serum lipids. Models for TBARS and 8-F_2-isoprostane were also adjusted for serum vitamin E, as it was significant in univariate comparisons and was considered a more accurate measure of current vitamin use than self-reported vitamin use. Though significant in univariate comparisons, education level was not included in the final models as it did not contribute significantly. The results of each model were combined using SAS MIANALYZE (SAS, 1999), which averages the point estimates from each dataset to obtain a pooled estimate and adjusts the variance for the within- and between-dataset variance. Independent imputations were done for each case–control comparison: (i) women with endometriosis versus women without endometriosis; (ii) women with endometriosis versus women with idiopathic infertility; and (iii) women with endometriosis versus women having tubal ligation. An attempt to compare the 17 women with mild, moderate or severe endometriosis to those with minimal endometriosis ($n = 15$) was made; however, given the small sample size the numbers were unstable and are not reported here. Sensitivity analysis for misclassification of outcome status was also undertaken. SAS 8.0 and Stata 8.0 were used for all analyses.

Ad hoc power for the study was ~87% for the detection of a 25% increase in TBARS in the case group, assuming an $\alpha = 0.05$ and that TBARS in the control group was ~1.3 nmol/ml (Trevisan *et al.*, 2001).

Results

Women with endometriosis were significantly more likely to have a college education, to have a lower mean BMI, to be taking vitamins, to have used hormones in the past 12 months, to have an older age at menarche, and to be nulligravida than women without endometriosis (Table I). Women with endometriosis were significantly less likely to be a current smoker than unaffected women.

Women with endometriosis had significantly higher mean levels of vitamin E (12.2 versus 10.1 mg/ml; $P = 0.04$), but appeared to have lower mean levels of paraoxonase activity (195.4 versus 223.1 IU/l; $P = 0.09$) compared to women without disease (Table II). Mean vitamin E levels were significantly higher ($P = 0.02$) and mean 8-F_2-isoprostane levels significantly lower ($P = 0.02$) among women with disease compared only to women undergoing tubal ligation ($n = 22$).

In multivariate logistic regression, increasing levels of TBARS were weakly associated with having endometriosis after adjusting for age, BMI, current smoking, hormone use in the past 12 months, gravidity, serum vitamin E, serum estradiol, and total serum lipids (Table III). A 0.5 nmol/ml increase in TBARS was associated with an 80% increased odds of endometriosis compared to women without

Table I. Characteristics of the study population

	Endometriosis ($n = 32$)		No endometriosis ($n = 52$)		*P*
Age (years), mean (SE)	32.7 (0.78)		31.6 (0.69)		0.30
Body mass index (kg/m^2), mean (SE)	23.7 (0.68)		26.9 (0.86)		0.006
Race					
White	29	(90.6)	46	(88.5)	
Non-White	3	(9.4)	6	(11.5)	0.76
Marital status					
Married	23	(71.9)	35	(67.3)	
Other	9	(28.1)	17	(32.7)	0.66
Education					
≤12 years	4	(12.5)	23	(44.2)	
13–16 years	19	(59.4)	24	(46.2)	
>16	9	(28.1)	5	(9.6)	0.004
Annual household income					
<$30 000	7	(21.9)	20	(38.5)	
$30 000–$59 999	8	(25.0)	14	(26.9)	
≥$60 000	17	(53.1)	18	(34.6)	0.19
Current smoker	4	(12.5)	24	(46.2)	0.002
Currently taking vitamins/mineral supplements	26	(81.3)	30	(57.7)	0.03
Hormone use in past 12 months	23	(71.9)	20	(38.5)	0.003
Age at menarche					
<12 years of age	2	(6.3)	16	(30.8)	
12years of age	8	(25.0)	10	(19.2)	
>12 years of age	22	(68.8)	26	(50.0)	0.03
Gravidity					
Nulligravida	22	(68.8)	19	(36.5)	
Gravida	10	(31.3)	33	(63.5)	0.004
Revised AFS endometriosis staging					
Minimal	15	(46.9)	NA		NA
Mild	5	(15.6)			
Moderate	6	(18.8)			
Severe	6	(18.8)			

Values are *n* (%) unless otherwise indicated.
AFS = American Fertility Society; NA = not applicable.

Table II. Mean (SE) values for measures of oxidative stress and antioxidants among women with and without endometriosis upon laparoscopy

	Endometriosis ($n = 32$)		No endometriosis ($n = 52$)	
TBARS (nmol/ml)	2.63	(0.38)	2.44	(0.32)
8-F_2-Isoprostane (pg/ml)	152.44	(15.41)	154.13	(9.73)
Paraoxonase activity (IU/l)[a]	195.42	(17.46)	223.07	(11.73)
Vitamin A (μg/ml)	0.56	(0.16)	0.56	(0.15)
Vitamin E (μg/ml)[b]	12.16	(0.91)	10.14	(0.73)
β-Carotene (μg/ml)	0.11	(0.12)	0.09	(0.10)
Lycopene (μg/ml)	0.25	(0.13)	0.27	(0.12)

[a]$P = 0.09$.
[b]$P = 0.04$.
TBARS = thiobarbituric acid-reacting substances.

Table III. Adjusted logistic regression estimates for the association between oxidative stress and endometriosis in comparison to all controls (cases $n = 32$; controls $n = 52$)

	β	(95% CI)
TBARS (nmol/ml)[a]	1.1763	(0.0400, 2.3926)
8-F_2-Isoprostane (pg/ml)[a]	0.0019	(−0.0040, 0.0079)
Paraoxonase activity (IU/l)[b]	−0.0002	(−0.0066, 0.0062)
Vitamin A (μg/ml)[b]	−2.9127	(−8.2809, 2.4556)
Vitamin E (μg/ml)[b]	0.2139	(−0.0624, 0.4902)
β-Carotene (μg/ml)[b]	0.3055	(−8.5686, 9.1796)
Lycopene (μg/ml)[b]	−0.0552	(−8.2635, 8.1532)

[a]Adjusted for age, body mass index, current smoking, serum vitamin E, serum estradiol, hormone use in the past 12 months, gravidity, and total serum lipids.
[b]Adjusted for age, body mass index, current smoking, serum estradiol, hormone use in the past 12 months, gravidity, and total serum lipids.

endometriosis (OR = 1.80; 95% CI 1.02, 3.18). No significant differences were found when separate analyses were conducted comparing women with endometriosis ($n = 32$) to women with tubal ligation ($n = 22$) and to women with idiopathic infertility ($n = 30$).

To determine the effect of potential misclassification of endometriosis status on the regression estimates, we assessed the β estimates by level of misclassification (5, 10 and 15%). There was no effect observed due to misclassification with the exception of lycopene and β-carotene, which both became more protective regardless of the direction of misclassification. We further evaluated the effect of misclassification by examining the effect on the β estimates if 20, 40 and 60% of our 'minimal' endometriosis cases ($n = 15$) were truly controls and found no substantial changes in the results.

Discussion

There is considerable evidence that retrograde menstruation is associated with endometriosis; however, it is not clear why some women with retrograde menstruation develop endometriosis whereas others do not. It may be that the presence of elements such as macrophages, iron or environmental contaminants disrupt the balance between ROS and antioxidants in the peritoneal fluid of some women, leading to oxidative stress and endometriosis (Arumugam and Dip, 1995a; Murphy *et al.*, 1998; Donnez *et al.*, 2002). Alternatively, overall circulating levels of oxidative stress in an individual may increase oxidative stress levels in the peritoneal fluid and further induce endometriosis. To this end, we observed an increased odds of endometriosis with increasing levels of TBARS in serum after adjusting for age, BMI, current smoking, hormone use in the past 12 months, gravidity, serum vitamin E, serum estradiol, and total serum lipids. However, other markers of oxidative stress and antioxidant status including 8-F_2-isoprostane, paraoxonase activity, vitamin A, lycopene, β-carotene and vitamin E were not found to be associated with endometriosis in adjusted analyses.

To date, published studies on the association between oxidative stress and endometriosis have been inconsistent. Two studies have found a positive association between oxidative stress and endometriosis. Szczepanska *et al.* (2003) reported that women with endometriosis had significantly lower levels of superoxide dismutase and glutathione peroxidase in peritoneal fluid compared to fertile control women. Both of these enzymes play an important role in the breakdown of free radicals and ROS, thereby preventing oxidative stress. Furthermore, women with endometriosis had significantly lower levels of antioxidants than women without endometriosis, and significantly higher levels of lipid peroxides. Shanti *et al.* (1999) found similar results in a study comparing women with endometriosis to women having tubal ligation, in which endometriosis was associated with significantly higher levels of lipid peroxide-modified rabbit serum albumin, malondialdehyde-modified low-density lipoprotein, and oxidized low-density lipoprotein as measured in serum compared to tubal ligation; however, no differences were detected in the peritoneal fluid. Ho *et al.* (1997) and Wang *et al.* (1997) found no association between oxidative stress markers (ROS and total antioxidants respectively) measured in peritoneal fluid and endometriosis compared to tubal ligation controls. Furthermore, Arumugam and Dip (1995a) found no significant differences in malondialdehyde levels measured in peritoneal fluid among women with moderate to severe endometriosis, women with minimal-to-mild endometriosis and women without endometriosis. While study results have varied, comparisons across studies have been difficult due to differences in eligibility criteria, selection of control groups, selection of oxidative stress markers, and the biological medium in which oxidative stress was measured. Furthermore, many studies have had limited power to detect a difference between cases and controls due to small sample sizes.

Our study was strengthened by our ability to control for potential confounders and preliminarily to investigate differences by comparison group. While some studies have excluded women who used hormones in the past 3 months (Ho *et al.*, 1997; Shanti *et al.*, 1999; Szczepanska *et al.*, 2003), or women with specific illnesses (Shanti *et al.*, 1999), in an attempt to control for potential confounders, none of the above studies controlled for age, smoking, parity or BMI, all of which have been associated with endometriosis and/or oxidative stress. Given our somewhat larger sample size, we were able to adjust for these important confounders in our analyses. However, we did not have sufficient power to compare women with mild, moderate or severe endometriosis to

women with minimal endometriosis. This comparison should be explored in future studies.

This is the first study to report on levels of 8-F_2-isoprostane, paraoxonase activity, vitamin A, β-carotene and lycopene in relation to endometriosis status. Furthermore, we measured multiple markers of oxidative stress and antioxidant status, while previous studies have only measured a select few.

Finally, it is unlikely our results can be explained by detection bias as the laparoscopic surgeons were blinded to exposure status and were instructed to do a complete examination for endometriosis on all women regardless of the indication for surgery (i.e. tubal ligation, infertility, pelvic pain). As a result, one woman indicated for tubal ligation was found to have endometriosis upon examination. The results are further strengthened by the sensitivity analysis that was undertaken to assess the potential effect of misclassification of disease status. The diagnosis and staging of endometriosis using the revised AFS staging is susceptible to both inter- and intra-rater variability and is dependent upon anatomical orientation during pelvic exploration, and therefore can result in misclassification of disease status, as well as staging of disease. However, we found no substantial differences when we examined how misclassification of endometriosis status would affect our results.

There are several limitations to the study, which must be considered when interpreting the results. First, in order to increase power, we combined two groups of women free of endometriosis for our control group including those having idiopathic infertility and those seeking tubal sterilization. If oxidative stress is associated with infertility regardless of endometriosis status, then any potential effect between oxidative stress and endometriosis may have been obscured by the inclusion of infertile controls. However, when we restricted analyses to women having tubal ligation and women with idiopathic infertility, independently, we found no significant differences for either group after adjustment, though small numbers limited the analyses.

Misclassification of exposure could have occurred for several reasons, thereby limiting our analysis. As serum samples were collected for other purposes, no agent was added to prevent auto-oxidation; therefore some auto-oxidation may have occurred, resulting in altered levels of oxidation and antioxidants given that our samples were ~4 years old. We believe such misclassification would have been non-differential and our results biased towards the null, as case and control specimens were stored and handled in a similar manner.

Levels of oxidative stress and antioxidant markers also may have been affected by the timing of blood collection, as it was not standardized in relation to the menstrual cycle and corresponding changes in estradiol, which is an antioxidant (Yagi and Komura, 1986; Sugioka *et al.*, 1987). We found that women with endometriosis had significantly higher levels of estradiol than women without disease, 249.54 and 197.12 pg/ml respectively ($P = 0.01$). If the higher estradiol levels observed in cases were related to menstrual cycle fluctuations and resulted in decreased oxidation, our results may have been biased towards the null; however, our results did not change after adjusting for estradiol levels. Alternatively, the higher estradiol levels may reflect the higher estradiol levels generally seen in endometriosis cases (Ho *et al.*, 1997; Bulun *et al.*, 2002). Furthermore, several studies have found no differences between follicular and luteal phase TBARS levels and certain antioxidants, suggesting they are not affected by changes in ovarian hormones (Chung *et al.*, 1999; Lutoslawska *et al.*, 2001, 2003).

The type of biospecimen (serum versus peritoneal fluid) may have resulted in potential exposure misclassification as well. Previous studies have found oxidative stress and antioxidant biomarkers present in both serum and peritoneal fluid (Murphy *et al.*, 1998). Oxidative stress in peritoneal fluid is initiated in inflammatory cells with cellular debris serving as a substrate, and products of this process are exported to serum/plasma where the oxidized metabolites are incorporated into carriers, for example ox-LDL where they modify lipids, proteins and carbohydrates in the peripheral circulation (Jahn and Spiteller, 1996; Steinberg, 1997). Murphy *et al.* (1998) found significantly lower levels of vitamin E in the peritoneal fluid than in plasma, suggesting that the peritoneal cavity has less antioxidant protection than serum. Consequently, peritoneal fluid might be more susceptible to oxidative stress than serum. As we measured markers of oxidative stress in serum and not peritoneal fluid, our results may have been biased towards the null. Furthermore, oxidative stress levels in serum may represent oxidative stress due to other causes in addition to endometriosis, while measures in peritoneal fluid provide a more localized measure of oxidative stress related to endometriosis. To our knowledge, there have been no studies on the correlation of the levels of oxidative stress in peritoneal fluid and in the blood.

Finally, there may be a more appropriate measure of oxidative stress in evaluating the association with endometriosis depending on the biological mechanism. We selected four biomarkers, which measure main points in the biochemical pathways involved in oxidative stress. These include: (i) TBARS which measures primarily malondialdehyde derived from lipid peroxidation, as well as other breakdown products from oxidatively modified proteins, carbohydrates and nucleic acids (Guichardant *et al.*, 2004); (ii) 8-F_2-isoprostane for a stable end-product of oxidized lipids derived from arachidonic acid (Fam and Morrow, 2003); (iii) fat-soluble antioxidants reflecting antioxidant protection in serum; and (iv) paraoxonase activity as an indicator of protection against lipid peroxidation. We chose 8-F_2-isoprostane and TBARS as they represent early and late markers of oxidative stress respectively. Furthermore, all the chosen markers were thought to be important markers of oxidative stress related to exposure to lipophilic, persistent organic compounds, which were of interest in the overall study (Prakasam *et al.*, 2001; Fadhel *et al.*, 2002; Costa *et al.*, 2003). We may not have found an association with 8-F_2-isoprostane as it has a short half-life in the peripheral circulation, since it is conjugated to protein (Poliakov *et al.*, 2004) or glutathione (Milne *et al.*, 2004) and is excreted. Maintaining a constant, elevated level of 8-F_2-isoprostane in conditions of low-to-moderate oxidative stress may have been difficult to demonstrate in our

samples. 8-F_2-Isoprostane represents primarily the end-product from peroxidation of arachidonic acid, with smaller contributions from linoleic acids (Rokach *et al.*, 2004). Linoleic acid (LA), the major polyunsaturated fatty acid in plasma, has been demonstrated to be the primary target of lipid peroxidation (Spiteller, 1998). Since LA has only two double bonds and therefore does not have an isoprostane structure (Fam and Morrow, 2003), it could have been elevated and would not have been detected by the 8-isoprostane assay. Thus in retrospect, a direct analysis of lipid hydroperoxides by HPLC (Browne and Armstrong, 1998, 2000), which identifies all species and regioisomers, might have provided more information on whether oxidative stress is actually associated with endometriosis and how specific and relevant 8-isoprostane is as a general screening methodology for the oxidation of lipids.

In conclusion, this study found a weak association between TBARS, a measure of overall oxidative stress, and endometriosis. This suggests that oxidative stress might play a role in the development and progression of endometriosis. Pathways (environmental or biological) leading to oxidative stress and endometriosis need further exploration using substantially larger samples sizes, and assessing markers of oxidative stress that might be more sensitive and specific for endometriosis.

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