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ORIGINAL ARTICLE

The Interaction of Serum Folate and Estradiol Levels in Alzheimer's Disease

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Abstract

OBJECTIVES: *In vitro* and *in vivo* animal studies suggest that sex steroids, such as estrogens and testosterone, could protect the brain. However, estrogen replacement therapy (ERT) for Alzheimer's disease (AD) in women has not been successful. We hypothesised that the lack of effect of ERT might be related to an interaction between estrogens and some other factor(s) associated with AD.

DESIGN, SETTING AND METHODS: We analysed total estrogen (TE) and testosterone (TT) levels in women diagnosed with AD and controls of the Oxford Project To Investigate Memory and Ageing. Because estradiol (TE2) after the menopause is largely derived from estrone (TE1), we computed the ratio TE2/(TE1+TE2) and a total steroid index (TT+TE2+TE1).

RESULTS: Women with AD (n=66) had significantly higher levels of TE2 (27±13 vs. 21±13) and a higher TE2/(TE1+TE2) ratio. Stepwise logistic regression analyses showed that the TE2/(TE1+TE2) ratio was the main sex steroid predictor for AD (O.R.=1.06, 95% C.I.=1.01–1.11, $p<0.01$). Multiple regression analyses revealed that dementia severity was associated with an interaction between the TE2/(TE1+TE2) ratio and serum folate (B=4.59 (SE=1.48), beta=1.22, 95% C.I.=1.62–7.56, $p<0.005$). None of the other potential mediators of this association (body mass index, sex hormone binding globulin, homocysteine levels, ApoE genotype, smoking, diabetes, blood pressure) was significant.

MAIN FINDINGS: A high ratio of estradiol to total estrogens is associated with AD but, in subjects with a high ratio, the dementia severity was lower in those with high serum folate levels.

CONCLUSIONS: If this association is causal, then supplementation with folic acid might be considered in future studies on ERT in AD.

Abbreviations:

AD	Alzheimer's Disease
E2	Estradiol
E1	Estrone
T	Testosterone
TT	Total Testosterone
TE2	Total Estradiol
TE1	Total Estrone
SHBG	Sex Hormone Binding Globulin
B.M.I.	Body Mass Index
ERT	estrogen replacement therapy
tHcy	total serum homocysteine
SBP	systolic blood pressure
DBP	diastolic blood pressure
ApoE allele genotype	apolipoprotein E allele genotype
OPTIMA	Oxford Project To Investigate Memory and Ageing
NINCDS/ADRDA	National Institute of Neurological Disorders and Stroke / Alzheimer's Disease and Related Disorders Association
CERAD	Consortium to Establish a Registry for AD
CAMDEX	Cambridge Examination for Mental Disorders of the Elderly
MMSE	Mini-Mental Status Examination
CT	computed tomography
MRI	magnetic resonance imaging
SPECT	Single Photon Emission Computed Tomography
C.V.	Coefficient of Variation
O.R.	Odds Ratio
LH	luteinizing hormone
EDTA	ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
CE	catechol estrogens
COMT	catechol-O-methyl-transferase
SAM	S-adenosylmethionine

Introduction

Animal and cell culture studies have shown there is strong biological plausibility [1] for estrogen replacement therapy (ERT) to treat Alzheimer's disease (AD). However, three recent randomised controlled trails did not show any positive effect of ERT in women with AD [2-4]. In addition, several studies using hormone assays of adequate sensitivity reported higher levels of estrogens in women with AD compared to controls [5-7]. There could be a confounding factor in AD cases which prevents them from profiting from the potential protective effects of estrogens.

In the present study, total estradiol (TE2), estrone (TE1) and testosterone (TT) were measured. After the menopause, only very small quantities of E2 are derived from E1 and from androgens such as testosterone [8]. E1 then becomes the predominant estrogen through conversion of androgens in adipose tissue, but it has less affinity for the estrogen receptors than E2 [8]. Because of these conversions, we calculated a ratio TE2/(TE2+TE1) and a total steroid index (TT+TE2+TE1).

Several potential confounds for the association between sex steroids and AD were measured and were covaried in the analyses, such as body mass index (B.M.I., [8], sex hormone binding globulin (SHBG), which determines the level of free or bioavailable hormones and changes with age and certain disease states [9], creatinine and albumin levels as markers for steroid clearance, and a number of risk factors for AD which have also been reported to interact with sex steroid levels, such as smoking [10, 11], having at least one ApoE e4 allele [12], high levels of serum homocysteine [13–15] and low serum folate and vitamin B12 [16–18].

Materials and methods

We studied 62 controls without objective memory impairment and 66 possible (n=15), probable (n=15) and definite (n=36) AD cases (according to the 'National Institute of Neurological and Communicative Disorders and Stroke' and the 'Alzheimer's Disease and Related Disorders Association' (NINCDS/ADRDA) criteria [19] and, for definite AD, according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria [20]) from the Oxford Project To Investigate Memory and Ageing (OPTIMA). Cases had usually been referred by a hospital consultant because dementia was suspected. Controls were community dwelling volunteers who had no progressive cognitive impairment and were either spouses of the patients or had been recruited through advertisements and lectures in the Oxfordshire region. All controls, patients and their closest relatives gave informed consent to the study, which had local ethics committee approval. We had excluded ERT users (9 controls and 5 AD cases) and cases with other types of dementia (n=24).

Clinical examination

All participants underwent medical examination, which included blood sampling, brain scans (CT or MRI and SPECT) and cognitive assessment using the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX which included a cognitive section, the CAMCOG and the Mini-Mental Status Examination or MMSE [21]). From this examination, we derived demographic characteristics (age and education), information on (current) smoking habits, supine blood pressure measurements, body mass index (B.M.I.=weight/height^2), medication use and disease status. We obtained routine albumin and creatinine from the John Radcliffe clinical biochemistry laboratory. ApoE allele genotyping was performed using standard methods [22] and coded as '0' ('no apoE (4 allele present') and '1' ('at least one apoE (4 allele present').

Hormone assays

We obtained non-fasting blood serum samples between 10:00 and 12:00 hours and then stored at –70°C for an average of 2.3 ± 2.1 years. Total testosterone (TT) was analyzed using a competitive enzyme immunoassay (Bayer(c), Bayer Corporation, 511 Benedict Avenue, Tarrytown, N.Y. 10591-5097 U.S.A.). The inter-assay reliability for the assay was good (correlation with DPC Coat-a-Count TT radio-immunoassay= 0.99). The within-run Coefficient of Variation (C.V.) varied between 1-8% and the between-day C.V.

varied between 2–5% for TT in the ranges from 2 to 67 nmol/L. The minimum sensitivity for this assay was 0.17 nmol/L [23]. In post-menopausal women, the ovary and adrenal glands secrete small amounts of testosterone in the range of 0.28 to 2.12 nmol/L. For TE2 the following protocol was used. Duplicate serum samples were extracted with ether. TE2 was then assessed by radio immunoassay using a highly specific rabbit antiserum. The lower limit or sensitivity of this assay was 3 pmol/L. The between assay C.V. was 12.8% (for a mean TE2 level of 27 pmol/L), the overall within assay C.V. was 9.4% (4% for a mean TE2 level of 14 pmol/L). Cross-reaction with estrone was low (0.03%). The validity of this assay when compared with other commercial assays was high ($r>0.99$) [24]. Estrone was assessed using a standard radio immunoassay. The sensitivity for this assay was 29.36 pmol/L. The inter-assay C.V. was between 9–11% and the intra-assay C.V. was 9%. The expected range for post-menopausal women was between 36.7 and 220 pmol/L. Because the level of E2 is determined by the amount of E1 available [8], we calculated a TE2/(TE1+TE2) ratio to control for this. SHBG levels were investigated using an immuno-enzymo-metric assay (IEMA) The inter-assay C.V. was between 7–12% and the intra-assay C.V. was 3–9%.

Other biochemistry

For other biochemistry variables (homocysteine, vitamin B12, serum folate) another protocol was employed. Non-fasting blood samples were collected into EDTA tubes, immediately refrigerated at 4 °C, and subsequently centrifuged and plasma aliquoted for storage at –70 °C. Total time from blood sampling to storage at –70 °C was always less than 2 hours. Serum total homocysteine (tHcy) was measured by HPLC with fluorescence detection [25] or by immunoassay with fluorescent polarization [26]. Folate and vitamin B12 were measured by Abbott Imx binding assay and immunoassay procedures, respectively.

Statistics

To test potential differences in demographic and clinical variables between groups, we did X^2 analyses for categorical variables and t-tests for continuous variables. Because the hormone data were skewed, we log transformed the sex steroid hormone levels. To assess the Odds Ratio (O.R.) and the predictive value of serum hormone levels with reference to the presence of AD, we performed logistic regression analyses entering the sex hormone data and potential confounds of the associations such as age, SHBG levels, B.MI, albumin, creatinine, homocysteine, vitamin B12 and serum folate. We also investigated whether cognitive function or dementia severity (MMSE) related to sex steroid hormone levels with stepwise linear regression. All analyses were done in SPSS [27] (version 10.0 for Windows) and the level of significance was set at 0.05.

Results

Demographic, clinical characteristics and sex steroid levels

Cases had significantly less years of education and lower MMSE scores than controls, but there was no difference in age (table 1). Having at least one ApoE e4 allele was twice as common in AD cases compared to controls. There was no difference in co-morbidity such as diabetes, smoking habits or (measured- or a history of elevated-) blood pressure between cases and controls. tHcy levels were significantly higher in cases, while vitamin B12, serum folate and albumin levels were significantly lower. There was no difference in SHBG, BMI or creatinine levels between cases and controls. Log transformed TE2 levels and the TE2/(TE1+TE2) ratio were both significantly higher in AD cases. There was no difference for TT and TE1 levels between AD cases and controls. Using general linear models which co-varied for age did not change results.

Potential mediating effects of the association between estradiol and AD

Spearman's rank correlations showed that the ratio TE2/(TE1+TE2) was associated with lower serum folate ($rho=–0.23$, $p<0.05$), which was associated with higher serum tHcy levels ($rho=–0.49$, $p<0.0001$). TE2 was positively associated with TE1 ($rho=0.46$, $p<0.0001$) and TT ($rho=0.45$, $p<0.0001$) levels. TE2

Table 1. Demographic and clinical characteristics of the cohort

Variable	CON (n=62)	AD (n=66)	p-value*
Demographics			
Age (years)	76±8	77±8	p=0.64
Education (years)	14±3	12±3	**p=0.002**
MMSE (0-30)	29±1	17±8	**p<0.0001**
Co-morbidity and risk factors			
Apoe E genotype (at least 1 e4 allele)	32%	64%	**p<0.0001**
Smoking (current)	7%	17%	p=0.12
Diabetes mellitus (medication use)	11%	8%	p=0.55
History raised blood pressure	25%	30%	p=0.72
Current SBP (mmHg)	156±23	152±25	p=0.44
Current DBP (mmHg)	85±12	86±13	p=0.77
Biochemistry			
tHcy (micromol/L)	13±3	15±5	**p<0.01**
Vitamine B12 (pmol/L)	346±116	261±108	**p<0.001**
Serum folate (microgram/L)	11±5	7±5	**p<0.001**
Albumin (g/dl)	45±4	43±4	**p=0.003**
Creatinine (mmol/L)	85±16	83±13	p=0.45
Sex steroids and related variables			
Estradiol (pmol/L)	21±13	26±13	**p=0.02**
Estrone (pmol/L)	60±22	66±37	p=0.31
Testosterone (nmol/L)	0.90±0.6	1.09±0.7	p=0.10
S.H.B.G. (nmol/L)	79±31	74±31	p=0.35
B.M.I.(weight /height²)	25±4	24±4	p=0.44

* these p-value are derived from Chi-square test for categorical variables and t-tests for continuous variables (mean values and SD).

Table 2. Linear regression analyses predicting the Mini-Mental Status Examination performance.

Model	B	St.Error	Beta	t-value	p-value	95% C.I.	
Constant	26.8	4.88		5.49	0	16.99	36.61
Ratio TE2/TE1+TE2 (%)	-0.49	0.15	-0.85	-3.21	0.002	-0.79	-0.18
Serum Folate	-0.92	0.41	-0.88	-2.28	0.027	-1.74	-0.11
Interaction ratio x serum folate	4.59	1.48	1.221	3.11	0.003	1.62	7.56
Education (years)	0.73	0.21	0.4	3.45	0.001	0.31	1.16

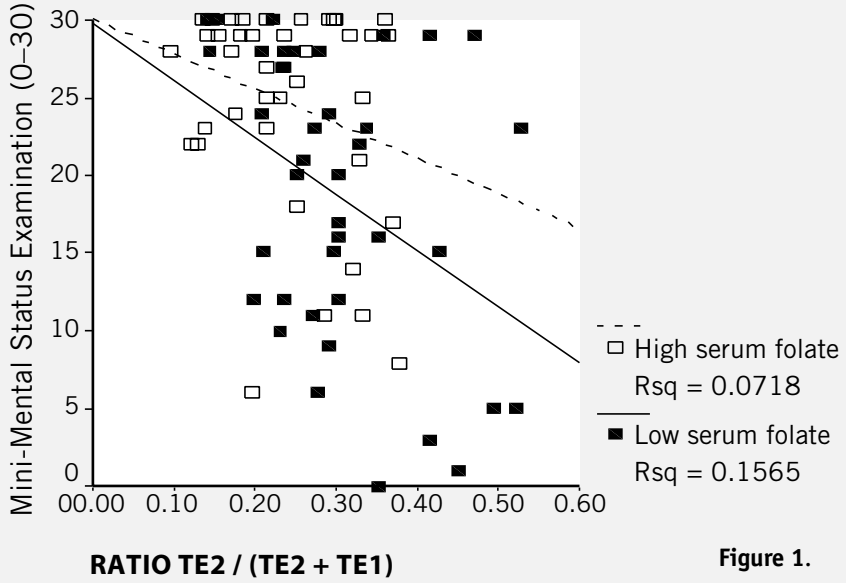

Figure 1. The performance on the Mini-Mental Status Examination (MMSE) and the ratio of TE2/(TE1 + TE2) in participants with high and low serum folate (based on the median split: serum folate higher than or less than 7.9).

Figure 1.

and TE1 were both negatively associated with SHBG levels (TE2: rho=–0.20, p=0.02, TE1: rho=–0.18, p<0.05). While SHBG was higher with increasing age (rho=0.22, p<0.01) and was lower with a higher B.M.I. (rho=–0.56, p<0.0001), neither age nor B.M.I. were significantly associated with sex steroid hormone levels or with dementia severity. None of the other variables had significant associations with the sex steroid levels.

Backward conditional logistic regression analyses was used to determine the best steroid predictor for AD, entering TE2, TE1 and TT and the TE2/(TE1+TE2) ratio. Only the ratio (in %) remained in the analyses (O.R.=1.06, 95% C.I.=1.01–1.11, p<0.01). Quadratic terms (to assess whether there was a curvilinear association) and the total steroid index were also not significant in this model.

We then performed backward conditional logistic regression analysis entering the ratio (%) and the potential confounds which were associated with AD or the sex steroids (age, vitamin B12, serum folate, homocysteine, albumin and SHBG levels, SBP, having at least one ApoE e4 allele). The ratio and having at least one ApoE e4 allele (O.R.=4.87, 95% C.I.=1.92–12.93) remained in the model as risk factors for AD.

Linear regression analyses showed that a lower MMSE performance was associated with a high TE2/(TE1 + TE2) ratio and lower levels of serum folate. The same result was obtained when the CAMCOG score was used instead of the MMSE score. None of the other variables had a significant association. Since the ratio correlated with serum folate (see above), we further investigated this association by entering the interaction term (serum folate x the ratio TE2/(TE1+TE2) and also controlled for the effect of education (see table 2). A lower MMSE was associated with a high TE2 (TE1+TE2) ratio over low levels of serum folate. Figure I shows that in subjects with low serum folate, a ratio of 0.30 (e.g. when TE2=60 and TE1=18 pmol/L) gives an average MMSE lower than 20, while high folate levels protect and maintain subjects above the cut-off score (>24) for dementia.

Discussion

In women, AD was associated with a higher TE2 level and with a higher TE2/(TE1+TE2) ratio. This association was not mediated through differences in age, body mass index, sex hormone binding globulin levels, indicators of liver and renal function, smoking, blood pressure or diabetes. This finding is in line with results of earlier smaller studies which reported non significantly higher E2 levels in the serum [5] and the brains of AD cases [28] when compared to controls.

It should be kept in mind that in the current study, the difference in TE2 levels between cases and controls was small. Furthermore, the values were all within the normal range, i.e. similar to those reported earlier in

postmenopausal females using assays of sufficient sensitivity [24]. Our findings could be in line with those of a study of girls with Down's syndrome (who have a higher risk of developing AD at an earlier age) which reported increased LH, but relatively normal E2 levels [29]. Increased LH levels could lead to an enhanced conversion from TE1 to TE2, which would explain the relatively low TE1 and high TE2 in the present study. Higher levels of LH have been found in women with AD not using ERT [30]. Raised LH [30], GH and TSH [31] in women with AD could be related to the large body of literature of increased activity of the hypothalamic-pituitary-(adrenocortical) axis in patients with AD [32, 33]

However, we also found that the degree of cognitive impairment (MMSE) was significantly associated with a higher TE2/(TE1+TE2) ratio, which appeared to be partly mediated by low serum folate levels. Estrogens can be converted into catechol estrogens (CE) which affect LH activity [34]. CE are potent competitive inhibitors of catechol-O-methyl-transferase (COMT). COMT can in turn convert CE to their methyl ethers. COMT catalyzes the O-methylation of catechol estrogens by using S-adenosylmethionine (SAM) as a methyl donor. Folate can inhibit the activity of COMT by demethylation of SAM [35] which would increase CE levels and which would then decrease LH levels.

Raised LH has been hypothesised to have detrimental effects in the brain and could be a risk factor for AD [36]. However, low folate is a known potentially modifiable risk factor for AD. Low folate is associated with high levels of homocysteine, which is increasingly recognised as a risk factor for AD and cognitive deficit [13, 37]. Although some ERT studies found a (transient) decrease in homocysteine levels [15, 38, 39] others found an increase in homocysteine after methionine loading and a trend for a decrease in serum folate ($p=0.06$) after 6 months of ERT treatment [40]. It could be hypothesized that when serum folate is maintained by adequate nutrition or supplementation, possibly, the protective effects of ERT could be maintained in AD patients. Three recent randomised controlled clinical trials did not find positive effects of ERT in patients with AD [2–4]. In fact, on some test outcome variables, the patients seemed to get worse [2]. Our data suggest that lower serum folate levels could perhaps explain this result. We suggest that taking serum folate into account, or perhaps even supplementing with folic acid, might be worth considering when investigating ERT for cognitive maintenance in elderly women with dementia.

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