

Adjuvant bisphosphonates in early breast cancer

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The writing of this thesis is entirely the work of Emma Jane Rathbone. The Clinical Trials Research Unit (Leeds) contributed to the design of the oral health-related quality of life study (advice and consultation, chapter 2) and the statistical analysis of incidence of osteonecrosis of the jaw (Helen Marshall undertook the cumulative incidence curves, chapter 2) and serum biomarkers (Helen Marshall, Samantha Hinsley and Walter Gregory undertook all statistical analysis in chapter 3). Fatma Gossiel (Bone Metabolic Unit, University of Sheffield) ran several of the automated bone markers assays and supervised my work on the manual assay 1CTP (chapter 3).

My own contributions, fully and explicitly indicated in the thesis, have been literature searching and writing of chapter 1; literature searching for quality of life measurement tool, leading of design of the study, statistical analysis and interpretation of all questionnaire data in chapter 2. Contributions to study design, biomarker choice, laboratory assay work, including the manual 1CTP assay in 872 samples, interpretation of results and write up in chapter 3. Significant contributions to the set-up of the quantitative bone scan sub-

study (meetings with medical physics, nuclear medicine, research nurses), identification, recruitment and consenting of participants, co-ordinating scheduling of scans, cannulation and blood-sampling during scans, compilation, statistical analysis and interpretation of data for chapters 4 and 5.

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Publications relating to work undertaken in this thesis:

Peer-reviewed Original Papers

Safety of zoledronic acid and incidence of osteonecrosis of the jaw (ONJ) during adjuvant therapy in a randomised phase III trial (AZURE: BIG 01-04) for women with stage II/III breast cancer.

Coleman R, Woodward* E, Brown J, Cameron D, Bell R, Dodwell D, Keane M, Gil M, Davies C, Burkinshaw R, Houston SJ, Grieve RJ, Barrett-Lee PJ, Thorpe H. *Breast Cancer Res Treat.* 2011 Jun;127(2):429-38

Osteonecrosis of the jaw and oral health-related quality of life after adjuvant zoledronic acid: an adjuvant zoledronic acid to reduce recurrence trial subprotocol (BIG01/04).

Rathbone EJ, Brown JE, Marshall HC, Collinson M, Liversedge V, Murden GA, Cameron D, Bell R, Spensley S, Agrawal R, Jyothirmayi R, Chakraborti P, Yuille F, Coleman RE. *J Clin Oncol.* 2013 Jul 20;31(21):2685-91

Adjuvant zoledronic acid in patients with early breast cancer: final efficacy analysis of the AZURE (BIG 01/04) randomised open-label phase 3 trial.

Coleman R, Cameron D, Dodwell D, Bell R, Wilson C, Rathbone E, Keane M, Gil M, Burkinshaw R, Grieve R, Barrett-Lee P, Ritchie D, Liversedge V, Hinsley S, Marshall H; AZURE investigators. *Lancet Oncol.* 2014 Aug;15(9):997-1006.

Associations Between Serum Bone Biomarkers in Early Breast Cancer and Development of Bone Metastasis: Results from the AZURE (BIG01/04) Trial.

Brown J, Rathbone E, Hinsley S, Gregory W, Gossiel F, Marshall H, Burkinshaw R, Shulver H, Thandar H, Bertelli G, Maccon K, Bowman A, Hanby A, Bell R, Cameron D, Coleman R. *J Natl Cancer Inst.* 2018 Feb 7. doi: 10.1093/jnci/djx280. [Epub ahead of print]

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Management of cancer treatment-induced bone loss.

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Prevention and treatment of bone metastases.

Woodward* EJ, Coleman RE. *Curr Pharm Des.* 2010;16(27):2998-3006.

*Denotes manuscripts written in my maiden name, Woodward.

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Abstract

Breast cancer is commonly associated with bone metastases, with approximately 70% of patients dying from breast cancer having radiological evidence of skeletal involvement. Median survival after diagnosis on bone metastases can be 2-3 years and therefore patients are at a high risk for the development of skeletal-related events. Consequently, research in both the laboratory and the clinic has addressed the potential for bone targeted agents to reduce the risk of developing skeletal metastases. The AZURE clinical trial is an international randomised phase III clinical trial that recruited 3360 early breast cancer patients in which participants received either 19 doses of zoledronic acid (ZOL) in 5 years or observation. No other clinical trial has undertaken such an intensive schedule of adjuvant bisphosphonates and therefore the safety and longer term sequelae were imperative to investigate should the drug become a standard of care.

This thesis describes sub-studies undertaken in AZURE participants to investigate i) the incidence of osteonecrosis of the jaw (a recognised complication of bisphosphonates) and oral health-related quality of life and ii) a quantitative bone scanning technique to describe the effects the intensive schedule of zoledronic acid on bone remodelling and how this changes with time. Finally, the use of bone-related biomarkers (1,25-OH vitamin D, P1NP, CTX and 1CTP) measured in serum collected at baseline (before commencing zoledronic acid) have been investigated for their prognostic and predictive potential.

The principal findings described in this thesis are: i) relatively low rate of osteonecrosis of the jaw (2.1%) with no significant impact on oral health related quality of life; ii) patients with elevated bone turnover markers at baseline are at increased risk of bone metastasis but these markers cannot be used to identify patients who will benefit from zoledronic acid; iii) bone turnover continues to be significantly suppressed in the axial skeleton 2 years after the cessation of zoledronic acid. The quality of the safety data presented in this thesis has contributed to the introduction of bisphosphonates into standard practice in the UK and across the globe.

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List of Abbreviations

AAOMS	The American Association of Oral and Maxillofacial Surgeons
AE	Adverse event
AI	Aromatase inhibitor
ALP	Alkaline phosphatase
ASCO	American Society of Clinical Oncology
ATP	Adenosine triphosphate
AUC	Area under the curve
AZURE	Does Adjuvant Zoledronic acid reduce recurrence in patients with high-risk, localised breast cancer?
BALP	Bone alkaline phosphatase
BCS	Breast-conserving surgery
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body Mass Index
BoHFAB	Bone Health in breast cancer survivors following adjuvant bisphosphonate therapy
BP	Bisphosphonate
BSP	Bone sialoprotein
CAPG	Macrophage-capping protein

CI	Confidence interval
CIF	Cumulative Incidence Function
CMF	Cyclophosphamide Methotrexate 5' Fluorouracil
CTC	Common Terminology Criteria
CTIBL	Cancer treatment-induced bone loss
CTRU	Clinical Trials Research Unit
CTX	C-terminal cross-linked telopeptide of type I collagen
CV	coefficient of variance
DCIS	Ductal carcinoma in situ
DFS	Disease-free survival
DPD	Deoxypyridinoline
DXA	Dual-energy x-ray absorptiometry
EBCTCG	Early Breast Cancer Trialists Collaborative Group
ELISA	Enzyme-linked immunosorbent assay
EORTC	European Organisation for Research and Treatment of Cancer
ER	Oestrogen receptor
ESMO	European Society of Medical Oncology
ESP	European Spine Phantom
FISH	Fluorescent in situ hybridisation
FN	Femoral neck
FPPS	farnesyl diphosphate synthase

GIPC1	PDZ domain-containing protein
GFR	Glomerular filtration rate
GnRH	gonadotrophin-releasing hormone
HER2	human epidermal growth factor receptor 2
HMDP	Hydroxymethylene diphosphate
HPLC	High-performance liquid chromatography
H-QoL	Health-related quality of life
HR	Hazard ratio
i.v.	intravenous
IDFS	Invasive disease-free survival
IL-1	Interleukin-1
IQR	interquartile range
LCIS	Lobular carcinoma in situ
LHRH	Luteinising hormone releasing hormone
LS	Lumbar spine
MDP	Methylene diphosphate
MM	Multiple myeloma
MMP	Matrix metalloproteinase
N-BP	Nitrogen-containing bisphosphonate
NICE	National Institute for Health and Care Excellence
nm	nanometre

NOS	Not otherwise specified
NPI	Nottingham Prognostic Indicator
NTX	N-terminal cross-linked telopeptide of type I collagen
OC	Osteocalcin
OHIP-14	Oral Health Impact Profile 14
OH-QoL	Oral health-related quality of life
ONJ	Osteonecrosis of the jaw
OPG	Osteoprotegerin
OS	Overall survival
p.o.	per oral
P1CP	procollagen type I C-propeptides
P1NP	Procollagen type I N-propeptides
pathCR	Pathological complete response
PR	Progestogen receptor
PS	Performance status
PTH	Parathyroid hormone
PYD	Pyridinoline
QA	Quality assurance
QBS	Quantitative bone scan
RANK	Receptor Activator of Nuclear factor-Kappa B
RANKL	Receptor Activator of Nuclear factor-Kappa B ligand

RFS	recurrence-free survival
RIA	Radioimmunoassay
ROI	Region of interest
rpm	revolutions per minute
RR	Relative risk
sBMD	Standardised BMD
SD	Standard deviation
SERM	Selective oestrogen receptor modulator
SNB	Sentinel lymph node biopsy
SOP	Standard Operating Procedure
SRE	Skeletal-related event
TH	Total hip
TNF	Tumour Necrosis Factor
TRAP 5b	Tartrate-resistant acid phosphatase type 5b
UVB	Ultraviolet B
VDR	Vitamin D receptor
WB	Whole body
WBR	Whole body retention
WHO	World Health Organisation
ZA	Zoledronic acid
ZOL	Zoledronic acid

Preface

The work for this thesis commenced in 2010 thanks to the encouragement and support of Professor Robert Coleman, Chief Investigator of the international, randomised phase III trial AZURE, a study designed to determine whether adjuvant zoledronic acid with (neo)adjuvant chemotherapy and/or (neo)adjuvant endocrine therapy is superior to (neo)adjuvant chemotherapy and/or(neo)adjuvant endocrine therapy alone in improving the disease-free and bone metastasis-free survival of stage II/III breast cancer patients. At the time, Professor Janet Brown was working in Leeds where I was an Academic Clinical Fellow in Medical Oncology exploring opportunities to undertake a higher research degree, preferably clinical research. She nurtured those interests and introduced me to Professor Coleman.

In collaboration with the Clinical Trials Research Unit (Leeds) and The Cancer Clinical Trials Centre (Sheffield) I have become experienced in the design, conduct and analysis of clinical trials, translation medicine, laboratory techniques and the preparation of manuscripts for peer-reviewed publication. The time spent in research has been immensely rewarding and afforded me valuable skills that I would not have otherwise have developed.

Life has changed a great deal since I embarked on this project, with 2 children and the appointment as a full-time NHS Consultant in Medical Oncology at Calderdale and Huddersfield NHS Foundation Trust, treating lung and pancreatico-biliary cancers. It has been with a great deal of dedication and support that I have managed to complete this thesis.

1 Introduction

1.1 Breast cancer

1.1.1 Background

Breast cancer is the most frequently diagnosed cancer among women in both the developed and developing world, representing 23% of all female cancers²⁻⁴. Within in the UK, there were nearly 55,000 new cases of female breast cancer and 11,433 deaths from female breast cancer in 2014⁵. While incidence of breast cancer has increased for a number of reasons, survival is also improving, with around 85% of women in England surviving their disease for 5 years or more⁵.

1.1.1.1 Pathology

Malignant tumours of the breast are overwhelmingly of epithelial origin. This thesis will only deal with invasive breast carcinoma but it is acknowledged here that non-invasive states (ductal and lobular carcinoma in situ, DCIS and LCIS, respectively) and tumours arising from other tissues including lymphomas and sarcomas exist. The majority of invasive breast carcinomas are classified as invasive ductal carcinoma, not otherwise specified (NOS), comprising a heterogeneous group of tumours that do not exhibit sufficient characteristics to be otherwise histologically classified⁶. Less common types include lobular, tubular, mucinous, inflammatory and medullary carcinomas.

However, it is well-recognised that the heterogeneity of breast cancer goes far beyond the traditional pathological staging and grading systems, as evidenced by the great variation in clinical behaviour. A greater understanding of the underlying biology of the disease has enabled some explanation as to the varying nature of the disease and progress in this area continues to be made⁷. Now widely established, the oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) confer both prognostic and predictive value and are routinely examined on pathological specimens by either immunohistochemistry or fluorescence in situ hybridization (FISH). ER has been identified as a hormone-regulated nuclear transcription factor that is present in two forms, α and β . The binding of oestrogen induces the expression of a number of genes including the progesterone receptor (PR)⁸. This signalling pathway has been exploited for therapeutic benefit through the development of drugs that antagonise the binding of oestrogens. For example tamoxifen, a selective oestrogen receptor modulator (SERM) substantially reduces recurrence rates in the first 10 years following diagnosis (relative risk (RR) 0.53 during years 0–4 and RR 0.68 during years 5–9; $p < 0.00001$)⁹. The drug also reduces breast cancer mortality by approximately a third at 15 years follow up. Aromatase inhibitors (AIs) were later developed to inhibit peripheral oestrogen biosynthesis and provide an alternative in post-menopausal women and have shown superiority over tamoxifen. For example, a meta-analysis of AIs versus tamoxifen reported a disease recurrence absolute risk reduction of 2.9% for the newer drugs in post-menopausal ER positive women in the adjuvant setting¹⁰.

The HER2 gene encodes a transmembrane tyrosine kinase receptor that is over expressed in 20-25% of breast cancers¹¹. Over expression of the gene is associated with a number of oncogenic processes including cell proliferation, angiogenesis and reduced apoptosis¹². The overexpression of the gene can be determined by immunohistochemistry performed on formalin-fixed paraffin-embedded tissue samples or, following equivocal or inconclusive results, FISH. Patients with HER2 positive breast cancer are considered to have a poorer prognosis, many clinical studies reporting HER2 gene amplification as an independent prognostic factor, including patients with node negative disease^{12, 13}. Like ER, HER2 is also a predictive marker, identifying patients who are likely to respond to targeted treatments. Trastuzumab is a monoclonal murine humanised antibody that binds to the extracellular portion of the HER2 receptor and is approved for treatment in both the adjuvant and metastatic setting. Since the work described in this thesis commenced, new anti-HER2 therapies have been developed including pertuzumab (anti-HER2 humanised monoclonal antibody that inhibits receptor dimerization), trastuzumab emtansine (conjugate of trastuzumab with the microtubule inhibitory agent TDM1) and lapatinib (oral anti-HER2 and anti-HER1 tyrosine kinase inhibitor)¹⁴.

Perou et al proposed that breast tumours could be further classified into molecular sub-types, including the separation of ER positive tumours, according to different gene expression profiles and that these correlate with different clinical outcomes^{15, 16}. These 5 initial sub-types (basal, ERBB2+, luminal A, luminal B and normal) were determined by hierarchical clustering

on patterns of expression of over 500 genes showing that luminal A breast cancer patients had considerably longer survival outcomes compared with basal and ERBB2+ subtypes^{16, 17}. The molecular sub-types have also been shown to respond differently to neoadjuvant chemotherapy in terms of pathological complete response rate (pathCR; 34% with triple negative tumours, 8% with low grade ER positive tumours)¹⁸. However, the molecular subtypes were not independent of more conventional predictors of response, including ER status.

Assessment of molecular sub-types did not easily translate into routine clinical practice and therefore surrogate biological sub-types determined using standard immunohistochemistry markers (ER, PR, HER2, Ki67) were proposed^{19, 20}. These sub-types have shown prognostic significance for OS and DFS in addition to predictive value for response to chemotherapy, and represent a convenient, if not identical, approximation^{19, 21}.

1.1.1.2 Staging

Prognosis and management of breast cancer depends particularly on the stage of the disease (figure 1-1) in addition to other factors including histological grade, hormone receptor status, human epidermal growth factor receptor (HER2) status, menopausal status and co-morbidities²². Initial stage of disease is significant in terms of overall survival. For example, in England, more than 90% of patients with a stage I cancer are expected to live for 5 years of more while this figure drops to around 50% for those diagnosed at stage III (figure 1-2)⁵. Asymptomatic distant metastases from breast cancer

are rare and therefore routine full radiological staging is not frequently undertaken.

Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Tx Primary tumour cannot be assessed

T0 No evidence of primary tumour

Tis Carcinoma in situ

T1 Tumour ≤ 20 mm in greatest

T1mi Tumour ≤ 1 mm in greatest dimension

T2 Tumour > 20 mm but ≤ 50 mm in greatest dimension

T3 Tumour > 50 mm in greatest dimension

T4 Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)

* T1 includes T1mi

** T0 and T1 tumours with nodal micrometastases only are excluded from Stage IIA and are classified Stage IB

Nx Regional lymph nodes cannot be assessed

N0 No regional lymph node metastases

N1 Metastases to movable ipsilateral level I, II axillary lymph node(s)

N2 Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected* ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases

M0 No clinical or radiographic evidence of distant metastases

Mx Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

Figure 1-1 Breast cancer staging system (7th Edition TNM) AJCC

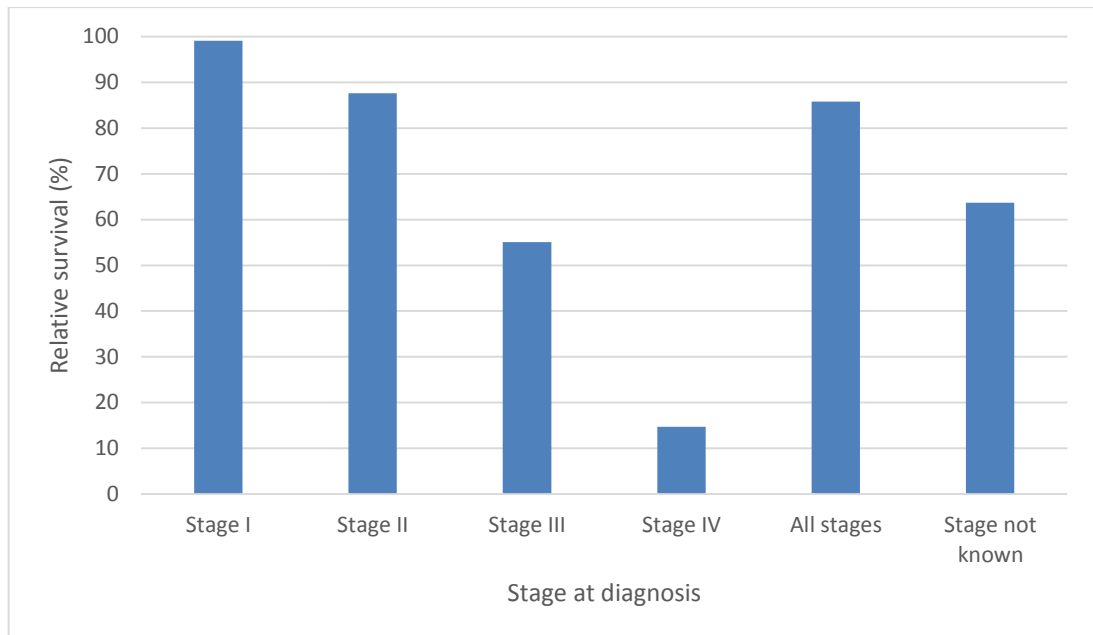


Figure 1-2 Five-Year Relative Survival (%) from breast cancer by Stage, Adults Aged 15-99, CRUK website⁵

1.1.1.3 Management

Management of early stage breast cancer typically includes a combination of surgery, radiotherapy and systemic therapies, the latter comprising cytotoxic chemotherapy, endocrine treatments and other targeted drugs.

1.1.1.3.1 Surgery

In 2009 the British Association of Surgical Oncology (BASO) produced comprehensive guidelines on the surgical management of breast cancer which have been widely endorsed and adopted into local policy²³. Regarding operable primary breast cancer, surgery aims to achieve local control of the

tumour and regional lymph nodes, either by mastectomy or breast-conserving surgery (BCS) followed by adjuvant radiotherapy. Randomised trials confirm that survival outcomes for either approach are equivalent for tumours up to 2cm in maximum diameter, with no significant differences up to 22 years follow-up²⁴⁻²⁷. The surgical team must additionally assess the axillary lymph nodes, either by sampling, clearance or sentinel lymph node biopsy (SNB)²³. SNB is often the preferred method for clinically node negative disease as it allows accurate assessment of the axilla with lower rates of morbidity²⁸.

Surgery does also play a role in the management of breast cancer that is not operable from the outset however, in the case of locally advanced disease, systemic therapy may be the most appropriate initial therapy. Where the disease is already metastatic at presentation, surgery is rarely indicated.

1.1.1.3.2 Radiotherapy

Radiotherapy plays an important adjuvant role in the management of early breast cancer. Women who have undergone BCS with clear margins require breast radiotherapy to reduce the risk of local recurrence. The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) undertook a meta-analysis of 17 randomised trials of BCS alone versus BCS with adjuvant radiotherapy, reporting that radiotherapy reduces both the risk of local recurrence and the risk of death from breast cancer²⁹. NICE guidelines also recommend offering chest wall radiotherapy to patients who are at high risk for recurrence for local recurrence (4 or more positive axillary lymph nodes

or involved resection margins). In addition, radiotherapy to the supraclavicular fossa should be offered if 4 or more positive axillary lymph nodes, or 1 – 3 positive axillary lymph nodes with another poor prognostic factor, such as a T3 or grade 3 tumour. This guidance is supported by the EBCTCG meta-analysis of 5-year local recurrence risks and mortality in 8500 early breast cancer patients who underwent mastectomy and chest wall or regional lymph node radiotherapy³⁰. Local recurrence rates and breast-cancer mortality rates were significantly reduced in the groups receiving radiotherapy (6% versus 23% and 54.7% and 60.1%, respectively).

1.1.1.3.3 Systemic therapies

Standard adjuvant systemic therapies for early breast cancer include a combination of traditional cytotoxic chemotherapy, hormonal therapy and biological agents and must take predictive and prognostic factors, menopausal status, side effects and performance status into account.

Post-menopausal women with ER-positive disease should be offered treatment with an aromatase inhibitor (either anastrozole, exemestane or letrozole) unless considered low risk according to the Nottingham Prognostic Index (NPI; figure 1-3). Those considered at low risk should receive tamoxifen for 5 years. Premenopausal women should be offered tamoxifen in the first instance. However, NICE recommends that premenopausal women who have been offered chemotherapy, but declined it, may be offered ovarian ablation/suppression in addition to tamoxifen.

NPI = [0.2 x S] + N + G	
Where:	
S is the size of the index lesion in centimetres	
N is the node status: 0 nodes = 1, 1-4 nodes = 2, >4 nodes = 3	
G is the grade of tumour: Grade I =1, Grade II =2, Grade III =3	
Score	5-year survival
>/=2.0 to </=2.4	93%
>2.4 to </=3.4	85%
>3.4 to </=5.4	70%
>5.4	50%

Figure 1-3 Nottingham Prognostic Index formula and interpretation¹

The treating clinician must make an estimate of benefit from cytotoxic chemotherapy based on the patient's age and underlying prognosis. Tools are now available to assist in this decision such as Predict and Adjuvant! Online, calculating risk of relapse and death based on the EBCTGC meta-analyses. More recently, gene expression profiles that can provide additional prognostic and/or predictive information are available. An anthracycline-based regimen is used routinely, however taxanes (docetaxel) are now

recommended to be offered as part of the adjuvant regimen for patients with positive lymph nodes³¹.

Finally, trastuzumab should be offered to women with HER2-positive early breast cancer following their other adjuvant therapies for 1 year, or until disease recurrence, unless there are significant cardiovascular contraindications, while the combination of trastuzumab and pertuzumab is now used in the neoadjuvant setting³².

1.2 Normal bone physiology

1.2.1 Composition of bone

Bone is a highly specialised tissue combining rigidity for support and protection with a dynamic biological environment that allows for mineral and acid-base balance, blood forming and repair³³. Additionally, it acts as a reservoir for numerous growth factors and cytokines. There are 2 types of bone: cortical and trabecular. Cortical bone is dense and solid, surrounding the inner bone marrow, while trabecular bone is a honeycomb-like network within the bone marrow compartment.

At a cellular level, bone is composed of 3 main cell types: osteoblasts, osteoclasts and osteocytes. These cells lie within the osteoid, an organic matrix of collagen and non-collagenous proteins and inorganic mineral salt deposits.

1.2.2 Bone remodelling

Bone constantly undergoes a process of remodelling in response to biomechanical forces, removing old, microdamaged bone (resorption) and replacing it with new, mechanically stronger bone (formation)³³. This dynamic tissue is composed of an inorganic component and an organic matrix, largely comprised of collagen. Within the organic network, the primary bone cells, osteoblasts and osteoclasts, are constantly remodelling bone through this coupled process, allowing bone to meet its mechanical needs in addition to the regulation of calcium and phosphate homeostasis (figure 1-4).

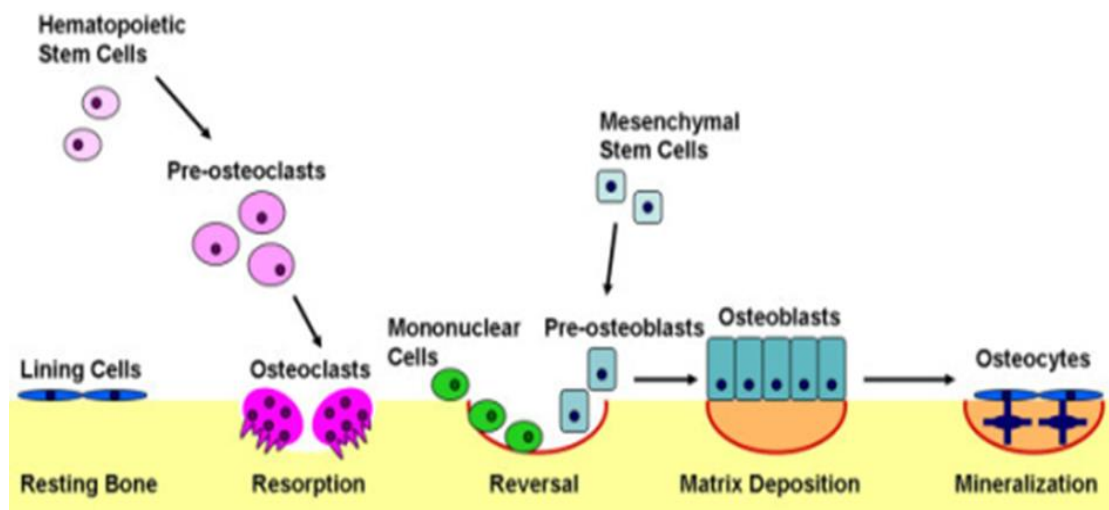


Figure 1-4 Drawing of bone remodelling³⁴

1.2.2.1 Osteoclasts

Osteoclasts are principally responsible for bone resorption and are closely related to macrophages, derived from mononuclear myeloid lineage. They are large, multinucleated cells found on the surface of bone with the unique capacity to degrade and remove bone. The process of resorption commences with the proliferation and differentiation of osteoclast precursors, allowing mature cells to fuse with and adhere to the bone surface, the site of bone resorption. Pits, or “basic multicellular units”, are formed on the bone surface³⁵. The process of resorption itself involves the secretion of acidic substances and lysosomal enzymes followed by apoptosis. Their key role in the process of bone resorption makes osteoclasts a principal target for the treatment of resorptive bone diseases such as osteoporosis and metastatic bone disease.

Regulation of osteoclasts begins with the recruitment and stimulation of osteoclast precursor in bone remodelling sites³⁶. Several steps in the resorption process are under the influence of the Receptor Activator of Nuclear Factor κ -B (RANK), a member of the tumour necrosis factor (TNF) superfamily, found on the surface of osteoclast precursors, chondrocytes and mature osteoclasts³⁷. The principal ligand for RANK, RANKL, is expressed by bone marrow stromal cells, activated T-cells and osteoblasts and is essential for promoting osteoclastogenesis, committing a precursor to the osteoclast phenotype³⁸. The binding of RANKL to RANK induces osteoclast differentiation, fusion and formation of mature osteoclasts, increases their activity and blocks apoptosis. Osteoclasts are further

regulated by osteoblasts through the production of osteoprotegerin (OPG) by the latter cell. OPG, also a member of the TNF family, is the decoy receptor for RANKL, blocking the RANKL-RANK interactions and the aforementioned processes³⁹. The RANK/RANKL/OPG pathway is an integral component of bone turnover, regulated by several cytokines and chemokines secreted within the bone microenvironment, including parathyroid hormone (PTH), interleukin-1 (IL-1), vitamin D derivatives and TNF- α ⁴⁰.

1.2.2.2 Osteoblasts

Osteoblasts, originating from mesenchymal stem cells of the bone marrow stroma, are the other key player in bone remodelling, forming new bone to fill the pits left by the osteoclasts. Osteoblast differentiation is promoted through the activation of the canonical Wnt signalling pathway, a branch of the ubiquitous wnt signalling system that is a key regulator of many developmental, physiological and pathological processes, including renewal of bone⁴¹⁻⁴³. Activated through factors secreted by osteoclasts they lay down osteoid that becomes mineralized and results in new bone⁴⁴. This bone formation process requires the osteoblast to secrete type I collagen, enzymes such as alkaline phosphatase (ALP) and non-collagenous proteins including bone sialoprotein (BSP)⁴⁵. Eventually, some of these osteoblasts become embedded in the osteoid and mature into terminally differentiated osteocytes. Osteocytes form cytoplasmic extensions that play an important role in the regulation of bone remodelling in response to mechanical forces⁴⁶. Any remaining osteoblasts undergo apoptosis⁴⁷.

The osteoclasts, osteoblasts and osteocytes in combination with the mineralized matrix and other cell types comprise the bone microenvironment and is under the control of many local and systemic factors. In relation to Paget's theory of metastasis, this environment provides a fertile "soil" for cancer cells, allowing them to survive, grow and expand⁴⁸. Tumour cells secrete a great variety of proteins that interact with the local cells and pathways, increasing resorption and releasing further growth factors into the system. This in turn feeds tumour growth in the so-called "vicious cycle"⁴⁹. An understanding of these interactions is crucial to the development of bone-directed therapies with many emerging treatments exploiting the vast array of potential targets⁵⁰.

1.2.3 Markers of bone turnover

The processes bone resorption and bone formation release a number of measurable factors that give an indication of current status of bone turnover. They may be either by-products of the process or secreted by the principal cells involved.

1.2.3.1 Bone resorption markers

Many of the measurable bone resorption markers are collagen degradation products. Type I collagen is the predominant collagen in bone and is composed of a triple helix of two α_1 chains and one α_2 chain, with proline and hydroxyproline accounting for approximately 25% of the total amino acid residues⁵¹. Urinary hydroxyproline was one of the first biochemical markers

used to monitor bone resorption rates in humans and animals⁵². However, its value is significantly limited due to several other sources of the peptide in addition to bone resorption, such as diet and turnover of soft connective tissues. Furthermore, much of the free hydroxyproline from collagen degradation is oxidised in the liver, further decreasing its usefulness. Hydroxyproline is additionally affected by age and circadian rhythm, with its peak excretion after midnight⁵³.

Pyridinoline (PYD) and deoxypyridinoline (DPD) cross-linking amino acids of collagen are also excreted in the urine during bone resorption and can be assayed relatively easily by high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA), providing some value in research and clinical situations. For example, multiple myeloma (MM) patients have significantly higher levels of PYD and PDP compared to healthy adults ($p < 0.0001$)⁵⁴. PYD and PDP levels are not affected by dietary collagen or metabolic degradation⁵³. However, while bone is the major reservoir of these molecules, the contribution from soft tissues does again lessen their accuracy and therefore their practical value⁵⁵.

N-terminal cross-linked telopeptide of type I collagen (NTX) and C-terminal cross-linked telopeptide (CTX) are peptides derived from bone collagen degradation. The NTX peptide exists in α_1 and α_2 isoforms, with the latter primarily derived from bone⁵⁶. The CTX peptide also exists in 2 isoforms, α and β , the latter primarily derived from bone. Assays developed using an antibody specific for the NTX α_2 chain can measure levels in either urine or

serum, however the urinary results must be adjusted for urinary dilution. The precision of the CTX assay to detect measurements lower than 200µg/L is poor so serum or plasma samples are preferable⁵⁵. Urinary NTX levels are able to identify individuals with bone metastases across a number of different tumour sites^{57, 58}. Perhaps clinically more useful however, is the ability of bone marker data to provide valuable prognostic information, which has been evaluated across many tumour sites. For example, high levels of urinary NTX in patients with bone metastases secondary to prostate cancer are indicative of an increased relative risk (RR) for disease progression (RR = 2.2; 95% CI 1.48, 2.74; p<0.001) and death (RR = 4.59; 95% CI 2.82, 7.46; p<0.001)⁵⁹. NTX and CTX are bone-specific and therefore not affected by diet, however are affected by circadian rhythm.

C-telopeptide cross-linking domain of type I collagen (1CTP), released during collagen degradation, can also be detected in the serum by immunoassay, correlating well with bone resorption⁶⁰. 1CTP is released by the action of matrix metalloproteinases (MMPs) and current assays are insensitive to physiological changes in bone turnover, such as those induced by oestrogen⁵⁵. Similar to the above markers, serum 1CTP does also display a circadian rhythm, with levels 20% higher between the hours of 0200 - 0550⁶⁰.

Tartrate-resistant acid phosphatase type 5b (TRAP 5b) is a specific marker for osteoclast number and activity that can be analysed in serum samples⁶¹. Expressed in high amounts by osteoclasts following attachment to the bone

surface, TRAP 5b is released into the circulation where it is inactivated and degraded. Therefore, catalytically active levels of the circulating enzyme reflect recently released enzyme as a result of bone resorption⁵⁵. Methods have been developed to measure only the intact, active TRAP 5b, producing consistent results that correlate strongly with other bone markers⁶¹.

1.2.3.2 Bone formation markers

Prior to the assembly of a triple helix, collagen is synthesised in a precursor form as procollagen, containing polypeptide extensions at both its amino (N-) and carboxy (C-) terminal ends⁶². Proteases then cleave these extensions, allowing the triple collagen molecules to spontaneously assemble into collagen fibres. The cleaved fragments, procollagen type I N-propeptides (P1NP) and procollagen type 1 C-propeptide (P1CP) are released into the system and can reflect osteogenesis. Both P1CP and P1NP can be analysed in serum by either ELISA or radioimmunoassay (RIA)⁵³. While both molecules are considered indices of collagen synthesis and thus bone formation, it is suggested that P1NP has greater diagnostic validity than P1CP⁶³.

Alkaline phosphatase (ALP) is a ubiquitously expressed, membrane-associated enzyme originating from various tissues⁶⁴. In healthy adults the bone alkaline phosphatase (BALP) isoform accounts for about 50% of total serum ALP⁶³. BALP is produced by osteoblasts in high amounts during bone formation and therefore is an excellent indicator of total bone formation

activity. It is likely that the enzyme participates in the mineralisation process, with BALP activity proportional to the inorganic phosphate concentration⁶⁰.

Osteocalcin (OC) is another marker commonly used as an indicator of bone formation and is the major non-collagenous protein found in bone. In addition to being produced by osteoblasts and found in significant amounts in bone, it is produced by odontoblasts and hypertrophic chondrocytes and consequently also found in dentin and calcified cartilage, as well as malignant tissues⁶³. However, most of the circulating OC originates from the bone as a product of osteoblastic activity. Interestingly, OC levels may reflect overall bone metabolism because it becomes incorporated into the bone matrix, allowing fragments to be released during the resorption process in addition to during osteogenesis^{55, 63}. OC can be analysed in serum however its value is limited by OC-lipid binding impairing detection at high lipid values while the existence of multiple isomers in the circulation causes further problems for current assays⁵⁵. OC can also be measured in urine as an index of bone turnover⁶⁵.

1.2.3.3 Regulators of bone turnover

In addition to the RANK/RANKL/OPG triad mentioned previously, there are a number of other key players in the regulation of bone turnover. Bone sialoprotein (BSP) is a phosphorylated glycoprotein, which accounts for 12% of the non-collagenous matrix protein of bones and is a member of the small integrin-binding ligand N-linking glycoprotein (SIBLING) family. BSP (as well as other glycoproteins such as osteonectin and osteopontin) plays a role in a

number of aspects of bone metabolism, for example the control of mineralisation in the formation of new bones and bone resorption. BSP contains an Arg-Gly-Asp (RGD)-sequence, which is essential for the attachment of cells to bone surfaces⁶⁶. BSP has important functions in the initiation of hydroxyapatite-crystallisation, and in the interaction between bone cells and the mineralised bone matrix. BSP can be detected in mineralising connective tissue, where the osteoblasts are primarily formed. It has however also been detected in trophoblasts and to a lesser extent in decidua cells. BSP can be measured in the serum by immunoassay, however results so far have been difficult to reproduce and validate with commercially available kits.

Parathyroid hormone (PTH) controls the homeostasis of calcium by direct action on the bone and kidney and an indirect action on the gut³³. PTH, secreted by the parathyroid gland, stimulates bone resorption at high doses through its action on osteoblasts to induce osteoclastogenesis, while at intermittent doses it stimulates bone formation.

1, 25 (OH) vitamin D is necessary for normal growth of the skeleton and calcium and phosphate metabolism. 80-90% is derived by the action of ultra-violet B (UVB) sunlight on the skin resulting in cholecalciferol (D3)⁶⁷. The remainder is derived from dietary sources (animal D3 or plant-derived ergocalciferol, D2). 25-hydroxylation takes place in the liver while further 1-hydroxylation occurs in the kidney, resulting in the active metabolite, 1.25 (OH) vit D. Mediated through the vitamin D receptor (VDR, steroid hormone

nuclear receptor superfamily), vitamin D has direct effects on bone resorption through induction of RANKL expression in osteoblasts, stimulating osteoclastogenesis^{68, 69}.

Cathepsin K is a protease that is highly expressed by osteoclasts and is primarily responsible for the degradation of the proteinaceous bone matrix. Cathepsin K therefore has a critical role in bone resorption and in recent times has become a drug target for metabolic bone diseases including malignant bone disease. The expression of cathepsin K is predominantly regulated by RANKL, as well as vitamin D, PTH, TNF and interleukins⁷⁰.

1.3 Bone health in breast cancer

1.3.1 Cancer treatment-induced bone loss (CTIBL)

The rate of bone loss increases with age, with the lifetime risk of a fragility fracture (hip, spine, distal forearm) without preventative treatment almost 40% for women older than 50 years⁷¹. Due to the critical role that oestrogen plays in the maintenance of bone mass in women, the substantial hormonal changes around the menopause result in an imbalance in bone remodelling⁷². For example, in the 3 years following the cessation of menstruation, net bone loss at the lumbar spine is 2-5% annually, slowing to 0.5% per year thereafter⁷². Many cancer survivors are at increased risk of bone loss due to their cancer treatment. As survival of patients with early breast cancer improves due to detection and the emergence of new therapies, the long-term implications of the various treatments has gathered

interest and importance. Many women can expect to live decades beyond their initial diagnosis but may be living with consequences of their cancer management. It is now widely recognized that cancer treatment-induced bone loss (CTIBL) is a complication of both hormonal and cytotoxic chemotherapies, affecting pre- and post-menopausal women.

Pre-menopausal levels of circulating oestrogen are known to be fundamental in the maintenance of normal bone mass in women⁷³. The transition from pre-menopausal to post-menopausal sees significant hormonal changes with the loss of ovarian follicular activity, including a fall in circulating oestrogens⁷⁴. The oestrogen-deficient environment present after menopause results in an imbalance in bone remodelling with a net loss of bone mass⁷³. Therefore, breast cancer survivors are at risk of bone loss due either to temporary amenorrhea or premature menopause from cytotoxic chemotherapy, reversible ovarian suppression or treatments to reduce circulating oestrogen levels, particularly third-generation aromatase inhibitors (AIs).

1.3.1.1 Cytotoxic ovarian failure

Cytotoxic cancer therapies can induce premature menopause and secondary amenorrhea in 25-100% of women receiving adjuvant treatment for breast cancer⁷⁵. Women older than 40 years at the time of chemotherapy are at greatest risk of induced ovarian failure, and consequently of BMD decline⁷⁶. Premenopausal women receiving cyclophosphamide, methotrexate and 5 fluorouracil (CMF) chemotherapy experience

menopause on average 10 years earlier than normal controls with a significant impact on their BMD⁷⁷. Most studies have investigated premenopausal women receiving a cyclophosphamide-containing regimen, commonly CMF, which is known to directly affect ovarian reserve in relation to age at treatment and cumulative dose. One such study reports that, where menstruation is preserved throughout chemotherapy, BMD is also maintained up to 5 years at the lumbar spine (LS) and femoral neck (FN) after initial treatment, with changes from baseline of -1.3% and -0.3% respectively⁷⁸. That is compared to significant losses at LS of -10.4% and FN of -5.8% in patients who became amenorrhoeic. It has been suggested that loss of ovarian function is not the sole cause of BMD declines in women undergoing adjuvant chemotherapy following a small study that reported mean losses in BMD were not significantly different between those who lost and those who maintained ovarian function 6 months after initial treatment⁷⁹. Loss in ovarian function during chemotherapy has been confirmed by a number of groups as detrimental to bone health^{76, 80-82}. The investigators suggest that during the administration of chemotherapy there may be a direct cytotoxic effect of chemotherapy on bone cells. However, after completion of chemotherapy, changes are related to ovarian function.

1.3.1.2 Hormonal manipulation

Aromatase inhibitors (AIs)

AIs are now widely considered the drug of choice for adjuvant oestrogen blockade in post-menopausal patients with hormone-receptor positive tumours due to their improved efficacy and favourable side effect profile

compared with tamoxifen. Aromatase converts androgens to oestrogens, the main source of endogenous oestrogen in post-menopausal women. In the adjuvant setting, all licensed third-generation AIs have demonstrated BMD declines which raises concern for osteoporosis and skeletal complications. This is the case whether upfront AI or a switch after 2-3 years of tamoxifen is used. For example, the large Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial randomized post-menopausal women to adjuvant tamoxifen or anastrozole reporting significantly improved disease-free survival outcomes in the group receiving the AI⁸³. A bone sub-study which assessed BMD at baseline, 1, 2 and 5 years after commencing treatment showed significant losses at the LS and total hip (TH) in the AI group (-6.08% and -7.24% respectively) compared with modest gains in the tamoxifen group (+2.77% and +0.74%, respectively)⁸⁴. This loss did not continue after the cessation of treatment⁸⁵. The Intergroup Exemestane Study (IES) which randomized post-menopausal women to either 5 years of tamoxifen or a switch to exemestane after 2-3 years observed that this loss of BMD in the AI group did translate to more fractures compared with the tamoxifen only group (7% versus 5%; $p=0.003$)^{86, 87}. However, 2 years after completion of treatment, the exemestane group partially recovered bone loss (+1.53% at LS and stabilization at TH) while the tamoxifen group saw a decline in BMD following the withdrawal of the drug (-1.93% at LS and -2.62% at TH)⁸⁸.

Of note in these studies and others, the comparison group receives tamoxifen which has been observed to exert moderate protective effects against bone loss in post-menopausal women, compounding the negative

effect observed in the AI groups⁸⁹⁻⁹¹. Tamoxifen is a selective oestrogen receptor modulator which can exert either oestrogen antagonistic effects (for example breast tissue) or agonistic effects (e.g. in the vasculature or bone)⁹². In contrast, tamoxifen has been associated with BMD losses in premenopausal women. It has been suggested that tamoxifen causes bone loss in pre-menopausal women by exerting an oestrogen antagonistic effect on bone in the presence of pre-menopausal oestrogen levels^{89, 93}.

LHRH analogues

LHRH analogues such as goserelin can be used for temporary ovarian suppression in the adjuvant management of premenopausal women. The Zoladex Early Breast Cancer Research Association (ZEBRA) study assessed the efficacy and tolerability of goserelin versus CMF in premenopausal women with node-positive breast cancer, observing equivalence comparing the 2 groups for hormone-receptor positive breast cancer⁹⁴. At 2 years follow-up in a bone sub-study, BMD losses in the goserelin group were significantly greater than the chemotherapy group at both the lumbar spine (-10.5% versus -6.5%) and femoral neck (-6.4% versus -4.5%)⁹⁵. One year after cessation of goserelin, however, there were no significant differences between the 2 groups due to partial recovery in the goserelin arm. Ovarian suppression was associated with BMD in both groups.

1.3.2 Treatment of CTIBL

1.3.2.1 Bisphosphonates

Mechanism of action

Bisphosphonates are a class of anti-resorptive drugs that have become established in routine clinical practice for both benign and malignant bone disease. They are stable synthetic analogues of pyrophosphate with a P-C-P backbone that allows avid binding to hydroxyapatite on the bone surface⁹⁶. The presence of a nitrogen atom on one of two covalently attached side chains generally separates bisphosphonates into 2 classes, either the more potent nitrogen-containing BPs (N-BPs; such as zoledronic acid) or those with less potent anti-resorptive activity, the non-nitrogen agents, including clodronate^{96, 97}. The 2 classes differ in their mechanisms of action. Non N-BPs are actively taken up by osteoclasts and metabolised to analogues of adenosine triphosphate (ATP) which consequently leads to osteoclast apoptosis. N-BPs, however, act through inhibition of the mevalonate pathway, blocking farnesyl diphosphate synthase (FPPS; figure 1-5). This results in a lack of required intermediates for the prenylation of signalling GTPases, including Ras, Rho and Rac, ultimately causing osteoclast dysfunction and apoptosis. In addition, the accumulated isopentyl diphosphate (IPP) is metabolised to a cytotoxic, intracellular ATP analogue, triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (Apppi)⁹⁸. Apppi inhibits mitochondrial ADP/ATP translocase, causing loss of mitochondrial membrane potential and direct induction of osteoclast apoptosis. Furthermore, Apppi stimulates $\gamma\delta$ T cells, thus modulating the immune system⁹⁹.

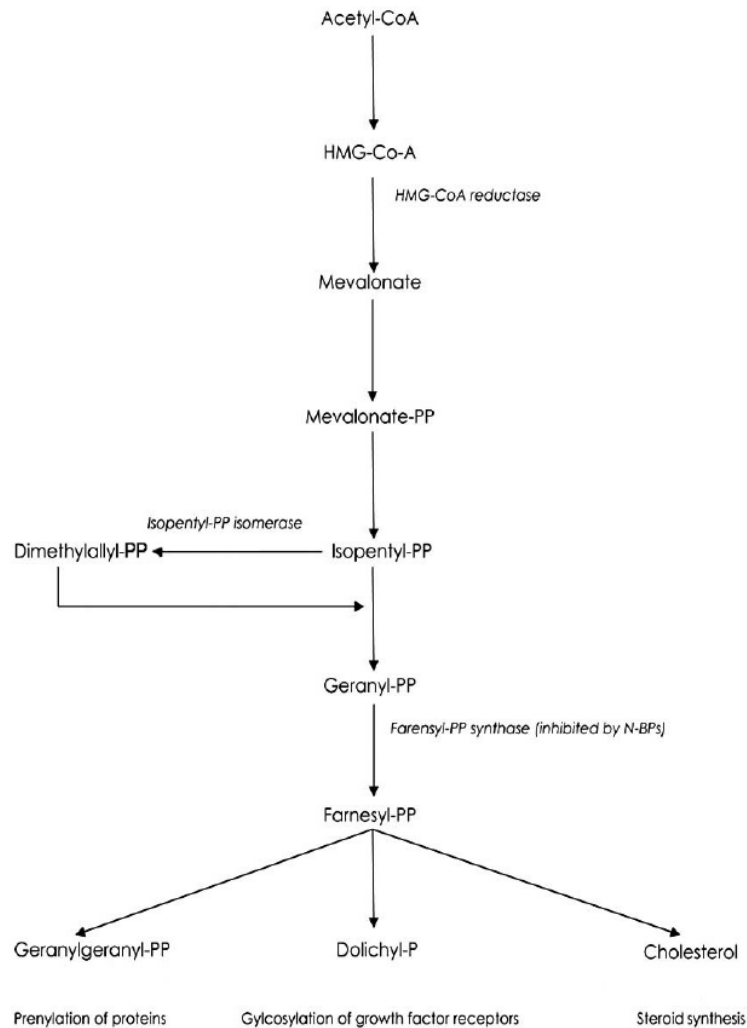


Figure 1-5 The mevalonate pathway. This pathway has a central role in cell metabolism producing isoprenoids that are incorporated into many essential end products

Safety and toxicity of zoledronic acid

The acute phase response is one of the most common side effects of zoledronic acid. This is an acute systemic inflammatory reaction characterised by fever, arthralgia and muscle pains, with or without nausea and oedema¹⁰⁰. This usually develops within 48 hours of administration and is short-lived and self-limiting.

Metabolic effects of zoledronic acid include hypocalcaemia, affecting 9-39% of patients¹⁰⁰. Whilst usually mild and transient, it is widely recommended that clinicians prescribe calcium and vitamin D supplements to prevent hypocalcaemia. Other electrolyte disturbances include hypomagnesaemia and hypophosphataemia and it is recommended that their levels are monitored during treatment.

Renal toxicity is a concern with zoledronic acid treatment, however significant nephrotoxicity is rare. Following intravenous administration, the drug is predominantly excreted unchanged by the kidneys and some transient effects may be observed on renal function. To minimise the risks, serum creatinine should be measured prior to each infusion, in addition to ensuring adequate hydration, dose-reducing in patients with pre-existing renal impairment and delaying treatment in the presence deteriorating renal function¹⁰¹.

Osteonecrosis of the jaw (ONJ) is a rare but serious complication of bisphosphonate therapy, first reported more than a decade ago by oral surgeons who noted painful exposed bone of the mandible, maxilla or both, in patients who had received intravenous bisphosphonate^{102, 103}. They report predominantly on cases in malignant disease and note that while the cancer may be under control, patients have poor quality of life due to their oral complications, with patients complaining of difficulty speaking, eating and performing oral hygiene. Neither antibiotics, surgical treatments nor hyperbaric oxygen proved effective treatments. The initial proposed underlying mechanisms included the anti-angiogenic effect of

bisphosphonates and the reduced bone turnover making the jaws vulnerable to the external environment.

Since those initial reports, a great deal has been published regarding ONJ. The American Association of Oral and Maxillofacial Surgeons (AAOMS) position paper states that a confirmed case of ONJ must fulfil 3 critical characteristics: 1) current or previous bisphosphonate treatment; 2) an area of exposed bone in the maxillofacial region that has not healed within 8 weeks after identification, and 3) no history of radiotherapy to the craniofacial region¹⁰⁴. Symptoms frequently occur at the site of a previous tooth extraction but may appear spontaneously. Patients may present with localised pain, soft tissue swelling, inflammation, loosening of teeth and exposed bone. Expert panels have come together to provide recommendations on the prevention, diagnosis and treatment of ONJ.

In their position paper, the AAOMS adopted the staging system proposed by Ruggiero^{104, 105}. This describes 3 stages of disease:

- Stage 1. Disease characterised by exposed bone that is asymptomatic with no evidence of any significant adjacent or regional soft tissue inflammatory swelling or infection.
- Stage 2. Disease characterised by exposed bone with associated pain, with adjacent or regional soft tissue inflammatory swelling or secondary infection.

Stage 3. Disease characterised by exposed bone with associated pain, adjacent or regional soft tissue inflammatory swelling or secondary infection that is difficult to manage with oral or intravenous antibiotic therapy.

Following their 2009 update, the AAOMS added stage 0, to include patients with “no clinical evidence of necrotic bone, but present with nonspecific symptoms or clinical or radiological findings”¹⁰⁶. This conflicts with their definition requiring exposed bone and has called into question whether exposed bone is required to confirm a case of ONJ or in fact earlier identification may facilitate more rapid management, and presumably more favourable outcome^{107, 108}. Either way, it is acknowledged that little is known about the early features of ONJ and the risk for progression to more advanced states of the condition, calling for more research into “stage 0” patients. The AAOMS definition and staging system remain those currently accepted in the field.

The underlying pathophysiology of ONJ remains poorly understood. Ruggiero outlines 4 principal theories: osteoclast-mediated bone remodelling suppression; anti-angiogenesis; local mucosal toxicity and, genetics¹⁰⁹. The significant suppression of bone remodelling mediated by osteoclast inhibition is the most widely accepted theory and has obtained recent support from the observation that other potent osteoclast inhibitors, in particular denosumab, can lead to ONJ. It is suggested that the effect of these drugs is greater in the jaw due to the higher basal rate of bone turnover in this region.

Bisphosphonates have been demonstrated as potent inhibitors of angiogenesis. In vitro, zoledronic acid inhibits the proliferation of human endothelial cells in addition to modulating their adhesion and migration properties¹¹⁰. Potent inhibition of angiogenesis has additionally been demonstrated in mice systemically administered zoledronic acid¹¹⁰. Should the vascular supply become compromised, a minor injury is at greater risk of developing into a non-healing wound with the potential to progress to necrosis and osteomyelitis. Additional support for this theory comes from the increased risk of ONJ when bisphosphonates and anti-angiogenic drugs are administered in combination¹¹¹⁻¹¹⁴. Furthermore, there are reports of ONJ in patients treated with anti-angiogenic compounds (bevacizumab) alone^{115, 116}.

It is postulated that high concentrations of bisphosphonate may accumulate in the jaw bone causing direct toxicity to the oral mucosa with consequent failure to heal and secondary osteomyelitis¹¹⁷. Preclinical studies have demonstrated direct toxicity to oral cell lines¹¹⁸⁻¹²⁰. However, in clinical practice the accumulation of sufficient levels to be directly toxic to the oral epithelium is unproven.

Finally, underlying pharmacogenetic factors may have a significant role in the pathophysiology of ONJ. Sarasquette et al identified a single nucleotide polymorphism in the cytochrome P450-2C gene that was associated with a significantly higher risk of ONJ development in a series of myeloma patients treated with bisphosphonate¹²¹. However, this finding was not validated in a further study¹²². Despite this, genetic susceptibility remains an attractive theory permitting new insights into the underlying mechanisms at play in the

occurrence of ONJ and the prediction of its development based on a genetic marker an appealing prospect¹²³.

Numerous risk factors for the development of ONJ have been identified and outlined in the AAOMS 2009 paper and for the purposes of this review have been summarised as a table.

Drug-related factors	<p>Bisphosphonate potency (zoledronic acid versus pamidronate)</p> <p>Route of administration (increased risk with i.v. versus oral)</p> <p>Duration of therapy (longer duration associated with increased risk)</p>
Local factors	<p>Dentoalveolar surgery (including extractions, dental implant surgery, periodontal surgery with osseous injury) while on bisphosphonates increases risk</p> <p>Local anatomy (increased risk mandible versus maxilla; thin mucosa overlying bony prominences; concomitant oral disease e.g. dental abscesses)</p>
Demographic factors	<p>Age (increasing risk with increasing age)</p> <p>Race (increased risk Caucasian versus black)</p> <p>Tobacco use increases risk</p>
Genetic factors	<p>Increased risk with single nucleotide polymorphisms in cytochrome P450-2C gene</p>

Table 1-1 Table of risk factors for ONJ, summarised from AAOMS 2009 Position Paper¹⁰⁶

In light of these known risk factors, a number of preventative measures are now routinely recommended. Dental examination before the first administration of BP is important to identify any required treatment and healing before the commencement of therapy. Additionally, patient education regarding dental hygiene and reporting of oral symptoms have contributed to a reported decrease in zoledronic acid-related ONJ^{124, 125}. It is recommended that dental procedures should be avoided on BP therapy with consideration given to withholding treatment for any invasive treatments to take place.

Clinical trials of BPs in CTIBL

Bisphosphonates are established for the treatment of benign and malignant bone disease. In the cancer setting they are used to prevent skeletal complications of bone metastases such as fracture and spinal cord compression⁹⁷. As anti-resorptive drugs with confirmed efficacy in the osteoporotic setting, several clinical trials have investigated their role in preventing CTIBL.

The oral bisphosphonate clodronate was amongst the first to be investigated in this setting. Saarto et al randomized post-menopausal women receiving tamoxifen or toremifene with or without clodronate for 3 years, observing increases in BMD at the LS (+2.9%) and femoral neck (+3.7%)¹²⁶. In a more recent study including pre- and post-menopausal women, the benefits of clodronate were confirmed, with LS BMD 1.92% higher and TH BMD 1.29% higher in the clodronate group compared with the placebo group¹²⁷. While

compliance with medication has been a concern around oral bisphosphonates due to their gastrointestinal side effects, clinical trials investigating both ibandronate¹²⁸ and risedronate¹²⁹⁻¹³¹ have similarly shown efficacy in preventing CTIBL.

The intravenous bisphosphonate zoledronic acid (ZOL) has been shown across a range of studies to prevent bone loss in both pre- and post-menopausal women. In a pre-menopausal population, 6-monthly (ZOL) has been shown to reverse the bone loss induced by a combination of goserelin plus tamoxifen or anastrozole. In this study (ABCSSG-12), the mean loss of bone in the lumbar spine over 3 years in the absence of a bisphosphonate was -11.3% while 2 years after the cessation of endocrine treatment, BMD was still significantly lower than baseline at -6.3%. However, BMD was protected following administration of 6-monthly zoledronic acid with values remaining stable over time¹³².

Three studies have specifically addressed upfront ZOL versus delayed treatment if osteoporosis or osteopenia occurs in postmenopausal women receiving the AI letrozole¹³³⁻¹³⁵. The Z-FAST study investigated 602 ER positive post-menopausal breast cancer patient receiving adjuvant letrozole who were randomised to either delayed or upfront ZOL, 4mg intravenously every 6 months for 5 years. The ZO-FAST study was of similar design in 1065 patients and finally the E-ZO-FAST study with 527 patients. All 3 studies observed higher BMD measurements in the upfront group compared with the delayed group. The Z-FAST study, which has the most mature data, reported at 5 years follow up the mean difference in LS and total hip BMDs

between the upfront and delayed groups was 8.9% and 6.7%, respectively¹³³. However, despite this positive effect on BMD, no significant difference in fracture rates has yet been demonstrated.

1.3.2.2 Denosumab

Denosumab is a relatively new anti-resorptive agent that has been investigated to protect against AI-induced bone loss. A fully humanised monoclonal antibody against receptor activator of nuclear factor-kappaB ligand (RANKL), denosumab blocks the interaction between RANKL and its receptor on osteoclasts, the binding of which would usually activate and maintain osteoclast-mediated bone resorption. In a 2 year double-blind, randomized controlled trial of denosumab versus placebo, osteopaenic postmenopausal women on an AI had significant gains in the denosumab arm at the LS compared with placebo¹³⁶.

While results from these studies are encouraging in terms of preserving skeletal health, no systemic treatments are approved to prevent CTIBL. A number of guidelines exist for patients at risk that generally require assessment of BMD at baseline with follow-up scans and advice regarding calcium, vitamin D and lifestyle changes. A recent economic evaluation has given support to this approach in terms of fracture prevention¹³⁷. The impact of lifestyle certainly has its place with a few small studies observing that regular resistance and weight-bearing activity can result in modest gains in BMD in addition to influencing bone turnover markers¹³⁸⁻¹⁴⁰. Baseline vitamin D levels are also of importance with sufficient supplementation shown to

improve BMD and reduce risk of fracture^{141, 142}. With the majority of new breast cancer patients reported to have insufficient or deficient levels of vitamin D, perhaps they are even at risk of bone loss and subsequent fracture before any treatment is commenced.

1.4 Breast cancer and bone metastases

1.4.1 Scope of the problem

1.4.1.1 Patterns of disease

Many patients with advanced cancer will develop metastatic bone disease accounting for considerable morbidity. The propensity for spread to bone varies depending on the primary site of disease, however breast, prostate and lung cancer account for approximately 80% of all bone metastases¹⁴³. With these being the 3 most common cancers in the U.K. the burden of disease from skeletal involvement is substantial¹⁴⁴. Consequently, the prevention and management of bone metastases is an important component of research and clinical agendas. Bone is the most frequent site of distant relapse among breast cancer patients, accounting for around 40% of all first distant recurrence and around 70% patients with advanced breast cancer develop bone metastasis¹⁴⁵.

Breast cancer patients in whom bone is the first site of metastasis have significantly better outcomes than those in whom the first site is the liver (median survival 2 years versus 3 months; $p < 0.001$)¹⁴⁶. Similarly, if disease

remains confined to the skeleton, median survival is 2.1 years versus 1.6 years if extraosseous sites are involved ($p < 0.001$)¹⁴⁷. Favourable outcomes for patients with “bone-only” disease has been confirmed in more recent studies¹⁴⁸⁻¹⁵⁰.

1.4.1.2 Skeletal-related events

While women with bone-only metastatic disease may have a prognosis measured in years, the potential complications of bone metastases, so-called skeletal-related events (SREs), can have a considerable impact of their lives. SREs include pathological fracture, spinal cord compression, hypercalcaemia and the need for radiotherapy or surgery to bone. Without bone-targeted therapy, a patient with bone metastases can experience up to an average of 4 SREs per year¹⁵¹. Zoledronic acid, amongst other bisphosphonates, has been investigated for its role in patients with bone metastases and is now established as standard treatment in this patient group to reduce frequency of SREs, improve bone pain and slow progression of disease in the bones. In a randomised, double-blind phase III trial of zoledronic acid versus pamidronate, zoledronic acid reduced the risk of SREs by 20% compared with pamidronate (2 years follow up; $p = 0.025$)¹⁵². A meta-analysis comprising 21 bisphosphonate trials conducted in advanced breast cancer patients with bone metastases (BP versus placebo or other BP) reported that zoledronic acid provides the greatest risk reduction for SREs (HR 0.59; 95% CI 0.42- 0.82)¹⁵³. The meta-analysis concludes that BPs, either i.v. or p.o. reduce the risk of developing a SRE, reduce the rate of SREs and increase the time to first SRE. The study reached no firm

conclusions regarding the optimal time to initiate treatment with a BP or the appropriate duration, an area that remains unconfirmed. However, an exploratory analysis of the above zoledronic acid study did show that if BP is administered before the onset of bone pain, outcomes may be improved¹⁵⁴.

The question around timing of initiation of BP may be in part resolved by identifying high risk individuals for SREs. Bone turnover markers have been investigated in this role. Breast cancer patients with a high level of the resorption marker NTX have a 3-fold increased risk for an SRE, in addition to poorer progression-free survival and mortality¹⁵⁵. If the marker is normalised during treatment with zoledronic acid, patients experience significantly lower risk for first SRE and improved survival^{156, 157}. While no marker of bone metabolism currently is able to definitively predict clinical outcomes in individual patients, there are emerging markers, including BSP and RANKL, which show encouraging results and warrant further investigation in the setting of clinical trials⁵⁵.

Recently, endocrine therapy has been combined with cyclin-dependent kinase (cdk) 4 and 6 inhibitors (palbociclib, ribociclib, abemaciclib) in advanced breast cancer with very promising results and are now NICE approved in the first line metastatic setting in combination with an aromatase inhibitor. For example, palbociclib combined with letrozole in advanced breast cancer demonstrated significant increased medical progression-free survival compared with letrozole plus placebo (24.8 months versus 14.5 months; $p < 0.001$ ¹⁵⁸). On subgroup analysis, a few studies have suggested a particularly promising role for the combination of cdk 4/6 inhibitors with

endocrine therapies amongst patients with bone-only disease at baseline and it may be that they have an emerging role in this specific population¹⁵⁹.

1.4.2 Underlying mechanism of bone metastasis

Tumour invasion into bone is associated with the recruitment of osteoblasts and osteoclasts, resulting in the release of growth factors from the bone matrix which further enhance tumour growth, the so-called “vicious cycle”^{160, 161}. The differentiation and activation of osteoclasts and osteoblasts result in increased bone turnover, a process to which the aforementioned RANK-RANKL-OPG triad is key. Additionally, the presence of tumour in the bone microenvironment modulates platelet function, myeloid cells, immune cells, nerve cells and angiogenesis⁵⁰. This specific variety of cell types provides a fertile soil for the attraction and survival of cancer cells. Cancer cells are able to inhabit the bone marrow, acting as a reservoir for dormant cells that may be able to resist cytotoxic therapy and either emerge later as full-blown bone metastases or seed to other sites¹⁶².

Whether a tumour spreads to bone or not is likely related to factors expressed by the primary tumour, its local environment and the metastatic site. The pre-metastatic niche refers to the concept that the primary tumour is able to prepare sites of metastasis⁵⁰. For example, breast cancer cells can secrete osteopontin, promoting bone marrow cell recruitment¹⁶³. Determining some of these factors when a patient initially presents would allow clinicians to make rational decisions regarding management and follow up.

Tumour cells that metastasise to bone can use the same physiological mechanism as those employed by haematopoietic stem cells homing to

bone. Chemokines and their receptors play a critical role in this process. Muller et al were among the first to investigate the similarities between tumour cell migration to preferential sites and leukocyte trafficking¹⁶⁴. They demonstrated that the chemokine receptor CXCR4 is strongly expressed on the cell surface in breast cancer cell lines and primary breast cancer cells and that CXCR4 mRNA was significantly upregulated in primary breast tumours compared with normal mammary tissue. Furthermore, the ligand for CXCR4, CXCL12, is expressed preferentially in bone, lymph nodes, liver and lungs. Finally they showed that neutralising the CXCR4/CXCL12 interactions significantly impaired metastasis of breast cancer cells. There are a few small clinical studies that suggest the primary tumour cells exploit this homing mechanism, however most are retrospective and of moderate size¹⁶⁵⁻¹⁶⁷. This mechanism has attractive therapeutic potential with some cell line work demonstrating that treatment of MDA-MB 231 inoculated mice with a CXCR4 antagonist can decrease the metastatic burden, though not the incidence of metastasis¹⁶⁸.

1.4.2.1 Prognostic and predictive markers

Some specific, conventional histopathological features have been associated with primary tumour preference for bone as a site of metastasis including low tumour grade, ER positivity and lymph node involvement^{147, 169-171}. Using immunohistochemistry to identify more novel markers of relapse in bone has so far failed to discover anything well-validated and robust enough to use either in clinical practice or stratify patients entering clinical trials. A proteomics discovery platform identified two novel biomarkers, macrophage-

capping protein (CAPG) and PDZ domain-containing protein (G1PC1), from primary breast tumours¹⁷². This revealed that co-expression of these two markers in primary tumours was prognostic for development of bone metastases and predictive of benefit from adjuvant zoledronic acid in women with early breast cancer. Furthermore, investigation of the transcription factor MAF has demonstrated some potential as a predicative marker¹⁷³. In patients with MAF-negative tumours, zoledronic acid was associated with higher invasive-disease-free survival than was control treatment (HR 0.74, 95% CI 0.56–0.98), but not in patients who had MAF-positive tumours. In fact, in this study, patients who were MAF positive experienced detrimental effects of zoledronic acid if they were not postmenopausal at the time of treatment.

Attempts at identifying a serum marker that predicts for bone metastasis development have also resulted in some interesting results however none so far reliable to move into clinical practice. In 1999 Diel et al used a radioimmunoassay to analyse serum BSP levels in 388 pre-operative breast cancer patients. With a median follow up of 20 months, Serum BSP was an independent prognostic factor for the development of skeletal metastases on multivariate regression analysis ($p, 0.001$)¹⁷⁴. Unfortunately, this work has never been reproduced. More recently, marker measurements in a couple of randomised, prospective clinical trials have shown some promise. Firstly, a subgroup analysis of a randomised, double-blind placebo-controlled trial of standard therapy with or without clodronate for 2 years suggested that early changes in P1NP levels was associated with the likelihood of developing

bone metastases¹²⁷. 230 of these patients had P1NP levels analysed at baseline and at 1 year. Those with an increase in P1NP, termed “progressive”, had higher rates of subsequent bone metastasis (20.8%) compared with those who had a decrease, or “responsive” (6.3%) or stable levels (7.8%; $p=0.011$). A second adjuvant phase III breast cancer trial measured pre-treatment β CTX levels in 621 primary breast cancer patients who were receiving tamoxifen with or without octreotide¹⁷⁵. With a median 7.9 years follow up, elevated serum β CTX levels were associated with shorted bone-only recurrence-free survival. Further work is required to establish whether any of these, or other markers, can reliably identify patients at high risk of subsequent bone metastases and to enable their appropriate management.

Employing modern genetic techniques has allowed genes to be identified that are differentially expressed between those that metastasise to bone and those that do not. Smid et al gene mapped the primary tumours from 107 breast cancer patients who experienced a relapse and identified 69 genes that were significantly differentially expressed between those with bone metastasis versus other disease of metastasis¹⁷⁶. Several others have attempted similar work however the results are largely non-conclusive. While we are gaining an increased understanding of factors associated with osteotropism, there is still no validated, reliable marker to predict elevated risk of subsequent bone metastases¹⁷⁷.

1.4.3 Does Adjuvant Zoledronic acid reduce recurrence in patients with high-risk, localised breast cancer? (AZURE clinical trial)

The AZURE trial was a prospective, randomised, open-label phase III trial designed to determine the role of ZOL 4mg i.v. combined with (neo)adjuvant chemotherapy and/or endocrine therapy in stage II/III breast cancer patients. The primary objective was to determine whether ZOL with chemotherapy and/or endocrine therapy was superior to chemotherapy and/or endocrine therapy alone in improving DFS. Secondary objectives were to determine superiority of the combination in terms of:

- Invasive disease-free survival (IDFS)
- Time to bone metastasis as first recurrence
- Time to bone metastasis per se
- Time to distant metastasis
- Overall survival
- Reducing SREs prior to and following bone metastasis development

The trial design and follow up are shown in figure 1-4 and the trial synopsis is available in appendix 1.

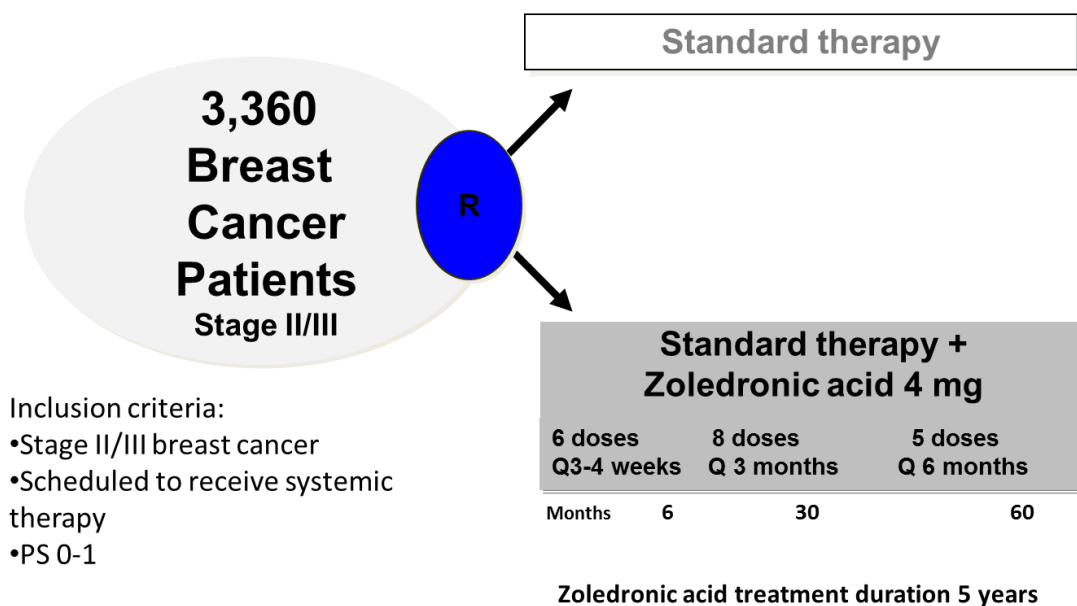


Figure 1-6 Basic schema of main AZURE study.

Full eligibility criteria available in Appendix 1.

The rationale behind the addition of the bisphosphonates to adjuvant therapy was based on pre-clinical and clinical evidence of their anti-cancer activity. In-vitro studies show that N-BPs inhibit adhesion, migration and growth of breast cancer cells in addition to inducing apoptosis⁹⁷. Further to this a sequence dependent synergy has been demonstrated in cell line work and in animal models. The combination of N-BP and anti-cancer drugs including doxorubicin more effectively suppresses metastases than either drug alone. Interestingly, sequential treatment with doxorubicin followed by ZOL produced a significant inhibition of tumour growth compared to ZOL followed by doxorubicin, simultaneous administration or either treatment alone.

At the time of writing the protocol, 3 clinical trials had investigated clodronate in the adjuvant setting with conflicting results (table 1-2)¹⁷⁸⁻¹⁸⁰.

Study	Number of participants	Intervention	Chemotherapy	Results
<i>Saarto T et al 2001</i>	299	Clodronate 1600mg daily for 3 years versus observation	All received chemotherapy (CMF)	Development of bone metastases; clodronate versus control: 21% vs. 17%, p=0.27 Development on non-skeletal metastases; clodronate versus control: 43% vs. 25%, p=0.0007
<i>Diel IJ et al 1998</i>	302 (all with tumour cells present on bone marrow aspirate)	Clodronate 1600mg daily for 2 years versus observation	Varying; only 80 patients received adjuvant chemotherapy	Development of bone metastases; clodronate versus control: 8% vs. 17%, p=0.003 Development on non-skeletal metastases; clodronate versus control: 8% vs. 19%, p=0.003
<i>Powles T et al 2002</i>	1069	Clodronate 1600mg daily for 2 years versus observation	All received chemotherapy (CMF or EC)	Development of bone metastases; clodronate versus control: 12% vs. 15%, p=0.127 Development on non-skeletal metastases; clodronate versus control: 21% vs. 24%, p=0.257

Table 1-2 Table summarising adjuvant clodronate clinical trials

A meta-analysis of these 3 studies concluded that the addition of clodronate probably does not improve outcome for patients¹⁸¹. It was hoped that the increased potency of ZOL would have beneficial effects compared to clodronate in terms of inhibition of bone resorption and reduction in growth factors and cytokines in the bone marrow microenvironment, but also through direct effects on tumour cells in the bone marrow. The overall results, however, did not show any advantage for the addition of ZOL to (neo)adjuvant therapy compared with standard therapy. At a median follow-up of 59 months, there was no significant difference in the primary end point, with a rate of disease-free survival of 77% in each group (adjusted hazard ratio in the zoledronic acid group, 0.98; 95% confidence interval [CI], 0.85 to 1.13; P=0.79). Rates of overall survival of 85.4% in the zoledronic acid group and 83.1% in the control group were also not significantly different (adjusted hazard ratio, 0.85; 95% CI, 0.72 to 1.01; P=0.07). Intriguingly, a protocol-defined sub-group analysis according to menopausal status did reveal significant heterogeneity of treatment effect. Patients who had gone through the menopause 5 or more years previously had improved IDFS with ZOL compared with standard treatment alone (78.2% versus 71.0%; HR 0.75CI, 0.59 to 0.96; P = 0.02) and improved 5-year overall survival (84.6% in the zoledronic acid group and 78.7% in the control group (adjusted hazard ratio, 0.74; 95% CI, 0.55 to 0.98; P = 0.04). Since the work described in this thesis was completed, a further publication has updated the efficacy data, demonstrating that there is continued reduction in occurrence of bone metastases at any time with zoledronic acid (HR 0.81, 0.68-0.97; p=0.022) and improved IDFS in those who were over 5 years since menopause at trial

entry (n=1041; HR 0.77, 95% CI 0.63-0.96)¹⁸². The role of reproductive hormones in the efficacy of bisphosphonates as anti-cancer drugs is now being investigated.

To conclude, the AZURE clinical trial, with its large and robust dataset of clinical information and samples for translation studies, provided a major opportunity to further the study of multiple factors affecting bone metastasis and the role of zoledronic acid in the adjuvant setting. Much of this thesis takes advantage of that significant opportunity. Chapter 2 investigates the occurrence of osteonecrosis of the jaw in patients within the AZURE study, dental health-related adverse events and oral health-related quality of life. The Oral Health Impact Profile-14 is used to compare oral health-related quality of life in patients who received zoledronic acid with those on observation. Chapter 3 utilises the blood samples collected for translational studies to identify prognostic markers in addition to markers predictive for benefit from zoledronic acid. The markers investigated as P1NP, CTX, 1CTP and vitamin D. Chapters 4 and 5 describe a novel quantitative bone scanning technique used to investigate the impact of 5 years of zoledronic acid on bone turnover in addition to serum markers of bone turnover and bone density data.

2 Osteonecrosis of the jaw and oral health-related quality of life in early breast cancer patients receiving adjuvant zoledronic acid

2.1 Background

The work considered in this chapter has been published in the Journal of Clinical Oncology¹⁸³. I undertook the literature search of various tools for measuring oral health-related quality of life, suggested additional questions, analysed the data and undertook all statistical analysis in this chapter, with the exception of producing cumulative incidence frequency for osteonecrosis of the jaw. Additionally, I undertook site visits to confirm cases of ONJ and reviewed all reported cases.

The association between bisphosphonate and ONJ has been outlined in Chapter 1. Following the initial reports of the association in 2003-2004^{102, 184}, the AZURE clinical trial patient information sheet was revised to highlight this possible risk and all patients already participating were required to re-consent. The trial protocol was amended to exclude patients with significant active dental problems or recent oral surgery. Dental hygiene advice was distributed to all patients while investigators were provided with guidance on diagnosis, prevention and treatment of ONJ. The AZURE trial collected detailed information on suspected and confirmed ONJ which allows direct comparison between the control arm and the ZOL arm.

The safety of administering zoledronic acid alongside chemotherapy has already been reported¹⁸⁵. In addition to the known risk of ONJ, this safety report also identified more frequent dental adverse events (AEs) among the patients receiving zoledronic acid compared with the control group. However, it is unknown what impact zoledronic acid has on patients' quality of life, specifically in relation to their oral health and the potential subclinical manifestations that may not be identified during routine oncology follow up.

2.1.1 Measuring oral health-related quality of life

The World Health Organisation (WHO) defines health as a “state of complete physical, mental and social well-being, not merely the absence of disease or infirmity”¹⁸⁶. Therefore, one cannot assume that purely providing curative treatment for breast cancer returns a patient to full health and that a more holistic approach must be adopted to monitor and manage the impact of the diagnosis and treatment on the individual. With that in mind, the assessment of patient's quality of life is also significant and has more recently been formally incorporated into clinical trials. The European Organisation for Research and Treatment of Cancer (EORTC) Study Group on Quality of Life identified the need for an integrated measurement system for evaluating the quality of life of patients participating in international clinical trials¹⁸⁷. Since their initial questionnaire EORTC QLQ-C36 in 1987, numerous tools have become available for measuring health-related quality of life directed at increasingly more specific patient populations.

A need to assess oral health-related quality of life (OH-QoL) was similarly identified by dentists and oral health surgeons resulting in a proliferation of

tools to investigate the quality of life of patients with various oral conditions. There are several definitions of OH-QoL in the published literature but one of the most common is that described by Locker as “the symptoms and functional and psychosocial impacts that emanate from oral diseases and disorders”¹⁸⁸. Many early tools were criticised for reflecting the values of health care professionals rather than patient concerns. Therefore, questionnaires were developed from in-depth, qualitative interviews with the target population. The patient survey that we chose, the Oral Health Impact Profile-14, is now one of the most widely used tools in the published literature with evidence for its use across many different populations worldwide including large national population studies¹⁸⁹. Its purpose is to assess the dysfunction, discomfort and disability caused by oral conditions¹⁹⁰. In its development, 535 statements were obtained from qualitative interviews with 64 dental patients. This was reduced to a set of 49 unique statements to represent the 7 domains of oral health as outlined in Locker’s conceptual model:

- functional limitation
- physical pain
- psychological discomfort
- physical disability
- psychological disability
- social disability
- handicap

Following the publication of the initial 49 item questionnaire, Slade derived and validated a shorter survey with only 14 questions as it was identified that many research settings did not permit the use of the full 49 item questionnaire¹⁹¹. This OHIP-14 allows 2 statements per domain and each question within the domain carries a weighting and has been found to have good validity, reliability and precision (full questionnaire available in appendix 2).

2.1.2 OH-QoL among patients with/at risk of ONJ

As mentioned previously, the reporting of ONJ has become a formalised requirement of clinical trials investigating bone-targeted agents such as ZOL and denosumab and consequently there has been rigorous assessment of patients oral health. Nevertheless, there remains minimal information on what this impact has on OH-QOL. Most of the reports focus on pain and general health-related quality of life (H-QoL). For example, a trio of parallel randomised phase III studies conducted in patients with bone metastasis from solid tumours or myeloma comparing 4-weekly ZOL with 4-weekly denosumab prospectively collected data on ONJ, H-QoL, using Functional Assessment of Cancer Therapy survey, and pain, using Brief Pain Inventory-Short Form⁴. However, it is not known what impact ONJ, or other dental AEs related to the study drugs, had on OH-QoL.

Two small studies have retrospectively investigated OH-QoL in patients with known ONJ. The first in a cohort of 34 patients with confirmed ONJ associated with bisphosphonates, using the OHIP-14, reported that ONJ causes a significant decline in OH-QoL¹⁹². However, there is no comparison

with patients who did not develop ONJ whilst receiving bisphosphonates. The second study compared patients with confirmed ONJ and metastatic breast cancer, metastatic breast cancer with no known ONJ and patients with cancer of the oral cavity but no ONJ¹⁹³. They used the EORTC-C30 and QLQ-HN35 (head and neck specific) questionnaires.

Our study, reported in detail below, has already been published¹⁸³ and at the time of this work is the only study that investigates the OH-QoL in patients receiving bisphosphonate treatment compared with patients who received no bisphosphonate treatment¹⁸³.

2.2 Aims of study

To describe the occurrence of ONJ in early breast cancer patients treated with standard therapy plus adjuvant ZOL and the outcomes of confirmed cases.

To describe the occurrence of dental AEs in early breast cancer patients treated with standard therapy plus adjuvant ZOL versus patients who received standard therapy alone.

To investigate OH-QoL in early breast cancer patients treated with standard therapy plus adjuvant ZOL versus patients who received standard therapy alone.

Endpoints of the study are:

- The safety and toxicity of ZOL in this clinical setting with regard to dental AEs and occurrence of ONJ.

- Dental health domain scores as derived from OHIP-14 questionnaire.

2.3 Methods

2.3.1 Patients

2.3.1.1 Patient population for ONJ assessments

All patients within the main AZURE study were required to have histologically-confirmed breast cancer with axillary node metastasis or a T3/T4 primary tumour. Patients were randomly assigned to receive either standard adjuvant therapy (control arm) or standard adjuvant therapy plus 19 intravenous administrations of ZOL 4mg over 5 years (see figure 1-1). All patients received oral supplements of calcium and vitamin D for the first 6 months and then continued thereafter at the local investigator's discretion. Following the emergence of a potential link between bisphosphonates and ONJ, the protocol was changed to mandate clinical review of the oral cavity and questions regarding any dental problems at every clinic visit, in addition to dental hygiene advice distributed to all patients on study.

2.3.1.2 Patient population for OH-QoL sub-protocol

486 (control, n=242; ZOL 244; see CONSORT diagram figure 2-1) AZURE trial participants from centres within the U.K. who recruited at least 10 patients to the main AZURE and were not involved in another AZURE sub-study, were invited to take part in the OH-QoL sub-protocol between February and November 2010. Eligibility criteria required patients to be 4.5 – 5.5 years past their randomisation date at the time of questionnaire

completion. This was to coincide with either final ZOL administration on study patients or similar follow up time point in control patients. Patients were ineligible if they had developed bone metastases as this might have produced symptoms affecting results.

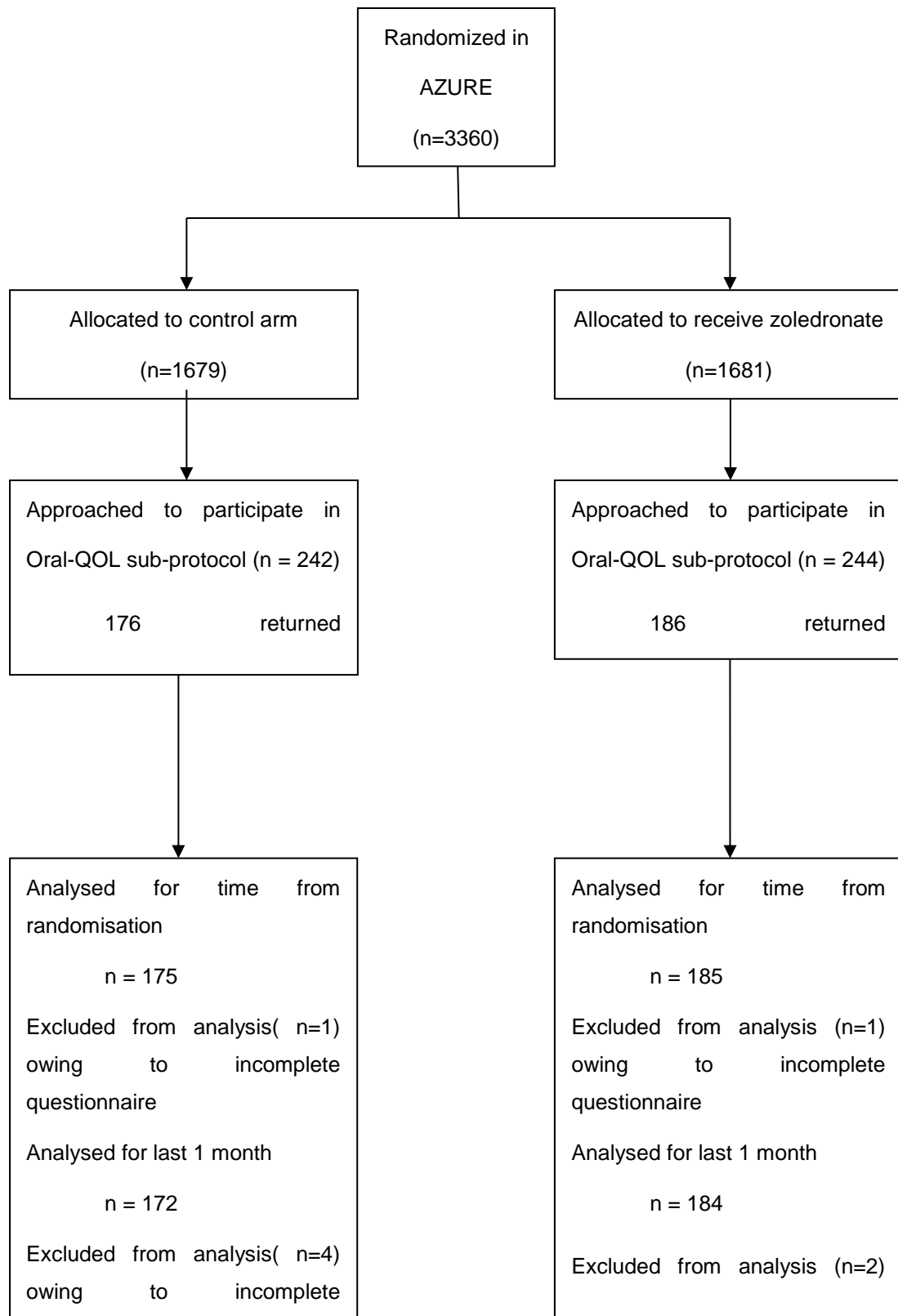


Figure 2-1 CONSORT diagram to show eligible and consented patients for the main AZURE study and OH-QoL sub-protocol.

2.3.2 Study design

Study design of the main AZURE study has been described in the introduction with further detail available in the protocol synopsis found in the appendix 1.

2.3.3 Oral-QoL questionnaire design and data collection

The study was designed as a one-off survey using the OHIP-14 questionnaire. Participants were asked to complete two identical questionnaires; the first to relate to their experience over the past one month and the second time to relate to their overall OH-QoL experience since randomisation. This was to separate out the time when all patients may have been receiving chemotherapy and steroids (which could in itself cause poor OH-QoL, for example from mucositis or oral infections), from time when only the study group were receiving ZOL but the control group receiving no intravenous anti-cancer therapy. Patients were asked to answer additional questions regarding their oral health that may influence OHIP-14 scores (see questionnaire in Appendix 2).

Responses to OHIP-14 questions were coded on a 5-point ordinal scale (0 = never, 1 = hardly ever, 2 = occasionally, 3 = fairly often, 4 = very often). Severity scores were calculated as the sum of all responses (maximum severity score = 56). Prevalence scores were represented as the percentage of participants responding with “fairly often” or “very often”.

Missing values were substituted by the mean for the specific item from the appropriate arm. The sensitivity of this approach was checked by

substituting the maximum and minimum possible values and re-running the analysis and ensuring that there was no significant difference in results. If more than 2 items were missing, the questionnaire was excluded from the analysis.

Participants provided informed consent by completing and returning the questionnaire. Approval from the appropriate ethical committee was obtained before patients were approached for the study (in appendix 3).

2.3.4 Statistical design

2.3.4.1 Analysis of ONJ incidence

Each case of reported ONJ underwent central review by clinical researcher (E Rathbone) including undertaking site visits to review clinical notes. The time to onset of ONJ was investigated using cumulative incidence function curves in which deaths without a diagnosis of ONJ were considered competing risk events. Additionally, each case is described descriptively in terms of number of ZOL administrations before diagnosis, median time to onset of ONJ from randomisation, site of ONJ and outcome. Date of onset of ONJ is defined as the date recorded by the local investigator of symptoms likely related to ONJ. Dental AEs are also reported descriptively according to CTC grading. This analysis was performed using SAS software, version 9.2 (SAS Institute, Cary, NC).

2.3.4.2 Sample size calculation

To allow an effect size of 0.3 to be detected between the 2 arms with a 5% (two-sided) significance level and 80% power using a two-sample *t* test of equal means 280 patients would be required to complete the questionnaire. A response rate of 50% was assumed. According to Cohen's operational definitions this would relate to detecting a small to medium difference in OH-QoL¹⁹⁴.

2.3.4.3 Analysis of OH-QoL

Mean severity scores were analysed for each treatment group with 95% C.I. obtained from a multivariable linear regression model adjusting for the following potential prognostic factors: age (at time of survey), smoking, dentures, and pre-existing dental conditions or procedures (missing teeth, numbers of tooth extractions or other surgical dental procedures, and the frequency of dental visits). Mean domain scores were calculated by the same linear regression model adjusting for the same prognostic variables. Additionally, an ad hoc analysis was carried out adjusting severity scores for the occurrence of dental AEs. Prevalence scores and dental AEs are reported descriptively. These analyses were performed using SPSS statistical software (PASW Statistics 17.0; SPSS, Chicago, IL).

2.4 Results

2.4.1 Occurrence of ONJ

The median follow up time from randomisation to time of analysis was 73.9 months (interquartile range, 60.7 – 84.2 months). During this time, 33 cases of suspected ONJ were reported, all in the ZOL arm. 26 of these have been centrally confirmed as cases of ONJ according to the AAOMS position paper definition. This equates to a cumulative incidence rate of 2.1% (95% C.I., 0.9% - 3.3%; see figure 2-2). Each case is described in table 2-1. Seven of the reported cases did not meet the criteria to be confirmed as cases of ONJ.

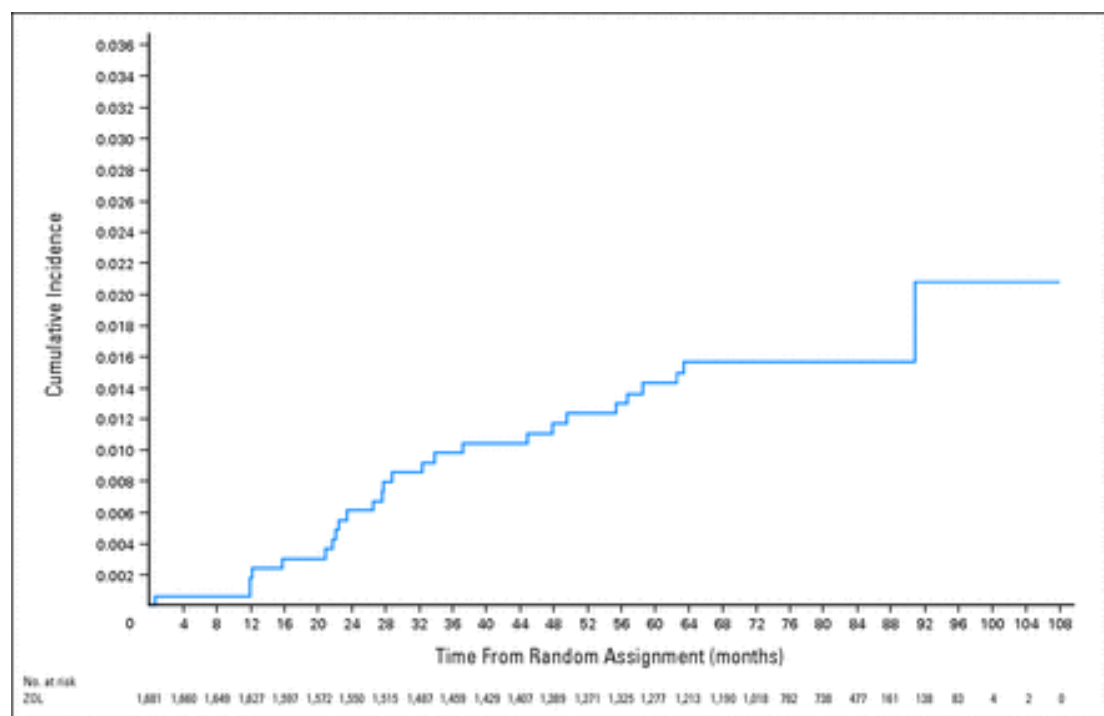


Figure 2-2 Time to confirmed ONJ for patients randomly assigned to ZOL.

Previously published, Rathbone et al¹⁸³.

The median number of zoledronate administrations before the onset of ONJ in confirmed cases was 13 (range, 1 – 19). The median time from randomisation to onset of ONJ in confirmed cases was 863 days (range, 21 – 2767 days). 85% of the confirmed cases (n=22) were known to have occurred following a dental extraction. Site of ONJ was confirmed in 25/26 cases. Of the 25, 16 cases involved the mandible (64%), 12 the maxilla (48%). Three cases involved both sites therefore these figures calculate to >100%.

Case	Age at onset	No. Zol administrations	Time from randomisation to onset (days)	Site	Extraction	Outcome (days to complete recovery, or to date of analysis if not completely recovered)
1	58	17	1780	Mandible	Yes	Completely recovered (420)
2	62	13	879	Maxilla	Yes	Completely recovered (985)
3	40	8	481	Mandible	Yes	Improving
4†	63	13*	1712	Maxilla	unknown	Present and unchanged
5†	51	16	1508	Maxilla	Yes	Present and unchanged
6	68	14	1029	Mandible and maxilla	Yes	Improving
7†	63	10*	840	Mandible	unknown	Unknown
8	58	15	1132	Unknown	unknown	Improving
9	54	8	636	Mandible	Yes	Present and unchanged
10	54	1	21	Mandible	Yes	Recovered with sequelae
11	42	11	647	Mandible	Yes	Completely recovered (615)
12	54	8	363	mandible and maxilla	Yes	Completely recovered (650)
13	39	11	670	Maxilla	Yes	Present and unchanged
14	55	19	1915	Maxilla	Yes	Improving
15	67	12	714	mandible and maxilla	No	Recovered with sequelae
16	68	11	672	Mandible	Yes	Recovered with sequelae
17	55	14	1364	Mandible	Yes	Present and unchanged
18	55	11	364	Maxilla	Yes	Completely recovered (542)
19	72	13	807	Mandible	Yes	Present and unchanged
20	65	16	1455	Mandible	Yes	Present and unchanged

21	54	7	369	Mandible	Yes	Completely recovered (1271)
22	46	14	846	Mandible	Yes	Completely recovered (914)
23	47	18	1904	Maxilla	Yes	Improving
24	46	18	1689	Maxilla	Yes	Completely recovered (280)
25	59	19	2767	Maxilla	Yes	Present and unchanged
26	52	13	988	Mandible	Yes	Completely recovered (1553)

Table 2-1 Table of all 26 confirmed cases of ONJ

†These patients have relapsed and continued a bisphosphonate off study. *Number of ZOL infusions on study prior to onset

The outcomes of the confirmed cases were: completely recovered, n=9; recovered with sequelae, n=3; condition improving, n=5; condition present and unchanged, n=8; outcome not known, n=1.

No case of suspected or confirmed ONJ was reported in the control arm (p<0.001).

2.4.2 Oral-QoL

2.4.2.1 Patient characteristics

362 patients of the 486 invited AZURE participants returned a completed questionnaire (control, n=176, response rate 72.7%; ZOL, n=186, response rate 76.2%. See CONSORT diagram in figure 2-2). The baseline characteristics were very similar between the total AZURE population and the questionnaire responders (table 2-2). The assigned arm of study did not influence response rates as these are very similar between the groups. Dental characteristics of those who completed a questionnaire are displayed in table 2-3. The mean age at time of completing the questionnaire was 57 years in both groups (control arm, range 36 – 79 years, SD 9.3 years; ZOL arm, range 38 – 79 years, SD 9.1 years).

Characteristic	Main AZURE study population		Oral QoL sub-study population	
	Zoledronic Acid	Control	Zoledronic Acid	Control
Axillary lymph nodes – no. (%)				
0	29 (1.7)	32 (1.9)	4 (2.2)	2 (1.1)
1-3	1041 (61.9)	1032 (61.5)	126 (67.7)	125 (71.0)
≥4	604 (35.9)	608 (36.2)	55 (29.6)	49 (27.8)
Unknown	7 (0.4)	6 (0.4)	1 (0.5)	0 (0)
Tumour stage – no. (%)				
T1	542 (32.2)	523 (31.2)	67 (36.0)	70 (39.8)
T2	851 (50.6)	867 (51.7)	88 (47.3)	87 (49.4)
T3	227 (13.5)	228 (13.6)	28 (15.1)	17 (9.7)
T4	58 (3.5)	59 (3.5)	2 (1.1)	2 (1.1)
TX	3 (0.2)	1 (0.1)	1 (0.5)	0 (0)
Oestrogen-receptor status – no. (%)				
Positive	1319 (78.5)	1316 (78.4)	161 (86.6)	144 (81.8)
Negative	349 (20.8)	355 (21.2)	24 (12.9)	30 (17.0)
Unknown	13 (0.8)	7 (0.4)	1 (0.5)	2 (1.1)
Menopausal status – no. (%)				
Premenopausal	751 (44.7)	752 (44.8)	83 (44.6)	71 (40.3)
Postmenopausal				
≤5 yr	247 (14.7)	244 (14.5)	28 (15.1)	31 (17.6)
>5 yr	519 (30.9)	522 (31.1)	56 (30.6)	56 (31.8)
Status unknown	164 (9.8)	160 (9.5)	18 (9.7)	18 (10.2)
Planned systemic therapy – no. (%)				
Endocrine therapy alone	76 (4.5)	75 (4.5)	9 (4.8)	2 (1.1)
Chemotherapy alone	362 (21.5)	360 (21.5)	24 (12.9)	30 (17.0)
Endocrine plus chemotherapy	1243 (73.9)	1243 (74.1)	153 (82.3)	144 (81.8)
Planned type of chemotherapy – no./total no. (%)				
Anthracyclines	1567/1605 (97.6)	1564/1603 (97.6)	174/177 (98.3)	167/174 (96.0)
Taxanes	390/1605 (24.3)	385/1603 (24.0)	9/177 (5.1)	13/174 (7.5)
Timing of chemotherapy – no./total no. (%)				
Neoadjuvant	104/1605 (6.5)	104/1603 (6.5)	7/177 (4.0)	3/174 (1.7)
Postoperative	1501/1605	1499/1603	170/177	171/174

	(93.5)	(93.5)	(96.0)	(98.3)
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Table 2-2 Table of baseline characteristics of whole AZURE populations and questionnaire responders

		Control (n=176)	Zol (n=186)
Mean age, years (range)		57 (36-79)	57 (38-79)
Dentures wearers (%)		35 (20)	39 (21)
Missing teeth (%)		124 (70)	136 (73)
Teeth or gums in need of attention		40 (23)	38 (20)
Number of tooth extractions (%)	None	115 (65)	145 (78)
	One	33 (19)	25 (13)
	2-5	22 (13)	13 (7)
	>5	5 (3)	1 (1)
	Missing data	1 (1)	2 (1)
Number of dental implants (%)	None	165 (94)	170 (91)
	One	6 (3)	6 (3)
	2-5	2 (1)	4 (2)
	>5	1 (1)	3 (2)
	Missing data	2 (1)	3 (2)
Number of surgical dental	None	136 (77)	142 (76)
	One	17 (10)	24 (13)
	2-5	19 (11)	12 (6)
	>5	2 (1)	4 (3)
	Missing data	2 (1)	4 (2)
Number of dental visits (%)	≥ 2 visits per year	123 (70)	137 (74)
	Once per year	30 (17)	27 (15)
	< Once per year	8 (5)	10 (5)
	Not in 5 years	14 (8)	11 (6)
	Missing	1 (1)	1(1)

Table 2-3 Dental characteristics of questionnaire responders

2.4.2.2 Last 1 month

Seven participants were excluded from this analysis due to more than 2 missing responses (control, n=4; ZOL, n=3). Mean severity scores did not significantly differ according to arm. The mean score in the control group was 4.86 (SD, 8.581; 95% CI, 3.58 to 6.14) compared with 4.21 (SD, 7.361; 95% CI, 3.14 to 5.28) in the ZOL arm ($p = .440$). Dentures worn ($p < .001$), teeth or gums in need of attention ($p < .001$) and number of tooth extractions ($p < .001$) significantly increased OHIP-14 scores. Severity of individual domain scores did not differ significantly by arm of study (table 2-4).

The prevalence scores for the control and ZOL groups were 16.3% and 13.7% respectively (difference, 2.6%; 95% CI -4.8% to 10%). The frequency of experienced impacts across the domains is shown in figure 2-3. Pain was the most commonly recorded impact.

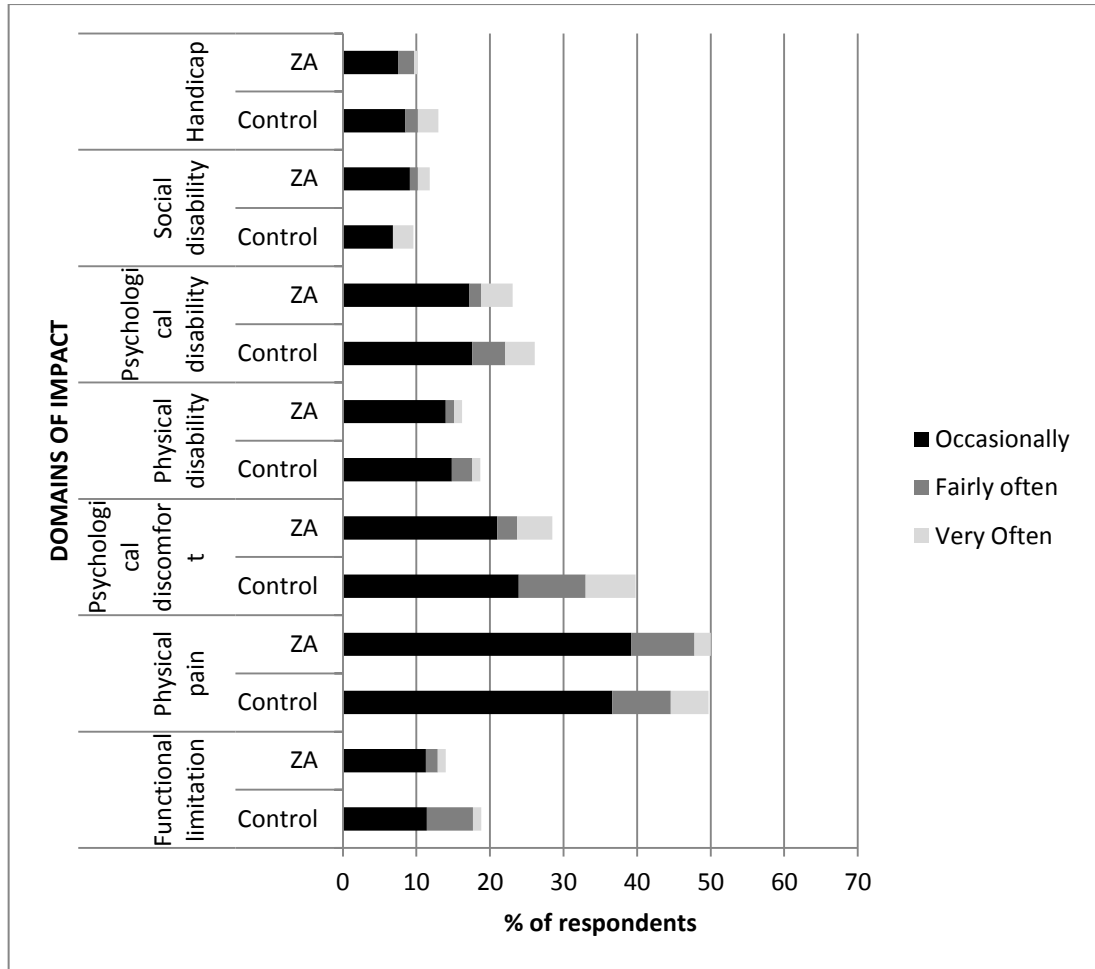


Figure 2-3 Prevalence of impacts at the three highest levels by arm for last 1 month

Domain	Last 1 month				Since randomisation			
	Control Group	Zoledronate Group	Mean difference (95% CI)	<i>P</i>	Control Group	Zoledronate Group	Mean difference (95% CI)	<i>P</i>
Functional limitation	0.295	0.217	0.078(-0.04-0.199)	0.481	0.386	0.259	0.127(-.001-.254)	0.177
Physical pain	0.806	0.748	0.058(-.132-.249)	0.637	0.857	0.784	0.073(-.117-.263)	0.641
Psychological discomfort	0.578	0.439	0.139(-.057-.335)	0.370	0.547	0.400	0.147(-.035-.237)	0.323
Physical disability	0.297	0.249	0.048(-.082-.178)	0.812	0.740	0.594	0.099(-.039-.237)	0.732
Psychological disability	0.419	0.364	0.056(-.110-.221)	0.945	0.509	0.383	0.126(-.043-.294)	0.512
Social disability	0.214	0.218	-0.004(-.126-.118)	0.145	0.298	0.252	-0.047(-.082-.175)	0.316
Handicap	0.259	0.187	0.072(-.045-.188)	0.891	0.312	0.209	0.103(-.019-.225)	0.565

Table 2-4 Individual domain scores from OHIP-14 by arm of study for both time periods

2.4.2.3 Time since randomisation

Four participants were excluded from this analysis due to more than 2 missing responses (control, n=2; ZOL, n=2). Mean severity scores did not significantly differ according to arm. The mean score in the control group was 6.46 (SD, 9.624; 95% CI, 5.03 to 7.89) compared with 5.06 (SD, 7.292; 95% CI, 4.01 to 6.11) in the ZOL arm ($p = .119$). Dentures worn ($p=.002$), teeth or gums in need of attention ($p<.001$) and number of tooth extractions ($p<.001$) significantly increased OHIP-14 scores. Severity of individual domain scores did not differ significantly by arm or study (table 2-4).

When occurrence of a dental AE was included in the statistical model, this was also shown to be a significant independent prognostic factor ($p<0.001$). Treatment with ZOL remained a non-significant factor when dental AEs were added to the model ($p=.109$).

The prevalence scores for the control and ZOL groups were 15.5% and 12.9% respectively (difference, 2.6%; 95% CI -4.7% to 9.9%). The frequency of experienced impacts across the domains is shown in figure 2-4. Pain was the most commonly recorded impact.

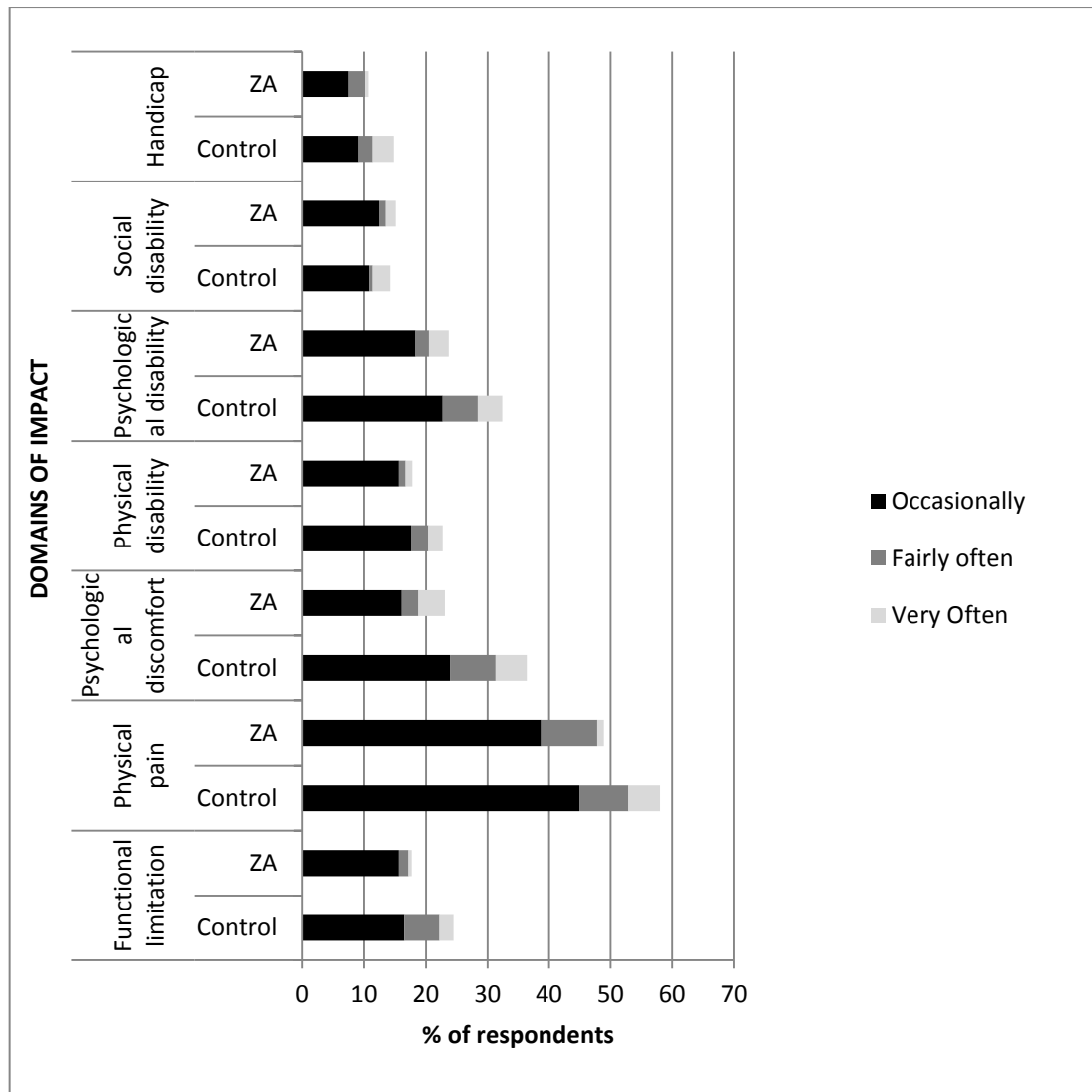


Figure 2-4 Prevalence of impacts at the three highest frequency levels by arm for time since randomisation

2.4.2.4 Dental AEs among responders

There were 55 dental AEs reported among 45 patients. 84% of these AEs were reported in the ZOL arm. There were only 2 CTC grade 3 events, an episode of jaw pain and a report of loose teeth. There were two grade 4 events. The mean OHIP-14 scores for patients who did experience a dental AE versus those who did not were 10.10 (95% CI 6.22 to 13.98) and 4.98 (95% CI, 4.15 to 5.81) respectively, since randomisation. For scores within

the last 1 month, the mean OHIP-14 scores for patients who did experience a dental AE versus those who did not were 7.95 (95% CI, 4.42 to 11.48) and 4.68 (95% CI, 3.84 to 5.52) respectively.

Of the patients invited to participate in the OH-QoL study, 3 had a reported case of suspected ONJ. Of these 3, one did not return the questionnaire and one did not meet the ONJ definition criteria. The remaining patient with a confirmed case of ONJ had OHIP-14 severity scores for the last 1 month and time since randomisation respectively of 32 and 28. The patient with suspected ONJ that did not meet the criteria also had higher than mean scores at both time points (scores for last 1 month and time since randomisation 18 and 19 respectively).

2.5 Discussion

ONJ is known to occur in both the benign and metastatic settings following treatment with either bisphosphonates or the RANK ligand inhibitor denosumab. Regarding intravenous bisphosphonates in the malignant setting, the cumulative incidence has been reported between 0.8% and 12%, though extreme value only reported in multiple myeloma¹⁰⁶. As more data has emerged, key risk factors for the condition have been identified, including potency of bisphosphonate, duration of treatment with the drug and dentoalveolar surgery. ZOL is the most potent bisphosphonate used in clinical practice and therefore the majority of cases of ONJ occur in patients who have received this treatment. The AAOMS position paper reports that patients receiving intravenous bisphosphonate treatment are at a 2.7 – 4.2-

fold increased risk of developing ONJ, that risk increases with longer duration and the patients who undergo dentoalveolar surgery are at least 7 times more likely to develop ONJ. Our study is in agreement with this, the majority of our cases developing on the background of dental extractions and after at least 13 intravenous ZOL administrations. It is reassuring that since the time of this analysis there have only been 4 further cases of ONJ confirmed within the AZURE cohort¹⁹⁵.

During the conduct of the AZURE clinical trial, guidance emerged regarding diagnosis, treatment and prevention of ONJ that was distributed to investigators in addition to dental hygiene advice sent to all patients. There is now increasing evidence that implementing preventative measures can reduce the occurrence of ONJ and these measures are strongly recommended to all patients receiving intravenous bisphosphonate treatment^{124, 125, 196, 197}. Such measures involve thorough oral investigation before starting intravenous bisphosphonate treatment, removal of any unsalvageable teeth, completion of any invasive dental procedures before commencing i.v. BP treatment and maintaining optimal periodontal health¹⁰⁶.

In the metastatic setting, the cumulative incidence of ONJ has been reported as 3.0% (95% CI 0 – 5.8%) with ZOL¹⁹⁸. The cumulative incidence of ONJ in our study is lower than this at 2.1% however it is higher than that reported in other adjuvant settings. This is likely due to the less intense schedule of ZOL in these studies. The ZO-FAST and Z-FAST studies administered ZOL 4mg i.v. 6 monthly for 5 years (total 10 administrations) and report 3 and 0 confirmed cases respectively^{133, 199}. The ABSCG-12 study administered ZOL 4mg i.v. 6 monthly for 3 years at no report no confirmed cases of ONJ²⁰⁰.

We also report an apparent increase in reported dental AEs in the ZOL arm. However, as this study was not blinded, it is likely that there has been differential reporting of dental-specific AEs, with a tendency to under-report oral issues in the control arm. Additionally, some of these patients with a reported dental AE may be considered stage 0 ONJ following the amendment to the staging system in 2009 by AAOMS with nonspecific symptoms that may result from bisphosphonate exposure and an unknown risk for advancing to higher disease stage. While the AAOMS does acknowledge that ONJ adversely affects quality of life, it is unknown whether the wider population of patients receiving potent i.v. bisphosphonates also experience negative impacts on their OH-QoL.

Our study remains the only published report of OH-QoL investigated in early breast cancer¹⁸³. The OHIP-14 has been used to assess OH-QoL in a retrospective cohort of 34 cancer patients with confirmed ONJ, before and after the diagnosis of ONJ. They show that ONJ significantly increases severity scores from a mean of 3.56 to 16.53 however there is no comparison with patients who did not go on to develop ONJ. In addition to the general tool EORTC-C30 survey, Kyrgidis et al used the EORTC QLQ Head and Neck 35 to investigate oral-specific impacts on quality of life related to ONJ¹⁹³. This module includes 35 items covering pain, swallowing, taste, smell, speech, social eating, social contact, sexuality, teeth problems, trismus, dry mouth, sticky saliva, cough and feeling ill. The authors conclude that use of the QLQ-HN35 module might be applicable to patients with any type of metastatic cancer who develop ONJ. However, there are many questions included in this survey that are inappropriate for early cancer patients who have received curative treatment and also may be considered

inappropriate for patients with ONJ for example, questions regarding feeding and cough. Furthermore, the questions regarding appearance and sexuality may put patients off from completing the questionnaire, as seen by the authors.

Our data are encouraging and reassuring to clinicians and patients, showing that ZOL does not seem to significantly affect OH-QoL. The mean scores we report are similar to those from both a general healthy adult population in the UK and the wider global community^{189, 201}. The relationship between reporting of a dental AE during the study and worse OH-QoL scores strongly suggests that the OHIP-14 is sensitive to oral health events in patients with early breast cancer. Given the widespread use of ZOL and the recent results in the adjuvant setting indicating that bisphosphonates may improve DFS and OS, the knowledge that there seems to be no demonstrable adverse impact on OH-QoL is reassuring encouraging.

A limitation of this study is that it evaluated OH-QoL at a single timepoint at completion of 5 years on study and relied on a retrospective evaluation by the patient of QoL at only 2 time frames. Ideally, a prospective longitudinal study would have been conducted, however the potential link between BP use and ONJ was not known when the study was designed and initially commenced accrual. It is acknowledged that there are other methods recognised for investigating quality of life amongst patients, such as conducting face-to-face or telephone interviews, however, this would have been more time consuming and may have prohibited some from participating.

It is now accepted that the non-bisphosphonate antiresorptive agent denosumab is also associated with the development of ONJ and at a frequency at least as high as seen with ZOL in the metastatic setting, providing further need for additional research. It seems unlikely that a bone-targeted agent will become available in the near future that is not associated with the development of ONJ. Whilst our study has provided important new information on both ONJ and oral health quality of life in patient receiving ZOL, it is desirable to understand the potential genetic, oral health and treatment risk factors, incidence and resolution of this uncommon problem somewhat better than we do at the present time. The adjuvant phase III clinical trial of denosumab as adjuvant treatment for women with early stage breast cancer (D-CARE; NCT01077154) has similar eligibility to AZURE, whereas the Austrian Breast and Colorectal Cancer Group-18 (NCT00556374) trial is evaluating an every 6-month osteoporosis dosing schedule of denosumab. The placebo-controlled, double-blind study design of these studies will provide additional and supplementary information on dental safety and ONJ incidence.

3 Serum biomarkers of bone metabolism as prognostic and predictive factors

3.1 Background

The work considered in this chapter has been published in the Journal of the National Cancer Institute²⁰². I undertook the laboratory work required for the manual assay 1CTP in over 800 samples, in addition to running some of the automated assays. Furthermore, I was a principal member of the group analysing the data, participated in numerous discussions on the statistical analysis and I am joint first author on the published manuscript to acknowledge my significant contribution.

As outlined in the Introduction, markers of bone metabolism can be easily measured in serum or urine and can give an indication of the state of bone health and turnover, but interpretation must take into account diurnal variation, seasonal changes and diet. Due to the known interactions between the bone microenvironment and breast cancer cells, we propose that markers of bone metabolism may provide useful prognostic information and additionally identify patients who may benefit from adjuvant bisphosphonates. As a planned translational component of AZURE, serum samples were collected from consenting participants to investigate the underlying mechanisms and status of the bone microenvironment, as reflected in the markers. Furthermore, we planned to investigate whether the markers are able to identify high-risk patients and if manipulation of the bone

microenvironment with bone-targeting agents in the adjuvant setting reduces the risk of recurrence.

Four serum markers were chosen for investigation: P1NP, CTX, 1CTP and total 25-hydroxyvitamin D (25-OH vitamin D). All four are markers that can be reliably tested in the laboratory and are well-validated. The 4 markers represent different aspects of bone metabolism. P1NP is a marker of bone formation while CTX is a marker of bone resorption. 1CTP is an additional marker of bone resorption however it is released by the action of matrix metalloproteinases (MMPs; proteolytic enzymes involved in the degradation of matrix) and is not affected by menopausal status. Finally, vitamin D is integral to bone metabolism; its levels are closely regulated by calcium and phosphate levels and parathyroid hormone, the latter also having a role in activating osteoblasts, stimulating the transformation of pre-osteoclasts into mature osteoclasts²⁰³. It is now widely accepted as playing a role in many cellular mechanisms related to cancer including differentiation, proliferation, apoptosis and angiogenesis.

Whilst it is known, to varying degrees, that these markers can display prognostic information, the evidence has never been robust enough to guide clinical practice. We had a unique opportunity within the AZURE clinical trial to significantly improve upon this by correlating our laboratory findings with the database collected at Leeds CTRU. Additionally, in light of the published efficacy data for adjuvant zoledronic acid, we were able to investigate whether it is possible to identify patients at baseline who may benefit from the treatment, other than on the basis of menopausal status.

3.2 Hypotheses tested

1. Markers of bone metabolism (P1NP, CTX, 1CTP, vitamin D) can identify patients at risk of developing bone metastasis from early breast cancer.
2. Markers of bone metabolism (P1NP, CTX, 1CTP, vitamin D) can identify patients with early breast cancer who will develop (any site) disease recurrence.
3. Patients who have elevated bone turnover at baseline (determined by levels of P1NP, CTX and 1CTP) benefit from adjuvant treatment with zoledronic acid, compared with controls, in terms of skeletal recurrence.
4. Patients who have elevated bone turnover at baseline (determined by levels of P1NP, CTX and 1CTP) benefit from adjuvant treatment with zoledronic acid, compared with controls, in terms of (any site) disease recurrence.
5. Levels of vitamin D cannot predict which patients may benefit from adjuvant treatment with zoledronic acid.

3.3 Methods

3.3.1 Patients and data collection

The AZURE trial design and methods have been published elsewhere²⁰⁴. In brief, women with histologically confirmed breast cancer and either lymph node metastasis or T3/T4 primary tumour were eligible to participate. Following written, informed consent, participants were randomised to either standard (neo)adjuvant therapy (control arm) or standard (neo)adjuvant therapy plus intravenous zoledronic acid (ZOL) 4mg (treatment arm) for a total treatment duration of 5 years (figure 1-4). Additionally, calcium and vitamin D supplementation was recommended for all trial participants for the first 6 months on study (until visit 6) to be continued thereafter at the discretion of the treating clinician.

In addition to the main trial, participants at UK centres were invited to take part in the translational studies by giving additional consent for the collection of serum samples at study entry. No serial samples were taken. Samples collected at UK centres were stored at -20°C or -80°C depending on local facilities. Following regular transfer to Sheffield, samples were kept at -80°C until central batch analysis.

3.3.2 Laboratory methods

All markers were measured according to strict SOPs in a fully accredited central laboratory (Metabolic Bone Unit, University of Sheffield).

Procollagen type I N-telopeptide propeptide (PINP), cross-linked c-telopeptide of type I collagen (CTX) and 25-hydroxyvitamin D (25-OH

vitamin D) were measured using Cobas e411 automated immunoassays (Roche Diagnostic, Germany). Pyridinoline cross-linked c-terminal telopeptide of type I collagen (1CTP) was measured by manual enzymeimmunoassay (Orion Diagnostica UniQ ICTP EIA, Finland).

The P1NP assay works on the sandwich principle, with an initial incubation of 20µl of sample with a biotinylated monoclonal mouse antibody specific for P1NP. This is followed by a second incubation of streptavidin labelled microparticles with a mouse monoclonal P1NP-specific antibody labelled with a ruthenium complex. The microparticles within the reaction mixture are captured magnetically onto the surface of the electrode, through which a voltage is applied, inducing a chemiluminescent emission which is captured by a photomultiplier. Results are determined via a calibration curve.

The P1NP assay has a lower detection limit of < 5ng/ml and values were categorised as “high” if greater than or equal to 70ng/ml base based on previous reports of a possible predictive role of PINP in the development of bone metastases¹³ and advice from Roche Diagnostics. Results lower than 70 ng/ml were categorised as “normal”.

The principle of the assay for CTX is the same as that for P1NP, except that the mouse monoclonal antibodies used are specific for CTX. The CTX assay has a measuring range of 0.010 – 6.00ng/ml. The upper limit of normal for premenopausal women (0.299 ng/ml) was used to categorise results as either “high” (≥ 0.299) or “normal” (< 0.299). An additional analysis with a higher threshold (high > 0.556) was carried out to allow a closer comparison with the earlier study by Lipton et al¹⁷⁵.

The total 25-OH vitamin D assay works on the competition principle. There are 3 incubations. The first is a pre-treatment dithiothreitol and sodium hydroxide to release bound 25-hydroxyvitamin D from the vitamin D binding protein. The pre-treatment sample is then incubated with ruthenium labelled vitamin D binding protein, forming a complex. Finally, after the addition of streptavidin-coated microparticles and 25-hydroxyvitamin D labelled with biotin, the free sites of the ruthenium labelled vitamin D binding protein become occupied and form a complex. The microparticles within the reaction mixture are captured magnetically onto the surface of the electrode, through which a voltage is applied, inducing a chemiluminescent emission which is captured by a photomultiplier. Results are determined via a calibration curve.

The vitamin D assay has a measuring range of 3.0-70.0 ng/ml and values were categorised as either “sufficient” or “insufficient”. A level of ≥ 30 ng/ml was chosen to classify participants as “sufficient” based on expert consensus that this is the desirable concentration required for good general health^{205, 206}.

1CTP was conducted as a manual assay which is based on the competitive immunoassay principle according to the following procedure.

1. All reagents, controls and patient samples were brought up to room temperature at least 30 minutes before use.
2. 50 μ l of calibrator, control and patient sample were pipetted in duplicate into appropriate microtitre wells coated with goat anti-rabbit antibodies. 2 wells were reserved for the substrate blank.
3. 50 μ l of peroxidase labelled 1CTP were pipetted into all wells except blanks

4. 50µl of rabbit antiserum were pipetted into all wells except blanks, within 3 minutes.
5. The plate was incubated on a plate shaker at room temperature for 2 hours using a shaking speed of 600 rpm.
6. All wells were washed 4 times with the provided wash solution. All remaining moisture was removed by tapping firmly against absorbent paper.
7. 100µl of 3,3,5,5'-tetramethylbenzidine was pipetted into all wells.
8. The plate was incubated on a plate shaker at room temperature for 30 minutes.
9. The enzyme reaction was stopped by adding 100µl of 0.5M H₂SO₄ into all wells. The plate was placed back on the plate shaker for a further 30 seconds to mix the reagents.
10. Absorbances of all wells were read at 450 nm on a plate reader within 10 minutes.

The assay has a lower detection limit of 0.3 µg/l. Upper limit or normal was 4.2 ng/ml.

The inter-assay coefficient of variance (CV) will be calculated for each assay.

All markers were also examined as continuous variables.

3.3.3 Statistical analysis

All analyses were performed using SAS version 9.2 or 9.4.

Statistical analysis was performed on the final analysis datalock for AZURE, after a median of 84.2 months follow up and 966 disease-free survival events in the 3359 AZURE participants. All analyses were performed on the intention-to-treat population and included 872 UK patients who consented to the collection of samples. Hypothesis testing was performed at the two-sided 5% level. Analyses were performed for all participants combined and according to menopausal status.

Cumulative incidence function (CIF) curves were used to investigate time to bone as first recurrence and distant recurrence, to take into account competing risks. Cox's proportional hazards model was used to assess the relationships between the bone biomarkers and prognosis and treatment effect with zoledronic acid. Bone marker data were analysed both as continuous variables (log transformed) and as categorical variables, using the pre-specified high versus normal cut-points above for both prognostic and predictive relationships.

Analyses were adjusted for factors which were found to have a statistically significant prognostic effect on the relevant outcome in the main AZURE analyses. For bone as first recurrence analysis was adjusted for lymph node involvement, tumour stage and treatment allocation. For distant recurrence, analysis was adjusted for lymph node involvement, tumour stage and ER

status. For IDFS and IDFS component analyses analysis was adjusted for lymph node involvement, tumour stage, ER status and neo-adjuvant therapy. Analyses are also adjusted for treatment allocation when assessing the interaction of the markers with treatment (i.e. the predictive analyses), where the interaction term is used to test for heterogeneity between the different markers levels.

Prognostic analyses of bone recurrence at any time (whether or not bone was the first site of recurrence), time to first recurrence being in bone (either in bone-only or with synchronous distant metastasis) and time to first distant recurrence were carried out. Predictive analyses of IDFS and its components for vitamin D, P1NP, CTX and 1CTP, overall and by menopausal status, were all pre-specified prior to the analyses taking place. Exploratory analyses with the composite P1NP/CTX biomarker were carried out, in terms of both markers high versus no both markers high.

Additional analyses of the vitamin D data were undertaken using normalised values. Vitamin D values were normalised to account for the seasonal variation in baseline vitamin D. The monthly means were calculated, and a log (base 10) was used for smoothing. These, along with the overall (log) mean, were used to normalise each patient's baseline vitamin D value by dividing their log vitamin D value by the monthly (log) mean and multiplying by the overall (log) mean, before raising 10 to the power of this log normalised result, to calculate their normalised vitamin D value.

3.4 Results

3.4.1 Baseline characteristics

Baseline samples of stored serum from 872 patients were available for translational studies (441 control arm, 431 treatment arm). The median follow up for these patients was 84.2 months (range, 0-107.6 months; IQR, 71.7-92.1 months). Baseline characteristics are displayed in table 3.1. Mean age at baseline was 51.2 years (range 25-79) and 51.6 years (range 28-79) in the control and treatment arms, respectively. The baseline characteristics of the serum biomarker population are compared with those of the whole main AZURE population in table 3-2 showing that they have similar demographics.

	Control arm		Treatment arm	
	Number	Percent	Number	Percent
Tumour stage				
T1	139	31.5	146	33.9
T2	227	51.5	200	46.4
T3	59	13.4	72	16.7
T4	16	3.6	13	3.0
Neo-adjuvant therapy				
Yes	24	5.4	28	6.5
No	417	94.6	403	93.5
Systemic therapy				
Endocrine therapy alone	20	4.5	13	3.0
Chemotherapy alone	95	21.5	95	22.0
Both	326	73.9	323	74.9
Menopausal status				
Pre-menopausal	209	47.4	200	46.4
≤5 years since menopause	63	14.3	60	13.9

		Control arm		Treatment arm	
		Number	Percent	Number	Percent
>5 years since menopause		134	30.4	132	30.6
Menstrual status unknown		35	7.9	39	9.0
ER status					
ER positive		338	76.6	338	78.4
ER negative		101	22.9	91	21.1
ER unknown		2	0.5	2	0.5
PR status					
Positive		181	41.0	180	41.8
Negative		114	25.9	91	21.1
Unknown		146	33.1	158	36.7
Missing		0	0.0	2	0.5
HER2 status					
Positive		59	13.4	49	11.4
Negative		154	34.9	164	38.1
Unknown		16	3.6	14	3.2
Not measured		211	47.8	201	46.6
Missing data		1	0.2	3	0.7
Lymph node involvement					
0		7	1.6	9	2.1
One - three nodes involved		277	62.8	257	59.6
≥ four nodes involved		157	35.6	163	37.8
Unknown involvement		0	0.0	2	0.5

Table 3-1 Baseline characteristics of 872 patients who had serum available for markers analysis

	Biomarker population, n (%)	Main AZURE population, n (%)
Lymph node status		
0	15 (1.7)	62 (1.8)
1-3	534 (61.2)	2075 (61.8)
≥ 4	320 (36.7)	1211 (36.1)
Unknown	3 (0.3)	11 (0.3)
T stage		
T1	285 (32.7)	1065 (31.7)
T2	427 (49.0)	1717 (51.1)
T3	131 (15.0)	456 (13.6)
T4	29 (3.3)	117 (3.5)
ER status		
Positive	676 (77.5)	2634 (78.4)
Negative	192 (22.0)	705 (21.0)
Unknown	4 (0.5)	20 (0.6)
Menopausal status		
Pre-menopausal	409 (46.9)	1504 (44.8)
≤ 5 years since menopause	123 (14.1)	490 (14.6)
> 5 years since menopause	266 (30.5)	1041 (31.0)
Unknown	74 (8.5)	324 (9.6)

Table 3-2 Table comparing the baseline characteristics of the serum biomarker population with the main AZURE population.

Baseline data for the three biomarkers (also broken down into menopausal status), revealed that the proportion of patients in each category who fall above the normal ranges for P1NP, CTX and 1-CTP for the whole population were 27.3%, 30.0% and 50.5% respectively (Table 2), confirming that the data were appropriate to test the relationship between accelerated baseline bone turnover and subsequent distant recurrence events. As expected for the transition through menopause, the median values for each of the three bone turnover biomarkers showed a stepwise increase from pre-menopausal through peri-menopausal to > 5 years post-menopausal women.

An analysis was performed to determine whether the outcomes of the subset of AZURE patients within the biomarker study (872 patients) are

representative of the whole AZURE population as shown in Figure 3-1 (analysis carried out by Helen Marshall and Walter Gregory at Leeds CTRU). For all analyses, the hazard ratios (HR) in the biomarker population are in the same direction and of similar magnitude to those seen in the main AZURE analyses. Although the confidence intervals are wider in the biomarker population, as expected due to the smaller number of patients compared to the whole AZURE population, the disease outcomes of the biomarker subset of patients are similar to the randomised study groups as a whole.

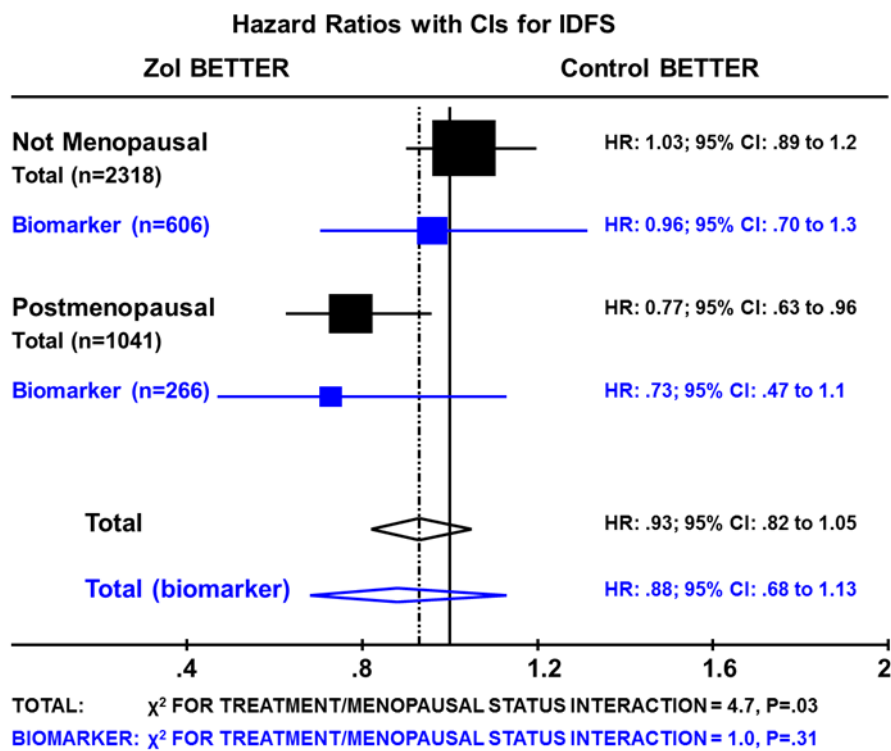


Figure 3-1 Forest plot of Invasive Disease Free Survival (IDFS) treatment hazard ratios and 95% confidence intervals (CIs) for all patients in the AZURE study (black) and patients in the biomarker population (blue).

Baseline results of the bone turnover markers (P1NP, CTX and 1CTP) are shown below in table 3-3.

Marker	Inter-assay CV (%)	Mean	SD	IQR
P1NP	4.1	59.1	26.92	41.2-72.7
CTX	4.0	.259	.153	.154-.324
1CTP	5.7	4.39	1.55	3.26-5.15

Table 3-3 Baseline data from bone turnover marker assays

P1NP values were not normally distributed, therefore they were log transformed using base 10 to approximate a normal distribution (figures 3-1 and 3-2).

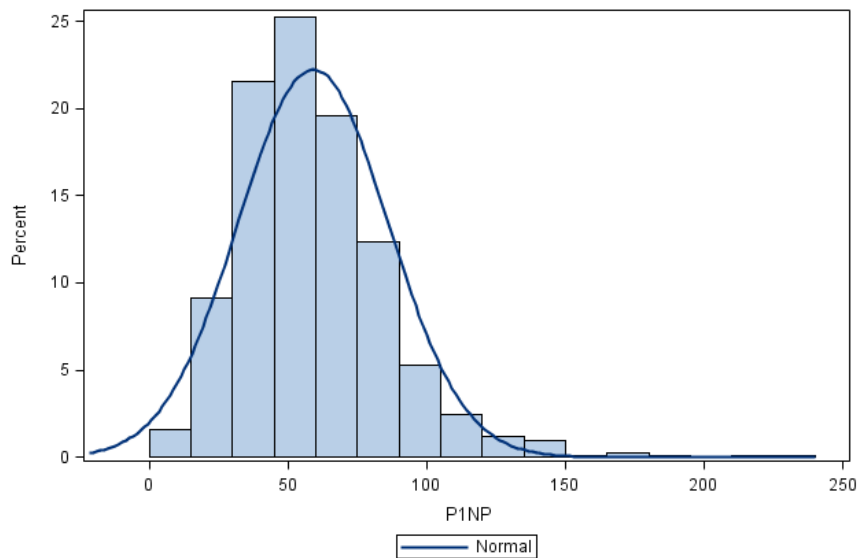


Figure 3-2 Distribution of P1NP values

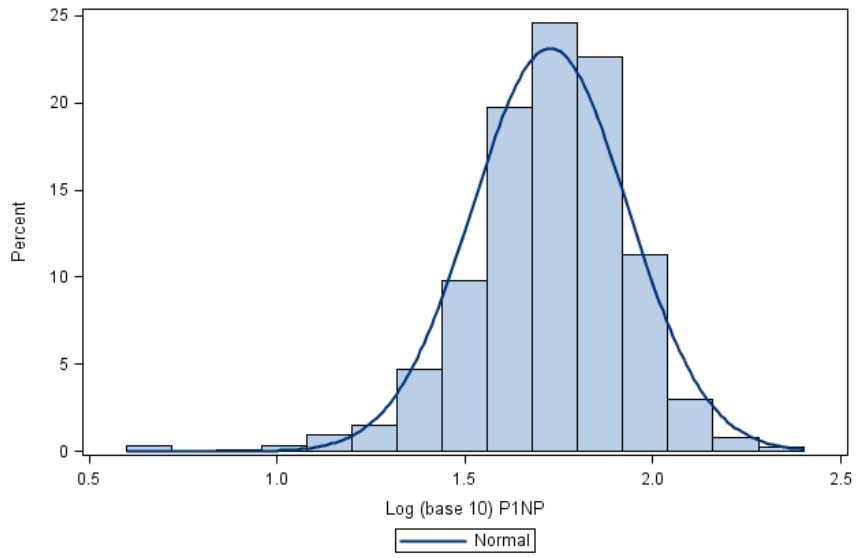


Figure 3-3 P1NP log transformed (using base 10) to approximate a normal distribution

	Menopausal status							
	Pre-menopausal		≤ 5 years since menopause		> 5 years since menopause		Menstrual status unknown	
	n	%	n	%	n	%	N	%
P1NP								
Normal	334	81.7	84	68.3	160	60.2	51	68.9
High	75	18.3	37	30.1	103	38.7	23	31.1
Missing	0	0.0	2	1.6	3	1.1	0	0.0
CTX								
Normal	337	82.4	74	60.2	140	52.6	50	67.6
High	71	17.4	46	37.4	122	45.9	23	31.1
Missing	1	0.2	3	2.4	4	1.5	1	1.4
1CTP								
Normal	226	55.3	57	46.3	101	38.0	37	50.0
High	182	44.5	61	49.6	164	61.7	33	44.6
Missing	1	0.2	5	4.1	1	0.4	4	5.4
Vitamin D								
≤30	361	88.3	103	83.7	236	88.7	66	89.2
>30	43	10.5	14	11.4	26	9.8	7	9.5
Missing	5	1.2	6	4.9	4	1.5	1	1.4

Table 3-4 Bone turnover markers by menopausal status (n, number).

CTX and 1CTP values were similarly not normally distributed, therefore they were log transformed using base e to approximate a normal distribution (figures 3-4 to 3-7).

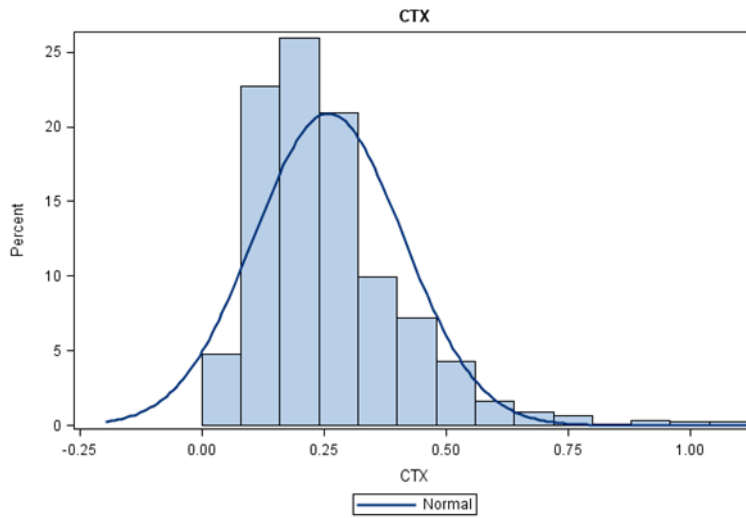


Figure 3-4 Distribution of CTX values

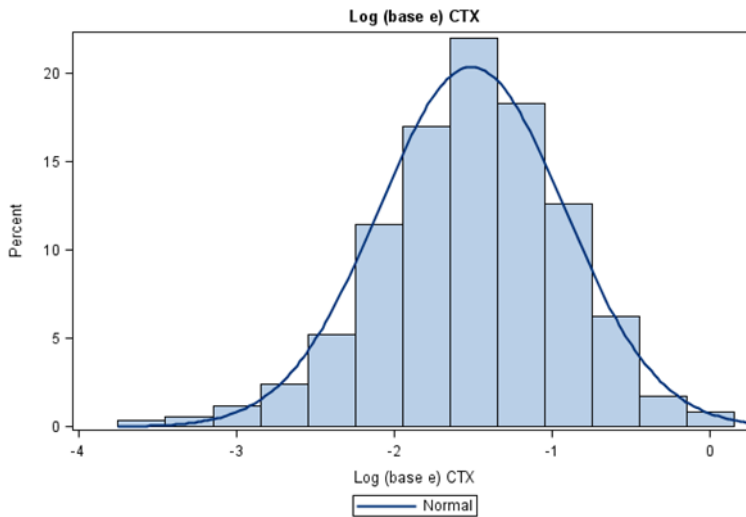


Figure 3-5 CTX log transformed (base e) to approximate normal distribution

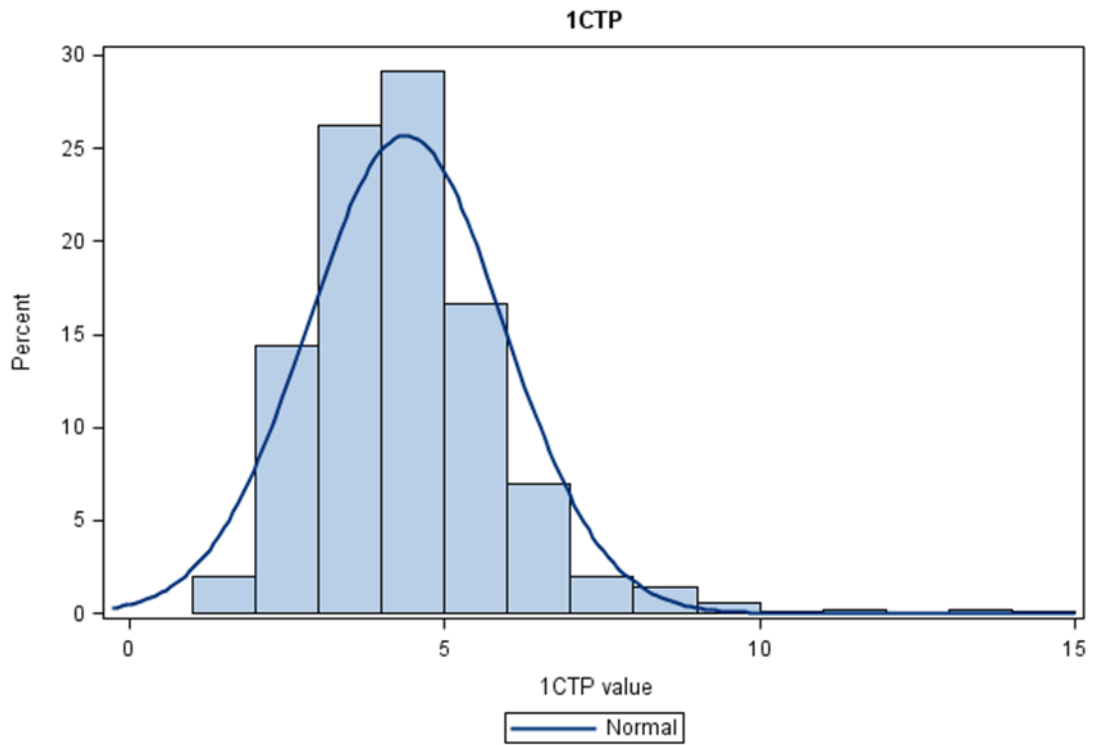


Figure 3-6 Distribution of 1CTP values

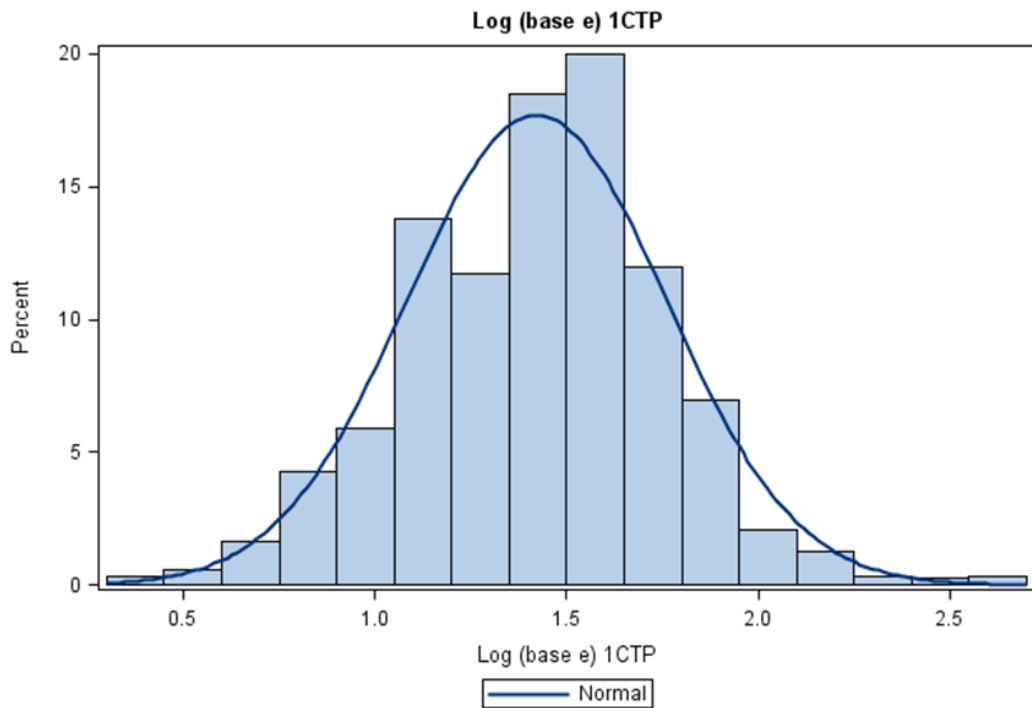


Figure 3-7 1CTP log transformed (using base e) to approximate a normal distribution

3.4.2 Bone recurrence at any time

When analysed as a continuous log transformed variable (adjusted for factors previously mentioned), all 3 bone turnover markers were associated with a statistically significant increased risk for development of bone metastasis (P1NP: $p=0.006$; CTX: $p=0.009$; 1-CTP: $p=0.008$; figure 3-8). When analysed as a categorical variable, both P1NP $>70\text{ng/ml}$ ($p=0.03$) and CTX >0.299 ($p=0.03$) were associated with statistically significant increased risk for development on bone metastasis at any time (table 3-5). This was not the case for CTX >0.566 ($p=0.12$) or 1CTP ($p=0.010$).

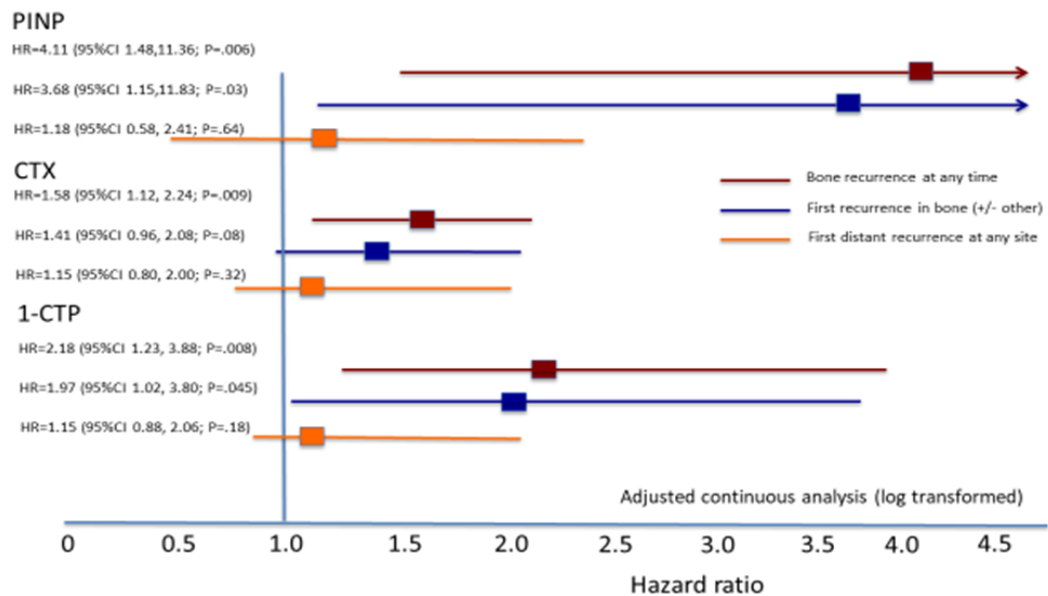


Figure 3-8 Hazard ratios and 95% confidence intervals (CI) for adjusted continuous analyses of log transformed data for baseline PINP, CTX and 1-CTP and disease outcomes.

3.4.3 First recurrence in bone

P1NP ($p=0.03$) and 1-CTP ($p=0.045$) were statistically significantly prognostic for first recurrence in bone in the adjusted continuous analyses (figure 3-8). However, the corresponding analyses for CTX showed that, although the HR values suggested increased risk, the results did not reach statistical significance. In adjusted categorical analyses, although the HRs for each marker were similar to bone recurrence at any time, the 95% CIs were wide and no statistically significant relationships between higher marker values and first disease recurrence in bone were seen. The number of bone-only first recurrence events was too small to justify separate analysis of this potential endpoint of interest.

Marker	Bone recurrence at any time HR (95% CI; p- value)	First recurrence in bone HR (95% CI)	First distant recurrence HR (95% CI)
P1NP <i>High vs. normal</i>	1.61 (1.07-2.42; p=0.03)	1.58 (1.00-2.50; p=0.06)	0.99 (0.72-1.37; p=0.96)
CTX <i>>0.299 vs. ≤0.299</i>	1.55 (1.05-2.31; p=0.03)	1.27 (0.80-2.00; p=0.32)	1.26 (0.93-1.71; p=0.13)
<i>>0.556 vs. ≤0.556</i>	2.17 (0.94-5.01; p=0.10)	1.95 (0.90-4.21; p=0.12)	0.94 (0.47-1.86; p=0.85)
1CTP <i>High vs. normal</i>	1.39 (0.94-2.05; p=0.10)	1.30 (0.84-2.02; p=0.24)	1.19 (0.89-1.59; p=0.25)

Table 3-5 Results from categorical analysis of bone markers for the 3 end-points

3.4.4 First distant recurrence

None of the 3 bone markers demonstrated statistical significance as a prognostic biomarker for distant recurrence, either as a categorical or continuous variable in an adjusted analysis (figure 3-8).

3.4.5 Composite P1NP and CTX biomarker analysis

Analyses were performed to assess risks of recurrence for patients where both P1NP and CTX were high (using the 0.299 ng/ml cutpoint for CTX) compared with all other patients. The adjusted analyses, along with the numbers of recurrence events for each endpoint for this composite categorical biomarker are displayed in table 3-6. No statistically significant relationships were identified between the composite marker and subsequent recurrence, although there was a borderline significant increased risk for bone recurrence at any time in the patients with elevation of both biomarkers (HR = 1.60, 95%CI 0.99, 2.48, p = 0.06).

Analysis	P1NP, CTX		Adjusted analysis		
	Both High Events/Censored (%)	Not Both High Events/Censored (%)	HR	95%CI	p- value*
Bone recurrence at any time	24/118 (16.9)	80/641 (11.1)	1.60	0.99 2.48	0.06
First recurrence being in bone	18/124 (12.7)	64/657 (8.9)	1.50	0.89 2.54	0.14
First distant recurrence (at any site)	34/108 (23.9)	154/567 (21.4)	1.00	0.68 1.45	0.99
First recurrence being in bone only	11/131 (7.7)	47/674 (6.5)	1.26	0.65 2.44	0.50

Table 3-6 Adjusted prognostic categorical analyses according to a composite P1NP-CTX marker for both P1NP and CTX high versus not both high.

3.4.6 Sensitivity analyses assessing optimum cut-points

Different cutpoints for categorical prognostic analysis of P1NP and bone metastasis at any time were explored. This analysis (figure 3-9) showed that the optimal cut-point for P1NP was approximately 64 nmol/ml, which we judged was sufficiently close to the pre-specified value of 70 nmol/ml, bearing in mind that the number of events was not sufficient to generate a smooth relationship. For 1-CTP and CTX, similar exploration yielded no clearly optimal cut-point or improvement to those pre-selected (data not shown).

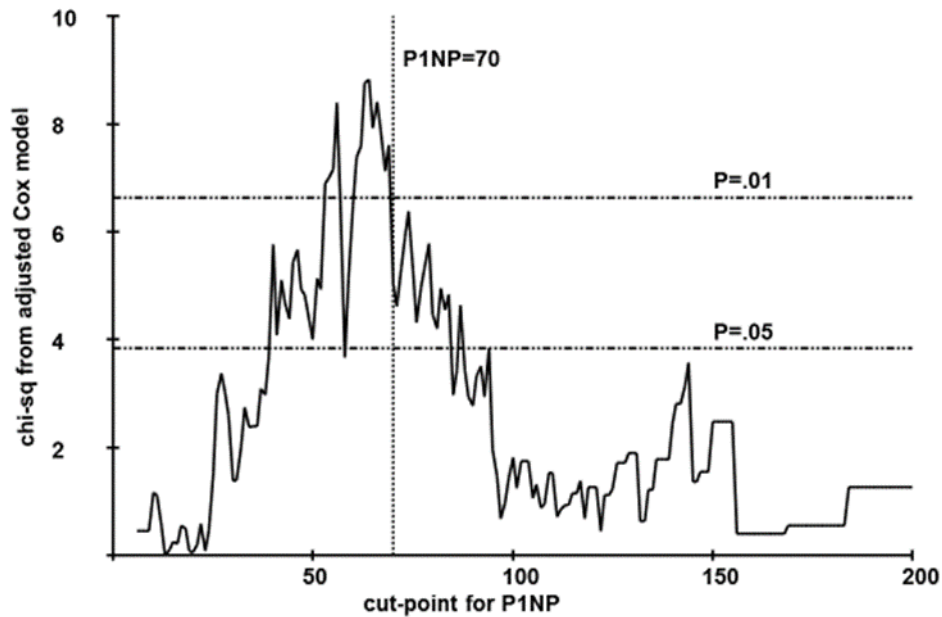


Figure 3-9 Identification of optimum cut-points.

χ^2 values from adjusted Cox proportional hazards model, analysing bone metastasis at any time by P1NP, with differing high vs. normal P1NP cut-points. Optimum cut-point observed at 64ng/ml with a corresponding p-value of 0.003. P1NP, N-terminal propeptide of type-1 collagen.

3.4.7 Analyses for treatment effect – test for predictive biomarkers

All 3 bone turnover markers and the composite P1NP/CTX marker were analysed to identify a potential predictive role in terms of benefit from zoledronic acid. In an adjusted analysis, no significant interaction with treatment allocation was identified for any of the three recurrence categories with any of the bone markers or the composite markers. This suggests that the bone markers do not predict for the benefits seen with zoledronate in postmenopausal women.

Although P1NP is higher in postmenopausal women and the benefits of zoledronate are largely restricted to this subset of patients, baseline P1NP did not predict benefit from zoledronate. For example, in cumulative incidence plots for effect of high P1NP on bone recurrence at any time or for bone as first recurrence (where we found a prognostic role for P1NP), there were no significant differences in outcome (HR 0.99 95%CI 0.52, 1.90, $p=0.693$; HR 0.84 95%CI 0.40, 1.75, $p=0.680$ respectively) between the zoledronate and control arms. Indeed, we found no significant interaction with treatment allocation for any of the four defined recurrence categories with any of the bone markers, or with the P1NP/CTX composite bone marker, suggesting that (data not shown).

Corresponding continuous (log transformed) analyses for bone metastases at any time found no statistically significant interaction with treatment allocation for any of the markers analysed: P1NP $p=0.74$; CTX $p=0.47$; 1-CTP $p=0.31$, confirming that these baseline markers are not predictive for the treatment benefits of zoledronate.

3.4.8 Vitamin D analyses

Inter-assay CV for this assay was 4.6%. Mean vitamin D was 18.2ng/ml (SD = 9.25; IQR, 11.1-23.7; range, <3 – 54.8ng/ml). Values below the limit of detection (<3ng/ml) were included in the analysis as 3ng/ml. 16 patients had a missing value and have been excluded from the analysis. Values were not normally distributed, therefore they were log transformed using base 10 to approximate a normal distribution (figures 3-10 and 3-11). In the control arm 8.6% had sufficient levels of vitamin D compared with 12.1% in the treatment

arm. Levels of vitamin D did not vary by menopausal status (tables 3-4 and 3-7).

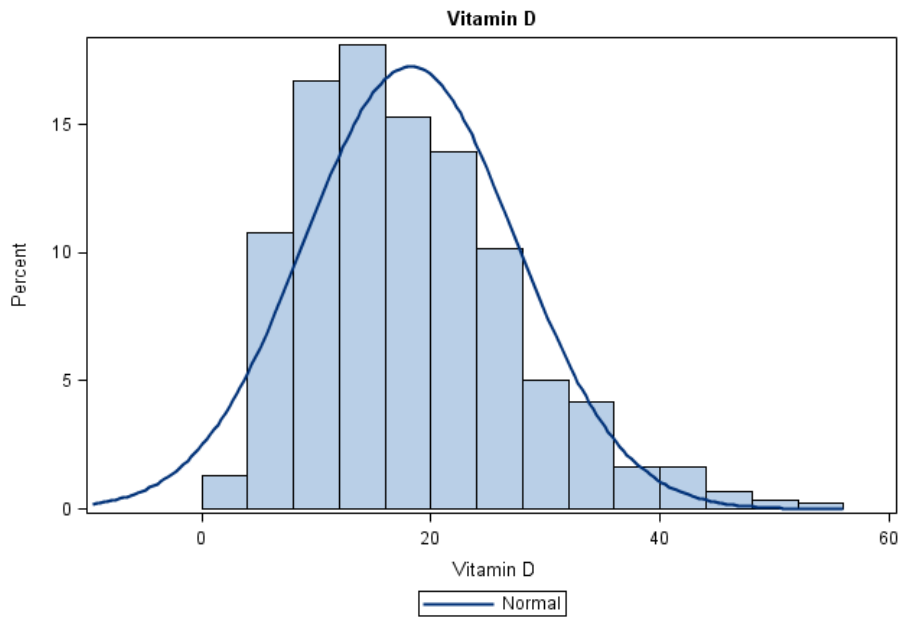


Figure 3-10 Distribution of vitamin D levels

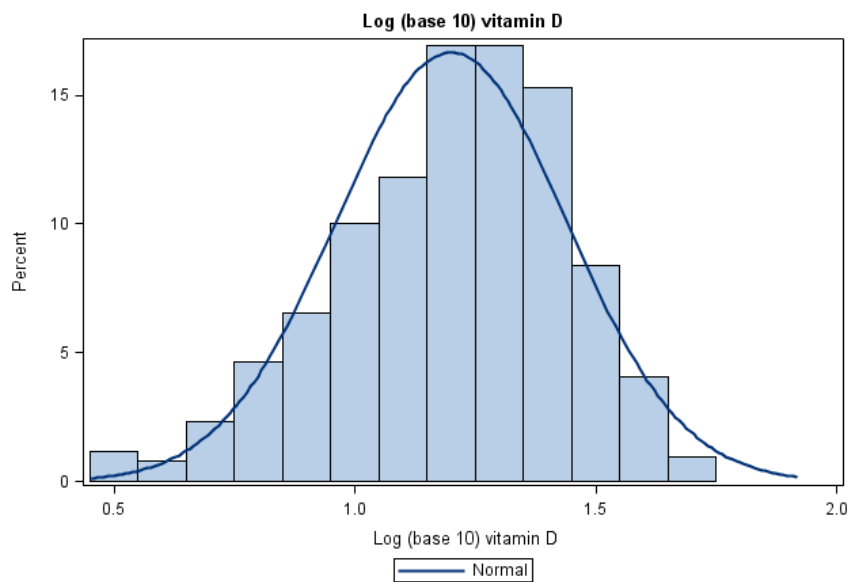


Figure 3-11 Vitamin D log transformed (base 10) to approximate normal distribution

Menopausal status	Number of patients	Mean	SD	IQR	Range
Pre-menopausal	404	18.03	9.35	10.89-23.12	<3-54.82
≤5 years since menopause	117	19.26	10.14	11.32-24.97	4.44-49.39
> 5 years since menopause	262	17.83	8.98	11.02-23.18	<3-49.28
Status unknown	73	18.70	8.10	12.85-23.66	7.52-42.43

Table 3-7 Vitamin D by menopausal status (measurements in ng/ml)

At visit 6 approximately 87% of patients in each arm were receiving calcium and vitamin D supplements. After this point, supplements were prescribed at the clinician's discretion and fell to 40.1% in the control arm and 51.1% in the treatment arm by visit 19 (final study visit).

3.4.8.1 Bone recurrence at any time

The numbers of events included in this analysis are shown in table 3-8. For time to bone recurrence at any time there is no significant difference between the sufficient and low vitamin D categories, however there is a trend towards lower risk in the sufficient group (HR 0.52, 95% CI 0.24 – 1.12; $p=0.066$).

3.4.8.2 First recurrence in bone

When adjusted for factors outlined in the methods, patients with sufficient levels of vitamin D at baseline have a lower risk for bone as a first recurrence but this does not reach statistical significance (HR 0.51; 95% CI, 0.20-1.26; $p=0.107$). The cumulative incidence function is seen in figure 3-12. This does not vary when additionally analysed by menopausal status. When analysed as a continuous variable vitamin D is not a significant prognostic marker for bone as a first recurrence ($p=0.5545$).

	Vitamin D						Total	
	Missing		≤30		>30			
	n	%	n	%	n	%	N	%
Bone only recurrence as first event								
Censored	16	100.0	710	92.7	88	97.8	814	93.3
Event	0	0.0	56	7.3	2	2.2	58	6.7
Bone recurrence as first event								
Censored	15	93.8	690	90.1	85	94.4	790	90.6
Event	1	6.3	76	9.9	5	5.6	82	9.4
Bone recurrence at any time								
Censored	15	93.8	670	87.5	83	92.2	768	88.1
Event	1	6.3	96	12.5	7	7.8	104	11.9
Distant recurrence								
Censored	15	93.8	595	77.7	74	82.2	684	78.4
Event	1	6.3	171	22.3	16	17.8	188	21.6
Total	16	100.0	766	100.0	90	100.0	872	100.0

Table 3-8 Number of events included in the vitamin D analyses

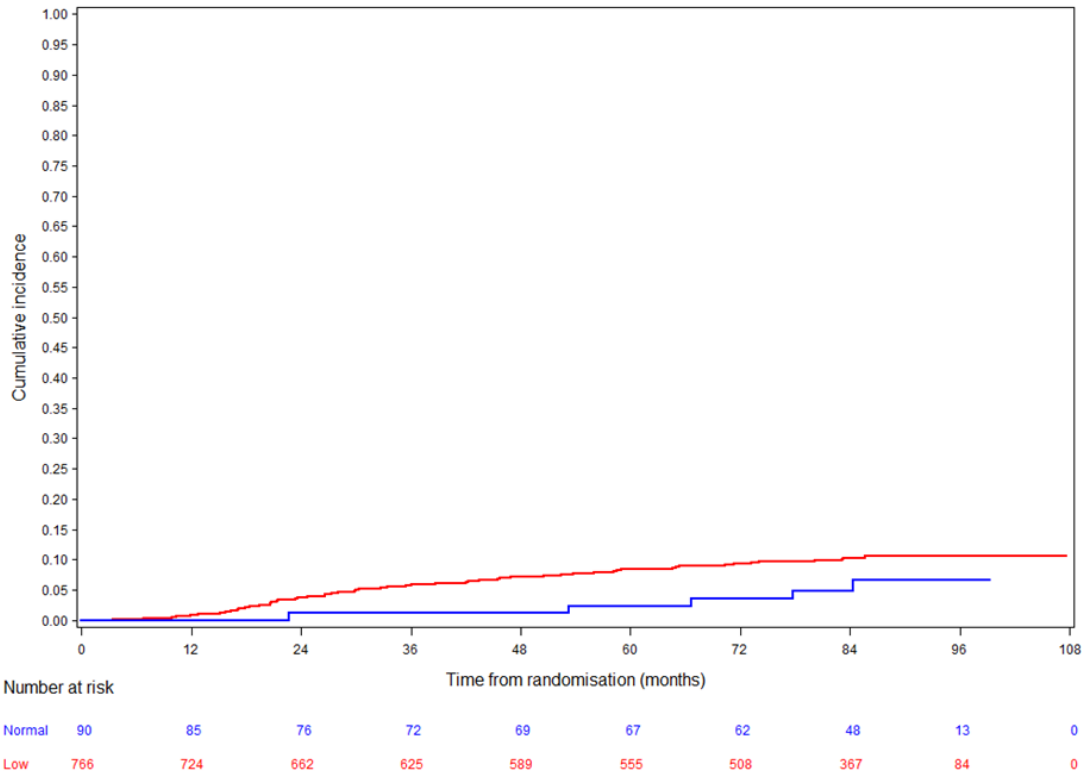


Figure 3-12 Cumulative incidence function for time to bone as first recurrence by vitamin D

3.4.8.3 First distant recurrence

The numbers of events included in this analysis are shown in table 3-6. Patients with sufficient levels of vitamin D have significantly lower risk for distant recurrence compared with those with low levels (HR 0.60; 95% CI 0.36 – 1.00; p=0.0378). See cumulative incidence function figure 3-16. When the analysis is performed additionally adjusting for menopausal status there remains a statistically significant difference (HR 0.6, 95% CI 0.36-1.10; p=0.042). When comparing different menopausal groups, the benefit is seen only amongst the non post-menopausal patients (see table 3-9).

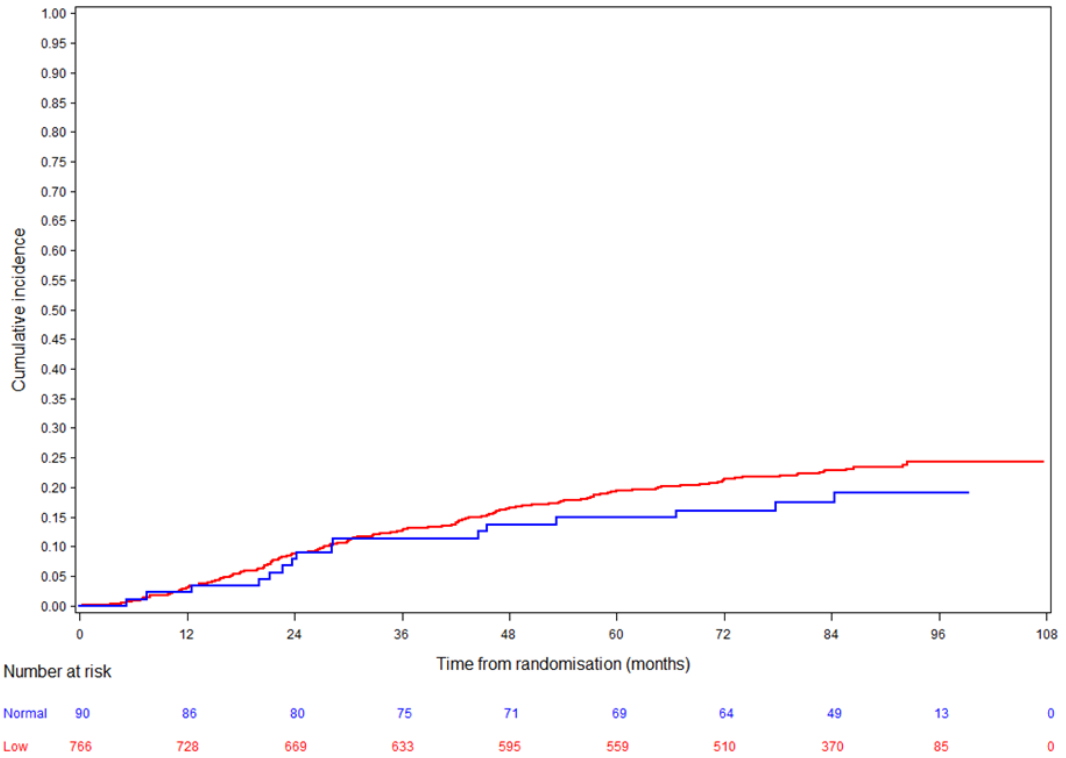


Figure 3-13 Cumulative incidence function for time to distant recurrence by vitamin D

Menopausal status	Analysis	Hazard ratio	Lower limit of 95% CI	Upper limit of 95% CI	Menopausal status interaction	
					X ² test statistic (DF)	p-value
Post-menopausal	>30 vs. ≤30 vitamin D (adjusted)	1.141	0.516	2.523	3.0717 (1 DF)	0.0797
Non post-menopausal	>30 vs. ≤30 vitamin D (adjusted)	0.437	0.221	0.866		

Table 3-9 Distant recurrence by vitamin D and menopausal status

3.4.8.4 Vitamin D as a continuous variable

Vitamin D as a continuous variable is not statistically significant as a prognostic marker for the development of bone as first recurrence (HR 0.99, 95% CI 0.97-1.02; p=0.555) or distant recurrence (HR 0.99, 95% CI 0.98-1.01; p=0.382).

3.4.8.5 Vitamin D treatment interaction

In terms of bone recurrence at any time and first recurrence in bone, there is a trend for increasing benefit from zoledronic acid in the sufficient vitamin D group however this is not statistically significant (table 3-10).

	Vitamin D level	HR	95% CI	Treatment interaction p value.
Bone recurrence at any time	≤30	0.985	0.657-1.477	0.287
	>30	0.425	0.094-1.913	
First recurrence in bone	≤30	0.823	0.521-1.301	0.386
	>30	0.365	0.060-2.207	

Table 3-10 Bone recurrence by randomised treatment arm – analysis of Zol vs. control by vitamin D category.

For distant recurrence, separate analyses were undertaken for non post-menopausal patients and post-menopausal patient. Following treatment interaction analysis, HR for non-post-menopausal patients with sufficient vitamin D treated with zoledronic acid was 0.712 (0.190 – 2.673) compared with 1.159 (0.803 – 1.672) for those with low vitamin D (p value for interaction = 0.4906). HR for post-menopausal patients with sufficient vitamin D treated with zoledronic acid was 0.081 (0.010 – 0.688) compared with 1.008 (0.572 – 1.778) for those with low vitamin D (p value for interaction = 0.0065).

For all components of the IDFS analysis, risk of recurrence is lower for patients with sufficient vitamin D levels compared with deficient levels (see table 3-11).

Component	Vitamin D	Hazard ratios			Treatment interaction	
		Hazard ratio	Lower limit of 95% CI	Upper limit of 95% CI	X ² test statistic (1 DF)	p-value
Skeletal distant recurrence	≤30	0.821	0.517	1.304	0.8970	0.3436
	>30	0.339	0.056	2.050		
Non-skeletal distant recurrence	≤30	1.386	0.914	2.103	2.2957	0.1297
	>30	0.537	0.171	1.685		
Local recurrence	≤30	0.890	0.486	1.631	1.2498	0.2636
	>30	0.354	0.077	1.615		
Second malignancy	≤30	1.408	0.682	2.907	1.5314	0.2159
	>30	0.305	0.028	3.383		
IDFS minus skeletal recurrence	≤30	1.067	0.788	1.446	3.2582	0.0711
	>30	0.468	0.201	1.091		
All IDFS events	≤30	0.984	0.754	1.284	4.4988	0.0339
	>30	0.392	0.173	0.889		

Table 3-11 Predictive IDFS component analyses – adjusted analysis of treatment arm versus control arm

3.4.8.6 Vitamin D analysis using normalised values

Mean normalised vitamin D values by menopausal status are shown in table 3-12.

Menopausal status	Number	Mean	SD	IQR	Range
Pre-menopausal	404	17.75	9.00	11.13- 22.86	3.11- 59.48
≤5 years since menopause	117	19.32	10.20	10.75- 26.19	4.38- 57.09
>5 years since menopause	262	18.07	9.58	11.32- 22.89	2.80- 56.96
Status unknown	73	19.06	8.43	13.00- 24.70	7.36- 43.10
All patients	856	18.17	9.31	11.30- 23.79	2.80- 59.48

Table 3-12 Summary statistics of normalised vitamin D by menopausal status and overall

Results for first recurrence in bone were similar to the analysis for the non-normalised vitamin D values, with a statistically non-significant reduced risk for women with sufficient levels of vitamin D (HR 0.56, 95% CI 0.22-1.39; $p=0.173$). The magnitude of risk reduction appears greatest among post-

menopausal patients (post-menopausal HR 0.369, 95% CI 0.050-2.739 vs non post-menopausal HR 0.642, 95% CI 0.231 – 1.782; p=0.612) however there appears to be a benefit to having sufficient levels of vitamin D for all groups. Patients with sufficient levels of normalised vitamin D appear to have lower risk for distant recurrence compared with those with low levels, however this is no longer statistically significant (HR 0.71; 95% CI 0.42 – 1.20; p=0.181). HRs are similar for post-menopausal and non-post-menopausal patients (0.782 vs. 0.677, p=0.803).

When normalised vitamin D was analysed as a continuous variable and as part of a treatment interaction, the results were very similar to those of non-normalised vitamin D (data not shown).

The significant treatment interaction seen with non-normalised vitamin D levels and the predicative “all IDFS events” component analysis is no longer significant when using the normalised vitamin D values (≤ 30 HR 0.921, 95% CI 0.705-1.203; >30 HR 0.640, 95% CI 0.289-1.418; p=0.391).

3.5 Discussion

The baseline characteristics of the serum marker sub-population are comparable to that of the main AZURE trial. This is reassuring with regards to the outcomes of the sub-population and translating the findings back to the main study.

This work has shown that, patients with early breast cancer and increased bone turnover, using bone turnover markers as a surrogate, are at increased risk of bone metastasis at any time. (P1NP $p=0.006$, 1CTP $p=0.008$, CTX $p=0.009$ when analysed as a continuous variable), with P1NP appearing to be the most sensitive of the markers studied. When analysed as a categorical variable, the HRs are all greater than 1, indicating the trend that high bone turnover can identify patients at greater risk for bone metastasis, however this only reached statistical significance for P1NP ($p=0.03$) and CTX ($p=0.03$). Using CTX and P1NP as a composite biomarker did not add to the sensitivity of the individual markers. This may be partially because the markers are not independent, reporting on linked metabolic processes, but may also be due to the relatively small numbers of events in the combined group.

This finding provides support for the hypothesis that the bone microenvironment, in which there is increased bone turnover in both formation and resorption, is a fertile soil for skeletal metastasis from breast cancer. By contrast with this clear association between baseline bone turnover markers and recurrence in bone, there was no association detectable between bone turnover markers and distant recurrence taken as a whole. It is acknowledged that, in some cases, elevation of baseline markers may be linked with active, but as yet undetected, bone metastases, however, the relatively long follow up (median 84 months) and few bone events in the first 2 years (<5%) when the cumulative incidence curves

diverge, makes it unlikely that the realised markers are simply an early diagnostic indication of bone metastases.

Whilst the work presented here has never been done before on such a scale and with such a robust dataset, there is some supporting evidence from the published literature. Lipton et al investigated CTX in 621 post-menopausal early breast cancer patients in a 5-year phase III trial of tamoxifen +/- octreotide, median follow up 7.9 years. They demonstrated that higher pre-treatment CTX (0.71 ng/ml cutpoint) was associated with shorter bone-only recurrence-free survival (RFS) as categorical variable (HR 2.8, 95% CI 1.05 – 7.48, $p=0.03$)¹⁷⁵. They also demonstrated its significance as a continuous variable. The trends in the present study are comparable with those of the Lipton study however the magnitude and significance of effect appears much greater in the latter work. This is likely due, in part, to the differences between the study populations; the Lipton study including only post-menopausal patients, almost entirely ER positive with lower risk disease and greater than two thirds did not receive adjuvant chemotherapy. In view of these characteristics it is likely that they were at greater risk from bone metastasis.

P1NP has previously been investigated in a relatively small group of mixed-risk early breast cancer patients not participating in a specific clinic trial²⁰⁷. 164 stage I-III breast cancer patients had their pre-treatment P1NP levels determined. The duration of follow up is unknown but a surprisingly high 55/164 patients developed bone metastases. Adjusting for factors including stage, grade, ER status and chemotherapy, P1NP was significant for early

bone recurrence (HR 2.9, 95% CI 1.2 – 6.0; p=0.03). There are a number of limitations of this study including its size, unknown follow up and heterogenous population but there is agreement between this and the present study.

A limitation of our study is that only baseline biomarker measurements were available for analysis and we are therefore unable to determine whether subsequent changes in bone turnover may also play a role. This was investigated by McCloskey et al who took paired serum samples at baseline and 1 year within the protocol of a large randomised clinical trial of oral clodronate versus placebo in early breast cancer¹²⁷. An increase in P1NP between the baseline and later sample was associated with significantly higher incidence of bone metastases compared with patients in whom the P1NP remained stable or reduced. They did not demonstrate prognostic significance for baseline P1NP alone.

Bone turnover markers are not prognostic for distant recurrence (any site). This finding suggests that the bone microenvironment status may play an important role in the development of skeletal metastasis from breast cancer but this does not necessarily translate into distant visceral metastasis. Whilst it has been suggested that cancer cells are attracted to the bone, perhaps initially as a sanctuary site where they may evade systemic therapies and then disseminate to the viscera, the mechanisms that are involved in the attraction to bone, maintaining their numbers low while dormant, reactivation, proliferation and then escaping are complex and dynamic^{50, 160}. Identifying a marker from this process is beyond the scope of this thesis, but an important area for future work.

The main AZURE study showed that there were significant reductions in the incidence of bone metastases either as a 1st recurrence (HR 0.78, 95% CI 0.63 – 0.96; p=0.020) or at any time (HR 0.81, 95% CI 0.68 – 0.97; p=0.022) in the treatment group¹⁸². The work presented here shows that bone turnover markers are unable to identify which patients may benefit from treatment with zoledronic acid. However, as the effect was seen in all menopausal groups, perhaps this is not surprising. It remains unclear what the mechanism is underlying the benefits seen in AZURE in post-menopausal women treated with zoledronic acid. We had thought that the benefits might be related to the higher rate of bone turnover that occurs at menopause however, the work in this thesis refutes this hypothesis as no association between bone turnover markers and the effects of zoledronic acid were detected. However, a number of factors may contribute to this result. Administration of multiple doses of a potent bisphosphonate can confidently be assumed to suppress bone turnover throughout the 5-year treatment period. This could render the baseline marker values less relevant in analyses of association. Additionally, bone turnover markers reflect activity across the skeleton as a whole whereas the amount of bone associated with disseminated tumour cells likely comprises only a very small fraction of the total skeletal metabolic activity. Finally, there is the intriguing possibility that the efficacy of zoledronic acid in the adjuvant setting may be due to a direct toxic effect on tumour cells in the bone microenvironment and independent of its action on bone turnover.

The overwhelming majority of women in this study had deficient levels of vitamin D. Only 8.6% and 12.1% in the control arm and treatment arm, respectively, had baseline levels ≥ 30 ng/ml, the level considered necessary for good bone health. This is consistent with other studies that have demonstrated a high prevalence of vitamin D deficiency amongst a breast cancer population^{208, 209}. Vitamin D deficiency is a recognised problem in Great Britain due to working indoors, northern climate, low dietary intake and obesity²¹⁰. A large population study reported that 90% of sampled adults had levels < 30 ng/ml in the winter and spring and 60% were deficient all year round²¹⁰. This is of particular concern due the finding in the present study that an “insufficient” level of vitamin D is prognostic for distant recurrence.

A recent meta-analysis has shown that patients with vitamin D levels of > 29.1 ng/ml have significantly lower breast cancer mortality compared with those with low levels (RR 0.58, 95% CI 0.40 – 0.85) and lower risk for breast cancer recurrence (RR 0.61, 95% CI 0.47 – 0.80)²¹¹. A number of mechanisms have been postulated for how vitamin D affects breast cancer risks and outcomes including through vitamin D receptors, which control a variety of cellular mechanisms such as differentiation, proliferation, apoptosis and angiogenesis²¹². These effects are seen in both oestrogen-dependent and oestrogen-independent tumours. In general, the work presented here agrees with the findings that patients at risk of breast cancer recurrence are more likely to relapse if their vitamin D levels are deficient. While the HR for bone as a first site of recurrence amongst women with sufficient levels of vitamin D is 0.5, this does not reach statistical significance, nor does this vary by menopausal status. However, with regards to any distant recurrence, women with sufficient levels are at

significantly lower risk, even when adjusted for menopausal status and treatment allocation (HR=0.6; p=0.0378). On further analysis, it appears that this is may be driven largely by the effect amongst non-post-menopausal women, though this is non-significant when analysed separately. Conversely, this study demonstrates that it is the post-menopausal women with sufficient levels of vitamin D that benefit from treatment with zoledronic acid but it must be borne in mind that the numbers at risk in this study are very low.

Although the mechanism is not clearly understood, it is increasingly accepted that the patients who may benefit from adjuvant bisphosphonates are those who are in established menopause, either natural or induced by GnRH analogues. However, it is uncertain how vitamin D levels may also interact in this oestrogen-deficient state to improve outcomes. It has been shown in pre-clinical studies that calcitriol inhibits the synthesis and biological action of oestrogens through suppressing aromatase expression²¹³. However, it is unknown whether the vitamin D-rich status promotes the underlying benefits of being oestrogen-deficient when receiving adjuvant bisphosphonates for early breast cancer.

4 Quantitative assessment of bone remodelling following adjuvant zoledronic acid in the AZURE study – methodology

4.1 Introduction

My role in chapters 4 and 5 included assisting set up of the QBS sub-protocol in Leeds and Sheffield, alongside medical physics and nuclear medicine, approaching and consenting patients, co-ordinating the timing of scans, present for most Leeds scans, undertaking blood sampling and limited laboratory work, in addition to all statistical analysis in chapter 5.

Despite the overall favourable safety profile of zoledronic acid, its toxicity has caused concern in recent years, particularly with regard to ONJ, as explored in chapter 2. A more recent concern is whether over-suppression of bone turnover has any detrimental effects, particularly in view of case report evidence of atypical fractures²¹⁴. While benefits of bisphosphonates are confirmed in terms of reduced skeletal fracture and expected improved bone mineral density, further exploration of the degree to which bone turnover is affected is required. It is currently unknown what effect an intense schedule of zoledronic acid will have on bone remodelling, particularly with regard to the skeletal regional differences, degree of difference from controls and the duration of any effects.

Studies demonstrating continued bone remodelling with the zoledronic acid dosing schedules used in osteoporosis have been reassuring²¹⁵. However, it is important to consider the issue of to what extent normal bone remodelling may be suppressed by the more intense adjuvant dosing schedules, since adjuvant zoledronic acid is now becoming the standard of care for postmenopausal women^{216, 217} and many of these women will be long-term survivors from their breast cancer.

4.1.1 Quantitative assessments on bone remodelling

4.1.1.1 Bone histomorphometry

The gold standard method for quantifying bone turnover is bone histomorphometry following double tetracycline labelling. The procedure for bone biopsy is invasive, involving extraction of a bone sample from the iliac crest using a trephine to obtain a cylindrical sample. This sample should contain internal and external layers of cortical bone in addition to an intermediate region of trabecular bone. The sample then undergoes an extensive process of preparation, cutting and staining before analysis can take place. The administration of tetracycline (orally or parenterally) allows dynamic assessment of bone metabolism. This fluorescent compound binds to mineralisation fronts, labelling them yellow-green under fluorescent light and acting as a marker for bone formation and mineralisation. Given 10-14 days apart, 2 doses of tetracycline will allow the amount of bone formed during that interval to be calculated by measuring the distance between the 2 fluorescent labels²¹⁸.

The primary limitation of this technique is the invasiveness of the procedure which does have potential complications including pain, bleeding, infection

and neuropathy. The technique only allows a single “snap-shot” in time, repeated biopsies would be required for assessment of response to treatment. In practice, double labelling with tetracycline is less useful in patients with highly suppressed bone turnover because of the significant percentage of patients showing either single labels or complete absence of labels. Finally, this technique only allows assessment of the specific region of the iliac crest.

4.1.1.2 Biochemical markers

As discussed in chapter 1, biochemical markers in both blood and urine are able to detect changes in bone turnover. To briefly recap, markers may either reflect resorption, including peptides from collagen degradation for example NTX, or formation, including peptides released during collagen synthesis such as P1NP. These markers have been extensively studied, are relatively easily measured and have reference ranges established.

More recently, markers have been developed to reflect more specific aspects of bone metabolism, including TRAP 5b and α/β CTX ratio. The 5b isoenzyme of tartrate-resistant acid phosphatase (TRAP 5b) was originally developed as a cytochemical test for hairy cell leukaemia as it was the only blood-derived cell to exclusively express type 5 TRAP²¹⁹. Shortly after, it was also identified as a marker of osteoclast function, with increased activity in metabolic and metastatic bone diseases. TRAP 5b is highly specific for osteoclasts, reflecting their number and thus providing complementary information to collagen degradation products which reflect the destruction of bone matrix²²⁰. It has been shown to be the most sensitive marker for measuring change, allowing precise measurements in individuals²²¹. Serum

TRAP 5b has been reported as responding to bisphosphonate therapy in post-menopausal women²²², in addition to being elevated amongst breast cancer patients with bone metastases²²³⁻²²⁶.

Once laid down, type I collagen is subject to a series of modifications that may influence bone strength including enzymatic cross-link formation and non-enzymatic glycation cross-linking, racemisation and isomerisation²²⁷. The ratio between the native α and isomerised β CTX measured in urine by specific immunoassays gives an estimate of the extent of type I collagen isomerisation in bone tissue²²⁸. In children, equilibrium between the 2 forms is not achieved due to the high rate of bone modelling, however, in adults, the rate of remodelling is slower than the rate of isomerisation, allowing equilibrium to be achieved. There are some clinical situations in which there is a localised increase in bone turnover for example Paget's disease and malignant bone disease, where again the equilibrium cannot be achieved resulting in higher α/β CTX ratio^{229, 230}. This alteration is associated with a disorganised collagen matrix and increased fragility which may consequently cause increased fracture risk^{231, 232}. Pre-clinical and clinical studies have demonstrated a decrease in α/β CTX ratio following bisphosphonate administration²³³. Thus, monitoring changes of α/β CTX may provide information on bone quality under long term zoledronic acid treatment which is not captured by BMD and conventional bone turnover markers.

Whilst bone markers are relatively easily measured in blood or urine using reliable, well-validated assays, they do have some disadvantages. Measurements on individual patients can be unreliable due to the day-to-day variations. Additionally, measurements in serum or urine reflect the degree

of bone remodelling occurring throughout the entire skeleton but are not useful for investigating specific skeletal sites of interest.

4.1.1.3 Dual-energy x-ray absorptiometry (DXA)

DXA has an important role in the early detection and monitoring of osteoporosis. It obtains a quantitative assessment of bone mineral density (BMD) and was a significant improvement on the assessment of bones compared to plain radiographs which rely purely on subjective, visual interpretation. Bone densitometry calculates bone mineral density in numerical units, providing a quantitative representation on bone mineral losses.

DXA uses highly collimated beams of low-energy x-rays. These beams are able to pass through soft tissues and bone and are captured by a detector placed on the opposite side. The intensity of the beam exiting the body is captured on the detector and is inversely related to the areal density (g cm^{-2}) of the body part being visualised. By measuring the attenuation of X-ray beams at two different energies, the areal densities of two types of tissue (bone and soft tissue) can be measured. For quality control processes, phantoms are scanned regularly. For example, the European Spine Phantom consists of 3 simulated vertebrae and is constructed to give BMD values of 0.5, 1.0 and 1.5g/cm² ²³⁴.

The hip and spine are usually chosen to evaluate fracture risk. As the spine has the most trabecular bone content, it best represents the patient's bone metabolism and therefore, vertebral bodies are generally regarded as being better for monitoring response to treatment than other skeletal sites²¹⁵. DXA devices compare the BMD result of an individual patient with the BMD data of the young normal populations (T-score) or with the BMD data of an age-matched control group (Z-score). These are expressed as units of standard deviation from the mean. Patients can then be classified according to whether they are normal, osteopaenic or osteoporotic at that particular site (WHO Classification, figure 4-1).

Normal	T-score \geq -1.0
Osteopaenic	T-score -1.0 to -2.5
Osteoporotic	T-score \leq -2.5

Figure 4-1 WHO Classification based on BMD

The National Health and Nutrition Examination Survey III (NHANES III) compiled a standard set of normal bone density measurements of the hip for different gender, ethnicity and age groups within the U.S. with which a test result can be compared²³⁵. More recently, the FRAX tool has been developed by Professor John Kanis and colleagues at the University of Sheffield to evaluate risk of fracture, integrating clinical risk factors specific

to the patient with BMD at the femoral neck to give a 10 year probability of fracture (hip or major osteoporotic fracture)²³⁶. Several risk factors are taken into account: low body mass index (BMI), previous fragility fracture, parental history of hip fracture, glucocorticoid treatment, current smoking, alcohol ≥ 3 units per day, rheumatoid arthritis, other secondary causes of osteoporosis.

DXA scanning along with appropriate use of risk scoring can provide extremely useful diagnostic and treatment response information. The scans are quick, give a low radiation dose and can be used to measure sites such as spine, hip and forearm. However, rates of change of DXA measured BMD are slow and even at a site such as the spine it can take several years to measure the rate of change. It is also possible to measure total body DXA which is of interest by providing a comprehensive view of changes across the whole skeleton (figure 4-2). Whole body scans measure bone mineral content (BMC) and average BMD in the total skeleton in addition to subregions skull, spine, arm, legs and pelvis. Furthermore, they can measure body composition, including total body and regional measurements of fat and lean.

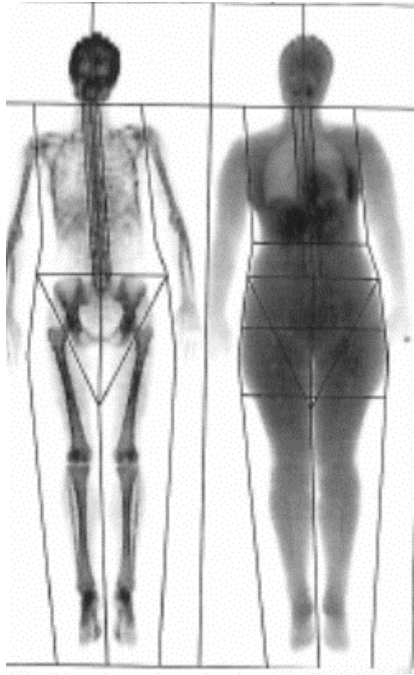


Figure 4-2 Example of image capture from whole body DXA scan

4.1.1.4 Quantitative radionuclide studies

Due to the affinity of bone for phosphate and phosphate tracers, their skeletal uptake has been investigated for their role in the detection of metabolic bone disorders. Quantitative radionuclide studies use short half-life radiopharmaceuticals such as ^{99m}Tc -methylene diphosphate (^{99m}Tc -MDP) to reflect the combined effects of bone blood flow and osteoblastic bone activity on the bone tracer kinetics²³⁷. The combination of blood sampling and gamma camera imaging allows quantitative investigation of bone tracer kinetics and there have been many developments in techniques over the last 3 decades²³⁸.

Initial methods included the 24-hour ^{99m}Tc -MDP whole body retention (WBR) test²³⁹. This technique is based on the compartmental model shown in figure 4-3.

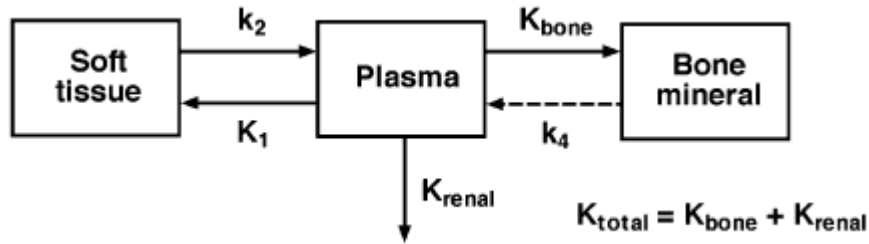


Figure 4-3 Compartmental model of tracer kinetics following intravenous injection

Approximately 2 hours following intravenous (i.v.) injection of $^{99\text{m}}\text{T-MDP}$, the tracer will have reached equilibrium with the extracellular, extravascular fluid compartment and is either cleared to bone or renally excreted. K_{bone} (mls min^{-1}) is the rate constant of the plasma clearance of the tracer to the bone mineral compartment (of whole skeleton) while K_{renal} (mls min^{-1}) reflects the clearance through the kidneys.

The value of k_4 (see figure 4-3) has been shown to be negligible²⁴⁰. Therefore, by 24 hours the WBR will approximate to the figure calculated from the division of the available tracer between bone and kidneys in the ratio of their respective plasma clearances.

$$\text{24-hour WBR} = K_{\text{bone}} / (K_{\text{bone}} + K_{\text{renal}})$$

The 24-h WBR therefore depends on the patient's GFR as well as the plasma clearance to bone.

4.1.1.4.1 Use of gamma camera

The previous method described used a whole-body counter, taking a baseline count at 5 minutes after injection and repeated at 24 hours.

However, these counters are now no longer widely available. Consequently, a number of methods using a dual-headed gamma camera have been described which combine counts from anterior and posterior views. This combination of counts reduces the attenuation errors due to the redistribution of tracer in the patient's body during the period of measurement. Quantifying the bone scan image must take into account the fact that by 4 hours post injection, around half of the non-excreted ^{99m}Tc -MDP is in the soft tissue, not bone. A method for this was described by Brenner et al ²⁴¹. By drawing a region of interest (ROI) around the adductor muscles of both thighs, an area that excludes any signal from bone can be selected. This count is performed at the time of the baseline whole body scan, commenced 3 minutes after injection, when the assumption is made that 100% of injected tracer is still in the soft tissue. These counts can be used to infer the whole body soft tissue retention on later scans and subtracted from the WBR to derive the bone uptake (see figure 4-4).

Soft tissue retention

$$ST(t) = 100\% \times Add(t)/Add(3 \text{ min})$$

Urinary excretion

$$U(t) = 100\% - WBR(t) + Bladder(t)$$

Bone uptake

$$B(t) = 100\% - ST(t) - U(t)$$

t, time since injection; ST, soft-tissue retention as a percentage of injected dose; Add, counts in adductor muscle ROI decay corrected to time of injection; U, urinary excretion as a percentage of injected dose; WBR, whole-body retention as a percentage of injected dose calculated from the decay corrected whole-body counts; Bladder, urinary bladder activity as a percentage of injected dose; B, bone uptake as a percentage of injected dose.

Figure 4-4 Equations used for the calculation of soft-tissue retention, urinary excretion and bone uptake of ^{99m}Tc-MDP using the standard Brenner gamma camera method.

4.1.1.5 Measurement of skeletal plasma clearance

As mentioned previously, the measurement of ^{99m}Tc-MDP bone plasma clearance (K_{bone}) to either whole skeleton or a defined region of bone is a more flexible and more informative measurement than the 24-hour WBR for monitoring response to treatment because it can be measured regionally and is independent of GFR. There are several methods for measuring this.

4.1.1.5.1 The area under the curve (AUC) method

This method uses the equation $K_{total} = (K_{bone} + K_{renal})$ and assumes that the rate constant k_4 is sufficiently small to be disregarded and therefore the total clearance of the free ^{99m}Tc-MDP can be calculated by dividing the amount of

tracer injected (Q) by the area under the plasma clearance curve, analogous to the calculation of GFR from a $^{51}\text{Cr-EDTA}$ plasma clearance curve²⁴². The measurement is complicated by the degree of protein binding, which can reach 70% at 24 hours following injection²⁴³. It is crucial to measure free MDP as the bound fraction is not available for skeletal uptake.

$$K_{\text{total}} = Q / \int_0^{\infty} P_{\text{FreeTracer}}^{99\text{mTc-MDP}}(t) dt = Q / \text{AUC}$$

where $P_{\text{free tracer}}^{99\text{mTc-MDP}}$ represents the plasma concentration of free $^{99\text{mTc-MDP}}$ at time t . The use of ultrafiltration as a method of measuring the plasma concentration of free $^{99\text{mTc-MDP}}$ has been validated by Moore et al²⁴⁴. As the renal clearance of free $^{99\text{mTc-MDP}}$ is the same as that of $^{51}\text{Cr-EDTA}$ the value of K_{bone} can be found by subtracting the GFR figure measured for $^{51}\text{Cr-EDTA}$ from the K_{total} figure measured using the previous equation.

$$K_{\text{bone}} = K_{\text{total}} - \text{GFR}$$

These plasma clearance curves are measured by multiple blood sampling between 5 minutes and 4 hours after tracer injection.

4.1.1.5.2 Modified Brenner method

In the Brenner method, soft tissue retention of $^{99\text{mTc-MDP}}$ is measured by imaging the adductor muscles in both thighs. In the modified method a dynamic study of this ROI is performed at the time of injection followed by a

series of 2 minute static images acquired at 1, 2, 3 and 4 hours with the same ROI copied onto the anterior and posterior images. A plot of the geometrical mean counts, after correction for decay, as a function of the area under the free ^{99m}Tc -MDP plasma curve can be constructed (figure 4-5)

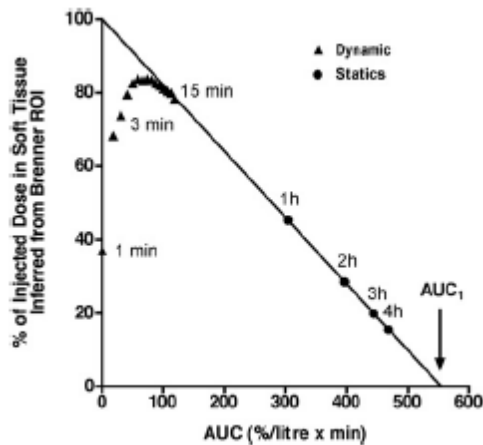


Figure 4-5 Curve showing the measurement of the total (renal plus bone) plasma clearance of free ^{99m}Tc -MDP using the modified Brenner method.

Taken from *Quantitative Studies of Bone Using ^{99m}Tc -MDP Skeletal plasma Clearance*²⁴⁵, with permission.

As Moore et al point out, there are 2 problems with the assumptions required for the original Brenner method. Firstly, the baseline whole body scan, even if started only 3 minutes after injection, will be misleading, as already about 10% of injected tracer will have been excreted via kidneys or cleared to bone due to the time it takes for the scan to reach the adductor muscles. Secondly, at such an early time-point, the tracer in soft tissue will have not yet reached equilibrium with tracer in the vascular compartment. They therefore developed a modified method in which the first soft tissue image is delayed until 1 hour after injection to ensure full equilibrium (figure 4-4)²³⁸.

The value of K_{total} can be found by extrapolating the straight line fit of linear decrease in percentage injected dose to find the intercept on the horizontal axis. The total plasma clearance through the kidneys and the skeleton can be calculated from the equation:

$$K_{total} = Q / AUC1$$

Renal plasma clearance of free ^{99m}Tc -MDP can be measured from a similar plot of the gamma camera measurements of WBR corrected for counts in the bladder and kidneys against AUC to find the intercept on the horizontal axis AUC2. Renal clearance can be calculated as

$$K_{renal} = Q / AUC2$$

K_{bone} can be found by $K_{total} - K_{renal}$ (see figure 4-6).

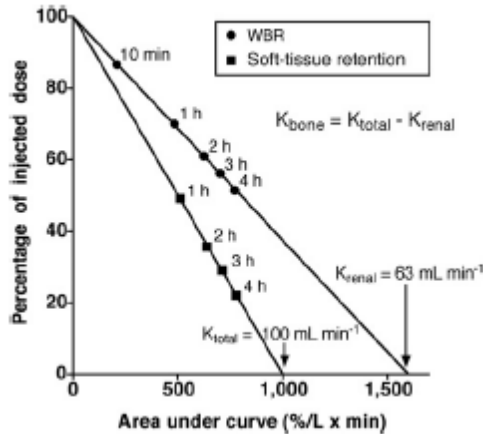


Figure 4-6 Plots of WBR and soft-tissue retention of ^{99m}Tc -MDP against AUC to estimate K_{total} and K_{renal} using the modified Brenner method

Taken from Quantitative Studies of Bone Using ^{99m}Tc -MDP Skeletal plasma Clearance²⁴⁵, with permission

4.1.1.5.3 Patlak Plot method

This method allows values for K_{bone} to be determined for either the whole skeleton or any chosen subregion. The method was originally developed as a theoretical model of blood-brain exchange, but the model is general and assumes linear transfer kinetics²⁴⁶. A simplified description of the mathematical principles is given elsewhere²⁴⁷. In brief, tracer uptake at time T can be measured for a ROI drawn on the bone scan (figure 4-7).

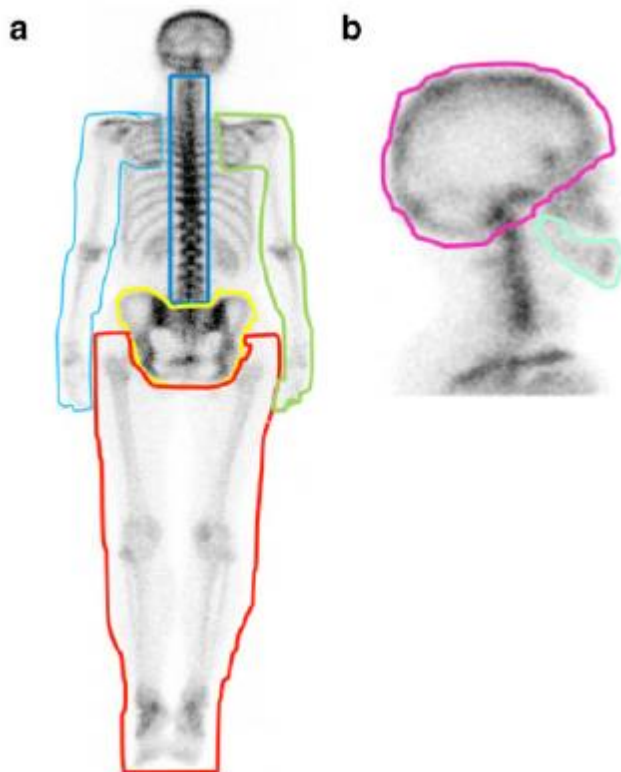


Figure 4-7 Displays regions of interest for measurement of K_{bone} values for: spine, pelvis, spine, arms and legs (a) and; mandible and calvarium (b).

Taken from Moore A et al²⁴⁸, with permission.

This uptake is composed of both tracer uptake in bone for the ROI and tracer uptake in soft tissue:

$$\text{Total Uptake} = \text{Bone Uptake} + \text{Soft Tissue Uptake} \quad (\text{Equation 1})$$

Bone uptake in equation 1 is equal to K_{bone} multiplied by the integral of the plasma concentration of free tracer from $t = 0$ to $t = T$. Once equilibrium is reached between tracer in the circulation and tracer in soft tissue (at about 2 hours after injection) then the soft tissue uptake in equation 1 is equal to the volume of distribution of tracer in the ROI multiplied by the total plasma concentration of tracer (bound plus free tracer). By substituting these two terms for bone and soft tissue uptake in equation 1 and dividing through by the total plasma concentration, the result is an equation for a straight line:

$$Y = m X + c$$

The slope m is the value of K_{bone} and the intercept c is the volume of distribution (figure 4-8).

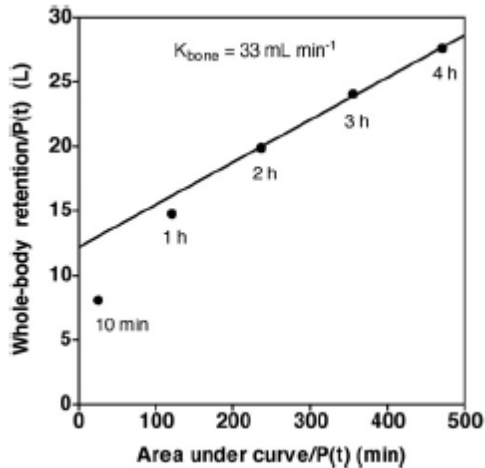


Figure 4-8 Patlak plot using ^{99m}Tc -MDP WBT and plasma data. K_{bone} was estimated from the straight-line fitted to the 2-, 3- and 4-hour time points. Points at 10 minutes and 1 hour deviate from the line as equilibrium has not yet been reached.

The 3 methods have been compared in a study reporting the baseline K_{bone} values in post-menopausal women participating in a clinical trial of teriparatide ²³⁸. They report mean values of $K_{bone} \pm \text{SD}$ as: i) 30.3 ± 6.4 mls/min using the AUC method; ii) 31.3 ± 5.8 mls/min using the modified Brenner method and ; iii) 35.7 ± 5.8 mls/min using the Patlak plot method. They conclude that there is close agreement between the AUC and modified Brenner methods, with no statistically significant difference between their results. However, the Patlak figure was higher than the other measurements ($p = 0.001$). When they re-examined the graphs, they identified that the slope for the 3-4 hours points was shallower than the 2-4 points. Recalculation based on using the 3-4 hours points brings the mean K_{bone} value to 33.0 ± 6.9 mls/min which was no longer significantly different. While this may be more accurate, the authors do point out that it would require a 6-hour measurement to compensate for the shorter time baseline, which would make the method less feasible.

4.2 Trial design

4.2.1 Main BoHFAB study

As the main BoHFAB study does not form part of this thesis, it will not be described in detail. However, it is summarised below to put the quantitative bone scan (QBS) sub-study into context.

244 patients (approximately equally drawn from the zoledronic acid and control arms) who have completed the 5-year main AZURE trial were recruited from UK centres. It is estimated that 60% patients who entered the main AZURE study were eligible for the main BoHFAB study. Patients were to be recruited within 3 months of completion of the main study.

Inclusion criteria:

- Participation in either the control arm or the zoledronic acid arm of the main AZURE study.
- Ability to perform first DXA lumbar spine and total hip measurement on this study within 3 months of the 5 year follow-up visit on the AZURE study.

Exclusion criteria:

- Presence of metastatic or recurrent breast cancer.
- Use of bisphosphonates other than on the AZURE study.
- Severe physical or psychological concomitant diseases that might impair compliance with the study protocol.

- Inability to obtain reliable DXA information due to pre-existing pathology or prior surgery
- Pregnancy or breast-feeding at study entry.

At entry to the main BoHFAB study, patients in the control arm will have a DXA BMD assessment of lumbar spine and total hip. These scans were performed on either a GE-Lunar (GE-Lunar, Madison, WI) or Hologic (Hologic Inc., Bedford, MA) machine, depending on site. GE-Lunar and Hologic machines are calibrated differently and the spine and hip BMD data were pooled after converting them to a common scale (referred to as standardised BMD) based on the scans of the European Spine Phantom. The femoral neck BMD, together with the clinical risk factors which are recorded on the FRAX Questionnaire (Appendix 4) permits their 10-year fracture risk to be calculated by the FRAX algorithm with treatment as indicated by reference to the National Osteoporosis Guideline Group (NOGG, www.shef.ac.uk/NOGG). Patients identified on study as being at high fracture risk were referred to their local osteoporosis centre for treatment advice and were followed up, but excluded from further data point analyses. If osteoporosis is detected in a patient from the zoledronate arm, they will be referred to their local osteoporosis centre for treatment advice and will be followed up, but excluded from further data point analyses.

The following assessments will be carried out:

- Measurement of BMD by DXA scan at lumbar spine and total hip (including femoral neck) at study entry, 12, 24 and 60 months

- Bone markers (NTX, P1NP, TRAP-5b and α/β CTX) at study entry, 6, 12, 24, 36, 48 and 60 months.
- Skeletal health questionnaire at study entry
- Recording of symptomatic fractures occurring on study (vertebral and non-vertebral, the latter to include femoral neck, radius or other).

Endpoints: The primary endpoint is a 2.5% difference in the mean percentage change in lumbar spine (L1-4) BMD at 24 months between the zoledronic acid and control arms.

Secondary endpoints are:

- The difference in mean percentage change in total hip BMD at 24 months between the zoledronic acid and control arms
- The difference in mean percentage change in lumbar spine BMD and in total hip BMD at 12 and 60 months
- The difference in mean percentage change in bone markers at 6, 12 and 24, 36, 48 and 60 months between the zoledronic acid and control arms
- The difference between the zoledronic acid and control groups at study entry in the following: mean BMD at lumbar spine; mean BMD at total hip; mean NTX, mean P1NP, serum Trap 5b and urinary α and β CTX

The study schema (including sub-study) is shown in figure 4-9.

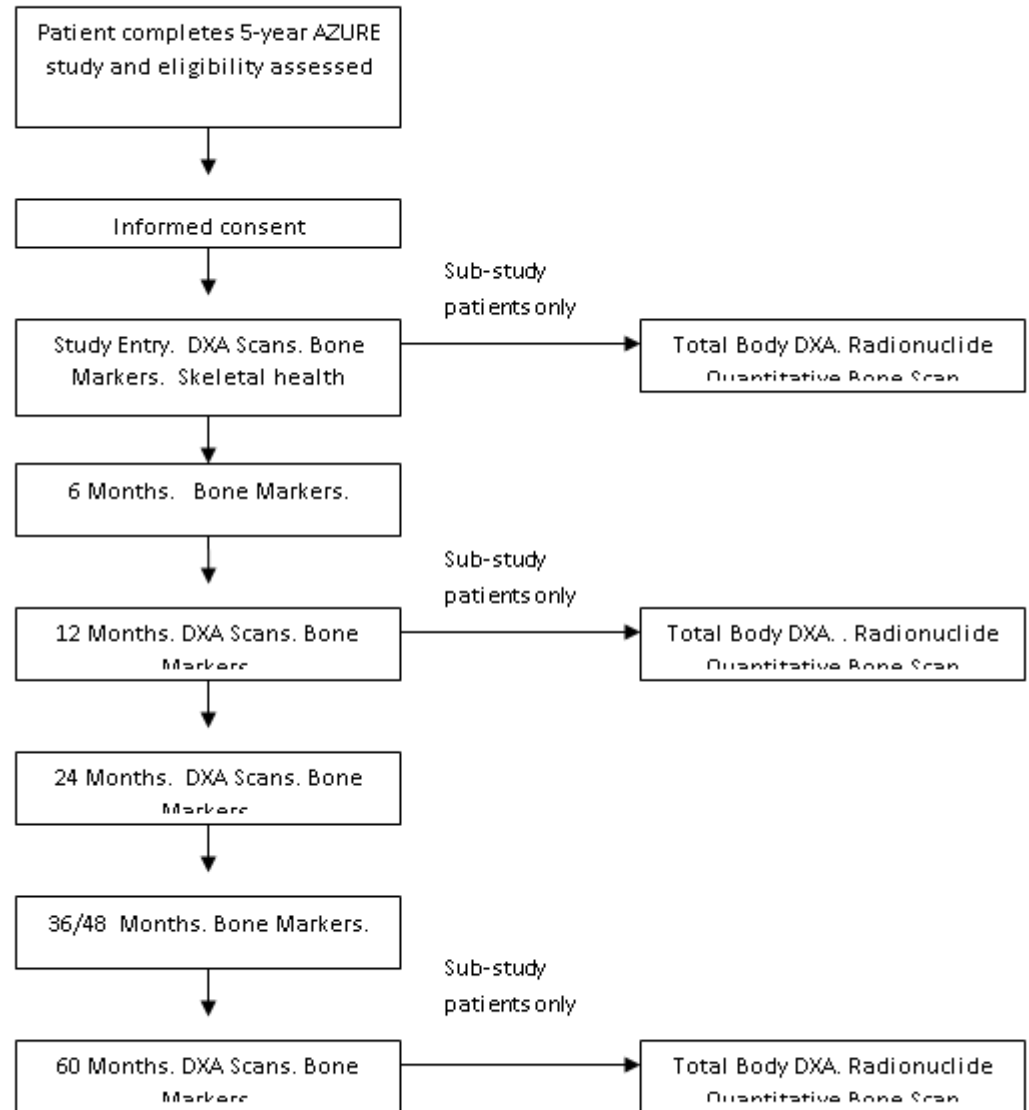


Figure 4-9 BoHFAB study schema including main and sub-studies

4.3 Quantitative bone scan sub-study design

4.3.1 Aims of study

Whilst studies with the zoledronic acid dosing schedules used in osteoporosis have been reassuring, it is important to consider the issue of to what extent normal bone remodelling may be suppressed by the more intense adjuvant dosing schedules. This will be addressed in the quantitative bone scan (QBS) sub-study.

4.3.2 Patients and recruitment

This sub-study was carried out in 40 patients (equal numbers from the control and zoledronic acid arms) drawn from those patients in the main BoHFAB study who were recruited from the Sheffield/Leeds centres. Written informed consent was taken specifically for the sub-study, in addition to the consent taken for the main BoHFAB study. Although the sub-study will be continued for 5 years, this report refers to the first two years, which includes the primary endpoint.

4.3.3 Assessments

4.3.3.1 Quantitative bone scan methodology

Patients undergo QBS at baseline, 12 months, 24 months and 60 months from consent to the sub-study. The following procedure was used for each QBS investigation:

Pre-scan checks

1. Identify the patient following the local protocol.
2. Measure the patient's height and weight – these are used to estimate their plasma volume and subsequently the initial plasma activity concentration of ^{99m}Tc -MDP.
3. Measure the activity of the ^{99m}Tc -MDP syringe in an assay calibrator, recording the exact time of the measurement.

After the study commenced it was realised that the Leeds and Sheffield centres were using different ^{99m}Tc diphosphonates. Leeds were using ^{99m}Tc -HMDP while Sheffield were using ^{99m}Tc -MDP. The statistical plan was therefore altered to allow for the systematically higher values of K_{bone} measured using ^{99m}Tc -HMDP.

Radioisotope administration

1. Administer the tracer, ^{99m}Tc -MDP (~600 MBq) peripherally via an indwelling venous cannula with a 3-way tap. In the event of extravasation the test should be abandoned, as this will make the kinetics of the tracers unpredictable.
2. Record the exact mid-point time of the administration.
3. To promote voiding the participant should be encouraged to drink ~300mL of fluid per hour
4. Measure the residual activity left in the ^{99m}Tc -MDP syringe in an assay calibrator, recording the exact time of the measurements. Dispose of the syringe in a designated sharps bin.

Blood sampling

1. Venous blood samples are taken at, or as close as possible to: 5, 20, 60, 120, 180 and 240 minutes post injection. To ensure there is no contamination of the samples they should be taken from a location other than the injection site, preferably from the contralateral arm
2. 6 - 7mL whole blood taken to ensure 1mL of whole plasma and 1mL of protein free plasma can be assayed – place the blood in a green top (sodium heparin) collection tube.
3. Record the exact mid-point time of each blood sample.
4. Disposed of all contaminated waste in the designated bins.

Gamma camera imaging

This QBS protocol should be adopted on a dual-headed gamma camera.

1. Scans were acquired at, or as close as possible to: 10, 60, 120, 180 and 240 minutes post injection.
2. The patient encouraged to empty their bladder before each scan is started.
3. Immediately before acquiring each whole body scan, a simultaneous anterior and posterior two-minute static scan of the thighs was acquired. This is used to assess soft-tissue retention and is required for the modified Brenner method of analysis.
4. Then acquire a simultaneous anterior and posterior whole body bone scan - note a quicker than normal scan speed will be adopted 25cm/min.

5. Immediately after acquiring each whole body scan a 5-minute static lateral skull view was acquired.
6. The exact start time of each study was recorded.
7. For the later scans (i.e., 60 minutes scan onwards) the participant was reproducibly positioned, aided by:
 - a. Noting down the position of their head on the couch
 - b. Providing the same head (lower back, leg etc) support during each scan
 - c. Tying their feet together
 - d. Using a Velcro wrap to 'restrain' their arms by their side.
8. A diagnostic level scan was acquired at 3.5 hours post injection, with a scan speed of 10cm/min.

Sample counting

1. Centrifuge the whole blood for 10 minutes at 2000 rpm.
2. To allow corrections for pipetting inaccuracies, all counting tubes (and their tops) were weighed empty and then again after pipetting.
3. Pipette 1mL of plasma into a counter tube and label appropriately.
4. Pipette at least 2mL of plasma into a 10-kDa filtered tube (Amicon®-Ultra: Cat No. UFC801096)
5. Centrifuge all the filtered tubes for 40 minutes at 2000g.
6. Pipette 1mL of ultrafiltrate (i.e. protein free plasma) into a counter tube and label appropriately.
7. All contaminated waste was disposed of in the designated bins.

Preparing the standard

1. Radiopharmacy to dispensed ~10-80 MBq from the stock solution used for the patient's administration, into a syringe.
2. The activity was measured in an assay calibrator, recording the exact time of the measurement.
3. Introduce the standard solution into a 1 litre volumetric flask, add water until the bottom of the meniscus is at the 1 litre reference point and mix well.
4. To allow corrections for pipetting inaccuracies, weigh all counting tubes (and their tops) empty and then again after pipetting.
5. Pipette 1mL of standard solution into three counting tubes and label appropriately.
6. Measure the residual activity left in the ^{99m}Tc -MDP standard syringe in an assay calibrator, recording the exact time of the measurement.

Gamma counter

1. Pipette three 1mL water samples for background sample counting and label appropriately – pipetting accuracy is not essential so the samples do not need to be weighed.
2. Batch the following samples into different trays, placing them in the counter in the following order:
 - i. Whole plasma,
 - ii. Protein free plasma,
 - iii. Standard
 - iv. Water i.e. background

3. Count the samples using protocol 4 – i.e.140keV +/-10% for 20 minutes.

Preparation of individual spreadsheets for data analysis

An individual spreadsheet was prepared for each QBS study performed comprising all collected data, timings of interventions and required corrections to allow calculation of K_{bone} by the 3 different methods and regional values by Patlak Plot method (please see sample in appendix 5). ROIs are drawn around the skull, pelvis, spine, arms, legs, calvarium and mandible. The numbers of pixels and counts for each ROI, and whole skeleton, are recorded in the spreadsheet.

4.3.3.2 DXA scan methodology

Measurements were made at lumbar spine, total hip (which also allows BMD at femoral neck to be calculated) and whole body for BMD assessment and BMC. Matching the QBS ROIs with the whole body DXA regions allows the normalisation of bone plasma clearance to BMC (mls min^{-1} per gram of bone), considered a more accurate assessment than K_{bone} (mls min^{-1}) alone. All participating centres already had access to a DXA bone densitometry service using either Hologic or GE-Lunar densitometers. Spine and hip DXA scans were carried out locally, but centrally coordinated by the Osteoporosis Centre in Sheffield. This centre provided standard operating procedures to each centre, as well as the standard European Spine phantom (ESP) to allow cross-calibration. However, all total body DXA scans were performed on GE-Lunar densitometers in either Leeds or Sheffield on the day of the

QBS scan and the BMD and BMC values from these scans were not cross-calibrated with the ESP.

The DXA scanners were regularly checked by scanning of phantoms according to the quality control standards of the DXA scanner manufacturer. It is important that follow-up BMD scans are performed on the same machine as used for the baseline scans. Where this was not possible, for example, because of centres upgrading or changing their densitometer, cross-calibration was performed to enable interpretation of follow-up results. A single ESP calibration phantom was used for the study and was sent to each centre in turn. In addition, each centre received a detailed SOP in DXA measurement for the study and was asked to send regular QA measurements from their DXA scanner to Sheffield for the duration of the study. All DXA scans were transmitted electronically in a fully anonymised format to the Sheffield Osteoporosis Centre.

4.3.3.3 Bone marker methodology

Samples for bone marker measurement were collected and stored according to strict SOPs provided by the Sheffield Metabolic Bone Unit. Samples were stored locally (-80°C) for up to one year, before being transferred to central storage (-80°C) at the Sheffield Metabolic Bone Unit (Fatma Gossiel). Measurements in Sheffield were carried out in batches, as this was most economical and subject to least intra-assay variation. NTX was measured in second morning voided urine samples by a chemiluminescent assay using a Vitros ECI analyser⁵² and expressed relative to urinary creatinine. P1NP

was measured in serum using radioimmunoassay as described in chapter 3 (Orion Diagnostics Oy, Finland).

Serum Trap 5b was measured by a specific ELISA (Immunodiagnostic Systems) and Urinary α and β CTX was measured by the Urine ALPHA CrossLaps ELISA® and the Urine BETA CrossLaps® ELISA, respectively. These are based on highly specific monoclonal antibodies against a specific amino acid sequence.

4.3.3.4 Other assessments

As part of the main study, a skeletal health questionnaire, developed in the Sheffield Osteoporosis Centre which has been verified over several years, was used (FRAX), combining the recorded family history of osteoporosis, previous fractures, concomitant medication and other data from the WHO Risk Factor Questionnaire (Appendix 4) with the femoral neck BMD to calculate 10-year fracture risk. The questionnaire is attached in appendix 4. Additionally, symptomatic fractures were recorded as part of the main AZURE study for comparison between the zoledronic acid-treated patients and control patients. Symptomatic fractures occurring during the study were recorded and the recording included if traumatic or low trauma fractures and site of fracture (vertebral and non-vertebral, the latter to include femoral neck, radius or other).

Ethical approval was sought and obtained from the West Midlands Research Ethics Committee (approval letter in appendix 6).

4.3.4 Endpoints

- A significant difference in the baseline measurements of $K_{\text{bone}}/\text{BMC}$ between the treatment and control groups
- A significant change in $K_{\text{bone}}/\text{BMC}$ from baseline at 1 and 2 years (and 5 years within the trial but out with the scope of this thesis) using patients as their own controls.
- A significant difference in BMD between the two groups
- A significant difference in mean NTX, P1NP, TRAP5b and α/β CTX NP, CTX, 1CTP at baseline, 12 months and 24 months between the two groups.

4.3.5 Statistical plan

4.3.5.1 Sample size calculations

From earlier studies in which the technique was developed for osteoporosis²³⁸, using a population standard deviation of 30%, and 80% power, 30 patients (both groups) are required to show a difference in K_{bone} of 20% ($p < 0.05$) at baseline. Similar power calculations based on a measurement precision of 20% show that 30 patients are also required to detect 20% change in K_{bone} from baseline, using patients as their own controls at 1,2 or 5 years. Allowing for 25% drop out, 40 patients will therefore be recruited.

4.3.5.2 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics 21. All endpoints were subjected to statistical significance testing with a 5% (2-sided) significance level. Comparisons with the control group at each timepoint (baseline, 12 month, 24 months) were analysed using a linear regression model adjusting for treatment allocation (control versus treatment arm) and tracer (HMDP versus MDP). The regression beta coefficient is used to estimate the degree of difference between the 2 groups and 95% CI for the coefficients are also reported. The Student's T-test was additionally used to compare mean values where necessary.

Change from baseline was subject to similar statistical testing, using patients as their own controls where appropriate.

5 Quantitative Bone Scan – results

5.1 Patient participation

The recruitment period was from September 2009 until January 2011. Patients were eligible if they participated in AZURE at centres in Leeds, Huddersfield, Sheffield or Chesterfield. There were 73 eligible patients across the 4 sites that were all approached, excluding 1 who was lost to follow up. Of the remaining 72, 14 declined both the main study and sub-study, 18 consented for the main study only (2 due to lack of availability for QBS) and 40 consented to the QBS sub-study. Of the 40 patients who consented, 37 were included in the analysis. 1 was excluded due to poor venous access and 2 were excluded due to relapse disease. Baseline characteristics for these 37 patients are shown in table 5-1. There was no significant difference between the two groups, with the exception of age and bone mineral density. Participants in the treatment arm were significantly older than patients in the control arm by a mean of 6.5 years. At baseline, participants in the treatment arm had a significantly higher T-score at both the hip (0.28 vs. -0.59, $p=0.029$) and the spine (0.21 vs. -1.04, $p=0.003$). Significantly fewer patients in the treatment arm were classified as either osteoporotic or osteopaenic compared with the control arm (26% vs. 73%, $p=0.017$).

Characteristic	Control (n=18)	ZOL (n=19)	Significance
Age in years (range)	53.0 (39-71)	59.5 (45-73)	P=0.030
Menopausal status, n (%)			
≥ 5 years postmenopausal	11 (61)	15 (79)	
< 5 years postmenopausal	4 (22)	3 (16)	P=0.421
Premenopausal	3 (17)	1 (5)	
Endocrine therapy use, n (%)			
AI	11 (61)	14 (74)	P=0.321
Tamoxifen	9 (50)	9 (47)	P=0.567
No endocrine therapy	5 (28)	5 (26)	P=0.605
Chemotherapy use, n (%)			
Yes	17 (94)	19 (100)	P=0.486
No	1 (6)	0	
BMI (kg/m²; mean ± SD)	26.1 ± 4.0	27.2 ± 4.5	P=0.436
Mean T score			
Hip	-0.59	0.28	P=0.029
Spine	-1.04	0.21	P=0.003
Mean Whole body BMD (mg m⁻²; SD)	1021 (114)	1155 (134)	P=0.003
WHO Classification, n (%)			
Osteoporotic	1 (6)	0	
Osteopaenic	12 (67)	5 (26)	P=0.017
Normal	5 (28)	14 (74)	
GFR (mls min⁻¹; SD)*	74.7 (13.5)	71.9 (14.4)	P=0.538

Table 5-1 Baseline characteristics of 37 patients include in baseline analysis.

5.2 Baseline results

37 baseline QBS were performed between Leeds and Sheffield. The tracer used by the Sheffield nuclear medicine department was MDP while Leeds nuclear medicine used HMDP, as discussed in chapter 4.

5.2.1 Whole body k_{bone} as calculated by 3 methods

Mean K_{bone} was significantly suppressed at baseline in the treatment arm compared with the control arm when using the modified Brenner method, observed with both MDP and HMDP studies and using the Patlak plot method for HMDP studies (table 5-2). The trend for suppressed K_{bone} in the treatment arm compared with the control arm was also observed when using the AUC method, however this was non-significant.

WHOLE SKELETON			Control (SD)(n)	ZOL (SD)(n)	Significance
<u>Mean</u>	<u>Mod.</u>	<u>Brenner</u>			
<u>K_{bone}(ml min⁻¹)</u>					
		<i>MDP</i>	28.7 (5.3)(9)	20.7 (6.7)(9)	P=0.013
		<i>HMDP</i>	34.3 (5.9)(9)	26.7 (3.9)(10)	P=0.006
<u>Mean Patlak Plot K_{bone}(ml min⁻¹)</u>					
		<i>MDP</i>	30.6 (5.8)(9)	26.5 (6.4)(9)	P=0.173
		<i>HMDP</i>	39.2 (5.3)(9)	33.6 (6.9)(10)	P=0.061
<u>Mean AUC K_{bone}(ml min⁻¹)</u>					
		<i>MDP</i>	30.0 (6.0)(9)	23.6 (11.0)(9)	P=0.149
		<i>HMDP</i>	35.1 (7.3)(9)	32.1 (7.1)(10)	P=0.372

Table 5-2 Mean baseline whole skeleton k_{bone}

5.2.2 Whole body k_{bone}/BMC by 3 methods

K_{bone}/BMC was significantly suppressed in the treatment arm compared with the control arm when calculated by all 3 methods and observed for both MDP and HMDP studies (table 5-3).

WHOLE SKELETON			Control (SD)(n)	ZOL (SD)(n)	Significance
<u>Mean</u>	<u>Mod.</u>	<u>Brenner</u>			
<u>K_{bone}/BMC (ml min⁻¹ g⁻¹)</u>					
		<i>MDP</i>	0.012 (0.002)(9)	0.008 (0.003)(9)	P=0.001
		<i>HMDP</i>	0.015 (0.002)(9)	0.011 (0.001)(10)	P<0.001
<u>Mean</u>	<u>Patlak</u>	<u>Plot</u>			
<u>K_{bone}/BMC (ml min⁻¹ g⁻¹)</u>					
		<i>MDP</i>	0.013 (0.003)(9)	0.010 (0.002)(9)	P=0.018
		<i>HMDP</i>	0.018 (0.002)(9)	0.014 (0.002)(10)	P=0.001
<u>Mean AUC</u>	<u>K_{bone}/BMC</u>	<u>(ml</u>			
<u>min⁻¹ g⁻¹)</u>					
		<i>MDP</i>	0.013 (0.002)(9)	0.009 (0.004)(9)	P=0.020
		<i>HMDP</i>	0.016 (0.003)(9)	0.013 (0.003)(10)	P=0.056

Table 5-3 Mean K_{bone}/BMC for whole skeleton by the 3 methods

In addition to the suppression observed in the whole skeleton, all sub-regions studied by the Patlak plot method displayed suppression of K_{bone}/BMC the treatment arm compared with the control arm (figures 5-1A and B and 5-2; K_{bone}/BMC data not available for the mandible and calvarium due to no BMC data possible, therefore K_{bone} data displayed). This was observed for both tracers.

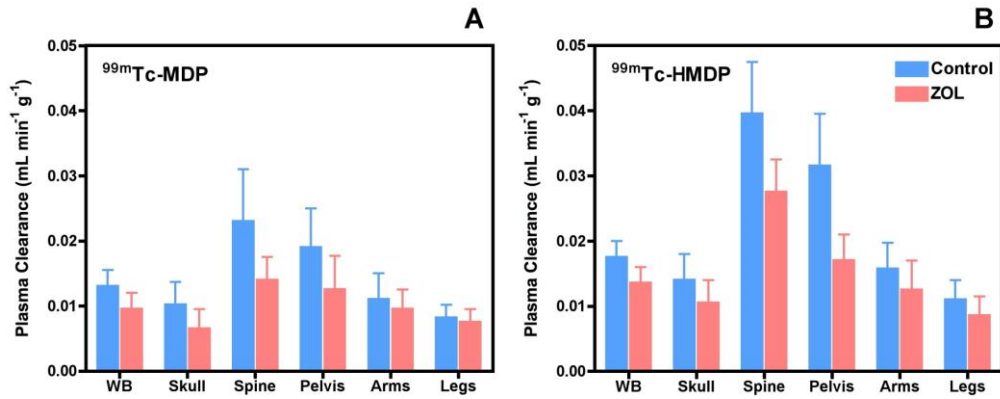


Figure 5-1 K_{bone}/BMC results for whole body (WB) and regions by arm of study for ^{99m}Tc -MDP (A) and ^{99m}Tc -HMDP (B) calculated by Patlak Plot method.

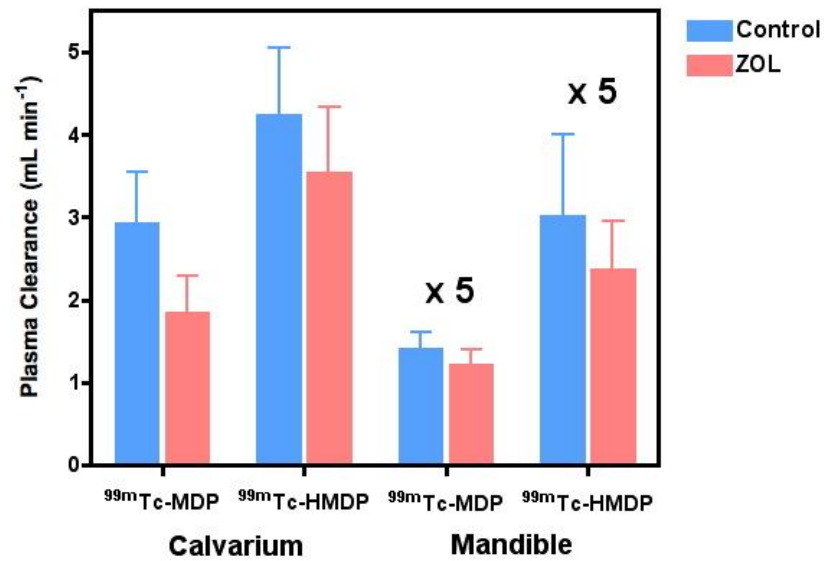


Figure 5-2 K_{bone} results for calvarium and mandible. (Mandible results have been scaled up by x 5)

The degree of suppression in K_{bone}/BMC amongst treatment patients was statistically significant at all sites apart from the legs: skull ($p=0.002$); spine ($p<0.001$); pelvis ($p<0.001$); arms ($p=0.020$); legs (0.096); calvarium and

mandible (no data available) (figure 5-3A). This is most profound in the pelvis and spine, with the least effect in the legs.

The tracer used was also an independent variable predicting for K_{bone}/BMC values using the Patlak plot method ($p < 0.001$) and modified Brenner method ($p < 0.001$). K_{bone}/BMC was statistically significantly greater in studies using HMDP compared with those using MDP for the whole body and all skeletal regions, with the exception of the legs (figure 5-3B).

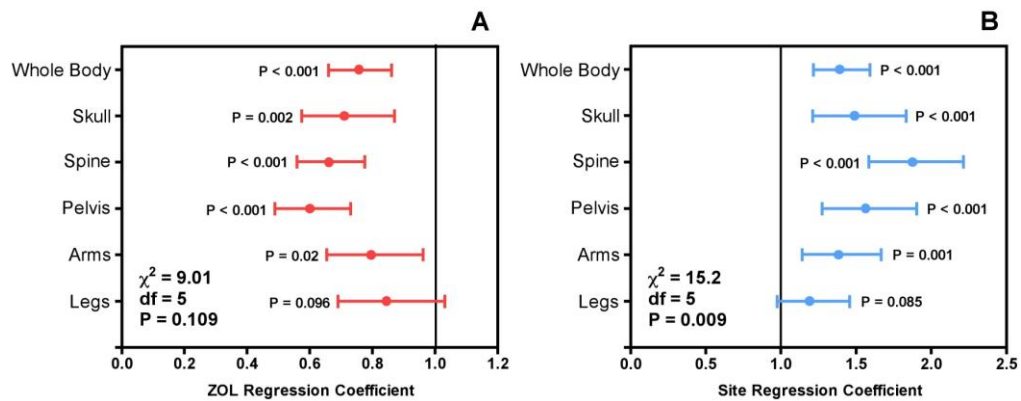


Figure 5-3 Regression coefficients for treatment arm (A) and tracer (B) calculated by linear regression analysis on baseline K_{bone}/BMC studied by Patlak analysis

5.2.3 Bone markers

Mean baseline NTX, P1NP, TRAP5b and α/β CTX values were all statistically significantly suppressed in the treatment arm compared with the control arm (table 5-4). Linear regression analysis confirmed this finding, the degree of suppression in the treatment arm illustrated in figure 5-4.

	Control (SD)(n)	ZOL (SD)(n)	Significance

<u>Mean NTX (nmol BCE mmol Cr⁻¹)</u>	42.5 (17.6)(17)	18.3 (6.2)(17)	P<0.001
<u>Mean P1NP (ng ml⁻¹)</u>	53.0 (20.7)(18)	17.6 (9.1)(18)	P<0.001
<u>Mean TRAP5b (µg l⁻¹)</u>	2.37 (0.77)(18)	1.61 (0.17)(18)	P=0.006
<u>Mean α/β CTX ratio</u>	0.36 (0.15)(18)	0.25 (0.06)(18)	P=0.016

Table 5-4 Baseline bone marker results

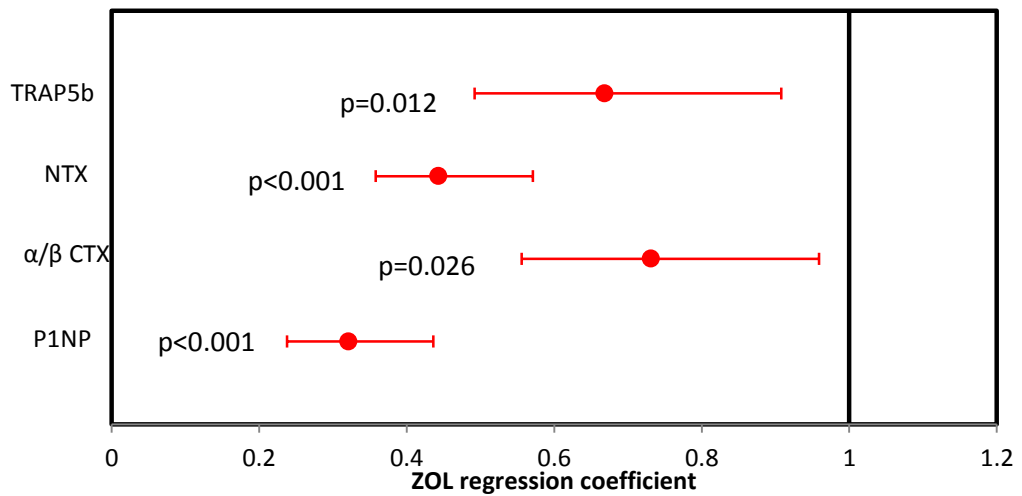


Figure 5-4 Regression coefficients for the treatment arm calculated by linear regression analysis on baseline bone marker results

5.2.4 Bone densitometry

Standardised BMD (sBMD) at the lumbar spine was statistically significantly higher in the treatment arm compared with the control arm on univariate analysis (p=0.006). This remained significant when age, AI use and menopausal status were added as variables to a linear regression model

($p=0.035$). At the hip, BMD was again statistically significantly higher in the treatment arm compared with the control arm on univariate analysis ($p=0.018$) and of borderline significance on linear regression ($p=0.058$).

At the hip, the T-score for patients in the treatment arm was significantly higher when compared with patients in the control arm (0.30 versus -0.60; $p=0.014$). Similarly at the spine, the T-score for patients in the treatment arm was significantly higher when compared with patients in the control arm (0.15 versus -1.07; $p=0.002$).

According to WHO classification, 72% of control patients and 84% of treatment arm patients had normal bone density at the hip. 50% of control patients and 83% of treatment arm patients had normal bone density at the spine. At the hip 28% of control patients were classified as osteopaenic compared with only 16% of treatment patients. At the spine, 44% of control patients were classified as osteopaenic compared with only 17% of treatment patients. Amongst the total cohort only 1 patient was classified as osteoporotic, their allocation was in the control arm.

5.3 Follow up results

5.3.1 Patient numbers

29 scans were included in the 1-year analysis. Of the 37 patients who had baseline QBS, 3 patients relapsed and 4 declined to participate further in the study. A further 1 patient who underwent a 1-year QBS had invalid results that cannot be interpreted due to tissue of tracer at the injection site and the scan was not repeated. Of the 29 patients who had a year 1 QBS, 3

further patients left the sub-study, 2 in the control arm and 1 in the treatment arm. Therefore, 26 scans were included in the 2-year analysis.

5.3.2 Whole body $K_{\text{bone}}/\text{BMC}$

Whole body $K_{\text{bone}}/\text{BMC}$ was significantly suppressed at the 1-year follow up by Patlak plot method ($p=0.003$) and the modified Brenner method ($p=0.011$), and at 2-year follow up by the modified Brenner method ($p=0.018$) but not the Patlak method ($p=0.100$) (figure 5-5). Whole body $K_{\text{bone}}/\text{BMC}$ was not significantly differently from baseline at 1 or 2 years of follow up amongst patients in the treatment arm.

5.3.3 $K_{\text{bone}}/\text{BMC}$ for skeletal ROIs

$K_{\text{bone}}/\text{BMC}$ remains significantly suppressed at the spine and pelvis at both 1-year and 2-year follow up scans as calculated by Patlak plot method ($p<0.001$ for all results; figure 5-5). A trend is observed for increasing $K_{\text{bone}}/\text{BMC}$ amongst treatment patients, becoming more similar to patients in the control arm, for the legs, arms and skull, however this does not reach statistical significance.

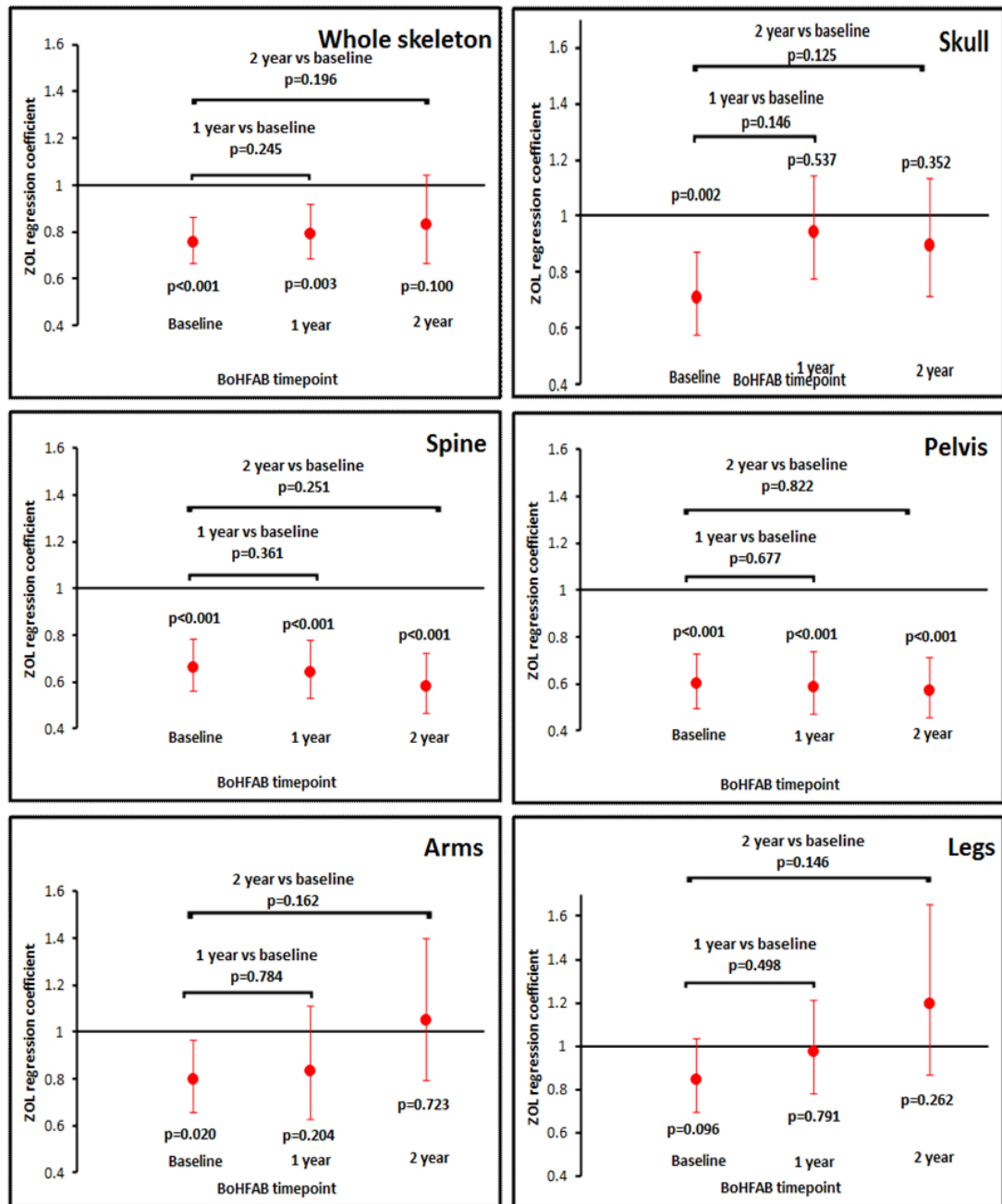


Figure 5-5 Plot displaying change in K_{bone}/BMC measured by Patlak analysis with time (x axis) and by treatment arm. Y axis represents coefficient for treatment with ZOL from linear regression analysis. P-values related to vertical bars represent degree of significance from control group. P values related to horizontal bars represent significance in change of K_{bone}/BMC from baseline to 1 or 2 years in ZOL group.

5.3.4 Bone markers- follow up results

NTX remained significantly suppressed in the treatment arm compared with the control arm at 6 months ($p=0.014$) and 2 years ($p=0.005$) of follow up, with a similar trend observed at 1 year that did not reach statistical significance (table 5-5). P1NP results displayed similar continued suppression through at 6 months ($p=0.008$), 1 year ($p=0.053$) and 2 years ($p=0.049$) of follow up. A trend for continued suppression at 2 years of follow up was also observed for TRAP5b and α/β CTX but did not reach statistical significance.

Bone marker	Control (SD)(n)	ZOL (SD)(n)	Significance
<u>NTX</u>			
<i>Baseline</i>	42.5 (17.6)(18)	18.3 (6.2)(18)	P<0.001
<i>6 month</i>	37.2 (16.0)(16)	23.6 (12.7)(15)	P=0.014
<i>12 month</i>	35.4 (13.4)(16)	26.8 (10.0)(15)	P=0.073
<i>24 month</i>	41.1 (20.2)(14)	20.6 (8.9)(11)	P=0.005
<u>P1NP</u>			
<i>Baseline</i>	53.0 (20.7)(18)	17.6 (9.1)(17)	P<0.001
<i>6 month</i>	51.0 (24.5)(16)	31.5 (10.9)(15)	P=0.008
<i>12 month</i>	45.1 (16.3)(16)	32.6 (17.5)(14)	P=0.053
<i>24 month</i>	46.5 (21.8)(15)	31.9 (15.5)(14)	P=0.049
<u>TRAP5b</u>			
<i>Baseline</i>	2.37 (0.77)(16)	1.61 (0.17)(13)	P=0.006
<i>6 month</i>	2.00 (0.75)(16)	2.07 (0.55)(11)	P=0.795
<i>12 month</i>	2.30 (0.71)(15)	1.79 (0.52)(13)	P=0.039
<i>24 month</i>	2.56 (0.73)(16)	1.84 (0.47)(14)	P=0.071
<u>α/β CTX</u>			
<i>Baseline</i>	0.36 (0.15)(14)	0.25 (0.06)(14)	P=0.016
<i>6 month</i>	0.34 (0.12)(13)	0.25 (0.08)(12)	P=0.029
<i>12 month</i>	0.38 (0.17)(13)	0.26 (0.06)(13)	P=0.034
<i>24 month</i>	0.32 (0.11)(13)	0.24 (0.08)(13)	P=0.06

Table 5-5 Baseline and follow up mean bone marker values by arm of study

The plots in figure 5-6 show P1NP amongst the treatment arm significantly changes with time, the regression coefficient moving towards 1. This was statistically significant at both 1 year and 2 years of follow up. NTX shows a similar trend at 1 year however, at 2 years of follow up the regression

coefficient decreased again. TRAP5b and α/β CTX did not change significantly with time up to 2 years of follow up.

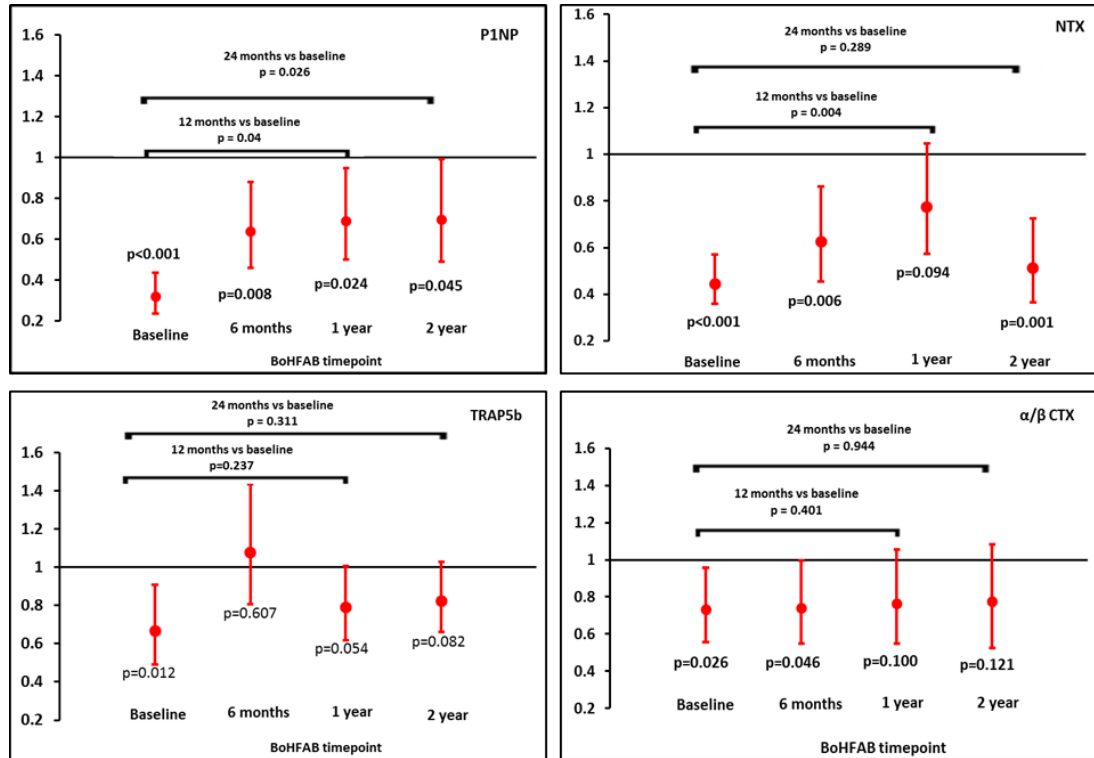


Figure 5-6 Change in bone markers with time (x axis) and by treatment arm. Y axis represents coefficient for treatment with ZOL from linear regression analysis (ZOL regression coefficient). P-values related to vertical bars represent degree of significance from control group. P-values related to horizontal bars represent significance in change of markers from baseline to 12 or 24 months in ZOL group.

5.3.5 Bone densitometry

At 1 year of follow up, mean sBMD at the lumbar spine had increased from baseline in the control group and decreased in the treatment arm. The difference between the 2 groups was significant at 1 year ($p=0.038$; figure 5-7). By 2 years of follow up the control group mean sBMD continued to increase from baseline to 1.6%, while mean sBMD had decreased from baseline in the treatment arm by -1.3%. At 2 years this difference was no

longer statistically significant ($p=0.137$). At the hip there was an observed mean decrease in sBMD from baseline in both treatment arms at 1 year and 2 years. The sBMD changes at the hip were not statistically different at either 1 or 2 years of follow up.

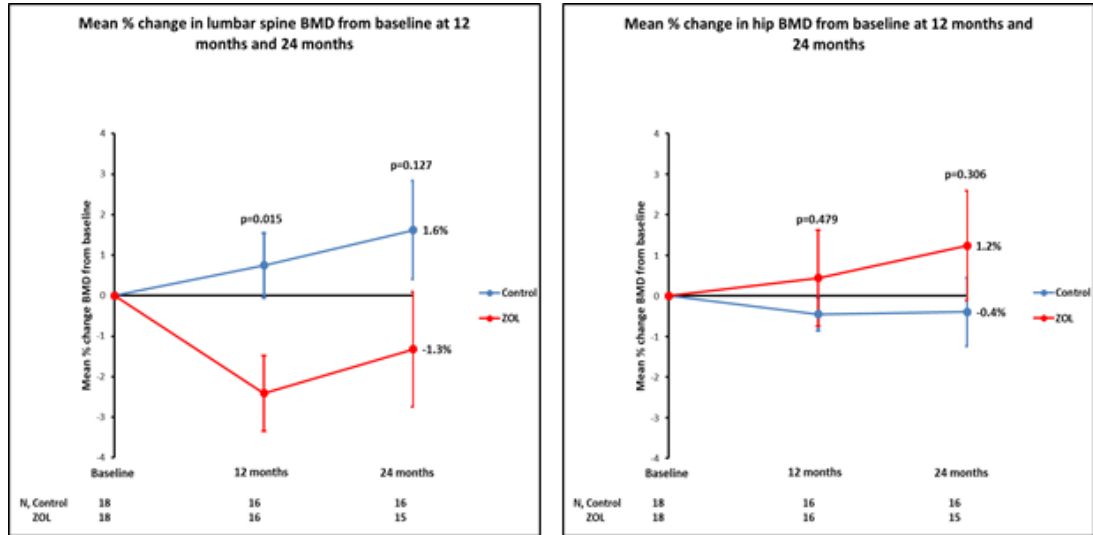


Figure 5-7 Mean percentage change in sBMD from baseline at 1 year and 2 years follow up. Error bars show 95% confidence interval for lumbar spine and hip.

5.4 Discussion

The present study confirmed that, following 5 years of ZOL, K_{bone} and $K_{\text{bone}}/\text{BMC}$ were significantly suppressed compared with the control group. Of particular interest, there was evidence that this suppression was not uniform throughout the skeleton and that the axial skeleton (pelvis and spine) may be more suppressed than the appendicular skeleton (arms and legs). Indeed, even after 5 years of ZOL, the $K_{\text{bone}}/\text{BMC}$ of the legs was not significantly different from that in the control group. This may be explained by the greater extent of trabecular bone in the axial skeleton, which is more influenced by ZOL and a greater impact of the drug was seen here.

The methodology used in this study provides us with greater information about the effect of ZOL on bone turnover than can be generated by bone markers or iliac crest bone biopsies and is a particularly novel aspect. Such a complex dynamic investigation has not previously been undertaken in a population of this size, nor in a population of early breast cancer patients with a control group for comparison. Furthermore, identifying suppression in regions of the skeleton in addition to the whole skeleton and normalising to bone mineral content has not previously been published and is a unique aspect of the study. Bone markers supported the QBS findings at baseline, were all significantly suppressed in the treatment arm compared with controls. This was most profound in the conventional turnover markers, NTX (resorption) and P1NP (formation).

BMD and T-scores at baseline were additionally significantly higher in the treatment arm compared with controls. This is an expected result but reassuring to confirm given the small numbers of subjects in this sub-study.

We believe that $K_{\text{bone}}/\text{BMC}$ gives us the most reliable estimate of skeletal plasma clearance of tracer given that it corrects for the differences in the mass of bone mineral in each sub-region of the skeleton. Using these calculations, by 1 year the treatment arm remained significantly suppressed compared with the control arm. Again, there was a differential effect across the regions. Only spine $K_{\text{bone}}/\text{BMC}$ and pelvis $K_{\text{bone}}/\text{BMC}$ remained significantly suppressed compared to controls. The other regions followed a similar trend but are approaching the levels seen in the control group.

Whilst still significantly suppressed compared to controls, the trend was that between baseline and 1 year, the WB $K_{\text{bone}}/\text{BMC}$ measurements in the treatment arm did rise, approaching the levels seen in the control group. Bone markers were also increased at 6 and 12 months compared with baseline in the treatment arm. These findings indicate that the degree of suppression among the treatment arm patients was wearing off, but the 2 groups did remain significantly different from each other at 12 months (P1NP, TRAP5b, α/β CTX).

The trends were similar at 2 years. WB $K_{\text{bone}}/\text{BMC}$ and the bone markers all increased from baseline, reflecting reduced degree of suppression in the treatment arm. However, the suppressing effect of ZOL was still seen at 2 years when compared with the control arm, particularly $K_{\text{bone}}/\text{BMC}$ spine and pelvis and bone markers (NTX and P1NP).

This study has shown that it is possible to recruit to interventional studies that are relatively complex and time-consuming for patients. However, there are some weaknesses identified. This sub-study successfully consented 40 patients to participate, achieving the sample size goal as per protocol and allowing confidence in the statistical interrogation of the data. 2 patients from the total sub-study cohort were identified at baseline as having relapse with bone metastases and therefore were excluded. There was a good balance of numbers between the control and treatment arms. However, by 1 year there were only 29 scans for analysis, due to drop out and relapse, and only 26 by 2 years. Our statistical analysis plan allowed for a 25% drop out, but this underestimated the actual number. It is possible that given greater numbers of subjects, some of the trends seen in this study may have reached statistical significance.

Another weakness was the different bone scan tracer used at the 2 sites. Whilst each of the individual tracers is adequate for performing the QBS, their detailed kinetics are different. Consequently, the patient investigations performed with MDP were not directly comparable with those performed with HMDP, requiring a statistical model that allowed for this discrepancy. Uniform use of tracer across both sites would have been preferable. This was not a problem when patients are used as their own controls, i.e. when investigating change over time and the data are paired. However, where necessary, data have been displayed separately, by tracer and arm. We have shown that HMDP consistently results in higher K_{bone} results than MDP by approximately 20-30%. For example, baseline WB K_{bone} as measured by

the modified Brenner method was $34.3 \text{ mls min}^{-1}$ for HMDP compared with $28.7 \text{ mls min}^{-1}$ for MDP, amongst the control group. This trend was seen throughout the skeleton and is displayed in figure 5-1 and figure 5-3B.

At baseline suppression was identified in K_{bone} in the mandible and calvarium. However, as it was not possible to collect BMC data for these regions, the analysis here was more limited. Furthermore, the drawing of these ROIs was particularly subjective and subject to great variation, resulting in less reliable data.

Final discussions

The entirety of the work in this thesis involved participants in the large randomised phase III AZURE trial. The efficacy data was most recently published in 2014 and demonstrates no overall benefit from the addition of zoledronic acid to standard adjuvant treatments for early breast cancer (HR 0.94, 95% CI 0.82-1.06; $p=0.30$)¹⁸². However, there is continued demonstration that zoledronic acid reduces bone metastases at any time (HR 0.81, 0.68-0.97; $p=0.022$) and improved IDFS in those who were over 5 years since menopause at trial entry ($n=1041$; HR 0.77, 95% CI 0.63-0.96). The study was incorporated into a meta-analysis of adjuvant bisphosphonates in early breast cancer ($n = 18, 766$) and concludes that the reduction in bone recurrence was convincing (HR 0.83, 0.73-0.94; $p=0.004$) and that for women who were postmenopausal at study entry, there were significant reductions in recurrence (RR 0.86, 95% CI 0.78-0.94; $p=0.002$), distant recurrence (0.82, 0.74-0.92; $p=0.0003$), bone recurrence (0.72, 0.60-0.86; $2p=0.0002$), and breast cancer mortality (0.82, 0.73-0.93; $p=0.002$).²⁴⁹ Adjuvant bisphosphonates have now been introduced as a standard of care and recommended in UK, European and American guidelines. The safety data in my thesis has contributed to the uptake of adjuvant bisphosphonates among UK and global oncologists (70% uptake amongst UK oncologists, personal communication). The UK Breast Cancer Group recommends i.v. bisphosphonates whilst on chemotherapy followed by 3 years of the oral bisphosphonate ibandronate, with calcium and vitamin D. It remains unknown the duration needed for benefit or, to what extent benefit reduces on stopping bisphosphonates.

The primary purpose of the QBS sub-study was to determine if there are any negative consequences of 5 years adjuvant zoledronic acid. We have shown that bone turnover remains significantly suppressed 2 years after the cessation of the bisphosphonate, most profoundly in the axial skeleton. The protocol design is a 5 year study and the data for the 5 year time point is now available for analysis, interpretation and preparation for presentation/publication. It is likely that if the QBS sub-study was being designed today it would be designed using hybrid positron emission tomography and computed tomography (PET/CT) dual modality system with the bone-imaging agent [^{18}F]NaF, rather than the gamma camera. PET imaging with [^{18}F]NaF is now recognised as the optimum radionuclide imaging technique for the investigation of metastatic and metabolic bone disease due to the tracers superior bone-seeking properties with exceptionally high and rapid uptake into bone, rapid clearance from soft tissue and absence of any protein binding²⁵⁰.

It is important to note that practice has changed significantly since AZURE was actively recruiting patients. In the initial phase of the study HER2 testing was not routinely carried out, where it is now a standard alongside ER and PR. Additional molecular testing is available to tailor treatment to a particular genetic profile, such as Oncotype, which has been widely taken up and changed decision making in early breast cancer. Emerging therapies in breast cancer, such as immunotherapy, are likely to change this even further and it is likely that the pattern of disease will consequently change. However, it is right that efforts continue to be made in the search for bone-modifying agents to ease the significant burden of this disease.

Appendices

Appendix 1

AZURE

**Does Adjuvant Zoledronic acid redUce
REcurrence in patients with high-risk,
localised breast cancer?**

PROTOCOL

Version 6.0

22 Aug 2008



01 - 04

Direct line for 24-hour randomisation +44 (0)113 343 1481

ISRCTN79831382

1. TRIAL SYNOPSIS

AZURE Protocol Version 6.0 (22nd August 2008)

5

It is the aim of this prospective, randomised, open label, parallel group trial to determine whether adjuvant treatment with 4mg zoledronic acid with (neo)adjuvant chemotherapy and/or (neo)adjuvant endocrine therapy is superior to (neo)adjuvant chemotherapy and/or (neo)adjuvant endocrine therapy alone in improving the disease-free and bone metastasis-free survival of stage II/III breast cancer patients. Patients will be randomly allocated to receive either zoledronic acid or allocation to a control group. 3300 patients will be included.

OBJECTIVES

Primary objective:

To determine whether zoledronic acid with chemotherapy and/or endocrine therapy is superior to chemotherapy and/or endocrine therapy alone in improving disease-free survival (events are death from any cause or disease recurrence, as detailed in Appendix 1).

Secondary objectives:

In addition, in light of proposals by Hudis et al²⁶ the Data Monitoring and Ethics Committee (DMEC) have recommended the inclusion of invasive disease-free survival (IDFS) as a key secondary endpoint. The secondary objectives are therefore:

To determine whether zoledronic acid with chemotherapy and/or endocrine therapy is superior to chemotherapy and/or endocrine therapy alone in terms of:

- invasive disease-free survival (please see section 12.2.2)
- time to bone metastases as first recurrence
- time to bone metastases per se
- time to distant metastases
- overall survival
- reducing skeletal-related events* prior to development of bone metastases
- reducing skeletal-related events* following development of bone metastases.

Additional secondary objectives are:

- to assess the safety and toxicity of zoledronic acid in this clinical setting
- to evaluate the influence of prognostic factors, such as ER/PR status, TNM stage, tumour grade, HER2/neu (if available) and menopausal status on treatment outcome
- to use proteomics, tissue micro-array and other modern techniques to identify more specific prognostic indicators for the development of bone metastases and factors that are able to predict specific benefit from bisphosphonate treatment (to be investigated via sub-studies).

* Defined as: fractures, spinal cord compression, radiation therapy to bone, surgery to bone and hypercalcaemia

ELIGIBILITY

Inclusion Criteria

- Female patients with Stage II / III primary breast cancer, with T stage \geq T1 (see Appendix 2)
- Patients should be receiving / scheduled to receive chemotherapy and/or endocrine therapy
- Patients receiving **neo-adjuvant** therapy
 - must have tumour size of >5 cm (T3), features of locally advanced disease (T4) or biopsy-proven lymph node involvement (N1)
 - should be scheduled to proceed to definitive surgery⁵ and/or radical radiotherapy with curative intent within six months of starting neoadjuvant therapy
 - time between commencement of neoadjuvant treatment and planned start date of study drug should be \leq 30 days

- Patients receiving **adjuvant therapy**
 - must have undergone complete primary tumour resection and treatment of the axillary lymph nodes*, without any prior neoadjuvant therapy[#].
 - must have evidence of lymph node involvement
 - time between definitive surgery[§] and planned start date of study drug should be ≤ 60 days
- Performance status: Karnofsky Index $\geq 80\%$ or ECOG 0 or 1
- Women of childbearing potential must be using a reliable and appropriate method of contraception
- Age ≥ 18 years
- Patient must have given written informed consent prior to any study-specific procedures.

[§] Final definitive surgery is considered to include re-operation for inadequate margins or another bona fide oncological indication.

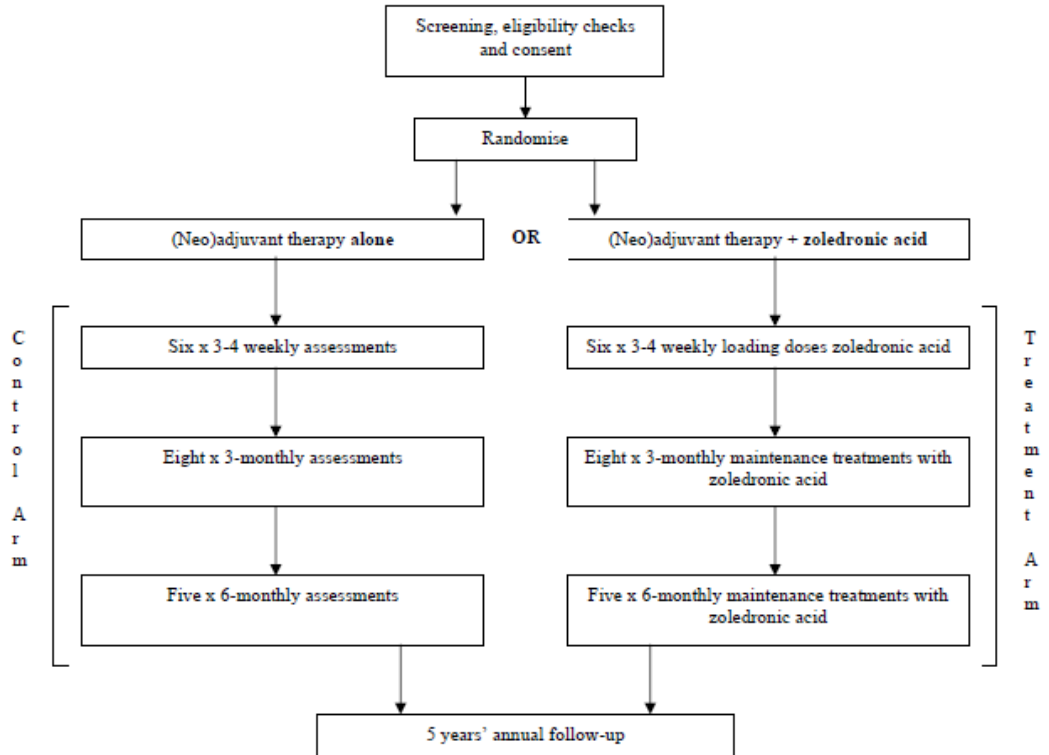
* Patients whose treatment plan is to proceed to further primary tumour resection and/or treatment of the axillary lymph nodes (e.g. clearance or radiotherapy) with curative intent after completion of chemotherapy would be eligible but this must be completed within 9 months of randomisation.

[#] Pre-operative endocrine therapy of less than 30 days would not be classed as prior neo-adjuvant therapy.

Exclusion Criteria

- Metastatic or recurrent breast cancer or a history of breast cancer (aside from DCIS or LCIS) prior to the currently diagnosed case
- History of prior cancers within the preceding five years (including previous contralateral breast cancer), aside from non-melanomatous skin cancer or carcinoma in situ of the uterine cervix treated with curative intent
- History of diseases with influence on bone metabolism, such as Paget's disease of bone, primary hyperparathyroidism or osteoporosis requiring treatment at the time of study entry or considered likely to become necessary within the subsequent six months
- Severe physical or psychological concomitant diseases that might impair compliance with the provisions of the study protocol
- Prior treatment with bisphosphonates within the past year
- Serum creatinine $> 1.5 \times$ Upper Limit of Normal
- Known hypersensitivity to bisphosphonates
- Current active dental problems including dental abscess or infection of the jawbone (maxilla or mandible), or a current or prior diagnosis of osteonecrosis of the jaw (ONJ)
- Recent (within 4 weeks of study entry) or planned dental or jaw surgery (e.g. extractions, implants). Recent dental fillings, teeth scaling and polishing or minor gingival surgery do not exclude the patient.
- Pregnancy or breast-feeding
- Use of other investigational drugs in the 30 days prior to study entry. (Patients may be receiving treatments within a clinical trial providing the treatment under test has a licensed indication within your country.)

TRIAL TREATMENT/ASSESSMENT FLOW DIAGRAM



Appendix 2

AZURE

**Does Adjuvant Zoledronic acid redUce
REcurrence in patients with high-risk,
localised breast cancer?**

Dental Health Quality of Life

SUB-PROTOCOL

Version 1.0

06 November 2009



01 - 04

ISRCTN79831382

AZURE Dental Health Quality of Life

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The Dental Health Quality of Life (QoL) sub-protocol is part of the AZURE trial protocol. The sub-protocol only includes sections relating specifically to the Dental Health QoL sub-study which can not be found in the AZURE protocol.

1. BACKGROUND

Following information that has become available regarding the association of bisphosphonates with osteonecrosis of the jaw (ONJ), it has been decided by the Trial Steering Committee (TSC) to assess AZURE participants' dental health-related quality of life. Therefore a questionnaire has been adapted from a published, validated tool, the Oral Health Impact Factor-14 (OHIP-14)^{2,3}, to measure patients' dental quality of life so as to enable an assessment of the effect of zoledronic acid on patients' dental health-related quality of life.

2. AIMS AND OBJECTIVES

The aim is to assess whether dental-related problems have impacted on the quality of life of a sub-set of patients randomised to AZURE (please see Inclusion Criteria below for patient selection criteria) and evaluate any differences between those receiving zoledronic acid and those who are not.

3. DESIGN

The sub-study will be co-ordinated by the Clinical Trials Research Unit (CTRU) at the University of Leeds and the Cancer Research Centre at Weston Park Hospital Sheffield. To assess dental health-related QoL a one-off postal survey will be carried out using the adapted patient self-reported OHIP-14 questionnaire which includes questions relating to whether problems with teeth, mouth or dentures have affected patients' quality of life. This is a simple questionnaire of 14 questions that can be easily completed by patients at home or in the clinic setting. Its use has been validated and extensively published on the oral health quality of life amongst several different populations. A sub-set of patients who are between 4.5 and 5.5 years since randomisation to AZURE will be asked to answer the set of questions twice; once relating to any problems experienced in the last month, and secondly relating to any problems experienced since taking part in the AZURE trial. Details regarding the subset of patients who will be asked to complete the questionnaire along with the method of distribution are described in the eligibility and recruitment sections.

4. ELIGIBILITY

4.1. Inclusion Criteria

- AZURE trial participants from UK centres which have recruited between 10-30 patients or from UK centres which have recruited greater than 30 patients who are not participating in the AZURE Bone Health sub-study.
- Patients between 4.5 and 5.5 years since randomisation to the AZURE study at the time of questionnaire completion.

4.2. Exclusion Criteria

- Participants who have transferred to a hospital not participating in the Dental Health Quality of Life Sub-protocol
- Participants who have withdrawn consent to further follow-up

- Participants who have died
- Participants with skeletal recurrence

5. RECRUITMENT

5.1. Number of centres

It is anticipated that approaching patients from all UK centres that recruited over 10 patients will provide questionnaire returns from a sufficient number of participants to enable an assessment of the effect of zoledronic acid on patients' dental health-related QoL (see Statistical Considerations). In order to avoid over researching sub-groups of patients, sites participating in the AZURE Bone Health sub-study will not be involved in the Dental Health Quality of Life sub-protocol.

5.2. Method of distribution

The questionnaires will be distributed as a postal survey from the appropriate centre. CTRU will provide each UK centre with a list of eligible participants. A pack including a Patient Information Sheet, the Dental Health QoL questionnaire and a pre-paid envelope to return the completed questionnaire will be sent out to participants. The participants will be informed that by completing and returning the questionnaire to the trials unit, they are providing informed consent to the Dental Health QoL sub-protocol. The centre will check the patient's status (using their medical records) before sending the questionnaire pack to ensure patients are still eligible. The centre will add the date the Dental Health QoL questionnaire was sent to the participant to the patient list sent by CTRU and will also add the reason why any participant was not sent this information. The centre will inform the trials unit once the questionnaires have been sent to all the eligible participants by returning a copy of the completed patient list.

6. ASSESSMENT/ DATA COLLECTION

Dental health-related QoL will be assessed using the patient self-reporting OHIP-14 questionnaire (adapted for use in AZURE) that allows an assessment of how dental-related problems have impacted on patients' quality of life. It includes 14 questions covering 7 domains that are felt to be important in oral health: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap. Responses to the individual questions are made on a 5-point scale (never, hardly ever, occasionally, fairly often and very often) and weights are applied to responses to those questions in each dimension to yield a subscale score. Patients will be asked to answer the set of questions twice; once relating to any problems experienced in the last month, and secondly relating to any problems experienced since taking part in the AZURE trial. This will allow assessment of both current dental health and a general overview of dental-related quality of life while on treatment within AZURE. The OHIP-14 questionnaire will be supplemented by questions relating to wearing of dentures, missing teeth, perceived need for dental treatment, tooth extractions, dental implants, dental surgical procedures, visiting a dentist and smoking. Details of the questionnaire can be found in the Appendix.

Participants should complete the dental health QoL questionnaire if they are between 4.5 years and 5.5 years since randomisation to the AZURE trial. All questionnaires received at the time of conducting the dental Quality of Life analysis will be included.

All data will be handled, computerised and stored in accordance with the Data Protection Act 1998.

7. ENDPOINTS

The dental health domain (subscale) scores, as derived from the OHIP-14 questionnaire using the relevant scoring system, for patients who have received zoledronic acid will be compared to the respective domain scores for patients who are in the control group. It must be noted that no specific dental health domain has been identified *a priori* to be of primary importance.

8. STATISTICAL CONSIDERATIONS

8.1. Number of patients

The sample size is determined by the number of patients randomised to the AZURE trial who were recruited to those UK centres described in the eligibility and recruitment sections and who are expected to be between 4.5 and 5.5 years since randomisation at the time of questionnaire completion. The number of patients expected to complete the questionnaire is based on the combined survival, bone metastases, withdrawal (from full follow-up) and transfer rates from AZURE and an estimated overall questionnaire response rate of 50%. It is therefore estimated that approximately 280 patients in total will complete the questionnaire, which will allow an effect size (ES) of approximately 0.3 to be detected between the two treatment groups in terms of their dental health domain scores (using a two-sided 5% significance level and 80% power). Using the operational definitions defined by Cohen (small ES = 0.2, medium ES = 0.5, large ES = 0.8), this will allow detection of a small to medium difference in dental health-related QoL.

8.2. Statistical analysis

Analysis will be performed on an intention-to-treat basis. All hypothesis tests will be two-sided and at the 5% significance level.

The OHIP-14 questionnaire will be scored according to the relevant guideline to allow calculation of domain (subscale) scores. Each dental health domain will be summarised for each treatment group using mean scores and 95% confidence intervals obtained from a multivariate linear regression model adjusting for the following potential prognostic factors: smoking; dentures, pre-existing dental conditions. Differences in the adjusted mean domain scores between the two treatment groups and their respective 95% confidence intervals will also be obtained from this linear regression model. The distribution of responses to each question of the OHIP-14 questionnaire and also to the supplementary questions will be summarised descriptively. Missing data will be examined and, if appropriate, sensitivity analyses will be carried out to test the robustness of the conclusions, the results of which will be fully reported.

9. REFERENCES

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2. Slade GD, Spencer AJ. Development and evaluation of the Oral Health Impact Profile. *Community Dent Health*. 1994 Mar; 11(1):3-11.
3. Slade, G.D. (1997) Derivation and validation of a short-form oral health impact profile. *Community Dentistry Oral Epidemiology* 25: 284-290.

10. APPENDICES

Appendix 1: Patient Information Sheet and Dental Health Quality of Life Questionnaire
(please see following page)



Dental Health Quality of Life Questionnaire

Many thanks for your ongoing participation in the AZURE trial. This trial is assessing whether or not adding a drug called zoledronic acid (also known as Zometa™) to standard treatments for breast cancer is beneficial.

You may remember that you have previously been given some information and advice regarding dental hygiene and dental procedures. Good dental hygiene is valuable in all breast cancer patients to help prevent standard dental problems. It is important that all patients in the trial maintain good dental hygiene. All patients have been monitored very closely for any dental related problems.

We would now like to assess whether the patients taking part in the AZURE trial felt their quality of life was affected by problems with their teeth, mouth or (where relevant) dentures and if this was related to their cancer treatment. You can help us with this by filling in a questionnaire about how any problems with your teeth, gums or dentures affect your life.

The questionnaire should only take 10 minutes to fill in. The first part involves some general questions regarding your dental health. The second part of the questionnaire relates to any problems in the **last month** and the third part relates to any problems **since your diagnosis of breast cancer and while taking part in the AZURE trial up to the present day**. (NOTE you will therefore be asked to answer the same questions twice, firstly for the last month and secondly, for the time since taking part in the trial)

Your decision to participate in this Quality of Life questionnaire study is voluntary. If you decide not to complete this questionnaire your medical care or legal rights will not be affected. Your continued participation in the AZURE trial will also not be affected.

By completing this questionnaire you are agreeing to take part in this study. By doing so you are agreeing to allow any information or results arising from this study to be used for healthcare and/or medical research purposes including monitoring the safety of the treatment that you will receive. Your identity will remain anonymous.

Once the questionnaire has been completed please return this to us in the pre-paid envelope provided. Please keep this information sheet in a safe place for future reference.

If you feel you would like to discuss the questionnaires in further detail, please feel free to contact the AZURE Clinical Co-ordinator on 0114 226 5772.

Many thanks



Dental Health Quality of Life Questionnaire

Page 1 of 5

Patient Initials		Date of Birth	Day	Month	Year	Patient ID	Centre no.	Trial no.
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PART 1
General questions regarding your dental health

Please complete today's date:
 ___ / ___ / _____

Please circle the most appropriate answer to the following questions

1. Do you wear dentures?

Yes No

2. Do you have any missing teeth?

Yes No

3. Do you feel your teeth/gums need dental attention?

Yes No

4. How many tooth extractions have you had in the past 5 years?

None One 2 – 5 More than 5

5. How many dental implants have you had in the past 5 years?

None One 2 – 5 More than 5

6. How many surgical procedures have you had on your teeth, gums or jaw in the past 5 years?

None One 2 – 5 More than 5

7. How often have you visited the dentist in the past 5 years?

Two or more times a year Once a year Less than once a year Not in 5 years

8. Do you smoke?

Yes Ex-smoker Never smoked

9. If you smoke, how many cigarettes per day?

Less than 5 5 – 10 11 – 20 More than 20

10. If you have any other comments regarding your dental health please write them in the space below:

For office use only	<i>Verified</i>	<i>Computerised</i>	<i>Checked</i>	Version 1.0 30/10/2009
	Date Initials	Date Initials	Date Initials	



Dental Health Quality of Life Questionnaire

Patient Initials		Date of Birth	Day	Month	Year	Patient ID	Centre no.	Trial no.
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PART 2
Regarding your dental health in the last month

Please can you answer the following 14 questions relating to the ***last month*** by circling the most appropriate response.

- 11. **Have you had trouble pronouncing any words because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

- 12. **Have you felt that your sense of taste has worsened because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

- 13. **Have you had painful aching in your mouth?**
 Never Hardly ever Occasionally Fairly often Very often

- 14. **Have you found it uncomfortable to eat any foods because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

- 15. **Have you been self-conscious because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

- 16. **Have you felt tense because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

- 17. **Has your diet been unsatisfactory because of problems with your teeth, mouth or dentures?**
 Never Hardly ever Occasionally Fairly often Very often

For office use only	<i>Verified</i>		<i>Computerised</i>		<i>Checked</i>		Version 1.0 30/10/2009
	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	



Dental Health Quality of Life Questionnaire

Patient Initials		Date of Birth	Day	Month	Year	Patient ID	Centre no.	Trial no.
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18. Have you had to interrupt meals because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

19. Have you found it difficult to relax because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

20. Have you been a bit embarrassed because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

21. Have you been a bit irritable with other people because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

22. Have you had difficulty doing your usual jobs because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

23. Have you felt that life in general was less satisfying because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

24. Have you been totally unable to function because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

For office use only	<i>Verified</i>		<i>Computerised</i>		<i>Checked</i>		Version 1.0 30/10/2009
	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	



Dental Health Quality of Life Questionnaire

Patient Initials		Date of Birth	Day	Month	Year	Patient ID	Centre no.	Trial no.
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PART 3

Regarding your dental health since your entry into the AZURE trial shortly after diagnosis of your breast cancer

Please can you answer the same 14 questions relating to the *time since your entry into AZURE to the present day*

25. Have you had trouble pronouncing any words because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

26. Have you felt that your sense of taste has worsened because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

27. Have you had painful aching in your mouth?

Never Hardly ever Occasionally Fairly often Very often

28. Have you found it uncomfortable to eat any foods because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

29. Have you been self-conscious because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

30. Have you felt tense because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

31. Has your diet been unsatisfactory because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

For office use only	<i>Verified</i>		<i>Computerised</i>		<i>Checked</i>		Version 1.0 30/10/2009
	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	



Dental Health Quality of Life Questionnaire

Patient Initials		Date of Birth	Day	Month	Year	Patient ID	Centre no.	Trial no.
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32. Have you had to interrupt meals because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

33. Have you found it difficult to relax because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

34. Have you been a bit embarrassed because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

35. Have you been a bit irritable with other people because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

36. Have you had difficulty doing your usual jobs because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

37. Have you felt that life in general was less satisfying because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

38. Have you been totally unable to function because of problems with your teeth, mouth or dentures?

Never Hardly ever Occasionally Fairly often Very often

For office use only	<i>Verified</i>		<i>Computerised</i>		<i>Checked</i>		Version 1.0 30/10/2009
	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	<i>Date</i>	<i>Initials</i>	

Appendix 3



Clinical Trials Research Unit

University of Leeds
Leeds
LS2 9JT
www.ctruleeds.co.uk

Ms Anne McCullough
West Midlands MREC
Osprey House
Albert Street
Redditch
Worcester
B97 4DE

06 November 2009

Dear Ms McCullough

AZURE Trial (EudraCT No: 2004-000608-42)
MREC Application Ref: 03/7/029 - Annex 2

Please find enclosed the following documents relating to an amendment for the addition of a Dental Health Quality of Life Sub-protocol to the Azure trial:

- Substantial Amendment Notification Form (Annexe 2)
- AZURE Dental Health Quality of Life Sub-Protocol Version 1.0, 06 November 2009

I would be very grateful if you could please provide confirmation of receipt of this update.

Thank you and best wishes

Geraldine Matthews
Senior Trial Manager

Tel: 0113 343 8091
Fax: 0113 343 1471
Email: g.a.matthews@leeds.ac.uk

03 DEC 2009



West Midlands Research Ethics Committee

Osprey House
Albert Street
Redditch
Worcestershire, B97 4DE
anne.mccullough@westmidlands.nhs.uk
Chairman: Mr Paul Hamilton

Tel: 01527 587688
Fax: 01527 587501

01 December 2009

Geraldine Matthews
Senior Trial Manager
Clinical Trials Research Unit
University of Leeds
Leeds
LS2 9JT

Dear Ms Matthews

Study title: A phase III randomised controlled trial to determine whether Adjuvant Zoledronic acid reduces REcurrence in patients with high risk localised breast cancer

REC reference: 03/7/029

Protocol number: ISRCTN79831382

EudraCT number: 2004-000608-42

Amendment number: AM28

Amendment date: 06 November 2009

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	1.0	06 November 2009
European Commission Notification of Substantial Amendment Form		06 November 2009
Covering Letter		06 November 2009

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

03 DEC 2009

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

03/7/029:	Please quote this number on all correspondence
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Yours sincerely



Mrs Jenny Tyers
Assistant Committee Co-ordinator

E-mail: jenny.tyers@westmidlands.nhs.uk

Enclosures: List of names and professions of members who took part in the review

Copy to: Prof Robert Coleman
University of Sheffield
Cancer Research Centre
Weston Park Hospital
Whitham Road
Sheffield
S10 2SJ

03 DEC 2009

West Midlands Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 25 November 2009

<i>Name</i>	<i>Profession</i>	<i>Capacity</i>
Mr Paul Hamilton	Local Government Officer (Chair)	Lay
Professor John Marriott	Pharmaceutical Chemist/Academic Pharmacist	Expert

#)

Appendix 4

WHO Risk factor Questionnaire

What is your date of birth? ___/___/___

dd mm yyyy

How tall are you? ___ ft and ___ inches OR ___ cm

How much do you weigh? ___ stones and ___ pounds OR ___ kg

Answer the following questions by circling the appropriate response:

Have you ever broken a bone after the age of 50 years that resulted from a low level of injury (e.g. a simple fall from standing height)?	Yes / No / Don't know
Have you ever taken glucocorticoids (steroids) (e.g. prednisolone) by tablets or suppository for more than a few weeks?	Yes / No / Don't know
Have either of your parents ever broken a hip following a low level of injury (e.g. a fall from standing height)?	Yes / No / Don't know
Have you ever been diagnosed with rheumatoid arthritis (not osteoarthritis)?	Yes / No / Don't know
On average, do you drink 3 or more units of alcohol (1 unit=1/2 pint of beer or 1 glass of wine or 1 short measures of spirits) each day?	Yes / No / Don't know
Are you a smoker?	Yes / No

Other conditions: Please tick the box beside any of the conditions listed below if they have or do affect you personally:

Menopause or prolonged absence of your periods (other than pregnancy) before age 45

Longstanding poor mobility (e.g. following a stroke, Parkinson's disease, spinal injury)

Crohn's Disease or Ulcerative Colitis

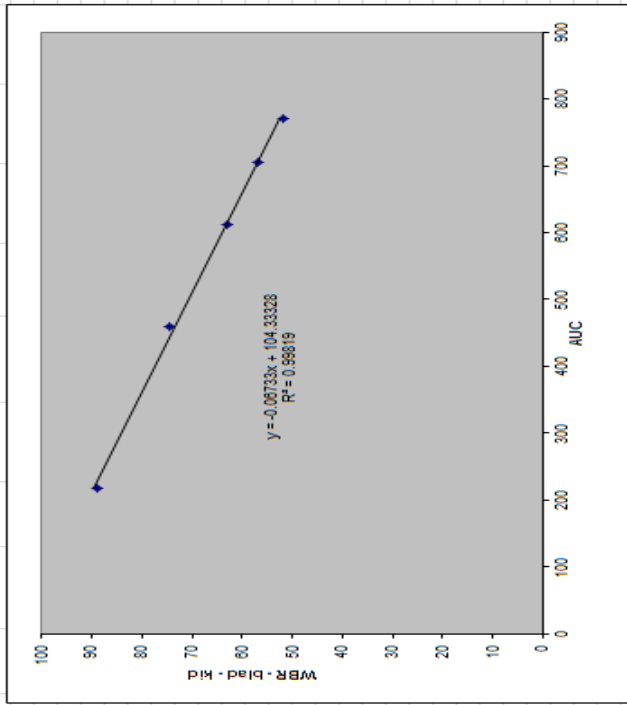
Major organ transplant

Insulin-dependent diabetes

Overactive thyroid gland

Coeliac disease

	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
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39	TodecaycoBladder/%		WB-blade	PK-ant	PK-post	Geom mean	Todecayco	PK-%	LK-ant	LK-post	Geom mean	Todecayco	LK-%	TotalK%	WBR-blad-k	AUC	Blenner GFR						
40	87534	6.6	93.4	23692	27465	25509	25509	1.931	27293	33600	30283	30283	2.293	4.224	89.1	216.78	Y-intercept	104.33328					
41	48079	3.6	76.8	11494	14523	12320	14289	1.082	12864	15797	14255	15766	1.194	2.275	74.5	458.48	X-intercept	1549.65	64.5				
42	29988	2.3	64.9	7696	10123	8826	10974	0.831	8147	11202	9553	11878	0.899	1.730	63.2	611.97							
43	19331	1.5	58.2	5097	6829	5900	8202	0.621	5600	7730	6579	9147	0.693	1.313	56.9	704.73							
44	14162	1.1	53.2	4092	5240	4631	7261	0.550	4597	5980	5243	8222	0.622	1.172	52.0	770.00							
45																							
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49	R arm %		L arm ant	L arm post	Geom mean	Todecayco	L arm %	LEGS		Both arms	legs ant	legs post	Geom mean	Todecayco	Legs %								
50	5.28	77195	70852	73956	73956	5.60	10.88	386148	340544	362630	362630	27.46											
51	4.72	66751	56242	61272	67764	5.13	9.85	331459	286050	307919	340546	25.78											
52	3.94	52922	35560	43381	53936	4.08	8.02	273349	219276	244824	304392	23.05											
53	3.53	42847	31524	36752	51093	3.87	7.40	215460	168820	190720	265142	20.07											
54	2.66	29980	24069	28862	42124	3.19	5.85	167999	133066	149516	234459	17.75											
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Appendix 6

Copy → *Frank Brown ✓*
Lesley Turner ✓
Shirley Fitt



West Midlands Research Ethics Committee

Osprey House
Albert Street
Redditch
Worcestershire, B97 4DE
anne.mccullough@westmidlands.nhs.uk
Chairman: Mr Paul Hamilton

Telephone: 01527 587528
Facsimile: 01527 587501

01 July 2009

Prof Robert Coleman
Professor of Medical Oncology
University of Sheffield
Cancer Research Centre
Weston Park Hospital
Whitham Road
Sheffield
S10 2SJ

Dear Prof Coleman

Study Title: Bone Health in Breast Cancer Survivors Following
Adjuvant Bisphosphonate Therapy
REC reference number: 09/H1208/31
Protocol number: ISRCTN79831382 V3

The Research Ethics Committee reviewed the above application at the meeting held on 24 June 2009. Thank you for attending to discuss the study.

Ethical opinion

Ethical Issues Discussed

1. The committee wanted to know if the DXA scan was equivalent to 15 days of sunshine was overall or individually – full body, lumbar spine, hip. You confirmed that this was inclusive.
2. The committee suggested that it would be useful for participants to describe the radiation dose from a DXA scan as being less than the exposure received from a conventional chest x-ray.
3. The committee wanted to know if the sub-group could have all their bloods taken at one time to prevent bloods being taken more than once during the day. You agreed that this should be possible.
4. The committee wanted to know if it was necessary to have fasting blood samples. You explained that this was necessary as it affects some of the markers being looked at. However, you will try to ensure that the samples are taken as early in the day as possible.

5. The committee suggested that the wording on Page 2 of the patient information sheet might refer to 'normal' bone breakdown, you agreed.
6. The committee wanted to know if the results of the DXA scan went back to the GP. You explained that the results went back to the patient's breast specialist and then the patient would go back to their GP for further treatment and follow-up as required.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

Other conditions specified by the REC

Sub-study patient information sheet - should, on page 1, state 'Zoledronic acid can inhibit normal bone breakdown....'.

Consent form should include the following standard NRES wording 'I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the sponsoring company, from regulatory authorities or from the NHS Trust where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records.'

Consent form should include a line giving permission to inform the participant's GP.

New documents with revised version numbers and dates should be sent for information purposes.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Covering Letter		28 May 2009
Protocol	ISRCTN7983 1382 V3	18 May 2009
Investigator CV		19 May 2009
Application		02 June 2009
GP Letter Main Bone Sub-study	1	12 May 2009
GP Letter Quantitative Bone Scan	1	12 May 2009
Participant Consent Form: Bone Health Quantitative Sub-study	1	06 May 2009
Participant Consent Form: Bone Health Sub-Study	1	06 May 2009
Participant Information Sheet: Quantitative Bone Sub-study	2	27 April 2009
Participant Information Sheet: Main Bone Sub-study	2	27 April 2009
External Review		
Clinical Trials Executive Minutes		12 January 2009
Medical Physics Expert Report		08 May 2009
Questionnaire: WHO Risk Factor		
AZURE TSC Minutes		18 June 2009
Statistician CV		
Letter from Sponsor		22 May 2009
Letter from Statistician		13 May 2009

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators

- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1208/31

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Mr Paul Hamilton
Chair

Enclosures:

*List of names and professions of members who were present at the meeting and those who submitted written comments
"After ethical review – guidance for researchers"*

Copy to: Mr Richard Hudson
Quality & Governance Manager
Research Office
Research Services, New Spring House
231 Glossop Road
Sheffield
S10 2GW

West Midlands Research Ethics Committee
Attendance at Committee meeting on 24 June 2009

Committee Members:

<i>Name</i>	<i>Profession</i>	<i>Present</i>	<i>Notes</i>
Dr Sylvia I J Chandler	Retired GP	Yes	
Mr Paul Hamilton	Local Government Officer (Chair)	Yes	
Dr Adrian Hamlyn	Consultant Physician & Hepatologist	No	
Mrs Theresa Hyde	Headteacher (Retired)	Yes	
Dr Ronald Jubb	Consultant Rheumatologist	Yes	
Dr Robert Law	Consultant in Anasesthesia and Intensive Care	Yes	
Professor John Marriott	Pharmaceutical Chemist/Academic Pharmacist	No	
Mrs Paulette Robinson	School Teacher (Retired)	No	
Mr Philip A Russell	Head Teacher (Retired)	Yes	
Mrs Ros Salter	Research Fellow	No	
Dr Eric Silove	Retired Paediatric Cardiologist	No	
Miss Heather Small	Heart Study Co-ordinator	Yes	
Dr Michael Wolffe	Optometrist	Yes	

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