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Loïc Lelièvre, Philippe Clézardin, Laurent Magaud, Laurent Roche, Nicole Tubiana-Mathieu, Jean-Dominique Tigaud, Delphine Topart, Nadia Raban, Marie-Ange Mouret-Reynier, Patrice Mathevet

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Comparative study of neoadjuvant chemotherapy with and without Zometa® for management of locally advanced breast cancer with serum VEGF as primary endpoint: the NEOZOL study

Loïc Lelièvre[1]*, Philippe Clézardin[2], Laurent Magaud[3], Laurent Roche[4], Nicole Tubiana-Mathieu[5], Jean-Dominique Tigaud[6], Delphine Topart[7], Nadia Raban[8], Marie-Ange Mouret-Reynier[9], Patrice Mathevet[10]

[1]Centre du sein, Centre Hospitalier Universitaire Vaudois, Avenue Pierre Decker 2, 1011 Lausanne, Suisse, Phone : +41 79 556 84 67 ; +41 21 314 32 69 ; Fax : +41 21 314 32 63

loic.lelievre@chuv.ch; ***Corresponding author**

[2]INSERM UMR 1033 – UFR de Médecine Lyon-Est - 2ème étage rue Guillaume Paradin, 69372 Lyon cedex08 – France ; philippe.clezardin@inserm.fr

[3]Hospices Civils de Lyon, pôle Information Médicale Evaluation Recherche (PIMER), and Université de Lyon, EA 7425 Health Services and Performance Research (HESPER),

Hôpital E. Herriot, Bat B12, 5 place d'Arsonval, 69437 LYON cedex 03;

laurent.magaud@chu-lyon.fr

[4]Hospices Civils de Lyon, Service de Biostatistique, 69424 Lyon, France

Service de Biostatistique des HCL, Site Lacassagne - 162, av. Lacassagne - 69424 Lyon Cedex 03 ;

laurent.roche01@chu-lyon.fr

[5]Department of Medical Oncology, University Hospital, 87000 Limoges, France

Hôpital Dupuytren 2 Av Martin Luther King, 87000 LIMOGES;

nicole.tubiana-mathieu@chu-limoges.fr

[6]Gynaecology Department, Hôpital Femme-Mère-Enfant, 59 Boulevard Pinel 69500 Bron, France ; jean-dominique.tigaud@chu-lyon.fr

[7]Department of Medical Oncology, Montpellier University Hospital, Hôpital Saint Eloi, 80 Avenue Augustin Fliche, 34090 Montpellier, France; d-topart@chu-montpellier.fr

[8]Department of medical oncology, Poitiers University Hospital, CHU La Miletrie, 2 Rue de la Miletrie, 86021, Poitiers, France; nadia.raban@chu-poitiers.fr

[9]Department of medical oncology, Centre Jean Perrin, 58 Rue Montalembert, 63011 Clermont-Ferrand, France; marie-ange.mouret-reynier@cjp.fr

[10]Centre du sein, Centre Hospitalier Universitaire Vaudois, Avenue Pierre Decker 2, 1011 Lausanne, Suisse; patrice.mathevet@chuv.ch

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MicroAbstract

Besides its effects on bone metastases, zoledronic acid has many other antitumor effects.

This randomized phase II trial studied the interest of adding zoledronic acid to neoadjuvant chemotherapy in breast cancer with serum VEGF as primary endpoint. We observed a trend of neoadjuvant zoledronic acid to lower serum VEGF. Further studies are needed with longer follow-up and additional relevant endpoints.

Abstract

Introduction: Neoadjuvant chemotherapy has become the treatment of choice for locally advanced breast cancer. Zoledronic acid (ZA) is a bisphosphonate initially used in the treatment of bone metastases due to its anti-bone resorption effect. Anti-tumour effects of ZA, such as the inhibition of cell adhesion to mineralized bone or the anti-angiogenic effect, have been demonstrated. However, the clinical significance of these effects remains to be determined.

Materials and Methods: We undertook a multicenter open-label randomized trial to analyze the value of adding ZA to neoadjuvant chemotherapy in TNM cT2/T3 breast cancer. The primary endpoint was the evolution of serum VEGF.

Results: 24 patients were included in the ZA group and 26 in the control group. Evolution of serum VEGF was slightly in favour of ZA at 5.5 months, - 0.7% versus + 7.5%, without reaching statistical significance (*p 0.52*). Secondary endpoints were breast conservation rate (higher with ZA, 83.3% vs 65.4%, NS), pathologic complete response (no effect), and circulating tumour cells (odds ratio of 0.68 in favour of ZA, 95% CI [0.02- 24.36]). No cases of jaw necrosis or severe renal failure were observed in both groups.

Conclusion: ZA is an anti-tumour drug of interest with multiple effects on tumour biology, and larger trials with longer follow-up would be of interest including additional endpoints such as relapse and survival rates.

Trial Registration: ClinicalTrials.gov, Identifier: NCT01367288.

Keywords: Breast cancer - Locally advanced breast cancer- Zoledronic acid - Neoadjuvant treatment- VEGF

Introduction

Zoledronic acid (ZA, Zometa°) is a bisphosphonate that has shown to have both direct and indirect anti-tumoural effects. Inhibition of tumour cell adhesion to mineralized bone and further inhibition of bone metastases, stimulation of gamma/delta T lymphocyte cytotoxicity, induction of tumour cell apoptosis and an anti-angiogenic effect have been demonstrated [1-8].

The side effects of bisphosphonates are relatively few, out of which we can mention mandibular osteonecrosis which is infrequent. We decided to carry out a prospective randomized multicenter study, the *NEOZOL* study, comparing two systemic neoadjuvant treatments in locally advanced cancers, chemotherapy with ZA versus chemotherapy alone.

Materials and Methods

Patients

Women aged 18 years and older, with newly diagnosed invasive breast cancer of maximal diameter greater than 2 cm, UICC stage IIa, IIb and IIIa, were invited to join the study. Multifocal or multicentric tumours and inflammatory cancers were excluded.

Absence of contraindication to ZA was checked: creatinine clearance < 30ml/min with Cockroft or MDRD method, pregnancy or concomitant dental problems.

Study design and procedures

This is an exploratory multicenter phase IIa clinical trial, randomized controlled (1:1) and open-label. Two systemic neoadjuvant treatments were evaluated, one with ZA, the other without. Nineteen French centres participated in the study. The trial was accepted by the Lyon IV Ethics Committee and patients were enrolled after having signed an informed consent.

Enrolment and Randomization

Work-up included complete physical examination, mammograms, breast sonograms, breast MRI and a tumour biopsy to determine the histological status, grade, estrogen and progesterone receptors and HER2 status. Minimal biopsy volume was 2 mm^3. Patients were checked for distant metastases (CT scan in addition to bone scintigraphy or FDG-PET). All patients were sent to a specialist for an oral examination and a jaw panoramic x-ray.

After the assessment was completed, patients were randomly allocated 1:1 to the experimental group with ZA or the control group by IWRS. The block randomization method and a stratification by SBR grading were used to ensure selection bias and to achieve balance in the allocation of treatments arms.

Neoadjuvant treatment and follow-up

Zoledronic acid (ZA, Zometa°, Novartis°) was delivered at the usual dose of 4 mg (in a 15 min intravenous infusion) every 3 weeks for a total of 6 injections. In the two arms, neoadjuvant chemotherapy was given through a minimum of 6 courses (with a minimum of 3 cycles of anthracycline) but did not exceed 8 courses. The interval in between each cycle was 3 weeks. The type of chemotherapy was in accordance with protocols in use at each centre, but the recommended treatment regimen was the one that demonstrated important efficacy in the NSABP B27 trial [9]: 4 injections of doxorubicin (60 mg/m²) combined with cyclophosphamide (600 mg/m²) every 3 weeks (+/- 2 days), followed by 4 injections of docetaxel (100 mg/m²) every 3 weeks (+/- 2 days).

Each ZA injection was given just after completion of the chemotherapy cycle.

Patients with overexpression of HER2 received trastuzumab (Herceptin°, Roche°), for a total of one year, starting concomitantly to chemotherapy. Surgery took place after completion of the antineoplastic treatment associated with trastuzumab.

Follow-up visits were planned once every 3 weeks, at the time of a chemotherapy cycle, and at surgery (V final). Two additional workups were also planned at mid-treatment and at chemotherapy completion, during which breast examination, breast imaging, oral and radiologic assessments by a dental specialist were carried out. At mid-treatment, a metallic clip was inserted (optional) to further localize the tumour site at the time of surgery. An assessment of osteonecrosis of the jaw (ONJ) was carried out at each visit (chemotherapy cycles and the 2 additional workups). In case of ONJ diagnosis, ZA was stopped permanently and the patient was discontinued from the study. Blood samples, used for the dosing inter alia of VEGF, platelet count, and creatinine and urine samples were collected at V0, at each chemotherapy cycle, and at V final.

In case of alteration of the renal function, ZA was stopped temporarily and given back only when creatinine level dropped down to baseline +/- 10%.

In case of tumour progression, patients were withdrawn from the study to receive appropriate therapy.

Endpoints

The primary endpoint was the evolution of serum Vascular Endothelial Growth Factor (VEGF) (in pg/mL). VEGF has been shown to be a good marker of neoadjuvant chemotherapy efficacy [10-17]. VEGF was measured in the collected blood samples using an ELISA kit (Human VEGF Quantikine° Kit DVE-00, R&D systems, Lille, France).

VEGF levels at V0 and at the first cycle were pooled and will herein be referred as 'baseline' VEGF level. Normal serum VEGF levels ranges from 62 pg/mL to 707 pg/mL (mean 220 pg/mL). This mainly reflects the platelet VEGF burden released during in vitro clotting of blood sample necessary to the serum collection. In order to diminish this range and focus on VEGF directly in relation to the tumor activity, i.e. free circulating VEGF not transported by platelets, we also analyzed the evolution of the VEGF/platelet count ratio (in pg/10^6 platelets) as previously reported [18].

The secondary endpoints were breast conservation rate, pathologic complete response (pCR) after final surgery, change in Circulating Tumour Cells (CTC) between V0 and V final, and therapeutic complications. Pathologic Complete Response was assessed according to the Sataloff's classifications on primary tumour site and regional lymph nodes (Sataloff's criteria for pCR: TA for tumour and Na or Nb for lymph nodes). Therapeutic complications are reported in this article as bucco-dental complications and renal failure, the two main potential side effects of ZA. Dedicated oral examination and imaging were carried out in ZA experimental group only; thus, bucco-dental complications are reported for the experimental group only. For the monitoring of renal function, creatinine clearance (in mL/min) was measured at V0 and V final, and at each chemotherapy cycle.

*Statistical analysis (see **Annex** for further information)*

As an exploration study, we initially set up the total population of this study to 76 patients and then estimated the power we would get with the enrolment of these 76 patients. The outcome of interest was the relative change in VEGF levels, that is: (value at V final - value at V0)/value at V0. With 76 patients (38 per group), using a two-sided 0.05-level t-test, a 0.35-point difference in VEGF relative change between arms will be detected with a power exceeding 95% (with a standard deviation of VEGF relative change equals to 0.4065).

All statistical tests were two-sided. P-value <0.05 was considered to indicate a statistically significant difference. The numbers in brackets [] following estimated values indicate their 95% confidence intervals. For baseline comparison between arms, Fisher's exact test and Wilcoxon test were respectively performed for categorical and quantitative characteristics.

Results

From April 2010 to October 2013, 259 patients were considered for enrolment, 71 were pre-registered with an informed consent signed, 53 were randomized and ultimately 50 went into analysis (***Figure 1***). The planned size of 76 patients was not achieved despite important extension of the enrolment period. The most frequent reason for exclusion, after patient's acceptance of the protocol, was dental examination prior to or at V0 in discordance with the protocol.

Patient characteristics and tumor characteristics are listed in ***Table 1***. We observed more clinical and pathologically proven lymph node involvement in the control group (57.7% Vs. 29.2% and 75% Vs. 60%, respectively). 10 patients were HER2 positive, 2 in the ZA group and 8 in the control group.

Evolution of serum VEGF (figures 3 and 4)

1/ Serum VEGF*

Median duration of follow-up and between chemotherapy initiation and surgery were 5.7 and 5.4 months, respectively. At baseline, no statistical difference was observed in serum VEGF levels between groups according to the SBR grade, HER2 status, clinical lymph node involvement, HR status, menopausal status or the tumor size.

Figure 2 shows the evolution of VEGF according to treatment arm (N=427 VEGF measurements). At baseline, VEGF levels were higher in the ZA group than in the control group, but the difference was not significant (p= 0.08). At the beginning of chemotherapy, we observed a similar evolution. Afterwards, the evolution of VEGF relatively to the baseline value was slightly better in the ZA group at the end of follow up. However, this difference of evolution between arms was not statistically significant (p = 0.52). Comparison of AUC (Area Under Curve) for the two evolution curves from baseline to 6 months showed no difference (p= 0.21).

We did not observe any clear significant effect of ZA on serum VEGF levels through treatment according to the menopausal status or the ER status (***figure 4***).

2/ Serum VEGF /Platelet count ratio*

At baseline, serum VEGF/Platelet count ratio was not different between groups (p= 0.11). We observed a regular increase of VEGF/Platelet ratio during treatment in each treatment group with linear evolution curves which contrasts with the evolution of VEGF (***Figure 3***; N=399 VEGF/platelets count values). Evolution of VEGF/Platelet ratio was slightly in favour of ZA. However, the difference between the two groups was not statistically significant, AUC comparison for the two evolution curves from baseline to 6 months showed no difference (p= 0.28). Sub-group analysis according to the menopausal status or the ER status revealed no significant difference either (***figure 4***).

Conservation rate

The rate of conservative treatment was higher in the ZA group: 83.3% (20/24 patients) versus 65.4% (17/26) in the control group, although this difference was not significant (NS, p = 0.2, Fisher's exact test).

Pathologic response *

No significant effect of ZA on the pCR rate was observed.

ZA seemed more effective in menopausal women than in pre-menopausal women (OR 4.32, 95% CI [0.85- 22.07]) on Sataloff's tumour criteria, and appeared significantly more effective in menopausal women than in pre-menopausal women as to the lymph node response (OR 12.06, 95% CI [1.54- 94.33]).

As expected, when studying the effects of the treatments on the Sataloff's tumour criteria according to the ER status, we observed that ER+ patients had a significant lesser response whatever the treatment arm: OR 0.07, 95% CI [0.01- 0.42]) in the ZA group and OR 0.09, 95% CI [0.01- 0.71] in the control group, when compared to ER- patients, but no effect of the ER status was observed on the Sataloff's node criteria. At the same time, we observed a significant benefit of trastuzumab on the pathological response on both Sataloff's criteria (data not shown, 10 patients only).

Circulating Tumour Cells (CTC)*

The number of patients with CTC was comparable at V0: ZA: 4/19 and Control: 4/18, and at V final: one patient in each arm, ZA had no effect on CTC.

Therapeutic complications

Bucco-dental:

Bucco-dental signs were observed in 5 patients in the ZA group: 1 patient suffered from dental pain, 4 were affected by loose teeth but no cases of osteonecrosis were observed. Two cases of stomatitis (grade I and II) were observed in this group.

Renal:

No occurrence of severe renal failure (clearance < 30 mL/min) was reported in both groups. A creatinine clearance of <60 mL/min was observed during treatment in 4 patients of the ZA group and in 3 patients of the control group (NS). One patient of the ZA group showed a creatinine clearance of 52 mL/min during treatment, the ZA dose had not been reduced as it should have been, with no implication. There were similar increases of the creatinine clearance in both groups during treatment, with a mean level of +2% per month in the control group and +1.2% in the ZA group (p 0.38).

Discussion*

Adjuvant zoledronic acid (ZA) has been proven to reduce bone metastases in addition to improving survival in post-menopausal women with early breast cancer [19].

Despite these benefits in adjuvant therapy, results from several randomized trials are controversial and often failed to reach statistical significance as to any advantage of ZA when concomitant to neoadjuvant chemotherapy (NAC). In 2010, Coleman reported results of the AZURE trial comparing neoadjuvant chemotherapy alone (n= 103) to chemotherapy and ZA (n= 102). ZA proved to have a significant effect on the residual tumour size but pathological complete response rates were not different [20]. Similarly, Charehbili in 2014 reported results of neoadjuvant ZA + NAC (n= 120) in stage II/III breast cancers with no difference in the pathological complete response rate when compared to NAC alone (n= 122), though he observed a slight benefit in post-menopausal women [21]. Hasegawa also reported positive effects of neoadjuvant ZA (NAC + ZA, n = 93) versus NAC alone (n= 95) in the pathological complete response rate, especially for post-menopausal women and in women with triple negative tumours, although statistically not significant [22].

The anti-tumoural effects of ZA are numerous and have been studied for almost 20 years, from inhibition of bone metastasis formation, through multiple effects including reduction of bone resorption due to osteoclastic activity [23; 24], to further studies that demonstrated implications of ZA in tumour cell biology and microenvironment that could affect tumour development not only in bone but in breast and in all possible secondary tumour locations as well via stimulation of gamma/delta T lymphocyte cytotoxicity, induction of apoptosis, inhibition of proliferation, angiogenesis, cell migration and invasion [1-8]. Of interest in breast oncology, these effects seem to be related to the estrogen environment, ZA being more effective in post-menopausal women [25] and in estrogen receptor negative (ER -) patients [26]. Our study failed to show a benefit of ZA for improving the pathologic response in post-menopausal and ER- patients. The

main trials that studied ZA in concomitance to neoadjuvant chemotherapy had residual tumour at surgery as primary endpoint [20; 21; 22]. We chose blood VEGF level as primary endpoint, as it has been shown of interest to assess antitumour treatment efficacy [13]. We did observe only a moderate and non-significant advantage of concomitant ZA on the VEGF levels. In a randomized study published in 2013, also comparing neoadjuvant ZA plus chemotherapy to chemotherapy alone in order to observe the short-term biologic effects of neoadjuvant ZA, Winter found that chemotherapy plus ZA was more efficient to reduce VEGF levels than chemotherapy alone but only from day 5 to day 21, thereafter the effect was lost [27]. Based on those results, much larger studies would be required to achieve detailed assessment of VEGF evolution with better precision.

In concordance with other published trials [20; 22], we failed to show any statistical effect of ZA on the residual tumour size at surgery. Conversely, like previously reported [28; 29], we did observe a significant effect of concomitant neoadjuvant trastuzumab on tumour shrinkage, even if the number of patients treated with trastuzumab was small, suggesting a higher efficiency of trastuzumab than ZA on reducing the tumour burden (breast tumour and lymph nodes). A recent meta-analysis pooling data from 4 trials that studied neoadjuvant ZA [25] showed no advantage of ZA on tumour volume regression overall, but in the post-menopausal women sub-group, ZA did show a significant effect on tumour regression (pCR in the breast: 10.8% (chemotherapy alone) versus 17.7% (chemotherapy + ZA), OR 2.14, CI [1.01-4.55].

Conclusion

ZA is an anti-tumour drug of interest with multiple effects on tumour biology. These effects might not be as efficient as trastuzumab at providing fast tumour regression, therefore neoadjuvant ZA should be studied in larger trials with longer follow-up with relapse and survival rates as endpoints in addition to pathologic response at surgery and serum VEGF levels. Moreover, the target population should be post-menopausal women and ER- patients.

Clinical Practice Points*

As the use of neoadjuvant chemotherapy has steadily increased, research studies are needed to improve its efficacy. Significant multiple biologic effects of zoledronic acid (ZA) in oncology have been reported, suggesting other possible uses of this bisphosphonate in addition to bone metastasis inhibition related to its anti-bone resorption properties. Interestingly, these effects have been reported to vary according to the menopausal status of the patients and the estrogen receptor expression of the tumour. Adverse effects of zoledronic acid rarely occur.

The main trials that studied ZA in concomitance to neoadjuvant chemotherapy (NAC) had residual tumour at surgery as primary endpoint. We chose blood VEGF level as primary endpoint, as it has been shown of interest to monitor treatment efficacy in a few previous studies. We found that choosing serum VEGF level as primary endpoint to monitor response to neo-adjuvant therapy is of little interest.

This study failed to show that adding ZA to NAC would increase tumour shrinkage and allow a significant higher rate of pathologic complete response.

In order to improve the efficacy of NAC, adding drugs of interest like ZA should be considered in trials with longer follow-up with recurrence and survival endpoints, in addition to short term pCR rates.

Annex : Detailed statistics

Sample size calculations

According to data previously published, the mean expected reduction of this biomarker was 25 % after neo-adjuvant chemotherapy [10;11;12;13;14;16;17].

The study from Colleoni et al. [13] demonstrated in 48 patients that the medial reduction was 0,75 (post/pre) with a 95% CI of (0.62-0.83) after neoadjuvant chemotherapy. These data allowed us to calculate an estimation of the standard deviation of the reduction (application of the normal rule and taking account that 95CI =+/- 1.96 x se and that

se = sd/√n, (n = sample size). Given the poor knowledge about the therapeutic efficiency of Zoledronic Acid (ZA), such as the standard deviation of the relative reduction of serum VEGF, it was impossible to accurately calculate the power of the statistical tests to be performed. The power of the test for the primary endpoint given below is thus only approximate and relies on hypotheses that may not be realistic. A preliminary experimental study by Ottewel et al.[30] showed that the adjunction of ZA to chemotherapy led to a 60 % reduction of serum VEGF in the experimental arm, to be compared with a 25% reduction in the chemotherapy alone group, i.e a difference of 35% (60%-25%). As there was no data concerning the standard deviation of the reduction with ZA, the same standard deviation as the one from Colleoni's study [13] has been chosen for sample size calculation with expected serum VEGF reductions according to Ottewel et al.[30]:

Arm A with ZA : expected reduction 60%

Arm B without ZA : expected reduction 25 %.

As an exploratory study, we set up the total population of this protocol to 76 patients. The table below gives the power of the test according to the "true" differences in serum VEGF reduction between arm A and arm B.

Differences in serum VEGF reduction between Arm A and Arm B	Power of the test
0.05	0.081
0.1	0.178
0.15	0.343
0.2	0.549
0.25	0.742
0.3	0.879
0.35 (the most probable, see above)	0.954
0.4	0.986
0.45	0.996
0.5	0.999

Following the previous hypothesis, for an expected difference of reduction of 35 % between the 2 arms (65 % vs 25 % reduction of serum VEGF), this population of 76 was expected to allow us to demonstrate this difference with a power of 95,4 %.

Analysis of VEGF evolution

For the analysis of the primary endpoint, the logarithm of VEGF levels was modelled using a linear hierarchical model to take into account the repetition of measurement per patient. This model included as fixed effects: treatment arm, a function of time specific to each arm, treatment by trastuzumab (trastuzumab received Vs. not received), and an interaction treatment by trastuzumab*time. It was expected that VEGF levels increased after initiation of chemotherapy and then decreased several weeks afterwards. To reflect this particular dynamics of VEGF, the effect of time was modelled using two restricted cubic splines, one per arm, with 1 interior knot set at time t=1.5 month and the two boundary knots set at times t=0 (time of 1st chemotherapy cycle) and t=5 months. Treatment by trastuzumab was included in this modelling because: i) trastuzumab was shown to be efficient in patients with overexpressed HER2, and ii) proportion of trastuzumab treatments differed between the two arms. The hierarchical model further

included 4 random effects, one intercept and one-time slope random effects per arm. The 4-dimension random effect vector was assumed to follow a centered Gaussian distribution with a 6-parameter variance-covariance matrix (4 variance parameters, one per random effect; one intercept-slope covariance per arm; covariance between random effects related to different arms were set to 0). Parameters were estimated by maximisation of the likelihood. Difference between arms of the dynamics of VEGF level was tested using a likelihood ratio test, by comparison with a model that included the same effects as previously described, except that it included only one restricted spline of time common to both arms. The fit of the model was checked by assessment of residual plots and plots comparing observed and predicted values. Furthermore, two additional analyses were carried out to study the evolution of VEGF according to estrogen receptor (ER) status and to menopausal status; for each status (ER or menopausal), this was done using a similar model, that further included the considered status, an interaction arm*status, and a time*arm*status interaction. Wald testing was performed to compare, between arms, the change in VEGF levels from baseline to 5.5 months, according to status. The same analyses were performed to study the evolution of VEGF/platelet ratio.

Analysis of secondary endpoints

A Fisher exact test was used for the comparison of breast conservation rate between arms. The analysis of the tumour response was performed taking into account all measures of the tumour diameter. We did not perform separate analysis per type of measurement (clinical or for one imaging technology) because of numerous missing values, which would have yield non-conclusive or non-interpretable results. Conversely, most patients had at least one clinical or radiological measurement. The logarithm of the tumour diameter was modelled using a hierarchical TOBIT model with the measurements lower than 0.5 cm being left-censored to 0.5 cm, the minimum observed positive value, to take into account a floor effect (see, for example, [31]). This model included as fixed effect: the type of measurement (clinical Vs. radiological),

treatment arm, an indicator for each visit (V0, mid-treatment, and chemotherapy completion), and, for each visit, an interaction visit*arm and visit*type of measurement. It included also two patient-random effects –intercept and slope- to account for between-subject variable regarding baseline tumour size and decrease in tumour size. For the analysis of pCR, the three outcomes (Chevallier, Sataloff's tumor, and Sataloff's node scores) are ordinary variables with 4 levels. For each pCR outcome, analysis was carried out using a logistic regression model for ordinal dependent-variables, assuming proportional odds [32]. Each model included the treatment arm and treatment by trastuzumab (trastuzumab received Vs. not received). Furthermore, two additional analyses were carried out to assess pCR according to estrogen receptor (ER) status and to menopausal status; this was done using a similar ordinal model, that further included either ER status and an interaction arm*ER status, or menopausal status and an interaction arm*menopausal status. For the change in CTC between V0 and V final, a logistic regression was carried out to model the probability of presence of CTC. Co-variables included in this model were: the treatment arm, the visit (V final Vs. V0) and an interaction arm*visit. Finally, the logarithm of the creatinine clearance was modelled using a hierarchical linear model that included treatment arm, time, and an interaction arm*time as fixed effects. 4 patient-random effects were included, similarly as the random effects for the analysis of primary endpoint.

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To the other members of the NEOZOL Group:

Jean-Philippe Jacquin, Department of Medical Oncology, Institut Lucien Neuwirth, 42270 St. Priest en Jarez, France

Céline Chauleur, Obstetrics and Gynecology Department, Saint Etienne University Hospital, 42055 Saint-Priest-en-Jarez, France

Philippe Dalivoust, Hôpital Ambroise Paré, 13291 Marseille, France

Jacques Salvat, Gynecologic Department, Hôpitaux du Léman, 74203 Thonon-les-Bains, France

Hubert Orfeuvre, Oncology Department, Centre Hospitalier de Bourg en Bresse, 01012 Bourg en Bresse, France

Pascale Dubray-Longeras, Department of Medical Oncology, Centre Jean Perrin, 63011 Clermont-Ferrand, France

Gilles Romieu, C.R.L.C. Val D'Aurelle, 34298 Montpellier, France

Carol Alliot, Hematology/Oncology Division, General Hospital of Annemasse, 74100 Annemasse, France

Jean-Paul Guastalla, Department of Medical Oncology, Centre Léon Bérard, 69373 Lyon, France

Francesco Del Piano, Gynecologic Department, Hôpitaux du Léman, 74203 Thonon-les-Bains, France

Koutir Mahour, Medical Oncology Department, Hôpitaux du Léman, 74203 Thonon-les-Bains, France

Laetitia Stefani, Oncology Department, 74374 CHG Annecy, France

Victoire Cabaud, Oncology Department, 73011 CHG Chambery, France

Isabelle Cauvin, Oncology Department, 73011 CHG Chambery, France

Gilles Freyer, Oncology Department, Lyon Sud Hospital, 69495 Pierre-Bénite, France

David Benayoun, Obstetrics and Gynecology Department, Lyon Sud Hospital, 69495 Pierre-Bénite, France

Xavier Fritel, Department of Gynecology and Obstetrics, La Milétrie University Hospital, 86021 Poitiers, France

René Ecochard, Hospices Civils de Lyon, Service de Biostatistique, 69424Lyon, France

Anne-Marie Schott-Pethelaz, Hospices Civils de Lyon, pôle Information Médicale Evaluation Recherche (PIMER), and Université de Lyon, EA 7425 Health Services and Performance Research (HESPER), 69424 Lyon, France

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References

1.Winter MC, Holen I, Coleman RE. Exploring the anti-tumour activity of bisphosphonates in early breast cancer. *Cancer Treat Rev* 2008;34:453-75.

2. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer* 2000;82:1459-68.

3. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. *Clin Cancer Res* 2004;10:4559-67.

4. Aft R, Watson M, Ylagan L. Effect of zoledronic acid on bone marrow micrometastases in women undergoing neoadjuvant chemotherapy for breast cancer. *J Clin Oncol* 2008;26:Suppl:46s. abstract.

5. Kohno N. Treatment of breast cancer with bone metastasis: bisphosphonate treatment - current and future. *Int J Clin Oncol* 2008;13:18-23.

6. Clézardin P, Ebetino FH, Fournier PGJ. Biphosphonates and cancer-induced bone disease: Beyond their antiresorptive activity. *Cancer Res* 2005;65:4971-4.

7. Coleman RE. Risks and benefits of bisphosphonates. *Br J Cancer* 2008;98:1736-40.

8. Daubiné F, Le Gall C, Gasser J, Green J, Clézardin P. Antitumor effects of clinical dosing regimens of biphosphonates in experimental breast cancer bone metastasis. *J Natl Cancer Inst* 2007;99:322-30.

9. Bear HD, Anderson S, Brown A, Smith R, Mamounas E, Fisher B, *et al.* The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide : preliminary results from national surgical adjuvant breast and bowel project protocol B27. *J Clin Oncol* 2003;21:4165-74.

10. Wu Y, Saldana L, Chillar R, Vadgama JV. Plasma vascular endothelial growth factor is useful in assessing progression of breast cancer post surgery and during adjuvant treatment. *Int J Oncol* 2002;20:509-16.

11. Caine GJ, Stonelake PS, Lip GY, Blann AD. Changes in plasma vascular endothelial growth factor, angiopoietins, and their receptors following surgery for breast cancer. *Cancer Lett* 2007;248:131-6.

12. Mills PJ, Parker B, Jones V, Adler KA, Perez CJ, Johnson S, *et al.* The effects of standard anthracycline-based chemotherapy on soluble ICAM-1 and vascular endothelial growth factor levels in breast cancer. *Clin Cancer Res* 2004;10:4998-5003.

13. Colleoni M, Rocca A, Sandri MT, Zorzino L, Masci G, Nolè F, *et al.* Low-dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. *Ann Oncol.* 2002;13:73-80:

14. Kümmel S, Eggemann H, Lüftner D, Thomas A, Jeschke S, Zerfel N, *et al.* Changes in the circulating plasma levels of VEGF and VEGF-D after adjuvant chemotherapy in patients with breast cancer and 1 to 3 positive lymph nodes. *Anticancer Res* 2006;26:1719-26.

15. Tang JH, Zhao JH, Gong JP, Qin JW, Pan LQ, Xu ZY. Effects of chemotherapy on circulating angiogenic factor levels in patients with breast cancer. *Zhonghua Zhong Liu Za Zhi* 2007;29:210-4.

16. Vincenzi B, Santini D, Dicuonzo G, Battistoni F, Gavasci M, La Cesa A, *et al.* Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. *J Interferon Cytokine Res* 2005;25:144-51.

17. Ferretti G, Fabi A, Carlini P, Papaldo P, Cordiali Fei P, Di Cosimo S, *et al.* Zoledronic-acid-induced circulating level modifications of angiogenic factors, metalloproteinases and proinflammatory cytokines in metastatic breast cancer patients. *Oncology* 2005;69:35-43.

18.O'Byrne KJ, Dobbs N, Propper D, Smith K, Harris AL. Vascular endothelial growth factor platelet counts, and prognosis in renal cancer. *Lancet* 1999;353:1494-5.

19. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Coleman R, Powles T, Paterson A, Gnant M, Anderson S, Diel I, *et al.* Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *Lancet* 2015;386:1353-61. Erratum in: *Lancet* 2016;387:30.

20. Coleman RE, Winter MC, Cameron D, Bell R, Dodwell D, Keane MM, et al. AZURE (BIG01/04) Investigators. The effects of adding zoledronic acid to neoadjuvant chemotherapy on tumour response: exploratory evidence for direct anti-tumour activity in breast cancer. *Br J Cancer* 2010;102(7):1099-105.

21. Charehbili A, Van de Ven S, Smit VT, Meershoek-Klein Kranenbarg E, Hamdy NA, Putter H, *et al.* Dutch Breast Cancer Research Group (BOOG). Addition of zoledronic acid to neoadjuvant chemotherapy does not enhance tumor response in patients with HER2-negative stage II/III breast cancer: the NEOZOTAC trial (BOOG 2010-01). *Ann Oncol* 2014;25(5):998-1004.

22. Hasegawa Y, Tanino H, Horiguchi J, Miura D, Ishikawa T, Hayashi M, *et al.* JONIE Study Group. Randomized Controlled Trial of Zoledronic Acid plus Chemotherapy versus Chemotherapy Alone as Neoadjuvant Treatment of HER2-Negative Primary Breast Cancer (JONIE Study). *PLoS One* 2015 ;10(12):e0143643.

23. Russell RG, Rogers MJ. Bisphosphonates: from the laboratory to the clinic and back again. *Bone* 1999;25:97-106.

24. Santini D, Galluzzo S, Vincenzi B, Schiavon G, Fratto E, Pantano F *et al.* New developments of aminobisphosphonates: the double face of Janus. *Ann Oncol* 2007;18:VI 164-7.

25. Kroep JR, Charehbili A, Coleman RE, Aft RL, Hasegawa Y, Winter MC, *et al.* Effects of neoadjuvant chemotherapy with or without zoledronic acid on pathological response: A meta-analysis of randomised trials. *Eur J Cancer* 2016;54:57-63.

26. Aft RL, Naughton M, Trinkaus K, Weilbaecher K. Effect of (Neo)adjuvant zoledronic acid on disease-free and overall survival in clinical stage II/III breast cancer. *Br J Cancer* 2012;107(1):7-11.

27. Winter MC, Wilson C, Syddall SP, Cross SS, Evans A, Ingram CE, *et al.* Neoadjuvant chemotherapy with or without zoledronic acid in early breast cancer - a randomized biomarker pilot study. *Clin Cancer Res* 2013;19(10): 2755-65.

28. Buzdar AU, Valero V, Ibrahim NK, Francis D, Broglio KR, Theriault RL, *et al.* Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin Cancer Res* 2007;13(1):228-33.

29. Untch M, Rezai M, Loibl S, Fasching PA, Huober J, Tesch H, *et al.* Neoadjuvant treatment with trastuzumab in HER2-positive breast cancer: results from the GeparQuattro study. *J Clin Oncol* 2010;28(12):2024-31.

30. Ottewel PD, Mönkkönen H, Jones M, Lefley DV, Coleman RE, Holen I. Antitumor effects of doxorubicin followed by zoledronic acid in a mouse model of breast cancer. J Natl Cancer Inst 2008;100(16):1167-78.

31. Berk KN, Lachenbruch PA. Repeated measures with zeros. *Stat Methods Med Res* 2002;11:303-16.

32. McCullagh P. Regression models for ordinal data. *Journal of the royal statistical society Series B (methodological)* 1980;42(2):109-42.

Table 1: Patient and tumour characteristics at V0

		ZA		Control		
		N	%	N	%	P value
Inflammatory cancer	All	24		26		0.2359
	No	24		23	88.5	
	Yes	0	100	3	11.5	
Multifocal cancer	All	24		26		0.6105
	No	23	95.8	23	88.5	
	Yes	1	4.2	3	11.5	
Clinical Lymph node invasion	All	24		26		0.0518
	No	17	70.8	11	42.3	
	Yes	7	29.2	15	57.7	
Pathologic proven Lymph node invasion prior to treatment	All	24		26		
	No	4	40	3	25	
	Yes	6	60	9	75	
	Undetermined	14		14		
UICC stage	All	24		26		0.1023
	IIB	10	41.7	6	23.1	
	IIIA	3	12.5	10	38.5	
	IIA > 2cm	11	45.8	10	38.5	
Mean age (years)		51.2 [35- 68]		50.5 [22- 72]		
Post-menopausal		9	37.5	13	50	
Clinical mean tumor diameter (mm)		48.4 [20- 170]		41.7 [21- 100]		0.8167
Ultrasonographic mean tumor diameter (mm)		31.8 [13- 63]		29.5 [0- 75]		
SBR Grade	All	24		26		1.0000
	I	1	4.2	1	3.8	
	II	12	50	15	50	
	III	11	45.8	12	46.2	
Histologic Type	All	24		26		
	Ductal	21	87.5	24	92.3	
	Lobular	2	8.3	2	7.7	
	Ductal and Lobular	1	4.2	0	0.0	
ER	All	24		26		0.7598
	Positive	16	66.7	19	73.1	
	Negative	8	33.3	7	26.9	
PR	All	24		26		1
	Positive	11	45.8	11	42.3	
	Negative	13	54.2	15	57.7	
ER Positive or PR Positive	All	24		26		0.7598
	Positive	16	66.7	19	73.1	
	Negative	8	33.3	7	26.9	
HER2	All	24		26		0.1498
	Positive	2	9.5	8	30.8	
	Negative	19	90.5	18	69.2	

Figure 1: Randomization diagram

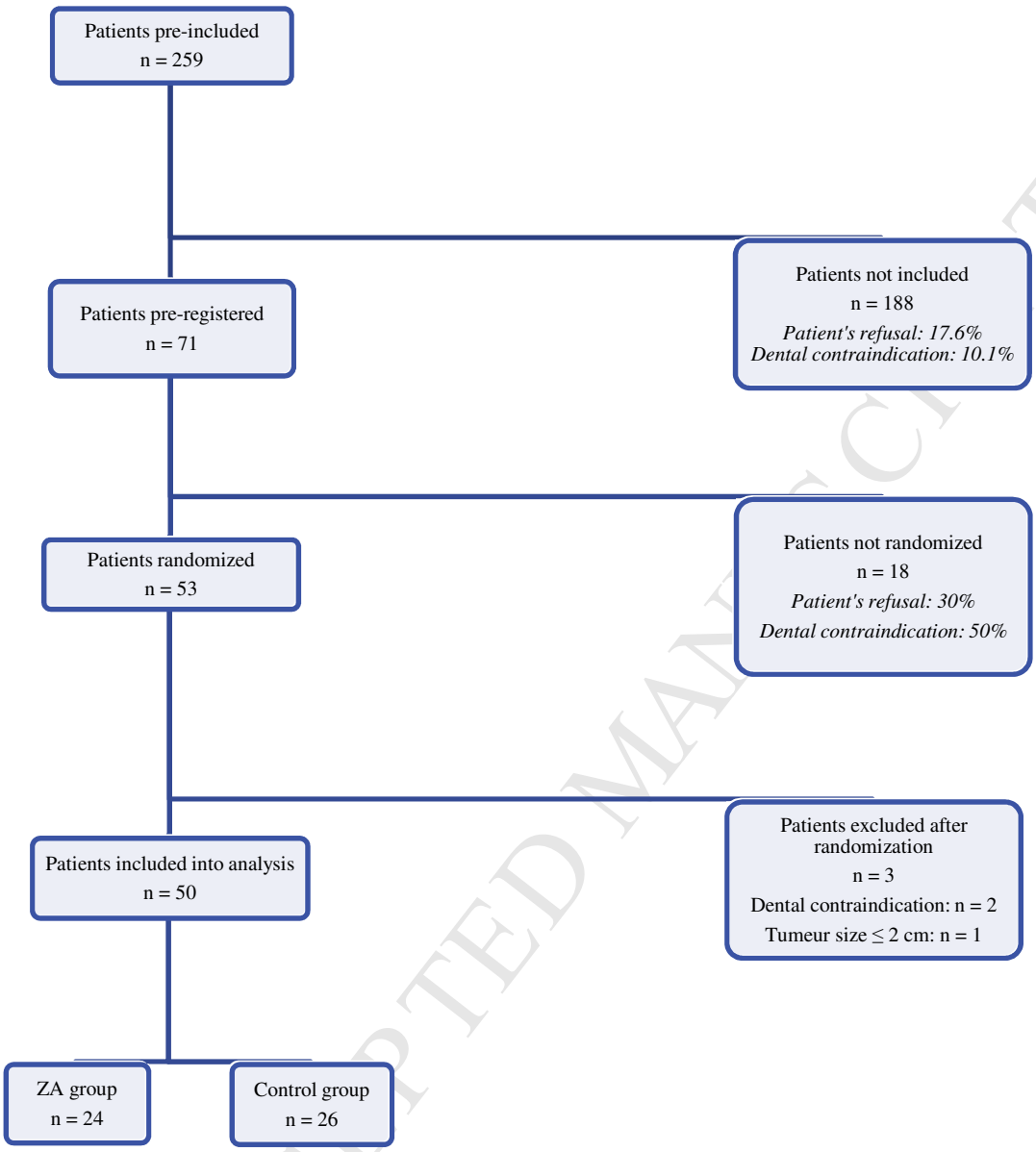

Figure 2: Mean Relative Evolution of serum VEGF (percentage) per treatment arm, the dotted lines show the estimate 95% CI

(Patients treated with trastuzumab excluded)

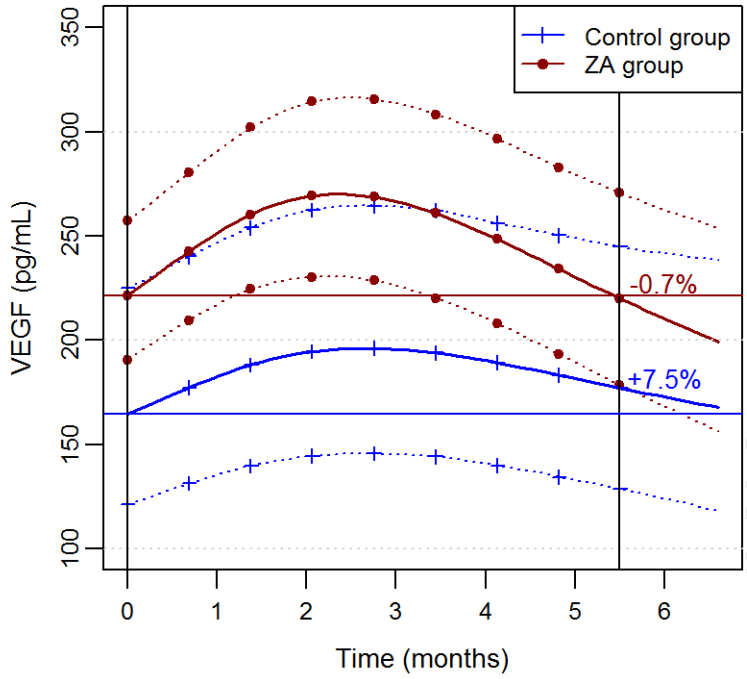

Figure 3: Mean Relative Evolution of serum VEGF/ platelet count ratio (percentage) per treatment arm, the dotted lines showing the estimate 95% CI

(Patients treated with trastuzumab excluded)

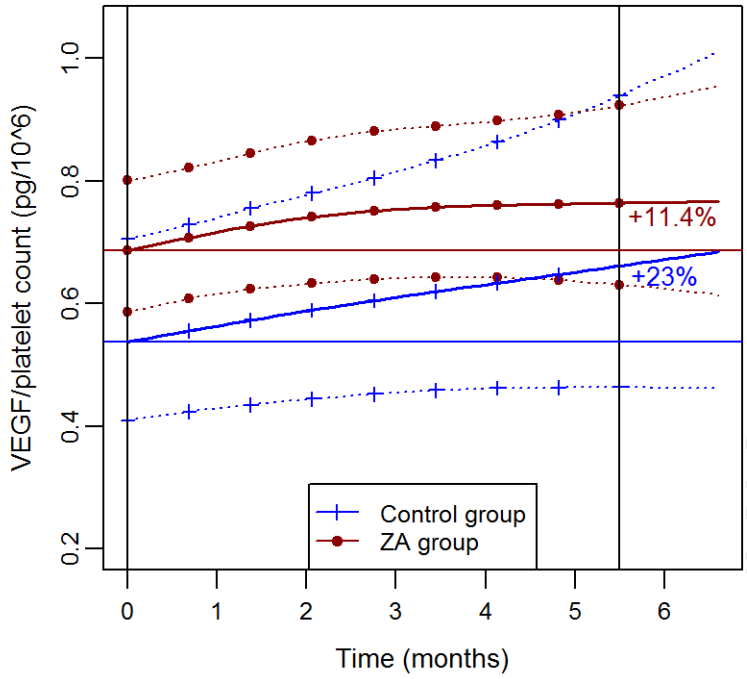

Figure 4: Relative evolution of VEGF and VEGF/Platelet between treatment groups according to menopausal and ER status

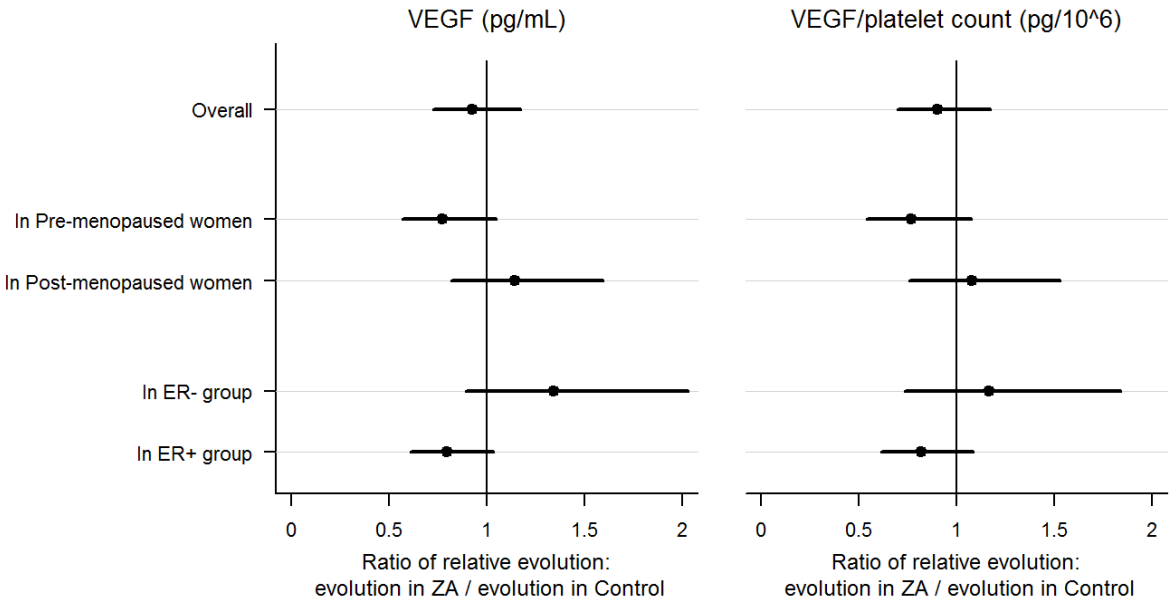

Figure 5: Odds ratio of the effect of the treatment arm on the pathologic response (Sataloff's criteria) according to the menopausal and the ER status; and Odds ratio of the menopausal and ER status on the pathologic response (Sataloff's criteria) according to the treatment arm