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ORIGINAL PAPER

Specificity proteins 1 and 4 in peripheral blood mononuclear cells in postmenopausal women with schizophrenia: a 24-week double-blind, randomized, parallel, placebo-controlled trial

Èlia Vila[1] · Elena Huerta-Ramos[2,3,4,5] · Christian Núñez[2,3,5] · Judith Usall[2,3,4,5] · Belén Ramos[1,3,4,6]

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Abstract

Accumulating evidence suggests that Specificity Protein 1 (SP1) and 4 (SP4) transcription factors are involved in the pathophysiology of schizophrenia. The therapeutic use of selective oestrogen modulators such as raloxifene added to antipsychotic drugs in the treatment of postmenopausal women with schizophrenia has been investigated in a few clinical trials, which reported an improvement in negative, positive, and general psychopathological symptoms. We aimed to investigate the possible association between peripheral SP protein levels and symptom improvement in postmenopausal women with schizophrenia treated with adjuvant raloxifene. In a subgroup of 14 postmenopausal women with schizophrenia from a 24-week, randomized, parallel, double-blind, placebo-controlled clinical trial (NCT015736370), we investigated changes in SP1 and SP4 protein levels in peripheral blood mononuclear cells. Participants were randomized to either 60 mg/day adjunctive raloxifene or placebo. Psychopathological symptoms were assessed at baseline and at week 24 with the Positive and Negative Syndrome Scale (PANSS). The expression of SP proteins was evaluated by immunoblot, and changes in PANSS scores and protein levels were compared at baseline and after 24 weeks of treatment. An improvement in symptoms was observed in the intervention group, but not in placebo group. Post-treatment protein levels of SP4, but not SP1, correlated with improvements in general and total PANSS subscales in the raloxifene intervention group. A reduction in SP4 levels was found after raloxifene treatment. These results suggest that SP4 may be involved in raloxifene symptom improvement in postmenopausal women and could be a potential candidate for future studies investigating blood-based biomarkers for raloxifene effectiveness.

Keywords Raloxifene · Schizophrenia · Blood biomarkers · SP4 transcription factor · Postmenopause, symptoms

Introduction

Oestrogens have a protective role in schizophrenia and could have a therapeutic potential in this disorder [1]. These hormones modulate neuroinflammation, neurotransmitter systems, and connectivity processes [1, 2]. They

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✉ Judith Usall
jusall@pssjd.org

✉ Belén Ramos
bramos@fsjd.org

1 Psiquiatria Molecular, Institut de Recerca Sant Joan de Déu, Santa Rosa 39-57, 08950 Esplugues de Llobregat, Spain

2 Intervencions en Salut Mental, Institut de Recerca Sant Joan de Déu, Santa Rosa 39-57, 08950 Esplugues de Llobregat, Spain

3 Parc Sanitari Sant Joan de Déu, Doctor Antoni Pujadas 42, 08830 Sant Boi de Llobregat, Spain

4 Instituto de Salud Carlos III, Centro de Investigación en Red de Salud Mental (CIBERSAM), Madrid, Spain

5 Catalan Group in Women's Mental Health Research (GTRDSM), Barcelona, Spain

6 Dept. de Bioquímica i Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

bind to oestrogen receptor (ER) to regulate transcriptional programs. The formation of dimers with only ER mediates the canonical genomic functions of oestrogens, while their binding with other transcription factors constitutes the non-canonical pathways [1, 3]. Specificity protein (SP) factors are transcription factors that interact with ER [3]. SP1 and SP4 associate with components of the neurotransmitter and inflammatory systems in the brain in schizophrenia and modulate dendritic architecture, and any alterations in them could have a possible impact on the development of schizophrenia [4–8]. SP1 and/or SP4 protein are increased in the hippocampus and peripheral blood mononuclear cells (PBMCs) and linked to negative symptoms in the cerebellum in chronic schizophrenia [6, 8, 9]. The use of hormone replacement therapy in postmenopausal women with schizophrenia is based on the greater vulnerability to the disorder during this period of oestrogen depletion and the exacerbation of symptoms [1]. However, the long-term use of oestrogens has potentially negative effects on breast and endometrium [10–12]. Selective Oestrogen Receptor Modulators, such as raloxifene, show neutral or antagonist effects on these tissues and maintain oestrogen agonist action in the brain [12]. Adjuvant treatment with raloxifene improves positive, general psychopathologic, and/or negative symptoms [13–16] in postmenopausal women with schizophrenia, and shows cognitive benefits [17–19], with distinct patterns of results among clinical trials. However, a little research to date has focused on the possible molecular players that could be linked to distinct improvements in symptoms observed in schizophrenia patients treated with adjuvant raloxifene.

We, therefore, hypothesized that SP1 and SP4 might have a role in the symptom improvement observed in raloxifene treatment and be a possible candidate for future studies on biomarkers of raloxifene effectiveness. We aimed to investigate the possible association between peripheral SP protein levels and symptom improvement in postmenopausal women with schizophrenia treated with adjuvant raloxifene.

Methods

Participants

Participating women were recruited from July 2011 to June 2014 from mental health centres and various long-stay hospital units (non-acute patients) at Parc Sanitari Sant Joan de Déu (PSSJD), Hospital Universitari Institut Pere Mata, and the Corporació Sanitària Parc Taulí. Seventy-eight patients were screened and randomized in the 24-week double-blind randomized parallel placebo-controlled clinical trial (Trial registration NCT01573637) [14], of whom seventy initiated the clinical trial. The reasons why eight patients retired are described elsewhere [14]. Only one of the participating centres (PSSJD) collected blood samples for PBMC isolation. Of the 21 patients that initiated the clinical trial in the PSSJD, 14 postmenopausal women with schizophrenia (DSM-IV) were included in the molecular study (Supplementary Fig. 1). Inclusion criteria were women with a schizophrenia diagnosis (DSM-IV-TR), postmenopausal status (age over 50 years and at least 1 year of amenorrhea; age between 45 and 50 years with FSH levels > 20 IU/L and at least 1 year of amenorrhea), receiving stable doses of their current antipsychotic medication for at least a month prior to study initiation and the presence of prominent negative symptoms, defined as one or more negative symptom scores greater than 4 on the Positive and Negative Syndrome Scale (PANSS). Exclusion criteria were substance abuse/dependence diagnosis in the previous 6 months, mental retardation, diagnosis of major depression (DSM-IV TR criteria), endocrine abnormalities related to sexual hormones, acute or chronic liver disease, impaired kidney function, history of thromboembolism, breast cancer, abnormal uterine bleeding, history of stroke, and the use of hormone replacement therapy. Patients gave written informed consent after a complete description of the study was provided.

The study received the approval of the institution's review board (IRB) and the Agencia Española del Medicamento. Patients provided informed consent in accordance with procedures established by the local IRB and were informed that they could drop out of the study at any time. The trial was performed in accordance with the Declaration of Helsinki and subsequent revisions, and registered at ClinicalTrials.gov (NCT01573637) on April 2012, and monitored by an external Clinical Research Organization (Sermes CRO).

Interventions

Participants were under regular antipsychotic medication and received 60 mg adjunctive raloxifene daily or placebo for 24 weeks. No changes in dosage were allowed throughout the duration of the study. The randomization and preparation of treatment compounds are described elsewhere [14]. Other psychotropic medications such as biperiden, benzodiazepines, and antidepressants were permitted. The antipsychotic drug doses are expressed in terms of their chlorpromazine equivalence, and sub-classified as typical, atypical, or combination. Moreover, none of the patients received any sex hormone therapy.

Clinical assessments

Diagnoses were established by means of SCID-IV. Psychopathological symptoms were assessed at baseline and at week 24 using the Spanish version of the Positive and Negative Syndrome Scale (PANSS) [20]. Neurocognitive measures were also collected at baseline and week 24. Briefly, the

tests used were The "España-Complutense Verbal Learning Test" (TAVEC) [21], the Continuous Performance Test (CPTII) [22], the Trail Making Test (TMT) A and B [23], the Stroop test [24], the Wechsler Adult Intelligence Scale (WAIS III), Spanish version [25], the Phonetic Fluency Test (letters "FAS"), and the Semantic Fluency Test (Animals) [26].

Peripheral blood mononuclear cell isolation

Blood was drawn by puncture of the cubital vein and collected in EDTA tubes at baseline and after 24 weeks. Peripheral blood mononuclear cells (PBMCs) from participants were isolated using the Ficoll-Paque gradient method (GE Healthcare) at baseline and at week 24 from the placebo and intervention groups as previously described [27]. We could not obtain blood samples at baseline from three patients in the raloxifene group; they were, however, included in the subsequent analysis. 6 ml of blood sample was added to 6 ml of Ficoll-Paque reagent and centrifuged at 2500 rpm for 30 min without brake. The PBMC intermediate cell layer was collected and washed with RPMI medium. The isolated cells were immediately stored at − 80 °C.

Immunoblotting

Protein extracts were prepared in NP40 lysis buffer as described in [27]. Cell extracts were then incubated on ice for 30 min, sonicated 8 s at 21% amplitude and centrifuged at 18,200 g for 15 min at 4 °C. Protein concentration was determined using the Bradford assay (BioRad). Equal amounts of 10 or 30 µg of protein for each sample were immunoblotted with polyclonal antibody against SP1 (Millipore) and SP4 (Santa Cruz Technology), respectively. Immunoblot with monoclonal antibody against actin (Sigma–Aldrich) was also included for all the analyses. Densitometric quantification was performed using Quantity One software (BioRad). Values were normalized to actin and to a reference sample to normalize between different sets of samples and repetitions. Only one band was observed at the correct molecular weight for all the proteins analysed.

Statistical analysis

The Kolmogorov–Smirnov omnibus test was carried out to test whether the variables followed a normal distribution. The unpaired *t* test (parametric quantitative variables), Mann–Whitney test (non-parametric quantitative variables), or Fisher's exact test (qualitative variables) was used to compare the sample baseline characteristics between the groups. The paired *t* test was used to assess differences between baseline and 24-week scale scores. To determine associations between protein levels and scales scores, Pearson (parametric variables) or Spearman (non-parametric variables) correlations were performed. Significance was set at $p < 0.05$. False discovery rate (FDR) was performed by the Benjamini and Hochberg method with a threshold set at 0.1.

Results

Demographic and clinical measures between our placebo and raloxifene subgroups are provided in Table 1. No differences were found between the groups. Comparison of the demographic data between our subgroup of patients ($n = 14$) and the whole clinical trial NCT01573637 ($n = 70$) is provided in Supplementary Table 1. Only significant differences were found regarding age in the raloxifene subgroup, with a mean age 3 years younger compared to the whole clinical trial raloxifene group, suggesting that our subgroup is similar to the whole clinical trial. An improvement in negative, general, and total PANSS scores has been described in the clinical trial NCT01573637 [14]. In our subgroup, we also observed a significant improvement in the same PANSS subscales when comparing the baseline and final scores in the raloxifene subgroup (significant threshold of FDR < 0.1): negative (uncorrected *p* value = 0.016; FDR-adjusted *p* value = 0.016), general (uncorrected *p* value = 0.006; FDR-adjusted *p* value = 0.010), and total (uncorrected *p* value = 0.005; FDR-adjusted *p* value = 0.010), but not in the placebo group (Fig. 1). Moreover, we observed better mean differences between baseline and final scores (dfPANSS), a measure of symptom improvement, for the total PANSS score (uncorrected *p* value = 0.016; FDR-adjusted *p* value = 0.023) and general PANSS subscale (uncorrected *p* value = 0.006; FDR-adjusted *p* value = 0.019) in the raloxifene group (Total: m.d. = 11.71, [3.85, 19.57]; General: m.d. = 6.29, [1.89, 10.68]) compared to the placebo group (Total: m.d. = − 0.86, [− 8.45, 6.74]; General: m.d. = − 1.86, [− 5.97, 2.26]). Using a significant threshold of FDR < 0.1, we report a significant inverse correlation between post-treatment SP4, but not SP1, protein levels and mean differences in the general (uncorrected *p* value = 0.011; FDR-adjusted *p* value = 0.054) and total (uncorrected *p* value = 0.018; FDR-adjusted *p* value = 0.054) PANSS subscales in the intervention group. No significant associations with symptom scores were found for SP proteins in the placebo group (Fig. 2a). Furthermore, only SP4 levels were significantly reduced in the raloxifene group at week 24 (uncorrected *p* value = 0.014; FDR-adjusted *p* value = 0.029, *p* value threshold = 0.050) (Fig. 2b, c). Significant differences were maintained after correction for multiple comparisons (Supplementary Table 2).

Table 1 Demographic data and baseline for the women in the raloxifene and placebo groups ($n=14$)

Characteristic	Raloxifene ($n=7$) Mean ± SD	Placebo ($n=7$) Mean ± SD	*Statistic*	*p* value
Age (years)	55.14 ± 3.13	58.43 ± 4.89	1.50; 12[a]	0.160
Education (years)	9.40 + 2.61	7.40 ± 4.45	7.50[b]	0.340
Age at onset of disease (years)	23.50 ± 5.07	29.00 ± 11.42	0.89; 7[a]	0.405
Baseline PANSS score				
Positive	17.43 ± 5.13	16.00 ± 4.62	0.55; 12[a]	0.594
Negative	22.86 ± 3.89	22.71 ± 4.46	0.06; 12[a]	0.950
General	42.14 ± 7.51	35.71 ± 3.55	2.05; 12[a]	0.063
Total	82.43 ± 14.75	74.43 ± 9.43	1.25; 12[a]	0.250
Participant medication type[c]				
Antipsychotic			N/A	N/A
First-generation AP	0	2 (14.28%)		
Second-generation AP	6 (42.85%)	5 (35.71%)		
Combination	1 (1.00%)	0		
Antidepressant (yes)	2 (14.28%)	2 (14.28%)	N/A	1.000
Biperiden (yes)	2 (14.28%)	3 (21.43%)	N/A	1.000
Dosage of AP, median, mg/d[d]	800 ± 610.62	750 ± 532.74	0.64; 11[a]	0.538

Mean and standard deviation or relative frequency are shown for each variable

Percentages based on the total *N* of 14

AP antipsychotic, *PANSS* positive and negative syndrome scale, *N/A* not applicable.

[a]*t* statistic and degrees of freedom are shown for parametric variables.

[b]Mann–Whitney *U* is shown for non-parametric variables

[c]Fisher's exact test for categorical variables.

[d]Last chlorpromazine equivalent dose was calculated based on the electronic records of drug prescriptions of the patients

Discussion

The main objective of this exploratory study was to investigate whether there was an association between SP proteins and raloxifene symptom improvement, contributing additional molecular data to the previous study of Usall and co-workers, in which an improvement in negative and general symptoms in postmenopausal women with schizophrenia treated with raloxifene was reported [14]. Our study shows that reduced SP4, but not SP1, levels correlate with the improvement in general and total PANSS scores in postmenopausal women with schizophrenia treated with raloxifene. These results suggest that SP4 transcription factor might be a biomarker for the effectiveness of raloxifene treatment in global symptom improvement and that it might be involved in the molecular mechanisms that underlie a better global outcome in these patients following treatment with adjuvant raloxifene. This is the first study to suggest an association between peripheral blood SP4 protein levels and symptom improvement in a clinical sample. However, further replication studies with a larger sample size are required to explore the possibility that SP4 might be a biomarker for raloxifene effectiveness.

Regulation of SP1 by oestrogen depletion or in response to oestradiol in postmenopausal animal models has been reported in different tissues (e.g., hippocampus, bone, and endometrium) [28–30]. However, to the best of our knowledge, no studies are available for SP4 protein in these contexts. In our study, we report that the oestrogen analogue raloxifene in postmenopausal women reduces SP4 levels in PBMCs. This finding is in agreement with the reported lower levels of SP4 in PBMCs in childbearing-age women compared to men [27]. It is possible that the depletion of oestrogens in postmenopausal women could increase SP4 levels and that supplementing with oestrogen analogues compensates for this increase in PBMCs. It is not clear the impact that SP4 changes in PBMCs could have on the brain. However, a reduction in SP4 in PBMCs has been associated with smaller right hippocampal volume in first-episode psychosis patients [31], suggesting that changes observed in PBMCs could reflect brain modifications linked to raloxifene treatment in schizophrenia. In fact, several reports show that raloxifene increases the activity of the hippocampus, inferior frontal gyrus, and prefrontal cortex during emotional face recognition, emotional inhibition, or probabilistic association learning in patients with schizophrenia [32–34].

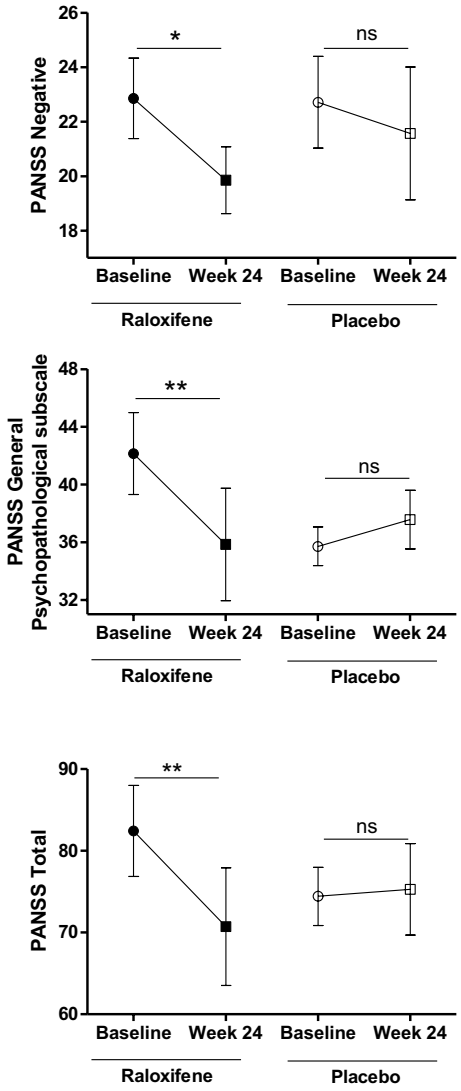

Fig. 1 Symptom scales in the subgroup of patients from the clinical trial. Means for Positive and Negative Syndrome Scale (PANSS) subscale scores are shown for raloxifene and placebo groups at baseline and after 24 weeks of treatment. Error bars indicate standard error of the mean. Statistical analysis was performed using paired *t* test (*$p < 0.05$; **$p < 0.01$: *n.s.* not significant)

Oestrogen effects in the brain include anti-inflammatory properties, modulation of the dopaminergic, GABAergic, serotoninergic and glutamatergic systems, and control of synaptic circuits. Deregulation of all these processes may contribute to the symptoms and cognitive deficits in schizophrenia [2]. Altered SP4 associates with nuclear transcription factor-κB and its pro-inflammatory genes cyclooxygenase-2 and inducible nitric oxide synthase in the brain in schizophrenia [5]. SP4 also correlates with NMDA receptor subunit NR1 in the brain [6]. SP4 modulates the trafficking of NMDA receptor through controlling the expression of its target gene Nervous Wreck-2, a pathway that impacts on SP4-dependent dendritic development and altered brain in schizophrenia [35]. Therefore, the mechanisms through which SP4 could be mediating raloxifene symptom improvement are complex and could involve different processes, including the activation of glutamatergic signalling, the compensation of neuroinflammation, and/or the modulation of dendritic architecture. SP4 also plays a role in ERα/Sp-mediated gene expression and hormone ligand-induced activation of these complexes [3], raising also the possibility that raloxifene could influence non-canonical ERα/SP4-dependent transcriptional programs to improve the symptoms in these patients. However, the exact process by which SP4 could contribute to the raloxifene symptom improvement should be further studied.

Several clinical trials using raloxifene have reported an improvement in symptoms in patients with schizophrenia treated with raloxifene added to their regular antipsychotic medication [14, 15, 36, 37]. In addition, some studies also suggest a positive effect of raloxifene in several cognitive domains such as attention, speed of processing, verbal memory, and executive function [17, 18]. Different treatment effects have been described among the clinical trials, which maybe due to sample characteristics such as baseline symptom severity as suggested in Weickert and Weickert [38]. In our study, we observed an association between SP4 and symptom improvement in patients with moderate baseline symptoms, suggesting that similar effects might be detected in cognitive functioning. Further studies will be needed to explore this possibility.

The limitations of the study include the small sample size. Negative findings should be interpreted with caution because of the limited power of our analyses. Replication with a larger sample should be carried out. Furthermore, it remains to be established whether SP4 changes in peripheral cells occur in parallel to the transcriptional changes in the brain responsible for symptom improvements. However, our study describes for the first time an association between SP4 and raloxifene symptom improvement in postmenopausal women with schizophrenia, suggesting that deregulation of SP4 may be of relevance for the worsening of symptoms in postmenopausal women. Moreover, our results suggest that SP4 might be a potential biomarker for raloxifene-dependent symptom improvement. Future clinical trials with larger samples will be of great interest to assess this possibility for SP4 transcription factor.

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	Raloxifene (n=7)		Placebo (n=7)	
	SP1	SP4	SP1	SP4
dfPANSS scale	r	r	r	r
Negative	-0.468[a]	-0.681[a]	0.167[a]	-0.151[a]
General	-0.351[a]	**-0.868[a]***	0.006[a]	-0.369[a]
Total	-0.422[a]	**-0.839[b]***	0.121[a]	-0.153[a]

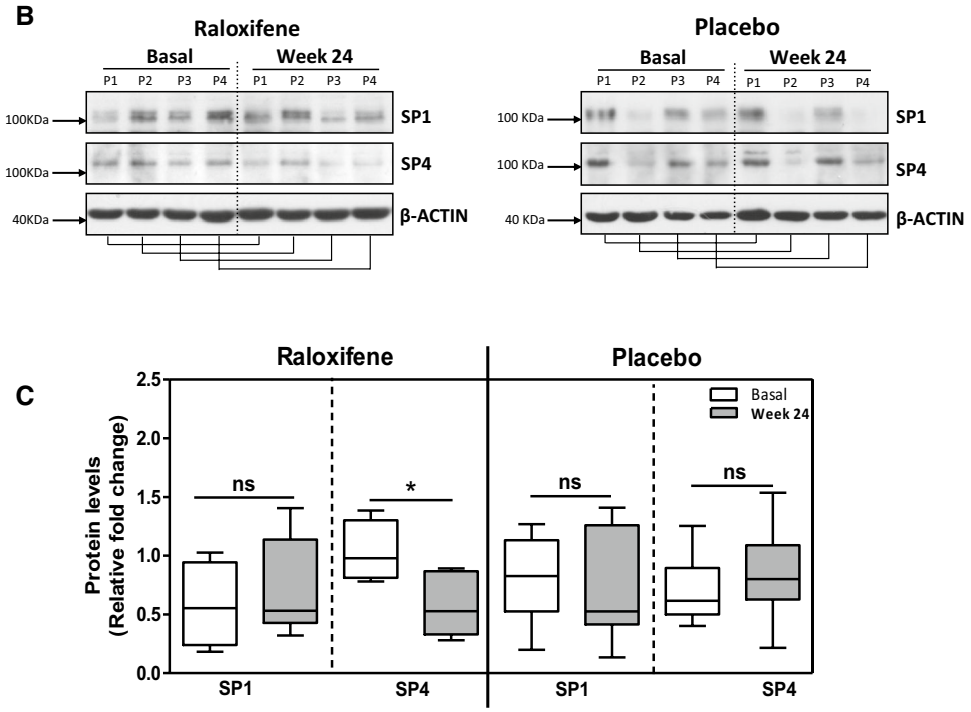

Fig. 2 Analysis of SP transcription factors in peripheral blood mononuclear cells of postmenopausal women with schizophrenia treated with adjunctive raloxifene or placebo. SP proteins from peripheral blood mononuclear cells of postmenopausal women with schizophrenia in raloxifene intervention and placebo groups were analysed at baseline and 24 weeks by immunoblot. Participants were evaluated by the Positive and Negative Syndrome Scale (PANSS) also at baseline and week 24. **a** Correlation between SP1 and SP4 protein levels after treatment (24 weeks) with the mean change from the baseline and final ratings of PANSS subscales (dfPANSS) in the raloxifene and placebo groups. r is shown for each comparison; [a]Pearson's r for parametric variables; [b]Spearman's r for non-parametric variables (*$p<0.05$). Significant associations were maintained after correction for multiple comparisons (SP4-dfPANSS-General: uncorrected $p=0.011$, q value = 0.054; dfPANSS-Total: uncorrected $p=0.018$, q value = 0.054, p value threshold = 0.033) with a false discovery rate (FDR) acceptance set at 0.1. **b** Images show representative SP1, SP4, and β-ACTIN immunoblots of four raloxifene and four placebo participants at baseline and week 24. Molecular marker weights are labelled and shown in KDa. The lines at the bottom of the panel indicate protein bands for the same individual (before and after treatment), **c** Comparison of SP protein levels between baseline and after treatment with raloxifene (baseline $n=4$; 24-week $n=7$) or placebo ($n=7$/group). Each box represents the median and interquartile range of protein levels at baseline and after 24 weeks of treatment. Protein levels were normalized to actin values. Statistical analysis was performed using unpaired t test (*$p<0.05$; ns, not significant). SP4 significant association was maintained after correction for multiple comparisons (SP4: uncorrected $p=0.014$; q value = 0.029, p value threshold = 0.050) with a false discovery rate (FDR) acceptance set at 0.1

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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