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| **Project Title** | The role of **ZOL**edronic acid and **MENO**pausal status on the tumour and bone microenvironment in patients with early breast cancer: a single center, randomised, proof of concept clinical study.  The **ZOLMENO** study. |
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| **Trial Title** | The role of **ZOL**edronic acid and **MENO**pausal status on the tumour and bone microenvironment in patients with early breast cancer: a single centre, randomised, proof of concept clinical study. |
| **Trial Acronym** | The **ZOLMENO** Study |
| **Research Question** | Does the administration of zoledronic acid alter levels of reproductive hormones and how does this affect the pre- and post-menopausal bone and tumour microenvironment? |
| **Trial Background** | In the UK, breast cancer is the 3rd most common cause of cancer death, after lung and prostate cancer, accounting for 7% of all cancer mortality (Cancer Research UK, 2014). The majority of the 12,000 UK women who die from breast cancer every year develop incurable bone metastases. Therefore, prevention of spread to the skeleton remains a major unmet clinical need (Walkington, 2011).  This study has arisen from a major international clinical trial, which was conceived and developed by a member of this research team and conducted from Sheffield. The AZURE trial was the first to demonstrate that menopausal status is a significant modifier of the effects of zoledronic acid (ZOL) in early breast cancer (Coleman R.E., 2014). Women who were post-menopausal significantly benefitted from adjuvant ZOL (with prevention of one death in every six); however this effect was not seen in pre-menopausal women. These findings have been substantiated in a recently published large meta-analysis of individual patient data from clinical trials involving >18,000 women which demonstrates a significant reduction in early breast cancer recurrence (RR 0·86, 2p=0·002), distant recurrence (RR 0·82, 2p=0·0003), bone recurrence (0·72, 2p=0·0002), and breast cancer mortality (RR 0.86, 2p=0.002) in post-menopausal women (EBCTCG, 2015). It is likely that, in light of these findings, adjuvant bisphosphonate treatment will become standard practice in postmenopausal women with early breast cancer, and this is already occurring in some centres including Sheffield.  There is a need for further research to identify the mechanisms responsible for this differential effect, which, we hypothesise, is caused by hormone-driven alterations of the tumour and bone microenvironment. Small clinical studies have shown that, in  addition to physiological levels of hormones such as activin and |

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|  | follistatin, breast cancer cells themselves produce high levels of activin and breast proliferative disorders result in higher expression of follistatin and follistatin related genes (Reis, et al., 2002; Bloise, et al., 2009). In pre-clinical studies ZOL has been shown to reduce follistatin levels in both tumour and bone *in vivo*, as well as in serum from patients following administration of a single dose of ZOL (see Appendix 1) (Winter, et al., 2013). These findings may have very different implications for the effect of ZOL on primary tumour and disseminated tumour cell growth, in the pre- and post-surgical and pre- and post-menopausal setting.  Understanding how the bone microenvironment differs between pre- and post-menopausal patients, and the implications for tumour growth and response to therapy, is critical for more effective tailoring of breast cancer treatment to individual patients. The ZOLMENO study will address this research gap and will help clinicians identify the patient population who will benefit most from adjuvant therapy with bone-targeted agents in the near future. We also hope that this research will suggest new approaches to treating early breast cancer patients who do not appear to benefit from bisphosphonates as adjuvant therapy, particularly as significant benefit is seen in both pre- and post-menopausal women in terms of reduction in skeletal related events in patients with metastatic disease (Coleman & McCloskey, 2011). |
| **Trial Design** | This is a proof of concept study which will recruit 80 patients (40 pre-menopausal and 40 post-menopausal women with newly diagnosed early breast cancer) from a single centre and randomise them to receive a single dose of zoledronic acid (ZOL) either 7 days pre-surgery (Group A) or 21 days post- surgery (Group B). Samples of serum, tumour tissue and bone marrow will be used to determine changes in reproductive hormone levels and in the bone and tumour microenvironment between pre- and post-menopausal women pre- and post- zoledronic acid administration. |
| **Trial Objectives** | The primary objective is to determine the changes in follistatin levels in pre-menopausal and post-menopausal women following zoledronic acid administration before and after surgical excision of the primary tumour.  In order to identify the underlying mechanisms leading to the differential effect of ZOL and to characterise the tumour and bone microenvironment in pre- and post-menopausal women in the pre- and post-ZOL setting, secondary objectives will be to:   * Compare changes in activin levels following zoledronic acid infusion and determine how these differ depending on   menopausal status and timing of ZOL administration (pre- |

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|  | vs. post-surgical)   * Identify histomorphological and immunohistochemical changes in the tumour microenvironment and determine how these relate to menopausal status and ZOL administration * Identify genetic and histomorphological changes in the bone marrow microenvironment and determine how these relate to menopausal status and ZOL administration   Through exploratory analyses, tertiary objectives will also be to:   * Determine the ability of serum from participants to modify breast cancer cells *in vitro* * Identify changes in pre-clinical biomarkers and proteomic profiles of serum, plasma, tumour and bone marrow samples relative to menopausal status. |
| **Outcome Measures** | The primary outcome measure will be the change in serum follistatin at day 28 post-ZOL administration.  The following secondary outcome measures, all compared relative to menopausal status (pre- vs. post-menopausal) and timing of ZOL administration (pre- vs. post-surgical), will include:   * Change in serum follistatin levels at day 7 and day 28 post- ZOL administration * Change in serum activin levels at day 7 and day 28 post- ZOL administration * Change in serum follistatin levels at day 21 and day 28 post-surgery * Change in serum activin levels at day 21 and day 28 post- surgery * Exploratory analysis of changes in the levels of follistatin and activin in tumour samples * Exploratory analysis of changes in the histomorphometry of the tumour microenvironment * Exploratory analysis of differences in the genetic expression of bone marrow cells * Exploratory analysis of changes in the histomorphometry of bone marrow   Tertiary outcomes, via exploratory analyses, also considered relative to menopausal status and timing of ZOL administration, will include:   * Differences in the effect of serum from participants on breast cancer cells *in vitro* * Changes in the proteomic profiles of serum, plasma and, where available, bone marrow samples * Changes in levels of pre-clinical biomarkers in the bone and |

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|  | tumour microenvironment |
| **Trial Population** | 80 patients comprising 40 pre-menopausal and 40 post- menopausal women with newly diagnosed early breast cancer scheduled to undergo primary breast surgery. |
| **Randomisation** | Pre- and post-menopausal women will be randomised on a 1:1 basis to receive zoledronic acid either 7 days pre-surgery (Group A) or 21 days post-surgery (Group B) using a computer- generated programme at the Cancer Clinical Trials Centre (CCTC), Sheffield. |
| **Trial Treatment** | A single intravenous infusion of 4mg zoledronic acid administered at either 7 days pre-surgery or 21 days post- surgery. |
| **Trial Duration** | Allowing for a 24 month recruitment period with a maximum 9 week participation period, the active study period will be 2 years and 2 months. Allowing for analysis of all data the total study duration will be 3 years and 2 months. |

1. **Trial Schema**

MDT

Potential participants identified

Breast clinic

Inital screening and study information

Research clinic

Day 7 pre-surgery

**Group A**

Blood samples Clinical assessment

+/- bone marrow sample

ZOLEDRONIC ACID

Screening and consent

Participants randomised

**Group B**

Blood samples

Surgery

Blood samples Tumour sample

Bone marrow sample

Blood samples Tumour sample

Bone marrow sample

Research clinic

Day 21 post-surgery

Blood samples

Clinical assessment

Blood samples Clincal assessment

ZOLEDRONIC ACID

Research clinic

Day 28 post-surgery

Blood samples

Clinical assessment

Blood samples Clinical assessment

+/- bone marrow sample

Research clinic

Day 49 post-surgery

Blood samples

Clinical assessment

1. **Introduction**
   1. **Background and Rationale**

In the UK, breast cancer is the 3rd most common cause of cancer death, after lung and prostate cancer, accounting for 7% of all cancer mortality (Cancer Research UK, 2014). The majority of the 12,000 UK women who die from breast cancer every year develop incurable bone metastases. Therefore, prevention of spread to the skeleton remains a major unmet clinical need (Walkington, 2011).

This study has arisen directly from a major international clinical trial which was conceived, developed and conducted from Sheffield and run through the Leeds Clinical Trials Research Unit. The AZURE trial (Chief Investigator RE Coleman) was the first to demonstrate that menopausal status is a significant modifier of the effects of zoledronic acid (ZOL) in early breast cancer (Coleman R.E., 2014). Women who were post-menopausal significantly benefitted from adjuvant ZOL (with prevention of one death in every six), however this effect was not seen in pre-menopausal women. These findings have been substantiated in a recently published large meta-analysis of individual patient data from clinical trials involving

>18,000 women which demonstrates a significant reduction in early breast cancer recurrence (RR 0·86, 2p=0·002), distant recurrence (RR 0·82, 2p=0·0003), bone recurrence (0·72, 2p=0·0002), and breast cancer mortality (RR 0.86, 2p=0.002) (EBCTCG, 2015). It is likely that, in light of these findings, adjuvant bisphosphonate treatment will become standard practice in postmenopausal women with early breast cancer and this is already occurring in some centres, including Sheffield.

There is, however, a need for further research to identify the mechanisms responsible for this differential effect, which we hypothesise is caused by hormone-driven alterations of the tumour and bone microenvironment. Understanding how the bone microenvironment differs between pre- and post-menopausal patients, and the implications for tumour growth and response to therapy, is critical for more effective tailoring of breast cancer treatment to individual patients. The ZOLMENO study will address this research gap and will help clinicians identify the patient population who will benefit most from adjuvant therapy with bone-targeted agents in the near future. We also hope that this research will suggest new approaches to treating early breast cancer patients who do not appear to benefit from bisphosphonates as adjuvant therapy, particularly as significant benefit is seen in both pre- and post-menopausal women in terms of reduction in skeletal related events (SRE) in patients with metastatic disease (Coleman & McCloskey, 2011).

## Menopause and Bone Metastases

The bone microenvironment that contributes to tumour growth and response to therapy differs markedly between pre- and post-menopausal women as a result of hormonal changes (Wilson, Holen, & Coleman, 2012). Falling oestrogen levels during menopause lead to increased bone turnover (Hale & Burger, 2009). This may create a microenvironment hospitable to disseminated breast cancer cells, with elevated bone resorption supporting their progression to overt metastases. Members of this research team published the first study demonstrating that the anti-tumour effect of ZOL differs in murine models of pre- and post-menopausal bone (Ottewell, et al., 2014). ZOL-sensitive (osteoclastic) bone resorption was shown to promote growth of disseminated tumour cells and development of bone metastasis in oophorectomised mice, but not in mice with intact ovarian function. This mirrors the findings in human patients, indicating that regulation of tumour growth in bone depends, at least in part, on menopausal status.

## Zoledronic Acid

Zoledronic acid is a bisphosphonate drug indicated in oncology for the prevention of skeletal related events (SRE’s), including pathological fractures, spinal cord compression and tumour-induced hypercalcaemia. It is also indicated for the treatment of the latter. In addition, the role of ZOL in the prevention and treatment of cancer treatment induced bone loss (CTIBL), such as that caused by the use of aromatase inhibitors in breast cancer, is well demonstrated by clinical trials (ZO-FAST, Z-FAST and E-ZO-FAST) and recommended in clinical guidelines (Reid, Doughty et al. 2008).

The mechanism of action of zoledronic acid in preventing bone loss involves inhibition of osteoclastic activity, thereby reducing bone turnover. Bisphosphonates bind to hydroxyapatite bone mineral surfaces around resorbing osteoclasts and are selectively internalised by osteoclasts where they inhibit their activity. Zoledronic acid inhibits the mevalonate pathway causing deregulation of signalling GTPases, ultimately leading to osteoclast apoptosis with consequent reduction in tumour cell-induced bone resorption and destruction (Russell, Watts et al. 2008). Pre-clinical studies also provide evidence that zoledronic acid has the potential for direct anti-tumour activities in patients, with direct *in vitro* and *in vivo* effects on tumour cell adhesion, invasion, apoptosis, proliferation and vascularisation demonstrated (Ottewell, Mönkkönen et al. 2008; Winter, Holen et al. 2008).

In clinical practice, zoledronic acid solution (4mg in 100ml 0.9% saline) for infusion is administered intravenously over 15 minutes. Zoledronic acid is excreted exclusively and untransformed via the kidneys. Within the first 24 hours one-third to two-thirds of the administered dose is rapidly excreted, with the remaining dose being internalised by osteoclasts and released at a much slower rate as bone remodelling occurs (Chen, Berenson et al. 2002). There is growing evidence that a single dose of ZOL can have clinically significant bioavailability in the context of osteoporosis for at least 2 years, although the regimes required for ongoing anti-tumour benefit are less clear (Brown et al, 2007).

The nitrogen containing structure of zoledronic acid results in higher potency of osteoclast activity inhibition when compared with other bisphosphonates, without adversely affecting the mineralisation of bone (Green and Rogers 2002). The bioavailability of intravenous (IV) bisphosphonates is significantly higher than oral bisphosphonates (<5%) whilst also avoiding potential, well-recognised, gastrointestinal adverse effects (Conte and Guarneri 2004). In addition, IV zoledronic acid has been shown to be 80-450 times more potent than IV pamidronate in pre-clinical trials (Green and Rogers 2002) and in the clinical setting reduces the risk of SREs in breast cancer patients by 20% compared to pamidronate, without any documented difference in tolerability (Rosen, Gordon et al. 2003). The mounting evidence for the greater efficacy of zoledronic acid has resulted in it being the agent of choice for many clinicians, both within the UK and internationally.

## The Inhibin, Activin and Follistatin Axis

Oestrogen and inhibin are ovarian hormones with a major impact on the bone microenvironment (Wilson, Holen, & Coleman, 2012; Nicks, Perrien, Akel, Suva, & Gaddy, 2009). Levels of both hormones fall during menopause, with inhibin becoming undetectable in post-menopausal women (Welt, McNicholl, Taylor, & Hall, 1999). Inhibin acts by inhibiting members of the TGFβ superfamily, including the tumour suppressor activin, which is also inhibited by follistatin (Reame, Lukacs, Olton, Ansbacher, & Padmanabhan, 2007). In pre- menopausal women, levels of bioavailable (tumour-suppressing) activin are low due to the high concentration of inhibin. In contrast, in post-menopausal women, follistatin concentration determines the level of bioavailable activin.

Small clinical studies have shown that, in addition to physiological levels of these hormones, breast cancer cells themselves produce high levels of activin and breast proliferative disorders result in higher expression of follistatin and follistatin related genes (Reis, Cobellis, Tameira, & Anania, 2002; Bloise, Couto, & Massai, 2009). In pre-clinical studies (appendix

1) ZOL has been shown to reduce follistatin levels in both tumour and bone *in vivo*, as well as in serum from patients following administration of a single dose of ZOL (Winter, et al., 2013). These findings may have very different implications for the effect of ZOL on primary tumour and disseminated tumour cell growth in the pre- and post-surgical and pre- and post- menopausal setting.

In pre-menopausal women, activin is neutralised by high inhibin levels and reducing follistatin levels makes little or no difference to activin bioavailability. In post-menopausal women, inhibin levels are negligible and a reduction in follistatin levels increases the availability of the tumour suppressor activin, potentially reducing tumour growth. Proteomic studies on breast cancer models by this group (Westbrook, Cairns et al. 2016), have also highlighted the potential importance of these molecules and other reproductive hormones in breast cancer bone metastasis. Overall, these studies provide the first evidence of the mechanisms responsible for the specific clinical benefit of ZOL seen in post-menopausal women with early breast cancer. It is these findings we aim to verify and expand in the proposed clinical study.

# Aims and Objectives

This study will address the following original research question: *Does the administration of zoledronic acid alter levels of reproductive hormones and how does this affect the pre- and post-menopausal bone and tumour microenvironment?*

We aim to identify the mechanisms responsible for the differential effect of zoledronic acid seen in pre- and post-menopausal women with early breast cancer and in doing so lead to effective administration of beneficial adjuvant bone-targeted therapy to appropriate patient groups.

## Primary Objective

The primary objective is to determine the changes in follistatin levels in pre-menopausal and post-menopausal women following zoledronic acid administration before and after surgical excision of the primary tumour.

## Secondary and Tertiary Objectives

In order to identify the underlying mechanisms leading to the differential effect of ZOL and to characterise the tumour and bone microenvironment in pre- and post-menopausal women in the pre- and post-ZOL setting, secondary objectives will be to:

* Compare changes in activin levels following zoledronic acid infusion and determine how these differ depending on menopausal status and timing of ZOL administration (pre- vs. post-surgical)
* Identify histomorphological and immunohistochemical changes in the tumour microenvironment and determine how these relate to menopausal status and ZOL administration
* Identify genetic and histomorphological changes in the bone marrow microenvironment and determine how these relate to menopausal status and ZOL administration.

Through exploratory analyses, tertiary objectives will also be to:

* Determine the ability of serum from participants in different treatment groups to modify the aggressiveness of breast cancer cells *in vitro*
* Identify changes in the bone and tumour biomarkers and proteomic profiles of serum, plasma, tumour and bone marrow samples from the different patient groups.

# Study Design and Methodology

## Study Design

This is a single centre, open label, randomised, proof of concept study. Pre-menopausal (N=40) and post-menopausal (N=40) women due to undergo primary surgery for early breast cancer will be randomised on a 1:1 basis to receive a 4mg infusion of zoledronic acid either pre-surgically (Group A) or post-surgically (Group B). This will create 4 patient groups for comparison: 20 **pre**-menopausal women receiving **pre**-surgical ZOL; 20 **post**-menopausal women receiving **pre**-surgical ZOL; 20 **pre**-menopausal women receiving **post**-surgical ZOL and 20 **post**-menopausal women receiving **post**-surgical ZOL (Table.1). By comparing samples before and after removal of the primary tumour we will be able to discriminate between changes in tumour-derived and host (including bone) sources of reproductive hormones and biomarkers. Allowing for a 24-month recruitment period, with a maximum of 9 weeks participation, the active study period will be 2 years and 2 months. Allowing for analysis of all data the total study duration will be 3 years and 2 months.

## Table 1. Patient Groups

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|  | **Group A Pre**-surgical ZOL | **Group B Post**-surgical ZOL |
| **Pre**-menopausal women | n=20 | n=20 |
| **Post**-menopausal women | n=20 | n=20 |

This will be an open label study as it is not ethically justifiable to offer or perform unnecessary additional bone marrow tests on patients, as would be required if patients or clinicians were to be blinded to the treatment schedule. However, analysis of biological samples and end-points will be performed without knowledge of treatment allocation, reducing the risk of analytical bias.

## Study Setting

This will be a single centre study based within Sheffield Teaching Hospitals NHS Foundation Trust (STH). Within this trust, patients are cared for at the Royal Hallamshire Hospital (RHH) under the Breast Surgical Team, followed by the Medical and Clinical Oncology Team at Weston Park Hospital. Potentially eligible patients will be identified at the local MDT. Following MDT discussion, patients attend a same-day appointment for confirmation of diagnosis and a Nurse-led clinic at RHH within the next 1-2 weeks. The study will be introduced to patients by their own care team at these appointments and discussion with a research team member will be offered if they are interested in receiving further verbal and written information relating to the study.

Further study visits, including research clinics for screening, consent, study investigations and treatment will take place at the Clinical Cancer Trials Centre (CCTC) as well as at RHH.

These settings provide the appropriate facilities required for patient contact at each of the time points, including private rooms for follow-up, clinical assessment, blood tests and bone marrow procedures in addition to nurse staffing and equipment for ZOL infusion.

# Study Participants

This study will recruit 80 patients from STH in total. This will comprise 40 pre- or peri- menopausal and 40 post-menopausal female patients (defined by menstrual history +/- baseline FSH / LH / oestradiol) with histologically diagnosed early breast cancer who are due to undergo primary breast surgery. Participation will have no impact on the planned local or systemic treatment of the underlying breast cancer.

This population reflects the patient groups recruited to the large clinical trials, described above, which have demonstrated a benefit of bisphosphonate treatment in post-menopausal participants. Our target population is similarly likely to be otherwise clinically well and therefore have fewer contra-indications to participation and ZOL infusion, in addition to representing the cohort of patients who are likely to benefit from the outcomes of this study in the future.

## Eligibility

Patients meeting all of the inclusion criteria and none of the exclusion criteria will be invited to participate in the study. Eligibility will be confirmed prior to randomisation by a medically qualified investigator listed in the delegation log, although patients may also been screened by named research nurses who will seek confirmation with a named investigator. In cases of unclear menopausal status, renal function and clotting screen (i.e, these tests have not been performed as part of standard care), consent will take place prior to confirmatory blood tests (sample processing time usually <4 hours at STH) but randomisation will only take place once menopausal status has been confirmed.

## Defining menopause

There is no universal definition of menopause. This study will use criteria which reflect recommendations made in *Menopause: diagnosis and management*, the NICE guideline published in November 2015, allowing for additional clarification using laboratory testing in unclear cases, given the significance of this variable in our study (National Institute for Health and Care Excellence, 2015). This document has been developed by a Guideline Development Group comprising 15 expert advisors in menopause management and post-reproductive care in association with the British Menopause Association and is likely to become the widely accepted national reference for determining menopausal status. The following criteria will be used to define menopausal status:

## Pre-menopausal:

* + - * Women 40-54 years of age **and**
      * regular or frequent menses without the use of oral contraceptives or hormone replacement therapy (HRT)

## Post-menopausal:

* + - * Women aged ≥55, or
      * Women with an intact uterus and absence of menses for ≥12 months, or
      * Women who have undergone bilateral oophorectomy

Women who do not meet any of these criteria, including those who have undergone hysterectomy, thyroidectomy and those who have been receiving HRT, cannot accurately have remaining ovarian function determined by clinical assessment and they will therefore have biochemical testing performed. This will include serum FSH and, where the FSH level is indeterminate, LH and oestradiol levels (these will be performed as extra tests on the sample already taken for FSH). The standard cut-off ranges for the STH laboratory will be used to classify participants as pre- or post-menopausal. Women who do not fit all the biochemical criteria of being post-menopausal (i.e. those who are peri-menopausal) will be classed as pre-menopausal.

## Inclusion criteria

* Female patients aged ≥ 40
* Histologically confirmed early breast cancer
* Tumour size more than 1cm (≥ T1)
* Any nodal status including unknown (≥N0)
* Scheduled for surgery as primary treatment
* Any tumour hormone receptor (ER/PR) or HER2 status
* ECOG performance status of 0,1 or 2 (appendix 2)
* Menopausal status defined clinically by menstrual and clinical history, or where this is indeterminate patient is willing to have biochemical profile testing following consent
* Clinical biochemistry:
  + Measured or calculated Glomerular Filtration Rate (GFR) ≥30 ml/min (Cockcroft and Gault formula, appendix 3)
  + Serum corrected calcium ≥2.2mmol/L
* Clotting screen:
  + APTT 30.5 seconds
  + PT 13.2 seconds or INR <1.5
  + Platelets 100 x 109/L
  + Or clotting abnormalities which are due to be reversed as part of standard care by the time of bone marrow sampling (e.g. stopping anticoagulants prior to surgery)
* Potentially fertile women must:
  + have a negative pregnancy test within 72 hours prior to randomisation, and not be breast-feeding
  + agree to use effective, medically approved, barrier contraception from the time of consent to 30 days after their zoledronic acid infusion
* Potential participants must:
  + be willing to have the required mandatory samples taken, including bone marrow aspiration and trephine at the time of surgery
  + have the mental capacity to understand the study information, make an informed choice regarding participation and to provide written informed consent.

## Exclusion criteria

* Any previous diagnosis or treatment of cancer that could confound results and endpoints (allowed situations include non-melanomatous skin cancer or superficial bladder cancer)
* Patients with an estimated life expectancy of <6 months
* Any diagnosis of a bone marrow disorder
* Any previous bisphosphonate treatment
* Use of hormone replacement therapy (HRT) or continued use of oral contraceptives / implant / depo injection in the past 30 days or a diagnosis of hormonal imbalance such as polycystic ovarian syndrome
* Current active dental problems including dental abscess or infection of the jawbone (maxilla or mandible), any open oral wounds or a current or previous diagnosis of osteonecrosis of the jaw
* Recent (within 4 weeks) or planned dental or jaw surgery (recent dental fillings, scale and polish or minor gingival surgery do not exclude the patient)
* Any other serious medical or psychiatric condition, which, in the opinion of the investigator, could affect participation in the ZOLMENO study. This may include dehydration, notable electrolyte disturbances, significant use of nephrotoxic, anti-angiogenic or hypocalcaemia-inducing drugs or history of significant renal failure, which would render the patient unsuitable for zoledronic acid or sample collection.

## Recruitment

The number of patients diagnosed with breast cancer at STH in 2014 was 497. We therefore do not anticipate any issues with availability of eligible patients over the 2 year recruitment period. Potentially eligible patients will be identified at weekly breast surgery MDT meetings at STH. The study will be introduced to patients at routine clinic appointments by their direct care team, following which interested patients will be introduced to a member of the research team for further written and verbal information and initial screening. Patients will be reassured that their decision to participate in the study or not will not impact on their future treatment; should they need to be given bisphosphonates as part of their ongoing treatment this will occur regardless of whether they have taken part in this study.

Limitations to recruitment are not expected to be substantial and are most likely to relate to the extra visits and bone marrow sampling required. The impact of these factors will be minimised by aligning study visits with routine appointments whenever possible and by performing the bone marrow sampling under general anaesthetic at the time of breast surgery.

It is recognised that while this is not likely to be a pre-morbidly vulnerable group, we are recruiting patients at a time-point close to a cancer diagnosis. We are therefore working closely with the Breast Cancer Specialist Nurses and, wherever possible, the study will be introduced to patients at the nurse-led holistic assessment clinic, rather than at the clinic at which they receive their diagnosis. Any psychological state that is likely to impair the ability to provide informed consent will exclude a patient from eligibility for the study. Administration of zoledronic acid as part of this study is not likely to adversely affect any future oncological treatment, and there will be no negative impact on future treatment planning to patients deemed suitable for further standard bisphosphonate therapy doses.

## Patient Screening

Patients will be screened for eligibility for entry into the study using the above inclusion and exclusion criteria by a named investigator or research nurse. Details to assess eligibility will be collected from patient records and clinical history will be obtained from the patient.

Where required investigations, such as biochemistry tests and clotting screens, have not been performed as part of standard care prior to consent or within the required time period, patients who are otherwise eligible for participation will be consented for participation, after which these investigations will be performed. Any patient who subsequently is deemed to be ineligible for participation will be withdrawn from the study and their number replaced during the recruitment period.

A Screening Log will be maintained in the Site File, which will include all patients screened for participation in the study. Patients will be identified by initials and date of birth. The screening log will document the following:

* Date screened
* Approached / not approached for participation in the study
* Reason for non-randomisation
  + Not eligible for trial participation and reason
  + Eligible but declined and reason (where available)
  + Other reason for non-randomisation/participation.

## Informed Consent

Patients interested in the study will be introduced to a member of the research team, by a member of their direct care team. Verbal and written information **(Participant Information Sheet version 1.3)** will be provided by a named member of the research team (investigator or research nurse). All research team members providing information and obtaining consent will have up to date Good Clinical Practice (GCP training), will be experienced in working with patient groups and obtaining consent for clinical studies, and will be well educated in the details and background of this clinical study.

Patients will be contacted at least 24 hours after receiving the participant information sheet by telephone and asked if they would be interested in taking part in the study. Those that do wish to participate will be booked into a research clinic at the CCTC at which point they will be screened for eligibility and given the opportunity to ask further questions and provide written, informed consent.

Consent will be obtained on the study-specific consent form by a medically qualified investigator. Should the patient be unable to sign or otherwise mark the consent form, but is able to make fully informed consent, provision for completion of the form by an appropriate witness will be made. This may be a carer, friend, family member or a local member of the clinical team who is independent of the research team.

The patient retains the right to refuse consent to participate in the study or to withdraw consent from the study at any time without giving reasons and without any impact on their future treatment or care.

The acquisition of consent will be documented in the participant’s medical records, including date, details of those present and randomisation arm. The original consent form (CF) will be held in the Investigator Site File and 2 copies will be distributed between the participant and the participant’s medical records. Following written consent participant identity will be anonymised by allocation of a unique study number.

## Randomisation

Randomisation will take place as soon as possible after eligibility has been confirmed and written consent obtained. Randomisation will take place in time to allow arrangements for the administration of ZOL to Group A at day 7 pre-surgery.

Patients will be randomised on a 1:1 basis to receive zoledronic acid at day 7 pre-surgery (Group A) or day 21 post-surgery (Group B). A computer-generated programme will be used to ensure balanced age characteristics as randomisation will involve the following age group stratification: 40-54 years and ≥55 years.

In order for patients to be randomised, a named investigator or research nurse will contact the Informatics Team in the Clinical Trials Office at Weston Park Hospital (Tel: 0114 2265217) Monday to Friday between the hours of 9am to 5pm. The following information will be provided:

* Name of person undertaking randomisation
* Patient initials and date of birth
* Confirmation of eligibility
* Confirmation and date of written informed consent
* Menopausal status

Following this the Informatics Team will provide the assigned study number and randomisation group for the patient. This will be recorded and stored in the Site File and will become the only identifier for participating patients.

A letter will be sent to the GPs of all participants informing them of their patient’s participation in the trial, their randomisation group, the nature of the samples to be taken and the side effect profile of zoledronic acid.

# Study Medicinal Product Management

The administration of zoledronic acid, within the remit of this study, will be as an Investigational Medicinal Product (IMP).

## Zoledronic acid

Zoledronic acid is widely used in the UK in the management of osteoporosis, hypercalcaemia, cancer treatment induced bone loss and metastatic bone disease, as discussed in section 3.3 above.

It is generally administered as zoledronic acid 4mg in 100ml 0.9% sodium chloride over 15 minutes. Details on the composition of zoledronic acid can be found in the current version of the manufacturer’s Summary of Product Characteristics (SPC), which can be accessed via the Electronic Medicines Compendium (eMC) website: [http://www.medicines.org.uk/emc.](http://www.medicines.org.uk/emc) A reference copy of this SPC will be kept in the Investigator Site File.

## Supply and handling

Provision of the study drug will be commercial drug supply via Weston Park Hospital Pharmacy Department. The study drug will be dispensed on receipt of a study-specific ChemoCare electronic prescription that will only be prescribed by a medically qualified investigator. Pharmacy will record details of the dispensing, including batch numbers, on Drug Accountability Logs.

## Formulation, storage, preparation and labelling

Any generic or branded formulation of zoledronic acid may be used. Formulation, storage, and preparation of zoledronic acid will be in line with the manufacturers’ recommendations. For further details, please refer to the relevant manufacturer’s SPC as above.

As per the conditions set out in Regulation 46(1) of the Medicines for Human Use (Clinical Trials) Regulations 2004, zoledronic acid will be labelled in accordance with Article 15 of Commission Directive 2003/94/EC. As zoledronic acid is being used outside of its licensed indication, trial labelling requirements apply and these will comply with paragraphs 26-30 of Annex 13 ‘Investigational Medicinal Products’, in Volume 4 ‘EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Vetinary Use’ of ‘The Rules Governing Medicinal Products in the European Union’ (EudraLex 2010). Labelling will occur at the point of dispensing by Weston Park Hospital pharmacy.

# Treatment Details

Participants will be randomised to receive a single intravenous infusion of zoledronic acid 4mg in 100ml 0.9% sodium chloride over 15 minutes on either day 7 pre-surgery or day 21 post-surgery.

## Pre-treatment and Post-treatment Investigations

See section 10.3 for full details of baseline and pre-treatment assessments required following written informed consent, and ongoing clinical assessment following administration of zoledronic acid.

## Concomitant Medications

There are no concomitant medications that preclude the use of zoledronic acid. There have been no reports of problems when zoledronic acid has been administered with commonly used anticancer drugs, diuretics, antibiotics and analgesics. However, to minimise risk to participants, a full drug history will be taken and consideration will be given to any additive risks for hypocalcaemia, nephrotoxicity or osteonecrosis of the jaw (ONJ) and reviewed in the context of biochemistry results, dental history and the current SPC for zoledronic acid. Based on these considerations exclusion from the study will be at the discretion of the co- investigator.

There is no requirement for supplementary calcium or vitamin D to be administered alongside a single dose of zoledronic acid. Participants will be advised to take a single dose of paracetamol following the zoledronic acid infusion to reduce the risk of an acute phase reactions.

## Side Effect Profile

The full side effect profile of zoledronic acid can be found in the current SPC, available as above. Both bisphosphonates as a group, and zoledronic acid itself, are widely used on a regular basis with most patients tolerating the drug well. Generally zoledronic acid has a low side effect profile, however one of the most common risks is a self-limiting acute phase reaction, affecting around one in ten patients. A rare, but well documented, risk is osteonecrosis of the jaw (ONJ). This is more likely to occur in patients undergoing invasive dental procedures, which is why these patients are excluded from participation. Large clinical trials have shown an incidence of ONJ of around 0.5-2% in early breast cancer patients receiving repeated doses of bisphosphonates. However, we anticipate this event to be very unlikely in our study due to the exclusion of patients with pre-existing dental problems and

the use of only a single dose of zoledronic acid. Other risks such as renal impairment and hypocalcaemia will be limited by careful screening of patients, as described above.

## Invasive Dental Procedures

Oral hygiene should be optimised before administration of a bisphosphonate. Invasive dental procedures should be avoided, if possible, in patients recently treated with a bisphosphonate (within the previous 28 days). Any significant previous dental issues should have completely resolved and any wounds healed prior to administration of zoledronic acid for the purpose of this study. Participants do not require a formal dental assessment prior to recruitment into the study, but medical and dental history, in addition to a basic oral examination by a medically qualified investigator, will be performed to screen any patients at increased risk of osteonecrosis of the jaw.

## Management of Toxicity and Dose Reductions

As per the SPC for zoledronic acid in single dosing regimens, no dose reductions will be made. All patients with a calculated creatinine clearance 30ml/min will be given a 4mg dose. This is more conservative than the SPC guidance for single dosing which advises that patients with creatinine levels of up to 400mol/l can be given the same dose, however this is on a risk/benefit assessment and in the context of a clinical study, benefit cannot be presumed. Deterioration in renal function following a single zoledronic acid infusion with renal function within the specified limits is unlikely but would be identified through blood tests performed at day 7 post-ZOL. Any identified renal toxicity will be managed as per local guidelines, for example with intravenous fluid infusion.

# Sampling Details

## Sample Collection

Participants will have 40ml of blood drawn at the time-points indicated in the trial schema. Collection will be by research or STH staff trained and experienced in phlebotomy. Where patients have had required tests performed (such as clinical biochemistry or clotting screens) as part of their standard care, and the results of these tests fall within the required timescales for the study, these tests will not be repeated.

Collection of bone marrow serum from aspirate will amount to a minimum of 1ml and maximum of 5ml. Bone marrow trephines will ideally be 2-2.5cm in length. Bone marrow sampling will take place under general anaesthetic in all participants, and any additional sampling agreed to by participants will take place under local anaesthetic with 1-2% lidocaine, as per standard practice.

Tumour sampling by the operating surgeon will occur in line with standard care with excised tissue being sent to the STH pathology for diagnostic purposes as would normally be the case; no additional tumour sample will be obtained for this study. Once STH pathology laboratories have concluded all diagnostic testing on the tumour samples they will prepare 6 fixed slides of 3-5 micron thick tumour specimen for use in the ZOLMENO study.

## Sample Handling

Serum and plasma will either be processed immediately by STH for biochemistry, clotting +/- FSH, or stored at the CCTC for study specific and translational study investigations. At the time of study specific testing, samples will be transferred to the University of Sheffield

Medical School laboratories for processing. Study samples will be identified and traceable via data matrix labels with a clear audit trail available.

Bone marrow samples will be stored in a similar manner at the CCTC laboratory before being transferred to a third party collaborator for genetic sequencing. The third party, *GenomeScan*, is a well-established company who have been used previously by this research team and who will sign agreements to comply with research standards as stipulated by the sponsor. Any ELISAs or immunohistochemistry performed on the bone marrow samples will be performed at the University of Sheffield Medical School laboratories.

Tumour specimen slides will be stored in the STH pathology laboratory until the time of analysis at the University of Sheffield Medical School laboratories.

## Sample Processing

Measurement of FSH, full blood count, urea and electrolytes and clotting screens will be performed by STH laboratories on the same day as collection. Other samples will be stored at the CCTC laboratory for batch analysis at the University of Sheffield Medical School, apart from the third party analysis for genetic sequencing of bone marrow as described above.

## Hormone and histomorphometry analysis

Follistatin and activin levels in serum samples will be measured using well validated ELISA techniques. Tumour samples will be stained using standard immunohistochemistry techniques for follistatin and activin levels and for general histomorphometry. Bone marrow trephines will also be stained for histomorphological assessment of the bone microenvironment. Commercially available kits, which have been well validated, such as those provided by R&D Systems, will be used to perform all of these assays.

## Genetic analysis

Next-generation sequencing will be performed by *GenomeScan* on an Illumina HiSeq2500 platform using standard operating procedures. We will compare gene expression profiles of the bone marrow of each patient before and after ZOL treatment and also compare these between pre-and post-menopausal women. RNA sequencing reads are aligned to the reference human genome, using standard bioinformatics tools (Tophat2), and gene expression is calculated by counting read coverage for each gene (HTSeq-Count). Statistical analysis is then performed to generate a list of genes differentially expressed between the conditions under study (DESeq). These results are used to draw conclusions at the pathway level.

## Biomarker analysis

Cell free DNA from plasma will be measured using commercially available circulating nucleic acid kits. It is intended that the blood samples will also be used for investigation of other known, conventional bone markers, such as bone-specific alkaline phosphatase, osteoprotogerin, RANKL and osteopontin. The University of Sheffield is an internationally recognised centre for the assay of such biomarkers, for which there are standard methods, validated for use in clinical samples. These are usually antibody- based (e.g. ELISA). It is expected that these measurements will be made in the University of Sheffield laboratories. By measuring these biomarkers before and after administration of zoledronic acid in post and pre-menopausal patients with breast

cancer, we will obtain further information on the differences in bone metabolism between pre and post-menopausal patients under the influence of zoledronic acid.

* + 1. ***In vitro* analysis**

The ability of the serum collected to modify breast cancer cells, and whether this is affected by ZOL, will be assessed in a panel of standard functional *in vitro* assays. Depending on levels of activin and follistatin, we will either use individual patient samples or pooled samples from patients in the same treatment group. MDA-MB-231 (ER-ve) and MCF7 (ER+ve) breast cancer cells will be cultured in 5-10% of the collected serum. Effects of the serum on proliferation (MTT assay), cell migration (scratch assay), adhesion (spheroid formation, adhesion to confluent monolayer) and chemotherapy-induced apoptosis (annexin V), will be assessed. All of these assays are run routinely in our laboratory. Experiments will be performed at least 3 times in triplicate using serum from 3 different patients and the results related to serum levels of activin/follistatin/TGF beta. Effects identified in the functional assays will be further investigated using reversal experiments where recombinant human activin, inhibin and follistatin (commercially available) are added to the cultures to identify factors driving the effects. The resulting data will show whether there is a difference in the ability of soluble factors in serum of pre- vs post-menopausal breast cancer patients to modify the aggressiveness of breast cancer cells and whether this is altered by ZOL.

## Proteomic analysis

Proteomic technology is evolving, with a number of proteomic methods available for use in analysing samples and the precise combination used in this study will depend on the initial data obtained. Examples of some of the techniques are listed below, but, in a typical procedure, the sample is immunodepleted of major proteins (such as albumin), which would otherwise interfere with the analyses of lower abundance proteins and then subjected to further laboratory preparation steps, before being analysed by mass spectrometry. The results will show the changes in protein expression profile between the groups of patients. The approaches that will be used include:

*Global protein profiling*: Several procedures exist for global protein profiling. One of the most commonly used techniques is label-free-quantification-mass-spectrometry (LFQ- MS) in which peptides are sequenced within the mass-spectrometer (to identify the proteins in question) and their levels determined by measuring the signal strength of each peptide. Following immunodepletion of the 14 most abundant proteins within serum/plasma LFQ-MS can identify and quantify hundreds of proteins within each serum / plasma sample. LFQ-MS can be applied to serum samples arising from the ZOLMENO protocol to identify alterations in circulating proteins as a participant progress through treatment. Comparison of these profiles between participants in the different groups of this study over time will be used to identify differences in circulating proteins, which would be potential markers of zoledronic acid response.

*Isobaric tagging for relative and absolute quantification (ITRAQ)* will also be used for quantification of protein expression, particularly for phosphoproteomics. ITRAQ reagents chemically label peptides providing quantitative read-out of peptide level in the mass spectrometer. The great advantage of using iTRAQ is the availability of 8-different isotopically labelled variants of the reagent (8-plex), thus 8 samples can be labelled, combined and all subsequent processing steps performed on the combined sample, eliminating concerns about technically variability within the comparison. 8-plex ITRAQ is

particularly useful when combined with phosphoproteomic enrichment for highly accurate assessment of phosphoprotein-levels within cells following different treatments and time-courses, for example.

*Targeted mass spectrometry (multiple-reaction monitoring - MRM):* Mass-spectrometry- based proteomics has recently advanced from being a tool for discovery of biologically- relevant proteins to one that can measure hundreds of previously identified proteins in a highly accurate, robust, rapid fashion within large numbers of patient derived samples. As such, targeted mass spectrometry is now another tool for biomarker validation. Multiple-reaction monitoring (MRM) accurately measures the level of up to a hundred proteins within a sample by precisely selecting intact peptides and measuring the signal from pre-defined fragments of the peptide formed within the mass spectrometer. By choosing appropriate combinations of both the intact peptide masses (and the fragments to record) absolute specificity for the target protein can be ensured. Up to a hundred different proteins can be accurately quantified in this way in one 1-hour run and thus this procedure can be applied to large numbers of patient derived samples. This procedure enables high throughput, highly accurate measurement of up to 100 pre- defined proteins within large numbers of patients derived samples and could be used to validate targets identified within the ZOLMENO study.

*Bioinformatics and Statistics*: These proteomic studies require considerable software analysis, both in terms of experimental design but also in terms of analysing the resulting data. The software required is either freely available online or already present within the university of Sheffield. Data from LFQ-MS can be extracted and analysed using MaxQuant (freely available online), iTRAQ data is planned to be analysed using ProteinPilot (also available within University of Sheffield) and peptides for measurement within MRM can be predicted by use of the free-software package Skyline. The University of Sheffield has access to biostatisticians for design, sample number determination and downstream analysis of the experimental results.

This methodology will yield the key proteins involved. Depending on the results, these may be investigated further, for example by use of commercially available antibodies through ELISA techniques. It is expected that these analyses will be performed in the specialised facilities in the University of Sheffield. Because we do not know in advance which are the key proteins, it is not possible to be more specific at this stage.

# Outcome Measures

Outcome measures will include assessment of the effect of ZOL in the different patient groups and also safety measures via assessment of patients for, and reporting of, adverse events. In order for these assessments to take place patient’s will have samples taken and be reviewed at the time-points indicated in the Visit Schedule below.

Outcome measures will be based on the following assessments:

* Study blood samples at each indicated time point:
  + Serum follistatin and activin
  + Cell free DNA and biomarkers
  + Serum and plasma for *in vitro* and proteomic studies
* Tumour samples processed for:
  + Immunohistochemical measurement of follistatin and activin
  + Histomorphometry of the tumour microenvironment
* Bone marrow samples processed for:
  + Genetic sequencing
  + Histomorphometry of bone marrow microenvironment
* Clinical assessment as outlined in sections 10.1, 10.3 and 10.4 below.

The primary outcome measure will be the change in serum follistatin level at day 28 post- ZOL administration.

The following secondary outcome measures, all compared relative to menopausal status (pre- vs. post-menopausal) and timing of ZOL administration (Group A vs. Group B), will include:

* Change in serum follistatin level at day 7 and 28 post-ZOL infusion
* Change in serum activin level at day 7 and day 28 post-ZOL infusion
* Change in serum follistatin level from day 0 (surgery) to day 21 and day 28 post-surgery
* Change in serum activin level from day 0 (surgery) to day 21 and day 28 post-surgery
* Exploratory analysis of changes in the levels of follistatin and activin in tumour samples
* Exploratory analysis of changes in the histomorphometry of the tumour microenvironment
* Exploratory analysis of differences in the genetic expression of bone marrow cells
* Exploratory analysis of changes in the histomorphometry of bone marrow

Tertiary outcomes, via exploratory analyses, also considered relative to menopausal status and timing of ZOL administration, will include:

* Differences in the effect of serum from participants on breast cancer cells *in vitro*, as measured by standard functional assays for migration, proliferation, adhesion and apoptosis
* Changes in the proteomic profiles of serum, plasma and, where available, bone marrow samples
* Changes in levels of biomarkers in the serum, bone and tumour microenvironment

## Visit Schedule

Patients will have contact with the research team, be reviewed and have samples taken at the time-points indicated in Table 2. Acceptable time windows are discussed below. The time between diagnosis and planned surgery (pre-surgical window) will be used to identify, screen, recruit and consent all patients, administer ZOL and perform bone marrow procedures in Group A (the pre-surgical ZOL group), and to assess the changes in serum, bone marrow and tumour biomarkers compared to a control group (which will comprise Group B, the post-surgical ZOL group). ZOL will be given 7 days prior to surgery (Group A) or 21 days after surgery (Group B) with serum sampling 7 days later. The time between surgery and commencing adjuvant treatment (the post-surgical window) will be used to administer ZOL to Group B, the post-surgical ZOL group, and assess the changes in serum biomarkers following surgery and following post-op ZOL.

All patients are followed up at day 28 post-ZOL administration regardless of their allocated group and this marks the end of their active involvement in the study. There is no indication to continue monitoring participants for side effects more than 28 days after administration of zoledronic acid as side effects associated with a single dose are very unlikely to occur after this time point.

Standard surgical care will remain under the relevant breast surgeon and at the end of active participation patients will go on to have standard adjuvant therapy as planned by the Medical

and Clinical Oncology team. This will include chemotherapy, endocrine therapy, radiotherapy and/or biological therapy as deemed clinically appropriate. Standard treatment will not be affected by participation in the study.

## Table 2. Schedule of Events

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Contact point** | **Breast clinic** | **Research clinic** | **Surgery** | **Research clinic** | **Research clinic** | **Research clinic** |
| ***Day*** | ***Day ≥-8*** | ***Day -7*** | ***Day 0*** | ***Day +21*** | ***Day +28*** | ***Day +49*** |
| Identifying patients | A+B |  |  |  |  |  |
| Trial information | A+B |  |  |  |  |  |
| Screening |  | A+B |  |  |  |  |
| Consent |  | A+B |  |  |  |  |
| Study blood samples |  | A+B | A+B | A+B | A+B | B |
| Zoledronic  acid |  | A |  | B |  |  |
| Tumour samples |  |  | A+B |  |  |  |
| Bone marrow  sample |  | +/-A | A+B |  | +/-B |  |
| Clinical assessment |  | A |  | A+B | A+B | B |
| Pre-ZOL  safety bloods  (+\- urine pregnancy  test) |  | A |  | B |  |  |
| Post-ZOL safety bloods |  |  | A |  | B |  |
| SAE reporting |  |  | A | A | A+B | B |

A= Group A, pre-surgical ZOL group, B= Group B, post-surgical ZOL group

## Acceptable Time Windows

In order to align with NICE guidelines surgery must take place within 31 days of diagnosis, therefore the pre-surgical window will be no more than 31 days. Patients will usually be seen within 7 days of diagnosis by the specialist nurses at a holistic ‘needs assessment’ breast clinic. This clinic, at which patients will be given information on the study, may take place at any time point in the pre-surgical window up to day 8 pre-surgery, after which point patients randomised to Group A, the pre-surgical ZOL group, would need to receive their study treatment.

Acceptable time windows for subsequent events are shown below. The breast surgery department at STH has confirmed that study patients can be highlighted within their system to prevent them from having their surgery brought forward or delayed by time periods outside of these windows.

## Table 3. Acceptable time windows and corresponding events

|  |  |  |  |
| --- | --- | --- | --- |
| **Day (D) relative** | **Corresponding Event** | **Acceptable** | **Essential Criteria** |

|  |  |  |  |
| --- | --- | --- | --- |
| **to surgery** |  | **Time Window** |  |
| **D-7** | Research clinic (Group A+B) | +/- 3 days (D-10 to D-4) |  |
| **D0** | Surgery (Group A+B) | +/- 3 days (D-3 to D+3) | Must be 5 and 9 days post-ZOL infusion |
| **D+21** | Research clinic (Group A+B) | +/- 3 days (D+18 to D+24) |  |
| **D+28** | Research clinic (Group A+B) | +/- 2 days (D+26 to D+30) | Must be 5 and 9 days post-ZOL infusion |
| **D+49** | Research clinic (Group B) | +/- 5 days (D+44 to D+54) |  |

In exceptional circumstances, minor changes in these times may be agreed by the Chief Investigator or her delegated investigator.

## Baseline Assessments

Following screening and written informed consent, the following baseline investigations (including safety assessments, and where required, study specific screening investigations) will be carried out (existing assessments may be used if within the specified time limits).

* FSH to determine menopausal status (results ≤28 days prior to randomisation acceptable) unless clear from clinical history
* Negative urine pregnancy test ≤72 hours prior to registration for the study and confirmation of the use of effective barrier contraception at the time of assessment if pre-menopausal
* Clinical biochemistry ≤9 days prior to administration of zoledronic acid:
  + Measured or calculated Glomerular Filtration Rate (GFR) ≥30 ml/min (Cockcroft and Gault formula, appendix 3)
  + Serum corrected calcium ≥2.2mmol/L
  + Serum magnesium and potassium within normal STH laboratory ranges
* Clotting screen ≤9 days prior to bone marrow sampling:
  + APTT 30.5 seconds
  + PT 13.2 seconds or INR <1.5
  + Platelets 100 x 109/L
* Dental history and basic oral and dental examination
* Medical history, physical examination and ECOG performance status score
* Documentation of any radiological or clinical assessment of nodal and metastatic status.

Any participant who subsequently becomes ineligible for participation as a result of these assessments will be withdrawn from the study and their number will be replaced as required during the recruitment period.

## Treatment and Follow-up Assessments

Participants will be assessed clinically for symptoms and toxicity at each contact point following administration of zoledronic acid. The Case Report Form (CRF) will include specific questions about expected side effects associated with zoledronic acid infusion, plus any other toxicity experience or side effects related to bone marrow sampling.

Assessment will include:

* Clinical assessment including ECOG score and vital signs (HR and BP)
* Adverse event reporting/toxicity assessment
* Medical and dental history
* Documentation of confirmed nodal status, hormonal receptor and HER2 status as they become available
* Clinical biochemistry ≤9 days following administration of zoledronic acid, including:
  + Calculated GFR (Cockcroft and Gault formula, appendix 3)
  + Serum corrected calcium
  + Serum magnesium and potassium

## End of trial

The end of the study is defined as the date of completion of the planned laboratory analysis of collected samples. Following the end of the study all remaining stored blood samples will be transferred to the Human Tissue Act compliant STH biorepository at the Royal Hallamshire hospital in order that they can be recovered for future translational research (patients will consent to this prior to participation in the study). It is not expected that any bone marrow trephine or tumour sample material will be left over at the end of the trial. If any tissue samples remain these will also be transferred to the STH biorepository in line with patient consent. The end of participant involvement in the study is discussed above in section 10.1.

# Safety Assessments

The reporting period for the ZOLMENO study will be from the date of administration of zoledronic acid up to 28 days thereafter. The recording, management and reporting of these events will be an agreed delegation to the investigators, research nurse or data manager and this will be documented in the Project Delegation Log in the Site File.

Suspected adverse reactions to drugs other than zoledronic acid will be reported to the appropriate competent authority as per the standard procedures for licensed drugs.

The Development Safety Update Report will be completed by the Chief Investigator and submitted to the MHRA on an annual basis. This may be delegated to the Trial Co-ordinator, in which case it will be checked and countersigned by the Chief Investigator.

## Adverse Events

An Adverse Event (AE) is defined as any untoward medical occurrence in a subject during the course of the trial. Pre-existing conditions, although they will be recorded, will not be regarded as AEs unless they worsen significantly. Recognised side effects of this widely used IMP, zoledronic acid, are well reported and are fully documented in the Summary of Product Characteristics, available as above. As such, data on non-serious adverse events occurring during the study period will not be collected except in the case of adverse events

of particular interest in this patient group as defined within the study CRF, on which they will be recorded following assessment for seriousness, expectedness and causality. Where possible, adverse events of interest will be described on the CRF by duration (start and end dates) and severity grade (1-4) and relationship to study drug using Common Toxicity Criteria for Adverse Events version 4.0 (published May 28th 2009, [http://www.eortc.be/services/doc/ctc/CTCAE\_4.03\_2010-06-14\_QuickReference\_5x7.pdf).](http://www.eortc.be/services/doc/ctc/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf))

## Serious Adverse Events

A Serious Adverse Event (SAE) will be defined as an AE which either

1. results in death
2. is life-threatening
3. requires hospitalisation or prolongation of existing hospitalisation
4. results in persistent disability or incapacity
5. consists of a congenital abnormality or birth defect

Important medical events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed in the definition above will also be considered serious. The investigator will determine the seriousness of each adverse event as per these criteria.

All participants will undergo breast cancer surgery as part of their standard care. This will involve hospitalisation and carries associated well-documented risks. The most common of these, which can be reliably attributed to the surgery rather than zoledronic acid administration, will be reported in the adverse events section of the CRF, but will be exempt from immediate reporting and formal SAE reporting. This will include treatment, assessment or hospitalisation related to breast surgery, including:

* + Elective hospital admission for surgery
  + Pain
  + Infection
  + Bleeding
  + Anaemia
  + Haematoma
  + Seroma
  + Lymphoedema
  + Reactions to anaesthetic drugs
  + Poor wound healing.

Any other SAE occurring in a participant from the point of administration of ZOL until 28 days later will be reported. These non-exempt SAEs will be promptly reported to the Sponsor within 24 hours of the investigator/clinical team becoming aware of the event. Information (seriousness, frequency, intensity, relationship to study drug, action and outcome) about these SAEs will be collected and recorded on the SAE Case Report Form. The Sheffield Teaching Hospitals SAE report form will be completed, signed by the Chief Investigator (CI) and faxed or emailed by a member of the research team as listed above within 24 hours of discovery of the event to the Sponsor (STH SAE reporting Fax number 0114 22 65937, email [sth.sae@nhs.net](mailto:sth.sae@nhs.net)). The SAE form will be sent without a signature if it is not possible to complete before the 24-hour timeframe and the form will be re-sent when the signature for

the CI is added. Further information on the event will be provided to the Sponsor when it becomes available using additional SAE report forms.

Any participants experiencing an ongoing serious adverse event (SAE) at the time of their last study visit (28 days following administration of ZOL), will continue to be followed up by the research team. This may take the form of further clinic visits, telephone follow-up or assessment of the patient at one of their routine appointments, depending on the nature of the event. Follow-up will be continued until the SAE resolves or stabilises, even if the patient is withdrawn from the study.

## Suspected Serious Adverse Reaction (SSAR)

A suspected serious adverse reaction (SSAR) is any adverse reaction that is classed as serious in nature and which is consistent with the SPC for the IMP (zoledronic acid). Additional information will be provided to the Sponsor as requested.

## Suspected Unexpected Serious Adverse Reaction (SUSAR)

A suspected unexpected serious adverse reaction (SUSAR) is any event which qualifies as an SAE and meets the criteria of being judged as possibly, probably or definitely related to study IMP and has a nature and/or severity of which is not consistent with the information about the medicinal product in question as set out in the summary of product characteristics, investigator brochure or IMP dossier for that product (i.e. it is ‘unexpected’).

A SUSAR will require expedited reporting as per the Clinical Trials Regulations. All serious adverse events that fall or are suspected to fall within these criteria shall be treated as a SUSAR until deemed otherwise.

All SUSARs will be reported by the sponsor to the MHRA and Research Ethics Committee (REC) and this responsibility will lie with the STH Research and Development department.. Fatal or life-threatening SUSARs will be reported within 7 days, and will be followed up with an additional report within a further 8 days. All other SUSARs will be reported as soon as possible to the MHRA and REC within 15 days at the latest.

## Causality

The investigator will determine the causality of each adverse event as defined below.

*Unrelated or unlikely*: a clinical event including laboratory test abnormality with temporal relationship to zoledronic acid administration, that makes a causal relationship incompatible or for which other drugs, chemicals or disease provide a plausible explanation. This will be counted as “unrelated” for notification purposes.

*Possible*: a clinical event, including laboratory test abnormality, with temporal relationship to zoledronic acid administration which makes a causal relationship a reasonable possibility, but which could also be explained by other drugs, chemicals or concurrent disease. This will be counted as “related” for notification purposes.

*Probable*: a clinical event, including laboratory test abnormality, with temporal relationship to zoledronic acid administration which makes a causal relationship a reasonable possibility, and is unlikely to be due to other drugs, chemicals or concurrent disease. This will be counted as “related” for notification purposes.

*Definite*: a clinical event, including laboratory test abnormality, with temporal relationship to zoledronic acid administration which makes a causal relationship a reasonable possibility,

and which can definitely not be attributed to other causes. This will be counted as “related” for notification purposes.

A SAE whose causal relationship to zoledronic acid is assessed by the Chief Investigator as “possible”, “probable” or “definite” will be considered ‘related’ and is an Adverse Drug Reaction.

## Withdrawal of Participants from the Main Analysis

Participants will be withdrawn from the study if:

* + - They are enrolled on the study but have not yet received ZOL treatment and they:
      * Develop active dental problems including dental abscess or infection of the jawbone or osteonecrosis of the jaw
      * Require or have dental or jaw surgery
      * Are of childbearing age and subsequently decline to use reliable and appropriate methods of contraception, or become pregnant
    - They develop significantly deranged clotting results which will not be corrected to within acceptable limits (as part of standard care) by the time of bone marrow sampling
    - The patient is inadvertently enrolled without meeting the eligibility criteria
    - They wish to cease participation in the study at any time

Documentation will record the reason(s) for withdrawal and whether this was at investigator’s or the patient’s request.

Patients who receive ZOL infusion but who do not have successful bone marrow sampling (for example due to surgical complications or failed procedure) will not be withdrawn from the study and will continue to have serum and tumour samples analysed, but an additional participant will be recruited to maintain the statistical power of the study.

## Pregnancies

There is no data available relating to the risks of zoledronic acid administration in pregnant patients. It is unlikely, however, that pregnancies occurring after administration would be affected in anyway. All pregnancies and suspected pregnancies occurring from the date of consent to 28 days following administration of zoledronic acid will be reported to the CCTC and the sponsor within 24 hours of the research team becoming aware. Protocol treatment will be stopped immediately if a pregnancy occurs or is suspected. If pregnancy occurs after the administration of zoledronic acid the participant will be allowed to continue participation in the study for follow-up and blood sampling. The CCTC will report all pregnancies occurring during treatment to the Sponsor along with any follow-up information. Any pregnancies will be followed up to determine outcome, including spontaneous or voluntary termination, details of birth, and the presence or absence of any birth defects, congenital abnormalities or maternal and newborn complications.

## Deaths

All deaths occurring from the date of randomisation to 28 days after the last patient has received zoledronic acid will be recorded and reported to the CCTC and the sponsor within

24 hours of the research team becoming aware of the death. In addition, suspected treatment-related deaths will be notified immediately to the CCTC via SAE or SUSAR CRFs in accordance with section 12.2 and 12.4 above.

## Premature Termination of the Study

Premature termination of the study may occur if

* there is evidence that the safety of the trial participants is no longer assured
* the study objectives can be answered distinctly after an intermediate evaluation
* the recruitment of trial participants is insufficient and the successful conclusion of the clinical trial does not seem to be possible

Any decision to prematurely terminate or temporarily halt the study will be made by the Trial Management Group in discussion with the study sponsor, STH.

In the event of premature termination or an unforeseen temporary halt of the study the Chief Investigator will provide a written statement stating the reason for the termination of the study and the sponsor will notify the REC and MHRA within 15 days of study closure if this relates to safety concerns, otherwise this will be within 90 days.

# Statistical Considerations

## Sample Size

The primary objective and outcome measure for this study is the change in serum follistatin 28 days after zoledronic acid administration. This variable has been chosen as the primary objective as it is the outcome which is most likely to address the research question and inform future research and clinical practice.

The only data (both published and unpublished) relating to zoledronic acid effects on hormone levels comes from unplanned analysis of samples from the ANZAC study conducted previously by this group (Winter, et al., 2013). In this retrospective analysis of follistatin and activin levels in 40 patients receiving chemotherapy plus zoledronic acid treatment for early breast cancer, compared to chemotherapy alone, there was a significant reduction in follistatin levels seen in the chemotherapy + ZOL group (n=20) at day 21 (p=0.004). The ZOLMENO study has been designed to confirm or refute the seemingly highly significant results of this unplanned analysis.

The preliminary data from the ANZAC study has been used to calculate a sample size using nQuery software. With our proposed sample size of 80 patients, the Wilcoxon Paired Signed Rank test will have 90% power to detect the difference between a null hypothesis mean of 0.0pg/ml and an alternative mean of -387.0pg/ml, assuming that the standard deviation is 970.80pg/ml. Should the data satisfy the normality assumptions required for the t-test, then, given the same parameters, a single group t-test with a 0.050 two-sided significance level will have an adjusted power of 94%.

The stipulated inclusion and exclusion criteria do not have any significant components which would result in this sample not being largely representative of the general population of female early breast cancer patients.

As this study involves administration of a single dose of ZOL, we do not anticipated issues with non-compliance. Any patients who drop-out prior to administration of ZOL will be replaced in number by an additional participant. Any patients who do not have bone marrow sampling performed at the time of surgery, for whatever reason, will also be replaced in number. This study will not involve crossover of participants. These considerations will minimise the risk of compromising the power of the study.

## Statistical Analysis

Statistical analysis will be performed using appropriate computer programmes. Data will be double entered by members of the data management team at the CCTC. The primary outcome measure is the change in serum follistatin from baseline to day 28 following ZOL infusion relative to menopausal status. This change will be calculated, where possible, using paired sample t-test for each patient. Independent sample t-test will be used to compare the change between groups (pre- vs post-menopausal and pre- vs post-surgery groups). These results will be presented with 95% confidence intervals and a p value of 0.050 will be used as the threshold for statistical significance. Any data which cannot be transformed to a normally distributed variable will be analysed using a suitable non-parametric alternative (e.g Wilcoxon Paired Signed Rank test, Mann Whitney U test). Analysis of variance (ANOVA) approaches will also be used for comparing data between the pre- and post-surgical group in relation to menopausal status.

The same methodology will be used to analyse changes in reproductive hormones, circulating tumour DNA and other serum and tumour biomarkers at the other time points specified in the study schedule.

The statistical analysis plan has been approved by a Medical Statistician (Mrs Kathleen Baster) via the STH Clinical Research Office. Data analysis will also be performed in consultation with these statisticians.

# Expertise of the Research Team

Sheffield is a global leader in bone oncology research and this research team includes internationally recognised experts in the field of both pre-clinical and clinical breast cancer and bone oncology. Significant experience has been accrued by members of the team in the development and running of large-scale national clinical trials. Preliminary data relating to this study has been gathered in previous research performed by members of this team and available expertise covers all aspects of the clinical and laboratory techniques stipulated in this protocol. All research team members regularly undertake GCP training. The support of those named in the protocol, in addition to colleagues within the relevant departments and the availability of facilities at the CCTC has all been confirmed.

# Project Management, Quality Control and Assurance

The ZOLMENO study will be conducted in accordance with GCP recommendations and relevant STH Standard Operating Procedures. Overall responsibility for this project will be shared between the Chief Investigator and the study sponsor (STH). The study will be supervised, monitored and reviewed by the sponsor, Sheffield Teaching Hospitals’ Research and Development Office (RDO), and the Cancer Clinical Trials Centre (CCTC). Auditing of the study will be conducted by STH as the study sponsor. The Data Management team at the CCTC will audit the site file and monitor CRFs against patient notes and against data entered into the study database. The database will have an integrity check prior to the start of the study and has an internal audit trail for all entries or changes.

A Trial Management Group (TMG) will meet every quarter (3 monthly) and ensure regular contact between the research team, the RDO and the CCTC. The TMG will assess trial

progress and address any issues arising, thus supporting the project in reaching its intended outcomes on time. Review and reporting mechanisms will include case report forms, SAE reporting forms and the study site file. A DMEC will not be used for this study as new safety concerns are very unlikely in a drug so widely used as zoledronic acid.

The Trial Management Group (TMG) will include: Prof Janet Brown, Chief-Investigator, Weston Park Hospital Consultant; the appointed Clinical Research Fellow as designated Trial Co-ordinator and Investigator; Professor Rob Coleman, Expert Adviser; and Professor Ingunn Holen, Scientific Lead and Investigator.

The wider research team, outlined in the Key Roles at the start of this document, have been consulted in the development stages of this study and have agreed to their relative responsibilities in relation to the ZOLMENO study. They will be invited to a start-up meeting prior to study initiation, and will be sent electronic study updates following the quarterly meeting of the TMG. Prior to and following the TMG quarterly meeting we will invite feedback from all those involved in the study to identify any issues that may need addressing.

Specifically, the following quality assurance activities will be conducted by the Trial Co- ordinator and the Data Management team:

1. Checking that there is an up-to-date trial Master File
2. Checking that investigators adhere to the current version of the protocol
3. Co-ordination and registration of all eligible patients
4. Maintenance of a central database of registered patients
5. Confirmation of each participant’s existence and review of source documents including 100% review of pathology reports, consent forms and eligibility criteria documents
6. Performing data entry (database) checks for validity and consistency
7. Querying missing or questionable data and chasing missing data until it is received, confirmed as unavailable or the trial is in analysis
8. Checking that CRFs are completed by authorised persons
9. Checking adverse events, including SAE/SUSARs are reported in accordance with the protocol and CRF
10. Reviewing recruitment rate during the recruitment period of the trial.

## Project plan

The following project timeline takes in to consideration the number of patients diagnosed with early breast cancer at STH, the likely recruitment rate based on previous studies conducted by this research team, and the availability of a named Clinical Research Fellow responsible for this study and with designated time allocated for data analysis, write-up and dissemination of results.

## Table 4. Project timeline

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Months** | **-6 to -1** | **0 to 6** | **7 to 26** | **27 to 33** | **33 to 38** |
| R&D approval, Ethical approvals |  |  |  |  |  |
| Recruitment and sample collection |  |  |  |  |  |
| Processing of serum, tumour and bone marrow samples |  |  |  |  |  |
| Gene expression studies |  |  |  |  |  |
| Proteomics and *in vitro* studies |  |  |  |  |  |
| Data analysis |  |  |  |  |  |
| Study write-up |  |  |  |  |  |
| Dissemination of results |  |  |  |  |  |
| Regular meetings between research team, RDO and CCTC |  |  |  |  |  |

1. **Ethical considerations**

This study will be performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964, and amended at the 52nd World Medical Association General Assembly, Edinburgh, Scotland, October 2000. The trial will be submitted to and approved by an NHS REC prior to entering participants into the trial.

## Good Medical Practice

This study will be conducted in accordance with the principles of GCP in clinical trials, as applicable under UK regulations, and the NHS Research Governance Framework (and Scottish Executive Health Department Research Governance Framework for Health and Social Care 2006 for studies conducted in Scotland). The CCTC and the sponsor have systems in place to ensure that serious breaches of GCP or the study protocol are identified and reported. Investigators are required to promptly notify the CCTC and sponsor of a serious breach (as defined in Regulation 29A of the Medicines for Human Use (Clinical Trials) Regulations 2004 [Statutory Instrument 2004/1031], as amended by Statutory Instrument 2006/1928) that they become aware of. A “serious breach” is a breach which is

likely to effect to a significant degree: (a) the safety or physical or mental integrity of the subjects of the trial; or (b) the scientific value of the trial. All breaches that could meet the criteria for being serious will be reported to the CCTC and the sponsor in accordance with the sponsor’s SOP for managing protocol non-compliance and management of serious breaches.

## Protocol Amendments

The protocol will be adhered to and any amendments will be made by a formal procedure to receive approval from the Ethics Committee that approved the original protocol prior to implementation.

## Informed consent

Full details on the process for obtaining informed consent are described in section 7.4. All patients will receive a detailed participant information sheet prior to attending the first research clinic. Informed written consent will be obtained from the participants prior to randomisation/registration into the study and prior to any study samples being taken. The right of a patient to refuse participation without giving reasons will be respected. The participant will remain free to withdraw consent at any time from the trial without giving reasons and without prejudicing her further treatment.

## Intervention

Blood, tumour and bone marrow samples will be taken by an appropriately trained member of research or surgical staff. Consent to use and store the samples will be obtained according to the Human Tissue Act 2004. Zoledronic acid will be prescribed by clinical members of the research team and administered by appropriately trained nursing staff.

Surgical samples will be obtained as part of routine care. No additional samples will be taken.

Venepuncture carries minimal risk for participants. A small number of patients may experience bruising, bleeding or pain at the site of venepuncture. A smaller number may feel faint during the procedure. Infection at the site of venepuncture is very rare.

Bone marrow sampling is also a low risk procedure. Aspiration in particular is very safe, with superficial bleeding being a small risk. Infection is very rare, as the puncture site is only minimally larger than that for venepuncture and significant bleeding is extremely rare. These risks are only slightly increased by trephine sampling, and are all minimised by operator experience. The main risk from bone marrow sampling is that of discomfort or pain. This will be minimised by performing the procedure under general anaesthetic. Those patients who agree to additional bone marrow sampling will have the procedure performed under local anaesthetic. Very few patients experience pain after bone marrow aspiration, with those that do feeling a mild bruised sensation at the site of sampling. Again, this risk is slightly increased by trephine sampling, but discomfort usually resolves within 24 hours of the procedure.

The side effect profile of zoledronic acid is outlined above in section 9.3, as are the approaches taken to minimise risk of ZOL related adverse events. The administration of this drug is not felt to confer a significant additional risk to participants.

## Confidentiality and storage methods

Confidentiality and security will be managed in accordance with Caldicott principles and the 1998 Data Protection Act. All research personnel who have participant contact and who are on University of Sheffield contracts of employment have STH honorary contracts.

Patient data will be anonymised using unique study numbers. The only identifying documentation will be the consent forms and CRFs. These will be stored separately to the Site File at the CCTC. All documents, including the Site File, will be kept in a locked drawer in an office that is locked out of hours. Electronic data will be stored in a password-protected database stored on the password protected hospital network. Data is maintained on a password-protected server which is kept in a locked cage in the CCTC and backed up hourly.

Consent forms will include permission to inform the participants GP of their involvement in the trial.

# Involvement of Service Users

This study has been reviewed by the Cancer Clinical Trials Executive (CTE) at STH. Patient representatives on this panel have been involved in the review of the protocol and the patient information sheet. The patient representative of the CTE panel have reviewed and approved the documentation prepared for dissemination of study results to participants.

Members of the surgical team including the Breast Care Cancer Nurses have been involved in the design of this study and the patient information sheet to ensure that the study schedule complements the standard patient journey and to minimise the chance of study components causing any patient distress.

# Archiving and Dissemination of Results

At the end of the study all paper and electronic data will be securely archived in line with the sponsor’s procedures for a minimum of 15 years.

It is expected that the results will be presented at national and international bone and cancer conferences. A manuscript detailing the results of the study will be prepared for submission to high impact peer review journals.

The dissemination of results to participants will be conducted in consultation with patient representatives of the CTE.

# Strategy for the Future

The results of this study will be used to inform future clinical practice in the care of breast cancer patients through the pre-selection of patients who will benefit most from the administration of adjuvant zoledronic acid. Data will contribute to the understanding of the mechanisms by which zoledronic acid has its beneficial effect and this will be used to develop further clinical studies and approaches to improve outcomes for patients who do not currently benefit from this treatment. This research team has a track record of successfully identifying, developing and obtaining funding for continuing research projects in this subject area.

# Intellectual Property

It is not anticipated that any intellectual property will arise from this study.

# Funding Source

This study has been funded by a grant from the charity Yorkshire Cancer Research in the form of a Clinical Research Training Award. Funding includes the following costs:

* Additional blood tests
* Bone marrow sampling equipment
* Pharmacy costs for zoledronic acid
* 155 hours of data management input
* Patient travel expenses for up to 4 additional visits
* Additional laboratory studies.

A named clinical research fellow has been appointed to this award and, under the direction of the Chief Investigator, they will have day-to-day responsibility for the study protocol and associated documentation, ethical approval, patient recruitment, consent, study visits, bone marrow sampling, data collection, data analysis and write-up, with assistance from research team members including research nurses, investigators and staff at the CCTC.

# References

Brown JE, Ellis SP, Lester JE, Gutcher S, Khanna T, Purohit OP, McCloskey EV, Coleman RE (2007), Prolonged efficacy of a single dose of the bisphosphonate zoledronic acid: implications for management of bone loss in cancer patients. *Clinical Cancer Research*, 13 pp. 5406-5410.

Bloise, E., Couto, H. L., & Massai, L. (2009). Differential expression of follistatin and FLRG in human breast proliferative disorders. *BMC Cancer 2009, 9:320 , 320* (9).

Cancer Research UK. (2014). *Cancer Research UK*. Retrieved October 7th, 2015 from <http://www.cancerresearchuk.org/health-professional/cancer-statistics/mortality/common-> cancers-compared#heading-Zero

Chen, T., J. Berenson, et al. (2002). "Pharmacokinetics and Pharmacodynamics of Zoledronic Acid in Cancer Patients with Bone Metastases." The Journal of Clinical Pharmacology **42**(11): 1228-1236.

Coleman, R. E. (2014). Adjuvant zoledronic acid in patients with early breast cancer: final efficacy analysis of the AZURE (BIG 01/04) randomised open-label phase 3 trial. *The Lancet Oncology , 15* (9), 997-1006.

Coleman, R., & McCloskey, E. (2011). Bisphosphonates in oncology. *Bone , 49*, 71-6.

Conte, P. and V. Guarneri (2004). "Safety of intravenous and oral bisphosphonates and compliance with dosing regimens." The oncologist **9 Suppl 4**: 28-37.

Early Breast Cancer Triallist’s Collaborative Group. (2015). Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *The Lancet , 386* (10001), 1353-1361.

Green, J. R. and M. J. Rogers (2002). "Pharmacologic profile of zoledronic acid: A highly potent inhibitor of bone resorption." Drug Development Research **55**(4): 210-224.

Hale, G., & Burger, H. (2009). Hormonal changes and biomarkers in late reproductive age, menopausal transition and menopause. *Best Practice & Research Clinical Obstetrics & Gynaecology , 23*, 7-23.

McAllister, S., Gifford, A., Greiner, A., Kelleher, S., Saelzler, M., Ince, T., et al. (2008). Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell , 133*, 994-1005.

National Institute for Health and Care Excellence. (2015, October 9th). *Menopause: diagnosis and management, NICE guideline, Draft for consultation June 2015.* From https://[www.nice.org.uk/guidance/GID-CGWAVE0639/documents/menopause-draft-](http://www.nice.org.uk/guidance/GID-CGWAVE0639/documents/menopause-draft-) guideline-nice2

Nicks, K., Perrien, D., Akel, N., Suva, L., & Gaddy, D. (2009). Regulation of osteoblastogenesis and osteoclastogenesis by the other reproductive hormones, Activin and Inhibin. *Molecular & Cellular Endocrinology , 310*, 11-20.

Ottewell, P. D., H. Mönkkönen, et al. (2008). "Antitumor Effects of Doxorubicin Followed by Zoledronic Acid in a Mouse Model of Breast Cancer." Journal of the National Cancer Institute **100**(16): 1167-1178.

Ottewell, P., Wang, N., Brown, H., Reeves, K., Fowles, C., Croucher, P., et al. (2014). Zoledronic acid has differetial anti-tumour activity in the pre- and post-menopausal bone microenvironment in vivo. *Clinical Cancer Research , 20* (11), 2922-32.

Reame, N., Lukacs, J., Olton, P., Ansbacher, R., & Padmanabhan, V. (2007). Differential effects of aging on activin A and it's binding protein, follistatin, across the menopause transition. *Fertiliy & Sterility , 88*, 1003-5.

Reid, D. M., J. Doughty, et al. (2008). "Guidance for the management of breast cancer treatment-induced bone loss: a consensus position statement from a UK Expert Group." Cancer Treatment Reviews **34 Suppl 1**: S3-18.

Reis, F. M., Cobellis, L., Tameira, L. C., & Anania, G. (2002). Serum and Tissue Expression of Activin A in Post-menopausal Women with Breast Cancer. *The Journal of Clinical Endocrinology & Metabolism , 87* (5), 2277–2282.

Rudland, P., Platt-HIggins, A., El-Tanani, M., Rudland, S. D., Barraclough, R., Winstanley, J., et al. (2002). Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. *Cancer Research , 62*, 3417-27.

Rosen, L. S., D. Gordon, et al. (2003). "Long-term efficacy and safety of zoledronic acid compared with pamidronate disodium in the treatment of skeletal complications in patients with advanced multiple myeloma or breast carcinoma: a randomized, double-blind, multicenter, comparative trial." Cancer **98**(8): 1735-1744.

Russell, R. G., N. B. Watts, et al. (2008). "Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy." Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA **19**(6): 733-759.

Walkington, L. C. (2011). Advances in management of bone disease in breast cancer. *Bone, 48* (1), 80-7.

Welt, C., McNicholl, D., Taylor, A., & Hall, J. (1999). Female reproductive aging is marked by decreased secretion of dimeric inhibin. *Journal of Clinical Endocrinology & Metabolism , 84* (1), 105-11.

Westbrook, J., D. Cairns, et al. (2016). "CAPG and GIPC1: Breast Cancer Biomarkers for Bone Metsasteses Development and Treatment." Journal of the National Cancer Institute 108(4).

Wilson, C., Holen, I., & Coleman, R. (2012). Seed, soil and secreted hormones: Potential interactions of breast cancer cells with their endocrine/paracrine microenvironment and implications for treatment with bone targeted agents. *Cancer Treatment Review , 38* (7), 877-89.

Winter, M. C., I. Holen, et al. (2008). "Exploring the anti-tumour activity of bisphosphonates in early breast cancer." Cancer Treatment Reviews **34**(5): 453-475*.*

Winter, M., Wilson, C., Sydalll, S., Cross, S., Evans, A., Ingram, C., et al. (2013). Neoadjvant chemotherapy with or without zoledronic acid in early breast cancer - a randomised biomarker evaluation. *Clinicla Cancer Research , 19* (10), 2755-65.

# Appendices

**Appendix 1. Data from preliminary studies:**

## ZOL reduces follistatin in tumour in a breast cancer

**xenograft model**

1. **ZOL reduces follistatin in bone in a**

**murine OVX model**

1. **ZOL reduces serum levels of**

**follistatin in breast cancer patients**

3.0

Area

\*\*\*

NS

80

\*

NS

Folliststin levels normalised to cell count (ng/ml)

3000

Follistatin

60

CT alone

CT +ZOL

Average score

Median follistatin +IQR (pg/ml)

2.5 2000

40

2.0

1000

20

1.5

0

Control (n=5) Zoledronic acid (n=6)

OVX control (n=5)

0

Baseline Day 5 Day 21

OVX zol (n=5)

sham control (n=5)

sham zol (n=5)

1. ***ZOL reduces follistatin expression in subcutaneous breast tumour xenografts in vivo.*** Animals with subcutaneous breast tumour xenografts were treated with ZOL (equivalent to the clinical dose) weekly for 6 weeks. Tumours were isolated and stained using an antibody specific for follistatin. There was a significant reduction in tumour area positive for follistatin in the ZOL treated group compared to control.
2. ***ZOL reduces bone follistatin levels exclusively in ovariectomised animals***. Mice that had undergone ovariectomy (OVX, mimicking post-menopausal bone) or sham (mimicking pre-menopausal bone) were treated with 100ug/kg ZOL weekly for 4 weeks. Animals were sacrificed and calvaria cut in pieces in a fixed volume of PBS for measurement of (mouse) follistatin in bone by ELISA. ZOL caused a significant reduction in bone follistatin levels compared to control only in OVX animals, supporting that ZOL modifies levels of follistatin in the post-menopausal bone microenvironment.
3. ***A single dose of ZOL reduces serum follistatin levels in breast cancer patients.*** In the neo-adjuvant ANZAC trial, breast cancer patients were given chemotherapy with/without addition of a single 4mg dose of ZOL. Serum follistatin levels were reduced in the group receiving ZOL compared to the chemotherapy alone group, demonstrating that ZOL reduces follistatin in breast cancer patients. By reducing follistatin levels, ZOL may cause an increase in the bio-available level of the tumour suppressor activin.
   * In pre-menopausal women, high inhibin levels neutralise activin and a reduction by ZOL has no effect.
   * In post-menopausal women, inhibin levels are negligible, follistatin determines the bio-availability of activin and a reduction in follistatin levels by ZOL will increase levels of tumour suppressing activin.

# Appendix 2. ECOG performance status classification

Developed by the Eastern Cooperative Oncology Group, Robert L. Comis, MD, Group Chair.\*

|  |  |
| --- | --- |
| GRADE | ECOG PERFORMANCE STATUS |
| 0 | Fully active, able to carry on all pre-disease performance without restriction |
| 1 | Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work |
| 2 | Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours |
| 3 | Capable of only limited self-care; confined to bed or chair more than 50% of waking hours |
| 4 | Completely disabled; cannot carry on any self-care; totally confined to bed or chair |
| 5 | Dead |

\*Oken M, Creech R, Tormey D, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group.*Am J Clin Oncol.* 1982;5:649-655.

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| **Appendix 3. The Cockcroft-Gault equation** |  | |
| Creatinine Clearance (mls/min) = (140-age) x weight (kg)  Serum Creatinine (mol/l) | x | 1.23 (male)  1.04 (female) |

*Reference: Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16(1):31-41.*