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Version: Accepted Version

Article:
Coleman, R, Hall, A, Albanell, J et al. (2017) 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. The Lancet Oncology, 18 (11). pp. 1543-1552. ISSN: 1470-2045

https://doi.org/10.1016/S1470-2045(17)30603-4

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Abstract

Background: In early breast cancer (EBC), the adjuvant use of bisphosphonates reduces the incidence of bone metastases but only appears to improve overall recurrence and survival in postmenopausal patients. The mechanisms underlying these observations remain unclear. To address this, we tested the recently identified bone relapse biomarker, *MAF* gene gain (MAF+) in primary tumours from women included in the AZURE trial (ISRCTN79831382) to determine the prognostic value of MAF and its potential to predict the effects of adjuvant zoledronic acid (ZOL) on disease outcomes.

Methods: The AZURE trial is an academic, prospective, open label, randomised phase III multicentre, parallel-group trial, performed in 3360 women with stage II/III breast cancer to receive standard adjuvant systemic therapy alone or with ZOL every 3-4 weeks for 6 doses, then 3-6 monthly thereafter to complete 5 years treatment. Consenting patients were randomised on a 1:1 basis using a central automated 24-hour computer-generated telephone randomisation system that included 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. The primary endpoint was disease recurrence. MAF was detected in breast tumours in tissue microarray format using a validated (*MAF/D16Z3*) FISH test, MAFTEST (Inbiomotion, Spain) in a blinded fashion by a central laboratory (Targos, Germany). A copy number cut-off ≥2.5 was preset to define a MAF+ve tumour. Multivariate analyses of disease outcomes by MAF status were performed in control and ZOL patients separately on an intention to treat basis according to a pre-specified analysis plan. Interactions between MAF+ve and menopausal status on effects of ZOL were also evaluated.

Findings: Of the 1769 AZURE patients who donated primary tumour samples, 865 (49%) had 2 FISH evaluable cores of which 184 (21%) were MAF+ve. MAF+ve tumours were more likely to be of higher grade, ER-ve and Her2+. At a median follow-up of 84 months, MAF was not prognostic in control patients for invasive disease free (IDFS) or overall (OS) survival, but in ZOL treated patients those with a MAF-ve tumor had better IDFS (HR_{IDFS}=0.52 [0.36-0.75] and OS (HR_{OS}=0.48 [0.31-0.75]). In patients with MAF-ve tumours, ZOL was associated with improved IDFS (HR_{IDFS}=0.74 [0.56-0.98]). However in patients with MAF+ve tumours, ZOL did not improve disease outcomes and, in the 119 MAF+ve patients who were not postmenopausal at randomisation, ZOL was associated with much worse outcomes (HR_{IDFS}=2.47 [1.23-4.97] and HR_{OS}=2.27 [1.04-4.93]). The interactions between disease outcomes, ZOL use and menopausal status were driven largely by an excess of extraskeletal recurrences (E-IDFS) in women with MAF+ve tumours who initiated ZOL before the development of menopause ($HR_{E\text{-}IDFS}$=6.92, [2.44-19.6]).

Interpretation: FISH evaluation of MAF in EBC segregates patients likely to benefit from adjuvant ZOL (MAF-ve) from those who may be harmed (not postmenopausal MAF+ve) and merits further investigation as a potential companion diagnostic.

Academic grant support, and study drug were provided by Novartis Global (Summit, New Jersey). Biomarker measurements and statistical analyses were funded by Inbiomotion (Barcelona, Spain).

Introduction

Meta-analysis of clinical trials has shown that adjuvant bisphosphonates (especially zoledronic acid [ZOL] or clodronate) reduce bone metastases and improve survival in postmenopausal patients with early breast cancer[1]. The AZURE study of adjuvant zoledronic acid in early breast cancer was the first study to identify the benefits of adjuvant bisphosphonates in postmenopausal women[2] and prompted the confirmatory meta-analysis of this subgroup finding,[1] In addition, marked heterogeneity according to menopausal status in the rates of recurrence outside bone was seen, with an increase in extraskeletal metastases in women who were not postmenopausal at study entry. However, despite recapitulation of these clinical observations in preclinical animal models of bone metastases[3], the biological mechanisms underpinning these apparent interactions between treatment effects and menopausal status are unknown.

There are many clinical, pathologic and molecular biomarkers that have been used to assess prognosis in early breast cancer although none, with the possible exception of estrogen receptor status, are useful to specifically identify patients at high risk for bone metastases. Gene profiles that may specifically predict for bone recurrence[4] were first described a decade ago but none are currently used in clinical practice. In terms of predicting treatment benefits, menopausal status (and by association age) are recommended in clinical practice guidelines for selection of patients likely to benefit from adjuvant bisphosphonate[5,6]. However, the imprecise definition and timing of menopause, makes this a somewhat difficult criterion on which to select patients most likely to benefit. Recently, using a proteomic discovery platform, the co-expression of GIPC1 and CAPG proteins by primary tumours was shown to predict benefit from adjuvant ZOL in early breast cancer.[7] However, to date, there are no confirmatory datasets supporting the clinical application of this discovery and the reproducibility of these analyses awaits confirmation. Thus, there is currently no biomarker to select patients for treatment with adjuvant ZOL.

To address this, the recently identified early breast cancer bone relapse biomarker, *MAF* gene gain (MAF+)[8] was tested retrospectively in the large prospectively randomized AZURE trial[2,9], of standard adjuvant therapy +/- ZOL in high risk early breast cancer. Overexpression of MAF, a transcription factor of the AP-1 family and encoded within the 16q arm, supports the molecular processes that affect the metastatic course from the primary site to distal colonization[8]. MAF regulates the expression of a set of genes that collectively support several steps of breast cancer cell metastasis through a series of cell-autonomous and niche-related functions[8]. Collectively, these observations suggest that MAF accumulation (*MAF* gain) may enable the identification of patients at high risk of metastasis. In this report, we have determined the prognostic value of MAF and its potential to predict the effects of ZOL on disease outcomes.

Methods

Study Design and patients

3360 patients from 174 centres were recruited between September 2003 and March 2006 to the AZURE study. Eligibility criteria have been reported previously[2,9] but, in summary, patients had to have histologically confirmed invasive breast cancer of any subtype with either pathologically involved axillary lymph node metastasis or a T3/T4 primary tumour treated with curative intent. Prior complete resection of the primary tumour was necessary or had to be planned if patients were treated with neoadjuvant chemotherapy. Patients had to be age ≥18 with a Karnofsky performance status index ≥80 and neither pregnant or breast-feeding to be eligible. Patients were not eligible if there was clinical or imaging evidence of distant metastases prior to study entry, current or recent (previous year) use of bisphosphonates or pre-existing bone disease likely to require bone-targeted treatment.

All patients gave written informed consent and, in the United Kingdom only, additional specific consent to use of biological materials (primary tumour and blood samples was requested. Patients could participate in the main trial alone if they so chose. Prior to randomisation, haematological, renal and hepatic function tests were required. Staging imaging tests were performed in accordance with institutional protocols. The full protocol may be viewed at http://ctru.leeds.ac.uk/Azure

The outcome data for the AZURE study have been previously reported[2,9] and the planned final analysis data-lock[2] was used for the analyses described below.

Procedures

Eligible patients were randomized to receive (neo) adjuvant chemotherapy and/or endocrine therapy +/- ZOL 4mg iv every 3-4 weeks for 6 doses, then 3 monthly x 8 and 6 monthly x 5 to complete 5 years treatment. Calcium and vitamin D oral supplements were recommended for all trial subjects during the first six months on study, and continued thereafter at the discretion of the treating physician. Both the use of adjuvant systemic

treatments and loco-regional radiotherapy were given in accordance with standard protocols at each participating institution.

The follow-up schedule was similar in both arms of the study and included clinical assessment, adverse event monitoring and haematological, renal and hepatic function test measurements. Routine follow-up imaging was not mandated, with investigations for possible recurrence clinically directed as deemed appropriate by the treating physician. The date of recurrence was defined as the date on which relapse was first suspected, to reduce the risk of ascertainment bias. 91% of recurrences were independently validated by either on-site or telephone-based monitoring. Subjects were followed up on an annual basis after completion of the 5-year treatment phase (ZOL or control) for both disease and relevant safety endpoints.

Of the 3360 patients accrued in the trial, primary tumour tissue blocks were collected from 1769/2710 (65%) patients treated at participating sites in the UK. Site participation in the collection of tumour blocks was encouraged but not mandatory and, for logistical reasons, restricted to UK sites.

All tumour blocks were sent to Sheffield for tissue microarray (TMA) construction where the location of invasive tumour within the tissue blocks was indicated by a single breast pathologist as a guide to the technician extracting the tissue cores for construction of the TMAs. Quadruplicate cores of 1 mm in diameter of each of the tumour tissue sample were arranged in 4 sets of 13 TMAs (150 samples each). All analyses are restricted to study participants who gave specific patient consent for the use of tissue samples.

The MAF biomarker analysis was completed on TMAs from primary tumours. Sample handling, methodology, scoring and statistical analyses were pre-specified in a study charter and statistical analysis plan. 5 µm thick tissue sections were cut from each TMA block, orientated to ensure correlation with the TMA map to allow identification of each tissue core and mounted onto Superfrost plus glass slides (Thermo Fisher Scientific). TMA slices were first analyzed using haematoxylin and eosin (H&E) staining to determine the presence of evaluable tumour. MAF amplification was then assessed using a validated (*MAF/D16Z3*) FISH test, MAFTEST, (Inbiomotion, Barcelona, Spain). A central laboratory (Targos Molecular Pathology, Kassel, Germany) validated the assay for analytic and diagnostic performance, established acceptance criteria, included appropriate quality controls for each assay, and performed the analyses in a blinded fashion. The previously described immunohistochemical (IHC) test for MAF[8] was found to perform sub-optimally on the TMA samples available, presumably due to epitope decay in the >10 years since fixation and is not considered further in this report.

Briefly, TMA sections were deparaffinated in Xylene (2x for 10 min), rehydrated in ethanol series, washed with water and pretreated at 98ºC for 15 min. Samples were digested with pepsin (Poseidon Tissue Digestion Kit, Kreatech, Amsterdam, Netherlands) for 30 min, dehydrated in ethanol series and dried. After adding 10 µl of MAF/D16Z3 probe (Inbiomotion), slides were denatured at 80ºC and placed overnight in a hybridizer at 37ºC. After hybridization, FISH slides were washed in Wash Buffer I at 72ºC for 2 min and then Wash Buffer II (both Poseidon Tissue Digestion Kit, Kreatech) for 1 min at room temperature. After dehydration and air-drying, slides were incubated with 15 µl of DAPI solution (0.03 mg/ml) and stored at 4ºC in the dark until scoring.

Mean MAF copy number per nuclei was scored by blinded central laboratory personnel in 20 nuclei from regions of the tumour with the highest amplification. If MAF mean copy number was between 2.0 and 3.0, an additional 30 nuclei were scored. Replica cores were scored until 2 FISH amplification values were obtained for each tumour. The highest value of MAF amplification in 2 evaluable samples of a patient was chosen for statistical analysis. The tumours on TMAs were FISH analysed only once, with no optimization and no repetitions allowed by protocol. A patient was considered as positive for MAF amplification when at least one of the replicas had a mean number of MAF copies per nuclei equal or higher than 2.5. The copy number cut-off $\geq$2.5 was predefined for MAF positivity (MAF+ve) based on studies in a retrospective cohort[8] and set at a level that was unlikely to be artificially influenced by rapid proliferation.

Statistical analysis

Following eligibility confirmation, patients were randomised by the treating clinical team on a 1:1 basis, using a central automated 24-hour computer-generated telephone minimisation system to ensure the concealment of the next treatment assignment based at the Clinical Trials Research Unit (CTRU), University of Leeds, to either standard adjuvant therapy alone (control) or with zoledronic acid monohydrate (ZOL), Novartis Pharmaceuticals, Summit, New Jersey, USA. To reduce possible imbalances in tumour and treatment characteristics, a minimisation process was used 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.

The primary endpoint of the AZURE trial was disease free survival (DFS), defined as distant recurrence, any invasive loco-regional recurrence except for ipsilateral operable relapse within a conserved breast, and death without recurrence. As described more fully in previous reports,[2,9] invasive DFS (IDFS) was added as a key secondary endpoint to reflect the international standard definition for recurrence that had emerged since the trial began[10] and is the primary disease endpoint assessed in this report. Secondary endpoints included overall survival (OS) defined as death from any cause after randomisation, time to bone metastases and subgroup analyses based on variables included in the randomisation including menopausal status. This study is registered as an International Standard Randomised Controlled Trial, number ISRCTN79831382

For this report, our hypothesis was that MAF status would have potential value as a prognostic factor for disease recurrence, especially in bone and as a predictive factor for response to adjuvant ZOL. The relationships in the control group were specified as the primary endpoint with those in the ZOL group exploratory. IDFS, OS, time to first IDFS event in bone (as first event or at any time) and time to first IDFS event not in bone endpoints, all defined as in the main study,[2,9] were assessed on all patients in the AZURE safety population with an evaluable MAFTEST. Subsequently, because disease outcomes by menopausal status had been pre-specified analyses in the AZURE protocol and reported in the main study reports,[2,9] interactions between MAF+ve and effects of ZOL on disease outcomes by menopausal status were also pre-specified in the statistical analysis plan (SAP).

The SAP was defined before any data analysis was performed. All statistical analyses were performed at the Clinical Trials Research Unit, University of Leeds where the AZURE clinical trial database is held on behalf of the trial sponsor, the University of Sheffield. All statistical analyses were conducted using SAS statistical software version 9.4. The SAP may be found in appendix p2-4.

The prognostic value of MAF status for IDFS and OS were investigated using Kaplan-Meier survival curves, whilst the time to first IDFS event in bone endpoint was assessed using a cumulative incidence function curve utilising a Fine and Gray approach. Differences in outcomes between patients with MAF+ve or MAF-ve tumours were compared using multivariable modelling in control patients only (Cox's proportional hazards) to adjust for the AZURE minimisation factors described above (excluding treating centre). Hypothesis testing was two-sided at a 5% significance level. No adjustments have been made for multiplicity.

For the predictive analyses, the response to zoledronic acid treatment was tested comparing control and ZOL groups. A predictive analysis, assessing the interaction of MAF status with treatment allocation, was performed using a Cox proportional hazards model. Only minimization factors identified as statistically significant in the prognostic analysis were included in the model to reduce potential overfitting. In addition, predictive analyses were carried out for patients who were unequivocally post-menopausal (>5 years since last menses) at trial entry separately to patients who were not post-menopausal (pre-menopausal, ≤5 years since menopause and menopausal status unknown), given the significant heterogeneity of treatment effect between established post-menopausal patients and all other patients observed in the AZURE trial as a whole[9].

Additional exploratory analyses were conducted for IDFS in all patients using a Cox proportional hazards model including the prognostic factors specified for the predictive analysis. The model included a three-way interaction term between MAF, menopausal status and treatment. Sensitivity analyses were conducted including all prognostic factors as specified in the prognostic analysis 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 fit with age as a surrogate for the menopausal status term; with an arbitrary cut-point of ≥ or <50 at randomization.

Role of the Funding Source

The AZURE trial was sponsored by the University of Sheffield and academic grant support provided by Novartis, supplemented by the infrastructure of the National Cancer Research Network. Novartis funded the sample collection but had no input in the study design, analysis or interpretation of data, nor in writing of the report. Inbiomotion had no involvement in the AZURE study design, or data analysis but JJM and JT did provide input into the statistical analysis plan, commented on the interpretation of the data and reviewed and approved the manuscript as co-authors. Biomarker measurements and statistical analyses were funded by Inbiomotion. The corresponding author had full access to all of the data and the final responsibility for publication.

Results

The primary tumours from 1739 patients had been processed between August 2003 and February 2006 according to routine local laboratory standard operating procedures and the TMAs prepared in 2007 and 2008 from representative tumour blocks sent to the University of Sheffield. Sections from the TMAs were sent to the

independent laboratory (TARGOS) for all analyses. Despite marking of the tumour blocks by an experienced pathologist before TMA construction, only 3978 out of 6326 TMA cores (63%) had sufficient invasive tumour confirmed on an H&E stained slide within each individual core for FISH analysis . The MAFTEST FISH assay was performed on adjacent sections and could be reliably assessed according to the stringent quality standards of the independent laboratory (TARGOS) in 2067 of 3978 (56%) tissues cores with sufficient invasive tumour. To reduce the impact of tumour heterogeneity in such small fragments of the original tumour, duplicate evaluable results from each patient were mandated. 865 of 1739 patients (50%) providing primary tumour for assessment had 2 evaluable FISH results and of these, 184 of 865 patients (21%) had a MAF+ve tumour.

The median follow-up was 84.6 (IQR 72.0-95.8) months. 282 patients in this biomarker subset had experienced an IDFS event (147 control, 135 ZOL), 60 a first IDFS event in bone (39 control, 21 ZOL) and 193 an OS event (102 control, 91 ZOL). The proportions of MAFTEST analyzed patients with an IDFS or OS event were similar to the main study; 5 year IDFS probabilities for the ZOL and control arms respectively in this biomarker population were 74.1% (95%CI 69.8-78.3) and 73.7% (95%CI 69.6-77.8) compared with 75.1% (95%CI 73.0-77.2) and 73.9% (95%CI 71.7-76.0) in the overall study population.

Patient characteristics in those patients with TMA cores evaluable for MAF gain were similar to the entire cohort of patients included in AZURE (Table 1). More patients with MAF+ve tumours had high grade, ER negative and HER2 positive tumours than did those with MAF-ve tumours (Table 2). As a result of these factors, patients with MAF+ve tumours were more likely to have received taxanes and trastuzumab and less likely to require endocrine treatments than for the whole group. The frequency of MAF+ve tumours was similar across menopausal subsets and age. Given the heterogeneity of the prognostic factors in the baseline demographics, results are reported using the multivariable Cox modelling.

Pre-specified analyses

Relationships between MAF status and prognosis

In the control patient group, 118 of 360 MAF-ve (33%) and 29 of 85 MAF+ (34%) experienced an IDFS event and MAF was not prognostic for IDFS (HR:0.92, 95%CI 0.59-1.41) However, this result is not fully representative of the data as the impact of MAF status on disease outcome was found to be significantly influenced by menopausal status at randomization, test for interaction (TFI) $_{IDFS}$ by menopause, Chi^2=7.34, P=0.009 (Figure 1). In postmenopausal women, the hazard ratio for IDFS (HR_{IDFS}) for MAF-ve/MAF+ve was 0.47 [95%CI 0.25-0.88], suggesting MAF is indeed prognostic for IDFS in this group of patients. However, in contrast, for non-postmenopausal women, the HR_{IDFS} for MAF-ve/MAF+ve was 1.58 [95%CI 0.82-3.03]. Lymph node involvement, tumour stage, ER status and histological grade were found to be significant in the prognostic analysis and are included in the predictive analysis modelling.

In ZOL patients, MAF provided prognostic information, with worse IDFS in MAF+ve tumors (Figure 1). (HR_{IDFS} for MAF-ve/MAF+ve = 0.52 [95%CI 0.36-0.75] and HR_{OS} for MAF-ve/MAF+ve =0.48 [95%CI 0.31-0.75] (figure Appendix page 1).

There were insufficient bone events (13 bone only and 6 bone with other sites in MAF+ve, 47 bone only and 26 bone with other sites in MAF-ve) in this sample set to reliably assess the relationships between MAF status and relapse in bone.

Relationships between MAF status and treatment effects

There was an IDFS MAF/treatment interaction for patients overall χ^2=4.55, p=0.03; this result however is convoluted by the interaction between menopausal status and MAF found in the prognostic analysis above. In subgroup analyses for IDFS there was a MAF/treatment interaction for non-postmenopausal patients (χ^2=9.23, p=0.002), but not for postmenopausal patients (χ^2=0.09, p=0.76).

In patients with MAF-ve tumours, treatment with ZOL was associated with improved IDFS compared to control patients (HR_{IDFS} ZOL/CONTROL=0.74 95%CI 0.56-0.98) (Figure 2). The group sizes, number of events and hazard ratios for the pre-planned menopausal sub-group and exploratory age analyses are shown in Figure 3. Treatment benefits with ZOL compared to control were similar irrespective of menopausal status or age.

In patients with MAF+ve tumours the findings are more complex (Figure 3). Overall, zoledronic acid did not appear to improve IDFS (HR_{IDFS} ZOL/CONTROL=1.34 [95%CI 0.83-2.14]). There was, however, significant heterogeneity of the treatment effect by menopause (hypothesis test reported in additional analyses). In women who were not postmenopausal at the start of treatment, there were clear adverse effects on IDFS (HR_{IDFS} ZOL/CONTROL=2.47 [95% CI 1.23-4.97]). In postmenopausal women, there were insufficient events, from the subset of MAF+ve tumours (HR_{IDFS} ZOL/CONTROL for MAF+ve postmenopausal women=0.74 [95%CI

0.35-1.58]) to establish a definitive relationship between MAF status and treatment effect, although the point estimate for the hazard ratio (albeit with wider confidence intervals) was similar to that seen in MAF –ve women.

For OS, a similar relationship between treatment, menopause and MAF was seen. There were fewer deaths in patients with MAF-ve tumours treated with ZOL (ZOL 57 events in 321 [18%] patients; CONTROL 76 events in 360 patients [21%]; (HR_{OS} ZOL/CONTROL=0.78 [95%CI 0.55-1.10]). In patients with MAF+ve tumours, no effect of ZOL on OS was seen (ZOL 34 events in 99 [34%] patients; CONTROL 26 events in 85 patients [31%]; HR_{OS} ZOL/CONTROL=1.11 [95%CI 0.66-1.86]). However there is a clear adverse effect of ZOL in non-postmenopausal women with MAF+ve tumours (ZOL 24 events in 66 [36%] patients; CONTROL 9 events in 55 patients [16%]; HR_{OS} ZOL/CONTROL=4.64 [95%CI 1.33-16.25]) compared with a numerical beneficial treatment effect on OS ((ZOL 10 events in 33 [30%] patients; CONTROL 17 events in 30 patients [57%]; HR_{OS} ZOL/CONTROL=0.62 [95%CI 0.27-1.48]) in postmenopausal women with MAF+ve tumours.

In 190 patients with an IDFS event, this was at an extraskeletal site (92 control, 98 ZOL). When compared with the control group, treatment with ZOL in non-postmenopausal women with MAF+ve tumours was associated with a marked increase in relapse (Figure 4) at extraskeletal sites. The extraskeletal IDFS estimates at 60 months for MAF +ve tumors were 5.7% 95%CI 1.5-14.2%) for control patients and 38.8% (95%CI 27.1-50.3%) for ZOL patients, (HR_{IDFS} ZOL/CONTROL =6.92, 95%CI 2.44-19.6). ZOL had no effect on extraskeletal recurrences in non-postmenopausal patients with MAF-ve tumors; the extraskeletal IDFS estimates at 60 months for MAF -ve tumors were 18% 95%CI 13.5-23.1%) for control patients and 19.8% (95%CI 14.8-25.5%) for ZOL patients (HR_{IDFS} ZOL/CONTROL=0.81, 95%CI 0.57-1.13).

Additional exploratory analyses

The menopausal subgroup analyses indicated that menopausal status at randomization was playing a role in how MAF influences IDFS disease outcomes. The cox model specified for the predictive analyses did not include any parameters for menopausal status; an exploratory Cox model was fitted to better understand any potential interactions. The likelihood ratio test for the three-way interaction term between MAF, menopausal status and treatment yields a χ^2 of 5.71 (P=0.017, 1DF), The model allows a hypothesis test for heterogeneity of the treatment effect by menopause for MAF subgroups; Wald heterogeneity Chi square statistics: 6.98 (P=0.008, 1DF) and 0.38 (P=0.539, 1DF) for MAF+ve and MAF-ve patients respectively.

Hazard Ratios (with 95%CIs) are presented using non-postmenopausal control patients with MAF-ve tumours as the reference group (Table 3). The individual hazard ratios are consistent with the prescribed analysis that IDFS outcome is independent of menopausal status when patients are treated with ZOL. There is however heterogeneity in IDFS outcomes by menopausal status in addition to MAF status in control patients. MAF+ve postmenopausal control patients have significantly worse IDFS outcome in contrast to MAF+ve non-postmenopausal patients (HR non-post/post: 0.26, 95%CI 0.12-0.56). Interestingly non-postmenopausal patients with MAF+ tumours appear to have a better IDFS than do those that are MAF-ve (HR MAF+ve/MAF-ve: 0.63, 95%CI 0.33-1.20).

The exploratory model was fit using age as a surrogate for menopausal status, with an arbitrary cut-point of ≥ or <50 at randomization (Table 4). There is no difference in the interpretation of the results in IDFS when using age as a surrogate marker for menopause with a similar beneficial effect for ZOL in patients with MAF-ve tumours (Figure 3). Sensitivity analyses for the exploratory Cox model including all AZURE minimization factors were conducted and made no interpretable difference to the estimates (data not shown).

Discussion

Using an independent specialist biomarker laboratory and a pre-defined cut-off, blinded to patient demographic, treatment and outcome data, we have shown that tumour copy number of the MAF transcription factor encoded by *MAF* gene with a precisely developed *MAF/D16Z3* FISH test performed on archival primary breast tumours in tissue microarray format is able to predict treatment benefit and harm from adjuvant zoledronic acid. To our knowledge, this is the first time a biomarker has been described that can potentially identify patients who may benefit from treatment with an adjuvant bisphosphonate.

The tissues used in this study were collected from patients taking part in the prospectively randomized AZURE trial designed to evaluate the effects of adjuvant ZOL on disease outcomes in stage II/III breast cancer; the primary results from this trial have been reported previously.[2,9] The tissues had been fixed in paraffin for >10 years and the FISH test performed on 1mm cores within TMA format – a technically much more challenging exercise than would be the case if the MAFTEST could have been performed on contemporary tissue sections. Of note one third of cores had insufficient tumor in the cut section of the TMA block. All of these factors explain the attrition in patients with a MAFTEST result for the pre-planned statistical analyses. Despite these

technical challenges in obtaining a reliable confirmed FISH test on relatively old paraffin embedded fixed tissues in TMA format, our findings show that adjuvant ZOL improved disease outcomes in the 79% of patients with a <2.5 MAF copy number (MAF-ve) and importantly, unlike in the study as a whole, this beneficial treatment effect was independent of menopausal status at study entry suggesting that the use of adjuvant bisphosphonates could be extended to the 80% of premenopausal women with a MAF-ve tumour, equivalent to around 16% of the early breast cancer population.

Conversely, the use of adjuvant ZOL in women with a gain in *MAF* gene (MAF+ve tumours) was not associated with treatment benefit and in women who were not postmenopausal at the start of treatment, the use of ZOL in the context of a MAF+ve tumour was associated with more frequent extra-skeletal spread, resulting in significantly worse IDFS and OS. Findings were similar when age ≤ or > 50 years was used as a surrogate for menopause. Our data strongly suggest that such women should not receive an adjuvant bisphosphonate. There are limitations to our study. Firstly, this is a retrospective analysis of data from a prospective randomised clinical trial and requires confirmation in another data-set. Secondly, because of the complex interactions between MAF, bisphosphonate treatment and menopause, the number of MAF evaluable patients is relatively small to assess outcome reliably in some of the subgroups of interest. Thirdly, although we mandated assessment of MAF on two tissue cores per patient to reduce the impact of tumour heterogeneity, evaluation 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. Following the findings of treatment benefits with adjuvant ZOL in young women receiving ovarian suppression therapy for ER+ breast cancer[11] and the positive findings in a pre-planned subset analysis by menopause within the AZURE trial[9], a hypothesis was generated that adjuvant bisphosphonate efficacy is related to levels of reproductive hormones at the time of treatment initiation. This hypothesis was rigorously tested by a large individual patient meta-analysis conducted by the Early Breast Cancer Trialists Collaborative Group (EBCTCG)[1]. Data were collected from 18,766 women randomized in adjuvant bisphosphonate trials. There were no demonstrable benefits of adjuvant bisphosphonates in premenopausal women, but in 11,767 postmenopausal women, highly significant reductions in bone recurrence (RR=0.72; 95%CI 0.60-0.86, 2p=0.0002) and breast cancer mortality (0.82; 95%CI 0.73-0.93, 2p=0.002) were seen. These results have led to supportive clinical guidelines[12], and recommendations by both European and American expert groups to incorporate adjuvant bisphosphonates into routine clinical care.[5,6] However, despite the clinically important effects on breast cancer mortality, global acceptance has been slow, in part due to the opinion that these benefits relate only to a subset of patients that is somewhat imprecise in its definition and also that the mechanistic explanation for the findings are unclear[6].

Our findings should be considered as hypothesis generating but, clearly suggest that the beneficial effects of ZOL on the underlying breast cancer are associated with the presence of a non-amplified *MAF* gene within the primary tumour. On the contrary, MAF+ve tumours in non post-menopausal women experience a worse outcome with ZOL. In these younger, not postmenopausal women, there seem to be two distinct populations, MAF-ve who, like older MAF-ve patients, benefit from zoledronic acid and MAF+ve for whom use of zoledronic acid in the presence of reproductive hormones appears to stimulate the emergence of extra-skeletal metastatic disease and worse survival, resulting in a net nil effect in the non-postmenopausal subgroup both in the biomarker cohort and the AZURE study population as a whole. Additional mechanistic studies addressing the importance of MAF in cancer metastasis are in progress. It is hoped that these investigations may provide some biological insights into the differential effects of bisphosphonates on disease outcomes according to MAF status and menopause and help us understand how tumour biology, treatments that influence the metastatic niche and the endocrine milieu, that influence both host and tumour cell functions, all interact. Additionally, evaluation of MAF in another large randomized trial of adjuvant bisphosphonates is required before our findings could be considered for routine clinical practice. This is planned to take place in 2018 using the NSABP B34[13] tumor bank and data set of patients randomized to either treatment with the oral bisphosphonate, clodronate or placebo.

In this study of 865 patients and a median follow-up of 84 months, there were still only 60 IDFS events in bone, making interpretation of any relationships between MAF and bone relapse unreliable. We were thus unable to confirm the prognostic capability of MAF proposed by Pavlovic et al.[8] In this evaluation of the AZURE study population, MAF amplification was associated with other adverse biological characteristics such as ER negativity, high grade and Her2 positivity, but was unable to meet its primary objective of providing clinically useful independent bone metastasis prognostic information in the control population as a whole. Although MAF+ve tumours were associated with worse disease outcomes in the subset of postmenopausal breast cancer patients in the control group, these findings are not sufficient to recommend its use in the assessment of risk prognosis in routine practice. Because bone relapses typically occur late in the follow-up of

patients with early breast cancer, further evaluation is planned now that all patients have completed 10 years of follow-up; this is anticipated to increase the number of bone events by around one third. Other data sets are thus likely to be necessary to assess whether the originally described relationship between MAF+ve tumours and the development of bone metastases holds true. However, we believe the clinical interest in MAF relates to the predictive capabilities described rather than potential use as another prognostic factor.

The heterogeneity in IDFS by menopause for women with MAF-ve tumours within the control arm cannot be adequately explained by imbalance in other prognostic factors. Other than a slight excess of larger T2-4 tumours (72% versus 62%) in the MAF-ve non-postmenopausal and postmenopausal control populations respectively, the clinical and pathologic characteristics of this younger MAF-ve population appear similar.

Collectively, our observations point to MAF as a potential molecular target for the prevention or treatment of metastases from breast cancer[8]. Although required for metastasis, MAF is a very challenging pharmacological target because of its nuclear localization and lack of a catalytic domain. Dissecting the role of genes that are transcriptionally regulated by MAF may lead to the identification of potentially targetable proteins that are necessary for metastasis[8]. Amongst the genes transcriptionally regulated by MAF are potentially targetable proteins that contribute to support bone metastasis[14,15].

In conclusion, our findings suggest that MAF status may become a clinically useful biomarker for treatment selection. FISH testing for MAF copy number may allow better and more precise selection of patients for adjuvant treatment with ZOL. MAF-ve patients are likely to represent almost 80% of breast cancers who could benefit from the use of adjuvant ZOL. On the other hand, because the presence of a MAF+ve tumour appears to be associated with adverse disease outcomes when patients are treated with bisphosphonates - especially if treatment is initiated before menopause is complete - such patients with MAF+ve tumours should avoid exposure to bisphosphonates in the adjuvant setting.

Author contributions:

RRG, JA, JJ-M, JCT and FR developed the MAFTEST biomarker. RC, RRG, AH, HM and WG developed the study concept, wrote the protocol, performed and reviewed all analyses. The first author wrote the first draft of the manuscript and all authors were involved in interpretation of the data, revision and approval of the manuscript.

Acknowledgements:

We wish to acknowledge the Academic Unit of Pathology at the University of Sheffield for constructing the tissue microarrays from tumour blocks submitted from sites in the United Kingdom to enable a range of translational studies including the analyses reported in this manuscript. We also thank the patients who took part in the AZURE trial and the investigators within the United Kingdom who provide tissue samples for these analyses.

Conflicts of Interest:

Dr. Coleman reports grants from Bayer, grants from Amgen, personal fees from Eisai, personal fees from Astra Zeneca outside the submitted work; RB reports grants from The Cancer Council Victoria (Australia) during the conduct of the study; JJM owns < 0.25% of Inbiomotion S.L; J-CT reports a patent pending related to the work; WG reports personal fees from Celgene, personal fees from Janssen outside the submitted work; RRG declares shares of Inbiomotion SL for a value of less than 10,000$ and patents pending related to the submitted work. AH, AHan, JA, DC, HM, DD, FR have no conflicts to declare.

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Legends to Figures

Figure1: Impact of MAF copy number on invasive disease free survival (IDFS). Hazard ratios (HR) and 95% confidence intervals (CI) based on Cox multivariable analysis shown separately for control and zoledronic acid treated patients and by menopausal subgroup

Figure 2: IDFS by randomized treatment allocation (zoledronic acid or control) in MAF FISH negative patients. Output from Cox multivariable model shown to adjust for differences in the prognostic factors between groups.

Figure 3: Impact of MAF copy number (based on Cox multivariable analysis) on effects of adjuvant zoledronic acid on invasive disease free survival (IDFS) split by menopausal status (postmenopausal or not postmenopausal) and age (≤50 or >50).

Figure 4: Cumulative risk for first IDFS recurrence not in bone in women who were not postmenopausal at trial entry by treatment allocation (zoledronic acid or control) for patients with MAF FISH+ (A) or MAF FISH – (B) tumours. Cumulative incidence function figures do not adjust for differences in the prognostic factors between groups. Death and local/contralateral invasive disease are considered an event for this end point. First IDFS events that were in bone are considered a competing risk.

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

Variable	FISH evaluable result (n=865)	Tumour Provided (n=1739)	AZURE population (n=3359)
Menopausal Status			
Non post-menopausal	590 (68.2%)	1192 (68.5%)	2318 (69.0%)
Post-menopausal	275 (31.8%)	547 (31.5%)	1041 (31.0%)
Age			
<40	87 (10.1%)	198 (11.4%)	384 (11.4%)
40-49	299 (34.6%)	571 (32.8%)	1108 (33.0%)
50-59	281 (32.5%)	580 (33.4%)	1126 (33.5%)
60-69	162 (18.7%)	332 (19.1%)	628 (18.7%)
70+	36 (4.2%)	58 (3.3%)	113 (3.4%)
Lymph node involvement			
0	2 (0.2%)	17 (1.0%)	62 (1.8%)
1-3	563 (65.1%)	1122 (64.5%)	2075 (61.8%)
≥4	300 (34.7%)	598 (34.4%)	1211 (36.1%)
Unknown	0 (0%)	2 (0.1%)	11 (0.3%)
Tumour stage			
T1	274 (31.7%)	577 (33.2%)	1065 (31.7%)
T2	475 (54.9%)	901 (51.8%)	1717 (51.1%)
T3	99 (11.4%)	214 (12.3%)	456 (13.6%)
T4	17 (2.0%)	47 (2.7%)	117 (3.5%)
TX	0 (0.0%)	0 (0.0%)	4 (0.1%)
ER status			
ER positive	689 (79.7%)	1388 (79.8%)	2634 (78.4%)
ER negative	171 (19.8%)	341 (19.6%)	705 (21.0%)
ER unknown	5 (0.6%)	10 (0.6%)	20 (0.6%)
Systemic therapy plan			
Endocrine therapy (ET)	46 (5.3%)	89 (5.1%)	152 (4.5%)
Chemotherapy (CT)	166 (19.2%)	339 (19.5%)	719 (21.4%)
ET and CT	653 (75.5%)	1311 (75.4%)	2488 (74.1%)
Anthracyclines			
Yes	794 (91.8%)	1604 (92.2%)	3132 (93.2%)
No	71 (8.2%)	135 (7.8%)	227 (6.8%)
Taxanes			
Yes	126 (14.6%)	267 (15.4%)	775 (23.1%)
No	739 (85.4%)	1472 (84.6%)	2584 (76.9%)
HER2 status			
Positive	93 (10.8%)	186 (10.7%)	415 (12.4%)
Negative	250 (28.9%)	503 (28.9%)	1251 (37.2%)
Unknown / Not measured	522 (60.3%)	1050 (60.4%)	1693 (50.4%)
Histological grade			
1	61 (7.1%)	147 (8.5%)	285 (8.5%)
2	333 (38.5%)	748 (43.0%)	1439 (42.8%)
3	467 (54.0%)	820 (47.2%)	1552 (46.2%)
Not specified	4 (0.5%)	24 (1.4%)	83 (2.5%)
PR status			
Positive	308 (35.6%)	633 (36.4%)	1423 (42.4%)
Negative	159 (18.4%)	350 (20.1%)	806 (24.0%)
Unknown	398 (46.0%)	756 (43.5%)	1130 (33.6%)

Table 2: Relationships between MAF status and clinical and tumour characteristics by randomised treatment allocation

	Standard Treatment (n=445)		Standard treatment + Zoledronic acid (n=420)		All patients (n=865)	
Variable	**Negative (n=360)**	**Positive (n=85)**	**Negative (n=321)**	**Positive (n=99)**	**Negative (n=681)**	**Positive (n=184)**
Menopausal status						
Non post-menopausal	253 (70.3%)	55 (64.7%)	216 (67.3%)	66 (66.7%)	469 (68.9%)	121 (65.8%)
Post-menopausal	107 (29.7%)	30 (35.3%)	105 (32.7%)	33 (33.3%)	212 (31.1%)	63 (34.2%)
Age (years)						
<40	43 (11.9%)	5 (5.9%)	24 (7.5%)	15 (15.2%)	2 (0.3%)	0 (0.0%)
40-49	124 (34.4%)	28 (32.9%)	118 (36.8%)	29 (29.3%)	445 (65.3%)	118 (64.1%)
50-59	112 (31.1%)	33 (38.8%)	109 (34.0%)	27 (27.3%)	234 (34.4%)	66 (35.9%)
60-69	63 (17.5%)	17 (20.0%)	61 (19.0%)	21 (21.2%)	61 (19.0%)	21 (21.2%)
70+	18 (5.0%)	2 (2.4%)	9 (2.8%)	7 (7.1%)	9 (2.8%)	7 (7.1%)
Tumour stage						
T1	111 (30.8%)	31 (36.5%)	100 (31.2%)	32 (32.3%)	211 (31.0%)	63 (34.2%)
T2	200 (55.6%)	40 (47.1%)	179 (55.8%)	56 (56.6%)	379 (55.7%)	96 (52.2%)
T3	43 (11.9%)	12 (14.1%)	37 (11.5%)	7 (7.1%)	80 (11.7%)	19 (10.3%)
T4	6 (1.7%)	2 (2.4%)	5 (1.6%)	4 (4.0%)	11 (1.6%)	6 (3.3%)
Lymph node status						
0	2 (0.6%)	0 (0%)	0 (0%)	0 (0%)	2 (0.3%)	0 (0%)
1-3	231 (64.2%)	58 (68.2%)	214 (66.%)	(60 (60.6%)	445 (65.3%)	118 (64.1%)
≥4	127 (35.3%)	27 (31.8%)	107 (33.3%)	39 (39.4%	234 (34.4%)	66 (35.9%)
ER status						
ER positive	308 (85.6%)	55 (64.7%)	264 (82.2%)	62 (62.6%)	572 (84.0%)	117 (63.6%)
ER negative	50 (13.9%)	29 (34.1%)	57 (17.8%)	35 (35.4%)	107 (15.7%)	64 (34.8%)
ER unknown	2 (0.6%)	1 (1.2%)	0 (0.0%)	2 (2.0%)	2 (0.3%)	3 (1.6%)
Systemic therapy plan						
Endocrine therapy (ET)	24 (6.7%)	2 (2.4%)	16 (5.0%)	4 (4.0%)	40 (5.9%)	6 (3.3%)
Chemotherapy (CT)	50 (13.9%)	29 (34.1%)	54 (16.8%)	33 (33.3%)	104 (15.3%)	62 (33.7%)
ET and CT	286 (79.4%)	54 (63.5%)	251 (78.2%)	62 (62.6%)	537 (78.9%)	116 (63.0%)
Anthracyclines						
Yes	325 (90.3%)	79 (92.9%)	297 (92.5%)	93 (93.9%)	622 (91.3%)	172 (93.5%)
No	35 (9.7%)	6 (7.1%)	24 (7.5%)	6 (6.1%)	59 (8.7%)	12 (6.5%)
Taxanes						
Yes	49 (13.6%)	17 (20.0%)	44 (13.7%)	16 (16.2%)	93 (13.7%)	33 (17.9%)
No	311 (86.4%)	68 (80.0%)	277 (86.3%)	83 (83.8%)	588 (86.3%)	151 (82.1%)
HER2 status						
Positive	39 (10.8%)	17 (20.0%)	20 (6.2%)	17 (17.2%)	59 (8.7%)	34 (18.5%)
Negative	116 (32.2%)	18 (21.2%)	85 (26.5%)	31 (31.3%)	201 (29.5%)	49 (26.6%)
Unknown / Not measured	205 (56.9%)	50 (58.8%)	216 (67.3%)	51 (51.5%)	421 (61.8%)	101 (54.9%)
Histological grade						
1	34 (9.4%)	3 (3.5%)	21 (6.5%)	3 (3.0%)	55 (8.1%)	6 (3.3%)
2	158 (43.9%)	21 (24.7%)	137 (42.7%)	17 (17.2%)	295 (43.3%)	38 (20.7%)
3	168 (46.7%)	60 (70.6%)	161 (50.2%)	78 (78.8%)	329 (48.3%)	138 (75.0%)
Not specified	0 (0.0%)	1 (1.2%)	2 (0.6%)	1 (1.0%)	2 (0.3%)	2 (1.1%)
PR status						
Positive	133 (36.9%)	25 (29.4%)	121 (37.7%)	29 (29.3%)	254 (37.3%)	54 (29.3%)
Negative	53 (14.7%)	28 (32.9%)	46 (14.3%)	32 (32.3%)	99 (14.5%)	60 (32.6%)
Unknown	174 (48.3%)	32 (37.6%)	154 (48.0%)	38 (38.4%)	328 (48.2%)	70 (38.0%)

Table 3: Comparison of IDFS outcomes between non postmenopausal MAF FISH negative control population and other groups classified by menopausal status, MAF FISH status and treatment

Patient Group n= (number of events)	**Hazard ratio (HR)***	**Lower 95% CI**	**Upper 95% CI**
MAF- ve Control post-meno • n=107 (35)	1.25	0.80	1.95
MAF-ve Zol post-meno • n=105 (29)	0.85	0.53	1.36
MAF-ve Zol non post-meno • n=216 (61)	0.83	0.59	1.15
MAF+ve Zol post-meno • n=33 (12)	1.72	0.90	3.29
MAF+ve Zol non post-meno • n=66 (33)	1.61	1.06	2.44
MAF+ve Control post-meno • n=30 (18)	2.68	1.53	4.68
MAF+ve Control non post-meno • n=55 (11)	0.63	0.33	1.20

All compared with MAF –ve Control non post-menopausal group (n=253), number of events=83

Table 4: Impact of MAF FISH status on IDFS by age (≤50 or >50) in zoledronic acid and control patients.

Patient Group n= (number of events)	**Hazard ratio (HR)***	**Lower 95% CI**	**Upper 95% CI**
Zoledronic acid patients < age 50 • n= 142 (41) vs 44 (23)	0.473	0.281	0.797
Zoledronic acid patients ≥ age 50 • n= 179 (49) vs 55 (22)	0.533	0.719	0.890
Control patients ≤ age 50 • n= 167 (55) vs 33 (8)	1.410	0.666	2.985
Control patients > age 50 • n= 193 (63) vs 52 (21)	0.673	0.407	1.114

*HR for patients with MAF FISH negative / MAF FISH positive tumours

Research in context clean

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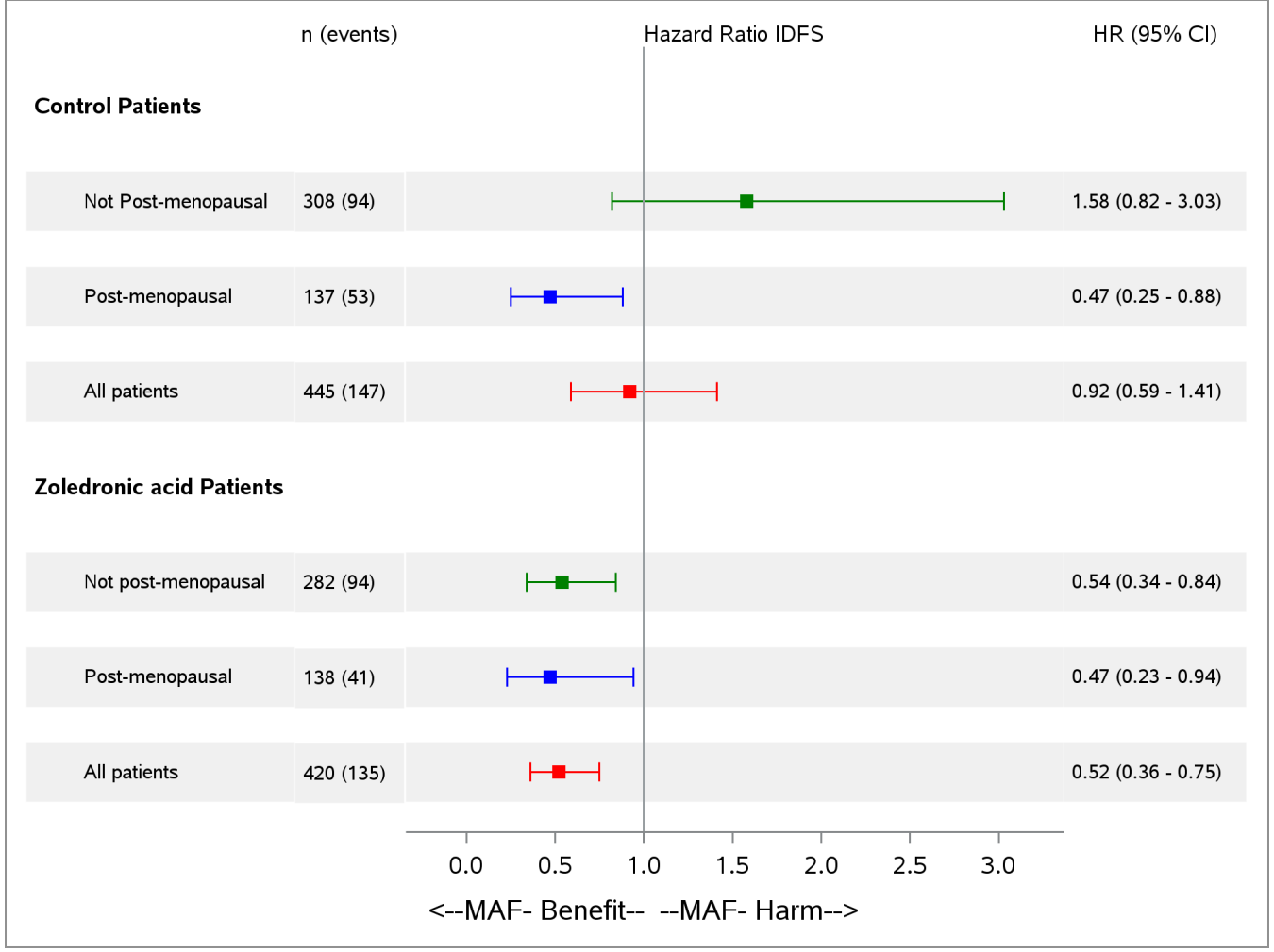
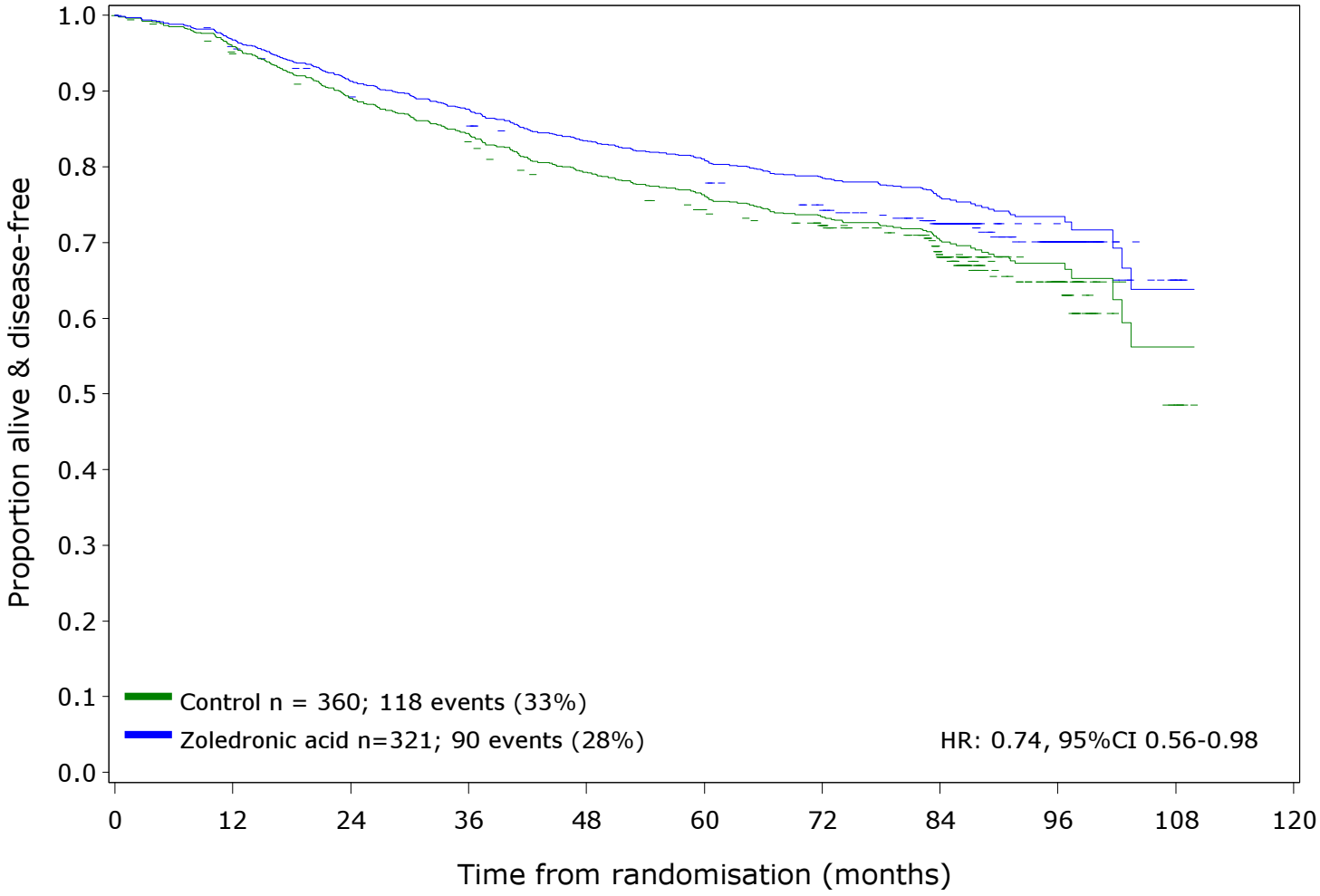

n (events)
Hazard Ratio IDFS
HR (95% CI)
Control Patients
Not Post-menopausal
308 (94)
1.58 (0.82 - 3.03)
Post-menopausal
137 (53)
0.47 (0.25 - 0.88)
All patients
445 (147)
0.92 (0.59 - 1.41)
Zoledronic acid Patients
Not post-menopausal
282 (94)
0.54 (0.34 - 0.84)
Post-menopausal
138 (41)
0.47 (0.23 - 0.94)
All patients
420 (135)
0.52 (0.36 - 0.75)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
<--MAF- Benefit-- --MAF- Harm-->

Figure 1

Figure 2



Number at Risk (number censored)											
Control	360 (0)	336 (24)	312 (48)	293 (67)	270 (90)	249 (111)	231 (129)	177 (183)	58 (302)	5 (355)	0
Zol	321 (0)	304 (17)	280 (41)	267 (54)	250 (71)	243 (78)	223 (98)	177 (144)	72 (249)	4 (317)	0

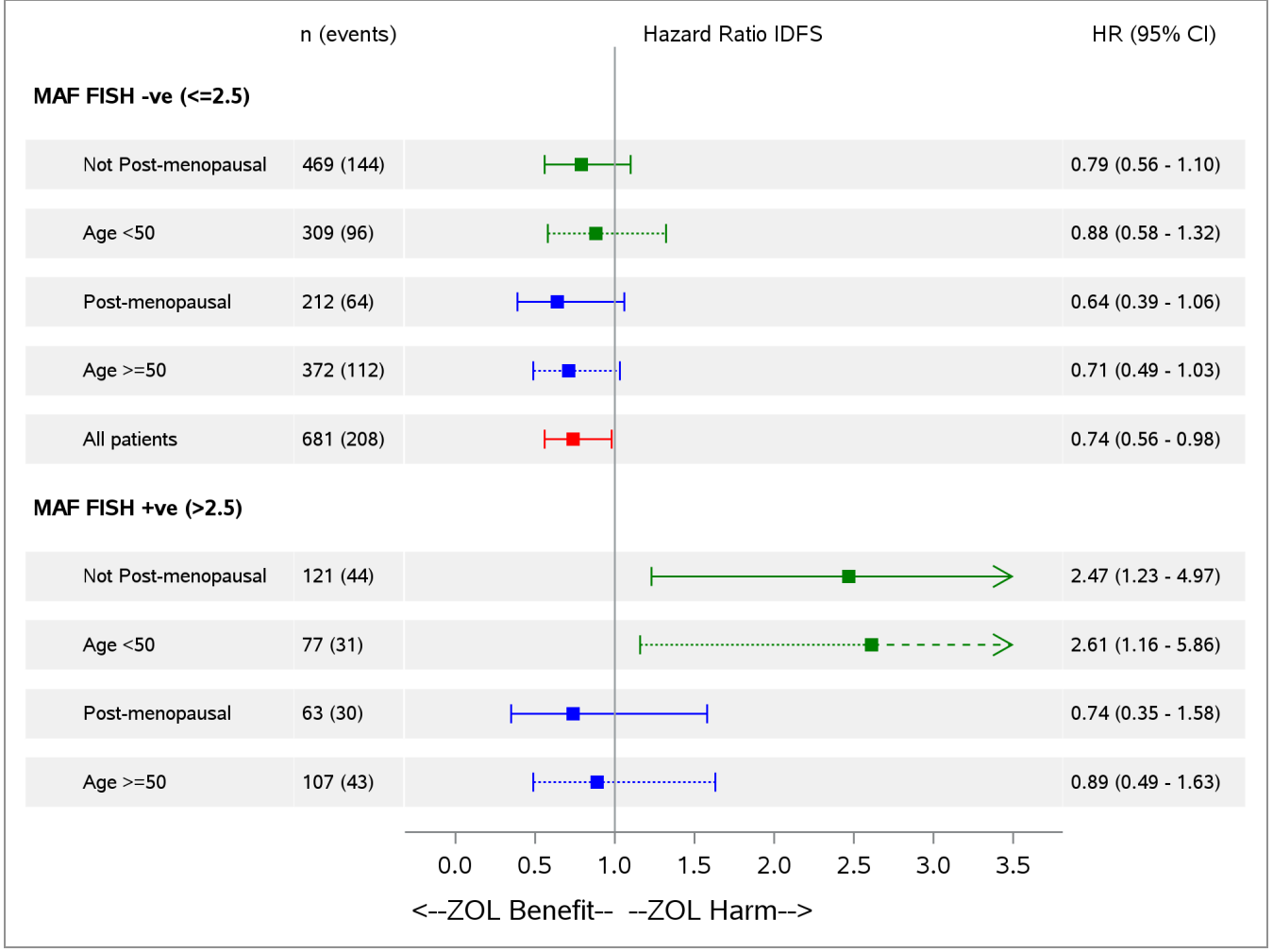
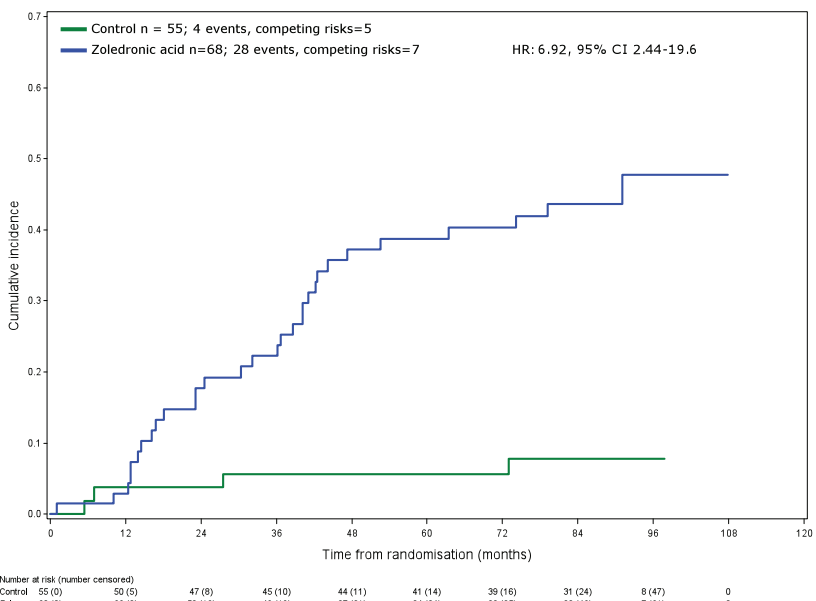
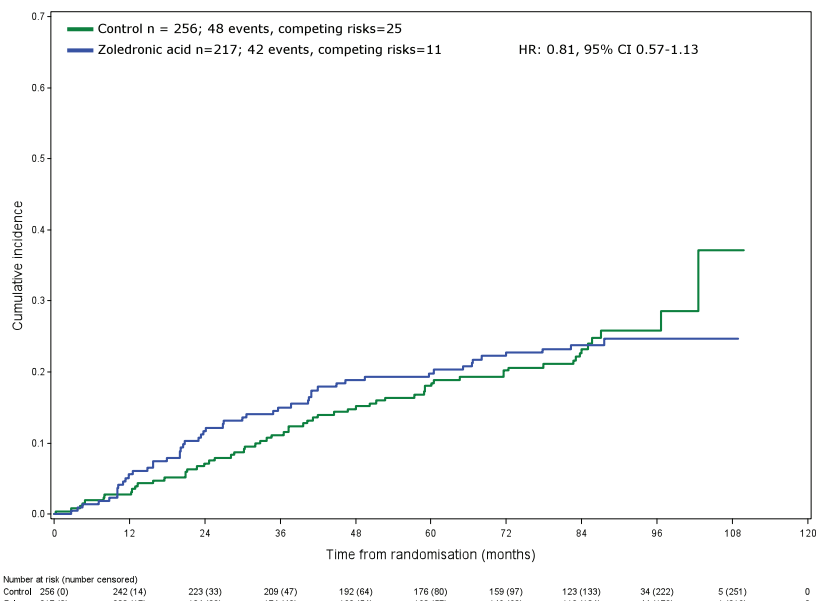

n (events)
Hazard Ratio IDFS
HR (95% CI)
MAF FISH -ve (<=2.5)
Not Post-menopausal
469 (144)
0.79 (0.56 - 1.10)
Age <50
309 (96)
0.88 (0.58 - 1.32)
Post-menopausal
212 (64)
0.64 (0.39 - 1.06)
Age >=50
372 (112)
0.71 (0.49 - 1.03)
All patients
681 (208)
0.74 (0.56 - 0.98)
MAF FISH +ve (>2.5)
Not Post-menopausal
121 (44)
2.47 (1.23 - 4.97)
Age <50
77 (31)
2.61 (1.16 - 5.86)
Post-menopausal
63 (30)
0.74 (0.35 - 1.58)
Age >=50
107 (43)
0.89 (0.49 - 1.63)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
<--ZOL Benefit-- --ZOL Harm-->

Figure 3

Figure 4





MAF FISH+ result and treatment covariate interaction Chi2=21.15, P=<0.0001

Necessary Additional Data

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*Manuscript with revisions highlighted

Influence of MAF gene amplification on treatment effects in the AZURE (BIG 01/04) prospective clinical trial of adjuvant zoledronic acid in early breast cancer

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Abstract

Background: In early breast cancer (EBC), the adjuvant use of bisphosphonates reduces the incidence of bone metastases but only appears to improve overall recurrence and survival in postmenopausal patients. The mechanisms underlying these observations remain unclear. To address this, we tested the recently identified bone relapse biomarker, *MAF* gene gain (MAF+) in primary tumours from women included in the AZURE trial (ISRCTN79831382) to determine the prognostic value of MAF and its potential to predict the effects of adjuvant zoledronic acid (ZOL) on disease outcomes.

Methods: The AZURE trial is an academic, prospective, open label, randomised phase III multicentre, parallel-group trial, performed in 3360 women with stage II/III breast cancer to receive standard adjuvant systemic therapy alone or with ZOL every 3-4 weeks for 6 doses, then 3-6 monthly thereafter to complete 5 years treatment. Consenting patients were randomised on a 1:1 basis using a central automated 24-hour computer-generated telephone randomisation system that included 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. The primary endpoint was disease recurrence. MAF was detected in breast tumours in tissue microarray format using a validated (*MAF/D16Z3*) FISH test, MAFTEST (Inbiomotion, Spain) in a blinded fashion by a central laboratory (Targos, Germany). A copy number cut-off ≥2.5 was preset to define a MAF+ve tumour. Multivariate analyses of disease outcomes by MAF status were performed in control and ZOL patients separately on an intention to treat basis according to a pre-specified analysis plan. Interactions between MAF+ve and menopausal status on effects of ZOL were also evaluated.

Findings: Of the 1769 AZURE patients who donated primary tumour samples, 865 (49%) had 2 FISH evaluable cores of which 184 (21%) were MAF+ve. MAF+ve tumours were more likely to be of higher grade, ER-ve and Her2+. At a median follow-up of 84 months, MAF was not prognostic in control patients for invasive disease free (IDFS) or overall (OS) survival, but in ZOL treated patients those with a MAF-ve tumor had better IDFS (HR_{IDFS}=0.52 [0.36-0.75] and OS (HR_{OS}=0.48 [0.31-0.75]). In patients with MAF-ve tumours, ZOL was associated with improved IDFS (HR_{IDFS}=0.74 [0.56-0.98]). However in patients with MAF+ve tumours, ZOL did not improve disease outcomes and, in the 119 MAF+ve patients who were not postmenopausal at randomisation, ZOL was associated with much worse outcomes (HR_{IDFS}=2.47 [1.23-4.97] and HR_{OS}=2.27 [1.04-4.93]). The interactions between disease outcomes, ZOL use and menopausal status were driven largely by an excess of extraskeletal recurrences (E-IDFS) in women with MAF+ve tumours who initiated ZOL before the development of menopause ($HR_{E\text{-}IDFS}$=6.92, [2.44-19.6]).

Interpretation: FISH evaluation of MAF in EBC segregates patients likely to benefit from adjuvant ZOL (MAF-ve) from those who may be harmed (not postmenopausal MAF+ve) and merits further investigation as a potential companion diagnostic.

Academic grant support, and study drug were provided by Novartis Global (Summit, New Jersey). Biomarker measurements and statistical analyses were funded by Inbiomotion (Barcelona, Spain).

Introduction

Meta-analysis of clinical trials has shown that adjuvant bisphosphonates (especially zoledronic acid [ZOL] or clodronate) reduce bone metastases and improve survival in postmenopausal patients with early breast cancer[1]. The AZURE study of adjuvant zoledronic acid in early breast cancer was the first study to identify the benefits of adjuvant bisphosphonates in postmenopausal women[2] and prompted the confirmatory meta-analysis of this subgroup finding,[1] In addition, marked heterogeneity according to menopausal status in the rates of recurrence outside bone was seen, with an increase in extraskeletal metastases in women who were not postmenopausal at study entry. However, despite recapitulation of these clinical observations in preclinical animal models of bone metastases[3], the biological mechanisms underpinning these apparent interactions between treatment effects and menopausal status are unknown.

There are many clinical, pathologic and molecular biomarkers that have been used to assess prognosis in early breast cancer although none, with the possible exception of estrogen receptor status, are useful to specifically identify patients at high risk for bone metastases. Gene profiles that may specifically predict for bone recurrence[4] were first described a decade ago but none are currently used in clinical practice. In terms of predicting treatment benefits, menopausal status (and by association age) are recommended in clinical practice guidelines for selection of patients likely to benefit from adjuvant bisphosphonate[5,6]. However, the imprecise definition and timing of menopause, makes this a somewhat difficult criterion on which to select patients most likely to benefit. Recently, using a proteomic discovery platform, the co-expression of GIPC1 and CAPG proteins by primary tumours was shown to predict benefit from adjuvant ZOL in early breast cancer.[7] However, to date, there are no confirmatory datasets supporting the clinical application of this discovery and the reproducibility of these analyses awaits confirmation. Thus, there is currently no biomarker to select patients for treatment with adjuvant ZOL.

To address this, the recently identified early breast cancer bone relapse biomarker, *MAF* gene gain (MAF+)[8] was tested retrospectively in the large prospectively randomized AZURE trial[2,9], of standard adjuvant therapy +/- ZOL in high risk early breast cancer. Overexpression of MAF, a transcription factor of the AP-1 family and encoded within the 16q arm, supports the molecular processes that affect the metastatic course from the primary site to distal colonization[8]. MAF regulates the expression of a set of genes that collectively support several steps of breast cancer cell metastasis through a series of cell-autonomous and niche-related functions[8]. Collectively, these observations suggest that MAF accumulation (*MAF* gain) may enable the identification of patients at high risk of metastasis. In this report, we have determined the prognostic value of MAF and its potential to predict the effects of ZOL on disease outcomes.

Methods

Study Design and patients

3360 patients from 174 centres were recruited between September 2003 and March 2006 to the AZURE study. Eligibility criteria have been reported previously[2,9] but, in summary, patients had to have histologically confirmed invasive breast cancer of any subtype with either pathologically involved axillary lymph node metastasis or a T3/T4 primary tumour treated with curative intent. Prior complete resection of the primary tumour was necessary or had to be planned if patients were treated with neoadjuvant chemotherapy. Patients had to be age ≥18 with a Karnofsky performance status index ≥80 and neither pregnant or breast-feeding to be eligible. Patients were not eligible if there was clinical or imaging evidence of distant metastases prior to study entry, current or recent (previous year) use of bisphosphonates or pre-existing bone disease likely to require bone-targeted treatment.

All patients gave written informed consent and, in the United Kingdom only, additional specific consent to use of biological materials (primary tumour and blood samples was requested. Patients could participate in the main trial alone if they so chose. Prior to randomisation, haematological, renal and hepatic function tests were required. Staging imaging tests were performed in accordance with institutional protocols. The full protocol may be viewed at http://ctru.leeds.ac.uk/Azure

The outcome data for the AZURE study have been previously reported[2,9] and the planned final analysis data-lock[2] was used for the analyses described below.

Procedures

Eligible patients were randomized to receive (neo) adjuvant chemotherapy and/or endocrine therapy +/- ZOL 4mg iv every 3-4 weeks for 6 doses, then 3 monthly x 8 and 6 monthly x 5 to complete 5 years treatment. Calcium and vitamin D oral supplements were recommended for all trial subjects during the first six months on study, and continued thereafter at the discretion of the treating physician. Both the use of adjuvant systemic

treatments and loco-regional radiotherapy were given in accordance with standard protocols at each participating institution.

The follow-up schedule was similar in both arms of the study and included clinical assessment, adverse event monitoring and haematological, renal and hepatic function test measurements. Routine follow-up imaging was not mandated, with investigations for possible recurrence clinically directed as deemed appropriate by the treating physician. The date of recurrence was defined as the date on which relapse was first suspected, to reduce the risk of ascertainment bias. 91% of recurrences were independently validated by either on-site or telephone-based monitoring. Subjects were followed up on an annual basis after completion of the 5-year treatment phase (ZOL or control) for both disease and relevant safety endpoints.

Of the 3360 patients accrued in the trial, primary tumour tissue blocks were collected from 1769/2710 (65%) patients treated at participating sites in the UK. Site participation in the collection of tumour blocks was encouraged but not mandatory and, for logistical reasons, restricted to UK sites.

All tumour blocks were sent to Sheffield for tissue microarray (TMA) construction where the location of invasive tumour within the tissue blocks was indicated by a single breast pathologist as a guide to the technician extracting the tissue cores for construction of the TMAs. Quadruplicate cores of 1 mm in diameter of each of the tumour tissue sample were arranged in 4 sets of 13 TMAs (150 samples each). All analyses are restricted to study participants who gave specific patient consent for the use of tissue samples.

The MAF biomarker analysis was completed on TMAs from primary tumours. Sample handling, methodology, scoring and statistical analyses were pre-specified in a study charter and statistical analysis plan. 5 µm thick tissue sections were cut from each TMA block, orientated to ensure correlation with the TMA map to allow identification of each tissue core and mounted onto Superfrost plus glass slides (Thermo Fisher Scientific). TMA slices were first analyzed using haematoxylin and eosin (H&E) staining to determine the presence of evaluable tumour. MAF amplification was then assessed using a validated (*MAF/D16Z3*) FISH test, MAFTEST, (Inbiomotion, Barcelona, Spain). A central laboratory (Targos Molecular Pathology, Kassel, Germany) validated the assay for analytic and diagnostic performance, established acceptance criteria, included appropriate quality controls for each assay, and performed the analyses in a blinded fashion. The previously described immunohistochemical (IHC) test for MAF[8] was found to perform sub-optimally on the TMA samples available, presumably due to epitope decay in the >10 years since fixation and is not considered further in this report.

Briefly, TMA sections were deparaffinated in Xylene (2x for 10 min), rehydrated in ethanol series, washed with water and pretreated at 98ºC for 15 min. Samples were digested with pepsin (Poseidon Tissue Digestion Kit, Kreatech, Amsterdam, Netherlands) for 30 min, dehydrated in ethanol series and dried. After adding 10 µl of MAF/D16Z3 probe (Inbiomotion), slides were denatured at 80ºC and placed overnight in a hybridizer at 37ºC. After hybridization, FISH slides were washed in Wash Buffer I at 72ºC for 2 min and then Wash Buffer II (both Poseidon Tissue Digestion Kit, Kreatech) for 1 min at room temperature. After dehydration and air-drying, slides were incubated with 15 µl of DAPI solution (0.03 mg/ml) and stored at 4ºC in the dark until scoring.

Mean MAF copy number per nuclei was scored by blinded central laboratory personnel in 20 nuclei from regions of the tumour with the highest amplification. If MAF mean copy number was between 2.0 and 3.0, an additional 30 nuclei were scored. Replica cores were scored until 2 FISH amplification values were obtained for each tumour. The highest value of MAF amplification in 2 evaluable samples of a patient was chosen for statistical analysis. The tumours on TMAs were FISH analysed only once, with no optimization and no repetitions allowed by protocol. A patient was considered as positive for MAF amplification when at least one of the replicas had a mean number of MAF copies per nuclei equal or higher than 2.5. The copy number cut-off $\geq$2.5 was predefined for MAF positivity (MAF+ve) based on studies in a retrospective cohort[8] and set at a level that was unlikely to be artificially influenced by rapid proliferation.

Statistical analysis

Following eligibility confirmation, patients were randomised by the treating clinical team on a 1:1 basis, using a central automated 24-hour computer-generated telephone minimisation system to ensure the concealment of the next treatment assignment based at the Clinical Trials Research Unit (CTRU), University of Leeds, to either standard adjuvant therapy alone (control) or with zoledronic acid monohydrate (ZOL), Novartis Pharmaceuticals, Summit, New Jersey, USA. To reduce possible imbalances in tumour and treatment characteristics, a minimisation process was used 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.

The primary endpoint of the AZURE trial was disease free survival (DFS), defined as distant recurrence, any invasive loco-regional recurrence except for ipsilateral operable relapse within a conserved breast, and death without recurrence. As described more fully in previous reports,[2,9] invasive DFS (IDFS) was added as a key secondary endpoint to reflect the international standard definition for recurrence that had emerged since the trial began[10] and is the primary disease endpoint assessed in this report. Secondary endpoints included overall survival (OS) defined as death from any cause after randomisation, time to bone metastases and subgroup analyses based on variables included in the randomisation including menopausal status. This study is registered as an International Standard Randomised Controlled Trial, number ISRCTN79831382

For this report, our hypothesis was that MAF status would have potential value as a prognostic factor for disease recurrence, especially in bone and as a predictive factor for response to adjuvant ZOL. The relationships in the control group were specified as the primary endpoint with those in the ZOL group exploratory. IDFS, OS, time to first IDFS event in bone (as first event or at any time) and time to first IDFS event not in bone endpoints, all defined as in the main study,[2,9] were assessed on all patients in the AZURE safety population with an evaluable MAFTEST. Subsequently, because disease outcomes by menopausal status had been pre-specified analyses in the AZURE protocol and reported in the main study reports,[2,9] interactions between MAF+ve and effects of ZOL on disease outcomes by menopausal status were also pre-specified in the statistical analysis plan (SAP).

The SAP was defined before any data analysis was performed. All statistical analyses were performed at the Clinical Trials Research Unit, University of Leeds where the AZURE clinical trial database is held on behalf of the trial sponsor, the University of Sheffield. All statistical analyses were conducted using SAS statistical software version 9.4. The SAP may be found in appendix p2-4.

The prognostic value of MAF status for IDFS and OS were investigated using Kaplan-Meier survival curves, whilst the time to first IDFS event in bone endpoint was assessed using a cumulative incidence function curve utilising a Fine and Gray approach. Differences in outcomes between patients with MAF+ve or MAF-ve tumours were compared using multivariable modelling in control patients only (Cox's proportional hazards) to adjust for the AZURE minimisation factors described above (excluding treating centre). Hypothesis testing was two-sided at a 5% significance level. No adjustments have been made for multiplicity.

For the predictive analyses, the response to zoledronic acid treatment was tested comparing control and ZOL groups. A predictive analysis, assessing the interaction of MAF status with treatment allocation, was performed using a Cox proportional hazards model. Only minimization factors identified as statistically significant in the prognostic analysis were included in the model to reduce potential overfitting. In addition, predictive analyses were carried out for patients who were unequivocally post-menopausal (>5 years since last menses) at trial entry separately to patients who were not post-menopausal (pre-menopausal, ≤5 years since menopause and menopausal status unknown), given the significant heterogeneity of treatment effect between established post-menopausal patients and all other patients observed in the AZURE trial as a whole[9].

Additional exploratory analyses were conducted for IDFS in all patients using a Cox proportional hazards model including the prognostic factors specified for the predictive analysis. The model included a three-way interaction term between MAF, menopausal status and treatment. Sensitivity analyses were conducted including all prognostic factors as specified in the prognostic analysis 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 fit with age as a surrogate for the menopausal status term; with an arbitrary cut-point of ≥ or <50 at randomization.

Role of the Funding Source

The AZURE trial was sponsored by the University of Sheffield and academic grant support provided by Novartis, supplemented by the infrastructure of the National Cancer Research Network. Novartis funded the sample collection but had no input in the study design, analysis or interpretation of data, nor in writing of the report. Inbiomotion had no involvement in the AZURE study design, or data analysis but JJM and JT did provide input into the statistical analysis plan, commented on the interpretation of the data and reviewed and approved the manuscript as co-authors. Biomarker measurements and statistical analyses were funded by Inbiomotion. ~~Inbiomotion reviewed the manuscript but all decisions on publication rested with the authors.~~ The corresponding author had full access to all of the data and the final responsibility for publication.

Results

The primary tumours from 1739 patients had been processed between August 2003 and February 2006 according to routine local laboratory standard operating procedures and the TMAs prepared in 2007 and 2008 from representative tumour blocks sent to the University of Sheffield. Sections from the TMAs were sent to the

independent laboratory (TARGOS) for all analyses. Despite marking of the tumour blocks by an experienced pathologist before TMA construction, only 3978 out of 6326 TMA cores (63%) had sufficient invasive tumour confirmed on an H&E stained slide within each individual core for FISH analysis . The MAFTEST FISH assay was performed on adjacent sections and could be reliably assessed according to the stringent quality standards of the independent laboratory (TARGOS) in 2067 of 3978 (56%) tissues cores with sufficient invasive tumour. To reduce the impact of tumour heterogeneity in such small fragments of the original tumour, duplicate evaluable results from each patient were mandated. 865 of 1739 patients (50%) providing primary tumour for assessment had 2 evaluable FISH results and of these, 184 of 865 patients (21%) had a MAF+ve tumour.

The median follow-up was 84.6 (IQR 72.0-95.8) months. 282 patients in this biomarker subset had experienced an IDFS event (147 control, 135 ZOL), 60 a first IDFS event in bone (39 control, 21 ZOL) and 193 an OS event (102 control, 91 ZOL). The proportions of MAFTEST analyzed patients with an IDFS or OS event were similar to the main study; 5 year IDFS probabilities for the ZOL and control arms respectively in this biomarker population were 74.1% (95%CI 69.8-78.3) and 73.7% (95%CI 69.6-77.8) compared with 75.1% (95%CI 73.0-77.2) and 73.9% (95%CI 71.7-76.0) in the overall study population.

Patient characteristics in those patients with TMA cores evaluable for MAF gain were similar to the entire cohort of patients included in AZURE (Table 1). More patients with MAF+ve tumours had high grade, ER negative and HER2 positive tumours than did those with MAF-ve tumours (Table 2). As a result of these factors, patients with MAF+ve tumours were more likely to have received taxanes and trastuzumab and less likely to require endocrine treatments than for the whole group. The frequency of MAF+ve tumours was similar across menopausal subsets and age. Given the heterogeneity of the prognostic factors in the baseline demographics, results are reported using the multivariable Cox modelling.

Pre-specified analyses

Relationships between MAF status and prognosis

In the control patient group, 118 of 360 MAF-ve (33%) and 29 of 85 MAF+ (34%) experienced an IDFS event and MAF was not prognostic for IDFS (HR:0.92, 95%CI 0.59-1.41) However, this result is not fully representative of the data as the impact of MAF status on disease outcome was found to be significantly influenced by menopausal status at randomization, test for interaction (TFI) $_{IDFS}$ by menopause, Chi^2=7.34, P=0.009 (Figure 1). In postmenopausal women, the hazard ratio for IDFS (HR_{IDFS}) for MAF-ve/MAF+ve was 0.47 [95%CI 0.25-0.88], suggesting MAF is indeed prognostic for IDFS in this group of patients. However, in contrast, for non-postmenopausal women, the HR_{IDFS} for MAF-ve/MAF+ve was 1.58 [95%CI 0.82-3.03]. Lymph node involvement, tumour stage, ER status and histological grade were found to be significant in the prognostic analysis and are included in the predictive analysis modelling.

In ZOL patients, MAF provided prognostic information, with worse IDFS in MAF+ve tumors (Figure 1). (HR_{IDFS} for MAF-ve/MAF+ve = 0.52 [95%CI 0.36-0.75] and HR_{OS} for MAF-ve/MAF+ve =0.48 [95%CI 0.31-0.75] (figure Appendix page 1).

There were insufficient bone events (13 bone only and 6 bone with other sites in MAF+ve, 47 bone only and 26 bone with other sites in MAF-ve) in this sample set to reliably assess the relationships between MAF status and relapse in bone.

Relationships between MAF status and treatment effects

There was an IDFS MAF/treatment interaction for patients overall χ^2=4.55, p=0.03; this result however is convoluted by the interaction between menopausal status and MAF found in the prognostic analysis above. In subgroup analyses for IDFS there was a MAF/treatment interaction for non-postmenopausal patients (χ^2=9.23, p=0.002), but not for postmenopausal patients (χ^2=0.09, p=0.76).

In patients with MAF-ve tumours, treatment with ZOL was associated with improved IDFS compared to control patients (HR_{IDFS} ZOL/CONTROL=0.74 95%CI 0.56-0.98) (Figure 2). The group sizes, number of events and hazard ratios for the pre-planned menopausal sub-group and exploratory age analyses are shown in Figure 3. Treatment benefits with ZOL compared to control were similar irrespective of menopausal status or age.

In patients with MAF+ve tumours the findings are more complex (Figure 3). Overall, zoledronic acid did not appear to improve IDFS (HR_{IDFS} ZOL/CONTROL=1.34 [95%CI 0.83-2.14]). There was, however, significant heterogeneity of the treatment effect by menopause (hypothesis test reported in additional analyses). In women who were not postmenopausal at the start of treatment, there were clear adverse effects on IDFS (HR_{IDFS} ZOL/CONTROL=2.47 [95% CI 1.23-4.97]). In postmenopausal women, there were insufficient events, from the subset of MAF+ve tumours (HR_{IDFS} ZOL/CONTROL for MAF+ve postmenopausal women=0.74 [95%CI

0.35-1.58]) to establish a definitive relationship between MAF status and treatment effect, although the point estimate for the hazard ratio (albeit with wider confidence intervals) was similar to that seen in MAF –ve women.

For OS, a similar relationship between treatment, menopause and MAF was seen. There were fewer deaths in patients with MAF-ve tumours treated with ZOL (ZOL 57 events in 321 [18%] patients; CONTROL 76 events in 360 patients [21%]; (HR_{OS} ZOL/CONTROL=0.78 [95%CI 0.55-1.10]). In patients with MAF+ve tumours, no effect of ZOL on OS was seen (ZOL 34 events in 99 [34%] patients; CONTROL 26 events in 85 patients [31%]; HR_{OS} ZOL/CONTROL=1.11 [95%CI 0.66-1.86]). However there is a clear adverse effect of ZOL in non-postmenopausal women with MAF+ve tumours (ZOL 24 events in 66 [36%] patients; CONTROL 9 events in 55 patients [16%]; HR_{OS} ZOL/CONTROL=4.64 [95%CI 1.33-16.25]) compared with a numerical beneficial treatment effect on OS ((ZOL 10 events in 33 [30%] patients; CONTROL 17 events in 30 patients [57%]; HR_{OS} ZOL/CONTROL=0.62 [95%CI 0.27-1.48]) in postmenopausal women with MAF+ve tumours.

In 190 patients with an IDFS event, this was at an extraskeletal site (92 control, 98 ZOL). When compared with the control group, treatment with ZOL in non-postmenopausal women with MAF+ve tumours was associated with a marked increase in relapse (Figure 4) at extraskeletal sites. The extraskeletal IDFS estimates at 60 months for MAF +ve tumors were 5.7% 95%CI 1.5-14.2%) for control patients and 38.8% (95%CI 27.1-50.3%) for ZOL patients, (HR_{IDFS} ZOL/CONTROL =6.92, 95%CI 2.44-19.6). ZOL had no effect on extraskeletal recurrences in non-postmenopausal patients with MAF-ve tumors; the extraskeletal IDFS estimates at 60 months for MAF -ve tumors were 18% 95%CI 13.5-23.1%) for control patients and 19.8% (95%CI 14.8-25.5%) for ZOL patients (HR_{IDFS} ZOL/CONTROL=0.81, 95%CI 0.57-1.13).

Additional exploratory analyses

The menopausal subgroup analyses indicated that menopausal status at randomization was playing a role in how MAF influences IDFS disease outcomes. The cox model specified for the predictive analyses did not include any parameters for menopausal status; an exploratory Cox model was fitted to better understand any potential interactions. The likelihood ratio test for the three-way interaction term between MAF, menopausal status and treatment yields a χ^2 of 5.71 (P=0.017, 1DF), The model allows a hypothesis test for heterogeneity of the treatment effect by menopause for MAF subgroups; Wald heterogeneity Chi square statistics: 6.98 (P=0.008, 1DF) and 0.38 (P=0.539, 1DF) for MAF+ve and MAF-ve patients respectively.

Hazard Ratios (with 95%CIs) are presented using non-postmenopausal control patients with MAF-ve tumours as the reference group (Table 3). The individual hazard ratios are consistent with the prescribed analysis that IDFS outcome is independent of menopausal status when patients are treated with ZOL. There is however heterogeneity in IDFS outcomes by menopausal status in addition to MAF status in control patients. MAF+ve postmenopausal control patients have significantly worse IDFS outcome in contrast to MAF+ve non-postmenopausal patients (HR non-post/post: 0.26, 95%CI 0.12-0.56). Interestingly non-postmenopausal patients with MAF+ tumours appear to have a better IDFS than do those that are MAF-ve (HR MAF+ve/MAF-ve: 0.63, 95%CI 0.33-1.20).

The exploratory model was fit using age as a surrogate for menopausal status, with an arbitrary cut-point of ≥ or <50 at randomization (Table 4). There is no difference in the interpretation of the results in IDFS when using age as a surrogate marker for menopause with a similar beneficial effect for ZOL in patients with MAF-ve tumours (Figure 3). Sensitivity analyses for the exploratory Cox model including all AZURE minimization factors were conducted and made no interpretable difference to the estimates (data not shown).

Discussion

Using an independent specialist biomarker laboratory and a pre-defined cut-off, blinded to patient demographic, treatment and outcome data, we have shown that tumour copy number of the MAF transcription factor encoded by *MAF* gene with a precisely developed *MAF/D16Z3* FISH test performed on archival primary breast tumours in tissue microarray format is able to predict treatment benefit and harm from adjuvant zoledronic acid. To our knowledge, this is the first time a biomarker has been described that can potentially identify patients who may benefit from treatment with an adjuvant bisphosphonate.

The tissues used in this study were collected from patients taking part in the prospectively randomized AZURE trial designed to evaluate the effects of adjuvant ZOL on disease outcomes in stage II/III breast cancer; the primary results from this trial have been reported previously.[2,9] The tissues had been fixed in paraffin for >10 years and the FISH test performed on 1mm cores within TMA format – a technically much more challenging exercise than would be the case if the MAFTEST could have been performed on contemporary tissue sections. Of note one third of cores had insufficient tumor in the cut section of the TMA block. All of these factors explain the attrition in patients with a MAFTEST result for the pre-planned statistical analyses. Despite these

technical challenges in obtaining a reliable confirmed FISH test on relatively old paraffin embedded fixed tissues in TMA format, our findings show that adjuvant ZOL improved disease outcomes in the 79% of patients with a <2.5 MAF copy number (MAF-ve) and importantly, unlike in the study as a whole, this beneficial treatment effect was independent of menopausal status at study entry suggesting that the use of adjuvant bisphosphonates could be extended to the 80% of premenopausal women with a MAF-ve tumour, equivalent to around 16% of the early breast cancer population.

Conversely, the use of adjuvant ZOL in women with a gain in *MAF* gene (MAF+ve tumours) was not associated with treatment benefit and in women who were not postmenopausal at the start of treatment, the use of ZOL in the context of a MAF+ve tumour was associated with more frequent extra-skeletal spread, resulting in significantly worse IDFS and OS. Findings were similar when age ≤ or > 50 years was used as a surrogate for menopause. Our data strongly suggest that such women should not receive an adjuvant bisphosphonate. There are limitations to our study. Firstly, this is a retrospective analysis of data from a prospective randomised clinical trial and requires confirmation in another data-set. Secondly, because of the complex interactions between MAF, bisphosphonate treatment and menopause, the number of MAF evaluable patients is relatively small to assess outcome reliably in some of the subgroups of interest. Thirdly, although we mandated assessment of MAF on two tissue cores per patient to reduce the impact of tumour heterogeneity, evaluation 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. Following the findings of treatment benefits with adjuvant ZOL in young women receiving ovarian suppression therapy for ER+ breast cancer[11] and the positive findings in a pre-planned subset analysis by menopause within the AZURE trial[9], a hypothesis was generated that adjuvant bisphosphonate efficacy is related to levels of reproductive hormones at the time of treatment initiation. This hypothesis was rigorously tested by a large individual patient meta-analysis conducted by the Early Breast Cancer Trialists Collaborative Group (EBCTCG)[1]. Data were collected from 18,766 women randomized in adjuvant bisphosphonate trials. There were no demonstrable benefits of adjuvant bisphosphonates in premenopausal women, but in 11,767 postmenopausal women, highly significant reductions in bone recurrence (RR=0.72; 95%CI 0.60-0.86, 2p=0.0002) and breast cancer mortality (0.82; 95%CI 0.73-0.93, 2p=0.002) were seen. These results have led to supportive clinical guidelines[12], and recommendations by both European and American expert groups to incorporate adjuvant bisphosphonates into routine clinical care.[5,6] However, despite the clinically important effects on breast cancer mortality, global acceptance has been slow, in part due to the opinion that these benefits relate only to a subset of patients that is somewhat imprecise in its definition and also that the mechanistic explanation for the findings are unclear[6].

Our findings should be considered as hypothesis generating but, clearly suggest that the beneficial effects of ZOL on the underlying breast cancer are associated with the presence of a non-amplified *MAF* gene within the primary tumour. On the contrary, MAF+ve tumours in non post-menopausal women experience a worse outcome with ZOL. In these younger, not postmenopausal women, there seem to be two distinct populations, MAF-ve who, like older MAF-ve patients, benefit from zoledronic acid and MAF+ve for whom use of zoledronic acid in the presence of reproductive hormones appears to stimulate the emergence of extra-skeletal metastatic disease and worse survival, resulting in a net nil effect in the non-postmenopausal subgroup both in the biomarker cohort and the AZURE study population as a whole. Additional mechanistic studies addressing the importance of MAF in cancer metastasis are in progress. It is hoped that these investigations may provide some biological insights into the differential effects of bisphosphonates on disease outcomes according to MAF status and menopause and help us understand how tumour biology, treatments that influence the metastatic niche and the endocrine milieu, that influence both host and tumour cell functions, all interact. Additionally, evaluation of MAF in another large randomized trial of adjuvant bisphosphonates is required before our findings could be considered for routine clinical practice. This is planned to take place in 2018 using the NSABP B34[13] tumor bank and data set of patients randomized to either treatment with the oral bisphosphonate, clodronate or placebo.

In this study of 865 patients and a median follow-up of 84 months, there were still only 60 IDFS events in bone, making interpretation of any relationships between MAF and bone relapse unreliable. We were thus unable to confirm the prognostic capability of MAF proposed by Pavlovic et al.[8] In this evaluation of the AZURE study population, MAF amplification was associated with other adverse biological characteristics such as ER negativity, high grade and Her2 positivity, but was unable to meet its primary objective of providing clinically useful independent bone metastasis prognostic information in the control population as a whole. Although MAF+ve tumours were associated with worse disease outcomes in the subset of postmenopausal breast cancer patients in the control group, these findings are not sufficient to recommend its use in the assessment of risk prognosis in routine practice. Because bone relapses typically occur late in the follow-up of

patients with early breast cancer, further evaluation is planned now that all patients have completed 10 years of follow-up; this is anticipated to increase the number of bone events by around one third. Other data sets are thus likely to be necessary to assess whether the originally described relationship between MAF+ve tumours and the development of bone metastases holds true. However, we believe the clinical interest in MAF relates to the predictive capabilities described rather than potential use as another prognostic factor.

The heterogeneity in IDFS by menopause for women with MAF-ve tumours within the control arm cannot be adequately explained by imbalance in other prognostic factors. Other than a slight excess of larger T2-4 tumours (72% versus 62%) in the MAF-ve non-postmenopausal and postmenopausal control populations respectively, the clinical and pathologic characteristics of this younger MAF-ve population appear similar.

Collectively, our observations point to MAF as a potential molecular target for the prevention or treatment of metastases from breast cancer[8]. Although required for metastasis, MAF is a very challenging pharmacological target because of its nuclear localization and lack of a catalytic domain. Dissecting the role of genes that are transcriptionally regulated by MAF may lead to the identification of potentially targetable proteins that are necessary for metastasis[8]. Amongst the genes transcriptionally regulated by MAF are potentially targetable proteins that contribute to support bone metastasis[14,15].

In conclusion, our findings suggest that MAF status may become a clinically useful biomarker for treatment selection. FISH testing for MAF copy number may allow better and more precise selection of patients for adjuvant treatment with ZOL. MAF-ve patients are likely to represent almost 80% of breast cancers who could benefit from the use of adjuvant ZOL. On the other hand, because the presence of a MAF+ve tumour appears to be associated with adverse disease outcomes when patients are treated with bisphosphonates - especially if treatment is initiated before menopause is complete - such patients with MAF+ve tumours should avoid exposure to bisphosphonates in the adjuvant setting.

Author contributions:

RRG, JA, JJ-M, JCT and FR developed the MAFTEST biomarker. RC, RRG, AH, HM and WG developed the study concept, wrote the protocol, performed and reviewed all analyses. The first author wrote the first draft of the manuscript and all authors were involved in interpretation of the data, revision and approval of the manuscript.

Acknowledgements:

We wish to acknowledge the Academic Unit of Pathology at the University of Sheffield for constructing the tissue microarrays from tumour blocks submitted from sites in the United Kingdom to enable a range of translational studies including the analyses reported in this manuscript. We also thank the patients who took part in the AZURE trial and the investigators within the United Kingdom who provide tissue samples for these analyses.

Conflicts of Interest:

Dr. Coleman reports grants from Bayer, grants from Amgen, personal fees from Eisai, personal fees from Astra Zeneca outside the submitted work; RB reports grants from The Cancer Council Victoria (Australia) during the conduct of the study; JJM owns < 0.25% of Inbiomotion S.L; J-CT reports a patent pending related to the work; WG reports personal fees from Celgene, personal fees from Janssen outside the submitted work; RRG declares shares of Inbiomotion SL for a value of less than 10,000$ and patents pending related to the submitted work. AH, AHan, JA, DC, HM, DD, FR have no conflicts to declare.

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Legends to Figures

Figure1: Impact of MAF copy number on invasive disease free survival (IDFS). Hazard ratios (HR) and 95% confidence intervals (CI) based on Cox multivariable analysis shown separately for control and zoledronic acid treated patients and by menopausal subgroup

Figure 2: IDFS by randomized treatment allocation (zoledronic acid or control) in MAF FISH negative patients. Output from Cox multivariable model shown to adjust for differences in the prognostic factors between groups.

Figure 3: Impact of MAF copy number (based on Cox multivariable analysis) on effects of adjuvant zoledronic acid on invasive disease free survival (IDFS) split by menopausal status (postmenopausal or not postmenopausal) and age (≤50 or >50).

Figure 4: Cumulative risk for first IDFS recurrence not in bone in women who were not postmenopausal at trial entry by treatment allocation (zoledronic acid or control) for patients with MAF FISH+ (A) or MAF FISH – (B) tumours. Cumulative incidence function figures do not adjust for differences in the prognostic factors between groups. Death and local/contralateral invasive disease are considered an event for this end point. First IDFS events that were in bone are considered a competing risk.

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

Variable	FISH evaluable result (n=865)	Tumour Provided (n=1739)	AZURE population (n=3359)
Menopausal Status			
Non post-menopausal	590 (68.2%)	1192 (68.5%)	2318 (69.0%)
Post-menopausal	275 (31.8%)	547 (31.5%)	1041 (31.0%)
Age			
<40	87 (10.1%)	198 (11.4%)	384 (11.4%)
40-49	299 (34.6%)	571 (32.8%)	1108 (33.0%)
50-59	281 (32.5%)	580 (33.4%)	1126 (33.5%)
60-69	162 (18.7%)	332 (19.1%)	628 (18.7%)
70+	36 (4.2%)	58 (3.3%)	113 (3.4%)
Lymph node involvement			
0	2 (0.2%)	17 (1.0%)	62 (1.8%)
1-3	563 (65.1%)	1122 (64.5%)	2075 (61.8%)
≥4	300 (34.7%)	598 (34.4%)	1211 (36.1%)
Unknown	0 (0%)	2 (0.1%)	11 (0.3%)
Tumour stage			
T1	274 (31.7%)	577 (33.2%)	1065 (31.7%)
T2	475 (54.9%)	901 (51.8%)	1717 (51.1%)
T3	99 (11.4%)	214 (12.3%)	456 (13.6%)
T4	17 (2.0%)	47 (2.7%)	117 (3.5%)
TX	0 (0.0%)	0 (0.0%)	4 (0.1%)
ER status			
ER positive	689 (79.7%)	1388 (79.8%)	2634 (78.4%)
ER negative	171 (19.8%)	341 (19.6%)	705 (21.0%)
ER unknown	5 (0.6%)	10 (0.6%)	20 (0.6%)
Systemic therapy plan			
Endocrine therapy (ET)	46 (5.3%)	89 (5.1%)	152 (4.5%)
Chemotherapy (CT)	166 (19.2%)	339 (19.5%)	719 (21.4%)
ET and CT	653 (75.5%)	1311 (75.4%)	2488 (74.1%)
Anthracyclines			
Yes	794 (91.8%)	1604 (92.2%)	3132 (93.2%)
No	71 (8.2%)	135 (7.8%)	227 (6.8%)
Taxanes			
Yes	126 (14.6%)	267 (15.4%)	775 (23.1%)
No	739 (85.4%)	1472 (84.6%)	2584 (76.9%)
HER2 status			
Positive	93 (10.8%)	186 (10.7%)	415 (12.4%)
Negative	250 (28.9%)	503 (28.9%)	1251 (37.2%)
Unknown / Not measured	522 (60.3%)	1050 (60.4%)	1693 (50.4%)
Histological grade			
1	61 (7.1%)	147 (8.5%)	285 (8.5%)
2	333 (38.5%)	748 (43.0%)	1439 (42.8%)
3	467 (54.0%)	820 (47.2%)	1552 (46.2%)
Not specified	4 (0.5%)	24 (1.4%)	83 (2.5%)
PR status			
Positive	308 (35.6%)	633 (36.4%)	1423 (42.4%)
Negative	159 (18.4%)	350 (20.1%)	806 (24.0%)
Unknown	398 (46.0%)	756 (43.5%)	1130 (33.6%)

Table 2: Relationships between MAF status and clinical and tumour characteristics by randomised treatment allocation

	Standard Treatment (n=445)		Standard treatment + Zoledronic acid (n=420)		All patients (n=865)	
Variable	**Negative (n=360)**	**Positive (n=85)**	**Negative (n=321)**	**Positive (n=99)**	**Negative (n=681)**	**Positive (n=184)**
Menopausal status						
Non post-menopausal	253 (70.3%)	55 (64.7%)	216 (67.3%)	66 (66.7%)	469 (68.9%)	121 (65.8%)
Post-menopausal	107 (29.7%)	30 (35.3%)	105 (32.7%)	33 (33.3%)	212 (31.1%)	63 (34.2%)
Age (years)						
<40	43 (11.9%)	5 (5.9%)	24 (7.5%)	15 (15.2%)	2 (0.3%)	0 (0.0%)
40-49	124 (34.4%)	28 (32.9%)	118 (36.8%)	29 (29.3%)	445 (65.3%)	118 (64.1%)
50-59	112 (31.1%)	33 (38.8%)	109 (34.0%)	27 (27.3%)	234 (34.4%)	66 (35.9%)
60-69	63 (17.5%)	17 (20.0%)	61 (19.0%)	21 (21.2%)	61 (19.0%)	21 (21.2%)
70+	18 (5.0%)	2 (2.4%)	9 (2.8%)	7 (7.1%)	9 (2.8%)	7 (7.1%)
Tumour stage						
T1	111 (30.8%)	31 (36.5%)	100 (31.2%)	32 (32.3%)	211 (31.0%)	63 (34.2%)
T2	200 (55.6%)	40 (47.1%)	179 (55.8%)	56 (56.6%)	379 (55.7%)	96 (52.2%)
T3	43 (11.9%)	12 (14.1%)	37 (11.5%)	7 (7.1%)	80 (11.7%)	19 (10.3%)
T4	6 (1.7%)	2 (2.4%)	5 (1.6%)	4 (4.0%)	11 (1.6%)	6 (3.3%)
Lymph node status						
0	2 (0.6%)	0 (0%)	0 (0%)	0 (0%)	2 (0.3%)	0 (0%)
1-3	231 (64.2%)	58 (68.2%)	214 (66.%)	(60 (60.6%)	445 (65.3%)	118 (64.1%)
≥4	127 (35.3%)	27 (31.8%)	107 (33.3%)	39 (39.4%	234 (34.4%)	66 (35.9%)
ER status						
ER positive	308 (85.6%)	55 (64.7%)	264 (82.2%)	62 (62.6%)	572 (84.0%)	117 (63.6%)
ER negative	50 (13.9%)	29 (34.1%)	57 (17.8%)	35 (35.4%)	107 (15.7%)	64 (34.8%)
ER unknown	2 (0.6%)	1 (1.2%)	0 (0.0%)	2 (2.0%)	2 (0.3%)	3 (1.6%)
Systemic therapy plan						
Endocrine therapy (ET)	24 (6.7%)	2 (2.4%)	16 (5.0%)	4 (4.0%)	40 (5.9%)	6 (3.3%)
Chemotherapy (CT)	50 (13.9%)	29 (34.1%)	54 (16.8%)	33 (33.3%)	104 (15.3%)	62 (33.7%)
ET and CT	286 (79.4%)	54 (63.5%)	251 (78.2%)	62 (62.6%)	537 (78.9%)	116 (63.0%)
Anthracyclines						
Yes	325 (90.3%)	79 (92.9%)	297 (92.5%)	93 (93.9%)	622 (91.3%)	172 (93.5%)
No	35 (9.7%)	6 (7.1%)	24 (7.5%)	6 (6.1%)	59 (8.7%)	12 (6.5%)
Taxanes						
Yes	49 (13.6%)	17 (20.0%)	44 (13.7%)	16 (16.2%)	93 (13.7%)	33 (17.9%)
No	311 (86.4%)	68 (80.0%)	277 (86.3%)	83 (83.8%)	588 (86.3%)	151 (82.1%)
HER2 status						
Positive	39 (10.8%)	17 (20.0%)	20 (6.2%)	17 (17.2%)	59 (8.7%)	34 (18.5%)
Negative	116 (32.2%)	18 (21.2%)	85 (26.5%)	31 (31.3%)	201 (29.5%)	49 (26.6%)
Unknown / Not measured	205 (56.9%)	50 (58.8%)	216 (67.3%)	51 (51.5%)	421 (61.8%)	101 (54.9%)
Histological grade						
1	34 (9.4%)	3 (3.5%)	21 (6.5%)	3 (3.0%)	55 (8.1%)	6 (3.3%)
2	158 (43.9%)	21 (24.7%)	137 (42.7%)	17 (17.2%)	295 (43.3%)	38 (20.7%)
3	168 (46.7%)	60 (70.6%)	161 (50.2%)	78 (78.8%)	329 (48.3%)	138 (75.0%)
Not specified	0 (0.0%)	1 (1.2%)	2 (0.6%)	1 (1.0%)	2 (0.3%)	2 (1.1%)
PR status						
Positive	133 (36.9%)	25 (29.4%)	121 (37.7%)	29 (29.3%)	254 (37.3%)	54 (29.3%)
Negative	53 (14.7%)	28 (32.9%)	46 (14.3%)	32 (32.3%)	99 (14.5%)	60 (32.6%)
Unknown	174 (48.3%)	32 (37.6%)	154 (48.0%)	38 (38.4%)	328 (48.2%)	70 (38.0%)

Table 3: Comparison of IDFS outcomes between non postmenopausal MAF FISH negative control population and other groups classified by menopausal status, MAF FISH status and treatment

Patient Group n= (number of events)	**Hazard ratio (HR)***	**Lower 95% CI**	**Upper 95% CI**
MAF- ve Control post-meno • n=107 (35)	1.25	0.80	1.95
MAF-ve Zol post-meno • n=105 (29)	0.85	0.53	1.36
MAF-ve Zol non post-meno • n=216 (61)	0.83	0.59	1.15
MAF+ve Zol post-meno • n=33 (12)	1.72	0.90	3.29
MAF+ve Zol non post-meno • n=66 (33)	1.61	1.06	2.44
MAF+ve Control post-meno • n=30 (18)	2.68	1.53	4.68
MAF+ve Control non post-meno • n=55 (11)	0.63	0.33	1.20

All compared with MAF –ve Control non post-menopausal group (n=253), number of events=83

Table 4: Impact of MAF FISH status on IDFS by age (≤50 or >50) in zoledronic acid and control patients.

Patient Group n= (number of events)	**Hazard ratio (HR)***	**Lower 95% CI**	**Upper 95% CI**
Zoledronic acid patients < age 50 • n= 142 (41) vs 44 (23)	0.473	0.281	0.797
Zoledronic acid patients ≥ age 50 • n= 179 (49) vs 55 (22)	0.533	0.719	0.890
Control patients ≤ age 50 • n= 167 (55) vs 33 (8)	1.410	0.666	2.985
Control patients > age 50 • n= 193 (63) vs 52 (21)	0.673	0.407	1.114

*HR for patients with MAF FISH negative / MAF FISH positive tumours

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