Effect of *MAF* amplification on treatment outcomes with adjuvant zoledronic acid in early breast cancer: a secondary analysis of the international, open-label, randomised, controlled, phase 3 AZURE (BIG 01/04) trial

Robert Coleman, Andrew Hall, Joan Albanell, Andrew Hanby, Richard Bell, David Cameron, David Dodwell, Helen Marshall, Joël Jean-Mairet, Juan-Carlos Tercero, Federico Rojo, Walter Gregory, Roger R Gomis

Summary

Background Adjuvant use of bisphosphonates can reduce the incidence of bone metastases in early breast cancer. Recurrence and survival seem to be improved only in postmenopausal patients, but the underlying mechanisms remain unclear. We investigated whether *MAF* amplification (a biomarker for bone metastasis) in primary tumours could predict the treatment outcomes of adjuvant zoledronic acid.

Methods The study population included patients enrolled in the international, open-label, randomised, controlled, phase 3 AZURE trial at eligible UK sites who had stage II or III breast cancer and who gave consent for use of their primary tumour samples. Patients were randomly assigned (1:1) to receive standard adjuvant systemic therapy alone (control group) or with zoledronic acid every 3–4 weeks for six doses, then every 3–6 months until the end of 5 years. Minimisation took into account the number of involved axillary lymph nodes, clinical tumour stage, oestrogen-receptor status, type and timing of systemic therapy, menopausal status, statin use, and treating centre. The primary endpoint was disease-free survival; the secondary endpoint, invasive-disease-free survival, was the primary disease endpoint for the analysis in this report. *MAF* amplification was assessed by fluorescence in-situ hybridisation of two cores of breast tumour tissue in a microarray, done in a central laboratory by technicians unaware of treatment assignment. We used multivariate analyses to assess disease outcomes by intention to treat. We also assessed interactions between *MAF*-positive status and menopausal status on efficacy of zoledronic acid. The AZURE trial is registered with the International Standard Randomised Controlled Trial Registry, number ISRCTN79831382.

Findings 1739 AZURE patients contributed primary tumour samples, of whom 865 (50%) had two assessable cores (445 in the control groups and 420 in the zoledronic acid group). 184 (21%) tumours were *MAF* positive (85 in the control groups and 99 in the zoledronic acid group) and the remaining tumours were *MAF* negative. At a median follow-up of 84·6 months (IQR 72·0–95·8), *MAF* status was not prognostic for invasive-disease-free survival in the control group (*MAF*-positive *vs* *MAF*-negative: hazard ratio [HR] 0·92, 95% CI 0·59–1·41), but was in the zoledronic acid group (0·52, 0·36–0·75). In patients with *MAF*-negative tumours, zoledronic acid was associated with higher invasive-disease-free survival than was control treatment (HR 0·74, 95% CI 0·56–0·98), but not in patients who had *MAF*-positive tumours. Additionally, among 121 patients not postmenopausal at randomisation with *MAF*-positive tumours, zoledronic acid was associated with lower invasive-disease-free survival (HR 2·47, 95% CI 1·23–4·97) and overall survival (2·27, 95% CI 1·04–4·93) than control treatment.

Interpretation *MAF* status can predict likelihood of benefit from adjuvant zoledronic acid and merits further investigation as a potential companion diagnostic.

Funding Novartis Global and Inbiomotion.

Lancet Oncol 2017; 18: 1543–52

Published Online October 13, 2017 http://dx.doi.org/10.1016/S1470-2045(17)30603-4

See Comment page 1437

Academic Unit of Clinical Oncology, Weston Park Hospital, Sheffield, UK (Prof R Coleman FRCP); **Clinical Trials Research Unit** (A Hall MSc, H Marshall MSc, Prof W Gregory PhD) **and Institute of Cancer and Pathology** (Prof A Hanby FRCPath), **University of Leeds, Leeds, UK; IMIM-CIBERONC, Barcelona** (Prof J Albanell MD, Prof R R Gomis PhD); **Medical Oncology Department, Hospital del Mar, Barcelona, Spain** (Prof J Albanell); **Pompeu Fabra University, Barcelona, Spain** (Prof J Albanell); **Deakin University, Geelong, VIC, Australia** (Prof R Bell FRACP); **Cancer Research UK Edinburgh Centre, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK** (Prof D Cameron FRCP); **St James Institute of Oncology, St James Hospital, Leeds, UK** (Prof D Dodwell FRCR); **Inbiomotion, Barcelona, Spain** (J Jean-Mairet PhD, J-C Tercero MD); **Fundación Jiménez Díaz, Madrid, Spain** (F Rojo MD); **CIBERONC, Madrid, Spain** (F Rojo); **Institute for Research in Biomedicine (IRB Barcelona), Barcelona, Spain** (Prof R R Gomis); **and Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain** (Prof R R Gomis)

Correspondence to: Prof Robert Coleman, Academic Unit of Clinical Oncology, Weston Park Hospital, Sheffield, UK **r.e.coleman@sheffield.ac.uk**

Introduction

Meta-analysis of clinical trials has shown that adjuvant bisphosphonates, especially zoledronic acid or clodronate, reduce the risk of bone metastases and improve survival in postmenopausal patients with early breast cancer.[1] The AZURE trial of adjuvant treatment with zoledronic acid in women with early breast cancer showed benefits in postmenopausal women[2] that were confirmed in a meta-analysis.[1] Additionally, striking heterogeneity was seen in recurrence outside bone by menopausal status, with an increase in extraskeletal mestastases in women who were not postmenopausal at study entry. However, although similar findings were seen in animal studies,[3] the underlying biological mechanisms for this interaction remain unclear.

Many clinical, pathological, and molecular prognostic biomarkers are used in early breast cancer, but none, except possibly oestrogen-receptor status,[4] can specifically identify patients at high risk of bone

Research in context

Evidence before this study

When we were planning this study, the Early Breast Cancer Trialists' Collaborative Group showed in a meta-analysis that adjuvant bisphosphonates help to prevent bone metastasis and extend survival in postmenopausal women with early breast cancer. We searched PubMed for studies of biomarkers to predict benefit from adjuvant bisphosphonates, using the search terms "metastasis", "biomarkers", "bisphosphonates", and "breast cancer". We restricted the language of publication to English but did not apply date restrictions. We also searched major conference websites for abstracts presented since 2005. We found no evidence of relevant biomarkers. Rather, patients are selected for treatment based on clinical criteria alone. As a result, the routine use of adjuvant bisphosphonates, which is now recommended by guidelines in Europe and North America, is hampered by the complexities of defining postmenopausal status in some patients. Additionally, there is no clear biological rationale for why treatment benefits seem to be confined to women with low concentrations of reproductive hormones. Among patients enrolled in the randomised, phase 3 AZURE trial of adjuvant zoledronic acid plus standard adjuvant treatment versus standard adjuvant treatment alone, expression of *MAF*, a bone-metastasis biomarker, has been measured in primary tumours.

Added value of this study

This study explored the association between treatment benefit, disease recurrence, and *MAF* amplification status. In patients with *MAF*-negative tumours, the use of adjuvant zoledronic acid was associated with improved disease outcomes, irrespective of menopausal status or age. By contrast, increased adverse outcomes, extraskeletal recurrence, and mortality were seen among women who were not menopausal at the start of the treatment with *MAF*-positive tumours and who received zoledronic acid. Assessment of *MAF* status, therefore, seems to provide an objective way to select breast cancer patients for adjuvant bisphosphonate treatment.

Implications of all the available evidence

Testing for *MAF* amplification in tumours is a potential companion diagnostic on which to base the decision of whether to use adjuvant zoledronic acid in patients with early breast cancer. Prospective validation of these findings in another randomised trial is warranted.

metastases. Gene profiles that might specifically predict for bone recurrence were first described a decade ago,[5] but are not used in clinical practice. In terms of predicting treatment benefits, menopausal status (and by association, age) are recommended in clinical practice guidelines as criteria to select patients most likely to benefit from adjuvant bisphosphonates,[6,7] but application in practice is hampered by the imprecise definition and timing of menopause. A proteomics discovery platform revealed that coexpression of PDZ domain-containing protein GIPC1 and macrophage-capping protein CAPG by primary tumours was predictive of benefit from adjuvant zoledronic acid in women with early breast cancer.[8] So far, however, no data have supported the clinical application of this discovery, and the reproducibility of these analyses awaits confirmation.

MAF, a biomarker of bone relapse in early breast cancer,[9] is a transcription factor of the AP-1 family encoded in the 16q arm. MAF regulates the expression of genes that collectively support several steps of breast-cancer-cell metastasis by various cell-autonomous and niche-related functions, and its overexpression supports processes that affect metastasis from the primary site to distant sites.[9] These observations suggest that *MAF* amplification in tumours could be a way to identify patients at high risk of metastasis. We retrospectively tested tumour samples from patients in the AZURE study to assess whether *MAF* status had prognostic value or could predict the treatment effects of adjuvant therapy with zoledronic acid.

Methods

Study design and patients

3360 patients from 174 centres worldwide were recruited to the AZURE trial between Sept 4, 2003, and Feb 16, 2006. Eligibility criteria have been reported previously.[2,10] Briefly, eligible patients had histologically confirmed invasive stage II or III breast cancer of any subtype, with either pathologically involved axillary lymph node metastasis or a T3 or T4 primary tumour treated with curative intent and complete resection of the primary tumour (or planned resection if patients were being treated with neoadjuvant chemotherapy). Other inclusion criteria were age 18 years or older, Karnofsky performance status score 80 or more, and not being pregnant or breastfeeding. Patients were excluded if they had clinical or imaging evidence of distant metastases, current or recent (<1 year) use of bisphosphonates, or pre-existing bone disease likely to require bone-targeted treatment.

All patients gave written informed consent. In the UK only, patients also provided voluntary specific consent for use of biological materials (primary tumour and blood samples). Before randomisation, patients underwent haematological, renal, hepatic function, and staging imaging tests according to institutional protocols. Those confirmed to be eligible were randomly assigned (1:1) to standard adjuvant systemic treatment alone or with zoledronic acid from a central computer-generated schedule held at the Clinical Trials Research Unit, University of Leeds, Leeds, UK, reached via an automated 24 h telephone line. A minimisation process that took into

account the number of involved axillary lymph nodes, clinical tumour stage, oestrogen receptor status, type and timing of systemic therapy, menopausal status, statin use, and treating centre was used.

Procedures

Patients received standard systemic therapy with (neo-) adjuvant chemotherapy, endocrine therapy, or both, alone (control group) or with 4 mg zoledronic acid given intravenously every 3–4 weeks for the first six doses, every 3 months for eight doses, and every 6 months for five doses to complete 5 years of treatment (zoledronic acid group). Oral calcium and vitamin D supplements were recommended for all patients in the first 6 months of treatment, and could be continued thereafter at the discretion of the treating physician. Adjuvant systemic treatments and locoregional radiotherapy were used in accordance with the standard protocols of each participating institution.

The follow-up schedule was similar in both study groups, and included clinical assessments, monitoring of adverse events, and haematological, renal, and hepatic function tests. Routine follow-up imaging was not mandated, but investigations were done for clinically suspected recurrence if deemed appropriate by the treating physician. Recurrence was defined by the date on which it was first suspected, to reduce the risk of ascertainment bias. 91% of recurrences were independently validated by either on-site or telephone-based monitoring. After treatment with zoledronic acid was completed, patients were followed up annually for disease and safety endpoints.

Site participation in the collection of tissue samples from primary tumours was, for logistical reasons, restricted to UK sites, and was encouraged but not mandatory. Tumour samples were sent to Sheffield for tissue microarray (TMA) construction. The locations of invasive tumour within samples were indicated by one pathologist as a guide to the technicians extracting tissue cores. Four cores, each 1 mm in diameter, were taken from each tissue sample. Cores were arranged into four sets of 13 blocks (holding 150 samples each) for assessment.

Measurement of *MAF* amplification was done according to the prespecified sample handling, methodological, scoring, and statistical protocols. We cut 5 µm thick sections from each TMA block, orientated to match the TMA map to allow identification of each tissue core. The slices were mounted onto Superfrost plus glass

	Two assessable FISH results (n=865)	Provided primary tumour samples (n=1739)	Whole AZURE population (n=3359*)
Menopausal status			
Not postmenopausal	590 (68%)	1192 (69%)	2318 (69%)
Postmenopausal	275 (32%)	547 (32%)	1041 (31%)
Age (years)			
<40	87 (10%)	198 (11%)	384 (11%)
40–49	299 (35%)	571 (33%)	1108 (33%)
50–59	281 (33%)	580 (33%)	1126 (34%)
60–69	162 (19%)	332 (19%)	628 (19%)
≥70	36 (4%)	58 (3%)	113 (3%)
Lymph-node involvement			
0	2 (<1%)	17 (1%)	62 (2%)
1–3	563 (65%)	1122 (65%)	2075 (62%)
≥4	300 (35%)	598 (34%)	1211 (36%)
Unknown	0	2 (<1%)	11 (<1%)
Tumour stage			
T1	274 (32%)	577 (33%)	1065 (32%)
T2	475 (55%)	901 (52%)	1717 (51%)
T3	99 (11%)	214 (12%)	456 (14%)
T4	17 (2%)	47 (3%)	117 (4%)
TX	0	0	4 (<1%)
Oestrogen-receptor status			
Positive	689 (80%)	1388 (80%)	2634 (78%)
Negative	171 (20%)	341 (20%)	705 (21%)
Unknown	5 (1%)	10 (1%)	20 (1%)
Systemic therapy plan			
Endocrine therapy	46 (5%)	89 (5%)	152 (5%)
Chemotherapy	166 (19%)	339 (20%)	719 (21%)
Both	653 (76%)	1311 (75%)	2488 (74%)

(Table 1 continues in next column)

(Continued from previous column)

	Two assessable FISH results (n=865)	Provided primary tumour samples (n=1739)	Whole AZURE population (n=3359*)
Taking anthracyclines			
Yes	794 (92%)	1604 (92%)	3132 (93%)
No	71 (8%)	135 (8%)	227 (7%)
Taking taxanes			
Yes	126 (15%)	267 (15%)	775 (23%)
No	739 (85%)	1472 (85%)	2584 (77%)
HER2 status			
Positive	93 (11%)	186 (11%)	415 (12%)
Negative	250 (29%)	503 (29%)	1251 (37%)
Unknown or not measured	522 (60%)	1050 (60%)	1693 (50%)
Histological grade			
1	61 (7%)	147 (9%)	285 (9%)
2	333 (39%)	748 (43%)	1439 (43%)
3	467 (54%)	820 (47%)	1552 (46%)
Not specified	4 (1%)	24 (1%)	83 (3%)
Progesterone-receptor status			
Positive	308 (36%)	633 (36%)	1423 (42%)
Negative	159 (18%)	350 (20%)	806 (24%)
Unknown	398 (46%)	756 (44%)	1130 (34%)

Some percentages might sum >100% because of rounding. FISH=fluorescence in-situ hybridisation. *3360 recruited and one withdrew consent.

***Table 1*: Clinical and tumour characteristics of test population and overall AZURE trial population**

See Online for appendix

slides (Thermo Fisher Scientific, Waltham, MA, USA) and stained with haematoxylin and eosin to confirm the presence of assessable tumour. *MAF* amplification was assessed with the validated MAFTEST fluorescence in-situ hybridisation (FISH) with the MAF/D16Z3 probe (Inbiomotion, Barcelona, Spain). A central laboratory (Targos Molecular Pathology, Kassel, Germany) validated the analytical and diagnostic performance of the assay, established acceptance criteria, included appropriate quality controls for each assay, and did the analyses masked to treatment assignment. Briefly, TMA sections were deparaffinated in xylene (twice for 10 min each), rehydrated in ethanol series, washed with water, and pretreated at 98°C for 15 min. Samples were digested with pepsin in a Poseidon Tissue Digestion Kit (Kreatech, Amsterdam, Netherlands) for 30 min, dehydrated in ethanol series, and dried. After adding 10 µL MAF/D16Z3 probe, slides were denatured at 80°C and placed overnight in a hybridiser at 37°C. After hybridisation, FISH slides were washed in Poseidon Tissue Digestion Kit Wash Buffer I at 72°C for 2 min and then Wash Buffer II for 1 min at room temperature. After dehydration and air-drying, slides were incubated with 15 µL 4′,6-diamidino-2-phenylindole solution (0·03 mg/mL) and stored at 4°C in the dark until scoring.

We also did immunohistochemical testing for MAF expression,[9] but the results were suboptimum in the available samples, presumably because of epitope decay in the time since fixation (>10 years). Therefore, these results are not considered further in this report.

Mean *MAF* copy number per nucleus was scored in 20 nuclei from the region of the tumour with the highest amplification, by laboratory staff who were masked to treatment group. If the mean copy number was between 2·0 and 3·0, 30 more nuclei were scored. To keep the effect of tumour heterogeneity in such small fragments to a minimum, duplicate results were mandated for each patient. Thus, replica cores were scored until two FISH amplification values were obtained for each tumour, of which the highest per patient was used in the statistical analysis. The TMAs were analysed by FISH without optimisation or repetition, as stipulated in the study protocol. Patients for whom the mean *MAF* copy number was 2·5 or greater in at least one replica were deemed to be *MAF* positive. This threshold was based on studies in a retrospective cohort[9] and judged unlikely to be artificially affected by rapid tumour cell proliferation.

	Control group (n=445)		Zoledronic acid group (n=420)	
	Negative (n=360)	Positive (n=85)	Negative (n=321)	Positive (n=99)
Menopausal status				
Not postmenopausal	253 (70%)	55 (65%)	216 (67%)	66 (67%)
Postmenopausal	107 (30%)	30 (35%)	105 (33%)	33 (33%)
Age (years)				
<40	43 (12%)	5 (6%)	24 (8%)	15 (15%)
40–49	124 (34%)	28 (33%)	118 (37%)	29 (29%)
50–59	112 (31%)	33 (39%)	109 (34%)	27 (27%)
60–69	63 (18%)	17 (20%)	61 (19%)	21 (21%)
≥70	18 (5%)	2 (2%)	9 (3%)	7 (7%)
Tumour stage				
T1	111 (31%)	31 (37%)	100 (31%)	32 (32%)
T2	200 (56%)	40 (47%)	179 (56%)	56 (57%)
T3	43 (12%)	12 (14%)	37 (12%)	7 (7%)
T4	6 (2%)	2 (2%)	5 (2%)	4 (4%)
Lymph-node status				
0	2 (1%)	0	0	0
1–3	231 (64%)	58 (682%)	214 (67%)	60 (61%)
≥4	127 (35%)	27 (32%)	107 (33%)	39 (39%)
Oestrogen-receptor status				
Positive	308 (86%)	55 (65%)	264 (82%)	62 (63%)
Negative	50 (13%)	29 (34%)	57 (18%)	35 (35%)
Unknown	2 (1%)	1 (1%)	0	2 (2%)
Systemic therapy plan				
Endocrine therapy	24 (7%)	2 (2%)	16 (5%)	4 (4%)
Chemotherapy	50 (14%)	29 (34%)	54 (17%)	33 (33%)
Both	286 (79%)	54 (64%)	251 (78%)	62 (63%)
Taking anthracyclines				
Yes	325 (90%)	79 (93%)	297 (93%)	93 (94%)
No	35 (10%)	6 (7%)	24 (8%)	6 (6%)
Taking taxanes				
Yes	49 (14%)	17 (20%)	44 (14%)	16 (16%)
No	311 (86%)	68 (80%)	277 (86%)	83 (84%)
HER2 status				
Positive	39 (11%)	17 (20%)	20 (6%)	17 (17%)
Negative	116 (32%)	18 (21%)	85 (27%)	31 (31%)
Unknown or not measured	205 (57%)	50 (59%)	216 (67%)	51 (52%)
Histological grade				
1	34 (9%)	3 (4%)	21 (7%)	3 (3%)
2	158 (44%)	21 (25%)	137 (43%)	17 (17%)
3	168 (47%)	60 (71%)	161 (50%)	78 (79%)
Not specified	0	1 (1%)	2 (1%)	1 (1%)
Progesterone-receptor status				
Positive	133 (37%)	25 (29%)	121 (38%)	29 (29%)
Negative	53 (15%)	28 (33%)	46 (14%)	32 (32%)
Unknown	174 (48%)	32 (38%)	154 (48%)	38 (38%)

Some percentages might sum >100% because of rounding.

Table 2: **Association between tumour *MAF* status and clinical and tumour characteristics**

Statistical analysis

The statistical analysis plan was prespecified (appendix) and used the data from the final analysis data cutoff of AZURE.[2] The primary endpoint of the AZURE trial was disease-free survival, defined as distant recurrence, any invasive locoregional recurrence except for ipsilateral operable relapse within a conserved breast, and death without recurrence. Invasive-disease-free survival, defined as death from any cause, invasive ipsilateral breast tumour recurrence, local or regional invasive recurrence, distant recurrence, invasive contralateral breast cancer, or second primary invasive cancer (non

breast, but excluding basal-cell or squamous skin cancers) was added as a secondary endpoint[2,10] to reflect the international standard definition for recurrence that had emerged during the trial,[11] and is the primary disease endpoint assessed in this report. Secondary endpoints included overall survival, defined as death from any cause after starting treatment, time to bone metastases, and subgroup analyses based on variables included in randomisation, including menopausal status.

For this report, we tested the hypothesis that *MAF* amplification status would be a useful prognostic factor for disease recurrence, especially in bone, and a predictive factor for response to adjuvant zoledronic acid. The primary objective was to test the prognostic associations in the control group. Invasive-disease-free survival, overall survival, time to first invasive-disease recurrence in bone (whether the first or a later event), and time to first extraskeletal invasive-disease recurrence, defined as in the main study,[2,10] were assessed in all patients in the AZURE population who had assessable *MAF* results on FISH (n=865).

We used Kaplan-Meier survival curves to assess the prognostic value of *MAF* status for invasive-disease-free survival and overall survival. Time to the first invasive-disease recurrence in bone was assessed with a cumulative incidence function curve and a Fine and Gray approach. Differences in outcomes between patients with *MAF*-positive and *MAF*-negative tumours were compared by a Cox proportional hazards multivariable model adjusted for the AZURE minimisation factors described above (excluding treating centre), and restricted to the control group. Analyses were done by intention to treat. Hypothesis testing was two-sided with significance at 5%. No adjustments were made for multiplicity.

The predictive analysis assessing the interaction of *MAF* status and treatment effect of zoledronic acid, was done with a Cox proportional hazards model. Only minimisation factors identified as significant in the prognostic analysis were included in the model to reduce potential overfitting. Additionally, we did predictive analyses for patients who were unequivocally postmenopausal (>5 years since last menses) at trial entry separately to those who were not (premenopausal, ≤5 years since last menses, and menopausal status unknown). Given the significant heterogeneity of treatment effect seen between patients established as being postmenopausal at the start of treatment and all other patients in AZURE,[10] and because menopausal status had been a prespecified analyses in the AZURE protocol and was reported in the main study reports,[2,10] we investigated interactions between *MAF*-positive status and treatment effects of zoledronic acid on disease by menopausal status.

Additional exploratory analyses were done for invasive-disease-free survival in all patients, with a Cox proportional hazards model that included the prognostic factors specified for the predictive analysis. The model included a three-way interaction term between *MAF* status, menopausal status, and treatment. We did sensitivity analyses that included all prespecified prognostic factors for this model. Because of the complexity of defining menopause, and in recognition that it is a biological process that occurs over years, the model was also fitted with age as a surrogate for menopausal status (≥50 or <50 years at start of treatment).

All statistical analyses were done at the Clinical Trials Research Unit, University of Leeds, with SAS version 9.4. The AZURE trial is registered with the International Standard Randomised Controlled Trial Registry, number ISRCTN79831382.

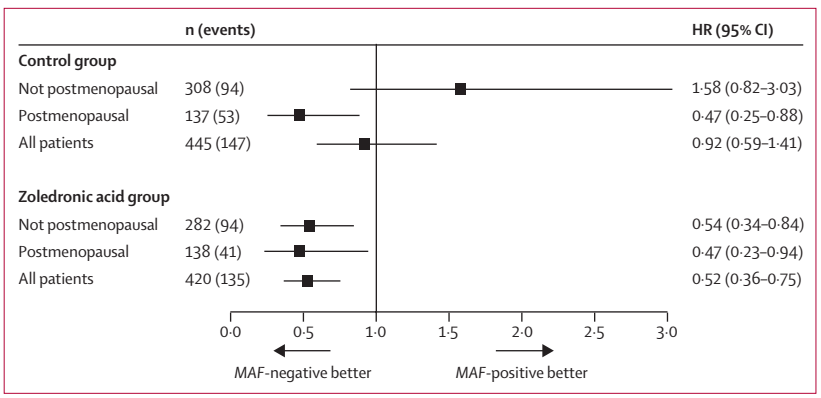

***Figure 1*: Effect of *MAF* copy number on invasive-disease-free survival**
HRs and 95% CIs are based on Cox multivariable analysis. HR=hazard ratio.

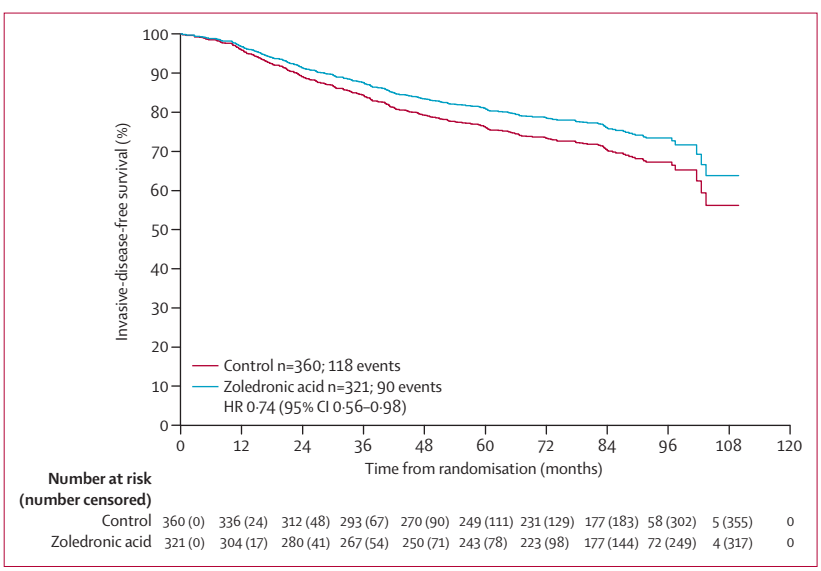

***Figure 2*: Invasive-disease-free survival by treatment group in *MAF*-negative patients**
Output from Cox multivariable model adjusted for differences in the prognostic factors between groups. HR=hazard ratio.

Role of the funding source

The funder Novartis had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The funder Inbiomotion contributed to data

interpretation and writing of the report, but had no role in study design, data collection, data analysis, or data interpretation. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

1739 (64%) of 2710 patients enrolled at eligible UK sites gave consent and had primary tumour samples sent for central analysis. Samples were processed between September, 2003, and March, 2006, and TMAs were prepared in 2007 and 2008. Despite marking of the tumour blocks by an experienced pathologist before TMA construction, only 3978 (63%) of 6326 TMA cores had sufficient invasive tumour for FISH analysis. The MAFTEST FISH assay could be reliably assessed in 2067 (56%) of these 3978 tissues cores. 865 (50%) of 1739 patients had two assessable FISH results (445 in the control group and 420 in the zoledronic acid group), and of these, 184 (21%) had *MAF*-positive tumours (85 in the control group and 99 in the zoledronic acid group).

The median follow-up was 84·6 months (IQR 72·0–95·8). 282 (33%) of 865 patients had an invasive-disease-free survival event (147 in the control group and 135 in the zoledronic acid group), 60 had a first event in bone (39 and 21), and 193 had died (102 and 91). 5-year invasive-disease-free-survival was 74·1% (95% CI 69·8–78·3) in the zoledronic acid group and 73·7% (69·6–77·8) in the control group; values were similar to those in the overall AZURE trial population.[2]

Characteristics of patients with two assessable TMA cores and those who provided primary tumour samples were similar to those of the entire AZURE cohort (table 1). More patients with *MAF*-positive tumours had high-grade, oestrogen-receptor-negative, and HER2-positive tumours than did those with *MAF*-negative tumours (table 2). Thus, patients with *MAF*-positive tumours were more likely to have received taxanes and trastuzumab and less likely to have received endocrine treatments than those with *MAF*-negative tumours. The frequency of *MAF*-positive tumours was similar across the menopausal and age subgroups.

Among patients in the control group, 118 (33%) of 360 with *MAF*-negative tumours and 29 (34%) of 85 with *MAF*-positive tumours had an invasive-disease-free survival event, suggesting that *MAF* status was not prognostic for this endpoint (figure 1). This result, however, is not fully representative of the data because the effect of *MAF* status on disease outcome was dependent on menopausal status at the start of treatment (χ^2=7·34, degree of freedom [df] 1, $p_{interaction}$=0·009). Among postmenopausal patients in the control group, *MAF*-negative status was associated with a shorter invasive-disease-free survival than was *MAF*-positive status, whereas among non-postmenopausal patients, the invasive-disease-free survival was longer for patients with *MAF*-negative tumours than those with *MAF*-positive tumours (figure 1). Lymph-node involvement, tumour stage, oestrogen-receptor status, and histological grade were significant in the prognostic analysis and were included in predictive analysis models (data not shown).

In the zoledronic acid group, invasive-disease-free survival was shorter in patients with *MAF*-positive tumours than in those with *MAF*-negative tumours, meaning that *MAF* status provided prognostic information (figure 1, appendix). A similar effect was seen for overall survival (appendix). Menopausal status did not substantially alter the effect of *MAF* status on disease outcome in the zoledronic acid group (figure 1).

13 bone-only invasive-disease-free survival events and six bone plus extraskeletal events were seen in patients with *MAF*-positive tumours, with 47 and 26, respectively, seen in patients with *MAF*-negative tumours. These numbers were insufficient to assess reliably the association between *MAF* status and invasive-disease-free survival in bone.

In the predictive analysis, we found an interaction between *MAF* status and treatment that affected invasive-disease-free survival (χ^2=4.55, df 1; p=0·033), but this result is convoluted by the prognostic interaction between menopausal status and *MAF* status. In subgroup analyses, the interaction remained significant for non-postmenopausal patients (χ^2=9·23, df 1; p=0·002), but not for postmenopausal patients (χ^2=0·09, df 1; p=0·76).

In patients with *MAF*-negative tumours, treatment with zoledronic acid was associated with longer invasive-disease-free survival than standard treatment alone (figure 2). Treatment benefits with zoledronic acid were similar irrespective of menopausal status or age (figure 3). By contrast, in patients with *MAF*-positive tumours, although zoledronic acid was not associated with longer invasive-disease-free survival,

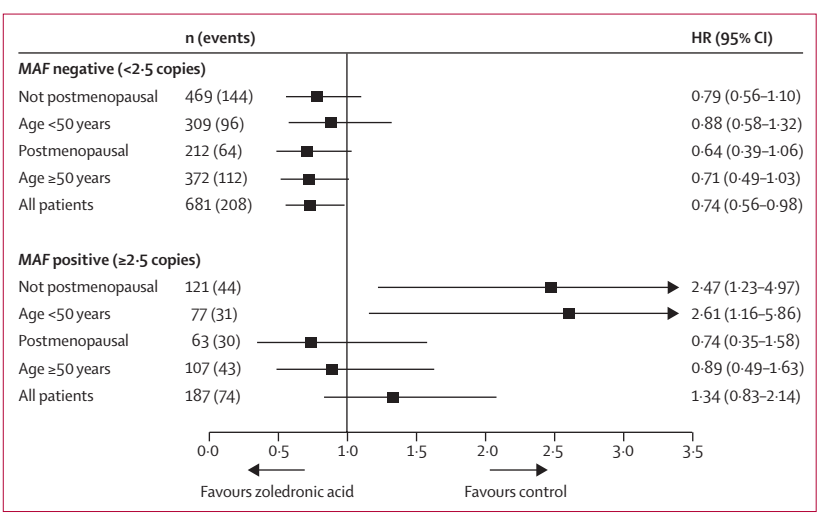

Figure 3: **Effect of *MAF* status on association between adjuvant zoledronic acid and invasive-disease-free survival, stratified by menopausal status and age**
HR=hazard ratio.

there was significant heterogeneity in the treatment effect by menopause status and age (figure 3). Not being postmenopausal at the start of treatment or age younger than 50 years had clear adverse effects on invasive-disease-free survival in patients treated with zoledronic acid (figure 3). In postmenopausal women, the number of events in those with *MAF*-positive tumours was insufficient to establish a definitive association between *MAF* status and treatment effect, although the HR was similar (albeit with wider confidence intervals) to that seen in *MAF*-negative women (figure 3).

For overall survival, a similar association between treatment, menopause, and *MAF* status was seen. Fewer patients with *MAF*-negative tumours treated with zoledronic acid died than did those in the control group (57 [18%] of 321 *vs* 76 [21%] of 360; HR 0·78, 95% CI 0·55–1·10). Among patients with *MAF*-positive tumours, no effect of zoledronic acid was seen on overall survival (34 [34%] of 99 died *vs* 26 [31%] of 85; HR 1·11, 95% CI 0·66–1·86). In women with *MAF*-positive tumours who were non-postmenopausal at the start of treatment there was, however, a clear adverse effect of zoledronic acid on overall survival (24 [36%] of 66 patients in the zoledronic acid group died *vs* nine [16%] of 55 patients in the control group; HR 2·27, 95% CI 1·04–4·93), which contrasts with the treatment effect of zoledronic acid on overall survival in postmenopausal women with *MAF*-positive tumours (ten [30%] of 33 patients in the zoledronic acid group died *vs* 17 [57%] of 30 in the control group; 0·62, 0·27–1·48).

190 patients had an extraskeletal invasive-disease-free survival event (92 in the control group and 98 in the zoledronic acid group). When compared with the control group, treatment with zoledronic acid in non-postmenopausal women with *MAF*-positive tumours was associated with a marked increase in relapse at extraskeletal sites (figure 4). The estimated extra-skeletal invasive-disease-free survival at 60 months for patients with *MAF*-positive tumours was 5·7% (95% CI 1·5–14·2) in the control group and 38·8% (27·1–50·3) in the zoledronic acid group (figure 4). We saw no effect of zoledronic acid treatment on extraskeletal recurrence in patients with *MAF*-negative tumours who were non-postmenopausal at the start of treatment (extraskeletal invasive-disease-free survival at 60 months was 18·0%, 95% CI 13·5–23·1 in the control group and 19·8%, 14·8–25·5 in the zoledronic acid group; figure 4).

The menopausal subgroup analyses indicated that menopausal status at the start of treatment played a part in the association between *MAF* status and invasive-disease-free survival (likelihood ratio test for the three-way interaction term between *MAF* status, menopausal status, and treatment: χ^2=5·71, df 1; p=0·017). We found heterogeneity in the treatment effect by menopausal status for *MAF*-positive tumours (χ^2=6·98, df 1; p=0·008), but not *MAF*-negative tumours (χ^2=0·38, df 1; p=0·539).

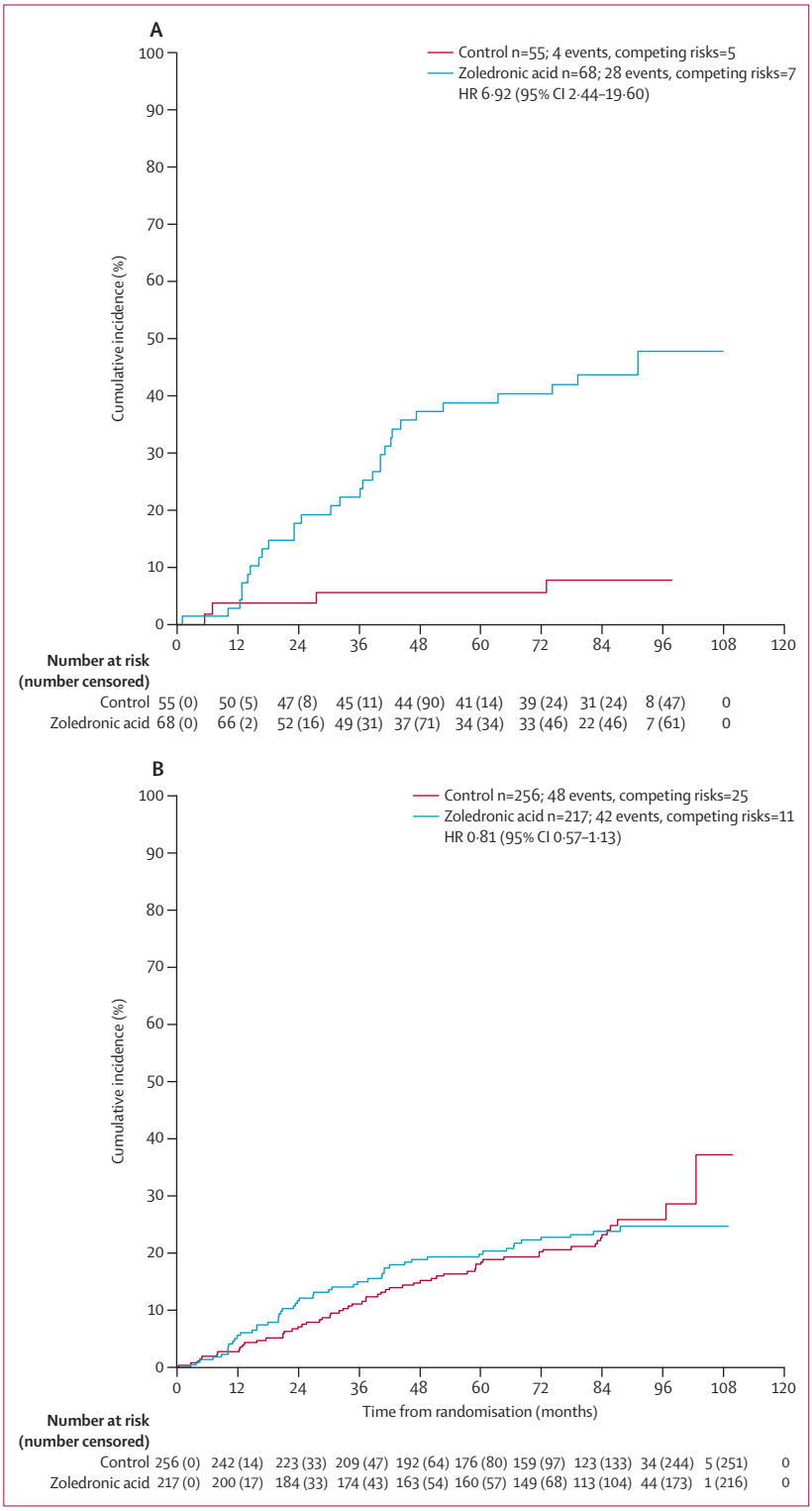

***Figure 4*: Cumulative risk of extraskeletal first recurrence in women not postmenopausal at trial entry, by treatment group**

(A) Patients with *MAF*-positive tumours. (B) Patients with *MAF*-negative tumours. Cumulative incidence unadjusted for differences in the prognostic factors between groups. Events for this endpoint were death and local or contralateral invasive disease. First events in bone were a competing risk. HR=hazard ratio.

	Number of patients (number of events)	Hazard ratio (95% CI)
***MAF*-negative**		
Non-postmenopausal control group	253 (83)	1
Postmenopausal control group	107 (35)	1·25 (0·80–1·95)
Non-postmenopausal zoledronic acid group	216 (61)	0·83 (0·59–1·15)
Postmenopausal zoledronic acid group	105 (29)	0·85 (0·53–1·36)
***MAF*-positive**		
Non-postmenopausal control group	55 (11)	0·63 (0·33–1·20)
Postmenopausal control group	30 (18)	2·68 (1·53–4·68)
Non-postmenopausal zoledronic acid group	66 (33)	1·61 (1·06–2·44)
Postmenopausal zoledronic acid group	33 (12)	1·72 (0·90–3·29)

Table 3: **Invasive-disease-free survival by *MAF* status, menopausal status, and treatment**

	Number of patients (number of events)	Hazard ratio (95% CI)*
Zoledronic acid group		
<50 years	142 (41) *vs* 44 (23)	0·473 (0·281–0·797)
≥50 years	179 (49) *vs* 55 (22)	0·533 (0·719–0·890)
Control group		
<50 years	167 (55) *vs* 33 (8)	1·410 (0·666–2·985)
≥50 years	193 (63) *vs* 52 (21)	0·673 (0·407–1·114)

**MAF*-negative *vs MAF*-positive tumours.

Table 4: **Effect of *MAF* status on invasive-disease-free survival, by age**

Compared with non-postmenopausal control patients with *MAF*-negative tumours, individual HRs were consistent with the primary analysis that invasive-disease-free survival is independent of menopausal status when patients are treated with zoledronic acid (table 3). We did, however, note heterogeneity in outcomes by menopausal status in addition to *MAF* status in patients in the control groups, as those with *MAF*-positive tumours who were not postmenopausal at the start of treatment had significantly better invasive-disease-free survival than those who were postmenopausal (HR 0·26, 95% CI 0·12–0·56). We found also that non-postmenopausal patients in the control group with *MAF*-positive tumours seemed to have a longer invasive-disease-free survival than patients in the control group with *MAF*-negative tumours (table 3).

We found no difference in invasive-disease-free survival when age was used as a surrogate marker for menopause (table 4), with similar beneficial effects in both age groups seen with zoledronic acid in patients with *MAF*-negative tumours (figure 3). Sensitivity analyses for the exploratory Cox model that included all AZURE minimisation factors showed no interpretable difference for any estimates (data not shown).

Discussion

Increased tumour copy number of *MAF*, when measured by FISH in primary breast tumour TMAs, predicted treatment benefit and harm associated with adjuvant zoledronic acid. This novel biomarker might, therefore, identify patients with early breast cancer who would benefit from treatment with an adjuvant bisphosphonate.

The tissues used in this study were collected from participants in the AZURE trial, which was designed to assess the effects of adjuvant zoledronic acid on disease outcomes in stage II or III breast cancer.[2,10] The tissues had been fixed in paraffin for more than 10 years, and FISH was done on 1 mm cores in a TMA format, which is technically much more challenging than would have been the case if contemporary tissue sections could have been used. Of note, a third of cores contained insufficient tumour for testing. These factors explain the attrition of patients with MAFTEST results meeting the criteria for the preplanned statistical analyses. Despite these technical challenges in obtaining reliable confirmed FISH results, we found that adjuvant zoledronic acid improved disease outcomes in the 79% of patients with *MAF*-negative tumours (mean copy number <2·5). Importantly, unlike in the AZURE trial population as a whole, this beneficial treatment effect was independent of menopausal status at study entry, which suggests that the use of adjuvant bisphosphonates could be extended to the 80% of premenopausal women who have *MAF*-negative tumours. This proportion is equivalent to around 16% of all women with early breast cancer. Conversely, the use of adjuvant zoledronic acid in women with *MAF*-positive tumours was not associated with treatment benefit. Additionally, among these women, the risk of extraskeletal recurrence was increased in those who were not postmenopausal at the start of treatment, resulting in significantly decreased invasive-disease-free survival and overall survival. Findings were similar when age (≤50 or >50 years) was used as a surrogate for menopausal status. Our data strongly suggest, therefore, that women who are not definitely postmenopausal and who have *MAF*-positive tumours should not receive an adjuvant bisphosphonate.

Our study has several limitations. First, we retrospectively analysed samples, and our findings require confirmation in another dataset. Second, because of the complex interactions between *MAF* status, bisphosphonate treatment, and menopausal status, the number of patients with reliable FISH results was too small to assess outcomes in some of the subgroups of interest. Third, although we mandated assessment of *MAF* in two tissue cores per patient to reduce the effect of tumour heterogeneity, assessment of routine tissue sections may reveal greater heterogeneity of expression than we could identify in replicate TMA cores.

The use of adjuvant bisphosphonates in early breast cancer and selection of appropriate patients remain areas of controversy. Treatment benefits with adjuvant zoledronic acid in young women receiving ovarian suppression therapy for oestrogen-receptor-positive breast cancer,[12] and the positive findings in a preplanned subset analysis of menopausal status in the AZURE trial[10]

suggested that adjuvant bisphosphonate efficacy is related to concentrations of reproductive hormones at the time of starting treatment. This hypothesis was rigorously tested by a large individual-patient meta-analysis done by the Early Breast Cancer Trialists Collaborative Group.[1] Data were collected from 18766 women in randomised trials of adjuvant bisphosphonate treatment. No benefits were found with adjuvant bisphosphonates in premenopausal women, but in the 11767 postmenopausal women, highly significant reductions were seen in bone recurrence (rate ratio 0·72, 95% CI 0·60–0·86, two-sided p=0·0002) and breast cancer mortality (0·82, 0·73–0·93, two-sided p=0·002). These results have led to supportive clinical guidelines,[13] and European and US expert groups recommend incorporating adjuvant bisphosphonates into routine clinical care.[6,7] Global acceptance, however, has been slow, partly because these benefits are thought to relate only to an imprecisely defined subset of patients, and the mechanistic explanation for the findings is unclear.[7]

Our findings should be viewed as hypothesis generating, but they clearly suggest that the beneficial effects of zoledronic acid on breast cancer are associated with the presence of a non-amplified *MAF* gene within the primary tumour. By contrast, in women with *MAF*-positive tumours who are not postmenopausal at the start of treatment, outcomes seem to be poor among those receiving zoledronic acid. Among non-postmenopausal women or those younger than 50 years, we identified two distinct subpopulations: women with *MAF*-negative tumours who, like older patients with *MAF*-negative tumours, benefited from zoledronic acid; and women with *MAF*-positive tumours in whom use of zoledronic acid was associated with extraskeletal metastatic disease and worse survival in the presence of reproductive hormones. This difference resulted in a net zero effect in the non-postmenopausal subgroup, in the biomarker cohort, and the AZURE trial population as a whole. Additional mechanistic studies addressing the importance of *MAF* in cancer metastasis are in progress. It is hoped that these investigations will provide some biological insights into the differential effects of bisphosphonates on disease outcomes according to *MAF* status and menopausal status and improve understanding of how tumour biology and treatments that affect the metastatic niche, the endocrine milieu, and host and tumour-cell functions interact. Before testing can be considered for routine clinical practice, *MAF* status needs further assessment in another large randomised trial of adjuvant bisphosphonates. Such a study is planned to take place in 2018 using the NSABP B-34 tumour bank and dataset for patients randomly assigned to treatment with the oral bisphosphonate clodronic acid or placebo.[14]

Among the 865 patients assessable for *MAF* status and during a median follow-up of 84 months, there were only 60 invasive-disease-free survival events in bone, which made interpretation of any association between *MAF* status and bone relapse unreliable. We were thus unable to confirm the prognostic capability of *MAF* status proposed by Pavlovic and colleagues.[9] We found that *MAF* amplification was associated with adverse biological characteristics, such as oestrogen-receptor negativity, high tumour grade, and *HER2* positivity, but could not show clinically useful independent prognostic value for bone metastasis. Although *MAF*-positive tumours were associated with worse disease outcomes than *MAF*-negative tumours in the control group who were postmenopausal at the start of treatment, our findings are not robust enough to recommend use of this biomarker in routine risk assessments. Because bone relapses typically occur late in the follow-up of patients with early breast cancer, further assessment is planned in the AZURE trial patients now they have completed 10 years of follow-up. This analysis is anticipated to increase the number of bone events by around a third. Use of other datasets is likely to be necessary to provide sufficient events to fully assess the association between *MAF*-positive tumours and the development of bone metastasis. We believe, however, that the clinical interest in *MAF* status will relate to the predictive capabilities we describe rather than its use as another prognostic factor.

The heterogeneity in invasive-disease-free survival by menopausal status among patients in the control group with *MAF*-negative tumours cannot be adequately explained by imbalances in other prognostic factors. Other than a slight excess of larger T2–T4 tumours in non-postmenopausal women than in postmenopausal women (72% *vs* 62%), the clinical and pathological characteristics were similar across these subgroups.

Collectively, our observations point to MAF as a potential molecular target for the prevention or treatment of metastases from breast cancer.[9] However, although required for metastasis, its nuclear localisation and lack of a catalytic domain make MAF a very challenging pharmacological target. Dissecting the role of genes that are transcriptionally regulated by MAF might lead to the identification of potentially targetable proteins that are necessary for metastasis,[9] including those that contribute to bone metastasis.[15,16]

Our findings suggest that *MAF* status could become a clinically useful biomarker for selection of patients who will benefit from adjuvant zoledronic acid. Women with *MAF*-negative tumours are likely to represent almost 80% of those with breast cancers who could benefit from the use of adjuvant zoledronic acid. *MAF* positivity seemed to be associated with adverse disease outcomes when women were treated with zoledronic acid, especially if treatment was started in women not definitely postmenopausal, and we recommend that exposure to bisphosphonates should be avoided in such women in the adjuvant setting.

Contributors

JA, JJ-M, J-CT, FR, and RRG developed the MAFTEST biomarker. RC, AHal, HM, WG, and RRG developed the study concept, wrote the protocol, and did and reviewed all analyses. All authors were involved in the

interpretation of the data. RC wrote the first draft of the paper, which was revised and approved by all authors.

Declaration of interests

RC has received grants from Amgen and Bayer and personal fees from AstraZeneca and Eisai. RB has received grants from Cancer Council Victoria. JJ-M owns less than 0·25% of Inbiomotion. J-CT has a patent pending related to this work. WG has received personal fees from Celgene and Janssen. RRG declares shares of Inbiomotion (<US$10 000) and has patents pending related to this work. The other authors declare no competing interests.

Acknowledgments

We thank the Academic Unit of Pathology at the University of Sheffield for constructing the tissue microarrays from tumour blocks submitted from sites in the UK, which enabled a range of translational studies, including the analyses reported in this Article. We also thank the patients who took part in the AZURE trial and the UK investigators who provided tissue samples for these analyses. This work was supported by grants from Novartis Global, Inbiomotion for biomarker measurements and statistical analyses, and University of Sheffield for sample collection. Novartis Global also donated the zoledronic acid used in the AZURE trial.

References

1 Early Breast Cancer Trialists' Collaborative Group. Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *Lancet* 2015; **386:** 1353–61.

2 Coleman RE, Cameron D, Dodwell D, et al. Adjuvant zoledronic acid in patients with early breast cancer: final efficacy analysis of the AZURE (BIG 01/04) randomized open-label phase 3 trial. *Lancet Oncol* 2014; **15:** 997–1006.

3 Ottewell PD, Wang N, Brown HK, et al. Zoledronic acid has differential antitumour activity in the pre- and postmenopausal bone microenvironment in vivo. *Clin Cancer Res* 2014; **20:** 2922–32.

4 Coleman RE, Rubens RD. The clinical course of bone metastases from breast cancer. *Br J Cancer* 1987; **55:** 61–66.

5 Smid M, Wang Y, Klijn JG, et al. Genes associated with breast cancer metastatic to bone. *J Clin Oncol* 2006; **24:** 2261–67.

6 Hadji P, Coleman R, Wilson C, et al. Adjuvant bisphosphonates in early breast cancer: consensus guidance for clinical practice from a European Panel. *Ann Oncol* 2016; **27:** 379–90

7 Dhesy-Thind S, Fletcher GG, Blanchette PS, et al. Use of adjuvant bisphosphonates and other bone-modifying agents in breast cancer: a Cancer Care Ontario and American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol* 2017; **35:** 2062–81.

8 Westbrook JA, Cairns DA, Peng J, et al. CAPG and GIPC1: breast cancer biomarkers for bone metastasis development and treatment. *J Natl Cancer Inst* 2016; **108:** djv360.

9 Pavlovic M, Arnal-Estapé A, Rojo F, et al. Enhanced *MAF* oncogene expression and breast cancer bone metastasis. *J Natl Cancer Inst* 2015; **107:** djv256.

10 Coleman RE, Marshall H, Cameron D, et al. Breast-cancer adjuvant therapy with zoledronic acid. *N Engl J Med* 2011; **365:** 1396–405.

11 Hudis CA, Barlow WE, Constantino JP, et al. Proposal for standardized definitions for efficacy end points in adjuvant breast cancer trials: the STEEP system. *J Clin Oncol* 2007; **25:** 2127–32.

12 Gnant M, Mlineritsch B, Stoeger H, et al. Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 62-month follow-up from the ABCSG-12 randomised trial. *Lancet Oncol* 2011; **12:** 631–41.

13 Coleman R, Body JJ, Aapro M, Hadji P, Herrstedt J. Bone health in cancer patients: ESMO Clinical Practice Guidelines. *Ann Oncol* 2014; **25** (suppl 3)**:** iii124–37.

14 Paterson AH, Anderson SJ, Lembersky BC, et al. Oral clodronate for adjuvant treatment of operable breast cancer (National Surgical Adjuvant Breast and Bowel Project protocol B-34): a multicentre, placebo-controlled, randomised trial. *Lancet Oncol* 2012; **13:** 734–42.

15 Blouin MJ, Bazile M, Birman E, et al. Germ line knockout of IGFBP3 reveals influences of the gene on mammary gland neoplasia. *Breast Cancer Res Treat* 2015; **149:** 577–85.

16 Pascual G, Avgustinova A, Mejetta S, et al. Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature* 2017; **541:** 41–45.