

Hormone replacement therapy-related changes in the early postmenopausal period (critical window): an in vivo brain proton magnetic resonance spectroscopy study

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Aim: Findings from clinical studies in postmenopausal women with late initiation of hormone replacement therapy (HRT) that test whether HRT protects cognitive functions in women are inconsistent. The aim of this study was to investigate the effects of HRT on brain metabolite ratios when initiated in the early postmenopausal period (critical window).

Materials and methods: Proton magnetic resonance spectrometry (1H MRS) was performed in 4 brain regions of 47 healthy postmenopausal women (21 received HRT, 26 did not). The subjects were aged between 45 and 65 years. The duration of HRT ranged from 1 to 12 years (mean: 6.3 years). The duration of menopause was 2–12 years (mean: 6.1 years) for HRT users and 1–20 years (mean: 7.8 years) for non-HRT users. Metabolite ratios [N-acetyl aspartate/choline (NAA/Cho), NAA/creatine (Cr), and Cho/Cr] were evaluated.

Results: Cho/Cr ratios were significantly increased and NAA/Cho ratios significantly decreased in all 4 regions in the HRT user group compared to the other group after elimination of the effects of age and menopause duration. Regression analysis revealed an association only between NAA/Cho and duration of menopause.

Conclusion: HRT-related changes in metabolite ratios are found in all brain regions. Decreased NAA/Cho and increased Cho/Cr levels do not support the neuroprotective role of HRT in the critical window.

Key words: Menopause, hormone replacement therapy, critical window, proton MR spectroscopy, metabolite ratios

1. Introduction

Hormonal replacement therapy (HRT) is a medical treatment to relieve the symptoms of menopause in surgically or naturally postmenopausal women (1–3).

HRT has a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous systems, as well as on cognitive processes (4,5). Estrogen receptors are widely distributed throughout the brain, involving cortical and limbic areas and the hypothalamus, which are important in the control of cognitive functions (6). Evidence from small, randomized controlled trials and cross-sectional studies indicate that estrogen may protect against cognitive decline with aging (7). However, the results of a recent, large

multicenter randomized controlled study unexpectedly showed an increased risk for dementia in women receiving estrogen plus progestin therapy (8).

This controversy helped develop the critical period, or window, hypothesis. This hypothesis suggests that estrogen has maximal protective benefits on cognition in women when it is initiated shortly after natural or surgical menopause, but not when treatment begins decades after menopause (9,10). There is some indirect evidence to support the idea that estradiol may prevent the deleterious effects of cognitive aging only when it is administered soon after the cessation of ovarian function (i.e. in the critical window) (7).

Magnetic resonance spectroscopy (MRS) is an in vivo method used to study brain metabolism; in particular, the

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changes that occur during aging and cognitive diseases have attracted interest. Biological data from animal studies and functional brain imaging studies have shown that estrogen has an effect on specific brain regions, including the striatum, hippocampus, basal forebrain, and prefrontal cortex, which have roles in cognitive processes (11,12).

However, brain MRS data on the effect of HRT in the postmenopausal period are lacking. We sought to determine whether there are metabolic changes related with HRT usage in the brains of healthy postmenopausal women with no cognitive complaints who started HRT in the early postmenopausal period (critical window).

2. Materials and methods

2.1. Patients

The cross-sectional study was conducted at the Zonguldak Karaelmas University (now Bülent Ecevit University) Department of Radiology between November 2003 and December 2004, enrolling postmenopausal women. Healthy, literate postmenopausal women, between 45 and 65 years old, were included in the study. Inclusion and exclusion criteria for the study are stated in Table 1. Postmenopausal women in the present study were assembled into 2 groups, as HRT users and non-HRT users. The 2 groups were matched for age, education, and postmenopausal period. All subjects underwent detailed cognitive tests. All HRT users had been taking 2.5 mg/day of tibolone (Livial), an oral form of HRT. Serum estrogen level was above 33 pg/mL in patients receiving HRT but under 25 pg/mL in the non-HRT user group.

2.2. MRS method

MR examinations were conducted with a 1.5 T scanner (Gyrosan Intera, Philips, Best, the Netherlands). A standard head coil was used for conventional MR imaging and MRS. Conventional imaging was performed in the

transverse plane, using T1 weighted (560/15 ms) and fluid attenuated turbo inversion recovery (FLAIR; 6000/100 ms) sequences. We obtained 3 plane T2 weighted (3000–3400/110 ms) sequences to localize voxels. The routine images were also used to identify any structural or signal intensity abnormalities.

In single-voxel proton MRS examinations, acquisition parameters were: PRESS sequence, TR 2000 ms and TE 136 ms. The number of measurements was 128 and spectral data points were 512. Four different target brain structures were studied in each subject. In 1 of them, a $30 \times 11 \times 12 \text{ mm}^3$ voxel was placed on the right hippocampus (HP) using the 3 orthogonal T2 weighted images. For the other 3 targets, voxel sizes were $30 \times 20 \times 10 \text{ mm}^3$ for the anterior cingulate gyrus (ACG), $30 \times 25 \times 10 \text{ mm}^3$ for the posterior cingulate gyrus (PCG), and $24 \times 17 \times 16 \text{ mm}^3$ for the left dorsolateral prefrontal cortex (DLPFC).

The methodology of MRS processing, including shimming and metabolite peak determination, is described elsewhere (13). The 3 major metabolites included in the study were N-acetyl aspartate (NAA) at 2 ppm, creatine (Cr) at 3 ppm, and compounds containing choline (Cho) at 3.2 ppm.

The study was conducted with the approval of our ethics committee and informed consent from participants was obtained.

2.3. Statistical analysis

Individual signal integrals were used to calculate metabolite ratios (NAA/Cho, NAA/Cr, and Cho/Cr). These 3 metabolite ratios were used in the statistics. Descriptive statistics of variables were computed as mean $\pm$ SD, count, and percentage. Before each significance test, the distributions of the metabolite ratios in each group (HRT users and nonusers) were tested for normality by the Kolmogorov–Smirnov normality test.

Table 1. Inclusion and exclusion criteria for the subjects enrolled in the study.

Inclusion criteria	Exclusion criteria
1- Female subjects aged between 45 and 65 years old	1- Any significant medical disorder that may affect the brain
2- No menstrual activity for at least 1 year after the last menstruation	2- Any ischemic or gliotic lesion suspicious for vascular disease in conventional brain MRI
3- Having no chronic diseases such as liver, renal, or cardiac diseases	3- History of drug use for any psychiatric reason
4- No symptoms of depression and anxiety disorder, gaining 8 or fewer points on the Hamilton Depression Scale	4- Exposure to severe stress factors leading to major depressive symptoms
5- Literate	5- Having neurologic diseases
	6- Alcohol abuse or substance use
	7- Diagnosed with schizophrenia or other psychotic disorders according to DSM-IV criteria
	8- Any prosthesis or other tool that may lead to MRI problems
	9- Severe claustrophobia

Factorial repeated measurement analysis of covariance with 2 factors was used for differences between groups, differences among the 4 brain regions, and their interaction by controlling the effect of age and duration of menopause with regard to metabolite ratios.

An interaction term was included to test whether the magnitude of the difference in each of the 3 metabolite ratios across HRT user and nonuser groups varied significantly by brain region.

The Student t-test was used for differences between HRT users and nonusers in age and duration of menopause.

The relationships among the ratios in each of the 4 brain regions and each group were evaluated using Pearson's correlation coefficient. If a P-value was equal to or less than 0.05, it was accepted as statistically significant. All data analysis was performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Subjects

Sixty-eight postmenopausal women aged between 45 and 55 years old were included in the study. Of these, 21 subjects were excluded from the study due to anxiety disorder (2 subjects), depression (5 subjects), vertebrobasilar insufficiency (1 subject), and hypertension or heart failure (13 subjects). Of the 47 subjects included in the study, 21 (45%) used HRT and 26 (55%) did not. The mean age of the HRT users was 52.81 (range: 48–60), and that of the non-HRT users was 53.73 years (range: 45–65). The duration of menopause was between 2 and 12 years (mean: 6.3 ± 4.1) for HRT users, while it was between 1 and 20 years (mean: 7.8 ± 5.9) for non-HRT users. The duration of use of HRT in subjects receiving HRT was 6.1 ± 2.8 years (range: 1–12 years). Fifteen women had been using HRT since the onset of menopause; however, 6 women had started using it between the first and sixth years of the onset of menopause.

The results of the comparison of HRT users and nonusers in terms of age and duration of menopause are given in Table 2. The differences between the 2 groups regarding age and duration of menopause were not statistically significant.

Table 2. Descriptive statistics of age and duration of menopause in the 2 groups. Results are shown as mean $\pm$ standard deviation.

	HRT (n = 21)	Non-HRT (n = 26)	P
Age	52.81 $\pm$ 4.11	53.73 $\pm$ 6.16	0.316
Menopause	6.48 $\pm$ 3.06	7.85 $\pm$ 5.97	0.543

3.2. Analysis of metabolite ratios

Descriptive statistics of NAA/Cr, Cho/Cr, and NAA/Cho ratios according to groups and brain regions are shown in Table 3. The NAA/Cr ratio was not affected by age and duration of menopause ($P = 0.765$ and 0.307 , respectively). In addition, the differences in NAA/Cr ratio between the 2 groups did not differ according to brain regions ($P = 0.415$), and this result did not change according to the 4 brain regions ($P = 0.560$). The 4 brain regions did not differ significantly in terms of NAA/Cr ratio ($P = 0.079$) and this result was the same for both groups ($P = 0.560$; Figure 1). In other words, there was no significant difference between the groups, irrespective of brain region.

The Cho/Cr ratio was not affected by age and duration of menopause ($P = 0.288$ and 0.118 , respectively). The level of the Cho/Cr ratio between the 2 groups did not differ according to brain regions ($P = 0.121$). The amount of difference between the 4 brain regions did not differ significantly when a comparison was made between groups ($P = 0.721$). However, the Cho/Cr ratio of the HRT-user group was significantly higher in all 4 regions when

Table 3. Descriptive statistics of NAA/Cr, Cho/Cr, and NAA/Cho ratios according to groups and brain regions (for the abbreviations, refer to the text).

		ACG	PCG	DLPFC	HP	P
NAA/Cr	Non-HRT	1.57 $\pm$ 0.32	1.52 $\pm$ 0.37	1.60 $\pm$ 0.36	1.37 $\pm$ 0.51	0.415
	HRT	1.45 $\pm$ 0.30	1.57 $\pm$ 0.35	1.55 $\pm$ 0.33	1.34 $\pm$ 0.48	
Cho/Cr	Non-HRT	1.119 $\pm$ 0.29	0.88 $\pm$ 0.30	1.02 $\pm$ 0.24	1.17 $\pm$ 0.36	0.05
	HRT	1.19 $\pm$ 0.27	0.98 $\pm$ 0.28	1.05 $\pm$ 0.23	1.28 $\pm$ 0.33	
NAA/Cho	Non-HRT	1.42 $\pm$ 0.43	1.74 $\pm$ 0.43	1.58 $\pm$ 0.36	1.20 $\pm$ 0.45	0.007
	HRT	1.23 $\pm$ 0.40	1.63 $\pm$ 0.40	1.42 $\pm$ 0.33	1.06 $\pm$ 0.42	

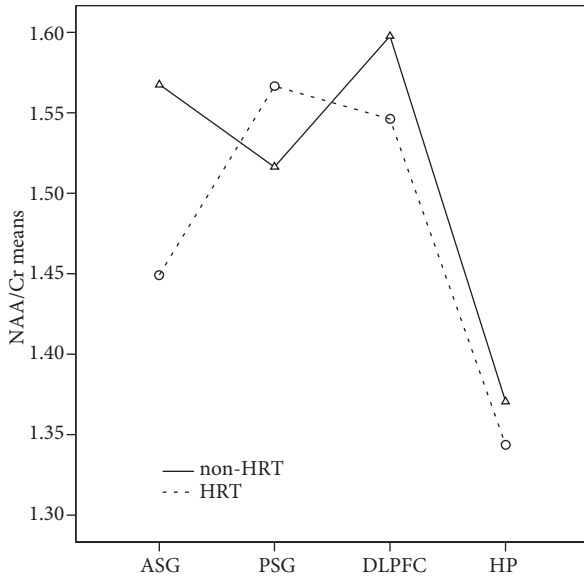


Figure 1. Comparison of mean NAA/Cr ratios according to groups and brain regions (for the abbreviations, refer to the text).

controlling for the differences between brain region and the group-by-region interaction ($P = 0.05$; Figure 2).

The relationship between age and NAA/Cho ratio was not significant ($P = 0.529$). However, NAA/Cho ratios correlated negatively with duration of menopause when controlling for the effect of age ($r = -0.350$, $P = 0.05$; Figure 3).

On the other hand, the level of difference in the NAA/Cho ratio between the 2 groups did not differ according

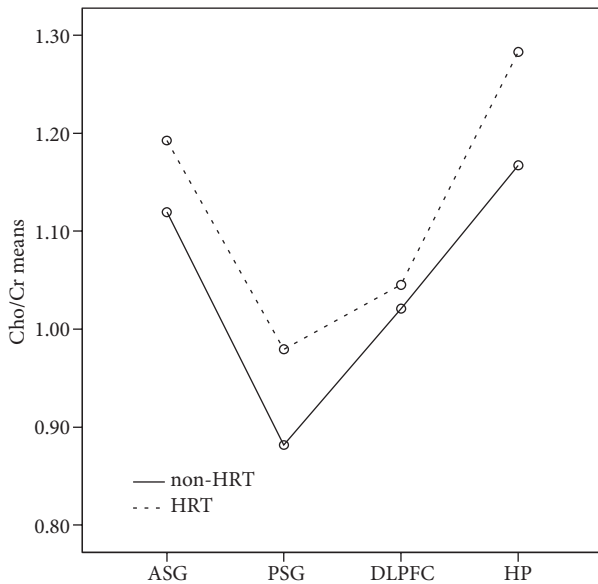


Figure 2. Means of Cho/Cr ratio according to groups and brain regions (for the abbreviations, refer to the text).

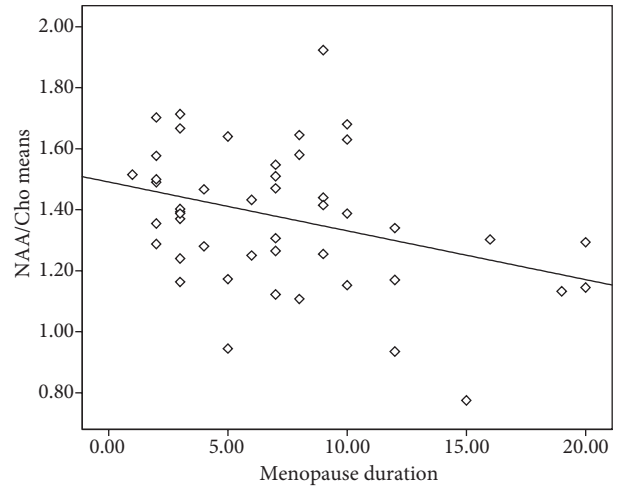


Figure 3. Scatter plot of the relationship between menopause duration and the NAA/Cho ratio.

to brain region ($P = 0.752$), and this result did not change between brain regions across the groups ($P = 0.940$). However, the NAA/Cho ratio of the HRT-user group was significantly lower in all 4 brain regions when controlling for the differences between brain region and the group-by-region interaction ($P = 0.007$; Figure 4).

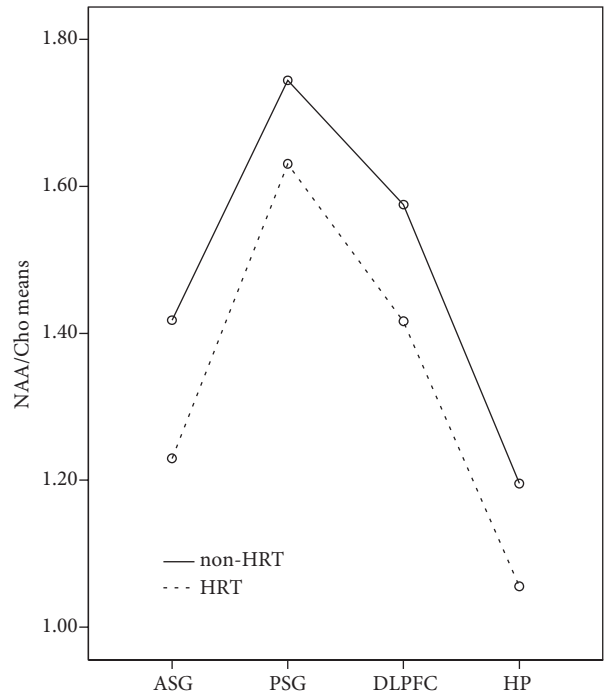


Figure 4. Means of NAA/Cho ratio according to groups and brain regions (for the abbreviations, refer to the text).

4. Discussion

Substantial biological evidence supports the importance of estrogen in cognitive function. Estrogen receptors have been identified throughout the brain. Estrogen actions are also widespread and affect many neurotransmitter systems including the catecholaminergic, serotonergic, dopaminergic, cholinergic, and γ -aminobutyric acidergic systems (6,14).

There are several mechanisms by which estrogen may influence cognitive functions. These mechanisms include increasing levels of neurotransmitters, enhancing neuron growth and formation of synapses, acting as antioxidants, and having regulatory effects on calcium homeostasis and second messenger systems (14,15).

In women, evidence suggests that postmenopausal hormone therapy (HT) is associated with improved or preserved cognitive function; however, not all studies support this (12,16).

Estrogen has an important role in maintaining and modulating neuronal integrity and cognitive function. The influence of HT on the brain and cognition in postmenopausal women is still debated. Evidence suggests that postmenopausal HT may preserve and improve cognitive function; however, not all studies support this (12,16).

The Women's Health Initiative Memory Study (WHIMS), the largest randomized controlled trial of HRT use for cognitive preservation, found an increased risk of dementia in hormone users (17) and a potential differential effect of combined estrogen/progestin (8,17,18). Specifically, the combined HRT group demonstrated a 2-fold increase in the risk of dementia (8), whereas the conjugated equine estrogen group demonstrated a trend, but no significant difference, for greater dementia risk compared with controls (3). This contrasts with previous prospective observational studies that suggested a protective role for HRT against dementia, specifically to Alzheimer's disease (19,20).

Morphometric brain magnetic resonance imaging (MRI) studies have yielded similar conflicting results. Some cross-sectional studies reported that women receiving HRT during menopause had significantly greater hippocampal volumes than their peers not receiving HT (21–23). Several others reported that high doses or levels of estrogen are associated with brain atrophy and smaller hippocampal volumes (24–27). The WHIMS-MRI Study on 1403 women aged 71–89 years revealed that specific areas of the brain involved in cognition, namely the hippocampus and frontal lobe, had smaller volumes on MRI after a 3-year follow-up in women assigned HRT compared to those receiving a placebo (27). Overall brain volumes were slightly lower, as well.

Contradictory results between these studies on the putative protective effect of estrogen on cognitive decline in women led to the development of the critical window hypothesis (28,29). This theory suggests that estrogen therapy effectively slows the cognitive decline associated with normal aging if the treatment is initiated at the time of menopause or very early in the postmenopausal period and is continued for several years thereafter. Furthermore, estrogen therapy has no effect, or might even cause harm, when treatment is initiated decades after the menopausal transition. Recent data from basic neuroscience and animal experiments support the idea that estradiol has a neuroprotective effect in young animals but has neutral or harmful effects when administered later in life (30,31).

Using a 3 T MRI scanner, Erickson et al. obtained 3D volumetric T1-weighted brain images from 102 healthy postmenopausal women. They found that HRT use, current or past, was associated with larger adjusted hippocampal volumes, but only when HT was initiated prior to or within 12 months of natural or surgical menopause (32). These MRI findings were interpreted as consistent with the critical period hypothesis, and they suggest that hippocampal volume loss may be reduced for women initiating HT during a critical window close to the time of menopause. HT exposure in this study was primarily from conjugated estrogens, with or without medroxyprogesterone acetate. However, larger hippocampal volumes in women who initiated hormone treatment at the time of menopause failed to translate into improved spatial memory performance. Furthermore, it is unclear how estrogen replacement therapy (ERT) exerts its effects on hippocampal hypertrophy and whether this effect is relevant at clinical or functional levels.

Given the available WHIMS data, neither unopposed estrogen nor combined estrogen-progestin therapy should be given to women over 65 years of age for preserving cognitive function and preventing dementia. The WHIMS trials do not address the question of the impact of estrogen administration in the early postmenopausal years on later dementia risk. It is still a matter of debate whether estrogen can have a protective effect on brain integrity within the critical window.

The present study evaluates neuronal integrity in the critical period by means of MRS. MRS is emerging as a powerful new tool in cognitive neuroscience research (33,34).

In this study, 3 markers of brain metabolism, NAA/Cr, NAA/Cho, and Cho/Cr ratios, were evaluated. NAA is one of the most abundant amino acids in the central nervous system, located predominantly in neurons, axons, and dendrites, and it is regarded as an indicator of neuronal viability, density, and functioning. A reduced concentration of NAA suggests neuronal loss or dysfunction (34,35).

Cho is present in all cell types. The Cho signal is a composite signal consisting mainly of phosphocholine, glycerophosphocholine, and, to a lesser extent, free choline itself. The MRS signal of Cho molecules is often interpreted as a measure of overall cellular density (e.g., astrogliosis, macrophage infiltration) and/or breakdown or turnover rate of membrane or myelin phospholipids as seen in demyelinating diseases (34,36,37).

Total Cr refers to the sum of Cr and phosphocreatine, which is assumed to be relatively constant in glia and neurons and is usually used as an internal reference to which ratios of other metabolites are expressed (38). Concentrations and signal intensity of Cr in the brain are relatively stable. Therefore, it is used as an internal standard to calculate metabolite ratios (38). Thus far, comparatively few spectroscopic studies have been carried out in postmenopausal women on HRT, and a clear pattern of neurochemical alterations associated with HRT usage has not yet been firmly established (39–41). Robertson et al. showed a significant increase in Cho concentrations in the hippocampus and parietal lobe, as well as an increase of Cho/Cr ratios in the hippocampus in ERT-naïve postmenopausal women compared to long-term ERT users and younger women (39). Craig et al. reported that young women, after ovarian suppression, had a significant increase in Cho concentration and Cho/Cr ratio in the DLPFC. They also showed a trend toward significant increase in Cho concentration in the hippocampus (40). Hu et al. reported a decreased NAA/Cr ratio in the area of the hippocampus in a non-HRT group compared to an HRT-user group (41).

Our results are discordant with those of Robertson et al. and Hu et al. (39,41). They reported a neuroprotective effect of ERT and HRT. A possible explanation for the discrepancy with our results resides in the difference in the ages and the duration of HRT in the respective samples. The mean age (63 vs. 53 in our study) and the duration of HRT (15.1 years vs. 5 years in our study) in our sample were lower than those reported by Robertson et al. (39). The study by Craig et al. evaluated young, healthy premenopausal women with acute loss of function during gonadotropin-releasing hormone agonist treatment. It can be assumed that natural menopause would have a different effect on brain neurochemistry than drug-induced ovarian suppression (40). Our study showed a negative effect of HRT on neural integrity. An explanation for this unexpected finding might be related to the hormone replacement regimen used.

Tibolone (Livial) is a synthetic steroid, with estrogenic, progestogenic, and androgenic properties, used extensively for the management of menopausal symptoms. It is structurally different from estradiol and selective estrogen-receptor modulators and, with its accompanying

progestogenic and androgenic effects on the brain, has been shown to improve mood and libido better than estrogen alone treatments (42–44).

Circulating levels of androgens, such as testosterone and dehydroepiandrosterone sulfate, gradually decrease with age in postmenopausal women. Maintaining an appropriate level of androgen by drug supplementation may play an important role in metabolic, psychological, and sexual functions in women. However, studies examining the effect of postmenopausal androgen replacement therapy on cognition yielded conflicting results (45).

Robertson et al. studied unopposed estrogen (39). In our trial, it is possible that the addition of progestogenic and androgenic properties negated a beneficial effect of the estrogenic type.

In vitro studies have shown that there are different mechanisms through which estrogens may exert their action on brain physiology, e.g., in affecting neurotransmitter synthesis and metabolism, in preserving cholinergic neurons, or in enhancing cerebral perfusion (46). However, progestagens have been shown to oppose some of the effects of estrogens when both are given together (46,47). In our study, negative effects of HRT might partly be due to the progestogenic and androgenic effects of tibolone in addition to its estrogenic-type effects. However, it is unclear whether progestins and androgens have such a negative effect. On the other hand, others failed to find a negative effect of the addition of progestagens and androgens (48–50).

The critical period or window theory suggests a beneficial effect of estrogen therapy in the early postmenopausal stage (9,10). The present findings of decreased NAA/Cho and elevated Cho/Cr do not support this theory and argue against the neuroprotective effect of HRT in early postmenopausal women during the critical period. It is unclear whether our results were affected by the addition of progestin to estrogen. Because all hormone-treated women in our study received tibolone, which has combined estrogenic and progestogenic properties, our study provides no evidence regarding the effect of unopposed estrogen therapy on neuronal integrity. Further comparative studies are necessary to resolve this issue.

Although we did not study women older than 65 years old, unlike the WHIMS study, we can suggest that changes in the metabolite ratios (decreased NAA/Cho and elevated Cho/Cr) would not be reversed at these older ages after much longer HRT use. Therefore, it can be suggested that our results would also be in keeping with the inverse association reported between HRT and cognition in elderly postmenopausal women in the WHIMS study (18).

Absolute concentrations were not calculated in our study, and so it is not clear if decreased NAA/Cho and increased Cho/Cr ratios are due to a decrease in NAA

and/or an elevation in Cho. Since NAA/Cr did not show a significant difference, it can be argued that changes in the relevant 2 metabolite ratios resulted from an increase in Cho. Increased choline levels (if true, as assumed) might be related to structural changes associated with membrane turnover/breakdown (34).

Most 1H MRS studies of healthy human brain aging have reported an age-related increase in the Cho signal, but no change in NAA or Cr (51). Analysis of covariance did not show any effect of age or menopause duration on metabolite ratios. There also was no correlation between age and metabolite ratios. Therefore, assumed metabolite changes in our study are expected to reflect a hormone-related effect. As the patients' ages are limited to a narrow range (45 to 65 years) in our study, it may explain the absence of an expected correlation between metabolite ratios and age and may account for the discrepancy between our results and previous studies displaying correlations (37,51,52). On the other hand, our results may reflect that normal aging between 45 to 65 years may not produce any significant change in metabolite ratios.

NAA/Cho ratios were correlated negatively with the duration of menopause in all subjects ($n = 47$) when controlling for the effect of age ($r = -0.350$, $P = 0.05$). The presence of an association for duration of menopause together with the absence of such an association for age can be explained by wider time range of menopause duration (20 years) compared to the narrow age range (10 years). This finding suggests that duration of menopause has an influence on the neuronal metabolite change during aging. It is of note that our study did not find any correlation between metabolite ratios and duration of HRT use. This is most probably due to the narrow time range (1–12 years) and the number of subjects in the HRT-user group ($n = 21$).

We studied all 4 cerebral locations, including the ACG, PCG, DLPFC, and HP, and found similar metabolic changes: specifically, significant elevations of the Cho/Cr and decrease of NAA/Cho ratios and no significant differences in the NAA/Cr ratios. This suggests that

the effect of HRT on the central nervous system may be diffuse. The effects of estrogens on cognitive processes may be explained by the wide distribution of estrogen receptors in a large number of regions, such as the cortical and limbic areas and the hypothalamus, which are involved in the processes of learning and memory (6).

One limitation in our study derives from the use of single-voxel spectroscopy, which prevented us from assessing a larger number of brain areas, as compared to multivoxel MR spectroscopy. However, one of the advantages of this study was the composition of our patient sample, spanning a narrow range of ages (45 to 65 years) before natural cognitive decline starts, and early initiation of HRT in the critical window period. Furthermore, we were unable to demonstrate a relationship between the age and metabolite ratios, which is most probably due to the narrow age range and the limited number of subjects in the HRT user group. A larger group is expected to provide an association between the age and the cerebral metabolic changes.

Another limitation of our study is the use of metabolite ratios rather than an absolute quantification of metabolites. Although measurement of metabolite concentration introduces less variability than metabolite ratio measurements, brain metabolites are frequently reported as ratios because quantification is more complex and the assumption is that brain creatine is relatively stable and unaffected by most neurological diseases. As the 2 groups in our study were homogeneous regarding age, menopause duration, education level, and absence of significant confounding medical factors, we think that systematic errors of ratio calculations did not affect the resulting differences of metabolic ratios significantly.

In summary, the current data suggest, but do not prove, that postmenopausal HRT with tibolone, a synthetic steroid with estrogenic, progestogenic, and androgenic properties, do not have a protective effect on the neurochemical structure of the brain in selected regions. Since this study is of a cross-sectional design, further longitudinal studies are needed to validate this finding.

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