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Original article

Effect of concomitant use of pitavastatin with neoadjuvant chemotherapy protocols in breast cancer patients: A randomized controlled clinical trial

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ABSTRACT

Introduction: Preclinical studies have demonstrated the possible anticancer effects of statins, but the synergistic effect of concomitant statin use with standard chemotherapy protocols in patients with breast cancer has not yet been investigated.
Aim: The current study aimed to evaluate the efficacy of concomitant pitavastatin use with neoadjuvant chemotherapy protocols in patients with breast cancer.
Methods: This study was a randomized controlled clinical trial. A total of 70 adult female patients with pathologically-proven invasive breast cancer were randomized to receive or not receive pitavastatin (2 mg) oral tablets once daily concomitantly with standard neoadjuvant chemotherapy protocols for 6 months. The primary outcomes of this study were changes in tumor size and changes to the Ki67 index. In addition, secondary outcomes were changes in cyclin D1 and cleaved caspase-3 serum levels. This study was registered at ClinicalTrials.gov (Identifier: NCT04705909).
Results: Patients in the pitavastatin group showed significantly higher median (IQR) reductions in tumor size [−19.8 (−41.5, 9.5)] compared to those in the control group [−5.0 (−15.5, 0.0), p = 0.0009]. The change in Ki67 from baseline to the end of therapy was similar between the two groups (p = 0.12). By the end of therapy, the cyclin D1 levels in the pitavastatin group were significantly decreased [median (IQR) change of − 10.0 (−20.2, −2.9) from baseline], whereas the control group showed an increase in cyclin D1 levels [14.8 (4.1, 56.4)]. The median (IQR) caspase−3 was elevated in the pitavastatin group 1.6 (0.2, 2.2), and decreased in the control group (−0.2 (−1.1, 0.0), p = 0.0002).

Subgroup analysis of the pitavastatin group revealed that patients with positive human epidermal growth receptor 2 (HER2) had higher median (IQR) reductions in Ki67 [−35.0 (−70.0, −12.5)] than those with negative HER2 [2.5 (−15.0, 10.0), p = 0.04]. All patients who achieved a complete pathological response (n = 9) exhibited an HER2-neu positive receptor at baseline.
Conclusion: Concomitant use of pitavastatin with standard neoadjuvant chemotherapy protocols may improve neoadjuvant chemotherapy responses in patients with breast cancer.

1. Introduction

Breast cancer is one of the most widespread types of cancer worldwide, with 2.3 million new cases per year and the fifth highest level of cancer-related mortality (Sung et al., 2021). In Egypt, 220,038 (32.4%) women develop breast cancer per year, and about 9148 (10.3%) new diagnostic cases result in death. Breast cancer is the second leading cause of cancer death after hepatocellular carcinoma (Sung et al., 2021).

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Treatment options available to breast cancer patients includes surgery, radiotherapy, chemotherapy, hormonal therapy, targeted therapy, and immunotherapy. It's preferable for locally advanced cases or cases with poor prognosis, such as triple negative and human epidermal growth factor receptor 2 (HER2)-enriched subtypes to precede surgery with neoadjuvant chemotherapy as well as its usage in selected cases with early breast cancer. In these cases, a regimen of double or triple chemotherapeutic agents is started immediately upon confirmed diagnosis (Łukasiewicz et al., 2021). Treating such tumors using cocktail of different agents working differently would provide much benefit to avoid the inherent genetic intrinsic instability of cancerous cells which is responsible for resistance to treatment (Mir et al., 2020). Despite the wide range modalities of breast cancer treatments, possible side effects and toxicities are frequent, including general fatigue, hair loss, neuropathy, cardiomyopathy, gastrointestinal symptoms (mouth sores, nausea/vomiting, or diarrhea), and bone marrow suppression resulting in anemia, neutropenia, and decreased immunity (Łukasiewicz et al., 2021).

Given these hindrances, there is a prominent need to repurpose drugs with accepted safety profiles and possible antitumor activities. Repurposing drugs shortens the drug development cycle time, which saves resources used for drug discovery and lessens the possibility of drug failure in early stages. Moreover, the repurposed drug is economically beneficial and only needs marketing for its optimal dosing (Tilija Pun and Jeong, 2021). Statins are attractive agents for drug repurposing from candidate pools (Tilija Pun and Jeong, 2021).

Statins are well established as anti-hypercholesterolemic agents and can be used for the primary and secondary prevention of cardiovascular events. They inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway to decrease cholesterol biosynthesis and intracellular isoprenoid intermediates, which leads to the alteration of different cellular signaling pathways that induce anti-inflammatory, antiproliferative, and apoptotic effects (Jiang et al., 2014).

In addition to their cardiovascular effects, in vitro and in vivo studies have pointed to the possible anticancer advantages of statins, given its role in controlling cancer progression and invasiveness (Alonso et al., 1998; Klawitter et al., 2010; Jiang et al., 2014). A *meta*-analysis found that cancer risk, including breast cancer, may be reduced by long term statin use (Wu et al., 2015). Another study reported that the use of statins after cancer diagnosis reduces the rate of cancer recurrence during the first five years (Kwan et al., 2008). Moreover, statins can sensitize cancer cells to radiotherapy (Lacerda et al., 2014).

Despite the persuasive preclinical evidence for the anticancer effects of statins, their clinical role is still not conclusive (Undela et al., 2012). The effect of statins in breast cancer patients has been investigated in two prospective clinical studies only (Garwood et al., 2010; Bjarnadottir et al., 2013). The outcomes in such studies differ according to the type of statin used, the time and duration of administration, the follow-up time, and patient characteristics (Van Wyhe et al., 2017). Furthermore, these studies were conducted based on the sole effect of statins (Garwood et al., 2010; Bjarnadottir et al., 2013). Although statins may have additive or synergistic effects with standard chemotherapy protocols (Stryjkowska-Góra et al., 2015), its efficacy in combination with neoadjuvant regimens in patients with breast cancer has not yet been evaluated. Consequently, it is worthwhile to study whether statins can potentiate the tumor response of neoadjuvant breast cancer therapy, especially pitavastatin, which has shown superiority in reducing the incidence of cancer cases in comparison to atorvastatin (Nagayama et al., 2021). This study aimed to evaluate the efficacy of pitavastatin as an adjuvant therapy added to neoadjuvant breast cancer therapy in patients with breast cancer in comparison to breast cancer patients who received only the neoadjuvant therapy.

2. Methods

2.1. Study design and ethical approval

This was a randomized controlled clinical study. Prospective breast cancer patients were recruited from the Oncology Center at Mansoura University (OCMU), Egypt. Patients who met the inclusion criteria were required to accept their enrollment in this trial and had to give their written, informed consent before any study intervention occurred. The study was performed according to the 1964 Declaration of Helsinki and its subsequent amendments (Rickham, 1964), and was approved by the Faculty of Pharmacy, Mansoura University Ethical Committee (number 2020–176). The study was registered at ClinicalTrials.gov (Identifier: NCT04705909).

2.2. Inclusion and exclusion criteria

Newly diagnosed adult female patients (age $\geq$ 18 years) with histologically confirmed primary invasive breast cancer who were due to start neoadjuvant chemotherapy were eligible for inclusion. The patients were excluded if they had renal impairment (defined as serum creatinine of more than 1.4 mg/dl), hepatic impairment (defined as aspartate transaminase, alanine transaminase, or alkaline phosphatase of more than 2.5 folds of the upper limit of the normal range), severe gastrointestinal disorder, cardiac failure, active infections, or major psychiatric diseases that may affect compliance to the study procedures. Patients were also excluded if they were pregnant or currently lactating. To avoid interactions with the pitavastatin therapy (intervention), patients on CYP3A4 inhibitors (erythromycin, clarithromycin, ritonavir, delavirdine, fluoxetine, fluvoxamine, diltiazem, and verapamil) were excluded. Patients currently or previously (within the last 3 months) on statins or fibrates for hypercholesterolemia were also excluded, as were patients on anticoagulant therapy. Patients with metastatic or non-invasive disease were also excluded.

2.3. Sample size

Twenty-two patients in each group were sufficient to achieve a power of 95% and to detect an effect size of 0.765 (based on the change in Ki67) (Garwood et al., 2010) with a two-sided test at the alpha level of 0.05 using G*power 3.1.9.7 software (Faul et al., 2007). A power of 90% would be achieved with a sample size of 20 patients in each group using the same calculation method. To account for any dropouts, the number of patients in the current study was increased to 35 patients in each group.

2.4. Patient allocation

After screening for inclusion and exclusion criteria, the eligible patients were randomly allocated in a 1:1 ratio to either the pitavastatin or control groups with 35 patients each, using a coin-flip method. Patients in the pitavastatin group received 2 mg pitavastatin oral tablets once daily for the treatment period before surgery. This dose was selected because pitavastatin can produce antitumor effect within its hypercholesterolemic dose (Jiang et al., 2014). Patients in the control group did not receive pitavastatin. Patients in both groups received the standard neoadjuvant chemotherapy protocol; dose dense doxorubicin (total dose/cycle = 60 mg/m^2) plus cyclophosphamide (total dose/cycle =

600 mg/m^2) (Citron et al., 2003) intravenously for four cycles with 21 days in between followed by 4 cycles of dose dense paclitaxel (total dose/cycle = 175 mg/m^2) with 2 weeks period between each two subsequent cycles (Sparano et al., 2008) followed by surgical intervention. Each patient received subcutaneous 2 or 3 doses of Filgrastim 5 μg/kg on days 4–6 of each cycle (National Comprehensive Cancer Network, 2022).

Before each cycle of the eight cycles, the patient received supportive antiemetic treatment regimens 8 mg intravenously serotonin antagonist (ondansetron (Zofran®)) and dexamethasone 8 mg intravenously 30 min before chemotherapy with or without neurokinin receptor antagonist (Aprepitant - Emend®) 125 mg orally on day 1, followed by 80 mg orally on days 2 and 3 or Granisetron 1 or 2 mg orally for 2 days after cycle in specific patients. As necessary, some of patients received proton pump inhibitors.

During the study, the patients were contacted weekly to enhance the adherence to pitavastatin and check if the patient suffered from any complaint after the chemotherapy cycle.

2.5. Baseline data collection

During patient screening, data on age, kidney function (serum creatinine), liver function (alanine transaminase, aspartate aminotransferase, albumin, bilirubin, and alkaline phosphatase), blood cell count, and comorbidities were collected. Upon trial recruitment, marital status, menopausal status, number of offspring, and tumor characteristics were also collected. Tumor characteristics included tumor side (right, left, or both sides), tumor type (invasive ductal carcinoma, invasive lobular carcinoma, or others), tumor grade, and molecular subtype (luminal A, luminal B, HER2-enriched, and triple-negative). The tumor stage was detected using TNM scoring from the American Joint Committee of Cancer (AJCC). In addition, a blood sample was collected from each patient at recruitment to measure their serum cyclin D1 and caspase-3 concentrations.

2.6. Outcome measures

After completion of the treatment course (6 months), the study outcomes were assessed. The primary outcomes of this trial were the change in radiological tumor size (expressed as the largest diameter) and changes in the Ki67 index. The change in tumor size was calculated by subtracting the initial tumor size at recruitment from the final tumor size after treatment completion. The change in the Ki67 index was calculated by considering the Ki67 immunohistochemistry expression in tumor tissue samples collected during definitive surgery in relation to tumor tissue collected during the diagnostic core biopsy.

The secondary outcomes included changes in the cyclin D1 serum levels as a marker of breast tumor proliferation and changes in cleaved caspase-3 as a marker of tumor apoptosis.

Response types were categorized as no response, partial response, and complete pathological response based on pathological assessment of the tumor tissues collected at the time of surgery after completion of the treatment course.

2.7. Details for performed measurements

2.7.1. Ki67 and hormone receptors

Staining was performed using ROCH automatic immunohistochemistry instrument model VENTANA BenchMark GX. Rabbit monoclonal primary antibodies "REF 790-4286" "REF 790-4324," "REF 790-2223," and "REF 790-4493" were used for Ki67, estrogen receptor (ER), progesterone receptor (PR), and HER2/neu, respectively. The Ki67 percent of positive staining cells were determined by an academic surgical pathologist by examination of the area with the highest mitotic activities. The Ki67 proliferation index was obtained by calculating the percentage of Ki67-positive tumor nuclei.

2.7.2. Tumor size

Tumor size was expressed as the largest tumor diameter after size detection using a sonomammogram or magnetic resonance imaging (MRI) before the start of the treatment protocol and after treatment completion during surgery.

2.7.3. Cyclin D1 and cleaved caspase-3

The serum cyclin D1 and caspase-3 levels were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) technique. Kits (code number; NBP2-75100, NOVUS biologicals, USA) and (code number; E4804Hu, Bioassay technology, China) were used for cyclin D1 and caspase-3, respectively, as directed by the manufacturers. The assays were based on the color intensity detected after the complete reaction. The microplates were precoated with the desired biomarker's (cyclin D1/caspase-3) specific antibody, which was then combined with the samples and standard. Next, the biotinylated detection and horseradish antibodies were subsequently added to each microplate well, then incubated (60 min for cyclin D1 and 10 min for caspase-3). The free components were washed away by a washing buffer after incubation. Afterwards, the substrate solution was added to each well. Only wells containing antigens for the desired biomarker resulted in a blue color. Enzyme substrate reaction was terminated by adding stop solution, causing the color to change to yellow. The concentration was detected by measuring the optical density of the resultant color at 450 nm.

2.8. Statistical analysis

The normality of a continuous variable distribution was tested via the Shapiro-Francia test. For normally distributed variables, the values were expressed as mean ± standard deviation (SD). Non-normally distributed variable values were presented as median and interquartile range (IQR). Differences between pitavastatin and the control groups were examined by t-tests or Mann-Whitney *U* test according to variables distribution. Differences within the same group before and after therapy were examined by paired t-tests or Wilcoxon Signed Rank test. Binary and categorical variables were presented as numbers and percentages and were compared using the chi-square test for independent groups. The significance level was set at $p \leq 0.05$. SPSS version 26, 2019 software (IBM SPSS statistics) was used to run all statistical analyses.

3. Results

From January to June 2021, a total of 282 patients were screened for recruitment eligibility. Of these, 70 patients were randomized to the pitavastatin and control groups (Fig. 1). During the 6 months follow-up period, 11 patients were dropped due to follow-up loss, death, study withdrawal, or the development of metastasis. The remaining 59 patients completed the study and were included in the analysis: 27 patients in the pitavastatin group; 32 patients in the control group.

3.1. Baseline demographics and biomedical data

The mean ± SD age of the patients was late forties (46.3 ± 10. 6 years). All the patients were married with a mean of three children. Twenty-five (42.4%) of them were postmenopausal. The patient demographics, comorbidities, regularly taken medicines, and laboratory tests did not significantly differ between the two

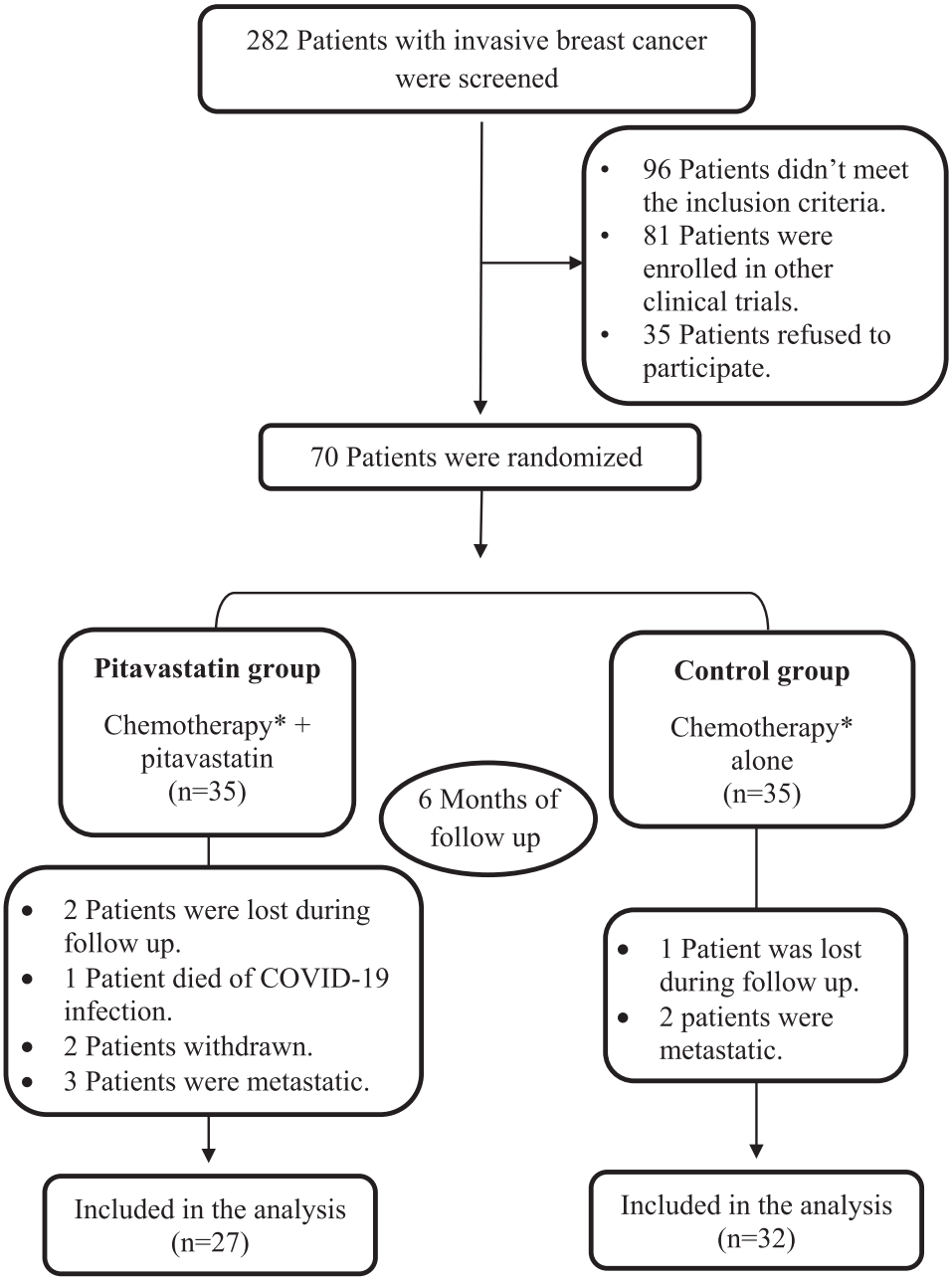

Fig. 1. Flow diagram of patients screening, recruitment, and follow up. * The standard neoadjuvant chemotherapy protocol (doxorubicin hydrochloride and cyclophosphamide followed by paclitaxel).

groups at baseline. About one-fifth (n = 12; 20.3%) of the patients had hypertension and 16.9% (n = 10) were diabetic. Diabetic patients were on either oral hypoglycaemic agent or on insulin regimen, hypertensive patients were treated with diuretics or angiotensin converting enzyme inhibitor or a combination of both agents where as patients with hypothyroidism were maintained on L-thyroxin. However, there were no significant difference between the two groups. In general, the kidney function, liver function, blood cell count, and hemoglobin levels of the patients were within normal range and were comparable between the two groups (Table 1).

3.2. Tumor characteristics

About one-third of the patients (n = 20; 33.9%) had a right-side tumor, 37 (62.7%) had a left-side tumor, and 2 (3.4%) had their tumor on both sides. Most of the patients (94%) were diagnosed with invasive ductal carcinoma. Invasive lobular carcinoma was diagnosed in only three patients (5.1%). Grade 2 tumors were seen in 40 (67.8%) patients, whereas 19 (32.2%) patients had grade 3 tumors. Tumor side, type, and grade did not differ between the two groups (Table 2).

A higher proportion of the patients in the control group were ER positive (n = 27; 84.4%) and PR positive (n = 24; 75.0%) compared to 13 (48.1%) and 11 (40.7%) in the pitavastatin group, respectively. The most prevalent molecular type among the patients was luminal B (n = 32; 54.2%). The molecular tumor type also differed across the two groups; the pitavastatin group had a higher proportion of patients (n = 9; 33.3%) with HER2-enriched tumors than the control group (n = 2; 6.3%).

Ki67 was high in most of the patients (n = 38; 71.7%) with a median (IQR) of 40.0 (15.0; 60.0). The control group showed a

Table 1
Baseline demographics and biomedical data.

	Pitavastatin group (n = 27)	Control group (n = 32)	Total (n = 59)	*P* value
Age, mean ± SD (year)	46.1±11.4	46.4±10.1	46.3±10.6	0.90*
Menopausal status, n (%)				
Premenopausal	16 (59.3)	18 (56.3)	34 (57.6)	0.82‡
Postmenopausal	11 (40.7)	14 (43.8)	25 (42.4)	
No. of offspring, mean ± SD	2.9 ± 1.7	3.2 ± 1.0	3.0 ± 1.4	0.44*
Comorbidities, n (%)				
Hypertension	5 (18.5)	7 (21.9)	12 (20.3)	0.10‡
Diabetes	5 (18.5)	5 (15.6)	10 (16.9)	0.09‡
Hypothyroidism	1 (3.7)	2 (6.3)	3 (5.1)	0.20‡
Hepatitis	1 (3.7)	0 (0.0)	1 (1.7)	1.21‡
Obesity	1 (3.7)	0 (0.0)	1 (1.7)	1.21‡
No. of comorbidities, median (IQR)	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)	0.97†
Medication profile				
Diuretic	1 (3.7)	0 (0)	1 (1.7)	0.46‡
ACE inhibitor	1 (3.7)	3 (9.4)	4 (6.8)	0.62‡
ACE inhibitor + diuretic	3 (11.1)	4 (12.5)	7 (11.9)	1‡
Insulin	2 (7.4)	4 (12.5)	6 (10.2)	0.68‡
Oral hypoglycemic agent	3 (11.1)	1 (3.1)	4 (6.8)	0.32‡
L-thyroxin	1 (3.7)	2 (6.3)	3 (5.1)	1‡
Liver function				
Alanine transaminase, median (IQR) (IU/L)	16.0 (12.9, 20.1)	18.9 (13.5, 28.0)	17.0 (13.0, 26.0)	0.40†
Aspartate aminotransferase, median (IQR) (IU/L)	19.0 (17.0, 23.1)	19.5 (16.0, 25.0)	19.0 (16.0, 24.0)	0.90†
Albumin, mean ± SD (g/dL)	4.2 ± 0.4	4.3 ± 0.4	4.2 ± 0.4	0.15*
Bilirubin, median (IQR) (mg/dL)	0.5 (0.3, 0.6)	0.5 (0.3, 0.6)	0.5 (0.3, 0.6)	0.28†
Serum alkaline phosphatase, mean ± SD (IU/L)	75.1 ± 22.3	68.8 ± 22.6	71.8 ± 22.4	0.33*
Serum creatinine, median (IQR) (mg/dL)	0.8 (0.7, 0.9)	0.7 (0.7, 0.8)	0.7 (0.7, 0.9)	0.11†
White blood cell count, mean ± SD ($\times 10^9$/L)	7.7 ± 1.9	6.9 ± 2.5	7.3 ± 2.3	0.16*
Platelet count, mean ± SD ($\times 10^9$/L)	278.2 ± 60.5	287.7 ± 92.9	283.3 ± 79.2	0.65*
Hemoglobin, median (IQR) (g/dl)	12.1 (11.1, 13.0)	12.2 (11.5, 12.6)	12.1 (11.3, 12.7)	0.84†

* *t*-test, † Mann-Whitney *U* test, and ‡ chi-square test. ACE. angiotensin converting enzyme.

Table 2
Baseline tumor characteristics.

		Pitavastatingroup (n = 27)	Controlgroup (n = 32)	Total(n = 59)	*P* value
Tumor side	Right	8 (29.6)	12 (37.5)	20 (33.9)	0.27‡
	Left	17 (63.0)	20 (62.5)	37 (62.7)	
	Both sides	2 (7.4)	0 (0.0)	2 (3.4)	
Tumor type	Invasive ductal carcinoma	25 (92.6)	31 (96.9)	56 (94.9)	0.99‡
	Invasive lobular carcinoma	2 (7.4)	1 (3.1)	3 (5.1)	
Tumor grade	Grade 2	15 (55.6)	25 (78.1)	40 (67.8)	0.07‡
	Grade 3	12 (44.4)	7 (21.9)	19 (32.2)	
Estrogen receptor	Positive	13 (48.1)	27 (84.4)	40 (67.8)	0.003‡
	Negative	14 (51.9)	5 (15.6)	19 (32.2)	
Progesterone receptor	Positive	11 (40.7)	24 (75.0)	35 (59.3)	0.01‡
	Negative	16 (59.3)	8 (25.0)	24 (40.7)	
HER2	Positive	18 (66.7)	15 (46.9)	33 (55.9)	0.28‡
	Negative	8 (29.6)	16 (50.0)	24 (40.7)	
	Equivocal	1 (3.7)	1 (3.1)	2 (3.4)	
Molecular type	Luminal A	2 (7.4)	6 (18.8)	8 (13.6)	0.04‡
	Luminal B	11 (40.7)	21 (65.6)	32 (54.2)	
	HER2-enriched	9 (33.3)	2 (6.3)	11 (18.6)	
	Triple-negative	4 (14.8)	2 (6.3)	6 (10.2)	
Ki67	Low	2 (8.7)	3 (10.0)	5 (9.4)	0.23‡
	Borderline	2 (8.7)	8 (26.7)	10 (18.9)	
	High	19 (82.6)	19 (63.3)	38 (71.7)	
Ki67, median (IQR) (%)		50.0 (30.0, 70.0)	27.5 (10.0, 40.0)	40.0 (15.0, 60.0)	0.03†
Tumor size, median (IQR) (mm)		36.5 (24.0, 53.0)	28.0 (21.8, 40.0)	30.0 (24.0, 49.0)	0.10†
Cyclin D1 levels, mean ± SD (ng/ml)		48.5 ± 21.9	39.1 ± 20.4	44.1 ± 21.5	0.17*
Caspase-3 levels, median (IQR) (ng/ml)		2.3 (2.0, 3.1)	2.7 (2.0, 3.6)	2.5 (2.0, 3.3)	0.24†
Radiological T score	T1	2 (7.4)	6 (18.8)	8 (13.6)	0.003‡
	T2	8 (29.6)	20 (62.5)	28 (47.5)	
	T3	3 (11.1)	3 (9.4)	6 (10.2)	
	T4	14 (51.9)	3 (9.4)	17 (28.8)	
Radiological N score	N0	1 (3.7)	10 (31.3)	11 (18.6)	0.02‡
	N1	18 (66.7)	19 (59.4)	37 (62.7)	
	N2	1 (3.7)	0 (0.0)	1 (1.7)	
	N3	7 (25.9)	3 (9.4)	10 (16.9)	

HER2. Human epidermal growth receptor 2. * *t*-test, † Mann-Whitney *U* test, and ‡ chi-square test.

lower Ki67 percentage than the pitavastatin group (p = 0.03; Table 2).

The median (IQR) greatest tumor size diameter was 30 (24–49) mm among patients and did not differ between the two groups (p = 0.10). Both cyclin D1 and caspase-3 levels did not differ between the two groups (Table 2).

3.3. Primary outcomes

3.3.1. Ki67

Table 3 shows the tumor characteristics at the end of therapy for both groups. Ki67 at surgery time was significantly lower than in the core biopsy at baseline within the pitavastatin group (p = 0.016), whereas there was no statistical difference within the control group (p = 0.18; Fig. 2A). No significant differences were found when the change in Ki67 from baseline to time of surgery was compared between the two groups (p = 0.12; Table 4). The reduction of Ki67 after receiving pitavastatin added to the standard neoadjuvant chemotherapy regimen is shown in supplementary material (Fig. S1).

3.3.2. Tumor size

Tumor size decreased significantly in both groups after neoadjuvant therapy according to the paired sample *t*-test (Fig. 2B). Patients in the pitavastatin group showed a more significant median (IQR) reduction in tumor size [−19.8 (−41.5, 9.5)] compared to those in the control group (−5.0 (−15.5, 0.0); Table 4). An example of complete response in the pitavastatin group as documented by MRI images is shown in supplementary material (Fig. S2).

3.4. Secondary outcomes

3.4.1. Cyclin D1

At baseline, the expression levels of cyclin D1 did not differ between the two groups (p = 0.17; Table 2); however, by the end of treatment, the pitavastatin group's mean level was significantly lower than the control group (p = 0.005). Upon analyzing the cyclin D1 levels in the same group before and after chemotherapy, the intervention group's level was significantly lower (p = 0.03), whereas the control group's level increased (p = 0.01) (Fig. 2C).

3.4.2. Caspase-3

After receiving therapy, the intervention group's caspase-3 levels increased significantly compared to the baseline (p = 0.003), whereas the control group's level decreased (p = 0.019; Fig. 2D). Although the caspase-3 levels were comparable at baseline (p = 0.24), the intervention group's median (IQR) expression level after treatment was significantly greater [4.1 (3.0, 4.5), p = 0.003] than that of the control group [2.2 (2.0, 2.6); Table 3].

Table 3
Tumor characteristics after therapy.

		Pitavastatin group (n = 27)	Control group (n = 32)	Total(n = 59)	*P* value
Estrogen receptor	Positive	13 (48.1)	21 (65.6)	34 (57.6)	0.19‡
	Negative	6 (22.2)	3 (9.4)	9 (15.3)	
	Pathological complete response	6 (22.2)	3 (9.4)	9 (15.3)	
Progesterone receptor	Positive	14 (51.9)	20 (62.5)	34 (57.6)	0.53‡
	Negative	7 (25.9)	6 (18.8)	13 (22.0)	
	Pathological complete response	5 (18.5)	3 (9.4)	8 (13.6)	
HER2	Positive	7 (25.9)	9 (28.1)	16 (27.1)	0.12‡
	Negative	9 (33.3)	18 (56.3)	27 (45.8)	
	Equivocal	2 (7.4)	2 (6.3)	4 (6.8)	
	Pathological complete response	5 (18.5)	3 (9.4)	8 (13.6)	
Type of surgery done	Mastectomy	15 (55.6)	19 (59.4)	34 (57.6)	0.05‡
	Conservative breast surgery	11 (40.7)	6 (18.8)	17 (28.8)	
Type of response	Complete response	6 (22.2)	3 (9.4)	9 (15.3)	0.19‡
	Partial response	18 (66.7)	27 (84.4)	45 (76.3)	
	No response	1 (3.7)	2 (6.3)	3 (5.1)	
Ki67	Low	6 (35.3)	4 (21.1)	10 (27.8)	0.59‡
	Borderline	5 (29.4)	8 (42.1)	13 (36.1)	
	High	6 (35.3)	7 (36.8)	13 (36.1)	
Ki67, median (IQR) (%)		10.0 (0.0, 30.0)	15.0 (10.0, 25.0)	10.0 (6.5, 27.5)	0.31†
Tumor size, median (IQR) (mm)		14.0 (4.5, 23.0)	23.5 (16.0, 32.5)	20.0 (12.0, 30.0)	0.01†
Cyclin D1 levels, mean ± SD (ng/ml)		38.1 ± 21.1	61.2 ± 28.9)	48.8 ± 27.3	0.005*
Caspase-3 levels, median (IQR) (ng/ml)		4.1 (3.0, 4.5)	2.2 (2.0, 2.6)	3.0 (2.2, 4.3)	0.003†
Radiological T score	T0	5 (19.2%)	5 (15.6%)	10 (17.2%)	0.10‡
	T1	10 (38.5%)	9 (28.1%)	19 (32.8%)	
	T2	4 (15.4%)	15 (46.9%)	19 (32.8%)	
	T3	1 (3.8%)	1 (3.1%)	2 (3.4%)	
	T4	6 (23.1%)	2 (6.3%)	8 (13.8%)	
Radiological N score	N0	16 (61.5%)	20 (62.5%)	36 (62.1%)	0.41‡
	N1	10 (38.5%)	9 (28.1%)	19 (32.4%)	
	N2	0 (0%)	2 (6.3%)	2 (3.4%)	
	N3	0 (0%)	1 (3.1%)	1 (1.7%)	
Pathological T score	T0	7 (25.9)	4 (12.5)	11 (18.6)	0.30‡
	T1	6 (22.2)	5 (15.6)	11 (18.6)	
	T2	9 (33.3)	18 (56.3)	27 (45.8)	
	T3	3 (11.1)	5 (15.6)	8 (13.6)	
	T4	1 (3.7)	0 (0.0)	1 (1.7)	
Pathological N score	N0	12 (44.4)	12 (37.5)	24 (40.7)	0.49‡
	N1	3 (11.1)	8 (25.0)	11 (18.6)	
	N2	9 (33.3)	11 (34.4)	20 (33.9)	
	N3	2 (7.4)	1 (3.1)	3 (5.1)	

HER2. Human epidermal growth receptor 2. * *t*-test, † Mann-Whitney *U* test, and ‡ chi-square test.

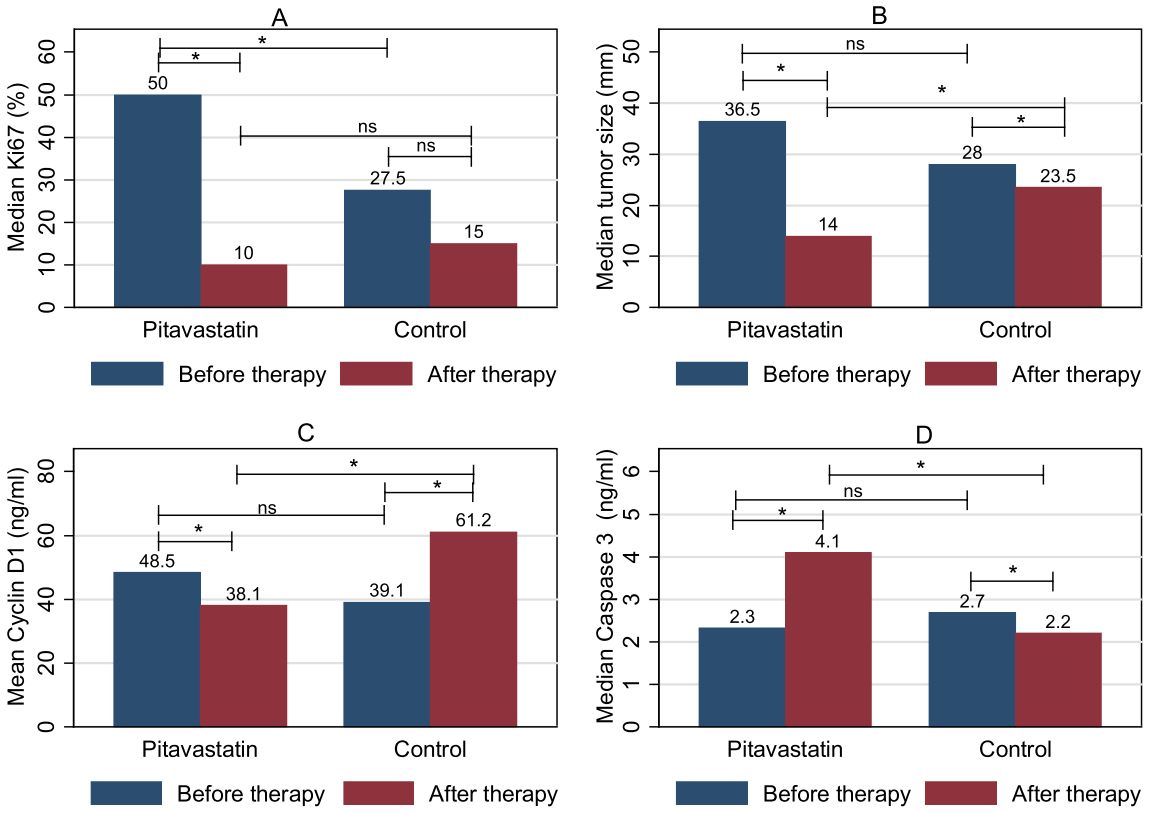

Fig. 2. Ki67 **(A)**, tumor size **(B)**, cyclin D1 levels **(C)**, and caspase-3 levels **(D)** before and after therapy in pitavastatin and control groups. * Significant difference ($p \leq 0.05$), and ns. non-significant.

Table 4
Primary and secondary outcomes of the study.

	Pitavastatin group			Control group			Total			*P* value*
	n	Median	(IQR)	n	Median	(IQR)	n	Median	(IQR)	
Change in Ki67, (%)	14	−20.0	(−50.0, 0.0)	18	−3.5	(−15.0, 4.0)	32	−5.0	(−25.0, 2.0)	0.12
Change in tumor size, (mm)	24	−19.8	(−41.5, 9.5)	32	−5.0	(−15.5, 0.0)	56	−10.0	(−22.5, −2.8)	0.0009
Change in cyclin D1, (ng/ml)	22	−10.0	(−20.2, −2.9)	19	14.8	(4.1, 56.4)	41	1.6	(-10.7, 16.1)	0.0002
Change in Caspase-3, (ng/ml)	22	1.6	(0.2, 2.2)	19	−0.2	(−1.1, 0.0)	13	0.2	(−0.5, 1.7)	0.0002

*Mann-Whitney *U* test.

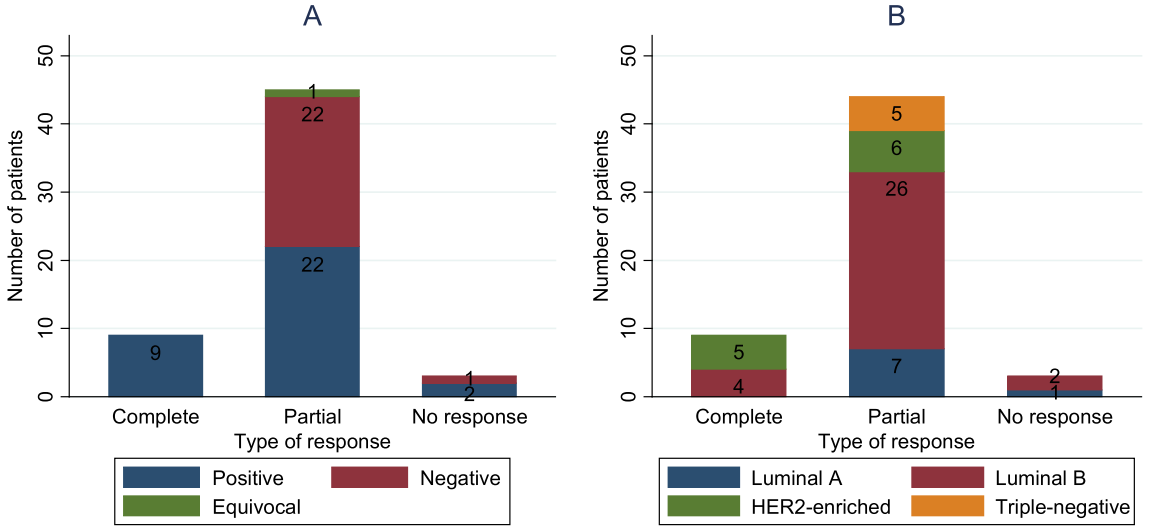

Fig. 3. Type of pathological response achieved in study patients according to baseline HER2 receptors **(A)** and the molecular tumor types **(B)**.

3.4.3. Type of response

Most of the patients showed a partial response [n = 45 (76.3%)] that was consistent between the two groups (p = 0.19; Table 3). A total of 26 (59.1%) of the patients who achieved partial response had the luminal B subtype. In total, 9 patients had a complete pathological response, 6 of which (22.2%) were in the pitavastatin group and 3 of which (9.4%) were in the control group. All of these 9 patients exhibited the HER2-neu positive receptor at baseline (Fig. 3A). According to tumor molecular type, 5 of these patients (55.6%) exhibited HER2-enriched luminal subtype, whereas 4 of them (44.4%) had the luminal B subtype (Fig. 3B).

After receiving therapy, the two groups showed similar pathological and radiological T and N scores (Table 3), although patients in the pitavastatin group had worse radiological T and N scores at baseline (Table 2).

3.5. Subgroup analysis according to HER2 receptor and molecular type

The median (IQR) Ki67 was significantly more reduced in HER2-positive patients – 25.0 (−40.0, 0.0) than in HER2-negative patients 0.0 (−7.5, 10.0; p = 0.005). This significant difference was also seen within the pitavastatin group (p = 0.04), but not within the control group (p = 0.06; Table 5). The change in tumor size, cyclin D1 levels, and caspase-3 levels did not differ according to HER2 receptor in both groups (Table 5).

The change in Ki67 index, tumor size, cyclin D1 levels, and caspase-3 levels did not differ according to molecular type or tumor grade in all patients regardless of group, except for the median (IQR) cyclin D1 level in the control group, which was significantly decreased in patients with grade 3 [−0.5 (−35.5, 9.2)] than in patients with grade 2 [20.0 (4.6, 57.0), p = 0.02; Table 6].

3.6. Pitavastatin related side effects

Throughout the trial, changes in the bowel habits were reported similarly in both pitavastatin and control groups. This didn't affect continuity of the treatment as the symptoms were resolved by the first two days of treatment and patients were able to regulate their symptoms by changing their diet. No additional side effects were reported.

Table 5
Primary and secondary outcomes of the study according to HER2 receptor.

	Pitavastatin group		P value	Control group		P value	Total		P value
	HER2 positive	HER2 negative		HER2 positive	HER2 negative		HER2 positive	HER2 negative	
Change in Ki67, (%)	−35.0 (−70.0, −12.5)	2.5 (−15.0, 10.0)	0.04	−20.0 (−25.0, 4.0)	0.0 (−5.0, 10.0)	0.06	−25.0 (−40.0, 0.0)	0.0 (−7.5, 10.0)	0.005
Change in tumor size, (mm)	−19.8 (−42.0, −7.0)	−20.5 (−37.0, −11.0)	0.95	−11.0 (−21.0, 0.0)	−3.5 (−5.5, 0.0)	0.19	−15.0 (−23.0, −4.0)	−5.0 (−18.5, −1.0)	0.13
Change in cyclin D1, (ng/ml)	−8.3 (−22.6, −2.9)	−10.0 (−15.9, 4.6)	0.59	20.0 (10.1, 68.2)	4.9 (3.7, 24.0)	0.14	−3.0 (−13.7, 18.0)	4.3 (−10.0, 13.8)	0.74
Change in Caspase3, (ng/ml)	1.2 (−0.2, 2.8)	1.7 (1.4, 1.8)	0.97	−0.2 (−1.1, 0.0)	−0.1 (−0.7, 0.1)	0.61	−0.0 (−0.6, 1.2)	1.4 (−0.1, 1.8)	0.22

Results are median (interquartile range). P values based on Mann-Whitney *U* test. HER2. Human epidermal growth receptor 2. Patients with equivocal HER 2 were dropped from the analysis due to very small size (2 patients).

Table 6
Primary and secondary outcomes of the study according to the molecular type and grade of the tumor.

	Molecular type					Tumor grade		
	Luminal A	Luminal B	HER2 enriched	Triple-negative	*P* value*	Grade 2	Grade 3	*P* value†
Change in Ki67, (%)								
Pitavastatin group	5.0(5.0, 5.0)	−20.0 (−65.0, 0.0)	−30.0 (−60.0, −25.0)	−2.5 (−32.5, 10.0)	0.31	0.0 (−40.0, 5.0)	−25.0 (−60.0, 0.0)	0.44
Control group	−2.5 (−5.0, 10.0)	−10.0 (−20.0, 4.0)	−20.0 (−20.0, −20.0)	15.0(15.0, 15.0)	0.23	−5.0 (−15.0, 4.0)	−5.0 (−20.0, 5.0)	0.73
Total	0.0 (−5.0, 5.0)	−10.0 (−25.0, 0.0)	−27.5 (−60.0, −20.0)	10.0 (−15.0, 10.0)	0.06	−2.5 (−20.0, 4.5)	−15.0 (−50.0, 0.0)	0.21
Change in tumor size, (mm)								
Pitavastatin group	−12.0 (−12.0, −12.0)	−27.0 (−50.0, −20.0)	−15.0 (−23.0, −5.0)	−13.5 (−30.5, −2.5)	0.29	−21.5 (−34.5, −14.0)	−13.0 (−44.5, −3.0)	0.20
Control group	−4.5 (−10.0, −3.0)	−5.0 (−16.0, 0.0)	−15.0 (−19.0, −11.0)	6.5(0.0, 13.0)	0.22	−5.0 (−19.5, 0.0)	−3.0 (−8.0, 11.0)	0.31
Total	−5.0 (−12.0, −3.0)	−13.0 (−24.0, −2.0)	−15.0 (−23.0, −5.0)	−5.0 (−17.0, 5.0)	0.42	−12.5 (−22.8, −3.5)	−6.0 (−22.5, −2.0)	0.48
Change in cyclin D1, (ng/ml)								
Pitavastatin group	−25.8 (−41.8, −9.9)	−16.2 (−22.6, −8.3)	−3.1 (−10.7, 1.6)	7.5(4.6, 10.3)	0.07	−9.1 (−19.5, −3.5)	−10.1 (−16.7, 4.6)	0.62
Control group	4.8(4.1, 5.4)	14.8(3.7, 49.8)	39.1(10.1, 68.2)	37.2(17.4, 57.0)	0.53	20.0(4.6, 57.0)	−0.5 (−35.5, 9.2)	0.02
Total	−2.9 (−25.8, 4.8)	−3.3 (−16.5, 20.0)	−2.9 (−6.5, 10.1)	13.8(7.5, 37.2)	0.30	4.5 (−8.3, 43.1)	−4.7 (−16.7, 4.6)	0.08
Change in caspase-3, (ng/ml)								
Pitavastatin group	0.8(0.2, 1.4)	1.7(0.2, 2.2)	1.6 (−0.1, 2.7)	1.8(1.5, 1.9)	0.90	1.6(0.2, 2.4)	1.6(0.2, 2.2)	0.90
Control group	−0.1 (−0.1, −0.1)	−0.1 (−0.9, 0.1)	−0.9 (−1.5, 0.2)	– –	0.40	−0.1 (−1.1, 0.0)	−0.6 (−0.8, −0.1)	0.64
Total	0.2 (−0.1, 1.4)	−0.1 (−0.6, 0.4)	0.7 (−0.5, 2.6)	1.8(1.5, 1.9)	0.30	0.1 (−0.2, 1.4)	0.2 (−0.6, 1.9)	0.61

* K-Wallis test, and † Mann-Whitney *U* test.

4. Discussion

This study aimed to identify whether the addition of pitavastatin to neoadjuvant chemotherapy protocols in patients with breast cancer could potentiate antitumor activity, induce cancer cells to undergo apoptosis, and inhibit proliferative activity through cell cycle regulation.

Pitavastatin was chosen rather than other statins due to its lipophilic nature, which enables its availability in extrahepatic tissues, and because its half-life is longer, which allows for the continuous inhibition of the targeted receptor (HMGCR) (Catapano, 2010; Barbalata et al., 2020). Pitavastatin has shown superiority over atorvastatin in preventing carcinogenesis (Nagayama et al., 2021). In addition, although much higher doses of statins are usually needed to produce antitumor activities, hypercholesterolemia doses of both cerivastatin and pitavastatin can produce anti-cancer activity (Jiang et al., 2014). In this study, a moderate dose of pitavastatin (2 mg daily) approved for hypercholesterolemia and cardiovascular disease prevention was used (Drugs.com, 2020). This dose was tolerated with no serious adverse effects reported by the patients.

Concomitant use of statins over the period of neoadjuvant chemotherapy resulted in increased tumor size reductions in comparison with the conventional chemotherapy protocol. The reduction in tumor size was higher in the pitavastatin group. Based on luminal subtype, luminal B and HER-2 expression tumors underwent the greatest size reductions, but this difference was not statistically significant. The pitavastatin treatment resulted in a reduction in tumor size regardless of estrogen receptor status. Although statins have been shown to reduce the cancer-specific mortality of ER-negative tumors, its effect on size reduction is universal (Garwood et al., 2010). On the other hand, the triple-negative of the bad repetition of worst prognosis decreased in size in the pitavastatin group. This indicates that patients with triple negative breast cancer may benefit from statin addition, not only to increase their survival as mentioned by Malgorzata et al., but also to control tumor size during neoadjuvant treatment (Nowakowska et al., 2021). This is of much benefit in improving response to treatment in triple-negative breast cancer as these tumors lack inter and intra tumoral heterogeneity and not easily respond to traditional chemotherapies (Mehraj et al., 2022). The tumor size was reduced in both grade 2 and 3 tumors equally.

In accordance with other window-of-opportunity clinical trials in which Ki67 was the primary endpoint as a proliferation marker, we used Ki67 as a primary endpoint (Garwood et al., 2010; Niraula et al., 2012; Bjarnadottir et al., 2013). Based on the recommendations of the international Ki67 in Breast Cancer Working Group of preanalytical, careful handling, and calibrated visual scoring use, the overall average proliferation was counted for the entire core and surgical patient biopsies to assess prognosis (Nielsen et al., 2021).

The median percentage of Ki67 staining cells in the pitavastatin group were significantly reduced in the surgical specimens (after treatment completion) compared to the core specimen cells. The decrease in the conventional neoadjuvant chemotherapy group was not significant; however, when we compared the change in Ki67 between the two groups, no significant difference was detected. This may be explained by the small sample size for calculating the difference between the core and surgical specimens. Fluvastatin and atorvastatin clinical trials showed similar results (Garwood et al., 2010; Bjarnadottir et al., 2013), although a similar outcome was not produced in a fluvastatin trial for prostate cancer (Longo et al., 2020). This suggests that the proliferation of tumor cells is significantly decreased by the addition of pitavastatin compared to conventional chemotherapy protocols, thus increasing the potential of the pitavastatin regimen against breast cancer.

For DNA replication and cell division, a strictly-controlled series of events are needed. Normally, this occurs via serin/sereonin kinases (i.e., cyclin-dependent kinases), which need to be activated by phosphorylation. By binding with their complementary elements (cyclins), subsequent regulatory proteins are also phosphorylated, thus leading to the initiation and regulation of the cell cycle phases (Morgan, 1997; Malumbres and Barbacid, 2005). Cyclin D1 is a transcriptional coordinator and a vital regulator for the G1/S phase bind to CDK4 and CDK6, which causes phosphorylation of the retinoblastoma protein (Rb-protein) and its inactivation. This interrupts the G1phase of the cell cycle and induces the expression of proliferation genes (Musgrove et al., 1994; Matthews et al., 2022). Since the expression level of the cyclin D1 gene is elevated in 50% of primary invasive breast cancer (Arnold and Papanikolaou, 2005), using a treatment that lessens cyclin D1 levels would hold much promise. In our study, cyclin D1 is attenuated upon the addition of the statin to conventional neoadjuvant chemotherapy, as shown by the significant decrease in the cyclin D1 levels in the pitavastatin group compared to the control. This finding was in concordance with a study using atorvastatin (Feldt et al., 2015). This may explain the underlying mechanism of antiproliferative effects in statins. It has been proposed that the antiapoptotic and proapoptotic effect of statins are derived from its inhibitory effect on isoprenoid intermediates, especially farnesyl pyrophosphate (FPP) and geranyl–geranyl-pyrophosphate (GGP). Both GGP and FPP are responsible for protein prenylation, which is the post-transcriptional process of adding hydrophobic moiety that allows it to anchor to the cell membrane for their normal function (Zhang and Casey, 1996; Cimino et al., 2007; Zhou and Liao, 2010). This process is necessary for the activation of different signaling pathways, such as the RAS/Rho subfamilies. RAS-dependent pathways regulate cyclin D1 expression and its subsequent action steps (Coleman et al., 2004). Thus, the arrest of the cell cycle through G1 suppression may be due to the reduction of cyclin D1 oncogene (Feldt et al., 2015).

Another potential mechanism responsible for the effect of pitavastatin addition to conventional protocols is its induction of the apoptotic pathway as evidenced by the increase of caspase-3 levels as apoptosis markers after neoadjuvant chemotherapy in the pitavastatin group, and its decrease in the control group. Although there wasn't a significant difference between the baseline levels in the two groups, caspase-3 after the neoadjuvant chemotherapy was significantly higher in the pitavastatin group. This matches the results of many other pitavastatin and statin family studies on various types of cancer (Park et al., 2010; Goc et al., 2012; Qi et al., 2013; Tsubaki et al., 2019; Chen et al., 2020; Goda et al., 2020; Otahal et al., 2020). This may suggest that statins can inhibit breast cell proliferation via induction of apoptosis and cell cycle arrest in human cases resulted from MDA231 cell line research (Yang et al., 2016).

In this study, most patients showed a partial response, with complete response achieved in only 9 patients (6 in the pitavastatin group and 3 in the control group). All 9 exhibited a HER2-neu positive receptor at baseline. This may suggest that pitavastatin provided additional benefits to the traditional chemotherapy of primarily HER2-positive tumors. These results were in accordance with a fluvastatin study that indicated that the most suitable subgroups for statin anticancer effects were ER-negative, and another cohort study that showed that patients using lipophilic statins were at a lower risk of developing ER-negative breast cancer (Kumar et al., 2008; Garwood et al., 2010).

The current study is the first to test the efficacy of pitavastatin as a concomitant treatment with neoadjuvant chemotherapy protocols in patients with breast cancer. One weak point of this study, in line with other prospective clinical trials of statin use in cancer,

is the limited dietary intake of geranylgeraniol, which in turn could suppress the proapoptotic activities and interfere with the antitumor activities of statins (de Wolf et al., 2017; Abdullah et al., 2018). Another weakness may be the single blind design, where the investigators were aware of the treatment assignment; however, most of the measurements were done by independent hospital staff. The patients in the control group did not receive a placebo; however, no patient-reported outcomes were collected in this study. Non-adherence to the treatment regimen may also limit the current results, but the patients were contacted weekly to remind them of the treatment.

In conclusion, the concomitant use of pitavastatin with neoadjuvant therapy can improve the tumor response to therapy and provide possible benefits for breast cancer patients. Further studies with larger sample sizes and multicenter designs are needed to confirm these findings. Future studies investigating and comparing the anticancer effects of other statins are also recommended.

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Author Contributions

Study concept: S.D., O.H., A.E G, M.E.-M, and M.S. Patient recruitment and follow up: S.D., O.H., and A.Et. Statistical analysis: S.D., O.H., M.E.-M and M.S. First draft writing: S.D., O.H., and M.S. Manuscript review and approval of its final version: All authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2022.07.011.

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