

Effect of Aromatase Inhibitors on Serum Calprotectin Levels in an Animal Experimental Model: Trial

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Abstract. *Background/Aim:* Utilizing an experimental animal model, we investigated the correlation between aromatase inhibitors (AIs) (anastrozole and letrozole) and Calprotectin levels. AIs have demonstrated superior efficacy when used as adjuvant endocrine therapy or monotherapy for postmenopausal patients with hormone receptor (HR)-positive early-stage breast cancer, although various side effects have been recorded. *Materials and Methods:* Fifty-five adult female Wistar rats were randomized and assigned into four groups. The control group received no intervention. The other three groups were subjected to ovariectomy, and serum Calprotectin levels were measured at baseline, 2, and 4 months. In addition, glucose, total cholesterol, very low-density lipoprotein- (VLDL-) cholesterol, low-density lipoprotein (LDL-) cholesterol, high-density lipoprotein- (HDL-) cholesterol, and triglyceride levels were measured. Histological analysis of liver tissue was carried out following rats'

euthanasia. Results: Aromatase inhibitors (anastrozole and letrozole) affect calprotectin levels in ovariectomized rats. Calprotectin, a marker of inflammation, was found to be affected by the use of the inhibitors. *Conclusion:* The potential of hepatotoxicity can be examined by assessing the elevation of inflammation markers such as Calprotectin, which is an indicator that should be strictly taken into consideration when administering aromatase inhibitors as treatment.

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Key Words: Anastrozole, letrozole, experiment, treatment for HR-positive breast cancer, side effects, hepatotoxicity, correlation of calprotectin with aromatase inhibitors.

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Aromatase inhibitors (AIs) block the conversion of androgen into estrogen by deactivating the enzyme aromatase. They are used as a primary prevention therapy for patients with high risk of breast cancer or any other hormone-related diseases (1). In adjuvant hormonal therapy and hormone receptor-related treatments for breast cancer, tamoxifen was the gold standard for more than 40 years (2, 3). Nowadays, AIs including letrozole (LTZ), anastrozole (ATZ), and exemestane are used as first-line therapy in postmenopausal women with breast cancer (4). Because AIs cannot regulate or stop the ovaries from producing estrogen, they tend to be more effective when they are used for treating postmenopausal women (5).

Despite their wide usage and very promising results, AIs have shown a variety of side effects (6). Amongst them are insomnia, aches of the joints, weight gain, hot flushes, mood swings, vaginal discharge and dryness, loss of libido, muscle aches and in some rare points even severe osteoporosis (7-9). In a very recent cohort study performed by Lee *et al.*, a correlation was found between AIs and increased risk and prevalence of non-alcoholic fatty liver disease (NAFLD), which is independent of the body mass index (BMI) or diabetes mellitus (DM) in postmenopausal patients (10).

There is still no clear evidence of hepatotoxicity for AIs (11). The purpose of this experimental study was to investigate the potential association between the administration of AIs and hepatotoxicity. For the purposes of this experiment, Calprotectin was selected as a hepatic inflammation marker because of its capacity to be activated when inflammation occurs. Calprotectin has a molecular size of 24 kDa and consists of two calcium-binding chains, which are linked non-covalently, in a calcium-dependent way (12, 13).

There are several synonyms of Calprotectin, including myeloid-histiocyte antigen, CP-10 when the light chain is concerned, calgranulin A/B, cystic fibrosis antigen, L1 protein, MRP8/14, 27E10 antigen, S100A8/A9 *etc.* (12, 14, 15). The terms S100A8, S100A9 and S100A8/A9 can also be used as alternatives to the term Calprotectin. S100A8 and S100A9 are both imperative for immune response and the regulation of inflammatory processes. Apart from the generic association of S100A8 and S100A9 with inflammation and the body's immune response to inflammation, there is evidence demonstrating a clear association between Calprotectin and liver damage in rats. More specifically, Koike *et al.* (16) administered recombinant human S100A8 (rh-S100A8) and S100A9 (rh-S100A9) intravenously in rats with LPS-induced liver damage. Then, ELISA was used for measuring the serum concentration of S100A9 in the rats. In cases where rh-S100A8 was administered intravenously in rats with liver failure, the concentration of rh-S100A9 in the serum was decreased, in comparison to rats with normal liver function. Therefore, Calprotectin is used as a diagnostic indicator. Using an experimental animal model, we examined alterations and variations in Calprotectin levels after administration of LTZ and ATZ in rats.

Materials and Methods

Animals. A total of 55 female Wistar rats were used for this experiment (Department of Animal Models for Biomedical Research, Hellenic Pasteur Institute, Athens, Greece). Following acclimatization, thirty-five 4-week-old female rats were assigned into 3 experimental subgroups groups of 15 rats each, while 10 rats were used as a control group. No surgical or pharmaceutical interventions had been applied to the control group. The animals were housed under conditions of controlled temperature ($20\pm 1^\circ\text{C}$) and humidity ($55\pm 5\%$) with 12 h light/dark cycle. Average mean body weight of animals of the three experimental subgroups was 214.13 g (range=169-248 g). Average mean body weight of rats belonging to the control group (without ovariectomy) was 275.9 g (range=225-326 g), with the same age as the other 3 subgroups. Rats were fed ELVIZ 510 food pellets (ELVIZ 510 food pellets from EL.VI. Z. S.A, Plati Imathias, Greece) to ensure full nutrient supplementation.

The experimental protocol was reviewed and approved by the Veterinary Directorate of Athens Prefecture (Approval number: 2901). It was performed according to the current EU and national legislation and taking into account the analytical description of the experimental procedure including anaesthesia methodology applied to the animals. The veterinarian of the animal facility as well as experienced animal assistants supervised the experimental procedure in order to prevent the induction of pain or distress in the animals.

Surgical procedures. Ovariectomy was performed surgically on 45 female Wistar rats following a 12-hour fasting period at the beginning of the experiment, time point T1. The surgical procedure was conducted during 8:00 am – 9:00 am. Xylazine (10 mg/kg, Rompun, Bayer AG, Leverkusen, Germany) and ketamine (75 mg/kg, Ketalar, Pfizer Inc, New York, NY, USA) were combined and administered intra-peritoneally in order to anesthetize the animals. An incision in the midline dorsal skin was performed and both were excised after ovarian vessels were clamped. Then, the skin and muscles were sutured in order to close the incision.

Animal treatment. Following ovariectomy, 45 rats were assigned into 3 subgroups of 15 rats each. The first group did not receive any drug regimen [control ovariectomy group (OVA)]. The second group was treated with letrozole (OVA-L) (Ariti SA, Acharnes, Greece) and the third group with anastrozole (OVA-A) (Specifar, Athens, Greece). Administration of AIs was performed previously described (17). Specifically, anastrozole was administered orally in drinking water following dissolution in dimethyl sulfoxide solution at a concentration determined to result in a daily uptake of ~ 0.1 mg/kg body weight; LTZ was similarly administered at a concentration determined to result in a daily uptake of ~ 2 mg/kg body weight (18).

Blood samples (1.0-1.5 ml) were collected at 9:00 am, after a 12-h fasting period at T1 (beginning of the study), T2 (60 days) and T3 (120 days – end of the study) from the retro-orbital venous plexus under light ether anesthesia by utilizing capillary tubes (Diethyl Ether, Sigma-Aldrich, Merck Millipore, Darmstadt, Germany). During anaesthesia, the tail pinch and the pedal withdrawal reflexes were assessed. Blood sampling was applied in the control group only at the end of the experimental period (T3).

At the end of the experiment (T3) all animals were sacrificed by cervical dislocation while under ether anesthesia and sedation with intramuscular injection of 5 mg/kg xylazine (Rompun™; Bayer AG). The flow of the experiment can be seen in Figure 1.

Histopathological analysis of the liver. We followed an experimental procedure described by Boutas *et al.* (19). Liver tissue samples were examined at the end of the experimental period following animal euthanasia. We diagnosed hepatic steatosis and ballooning following relevant indications including macro > micro, accentuated in zone 3, lobular inflammation (mixed, mild), and hepatocellular ballooning (most apparent near steatotic liver cells, typically in hepatic zone 3) (20).

Blood measures. Measurement of serum glucose, total cholesterol, very low-density lipoprotein- (VLDL-) cholesterol, low-density lipoprotein (LDL-) cholesterol, high-density lipoprotein- (HDL-) cholesterol, and triglycerides levels was performed using an auto-analyzer (StatPlus 2300, Yellow Springs Instruments, Yellow Springs, OH, USA) as recommended by the manufacturer (Bioclin, Quimbasa, Basic Chemistry Ltda, Brazil). Additionally, Calprotectin, serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT) levels were also measured.

Statistical analysis. The statistical analysis was performed using the SPSS (v23.0) tool (IBM, Armonk, NY, USA). The nominal variables were described based on the frequencies and their relevant percentages, along with the presentation of frequency diagrams. For the comparison of nominal variables and the corresponding categories the Pearson Chi-Square (χ^2) test was used.

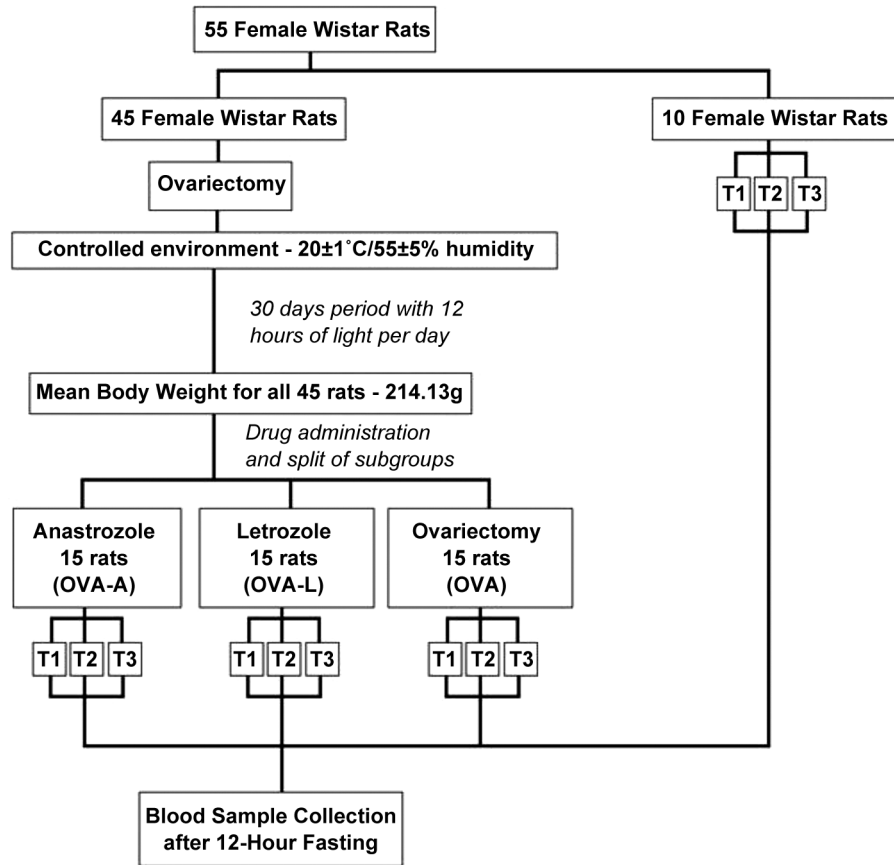


Figure 1. Experiment flow chart.

Table I. Calprotectin levels for rats that underwent ovariectomy.

	Ovariectomy	Anastrozole	Letrozole	Control	p-Value
Calprotectin_T1	100.16±28.82	43.68±22.74	59.99±39.98		0.002
Calprotectin_T2	68.40±42.59	108.73±24.82	131.34±31.85		<0.001
Calprotectin_T3	69.86±64.88	129.00±29.14	83.96±9.35	12.65±1.49	<0.001

Statistically significant p-Values are shown in bold.

In order to compare all continuous variables for the different subgroups, ANOVA tests were introduced along with an appropriate Post Hoc analysis or the equivalent non-parametric Kruskal–Wallis test, accompanied by the non-parametric Mann–Whitney *U*-test depending on the occasion. In order to compare all parameters in the three different time points (beginning of the study - T1, 2 months - T2 and 4 months - T3) the Repeated Measures ANOVA test or the non-parametric Friedman’s Two-Way analysis test were used, accompanied by the Paired Samples *t*-Test and Wilcoxon Signed Rank test (non-parametric). The Cramer’s V Test for nominal variables was also used in order to detect correlations between variables. Furthermore, for all continuous variables the non-parametric Spearman coefficient or the equivalent parametric Pearson correlation coefficient was calculated.

A statistically significant difference or correlation was observed when the calculated *p*-value was smaller than 0.05. The strength of the correlations is determined from the value of the corresponding test. All methods and tests carried out were in accordance with relevant regulations and guidelines.

Results

Average mean values (±S.D.) of serum Calprotectin levels in all experimental animal groups are summarized in Table I.

Comparing Calprotectin between groups. Serum baseline levels of Calprotectin were comparable among the three

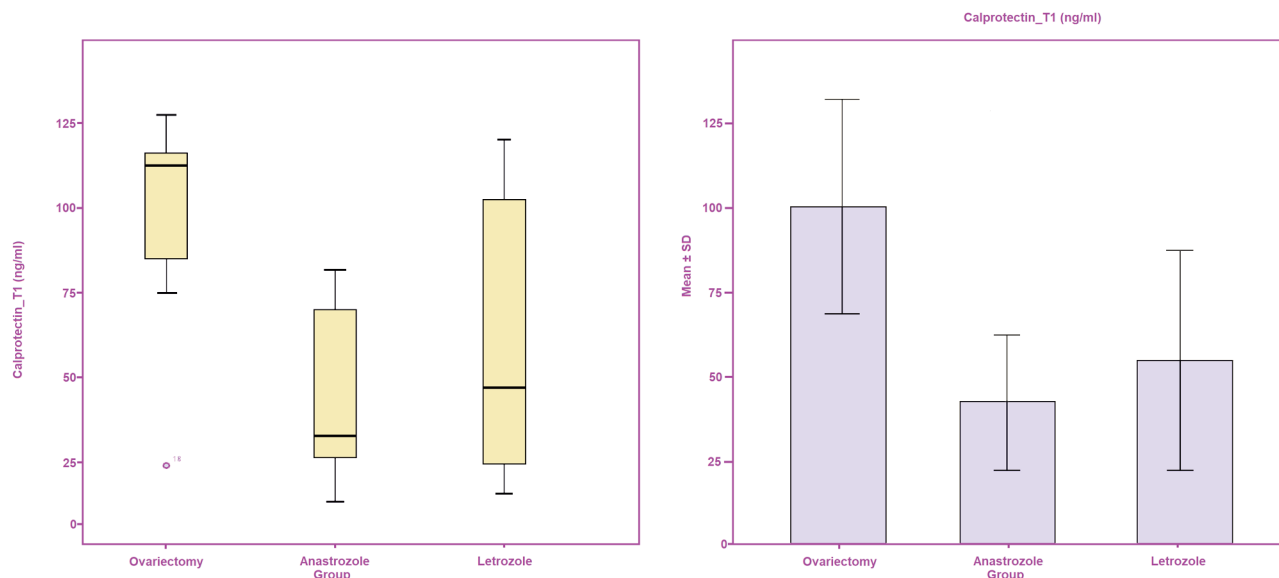


Figure 2. Calprotectin levels in all 3 groups at T1. OVA: Ovariectomy Group; OVA-L: Ovariectomy Letrozole Group; OVA-A: Ovariectomy Anastrozole Group.

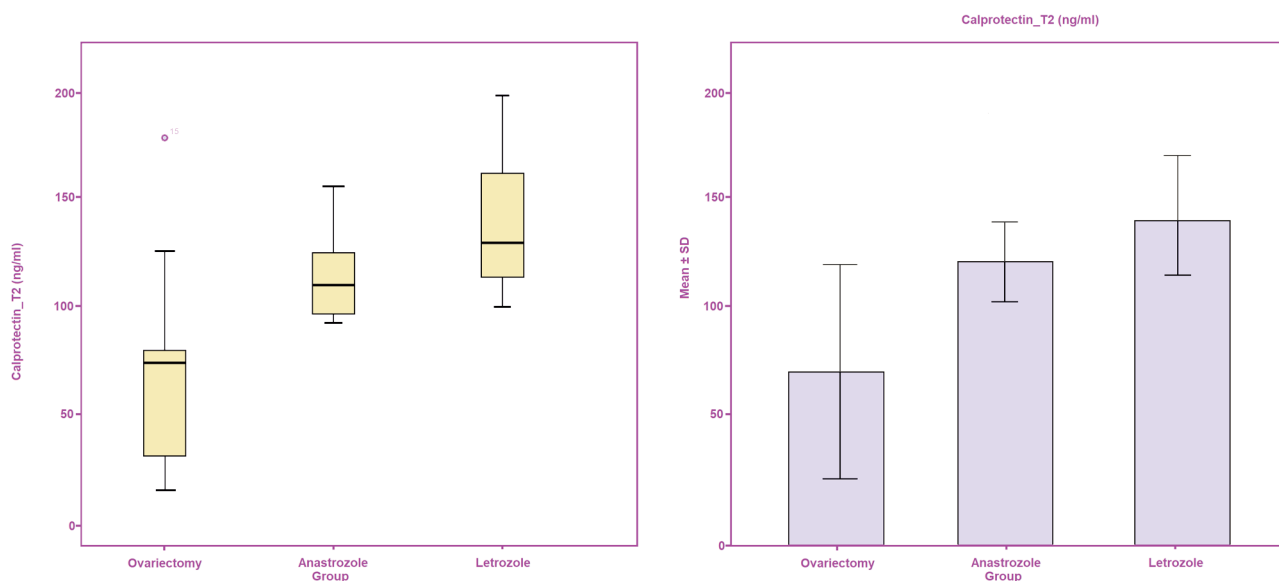


Figure 3. Calprotectin levels in all 3 groups at T2.

groups (Ovariectomy, ATZ, and LTZ) of animals analyzed in the present study. At the time point T1 (beginning of the experiment) the variation in the value of Calprotectin in all 3 groups was statistically significant ($p=0.002$) and more specifically, the difference is recognized in two comparisons. Between the group of rats that underwent ovariectomy and the group that was treated with ATZ ($p<0.0001$) and also between the group that underwent ovariectomy and the group that was treated with LTZ ($p=0.015$) (Figure 2).

At T2 (2 months after the beginning of the experiment), the values of Calprotectin also differed significantly ($p<0.001$). More specifically, a difference was detected between the ovariectomy group and the group that was treated with ATZ ($p=0.019$) and also between the group that underwent ovariectomy and the one that was treated with LTZ ($p<0.001$) (Figure 3).

At T3 (4 months after the beginning of the experiment), the values of Calprotectin differed significantly between the

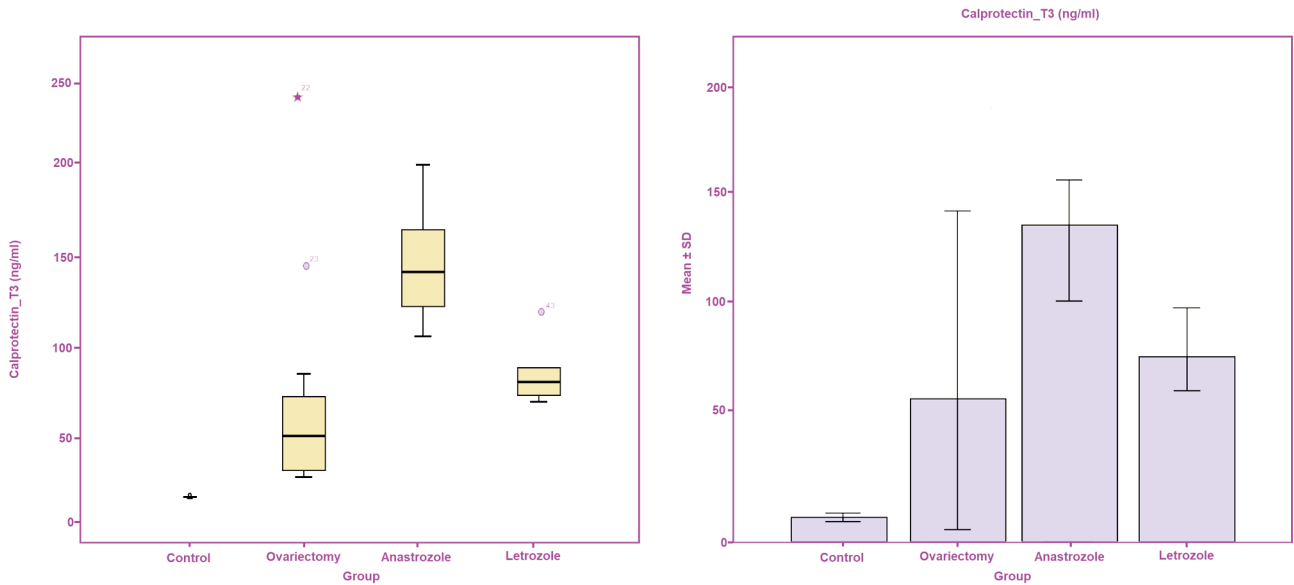


Figure 4. Calprotectin levels in all 3 groups at T3.

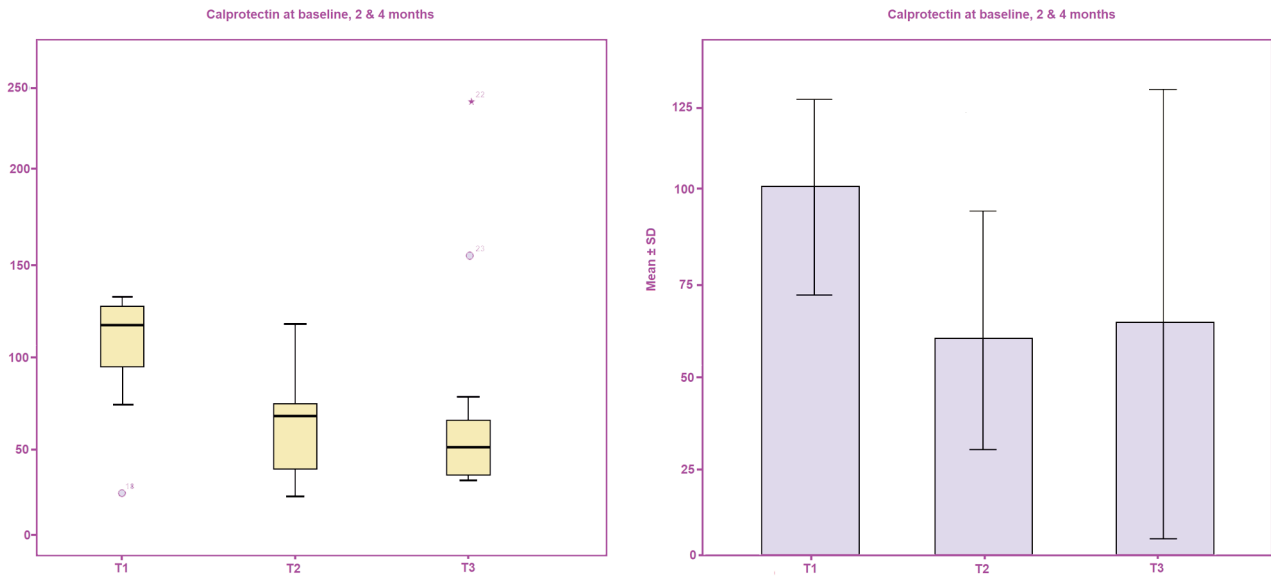


Figure 5. Calprotectin at baseline, 2 and 4 months in the ovariectomy group.

3 subgroups and the Control group ($p < 0.001$). Furthermore, there was a significant difference between the ovariectomy group and the ATZ group ($p = 0.005$), the ovariectomy group and the LTZ group ($p = 0.019$) and the anastrozole group and the letrozole group ($p < 0.001$) (Figure 4).

Differences in Calprotectin between time points for all subgroups. The value variation of Calprotectin levels was calculated for each subgroup separately for all three time points,

T1 (beginning of experiment), T2 (2 months) and T3 (4 months).

When comparing the difference in Calprotectin levels for the Ovariectomy group at all 3 time points, there was no statistically significant difference (Figure 5). Regarding the group that was treated with anastrozole, the difference between the 3 time points was statistically significant ($p < 0.001$). There was a significant difference between T1 and T2 and also between T1 and T3 ($p < 0.001$ in both occasions; Figure 6). Furthermore, for the subgroup that was

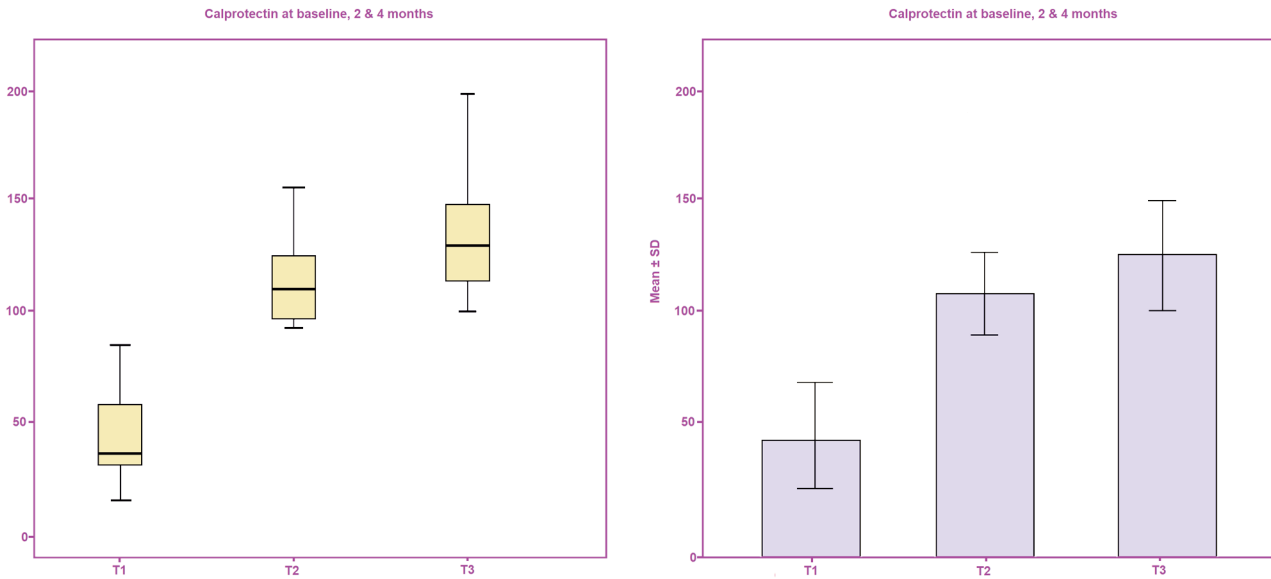


Figure 6. Calprotectin at baseline, 2 and 4 months in the anastrozole group.

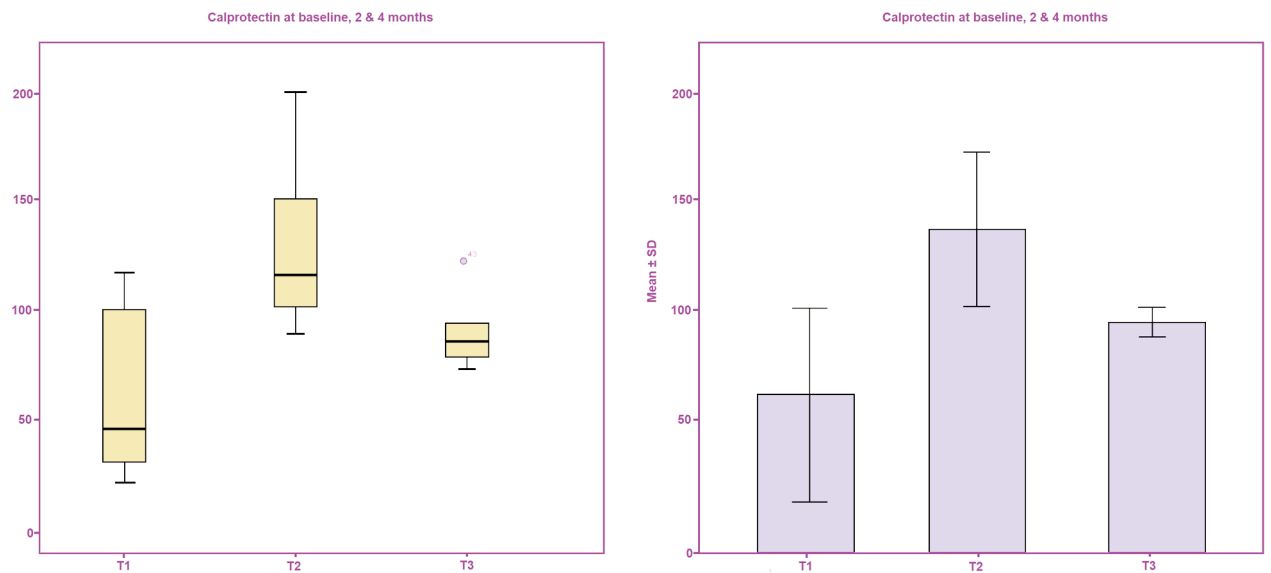


Figure 7. Calprotectin at baseline, 2 and 4 months in the letrozole group.

treated with LTZ, the difference in Calprotectin levels was statistically significant between the 3 time points ($p < 0.001$). Significant differences were observed between T1 and T2 ($p = 0.002$) and T2 and T3 ($p = 0.002$) (Figure 7).

Correlations. Liver damage is determined by examining steatosis, lobular inflammation, portal inflammation and ballooning, as clinical indicators. In our study, rats only showed steatosis. For the LTZ group at the time point T1 (beginning of

the experiment) there was a statistically significant, moderately negative linear correlation between Calprotectin levels and the LDL index ($R = -0.653, p = 0.021$). For the Control group at time point T3 (end of experiment) there was a statistically significant, strong positive linear correlation between Calprotectin levels and the LDL index ($R = 0.854, p = 0.002$).

In the subgroup of rats in which there was no manifestation of pathology in regard to the location of steatosis, there was a statistically significant moderate positive linear correlation

Table II. Alteration of Calprotectin T1-T2.

		Group			
		Ovariectomy	Anastrozole	Letrozole	Total
Decrease	Count	3	11	12	26
	Change in Calprotectin levels between T1 and T2	11.5%	42.3%	46.2%	100.0%
	Calprotectin levels within each Group	23.1%	100.0%	100.0%	72.2%
Increase	Count	10	0	0	10
	Change in Calprotectin levels between T1 and T2	100.0%	0.0%	0.0%	100.0%
	Calprotectin levels within each Group	76.9%	0.0%	0.0%	27.8%
Total	Count	13	11	12	36
	Change in Calprotectin levels between T1 and T2	36.1%	30.6%	33.3%	100.0%
	Calprotectin levels within each Group	100.0%	100.0%	100.0%	100.0%

Table III. Steatosis pathology T1-T2.

		Steatosis			
		No pathology	Mild pathology	Moderate pathology	Total
Decrease	Count	6	18	2	26
	Change in Calprotectin levels between T1 and T2 in mice with steatosis	23.1%	69.2%	7.7%	100.0%
	Calprotectin levels in mice with steatosis	37.5%	100.0%	100.0%	72.2%
Increase	Count	10	0	0	10
	Change in Calprotectin levels between T1 and T2 in mice with steatosis	100.0%	0.0%	0.0%	100.0%
	Calprotectin levels in mice with steatosis	62.5%	0.0%	0.0%	27.8%
Total	Count	16	18	2	36
	Change in Calprotectin levels between T1 and T2 in mice with steatosis	44.4%	50.0%	5.6%	100.0%
	Calprotectin levels in mice with steatosis	100.0%	100.0%	100.0%	100.0%

between Calprotectin levels and the Glucose index at time point T1 (beginning of experiment) ($R=0.457$, $p=0.006$). There was also a statistically significant positive linear correlation, of moderate potency at the time point T2, between Calprotectin levels and the Cholesterol Index ($R=0.396$, $p=0.021$). Moreover, at the time point T2, there was a statistically significant moderate positive linear correlation between Calprotectin levels and the HDL index ($R=0.365$, $p=0.034$). A statistically significant moderate positive linear correlation between Calprotectin levels and the weight of the animals at T2 was also present ($R=0.359$, $p=0.040$). Another statistically significant moderate positive linear correlation was found at the time point T3 between Calprotectin levels and the LDL index ($R=0.350$, $p=0.023$).

Regarding lobular inflammation, no pathological alterations were detected in any animal group. A moderate positive linear correlation was observed between Calprotectin levels and the Glucose Index at T1 ($R=0.437$, $p=0.007$), while at T2 a moderate positive linear correlation

was observed between Calprotectin levels and HDL index ($R=0.378$, $p=0.023$). Another statistically significant positive linear correlation was observed at time point T2, of moderate potency, between Calprotectin levels and the Cholesterol Index ($R=0.348$, $p=0.038$). Furthermore, at time point T3, there was a statistically significant moderate positive linear correlation between Calprotectin levels and the LDL index ($R=0.358$, $p=0.017$) and between Calprotectin levels and the weight of the rats ($R=0.535$, $p<0.001$). The same correlations were observed for portal inflammation.

The mice that underwent ovariectomy in the OVA, OVA-A, and OVA-L groups that did not show any Ballooning pathology, there was a statistically significant moderate positive linear correlation between Calprotectin levels and the Glucose Index at T1 ($R=0.495$, $p=0.007$) and at T2, a statistically significant moderate positive linear correlation was observed between Calprotectin levels and the Triglyceride Index ($R=0.483$, $p=0.011$), between Calprotectin levels and the Cholesterol Index ($R=0.400$, $p=0.039$) and between

Table IV. Alteration of Calprotectin T2-T3.

		Group			
		Ovariectomy	Anastrozole	Letrozole	Total
Decrease	Count	4	8	0	12
	Change in Calprotectin levels between T2 and T3	33.3%	66.7%	0.0%	100.0%
	Calprotectin levels within each Group	36.4%	80.0%	0.0%	36.4%
Increase	Count	7	2	12	21
	Change in Calprotectin levels between T2 and T3	33.3%	9.5%	57.1%	100.0%
	Calprotectin levels within each Group	63.6%	20.0%	100.0%	63.6%
Total	Count	11	10	12	33
	Change in Calprotectin levels between T2 and T3	33.3%	30.3%	36.4%	100.0%
	Calprotectin levels within each Group	100.0%	100.0%	100.0%	100.0%

Table V. Alteration of Calprotectin T1-T3.

		Group			
		Ovariectomy	Anastrozole	Letrozole	Total
Decrease	Count	3	11	7	21
	Change in Calprotectin levels between T1 and T3	14.3%	52.4%	33.3%	100.0%
	Calprotectin levels within each Group	25.0%	100.0%	58.3%	60.0%
Increase	Count	9	0	5	14
	Change in Calprotectin levels between T1 and T3	64.3%	0.0%	35.7%	100.0%
	Calprotectin levels within each Group	75.0%	0.0%	41.7%	40.0%
Total	Count	12	11	12	35
	Change in Calprotectin levels between T1 and T3	34.3%	31.4%	34.3%	100.0%
	Calprotectin levels within each Group	100.0%	100.0%	100.0%	100.0%

Table VI. Steatosis pathology T1-T3.

		Steatosis			
		No pathology	Mild pathology	Moderate pathology	Total
Decrease	Count	6	15	0	21
	Change in Calprotectin levels between T1 and T3 in mice with Steatosis	28.6%	71.4%	0.0%	100.0%
	Calprotectin levels in mice with steatosis	40.0%	83.3%	0.0%	60.0%
Increase	Count	9	3	2	14
	Change in Calprotectin levels between T1 and T3 in mice with Steatosis	64.3%	21.4%	14.3%	100.0%
	Calprotectin levels in mice with steatosis	60.0%	16.7%	100.0%	40.0%
Total	Count	15	18	2	35
	Change in Calprotectin levels between T1 and T3 in mice with steatosis	42.9%	51.4%	5.7%	100.0%
	Calprotectin levels in mice with steatosis	100.0%	100.0%	100.0%	100.0%

Calprotectin levels and the HDL index at T2 ($R=0.515$, $p=0.006$). Finally, a statistically significant positive linear correlation, of moderate strength, between Calprotectin levels and the weight of rats at T3 ($R=0.465$, $p=0.014$) was detected.

The mice that underwent ovariectomy in the groups OVA, OVA-A and OVA-L, with mild Ballooning pathology, there was a statistically significant strong positive linear correlation between Calprotectin levels and the Triglyceride index at time

Table VII. Summary table of measured values for steatosis markers in the four subgroups.

	Time	Group				Differences between groups
		OVA	OVA - A	OVA - L	Control	
Glucose	T1	161.71±23.26	147.64±17.03	140.75±5.36	-	OVA vs. OVA-L
	T2	137.46±16.18	149.72±10.85	151.17±21.57	-	N.S.
	T3	139.55±14.60	142.00±17.56	156.50±23.23	155.20±10.55	N.S.
Cholesterol	T1	56.29±8.62	55.36±10.605	54.83±4.47	-	N.S.
	T2	50.39±18.48	60.55±7.326	76.83±18.37	-	OVA vs. OVA-L & OVA-A vs. OVA-L
	T3	50.91±10.60	61.46±26.920	62.75±9.95	51.70±6.45	Control vs. OVA-L & OVA vs. OVA-L
Triglycerides	T1	67.71±25.45	64.18±21.15	67.00±8.40	-	N.S.
	T2	71.31±32.19	124.55±31.22	92.17±31.22	-	OVA vs. OVA-A & OVA-A vs. OVA-L
	T3	79.82±42.63	105.82±38.83	136.33±37.03	131.80±38.53	Control vs. OVA & OVA vs. OVA-L
HDL	T1	11.00±1.24	12.27±4.38	13.17±1.53	-	OVA vs. OVA-L & OVA-A vs. OVA-L
	T2	12.00±1.58	14.46±4.68	26.58±12.77	-	OVA vs. OVA-L & OVA-A vs. OVA-L
	T3	11.91 ±1.87	11.55±1.64	12.42±2.50	13.60±1.78	N.S.
LDL	T1	31.50±9.67	30.36±8.59	28.25±5.28	-	N.S.
	T2	25.69±13.94	21.27±7.90	31.83±13.12	-	N.S.
	T3	27.27±17.51	28.91±21.97	25.83±15.14	11.90±9.29	Control vs. OVA & Control vs. OVA-A & Control vs. OVA-L

OVA: Ovariectomy Group; OVA-L: Ovariectomy Letrozole Group; OVA-A: Ovariectomy Anastrozole Group.

point T1 ($R=0.811$, $p=0.027$) and a statistically significant strong positive linear correlation between Calprotectin levels and the weight of the rats at T3 ($R=0.758$, $p=0.011$). As for the subgroup with mild Ballooning pathology, at time point T3, there was a statistically significant strong negative linear correlation between Calprotectin levels and the HDL index ($R=-0.924$, $p=0.003$). Lastly, there was a statistically significant strong positive linear correlation between Calprotectin levels and the LDL index at T3 ($R=0.963$, $p<0.001$).

Steatosis is distinguished to mild and moderate for this experiment. For the group of rats with mild steatosis, Calprotectin levels in relation to the LDL index and weight at the beginning and the end of the experiment were assessed. For time point T1 there was a moderate negative correlation between LDL and Calprotectin levels ($R=-0.588$, $p=0.010$). At time point T3, there was a moderately significant positive linear correlation between Calprotectin levels and the LDL index ($R=0.40$, $p=0.048$). Finally, the correlation between Calprotectin and weight for this subgroup was evaluated for time point T3 and a moderate positive linear correlation was found ($R=0.551$, $p=0.004$).

Regarding the alteration in Calprotectin levels, the levels of Calprotectin in all groups at the 3 different time points (T1, T2 and T3) were measured. The changes of Calprotectin levels from time point T1 until time point T2 (can be seen on Table II and Table III, respectively).

The alteration of Calprotectin differed significantly between the groups ($p<0.001$). There was also a strong correlation (Cramer's $V=0.825$, $p<0.001$) of group and alteration in Calprotectin levels.

The alteration of Calprotectin also differed significantly between the OVA, OVA-A and OVA-L groups which presented mild or moderate steatosis ($p<0.001$). There was also a moderate to strong correlation (Cramer's $V=0.693$, $p<0.001$) of the pathology of steatosis and the alteration of Calprotectin levels.

The alteration of Calprotectin from time point T2 until time point T3 can be seen on Table IV. The alteration of Calprotectin differed significantly between the groups ($p=0.001$). There was also a moderate to strong correlation (Cramer's $V=0.676$, $p=0.001$) of group and alteration of Calprotectin levels. The alteration of Calprotectin between time point T1 and time point T3 can be seen on Table V and Table VI, respectively.

The alteration of Calprotectin differed significantly between the groups ($p=0.001$). There was also a moderate to strong correlation (Cramer's $V=0.620$, $p=0.001$) of group and alteration of Calprotectin levels.

The alteration of Calprotectin also differed significantly depending on the extent of the pathology of steatosis ($p=0.008$). There was also a moderate to strong correlation (Cramer's $V=0.523$, $p=0.008$) of the extent of the pathology of steatosis and the alteration of Calprotectin levels. A summary table of measured values for steatosis markers in animals from all subgroups can be seen in Table VII.

Discussion

Third-generation AIs, including ATZ and LTZ, have shown superior efficacy compared to previously administered medications, either as initial monotherapy or as adjuvant endocrine therapy, especially in postmenopausal patients with

HR-positive early-stage breast cancer (21-23). However, considering that endocrine therapy is a long-term treatment, drug toxicity is a very important factor to consider besides efficacy. Hepatotoxicity remains a strong consideration among clinicians regarding the AIs toxicity profile (21-23).

Hepatotoxicity is expressed by the elevation of inflammation markers, including Calprotectin. Calprotectin is an antimicrobial manganese sequestration protein complex (24). Many studies have indicated that Calprotectin is related to many pathologic and physiologic processes like pregnancy, the menstrual cycle, labor, vaginal and cervical physiology, and some types of cancer like cervical (25), ovarian (26), breast (27) and endometrial. It serves as an indication marker for inflammation, a prognostic, diagnostic and metastatic marker for several types of cancer and as a target for pharmaceutical treatment in a variety of conditions (28), one of them being hepatic inflammation.

Interestingly, some studies report that serum enzyme levels elevate in 2-4% of women treated with ATZ and LTZ, but this elevation tends to be asymptomatic or very mild and therefore in only very limited cases the dose will be modified by the treating physicians. In even more extreme circumstances, liver injury has been associated with ATZ or LTZ therapy (29). Due to the lack of literature in the field, some immune-allergic features that could potentially be indicative of this condition haven't been associated with it. It appears though that the injury that occurs via the usage of ATZ and LTZ is potentially due to an immune-allergic or toxic reaction while the inhibitor is being metabolized.

Strengths and limitations of the present study. The primary strength of this study is its randomized design and the duration of the experiment (4 months), which corresponds to 6.5 human years. A limitation to the study design is the control non-ovariectomized group that was added at the end of the experiment.

Implications for future research. According to our results, alterations occur in Calprotectin levels, liver architecture and biochemical parameters, between 0 and 4 months. It seems plausible to assume that Calprotectin levels may reveal other facets of the underlying molecular mechanisms in women treated with AIs. This parameter should be taken into account in future relevant studies.

Conclusion

In our study, the results indicated that the groups that received ATZ and LTZ had significantly higher levels of Calprotectin. To date, there are very few published studies on this field and undoubtedly, this is a literature gap that must be further filled. More research needs to be performed in order to determine the correlation between AIs and hepatotoxicity.

Conflicts of Interest

The Authors report no conflicts of interest in relation to this study.

Authors' Contributions

Ioannis Boutas - Conceptualization, methodology, data curation, writing, original draft preparation. Adamantia Kontogeorgi - Data curation, writing, original draft preparation, software. Nektarios Koufopoulos - Reviewing and editing. Constantine Dimitrakakis - Supervision, reviewing, and editing. Dionysios Dimas - Methodology, data curation. Sophia N. Kalantaridou - Supervision, writing, reviewing, and editing. Laskarina-Maria Korou - Supervision, methodology. Despoina Perrea - Supervision, reviewing, and editing. All Authors read and approved the final manuscript.

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