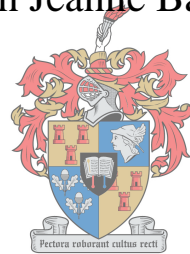


Bone health in post-menopausal patients with breast cancer treated with aromatase inhibitors: factors predicting the risk for osteoporosis.

by

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Dissertation presented for the degree of **Doctor of Philosophy** in the Department of Surgical Sciences

Faculty of Medicine and Health Sciences at Stellenbosch University



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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Aromatase inhibitors (AI), the gold standard for treatment of postmenopausal women with hormone-sensitive breast cancer, add an additional burden to the risk of osteoporosis in the postmenopausal population. Individual variation in AI associated bone loss is related to clinical risk factors as well as genetic variations in drug metabolism.

The aim of the study is to identify postmenopausal breast cancer patients at highest risk for AI-associated bone loss by utilizing clinical, biochemical and genetic parameters. In parallel, clinically meaningful patient reports were developed from a secure online genomics database resource, enriched during the study.

This prospective study was conducted at the Tygerberg Hospital Breast Clinic in affiliation with Stellenbosch University. Postmenopausal women with endocrine sensitive breast cancer, aged 50 to 80 years, were enrolled after obtaining informed consent. A baseline questionnaire documented demographic-, lifestyle- and medical history before commencing AI treatment. Clinical, biochemical and bone mineral density (BMD) measurements were obtained at baseline. Cytochrome P450 19A1 (*CYP19A1*) genotyping was performed using real-time polymerase chain reaction (PCR), and a screening algorithm applied to select patients for whole exome sequencing (WES). Results relevant to breast cancer diagnosis, comorbidities and treatment response were integrated into an adaptable report format for clinical application. Descriptive statistics were used to analyze the data.

A total of 101 participants were recruited, with a mean age of 61 ± 7 years. Thirty-two women fulfilled global criteria for bone protection at baseline [BMD T-score $\geq -2SD$ (n=18); BMD T-score $-1.5SD$ to $< -2SD$ with risk factors (n=14)]. In women with osteoporosis, significantly lower body

weight, body mass-, fat mass- and lean mass index were documented ($p < 0.001$). Low vitamin D status was present in more than 90% of the cohort tested ($n=95$). After one year of AI treatment, 72 patients remained in the study, of whom 10 (14%) experienced more than 5% bone loss at the lumbar spine. Genotyping for the *CYP19A1* rs10046 in 72 patients revealed that patients with two copies of the A-allele are 7,37 times more likely to have a higher percentage bone loss at the total hip compared to those without this allele (CI of 1.101- 49.336, $p=0.04$). At the lumbar spine, *CYP19A1* rs10046 AA homozygotes were 10.79 times more likely to have a higher percentage bone loss compared to patients with the GA or GG genotypes (CI of 1.771- 65.830, $p=0.01$). Extended genetic testing using Sanger sequencing and WES in the 10 patients with more than 5% bone loss supported the clinical findings. None of the 34 patients without bone loss at the lumbar spine at month 12 were homozygous for the functional *CYP19A1* polymorphism.

At baseline, a third of women fulfilled global criteria for bone protection. This highlights bone fragility associated with body composition variables of postmenopausal women in our predominantly Mixed Ancestry study cohort. Homozygosity for *CYP19A1* rs10046 provides additional information for individual risk stratification to optimize bone health maintenance. New insights gained into the mechanisms impacting bone health merit continued health outcome studies embedded in routine clinical practice.

Opsomming

Aromatase inhibitore (AI), die goue standaard vir die behandeling van postmenopausale vroue met hormoon-sensitiewe borskanker, dra by tot die risiko vir osteoporose in die postmenopausale bevolking. Individuele variasie in AI geassosieerde beenverlies is verwant aan kliniese risiko faktore asook genetiese variasie in middel metabolisme.

Die doel van die studie is om postmenopausale borskanker pasiënte te identifiseer wat die hoogste risiko het vir AI geassosieerde beenverlies deur gebruik te maak van kliniese, biochemiese en genetiese maatreëls. In parallel hiermee, is klinies betekenisvolle pasiënt verslae ontwikkel vanuit die aanlyn geslote genomiese databasis bron, wat verryk is tydens die studie.

Die prospektiewe studie het plaasgevind by die Tygerberg Hospitaal Borskliniek, geaffilieër met die Universiteit van Stellenbosch. Postmenopausale vroue met endokrien-sensitiewe borskanker, tussen die ouderdomme van 50-80 jaar, is opgeneem in die studie, na verkryging van ingeligte toestemming. 'n Basislyn vraelys het demografiese- leefstyl- en mediese geskiedenis gedokumenteer voor die aanvang van AI behandeling. Kliniese, biochemiese en been mineraal digtheid mates is geneem met basislyn. Sitochroom P450 19A1 (*CYP19A1*) genotipering is uitgevoer deur die gebruik van reël-tyd polimerase ketting reaksie (PCR) en 'n siftingsalgoritme is toegepas om pasiënte te selekteer vir heel eksoom volgorde bepaling (WES). Alle inligting is ingelees in 'n geslote aanlyn genomiese bron, op 'n aangaande basis. Resultate wat relevant is tot die borskanker diagnose, ko-morbiditeite en behandelingsrepons is geïntegreer in 'n aanpasbare verslag formaat vir kliniese toepassing. Beskrywende statistiek is gebruik om die data te analiseer.

'n Totaal van 101 deelnemers is gewerf, met 'n gemiddelde ouderdom van 61 ± 7 jaar. Twee-enderdig vroue het voldoen aan die internasionale kriteria vir been beskerming, met basislyn [BMD

T-telling $\geq -2SD$ ($n=18$); BMD T-telling $-1.5SD$ to $< -2SD$ met risiko faktore ($n=14$)]. In vroue met osteoporose is beduidend laer liggamsgewig, liggamsmassa-, vet massa- en spier massa indeks gedokumenteer ($p < 0.001$). Lae vitamin D status was teenwoordig in meer as 90% van die kohort wat getoets is ($n=95$). Na een jaar van AI behandeling, het 72 pasiënte oorgebly in die studie, van wie 14% ($n=10$) meer as 5% beenverlies ervaar het by die lumbale area. Genotipering van *CYP19A1* rs10046 in 72 pasiënte het geoon dat pasiënte met twee kopiee van die A-alleel, 7,37 meer geneig sal wees om 'n hoër persentasie been verlies by die heup te hê in vergelyking met die sonder hierdie alleel (CI of 1.101- 49.336, $p=0.04$). By die lumbale area, was *CYP19A1* rs10046 AA homosigote, 10.79 meer geneig om 'n hoër persentasie beenverlies te hê in vergelyking met pasiënte wat die GA or GG genotipes het (CI of 1.771- 65.830, $p=0.01$). Uitgebreide genetiese toetsing met Sanger volgorde bepaling en WES in die 10 pasiente met meer as 5% beenverlies, ondersteun die kliniese bevindinge. Nie enige van die 34 pasiënte wat geen been verlies getoon het by die lumbale area teen maand 12, was homosigote vir die funksionele *CYP19A1* polimorfisme nie.

By basislyn het 'n derde van vroue voldoen aan die internasionale kriteria vir beenbeskerming, wat die assosiasie van liggaamsamestelling in postmenopausale vroue van Gemengde Oorsprong, beklemtoon. Homosigote vir *CYP19A1* rs10046 verskaf bykomende inligting wat in oorweging gebring kan word ten einde individuele risiko bepaling vir optimale beengesondheid te verwesenlik. Nuwe insigte in meganismes wat beengesondheid beïnvloed, vereis voortgaande navorsing vasgelê binne 'n kliniese opset.

Dedication

I dedicate this PhD to my parents, Evelyn Hilary Baatjes and the late Fred Henry Baatjes, whom by their example and legacy, embedded a lifelong love for learning in me. They showed me that education is the most powerful instrument to bring about change in life.

Dankie Darra.

Dankie Mammie.

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The REDcap database web application was used to facilitate statistical analysis and the Gknowmix system to integrate clinical, pathology and genetic information into adaptable reports.

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List of Abbreviations

ABCSG 8	Austrian Breast and Colorectal Cancer Study Group 8
AEs	Adverse Effects
AIs	Aromatase Inhibitors
ASCO	American Society of Clinical Oncology
ATAC	Arimidex Tamoxifen, Alone or in Combination
BIG 1-98	Breast International Group 1-98
BP	Bisphosphonates
BMD	Bone mineral density
BMI	Body mass index
BS-ALP	Bone-specific alkaline phosphatase
CANSA	Cancer Association of South Africa
CR	Clinical response
CTX	C terminal telopeptide
CYP	Cytochrome P450 enzyme
CYP17	17-alpha-hydroxylase/17, 20-lyase
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid

DXA	Dual-energy x-ray absorptiometry
ER	Estrogen receptor
ESR1	Estrogenic response gene 1
EM	Extensive metabolizers
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FRAX	Fracture Risk Assessment Tools
GWAS	Genome wide association studies
HER 2	Human epidermal growth factor 2
HT	Hormone replacement therapy
ht-SNP	Haplotype tagging SNPs
HWE	Hardy Weinberg equilibrium
IES	Intergroup Exemestane Study
IMPACT	Immediate Preoperative Anastrozole, Tamoxifen or Combined with Tamoxifen
IOF	International Osteoporosis Foundation
IV	Intravenous
LD	Linkage disequilibrium

LOH	Loss of heterozygosity
LS-BMD	Lumbar spine bone mineral density
LVA	Lateral vertebral assessment
MAF	Minor allele frequency
MINDACT	Microarray in Node Negative and 1 to 3 Positive Lymph Node Disease May Avoid Chemotherapy
MRC	Medical Research Council
NCIC CTG	National Cancer Institute of Canada Clinical Trialist Group
NGS	Next generation sequencing
NHANES	National Health and Nutrition Examination Survey
NHGRI	National Human Genome Research Institute
NOF	National Osteoporosis Foundation
NTX	Cross-linked N-telopeptides of bone type I collagen
OCCR	Ovarian cancer cluster region
ONJ	Osteonecrosis of the jaw
PR	Progesterone receptor
PSGT	Pathology-supported genetic testing
PTH	Parathyroid hormone

PTT	Protein truncation test
RANKL	Receptor activator of NF- κ B ligand
RCT	Randomized controlled trial
SDs	Standard deviations
SERM	Selective Estrogen Receptor Modulator
SHIP	Strategic Health Innovation Partnerships
SNPs	Single nucleotide polymorphisms
SSRI's	Selective Serotonin release inhibitors
SULT	Sulfotransferase
TCL1A	T-Cell Leukemia/Lymphoma 1A
TEAM	Tamoxifen Exemestane Adjuvant Multinational trial
UTR	Untranslated region
UGT	Uridine diphosphate glucuronosyltransferase
25(OH) vitamin D	25 hydroxy vitamin D
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

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Outline of the dissertation

The thesis is presented in chapter format, starting with a general introduction (Chapter 1). A review of the clinical (Chapter 2) and genetic (Chapter 3) aspects relevant to the study is presented separately. In parallel with study recruitment, a database resource was developed to align the fragmented genetic research data with clinical service delivery information (Chapter 4). The characteristics of the study cohort was described at baseline (Chapter 5) and used to explore the role of genetics in aromatase inhibitor related bone outcomes, following 12 months of treatment (Chapter 6). A summative conclusion (Chapter 7) is provided as cohesion of the multidisciplinary research performed.

This dissertation includes 2 original papers published in peer reviewed journals. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case, a declaration is included in Appendix 1, indicating the nature and extent of the contributions of co-authors.

CHAPTER 1

General Introduction

Breast cancer is the most common malignancy in women worldwide (1). Endocrine therapy is an important modality in the treatment of postmenopausal women with hormone-sensitive breast cancer. Several trials have documented a significant reduction of in-breast recurrence and contralateral breast cancer, as well as a reduction in the risk of distant metastases with the use of aromatase inhibitors (AI)(2-4). The prolonged breast cancer survival rates necessitates attention to quality of life, most notably prevention of treatment-related bone health impairment (6).

Physiological changes in older women increase the risk of developing osteoporosis. The bone side effects of AIs add an additional burden of osteoporosis to an already at-risk population (5, 6), which accentuates the importance of maintaining bone health. In the post-menopause, estrogen production predominantly originates from adipose tissue, the adrenal glands, smooth muscle, and bone (7). AIs decrease estrogen levels by preventing estrogen production via aromatization of androgen precursors in peripheral tissues (6, 8). These low residual estrogen levels in healthy postmenopausal women are important in preserving bone health (9, 10).

Treatment-related bone loss in breast cancer may be different to the bone loss typically experienced in the postmenopausal woman (11). At the start of menopause, yearly bone loss is estimated at 2% annually; it plateaus at about 1% during the 2nd decade after menopause and beyond. However, the bone loss associated with AIs in postmenopausal women was estimated to be >2.5% per annum (6, 12). Although breast cancer patients treated with AIs are at increased risk of bone loss and fracture, only 25 – 50% of postmenopausal AI users develop bone loss and fractures (13). This vulnerable subgroup is undefined and risk factors remain unclear. The

American Society of Clinical Oncology and the UK Expert Group have established osteoporosis prevention and treatment algorithms for women initiating treatment with AIs (14). These involve close follow-up of bone mineral density (BMD) and a more aggressive pharmacotherapy compared to healthy postmenopausal women with osteoporosis (15).

BMD, an assessment of mineral content at specific skeletal sites is measured by dual energy X-ray absorptiometry (DXA) (16). DXA is accurate, non-invasive and can detect silent vertebral fractures and calculate body composition. It is also utilized to estimate BMD changes over time and evaluate response to therapy (17-19). As BMD decreases, the risk of fracture increases exponentially, two- to three-fold with every standard deviation (SD) decline in BMD (20, 21). Conventional risk factors for osteoporosis include modifiable and non-modifiable risk factors (22). Age, sex, genetic predisposition and ethnicity signify the most important non-modifiable factors. Adjustable factors such as low body weight, an inactive lifestyle, poor calcium diet and deficient vitamin D levels, smoking and excess alcohol use, may also impact bone density considerably. In women of all ethnicities, body weight is one of the most significant determinants of BMD at most skeletal sites (22, 23).

Bone health should be assessed by utilizing a mixture of parameters. These include the clinical risk factors, BMD measurement and biochemical markers of bone turnover. Bone formation biomarkers include serum bone specific alkaline phosphatase and osteocalcin, as well as parameters of bone resorption e.g. urine deoxypyridinoline, serum C terminal telopeptide. These markers of bone turnover can forecast postmenopausal bone loss rates and assess fracture risk independent of BMD. These indicators of bone turnover are more sensitive than BMD and changes can be identified within 4-6 months. In the osteoporosis-treatment studies (with alendronate, risedronate, raloxifene), bone turnover markers appear to have a stronger correlation with fracture

risk reduction than BMD (24, 25). This supports the use of bone turnover markers as substitutes for fracture risk reduction.

Osteoporosis has serious clinical and health systems implications. The addition of AIs as endocrine treatment of breast cancer compounds the problem. The prevalence of BMD and fractures have been described internationally, but data amongst diverse populations in Southern Africa is limited to black and white patients (26- 28). Expansion of knowledge in other ethnicities will optimize strategies towards prevention and management of osteoporosis as well as for the post-menopausal woman treated for breast cancer (29).

The causal mechanism of cancer treatment induced bone loss remains undefined. The aromatase enzyme plays a critical role in bone health. Pathogenic mutations in the Cytochrome P450 (CYP450) *CYP19A1* gene cause decreased BMD due to a significant effect on enzyme activity (30-32). Genetic polymorphisms in the aromatase gene have been associated with estrogen levels and bone mass in healthy postmenopausal women and men (33). Significant associations have been reported between several polymorphisms in the *CYP19A1* gene and bone health in postmenopausal women (33). Careful selection of clinically relevant single nucleotide polymorphisms (SNP) for pharmacogenetic studies is important and functionality should ideally be confirmed using *in vitro* studies as demonstrated for *CYP19A1* rs10046 (34). This SNP is associated with raised estrogen levels because of elevated enzyme activity, expected to be beneficial for bone health but detrimental to cancer outcomes. Advances in pharmacogenetics, moving from SNP genotyping testing to next generation sequencing, covering the entire gene, could overcome the limitations of incomplete genotyping, which may lead to incorrect risk allocation of polymorphic alleles.

To determine the genetic contribution to AI treatment-related bone loss, a comprehensive assessment of established baseline characteristics is necessary. International consensus recommends that all women with endocrine sensitive breast cancer should have a baseline osteoporosis risk assessment, prior to starting an AI (35, 36). A thorough evaluation allows for individualized risk stratification and bone protective measures to be introduced as clinically indicated (37, 38). Bone protective measures as globally agreed, suggest that all women with a BMD T-score of -2 SD or less at any measured site, should be protected with bone directed therapy. Furthermore, patients with a BMD T-score of -1.5 SD or less with additional bone risk factors, should also be considered for treatment (36). These include women above 65 years of age, smoking, low body weight, a family or personal history of fractures as well as a course of steroid therapy of longer than a 3-month period. Recommended pharmacological therapy for these at-risk patients includes vitamin D and calcium supplementation especially if nutritional intake is insufficient. Antiresorptive therapy with bisphosphonates are recommended in patients with a baseline T score of ≤ -2.0 or two or more clinical risk factors for fracture (5).

There is significant individual variation in AI associated bone loss, which could be related to clinical factors such as age, menopausal status, years since menopause and body mass index (BMI). It is clear that individual vulnerability to AI side effects differs and this unpredictability may partly be explained by diverse genetic profiles (39). Breast cancer pharmacogenetics are evolving with utilization of whole exome sequencing (WES) to identify genetically predisposed patients for AI adverse bone effects. In this study, the clinical value of AI pharmacogenetics as an additional bone risk factor postmenopausal breast cancer patients on AIs, was explored within the context of a pathology supported genetic testing algorithm developed in South Africa (40).

Aim

The aim of the study is to employ clinical, biochemical and genetic measures to improve the understanding of the pathophysiology of treatment-induced bone loss. An examination into the impact of AIs on bone health in a multi-ethnic postmenopausal cohort with endocrine responsive breast cancer resident in the Western Cape Province of South Africa was undertaken.

The goal is to identify a subgroup of women at highest risk of severe side effects, for effective implementation of preventive measures at the onset of treatment or by early modification of management.

Integrating research on AI pharmacogenetics with established clinical and biochemical bone loss risk factors could lead to improved individualized cancer care and simultaneously enrich an onco-genomic database resource, beyond a single study objective.

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CHAPTER 2

Postmenopausal breast cancer, aromatase inhibitors and bone health: what the surgeon should know

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Abstract

Breast cancer, as the most common malignancy in women, remains a major public health issue despite countless advances across decades. Endocrine therapy is the cornerstone of treatment of the hormone sensitive subtype of breast cancer. The use of aromatase inhibitors (AIs) in the postmenopausal women has extended the survival beyond that of Tamoxifen, but harbours a subset of side effects, most notably accelerated bone loss. This, however, does not occur in all women undergoing treatment. It is vital to identify susceptible patients early, to limit such events, employ early treatment thereof or to alter drug therapy. International trials on AIs, predominantly performed in North American and European females, provide little information on what to expect in women in developing countries. Here, surgeons often prescribe and manage endocrine therapy. The prescribing surgeon should be aware of adverse effect of endocrine therapy and be able to

attend to side effects. This review highlights clinical and biochemical factors associated with decrease in bone mineral density in an, as yet, unidentified subgroup of post-menopausal women. In the era of personalised medical care, appropriate management of bone health by surgeons based on these factors becomes increasingly important.

Abbreviations

AIs - Aromatase inhibitors; ATAC- Arimidex Tamoxifen, Alone or in Combination; AEs- adverse effects; BMD - Bone mineral density; BMI – Body mass index; BS-ALP- bone-specific alkaline phosphatase; BIG 1-98- Breast International Group 1-98; CYP 19- cytochrome P450 enzyme; CR- clinical response; CTX- C terminal telopeptide; DNA- deoxyribonucleic acid; DXA -dual-energy x-ray absorptiometry; ER- estrogen receptor; FRAX- Fracture Risk Assessment Tools; HER 2- human epidermal growth factor 2; IOF- International Osteoporosis Foundation; IES Intergroup Exemestane Study; LVA- Lateral vertebral assessment; NCIC CTG-National Cancer Institute of Canada Clinical Trialist Group; NTX- cross-linked N-telopeptides of bone type I collagen; NOF- National Osteoporosis Foundation; NHANES- National Health and Nutrition Examination Survey; PTH- parathyroid hormone; PR- progesterone receptor; RANKL- receptor activator of NF- κ B ligand; SNPs- single nucleotide polymorphisms; SDs- standard deviations; 25(OH) vitamin D- 25 hydroxy vitamin D; WHO- World Health Organisation

Introduction

Breast cancer is the most common female cancer, globally (1). In developing countries, it has replaced cervical cancer as the leading cause of cancer death in women (1). These patients ideally should be managed in multidisciplinary teams that coordinate surgical treatment in conjunction with the modalities of chemotherapy, irradiation, endocrine therapy, and biological therapy (2).

In developing countries, such as South Africa, this is often available only in major centres and mostly in tertiary hospitals affiliated with universities (3).

Determination of the molecular receptor status of tumours is standard in breast cancer classification. Routine testing for receptors in breast cancer includes the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) (2).

Hormone-receptor sensitive breast cancer is the most common breast cancer subtype and endocrine therapy is the cornerstone of systemic treatment (4).

Women with hormone receptor-positive disease have an excellent 5-year survival (5). Endocrine therapy in the adjuvant setting for the post-menopausal status consists of treatment with Tamoxifen or Aromatase inhibitors (4). Endocrine manipulation has systemic side effects. It is important to take cognizance of these and accurately quantify the potential for long-term morbidity (6).

In premenopause, ovaries are the principal source of estradiol. In post menopause, ovaries cease to produce estrogen and circulating estrogen levels fall precipitously. Extragonadal sites such as adipose tissue, breast, bone, vascular epithelium and brain produce estrogen locally from C19 steroid precursors via the aromatase cytochrome P450 enzyme. Circulating estrogen levels therefore do not accurately reflect concentrations in local tissue, where estrogen acts in a paracrine or intracrine fashion (7). In bone in particular, local estrogen production slows postmenopausal bone loss (7).

Bone health

Osteoporosis is characterized by compromised bone strength that predisposes to an increased risk of fracture (8). Osteoporotic fractures occur in nearly 40% of post-menopausal women. Menopausal women experience a sustained doubling of bone turnover (8) due to estrogen

withdrawal and a subsequent increase in bone resorption. An accelerated loss of BMD of 1–3% per year at the spine and 1–2% per year at the hip has been observed in the first 7 years after the onset of the menopause.

This weakening of the bone structure decreases resistance to low-energy trauma and coupled with a low BMD increases bone fragility and fracture risk (9). Major risk factors for osteoporosis comprise: age, female sex, a personal history of fracture as an adult, a history of a fragility fracture in a first-degree relative, low body weight, current smoking and excessive alcohol consumption, and use of corticosteroids (10). Other contributing factors are: excess height, poor general health and certain endocrine and systemic conditions. Poor depth perception and the use of drugs like benzodiazepines increase the risk of falling and so add to the fracture incidence.

The most common sites of fragility fractures are: vertebrae, femoral neck, and distal radius (8).

Methods used to assess fracture risk include bone mineral density (BMD), biochemical bone markers and the Fracture Risk Assessment Tools utilized in countries or regions with known hip prevalence figures.

Bone Mineral Density

Bone mineral density (BMD) is an assessment of the mineral content in key skeletal regions (11). It is measured with dual x-ray absorptiometry and expressed in absolute terms as grams of mineral per square centimeter scanned (g/cm^2). The T-score is the number of standard deviations that a patient's bone mineral density value is above or below the reference value for a healthy thirty-year-old adult.

Results are expressed as standard deviations (SDs) from age- and sex-matched standards (Z score) or from the population mean peak bone mass (T score). The reference range recommended by the

International Osteoporosis Foundation (IOF), World Health Organisation (WHO) and National Osteoporosis Foundation (NOF) for calculating the T-score is the National Health and Nutrition Examination Survey (NHANES) III reference database for femoral neck measurements in Caucasian women aged 20–29 years (12). Fracture risk increases roughly twofold for every standard deviation below the mean for a young adult. The WHO defines normal bone mass as $T > -1.0$, with osteopenia being $T < -1.0$ and $Z > -2.5$, and osteoporosis $T < -2.5$. Each SD represents a difference of 10%-15% in BMD. A T score of < -2.5 is indicative of a 25% loss from peak bone mass. Fracture risk increases exponentially with lower BMD. For T scores of -1.0, -2.0, and -3.0, the relative risks of fracture are 1.7-, 3.4- and 6.8-fold, respectively (8).

DXA measured BMD is accurate and reproducible. It uses x-rays to assess BMD by area (not volume). The radiation dose is approximately one-tenth of a standard chest x-ray. Patients should have repeat BMD measured by the same machine and by the same operator, to minimize error (8). It is the only bone density test that is currently useful for assessment of BMD changes over a time period and for determining the response to therapy (13).

Fracture Risk Assessment Tools (FRAX)

BMD provides the cornerstone for the diagnosis of osteoporosis, but it cannot be used in isolation as a determinant for the initiation of therapy (12). The WHO's Fracture Risk Assessment Tool (FRAX) is a risk prediction model that employs the femoral neck BMD as measured by DXA and includes clinical factors for bone loss. It estimates the 10-year probability of hip and other major osteoporotic fractures (spine, humerus and forearm). Clinical factors include: country or geographic region, the patient's ethnic origin, age, sex, weight, height, prior fragility fracture,

parental history of hip fracture, current smoking, excess alcohol intake, long-term use of oral glucocorticoids, rheumatoid arthritis, and secondary osteoporosis (11).

FRAX can be calculated for 4 ethnicities (white, Hispanic, Asian, and black) in a sex- and geographic-specific manner (8). It allows entry of ages 40 to 90 years; there is no validation of FRAX in younger or older patients. FRAX cannot be used to monitor therapy as it considers only femoral neck bone density in the calculation of risk and allows only yes / no input rather than gradations of secondary risk factors. In the United States, the National Osteoporosis Foundation recommends treatment of patients with a FRAX-calculated 10-year fracture probability of >3% for hip fracture and >20% for major osteoporotic fracture (11).

A similar web-based tool, the FORE 10-Year Fracture Risk Calculator (<http://riskcalculator.fore.org>), closely aligns with the US regional data from the WHO-FRAX model offering similar risk estimates for men and women older than 45 years. FORE also allows entry of glucocorticoid dosing; allows information on spine fracture; and adds a graphic display showing low, moderate, or high 10-year fracture risk for use in patient education (8).

Biomarkers of Bone Turnover

The common use of aromatase inhibitors led to an increased focus on cancer treatment-induced bone loss. Bone strength is a function of BMD and bone quality. Bone quality describes the set of characteristics that influence bone strength independently of BMD and include structural and material properties. Bone turnover is a function of the bone renewal process in which old or damaged bone is resorbed (bone resorption) and new bone is created (bone formation). Normally bone resorption and formation is tightly balanced to ensure that bone mass and quality is maintained. Excess resorption and sustained increases in bone turnover not only result in decreased

BMD, but may also adversely affect bone architecture and quality. These qualitative changes may decrease bone strength independent of BMD. Biomarkers are used to assess the rate of bone turnover and can thus provide information on bone quality. Combining BMD and bone markers allows for the identification of a subcategory of individuals at an increased risk of hip fracture compared to those identified by each test in isolation (14).

The role of estrogen in bone health

Estrogen plays an integral part in bone metabolism in women and is fundamental in the pathogenesis of osteoporosis in postmenopausal women. The bone loss associated with estrogen deficiency is a complex and multidimensional process (15). Estrogen is a systemic inhibitor of bone resorption by complex measures on bone cellular level (16). The reduction of serum oestradiol at the onset of the menopause leads to a negative balance at the bone remodelling unit level (17). The mechanisms by which estrogen regulates bone remodelling are not fully understood but estrogen is thought to affect osteoclastogenesis and osteoclast functioning through its effects on local cytokines and growth factors.

Endocrine therapy

There are two distinct subtypes of estrogen receptors, namely ER- α and ER- β . Tamoxifen has been used in the treatment of endocrine sensitive breast cancer for decades and it is the benchmark against which newer drugs are measured. Tamoxifen acts as a pure antagonist on ER α in breast tissue, resulting in a decrease in breast cancer cell proliferation (19). Conversely, it acts as an agonist on the estrogen receptor β expressed in bone and brain thereby promoting estrogen effects in these organs. This selective agonist effect of Tamoxifen in bone thus protects women

against accelerated postmenopausal bone loss attributable to cessation of ovarian estrogen production (20).

An overall 19% reduction in the incidence of fractures was seen in postmenopausal women receiving Tamoxifen therapy for a median of 5.75 years (18). Tamoxifen use in the switch trials as well as extended duration of treatment beyond 5 years are well documented (19, 20).

Aromatase inhibitors heralded a new strategy in the treatment of breast cancer. These agents are without the estrogenic effects and have an improved side effect profile compared to Tamoxifen (21). Today, it constitutes the gold standard in treatment of endocrine responsive breast cancers in postmenopausal women.

The use of aromatase inhibitors (AI) in post-menopausal patients is well-established. Several trials have documented a significant reduction of in-breast recurrence and contra-lateral breast cancer, as well as a reduction in the risk of distant metastases (22, 23). The third-generation aromatase inhibitors demonstrate greater efficacy and superior overall safety in the adjuvant treatment of women with hormone receptor-positive breast cancer, compared with the selective estrogen receptor modulator Tamoxifen (24, 25).

The near total suppression of oestrogen production by aromatase inhibitors has focused research on the aggravation of symptoms of menopause such as hot flashes and cardiovascular disease and has also raised significant concern regarding potential worsening of bone loss and the incidence of fragility fractures (26).

Bone loss and fracture

Aromatase inhibitors are the drugs of choice in post-menopausal breast cancer patients with endocrine responsive tumours. However, aromatase inhibitors enhance bone turnover and result in

the loss of bone mass (27). The general population risk factors for osteoporosis apply to breast cancer patients. However, cancer treatment causes additional bone loss that could increase the risk, above that seen in cancer-free women.

The level of bone loss and fracture risk is directly related to the further suppression of already low post-menopausal estrogen levels. In post-menopausal women, AIs decrease the serum levels of oestrogen beyond physiological levels and it is expected that bone loss would be augmented (28, 29). The ATAC (Arimidex, Tamoxifen, alone or in combination) bone sub-protocol confirmed that adjuvant Anastrozole therapy can lead to accelerated bone loss for postmenopausal women with early breast cancer (30), compared to the bone-protective effect seen with Tamoxifen. This confers a 2- to 3-fold higher risk of fractures versus women receiving Tamoxifen. Annual rates of bone loss from AI treatment range from 3%-4% at the spine and 1%-2% at the hip (31). Hip fractures, associated with greater morbidity than all other osteoporotic fractures combined, did not differ between treatment groups, even with follow-up extending beyond the 5-year treatment period. The relative increase in fractures in the Anastrozole group remained constant over the 5-year treatment period but was not evident in year 6 (32, 33).

Trials	Intervention	BMD changes (%)	p value	Fracture rate (%)
ATAC (6)	Arimidex	Hip: - 7.24 Spine: - 6.08	<0.01	11
	Tamoxifen, alone or in combination	Hip: 0.74 Spine: 2.77		
NCIC CTG MA.17/BIG 1-97(6)	Letrozole (post Tamoxifen)	Hip: -3.4 Spine: - 4.1	0.009	5.3
	Placebo	Hip: 2 Spine: 1.0		
IES (6, 24)	Exemestane post Tamoxifen	Hip: -2.9 Spine - 3.9	<0.001	7
	Tamoxifen (continued)	Hip: -1 Spine - 0.6		
Gonelli (6)	Tamoxifen	Hip: - 2.01 Spine - 3.0	<0.01	Not available
	Exemestane (post Tamoxifen)	Hip: 0 Spine: 0.0		
BIG 1-98 (6, 17)	Letrozole (L)	Not available	0.002	5.7
	Tamoxifen (T)			4.0

Table 2.1: Impact of endocrine therapy on BMD in postmenopausal women with breast cancer

Most of the large clinical trials have evaluated bone loss rates of AI therapy and reported significant bone loss at lumbar spine and hip. (Table 2.1) The rates of bone density change after 1 year of AI treatment ranged from -1.66 % to -7.40 %; a wide variation depending on the baseline characteristics of the patients studied (34).

Many trials lack data on baseline risk factors for fracture, including, older age, prior fracture, and other co-morbidities, as well as the longer-term effect on bones. The objective of treatment is not only to ensure cancer-free survival, but to limit detrimental effects of therapy (6).

Bone turnover

Measurement of bone turnover markers can be used to examine changes in bone turnover in the short term (35). ATAC and MA17 (20, 36) indicated statistically significant increases in both bone formation markers (e.g. osteocalcin) and bone resorption markers (e.g. cross-linked N-telopeptides of bone type I collagen [NTXs]) over the first 3 to 24 months of treatment of AI therapy. Studies examining AI-induced bone marker changes suggest a disparity between resorption and formation, leading to a net bone loss and increased fracture risk. Bone turnover marker profiles may be clinically useful in identifying those at highest fracture risk who require early intervention with anti-resorptive agents or potentially a change in treatment (35). The bone turnover changes occur early on in the initiation of AI treatment and in the ATAC, MA-17 and IES trials (18, 36), bone loss has translated into increased fracture rates with AI use compared to Tamoxifen use (25, 35).

Body weight

The relationship between body weight, breast cancer risk and breast cancer treatment is complex (37). Estrogen has long been suspected as the hormone responsible for increasing breast cancer risk in obese postmenopausal women (38). Aromatase resides in adipose tissue (among other tissues), leading to higher estrogen levels in heavier, postmenopausal breast cancer patients. This higher level of estrogen may thus worsen breast cancer outcome, but may be bone-protective in this subgroup of postmenopausal women (39).

The adjuvant use of adjuvant AIs have increased the concern about long-term bone health and fracture risk (40). Considering the bone protecting effect of estrogen (41) and the hypothesis that concentrations of estrogen differ among lean and obese women, it is important to investigate bone health in accordance with BMI (39). Endocrine therapies for breast cancer are not given by weight- or body-surface-area-related dosing: currently one standard dosage applies to all patients (42).

On the other side of the spectrum, low body mass index (BMI) has long been associated with an increased risk of fracture (43). The fracture risk associated with low BMI (<20 kg/m²) is the strongest for hip fracture and independent of age, sex and BMD (43).

In the post-menopausal breast cancer patient population, there is marked variation in BMI. The increased fracture risk in the lean patient, and the potential protective effect of estrogen in obese patients, may thus influence the outcome of BMD changes in patients on AIs.

Vitamin D

Vitamin D is essential for the maintenance of the human skeleton (44, 45). Wide variability in vitamin D levels occurs due to differences in geographic location, season, sun avoidance behaviours, sunscreen use, increasing age and skin pigmentation, obesity, and other lifestyle factors (46). The normal 25(OH)D values remain vague (47, 48). The International Osteoporosis Foundation recommends a desirable 25(OH)D serum level of 30 ng/mL or above (47).

Deficiency in Vitamin D can cause secondary hyperparathyroidism, high bone turnover, low bone mineral density and mineralization defects. Insufficiency can be a significant risk factor for osteoporosis (44) and could contribute to an increased fracture risk (34).

Vitamin D deficiency is very common among the general population, especially the elderly, (28) with up to 88% of post-menopausal breast cancer patients having levels <30 ng/mL (49). Adequate

dietary calcium and vitamin D intake is important for maintaining BMD, but supplementation alone is not sufficient to prevent the accelerated bone loss that occurs during AI therapy (43). Vitamin D repletion to a target threshold of >40 ng/ml can have a protective effect on bone loss among low-risk patients on AI treatment (34). Vitamin D level is currently not measured in a standard fashion, prior to initiation of AI therapy but is strongly recommended if resources allow (50).

Guidelines for initiation of bone therapy for surgeons prescribing aromatase inhibitors

The importance of maintenance of bone health during adjuvant breast cancer therapy has led to the formulation of multiple guidelines regarding the need for bone specific protection in the setting of AI therapy. These guidelines are very similar in their assessment of risk and recommendations (30, 40, 51). Bone-specific protection therapy with bisphosphonates as first line option is indicated in all women with a baseline bone mineral density in the osteoporotic range (T-score ≥ -2.5 SD below norm) and should be continued for the duration of AI therapy.

Patients with baseline BMD in the osteopenic range, i.e. a BMD T-score between -1 and -2.5 below norm, also qualify for bone specific protection if additional risk factors for bone loss are identified at baseline or if they display accelerated bone loss during follow-up.

Recommended calcium supplementation in postmenopausal women is a total daily intake of 1200 mg (dietary AND supplementation). Supplementation per se should not exceed 600 mg daily.

Recommended daily Vitamin D supplementation is 800 – 2000 IU.

Intravenous bisphosphonate therapy such as Zoledronic Acid is currently regarded the gold standard during adjuvant breast cancer therapy with AIs (52). Oral bisphosphonates and

Denosomab are other potential and very useful treatment options. Bisphosphonates suppress bone resorption. Side-effects with oral bisphosphonates are mostly limited to reversible gastro-esophageal irritation. Severe suppression of bone turnover with osteonecrosis of the jaw (ONJ) or atypical fractures are very unusual side-effects and almost exclusively seen with the more potent intravenous preparations and with longstanding use (beyond 5–10 years of therapy) (53, 54). The advantage of preventing excessive bone loss and fractures with bisphosphonate therapy far outweigh the potential risk of these very unusual complications.

An adjusted protocol based on guidelines in the setting of adjuvant AI therapy for postmenopausal breast cancer patients is illustrated in fig. 2.1 (51, 55).

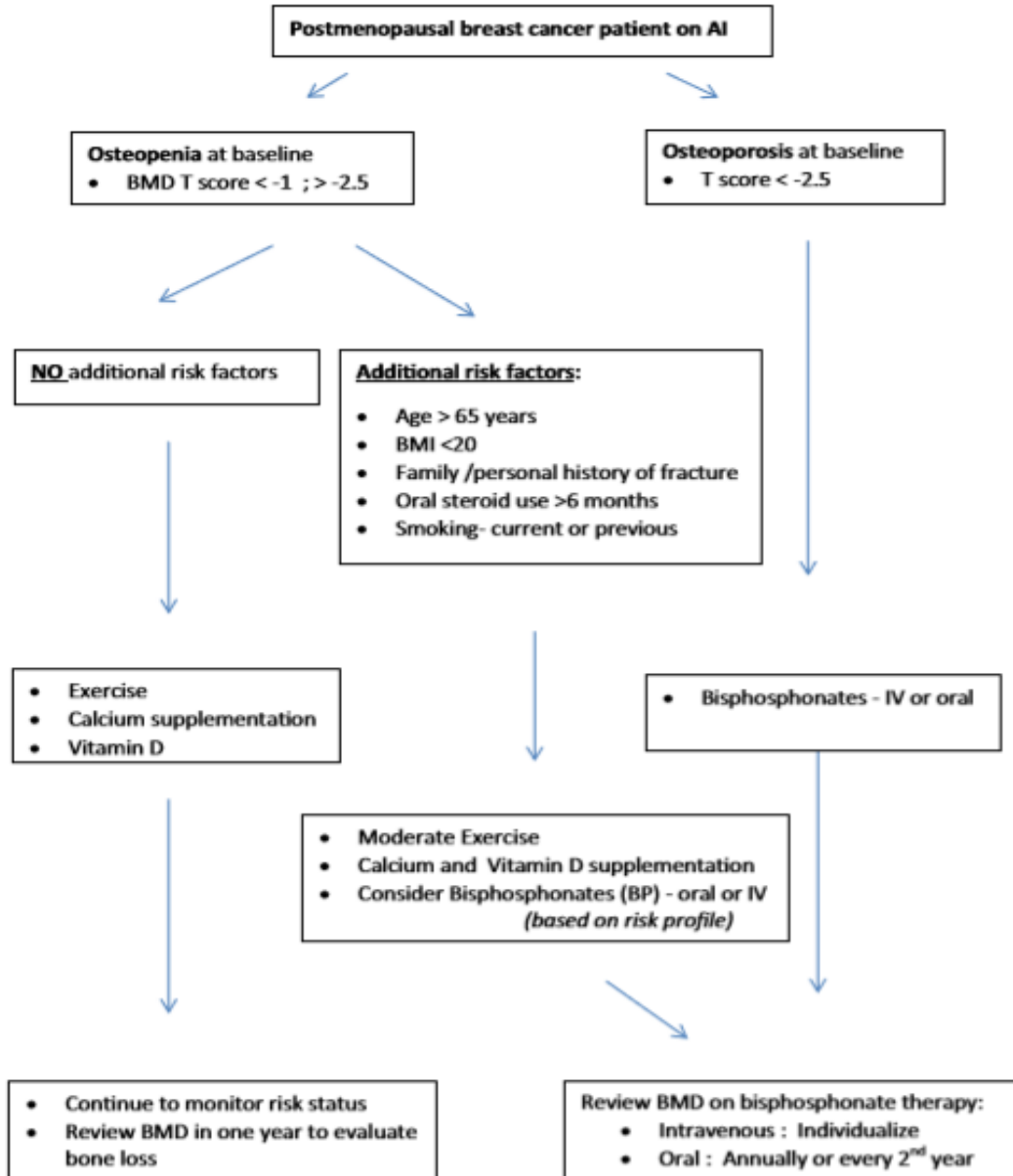


Figure 2.1. Recommended algorithm for bone protection in post-menopausal breast cancer patients, on Aromatase Inhibitors (AI). Adopted with permission of Hadji et al (40, 51).

Conclusion

The extended survival in breast cancer patients heightened interest in the side effect profile of therapies. The secondary aim of treatment should be to minimize morbidity for survivors and simultaneously maximize quality of life. In the era of personalized medicine, an early assessment of bone risk would facilitate individualized patient management decisions and provide an accurate estimate of disease outcomes and side effects. This would aid in implementation of measures to prevent or limit adverse events and to assist the clinician/ surgeon in early treatment modification to potentially avoid the side effect in this subgroup of susceptible women or to minimize harmful effects.

Declaration of interest

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CHAPTER 3

Pharmacogenetics of aromatase inhibitors in endocrine responsive breast cancer: lessons learnt from tamoxifen and *CYP2D6* genotyping.

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Abstract

Background

Genetics play a significant role in drug metabolism of endocrine therapy of breast cancer. These aspects have been studied extensively in patients on tamoxifen, but the pharmacogenetics of aromatase inhibitors are less established. In contrast to the protective effect of tamoxifen, aromatase inhibitors are linked with an increased risk for bone loss and fractures.

Objective

This review outlines key issues around implementation of pharmacogenetics of cytochrome P450 and tamoxifen as a model for optimal use of aromatase inhibitors in postmenopausal women with estrogen receptor positive breast cancer.

Methods

Lessons learnt from the association between tamoxifen and *CYP2D6* genotyping were applied to identify polymorphisms with the potential to change clinical decision-making in patients on aromatase inhibitors. The ability of next generation sequencing to supersede single-gene analysis was furthermore evaluated in a subset of breast cancer patients on aromatase inhibitors selected from a central genomics database.

Results

Methodological flaws in major randomized controlled trials and continued referral to incorrect results in expert consensus statements are important factors delaying the implementation of *CYP2D6* pharmacogenetics in tamoxifen treatment. This highlighted the importance of a clinical

pipeline including comprehensive genotyping, to define the target population most likely to benefit from aromatase inhibitor pharmacogenetics.

Conclusion

The clinical utility of *CYP2D6* genotyping is well-established in patients at increased risk of tamoxifen resistance due to cumulative risk. The pharmacogenetics of *CYP19A1* requires further clarification in terms of bone risk assessment for appropriate use in the treatment algorithm of high-risk patients at the onset of aromatase inhibitors.

Keywords: Breast cancer; Oncology, Pharmacogenetics, Tamoxifen, Aromatase inhibitors, Bone health

Introduction

Breast cancer is the most common malignancy in females worldwide (1), but the incidence varies significantly across continents (2). This global variation may partly be ascribed to differences in genetic background underlying the development of breast cancer and response to treatment. In South Africa, breast cancer is most prevalent among Caucasian and Asian women and the second most common cancer among Black and Coloured women (3). Population differences in drug metabolism supports individualized breast cancer treatment to replace a one-size-fits all approach (4). The high level of population admixture detected in genetically divergent ancestral clusters in Africa provides the ideal study ground for pharmacogenetic studies (5, 6).

Both germline and tumour genetics contribute to distinct immuno-phenotypes defined by estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) status. These histo-pathological parameters are assessed routinely in all breast cancer patients at diagnosis. Along with clinical variables such as tumour size and nodal status, assessment of ER, PR and HER2 aid risk stratification and are mandatory in guiding systemic treatment decisions (7). In patients with the most common ER-positive breast cancer, endocrine therapy has been used as the cornerstone of treatment for decades (8).

Tamoxifen was the first targeted treatment used in ER-positive breast cancer, signalling the era of personalized medicine. Aromatase inhibitors (AIs) are currently the gold standard for treatment of endocrine responsive breast cancers in postmenopausal women. Several trials verified improved overall survival, a substantial decrease in recurrence and contra-lateral breast cancer, as well as a decrease in distant metastases when compared to tamoxifen (9, 10). However, in contrast to the protective effect of tamoxifen on bone health, AIs are associated with a significantly increased risk of bone loss and fractures (11). The severity of side-effects may impact on treatment compliance and thereby reduce treatment efficacy (12, 13). Table 3.1 lists the most common side effects encountered with endocrine treatment, with some overlap noted between tamoxifen and AIs in relation to incidence and severity.

	Tamoxifen	Aromatase inhibitors	References
Bone health	Bone protective	Increased bone loss / fractures	(11,14, 15, 16)
Hot flashes	Frequent	Frequent	(13, 17, 18,19)
Gynaecological effects	Vaginal bleeding	Less vaginal bleeding	(17, 18)
Thromboembolic events	Increased risk	Rare	(18)
Cognitive Function	Brain Cognitive impairment in verbal memory and executive functioning	Similar to Tamoxifen	(20)
Lipid metabolism and cardiovascular disease	Decrease of low-density lipoproteins and total cholesterol	Increase of low-density lipoproteins and total cholesterol	(14, 15)
Endometrial cancer	Increased risk after long term use	No increased risk	(19)
Arthralgia/myalgia	Rare	Frequent	(19, 21, 22)

Table 3.1: Common side effects of tamoxifen and aromatase inhibitors.

Evaluation of fracture risk preceding the initiation of AI-treatment is essential. Lifestyle adjustments such as exercise and supplementation with calcium and vitamin D have a favourable impact on long-term bone health (23). However, thresholds to introduce preventative therapy and bisphosphonates as the first therapeutic option for AI-induced bone loss differ amongst available recommendations (24). The risk of side effects from bisphosphonates, such as gastro-esophageal irritation and rarely osteonecrosis of the jaw exists, but the benefit of limiting bone loss and reducing fracture risk prevails with the use of these agents in high-risk breast cancer patients.

We recently reviewed the clinical and biochemical risk factors associated with decreased bone mineral density and adopted a treatment algorithm for application in resource-limited environments (25). The potential role of genetics in this clinical management scheme has not previously been explored in South African breast cancer patients. The clinical usefulness of testing for common single nucleotide polymorphisms (SNPs) at critical control points within metabolic pathways affecting bone health, would depend on their effect on gene regulation or structure. Differences in SNP allele frequency across ethnic groups and haplotype associations also require careful consideration prior to inclusion of clinically validated gene targets in treatment algorithms (6).

An enhanced understanding of breast cancer pharmacogenetics has evolved over recent years. It has become clear that genetic heterogeneity necessitates the identification of therapeutic targets to decrease drug toxicity and improve compliance (4). The cytochrome P450 (CYP 450) enzyme system, which metabolizes 80-90% of all commonly prescribed drugs, has been studied in relation to both tamoxifen resistance and the AI side effect profile. The evidence supporting genetic testing before therapy is still considered too weak for incorporation in oncology practice (26). However, continued referral to flawed results in randomized controlled trials in expert consensus recommendations exemplifies issues of fundamental importance in breast cancer pharmacogenetics (27). The Austrian Breast and Colorectal Cancer Study Group Trial 8 (ABCSG 8) fully validated the association between *CYP2D6* genotype and increased recurrence rate or death in a subgroup of post-menopausal women with invasive ER positive breast cancer (28). These include comprehensive *CYP2D6* genotyping to minimize misclassification of poor metabolizer status and numerous pharmacologic features known to influence endoxifen levels comprising tamoxifen monotherapy, dose (20mg) and duration of 5 years with annual follow-up. Studies

without such strict selection criteria for target group identification, which should preferably include consideration of concomitant prescriptions influencing enzyme activity, cannot be used to either support or refute the *CYP2D6* hypothesis.

Delaying the implementation of *CYP2D6* pharmacogenetics despite evidence of clinical utility in a subgroup of patients, may have serious consequences in affected families (29). This is an important consideration in South Africa, due to an increased frequency of founder mutations in the *BRCA1* and *BRCA2* tumour suppressor genes in Afrikaner, Coloured and Xhosa breast cancer patients (30). A risk-benefit assessment of potential cumulative effects led to recommendation of *CYP2D6* genotyping in ER-positive breast cancer patients with defective *BRCA1/2* genes or concomitant use of anti-depressants associated with reduced *CYP2D6* activity (31).

Acceptance that genetic information may be insufficient to predict treatment response led to the development of a pathology-supported genetic testing platform for research translation in South Africa (32). Genetic testing service delivery is linked to the generation of a research database using an institutional review board approved protocol. Establishment of joint pathology and genomic facilities could overcome the limitations of single health disciplines and result in new models for data acquisition and earlier adoption of pharmacogenetic applications. The use of stored patient information for validation studies performed at the interface between the laboratory and clinic has gained acceptance as a possible alternative to randomized controlled trials, provided that patient selection criteria are well defined and adhered to (32). This approach was used to validate a microarray pre-screen algorithm as an appropriate strategy to reduce chemotherapy overtreatment in South African patients with early-stage breast cancer (33, 34). Over a 9-year period, after introduction of the Food and Drug Administration (FDA) approved MammaPrint test, more than 100 early-stage breast cancer patients in South Africa could safely avoid chemotherapy. This was

confirmed by recent level 1A evidence from the prospective Microarray in Node Negative and 1 to 3 Positive Lymph Node Disease May Avoid Chemotherapy (MINDACT) study (35,36). As demonstrated in this case, appropriate introduction of new companion diagnostics may outpace the reporting of randomized controlled trials that require lengthy follow-up for final assessment of clinical outcome.

Similar to microarray-based breast cancer gene profiling, many challenges have been encountered in the pursuit of *CYP 450* pharmacogenetics in patients receiving endocrine treatment for breast cancer (37, 38). Key issues addressed during incorporation of *CYP2D6* genotyping in clinical practice (31) served as a model in this study to determine the appropriateness of *CYP19A1* genotyping in patients treated with AIs.

Tamoxifen pharmacogenetics

CYP2D6 metabolizes tamoxifen, a Selective Estrogen Receptor Modulator (SERM). The principal mechanism of action of tamoxifen is mediated by ER binding and blocking of the proliferative effects of estrogen on mammary epithelium. Figure 3.1 illustrates the tamoxifen-endoxifen pathway with the *CYP 450* enzyme encoding genes, including *CYP2D6*, *CYP2B6*, *CYP2C9*, *CYP2C19* and *CYP3A4/5*, shown at each step. Except for *CYP2D6*, none of the other enzymes involved in tamoxifen metabolism appear to cause any meaningful differences in drug efficacy (39).

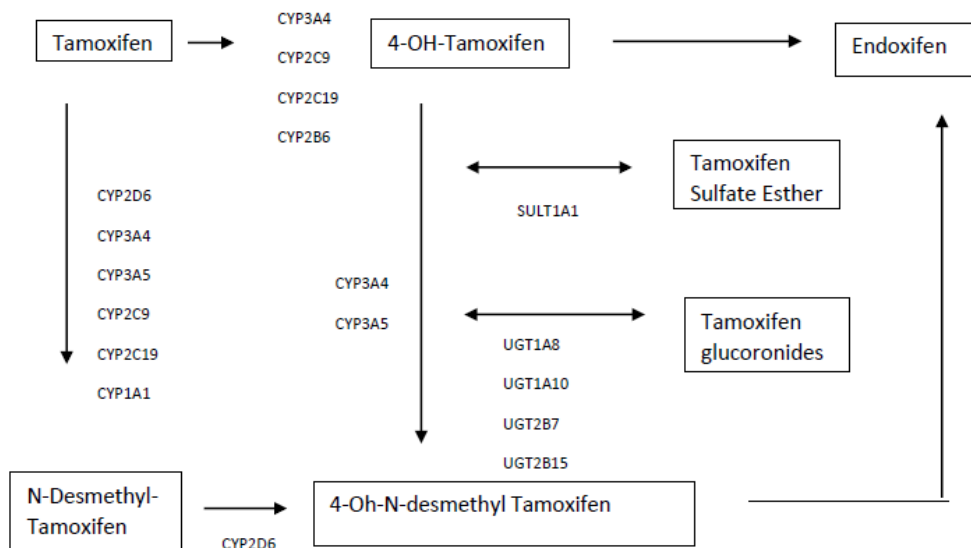


Figure 3.1: Major metabolic pathways for tamoxifen, with the key enzymes indicated at each step. CYP, cytochrome P450; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase

The relationship between *CYP2D6* and tamoxifen is intricate (40)(41). A defective *CYP2D6* gene may lead to slower metabolizing of tamoxifen, and could result in a greater risk for adverse events and lower efficacy of drugs requiring *CYP2D6* activation (42). The efficacy of tamoxifen is also influenced by co-prescription of *CYP2D6* inhibitors such as certain Selective Serotonin Release Inhibitors (SSRI's), commonly prescribed for depression and relief of hot flashes as a by-effect in breast cancer patients (43, 44). Polymorphic variation may furthermore lead to absence of a functional *CYP2D6* protein in approximately 5-10% of individuals of European ancestry and 1-2% of those of Asian and African ancestry (6, 43). The majority of *CYP2D6* genotyping studies were performed in Caucasian patients. As the frequencies of *CYP2D6* polymorphisms vary significantly between different ethnic groups, data from these studies cannot be extrapolated directly to non-Caucasian breast cancer patients (6, 45).

Several studies reported the association between *CYP2D6* and hot flashes as a possible marker for treatment efficacy (13, 42, 46). The Breast International Group 1–98 (BIG1-98) study described a link between *CYP2D6* genotype and tamoxifen-associated hot flashes (47). However, other studies reported conflicting results (48). This has partly been ascribed to the use of tumour-derived DNA extracted from formalin-fixed paraffin-embedded tissue in the BIG 1–98 study for *CYP2D6* genotyping (40). Significant deviation from the Hardy Weinberg equilibrium (HWE) raised concerns about quality and accuracy of genotyping and the consequential mistakes in data interpretation and conclusions drawn from these results (27). The HWE defines expected versus observed genotype frequencies in a randomly mating population. Similar discrepancies were detected in the Arimidex, Tamoxifen, Alone or in Combination (ATAC) and the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial (13, 49). These studies did not prove a link between *CYP2D6* and tamoxifen outcome, but elicited severe critique about genotyping errors with considerable departure from HWE for the most important *CYP2D6**4, causing conflicting results (50, 51).

Despite the fact that *CYP2D6* activity is largely dependent on polymorphic variation tested for in many laboratories worldwide, the American Society of Clinical Oncology (ASCO) maintains that data on the clinical utility of *CYP2D6* pharmacogenetics is insufficient to endorse testing for endocrine treatment planning (26). They nevertheless recommend counselling of breast cancer patients treated with tamoxifen to avoid co-prescription of *CYP2D6* inhibitors, which include a number of drugs frequently used for treatment of depression and other co-morbidities. The frequent co-prescription of certain antidepressants with tamoxifen may significantly impair the function of *CYP2D6*. This emphasizes the importance of appropriate eligibility criteria for selection of a subset of patients for whom the advantage of genetic testing offsets the risk (31).

The value of *CYP2D6* genotyping depends on many factors, including the appropriate target population identified as one of the most important factors to consider in clinical outcome studies. Clinical utility was confirmed in the ABCSG 8 trial in a subgroup of breast cancer patients by comparing *CYP2D6* poor metabolizers with extensive metabolizers according to different selection criteria (28). In this study, the observed *CYP2D6* genotypes were in HWE, which is important to exclude genotype errors. *CYP2D6* genotypes determined from tumor-derived DNA may be subject to inaccuracies due to loss of heterozygosity, known to affect the *CYP2D6* locus in up to a third of breast cancers (40, 52). Chromosomal instability in breast cancer tissue at the *CYP2D6* locus was an important source of error with use of breast cancer tissue to determine genotypes in previous randomized controlled trials (47, 49). Requests for retraction of BIG 1–98 from the scientific literature due to significant methodological flaws, were unsuccessful (52, 53). It impacted on the interpretation of side effect profiles and delayed proof of clinical utility of *CYP2D6*-tamoxifen pharmacogenetics. It is therefore important to ensure quality control measures for accurate germline genotyping (44, 52) as we embark into the era of AI pharmacogenetics.

Aromatase Inhibitor pharmacogenetics

In the light of the challenges faced in the evolution of pharmacogenetics of the tamoxifen–*CYP2D6* pathway (figure 1), it is imperative to critically review the literature for genetic determinants of AI response and side effects. By comparison, little is known about the pharmacogenetics of AIs and it is unclear whether impediments similar to the use of tamoxifen will be encountered.

AIs have replaced tamoxifen in endocrine therapy of women with ER-positive breast cancer due to improved outcome compared to tamoxifen (9, 54). In the post-menopause, estrogens are

produced by peripheral aromatization of androgen precursors to estrogen (55). This reaction is catalyzed by the aromatase enzyme (*CYP19*) (56). Aromatase is a CYP 450 enzyme that is encoded by *CYP19A1* located on chromosome 15q21.2. *CYP19A1* has a complex structure, with a long 5'-untranslated region that serves as the regulatory unit of the gene (57). Genetic variation could alter the levels of AIs available to inhibit aromatase and as such influence treatment efficacy and side effects such as bone loss (57).

The profound suppression of estrogen production by AIs has intensified study into the potential deterioration of bone quality and subsequent increase of fractures (58). Estrogen is vital in maintaining bone structure, and plays a crucial role in the development of postmenopausal osteoporosis, a systemic bone disease characterized by alterations in bone quality, leading to fragility and fracture risk (59). The pathogenesis of osteoporosis includes multiple genetic and environmental risk factors. Alterations in genes involved in estrogen metabolism, such as *CYP19A1*, *CYP11A1*, 17-alpha-hydroxylase/17,20-lyase (*CYP17*), T-Cell Leukemia/Lymphoma 1A (*TCL1A*) and estrogenic response (*ESR1*) genes are potential contributors to the abnormal pathophysiology of bone (60-63).

CYP19A1 and bone effects

The effects of genetic polymorphisms in the *CYP19A1* gene have been studied most extensively in breast cancer, prostate cancer and osteoporosis (59, 64). Susceptibility to side effects from AI-treatment differs between patients as a result of individual and ethnic variability in genetic traits (63, 65, 66). This supports the need for identification of biomarkers predicting clinical benefit and limitation of drug toxicity (65). Copy number variants and allelic variations of *CYP19A1* between population groups justify investigation into the gene effects on side-effect profiles and drug efficacy between subgroups taking AIs (65). The mechanism at the core of the association between

CYP19A1 alleles and bone mass is still unclear. A number of polymorphisms in the *CYP19A1* gene is associated with alterations in steroid hormone levels, aromatase activity, bone mineral density and risk of fracture (67). These polymorphisms may impact on a predisposition to skeletal effects from AIs leading to substantial variances in bone loss among patients (60).

Some studies observed no difference in treatment-related adverse effects when stratified according to *CYP19A1* genotypes for SNPs rs10046, rs4646 and rs700519 (68, 69). Napoli and colleagues observed that women with the AA genotype for *CYP19A1* rs700518 (G/A, Val80) developed substantial AI associated bone loss at the lumbar spine and total hip at 12 months compared to patients with GA/GG variants (64). *CYP19A1* rs700518 is a synonymous G/A (or C/T) polymorphism (at position 49,316,404) in exon 3 of the gene. In the BIG 1-98 trial including *CYP19A1* genotyping, SNP rs700518 AA homozygotes or AG heterozygotes exacerbated the risk of adverse bone effects, compared with patients who had the GG wild-type genotype, irrespective of treatment with tamoxifen or letrozole (70,71). Reasons provided for this discrepancy in allocation of the risk-associated allele focused on differences in sample size between these two studies. However, these contradictory results highlight inconsistencies that can be expected for silent mutations or synonymous SNPs such as *CYP19A1* rs700518 (Val80) due to the potential for chromosomal cross over events.

CYP19A1 rs700518 was found to be in complete linkage disequilibrium with allele 7 of the TTTAn repeat polymorphism in intron 4, known to be involved in bone homeostasis (61). Although this marker is considered unlikely to be functional due to its location outside the coding region of the *CYP19A1* gene, the allelic differences in gene expression summarized in table 2 favour potential clinical relevance. The influence of the TTTAn repeat polymorphism on lumbar spine bone mineral density difference was also assessed in response to hormone replacement therapy. A

higher number of TTTAn repeats were associated with higher lumbar spine bone mineral density and lower risk of spine fracture (62). Breast cancer patients with shorter alleles may be prone to these bone-related risks, which could potentially be worsened by AI therapy.

CYP19 repeat polymorphism alleles	Effect on gene expression
TTTA ₇ ; TTTA _{<9}	Decrease transcription
TTTA ₈ ; TTTA _{>9}	Increase transcription
3TCT del TTTA ₇	Decrease transcription

Table 3.2 Functional effects of the TTTAn repeat polymorphism rs60271534 in the *CYP19A1* gene.

From SNP analysis to next generation sequencing

Genotyping of the *CYP19A1* TTTAn polymorphism is complex and in contrast to high throughput SNP analysis, it usually requires Sanger sequencing for allelic discrimination (72, 73). This may be the reason why several studies used the synonymous *CYP19A1* rs700518 as a tagging SNP for genotyping of this repeat polymorphism (71). In an attempt to clarify whether this synonymous SNP or the TTTAn polymorphism in intron 4 of the *CYP19A1* gene is in linkage disequilibrium with a functional variant elsewhere in the gene as previously suggested (71), five AI-treated breast cancer patients formerly subjected to next generation sequencing due to ultra-low vitamin D levels (data not shown), were selected from the genomics database for variant calling of the *CYP19A1* gene. Table 3.3 shows seven *CYP19A1* SNPs identified in these patients and one control individual. The synonymous SNP rs700518 was found to be in linkage disequilibrium with other common SNPs (rs1065778, rs10046, rs4324076, rs1143704, rs17601241, rs2289105) with a minor allele frequency greater than 10%. *CYP19A1* rs17601241 with a minor allele frequency of 0.08 was only identified in one individual. This limits potential clinical utility in the context of

pharmacogenetics, as opposed to rare high impact variants applicable to familial risk. All of these SNPs except for the synonymous rs700518, occur in non-coding regions of the *CYP19A1* gene. SNP rs10046 located in the 3'untranslated region (UTR) of the *CYP19A1* gene, known to be associated with post-transcriptional gene regulation, was identified as the most likely functional variant among the 7 SNPs detected by whole exome sequencing. Indeed, *in vitro* studies previously demonstrated that this SNP is associated with a high estrogen profile, which correlates with the amount of tumor aromatase mRNA levels (74). SNP rs10046, together with rs727479 and rs4646, furthermore covers 88% of haplotype diversity in Caucasians (75, 76). In our opinion, these findings identify rs10046 as the best candidate SNP for validation as an additional risk factor for bone loss in AI pharmacogenetic studies. Additional studies which take different clinical settings into account, is warranted in the high risk South African population, using genotype strategies that include both founder mutations, underlying familial risk, as well as pharmacogenetics influencing clinical outcome (31,77).

Location	dbSNP ID	dbSNP P ref	Minor Allele Frequency	Control	BC1	BC2	BC3	BC4	BC5
syn exon3	rs700518	C	0,3259 T	C	T	T	C/T	T	T
				412	1135	1065	17/16	54	44
intron 3	rs1065778	A	0,3259 C	C	T	T	C/T	T	T
				911	2349	2554	24/34	100	92
intron 5	rs4324076	A	0,3672 C	C	A	A	C/A	A	A
				310	724	826	3/10	30	24
intron 6	rs1143704	A	0,3662 A	A	T	T	A/T	T	T
				160	268	271	2/3	17	7
intron 7	rs17601241	G	0,0857 A	G	G/A	G	G	G	G
				388	395/476	877	16	34	26
intron 7	rs2289105	T	0,3718 C	C	T	T	C/T	T	T
				176	253	285	11/12	15	6
3' UTR exon 10	rs10046	G	0,3628 G	A	G	G	G/A	G	G
				670	1188	1176	21/29	79	49

Table 3.3 Next generation sequencing results of the *CYP19A1* gene in 5 breast cancer cases and a control individual.

BC-breast cancer sample number; dbSNP-database Single Nucleotide Polymorphism

Table 3.3 supports the findings in previous studies indicating that the functional SNP rs10046 is in linkage disequilibrium with the synonymous *CYP19A1* rs700518. The minor G allele of rs10046 assigned as the major allele in the standard human genome reference sequence (hg19) is the most common allele in some populations. Notably, the six-SNP (TTATTG) haplotype identified in all the vitamin D deficient breast cancer patients from three different population groups in South Africa, was not identified in a control individual (CCCACA). Figure 3.2 shows the alignment view

of next generation sequencing reads encompassing the 3'UTR SNP rs10046 in exon 10 of the *CYP19A1* gene, identified as the functional SNP most likely to be clinically useful for future studies in an extended patient sample. Whole exome sequencing could not detect the TTTAn polymorphism due to its position outside the coding region (intron 4) of the *CYP19A1* gene. Failure to observe the expected similar clinical association of *CYP19A1* rs700518 and rs10046 occurring in linkage disequilibrium, impedes clinical application of the BIG 1-98 randomized control trial results (71). The finding that rs10046 is associated with increased risk of bone AEs in patients on tamoxifen, not observed for patients assigned on an AI, is clinically divergent.

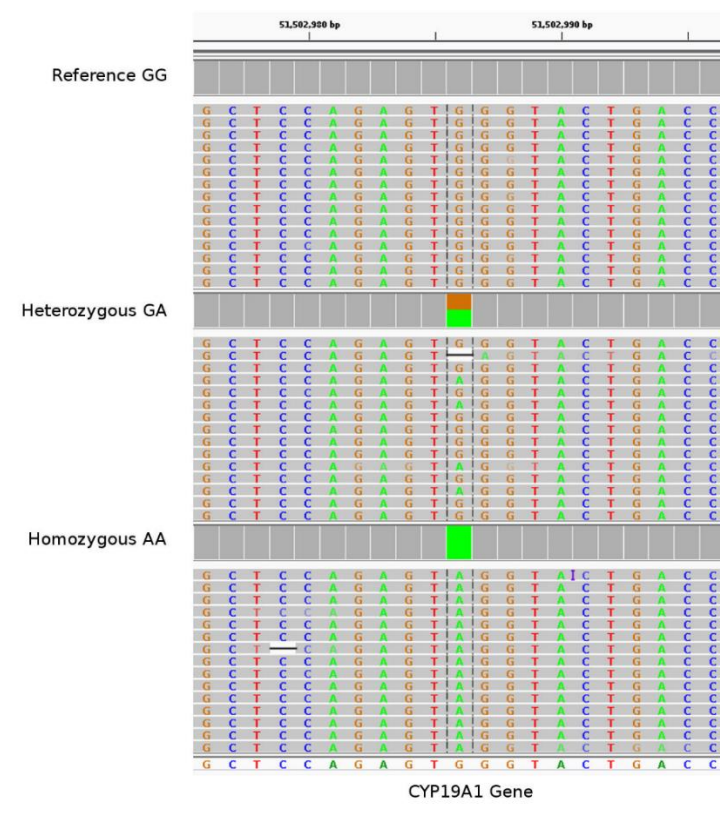


Figure 3.2: Alignment view of next generation sequencing reads encompassing the 3'UTR SNP rs10046 in exon 10 of the *CYP19A1* gene.

Conclusion

Application of breast cancer pharmacogenetics into the clinical scenario remains challenging as management recommendations cannot be based on genotype alone. It requires the definition of a target group most likely to benefit from translation of research into a clinical management pipeline, as outlined in figure 3. This pathology supported genetic testing approach facilitates inclusion of pharmacogenetics in the treatment algorithm (78), utilizing whole exome sequencing to identify patients with a genetic predisposition for AI adverse bone effects. Arguments around the implementation of *CYP2D6* genotyping at the onset of treatment with tamoxifen as part of the clinical work up and decision making are constantly developing (39, 66, 79). In South Africa, with an increased frequency of founder mutations in the *BRCA1* and *BRCA2* genes (30), *CYP2D6* genotyping has already been integrated into clinical practice for high risk patients on tamoxifen (31). The clinical value of incorporating AI pharmacogenetics as an additional risk factor for bone adverse events in post-menopausal breast cancer patients on endocrine therapy, at highest risk for further bone loss on long term AI therapy, merits further investigation.

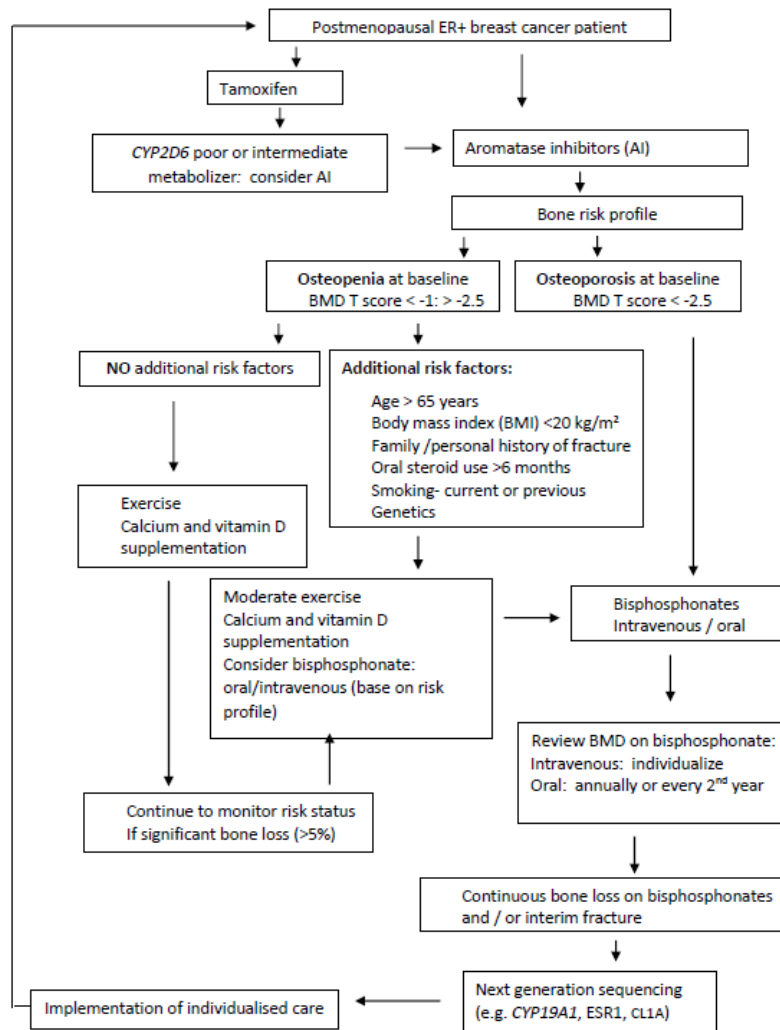


Figure 3.3: Clinical pipeline for identification of genetically predisposed postmenopausal estrogen receptor positive (ER+) breast cancer patients on aromatase inhibitors with severe bone events despite optimal treatment.

Conflict of Interest

Prof. M. J. Kotze is a director and shareholder of Gknowmix (Pty) Ltd. that has developed a database tool for research translation under the auspices of the Innovation Centre of the South African Medical Research Council (MRC).

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CHAPTER 4

Utilizing a clinically enriched breast cancer genomics database resource for development of adaptable patient reports, incorporating whole exome sequencing.

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Abstract

Background

Incorporating fragmented clinical and genomic data of individual patients into medical management is a major challenge. This study describes the conversion of patient information collated at the interface between the research laboratory and clinical practice into a comprehensive report for real-time service delivery.

Methods

Data from postmenopausal breast cancer patients treated with aromatase inhibitors (AIs) were entered into a central genomics resource, developed in an ongoing manner. A pathology-supported genetic testing algorithm was used to select patients for whole exome sequencing (WES). Results relevant to breast cancer diagnosis, comorbidities and treatment response were integrated into an adaptable report format.

Results

The reports generated for 101 patients at the start of AIs revealed a case with an unanticipated *BRCA2* c.3881T>A (L1294*) mutation in the presence of *MTHFR* rs1801133 and rs1801131 considered relevant to osteoporosis diagnosed at baseline. The report is presented in a summarized format to serve as an example of recommendations that can be provided for genetic counselling and clinical monitoring.

Conclusion

The database resource enabled integration of genomic research findings for responsive health care delivery, an approach rarely applied in routine clinical management. The ethical framework

developed for this purpose provides a sound basis for clinical intervention during and beyond the course of a single research project.

Introduction

In today's data-centered medical world, patient management and research are heavily influenced by the availability of accurate patient information in a secure digital format. Databases developed for a specific disease usually contain a summary of associated comorbidities and co-prescribed medication, but may lack information on the genetic background of study participants (1-3). Development of a clinically enriched genomics database necessitates an in-depth understanding of the effect of individual and population genomic variation on health outcomes, disease and drug predisposition (4, 5). Literature curation for application of personalized medicine is a time-consuming process that may best be achieved as part of translational research projects (4).

In an effort to keep up with new discoveries on the relationship between disease, genetic variation and environmental triggers, we developed a pathology-supported genetic testing (PSGT) service linked to the establishment of a genomics database for research translation across diagnostic boundaries (5). Extension of PSGT to whole exome sequencing (WES) facilitated the identification and clinical interpretation of genetic risk factors of relevance to both cancer development and tailored therapeutic intervention in a single test (6). Lifestyle factors acting in combination with modifier genes or low-penetrance mutations are evaluated as part of a chronic disease screen, routinely applied as part of the PSGT algorithm before commencing WES (7). The creation of a clinically enriched patient registry allows for generation of medically meaningful

dynamic patient reports for real-time intervention during and beyond the duration of a single research project.

The need to ensure long-term sustainability of database resources was highlighted by our first attempt at utilizing banked information from breast cancer patients at Tygerberg hospital. Van der Merwe et al.(8) integrated information of a 48-year old patient with a pathogenic *BRCA2* mutation and the cytochrome P450 D6 (*CYP2D6*) poor metabolizer status into an informative report, provided to the treating physician. It led to a change of treatment from tamoxifen to an aromatase inhibitor (AI) in this case. When clinical outcome studies of the entire cohort were pursued, only a small proportion of patient information was available due to poor record-keeping (9). This hampered research progress and delayed clinical implementation dependent on defining a high-risk patient group most likely to benefit from pharmacogenetics (4).

Genomic data generated through research is rarely used in routine clinical decision-making. In response to the challenges encountered with translation of database information into clinical interpretation and management, three aspects relating to familial risk, treatment side effects and comorbidities were identified for simultaneous appraisal in South African breast cancer patients (7). Research data relating to all three aspects are deemed important to inform the development of dynamic WES reports as a clinical intervention tool for personalized treatment and monitoring of high-risk breast cancer patients. In this article, a representative case is used as an example to reflect our own experience of translating genomic research findings into clinical practice.

Materials and Methods

Ethics approval was obtained from the Stellenbosch University Health Research Ethics Committee to develop a secure online research database, in parallel with patient recruitment for a bone health

study at Tygerberg Hospital (project reference number S13/05/103). Study participants included postmenopausal women between the ages of 50-80 years with newly-diagnosed, hormone-sensitive breast cancer, due to start endocrine therapy with AIs. All participants signed informed consent for WES and were given the choice for sample storage in a biobank linked to this study. Patients were also given the option to receive feedback on lifestyle assessment and special investigations performed. These included a chronic disease screen incorporated as part of the PSGT algorithm for WES, as previously described by van der Merwe et al. (7). The questionnaire initially developed for this purpose was used to document the family history, personal medical conditions, medication use/side effects and lifestyle factors relevant to the genes tested, after minor modification.

Anthropometric dimensions were measured, and dual-energy X-ray absorptiometry used to quantify bone mineral density. Biochemistry testing included determination of calcium, phosphate, parathyroid hormone and 25 hydroxy-vitamin D levels as well as bone-specific alkaline phosphatase and C terminal telopeptides status. Tumor histopathology was recorded and immunohistochemistry of estrogen receptor (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) status documented. Results of *BRCA1/2* mutation screening performed as part of routine clinical practice at Tygerberg Hospital (10), were also documented in the REDcap (Research Electronic Data capture) research database (11). It is a secure, web-based application used to store and update project-specific clinical and translational research data as shown in table 4.1.

Schedule of assessments	Baseline	Follow-up
Written informed consent	Yes	n/a
Questionnaire administration	Yes	n/a
Medical and surgical history from hospital records	Yes	n/a
Biochemistry testing	Yes	No
Bone mineral density assessment	Yes	Yes
DNA extraction	Yes	n/a
Sample storage for whole exome sequencing	Yes	n/a
Data capture and input	Yes	Yes

Table 4.1: Information entered into the databases

Figure 4.1 illustrates the adaptation of the clinical pipeline previously described by Baatjes et al. (4), used to select patients for next-generation sequencing. Whole exome sequencing (WES) was performed according to van der Merwe et al (9) using the Ion Proton apparatus. The sequencing data was generated and stored at the Central Analytical Facility, Stellenbosch University. The Ion AmpliSeq™ Exome RDY kit was used for library construction and samples were sequenced with the One Touch workflow, followed by variant calling against the major allele reference sequence (12) to screen for deleterious variants in genes relating to bone health pathways (13)(14). Mutations considered to be pathogenic according to ClinVar (15), were confirmed by Sanger sequencing and added into the research database. It is acknowledged that the several variants previously associated with osteoporosis are located outside of the genomic regions covered by WES (15, 16).

The information obtained from routine diagnostic workup of patients at Tygerberg Hospital and entered into the database at baseline were uploaded for integration with genetic research data. Information relevant to breast cancer diagnosis, comorbidities and treatment response were

extracted from the database (<https://www.gknowmix.org>) and compiled into an adaptable report format for clinical application by the treating clinician and supporting genetic counsellor.

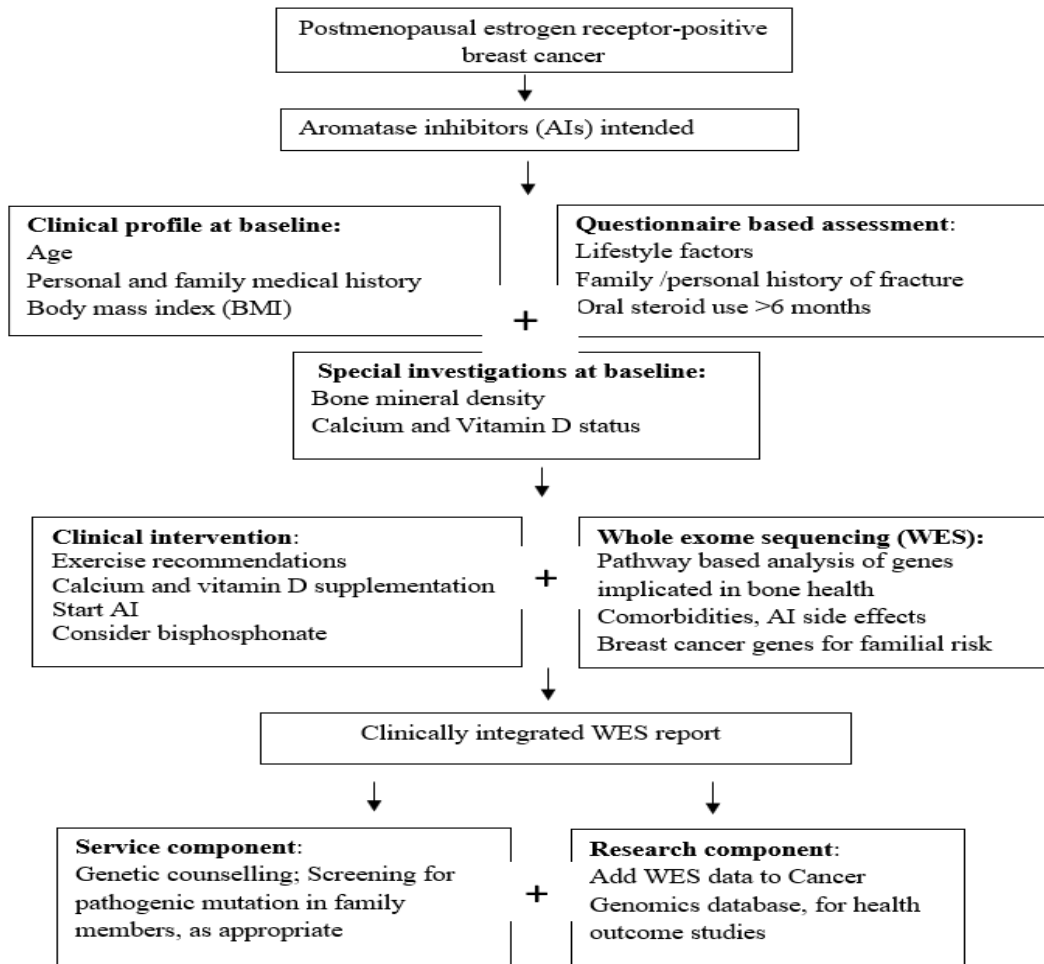


Figure 4.1: Incorporating research generated WES findings in clinical management within an adaptable report, generated from the genomics database.

Results

The clinical, pathological, biochemical and genetic characteristics of ER-positive postmenopausal breast cancer patients at the start of AIs, are captured on an ongoing basis. Figure 4.2 illustrates study enrollment and attrition of the cohort. Routine testing for familial breast cancer risk

previously identified a founder mutation (*BRCA2* c.6449_6450insTA) in one patient included in the database, as well as a variant of uncertain clinical significance in another (data not shown).

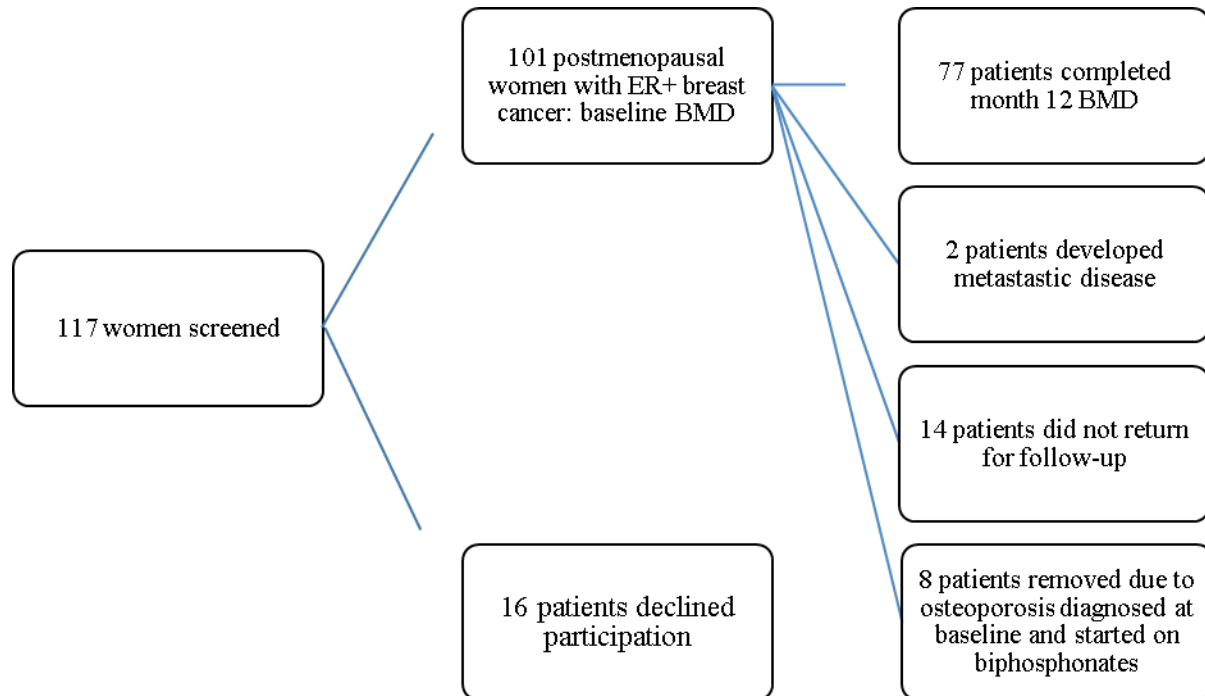


Figure 4.2: Study enrolment and attrition of the breast cancer cohort evaluated by dual-energy X-ray absorptiometry to quantify bone mineral density (BMD) at baseline and 12 months after initiation of AI treatment.

WES preceded by PSGT identified the *BRCA2* c.3881T>A (p.L1294*; rs80358632) mutation in a patient found to be a compound heterozygote for *MTHFR* rs1801133 and rs1801131. The collective information of this patient was extracted from the database and assimilated into a clinical management report as an illustrative example. Table 4.2 shows the patient data focused on identification of 1) pathogenic mutations associated with familial risk, 2) genetic underpinnings of biochemical abnormalities and comorbidities influenced by modifiable risk factors, and 3) increased risk of medication side effects/failure possibly due to genetic variation.

CASE	PRE-WES EVALUATION	PROPOSED ACTION
1.DIAGNOSIS	<p>Age at breast cancer diagnosis (postmenopausal)</p> <ul style="list-style-type: none"> • 68 years <p>Tumor histology</p> <ul style="list-style-type: none"> • Invasive lobular carcinoma 	<p>Genetic counselling</p> <ul style="list-style-type: none"> • Pathogenic <i>BRCA2</i> c.3881T>A mutation identified in germline DNA
Familial risk	<p>Family members diagnosed >50 years (late-onset)</p> <ul style="list-style-type: none"> • Breast cancer, Sister; Pancreatic and Lung, Sister; Prostate, Brother; Stomach, Mother; Lung, Father <p>Family members diagnosed <50 years (early-onset)</p> <ul style="list-style-type: none"> • None reported 	<p>Eligible for cascade testing of <i>BRCA2</i> c.3881T>A</p> <ul style="list-style-type: none"> • Test family members previously diagnosed with cancer • Test unaffected relatives on maternal or paternal side of the family, depending on whether the pathogenic mutation is detected in the mother or father of the index patient
2.PATHOLOGY	<p>Immunohistochemistry</p> <ul style="list-style-type: none"> • Estrogen receptor-positive, progesterone receptor-positive, human epidermal growth factor receptor 2-negative (luminal-type) <p>Biochemistry</p> <ul style="list-style-type: none"> • Vitamin D 17.4 ng/ml (deficient) <p>Bone mineral density</p>	<p>Pathway analysis</p> <ul style="list-style-type: none"> • Monitor homocysteine levels as a marker of folate status due to genetic variation detected in the <i>MTHFR</i> gene, implicated in both cancer risk and osteoporosis <p>Optimize bone health</p> <ul style="list-style-type: none"> • Increase vitamin D levels >30 ng/ml • Consider calcium, vitamin D

	<ul style="list-style-type: none"> • T score -3 (osteoporosis) 	supplementation and Bisphosphonates
Lifestyle risk	Body mass index: 28.3 kg/m2 (overweight)	Weight management
3. TREATMENT	<p>Current</p> <ul style="list-style-type: none"> • Hypertension and type II diabetes <p>Completed</p> <ul style="list-style-type: none"> • Chemotherapy <p>To commence</p> <ul style="list-style-type: none"> • Aromatase inhibitor (↑risk of bone loss) 	<p>Implementation of clinical management pipeline (Figure 4.1)</p> <ul style="list-style-type: none"> • Extended WES data analysis if considered clinically important by the referring clinician • Research: Extended pharmacogenetic analysis as appropriate
Side effects	None reported at baseline	Monitoring

Table 4.2. Patient data integration for risk management in a representative case report using a 3-pronged approach to whole exome sequencing (WES), which enables simultaneous assessment of high-moderate risk genes and low-penetrance mutations in key disease pathways for consideration of familial or personal risk reduction intervention.

Discussion

This study describes the development of an adaptable patient report from integrated database resources. The availability of genome-scale sequencing allows integration of genetic knowledge with disease information, comorbidities and medication side effects across health disciplines (19, 20). The PSGT approach applied in this study permits actionable interventions on both a personal and family level, which requires careful consideration of established clinical guidelines applicable to high- moderate risk genes and low-penetrance mutations in key disease pathways. Given the lack of reporting guidelines for WES used in multifaceted conditions such as breast cancer (20), we developed an integrated clinical and genetic pipeline, to permit continued monitoring of outcome and medication side effects (4). WES using a three-pronged approach as presented here, is based

on at least one of three indications for testing, namely 1) cancer in the family that cannot be explained by previous genetic evaluation, 2) abnormal pathology test results known to be associated with gene-environment interaction and 3) treatment failure or toxicity associated with prescription medication.

These benefits of genetic testing were demonstrated in the representative case. Co-existence of breast cancer and osteoporosis could partly be explained by detection of genetic variation in the *MTHFR* gene, previously identified as a *BRCA1/2* modifier and risk factor for many chronic, non-communicable diseases with a genetic component (7). *MTHFR* genotyping forms part of the PSGT algorithm applied by van der Merwe et al. (7) to determine eligibility for WES.

The pathogenic *BRCA2* c.3881T>A (p. L1294*; rs80358632) mutation detected by WES creates a premature stop codon in the ovarian cancer cluster region in exon 11. Mutations in this gene region were previously identified in families with multiple types of cancer among first-degree relatives of *BRCA2* mutation carriers (21). The finding was consistent with the breast, prostate, pancreas and stomach cancers diagnosed at an advanced age, in 5 first-degree relatives of the patient studied. This familial risk profile did not fulfil the standard *BRCA1/2* testing criteria of the institute at the time of breast cancer diagnosis, nor meet the minimum of 15 points signifying a 10% likelihood of detecting a pathogenic mutation according to the updated Manchester scoring system (22). Although the *BRCA2* mutation was detected at a relatively advanced age in the patient, the potential value to the extended family would not have come under the attention for genetic counselling, without WES. This unanticipated genetic finding was communicated to the genetic counsellor for return of research results, to the individual patient (23).

While this study presented a single example as proof of concept for the value of WES preceded by PSGT, the expansion of the onco-genomic research database within a real-life clinical setting allows for continuation of future comparative effectiveness studies centred on an ethical framework. WES using a three-pronged approach as presented here, requires careful consideration of multiple factors, including clinician education and policy development towards future adoption of personalized medicine (7, 18).

Conclusion

Adaptable reports generated from the genomics research database embedded within routine health care delivery, augment personalized patient care and supplement the clinician and counsellor's decision-making process.

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Conflict of Interest

Prof. MJ Kotze is a director and shareholder of Gknowmix (Pty) Ltd.

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CHAPTER 5

Baseline bone health status in multi-ethnic South African postmenopausal breast cancer patients at initiation of aromatase inhibitor therapy: A descriptive study.

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Abstract

Purpose

Osteoporosis (OP) risk factor assessment and bone mineral density (BMD) testing are frequently omitted at baseline in AI studies, which may lead to misinterpretation of AI associated bone loss. The present study describes bone health of South African postmenopausal women of predominantly mixed ancestry, prior to AI treatment.

Methods

This descriptive baseline study, nested in a prospective AI intervention study, included postmenopausal women with endocrine sensitive breast cancer, aged 50 to 80 years. A baseline questionnaire documented demographic-, medical-, lifestyle- and fracture history. Body weight was assessed clinically, and body composition and BMD measured via dual energy absorptiometry. Descriptive statistics were used to summarize the data (STATA 14).

Results

101 participants were recruited, with a mean age of 61 ± 7 years. Near one-third ($n=32$) of women at baseline fulfilled global criteria for bone protection (BMD T-score $\geq -2SD$ ($n=18$); BMD T-score $-1.5SD$ to $< -2SD$ with risk factors ($n=14$). Lower body weight, body mass index (BMI), fat mass index and lean mass index was documented in women with OP ($p < 0.001$). Low vitamin D was present in 93% of the cohort tested ($n=95$), whilst deficient vitamin D status ($< 20\text{ng/ml}$) was documented in 52 women (55%).

Conclusions

In this study, a third of postmenopausal women considered for AI therapy fulfilled international criteria for bone protective pharmacological intervention. This emphasizes the need for baseline clinical risk and BMD assessment in postmenopausal breast cancer patients considered for AIs. Body composition and bone health associations highlight bone fragility associated with lower body weight.

Keywords

breast cancer; osteoporosis; aromatase inhibitors; body composition

Introduction

Postmenopausal women have a significantly increased risk of developing osteoporosis, which relates to physiological changes in the ageing female body. Osteoporosis (OP) is a potentially debilitating condition with high morbidity in elderly populations especially women due to the increased risk of fracture, especially of the spine and hip (1). Osteoporotic fractures can cause severe morbidity with impairment of function and quality of life (2).

Bone mineral density (BMD) measured by dual energy X-ray absorptiometry (DXA) is a two-dimensional measure of mineral content in specific skeletal regions (3). It is useful for evaluation of BMD changes over time and to assess response to therapeutic interventions (4, 5). The risk of fracture increases two- to three-fold with every standard deviation (SD) decline in BMD (1,6).

Endocrine treatment is indicated for estrogen receptor (ER) positive breast cancers. Tamoxifen, a selective estrogen receptor modulator has been used in the treatment of endocrine responsive breast

cancer for several decades (7). It protects against accelerated postmenopausal bone loss, as it maintains selective estrogenic effects on skeletal tissue (8). Currently, aromatase inhibitors (AIs) are the gold standard in the treatment of endocrine sensitive breast cancer with an improved clinical outcome compared to Tamoxifen (9, 10).

Estrogen is integral in bone metabolism with a multidimensional role in the pathogenesis of postmenopausal OP (11). In postmenopausal breast cancer patients, AIs further decrease the already low circulating and tissue levels of estrogen by inhibition of the aromatase enzyme (12, 13). Numerous studies have documented accelerated bone loss and heightened fracture risk in postmenopausal women on AI therapy. The most pronounced loss is noted in the first two years of AI treatment and in early menopause (< 4 years). AI therapy predominantly affects the axial skeleton (7, 9).

Many conventional risk factors for the development of OP have been identified in the general population (14). Age, sex, genetic predisposition and ethnic origin represent the most important non-modifiable risk factors. Modifiable factors such as low body weight, a sedentary lifestyle, poor calcium nutrition and deficient vitamin D levels, smoking and alcohol excess, may also significantly impact on bone density. Body weight is one of the most important determinants of BMD at most skeletal sites in women of all ethnicities (14, 15).

In randomized controlled trials of AIs, the baseline state of BMD was not always reported. The conventional risk factors for fracture were not quantified and the prevalence of osteoporotic fractures (an important risk factor for development of incident fractures) prior to AI therapy remains unknown (16). The presence of conventional risk factors for osteoporosis and baseline

BMD must be considered to accurately calculate the excessive fracture risk attributable to AI therapy per se.

The present study describes the baseline bone health status, prior to initiation of Aromatase Inhibitor therapy, of a multi-ethnic postmenopausal women cohort with endocrine responsive breast cancer, resident in the Western Cape Province of South Africa. This study is nested within a cohort study aiming to prospectively examine the impact of AIs on bone health. The associations between BMD and body composition as well as certain lifestyle factors, known to potentially adversely affect bone mineral status, will be examined.

Methods

Study Population

This descriptive study, nested within a larger prospective cohort study was conducted at the tertiary breast clinic of Tygerberg Hospital, affiliated to the University of Stellenbosch. Postmenopausal women with newly diagnosed, histologically confirmed endocrine sensitive breast cancer, stage 0-III were eligible for study entry. All women were between 50 to 80 years of age and were consecutively enrolled from August 2014 until February 2017. Race determination was made by self-declaration. Patients were excluded if they had known metabolic bone disease, if they suffered from any disease (other than breast cancer), or were taking medication known to adversely affect BMD. The research complied with the World Medical Association Declaration of Helsinki (ethical principles for medical research involving human subjects). The study was approved by the Ethical Review Board of the Faculty of Medicine, University of Stellenbosch (S13/05/103).

Demographics

A demographic questionnaire was administered at baseline and included questions related to age, family medical history, personal health, lifestyle, reproduction and falls and fractures. Lifestyle questions included the use of alcohol (abstain, 1-7 units per week or >7 units per week), smoking (ever, current or never) and activity level (nil, in-house only or in-house and outdoors). The use of progesterone-only hormonal contraception and years since menopause (YSM) were documented. A history of prior fragility fractures, fall propensity indicated by falls in the last year and prolonged immobilization (>1 month) (14) was obtained. The medical, pharmacological and surgical history, as well as pathological information of the tumor, were collected at baseline.

Anthropometry

Basic anthropometric measurements including weight (in light clothing without shoes), height, waist (at level of umbilicus) and hip circumference (largest gluteal area), were taken. Body mass index (BMI) values were divided into weight categories (low/normal, overweight, obese and morbidly obese) according to the World Health Organisation (WHO) classification (17).

Densitometry

The DXA Hologic Discovery-W, S/N 70215; software Version 13.1 was employed in this study to measure BMD and body composition. A spine phantom was scanned daily to determine the intrinsic coefficient of variation of the machine. During the course of the study, coefficients of variation for BMD were < 1.5%. A single trained DXA technician (MM Conradie) performed scans on all study subjects and the intra-operative variation was found to be below 1% for all skeletal sites.

Body composition

Whole body DXA was used to measure and calculate total fat percentage, fat mass index (FMI), lean mass index (LMI), appendicular skeletal muscle mass as well as an android/gynoid fat ratio. Fat mass and lean mass measured by DXA are normalized for height (just like BMI) to calculate a fat mass index (fat/height²), a measure of obesity and a lean mass/height² as an index of total body muscle mass. The appendicular skeletal muscle mass normalised for height², is a good surrogate marker of sarcopenia, if found to be low. No local normative data for DXA measured body composition exist. The National Health and Nutrition Examination Survey (NHANES) reference dataset was thus used, which allowed comparison of the measured indices of body composition of our study subjects to the NHANES normal young adult female population aged 18-25 years with a BMI within the normal WHO range (17). In the present NHANES database, fat comprises approximately 38% of body weight in females at age 25 years and this value will be regarded as the normal reference value for our study. The fat mass index was used to categorize study participants into weight classes similar to those mentioned for BMI (low/normal fat, excess fat, obese and morbidly obese). The normal fat mass index range is 5-9 kg/m², excess fat 9.1-13 kg/m², obese 13.1-21 kg/m² and morbid obesity indicated by a FMI in excess of 21 kg/m². LMI and appendicular lean mass/height² was categorized as being 2SD below or above expected with the cut-off values being 12.5 kg/m² and 4.36 kg/m², respectively.

Bone Mineral Density

Femoral neck (FN), total hip (TH) and lumbar spine (LS) BMD was measured. No normative data for South African women of mixed or black race exist. In this study, we therefore used a white female reference population to calculate T-scores and to define osteopenia and osteoporosis subgroups for all ethnicities. The use of white women as a reference for all persons in a multi-

ethnic study may well not be appropriate, but until these ethnic specific reference ranges become available in our country, it is recommended to diagnose osteoporosis in all women by using the uniform normative database for whites. A lateral vertebral assessment was done to detect prevalent morphometric vertebral fractures.

Biochemistry

Early morning blood samples were drawn for the evaluation of calcium homeostasis (serum calcium, phosphate, parathyroid hormone (PTH) and 25-OH Vitamin D levels) and to determine biochemical bone turnover markers (serum bone specific alkaline phosphatase and C-terminal telopeptides: Beta-CrossLaps/serum assay). Commercially available assays were used according to the manufacturer's protocol.

Statistical analyses

Data management and analysis were conducted in STATA 14. Descriptive statistics were used to summarize the data including baseline characteristics and outcomes. Continuous data were tested for normality using descriptive statistics (e.g. histograms) where normally distributed data were presented as means and standard deviations or as medians and interquartile ranges (IQR), for non-normally distributed data. Categorical data were presented as proportions and 95% confidence intervals. The associations between biological parameters and BMD was determined using one-way ANOVA and χ^2 tests. To account for confounding, significant univariate predictors were included in a final multinomial logistic regression model at $p < 0,2$. An alpha of 0.05 was considered statistically significant. Associations were reported as relative risks with 95% confidence intervals. Missing data was assumed to be missing at random and no inputting performed.

Results

Clinical demographic characteristics

From August 2014 until February 2017, 101 postmenopausal participants were recruited with a mean age of 61 ± 7 years. Near half ($n=48$, 48%) of women were in the 50-59 year age group, with only a minority of the study cohort 70 years of age and older (~10%). Eighty-two percent of the study population were of mixed ancestry, in accordance with the hospital's reference population. White women represented 13.4% of the total cohort and two black and three Indian women were included. Demographics and lifestyle data are presented in Table 5.1.

Clinical characteristics (n=101)	
Age (years)	61 ± 7
• 50 – 59 yrs	48 (48)
• 60 – 69 yrs	43 (48)
• 70 yrs ⁺	10 (10)
Smoking	
• ever	42 (42)
• current	28 (28)
Alcohol	
• abstain	79 (79)
• 1-7 units per week	22 (22)
• >7 units per week	0
Activity level	
• In-house	20 (20)
• In-house and Outdoors	81 (80)
Falls in last year	
• Any fall	0
Clinical fractures	
• non-vertebral	7 (7)
Family history of OP	
• positive	1 (1)
Age at menopause (years)	48 ± 5 years
Duration of menopause (n=72)	12 ± 8 years

• 0 - 4 yrs	15 (21)
• 5 - 10 yrs	15 (21)
• > 10 yrs	42 (58)
Hot Flashes	
• ever	64 (63)
Hormonal contraception (ever)	
• Depot Provera	19 (19)
• OCP	30 (30)

Table 5.1. Summary of lifestyle and menstrual data of breast cancer patients at baseline

Values for age, age at menopause and duration of menopause expressed as mean \pm SD, rest of data expressed as n (%). Cohort n = 101 for all clinical characteristics tabulated unless otherwise specified. OCP = oral estrogen containing contraceptive preparation

Near half of the study population (42%) smoked at some stage in their lives and 28% of women reported to be current smokers. Alcohol consumption was minimal with 79% abstaining from any alcohol use and no intake in excess of 7 units per week reported. Most women lead a moderately active lifestyle with out-of-house activities documented in 81%. No falls in the last year were reported amongst this relatively young cohort of postmenopausal women. Low trauma non-vertebral fractures were documented in seven women. Only one woman reported a family history of osteoporosis.

Menopause occurred at a mean age of 48 ± 5 years, within the expected normal range (45 yrs and older) in the vast majority. Eight women experienced an early menopause, of which six became menopausal between 40–45 years of age. The duration of menopause (n=73) was short (less or equal to 5 years) in 23% of women. Hot flashes were reported by 64% of women at some stage during their menopause. Hormonal contraception was used by 49% of the cohort during their reproductive years, of these 19% used an injectable progesterone containing preparation only.

Mineral homeostasis, calcitropic hormones and biochemical bone turnover markers

Normal vitamin D status was only documented in 7% of the cohort. Insufficient vitamin D levels (20-30ng/ml) were present in 38% (n=36) of the cohort, whilst deficient vitamin D status (<20ng/ml) was documented in 55% (n=52). Bone disease caused by vitamin D deficiency is usually associated with values below 10-12ng/ml. This severe degree of deficiency was only evident in two participants, interestingly both these women had a normal BMD. Despite the almost universal 25-OH-Vitamin D deficiency, only twenty-four subjects (25%) had secondary hyperparathyroidism, a marker of poor vitamin D nutrition and/or a negative calcium balance.

Bone specific alkaline phosphatase and β -cross laps, biochemical parameters of bone turnover, were normal (95% and 97% respectively) in most women, indicative of normal bone turnover at baseline in this cohort of postmenopausal women.

Body composition

Clinical and densitometric parameters of body composition are tabulated in Table 5.2. A concerning 85% of our cohort were overweight, with 59% falling into the obese categories of WHO-BMI (17). A waist/hip ratio in excess of 0.85, indicating excess metabolic risk, was present in the majority (79%). Densitometric assessment of body composition was in accordance with our clinical assessment. The mean total body fat mass ($14.6 \pm 6\%$) and the FMI ($14.4 \pm 4.8\text{kg/m}^2$) were significantly above normal. A high FMI was documented in 89% and a FMI indicative of obesity was present in 61% of the cohort.

Clinical parameters (n = 101)	Category values	Measurement
Weight (kg)		81.2 ± 19.4
Height (cm)		158.6 ± 6.0
BMI (kg/cm ²)		32.4 ± 7.8
BMI weight categories		
• Low/normal body weight	≤ 25 kg/m ²	15 (15)
• Overweight	25.1–29.9 kg/m ²	26 (26)
• Obesity	30–39.9 kg/m ²	42 (41)
• Morbid obesity	≥40 kg/m ²	18 (18)
Waist circumference (cm)		102.1 ± 15.8
Waist/Hip circumference (cm)		0.9 ± 0.1
• > 0.85*		79 (78)
Densitometric parameters (n = 101)	Category values	Measurement
Mean Total Body Fat Mass (%)		45 ± 6
• Normal	≤ 38%	6 (6)
• Increased	> 38%	95 (94)
Mean Fat Mass Index (FMI) (kg/m²)		14.6 ± 4.9
• Normal	5 - 9	11 (11)
• Excess fat	9.1 - 13	30 (30)
• Obese	13.1 - 21	53 (52)
• Morbid obesity	>21	7 (7)
Mean Lean Mass Index (LMI) (kg/m²)		16.7 ± 2.8
• Above third centile (>2SD)	≥ 12.5	98 (97)
• Normal range	< 12.5	3 (3)
Mean Appendicular Lean mass/height² (kg/cm²)		7.2 ± 5.5
• Above third centile (>2SD)	≥ 4.36	99 (98)
• Normal range	< 4.36	2 (2)
Mean Android/Gynoid ratio		1.0 ± 0.1
• Gynoid dominant fat distribution	≤ 1	44 (44)
• Android dominant fat distribution (visceral)	> 1	57 (56)

Table 5.2. Body composition in postmenopausal breast cancer patients at baseline

Body composition evaluated clinically and with DXA. Mean values presented as means ± standard deviation unless otherwise specified. Cohort sub-classified into WHO weight categories based on BMI and

percentage of cohort with waist/hip circumference indicative of metabolic syndrome (17)(18) noted. Cohort also sub-classified into Fat Mass Index classification ranges in accordance with BMI weight categories. LMI and appendicular lean mass/height² divided into categories below and above the third centile (2SD) for specific measurement in young NHANES females. All densitometric measured categories defined based on NHANES data base for young normal females within normal BMI range (17) Data expressed as n(%) of subjects within all weight categories*

Lean mass appeared well maintained with a significantly lowered appendicular lean mass (< 12.5 kg/m²) indicative of sarcopenia i.e. loss of muscle strength, only documented in 2 subjects. It is noteworthy that both these subjects had osteoporotic range BMD, which infers a significant fracture risk based on low BMD and excess fall risk due to sarcopenia.

The clinically determined BMI and the densitometric FMI were remarkably similar in their classification of women within the different weight categories. In the BMI determined normal to low body weight category, three of the women had a FMI marginally in excess of 9 (10.3 kg/m² in two subjects and 10.5 kg/m² in the third subject) and only four of the women in the obese category, had a FMI below 13.1 kg/m² (1.7%).

Bone mineral density

Baseline BMD was assessed at the lumbar spine in all participants (n=101) and in all but one study participant at the femoral neck and total hip region (bilateral hip replacement in one participant) (Table 5.3). BMD is expressed as an absolute density in g/cm² and the deviation from expected peak value for the specific individual i.e. as a T-score to determine the patient's specific BMD category as either within the normal, osteopenic or osteoporotic range (19). The mean BMD at all the measured sites for the total cohort was within the normal range (T-scores less -1 SD below expected peak).

BMD SUBCATEGORIES	Lumbar Spine BMD (n= 101)	Femoral neck BMD (n=100)	Total hip BMD (n=100)
Absolute value g/cm ²	0.982 ± 0.171	0.780 ± 0.119	0.913 ± 0.142
T-score	-0.5 ± 1.6	-0.65 ± 1.1	-0.2 ± 1.1
• Normal	58 (58)	61 (60)	75 (75)
• Osteopenia (< -1.0 > -2.5)	26 (26)	32 (33)	21 (21)
• Osteoporosis (≤ -2.5)	13 (13)	6 (6)	3 (3)
• High BMD (> 2.5)	3 (3)	1 (1)	1 (1)

Table 5.3. Bone Mineral Density in postmenopausal breast cancer patients at baseline

Absolute BMD values and T-scores at all the measured sites are presented as means ± standard deviation for the total study population. The cohort is then sub-classified into WHO-BMD categories (20). Number of patients and percentage of study population within subgroups for all measured sites is noted.

A concerning 50% of participants displayed osteopenia at one or more measured site i.e. BMD T-score deviations of -1 SD or more. Only two women with osteopenia, had a BMD in keeping with severe osteopenia i.e. a BMD T-score between -2.0 and -2.5 SD. BMD in keeping with osteoporosis was present in fourteen women (14%) prior to any hormonal intervention for breast cancer. Osteopenia and osteoporotic range BMD were most prevalent in the axial skeleton (all but one study subject with osteoporosis). In this group of relatively young postmenopausal women, the dominant loss of bone was at the lumbar spine region. This finding is not unexpected but noteworthy in a population expected to commence treatment with anti-estrogenic medication. A supernormal BMD (> +2.5 SD) was noted in three participants (3%).

A lateral vertebral assessment with DXA raised the suspicion of mild morphometric vertebral abnormalities in 15 women. Conventional radiology with a lateral lumbosacral X-ray, excluded significant vertebral compression ($\geq 20\%$ of vertebral height) in all of these women.

Clinical characteristics, body composition and biochemistry within BMD sub-categories (normal, osteopenia, osteoporosis)

Chronological age did not significantly differ amongst the BMD categories. Years since menopause (YSM) increased with worsening bone profile, with a mean duration of menopause 5 years longer in the osteoporotic BMD subgroup compared to the normal BMD subgroup. No significant association between YSM and the BMD subgroups was noted ($p = 0.14$). In all the patients with OP, in whom the duration of menopause was documented ($n=6$), YSM exceeded 5 years.

Body composition differed significantly amongst BMD subcategories, with significantly lower total body weight, BMI, FMI, total fat percentage and LMI documented in the women with OP ($p < 0.001$; Table 5.4). The waist/hip ratio was not significantly associated with BMD subcategories ($p=0.48$). The lack of association of BMD measurements with the waist/hip ratio may indicate difficulty to accurately determine this anthropometric parameter in a dominantly obese population.

Clinical characteristics, body composition and biochemistry	BMD subcategories			p-value
	Normal BMD (n=50)	Osteopenia (n=37)	Osteoporosis (n=14)	
<i>Clinical characteristics</i>				
Age (yrs)	59 ± 6	62 ± 7	64 ± 8	0.074
Years since menopause (yrs)	11 ± 9	14 ± 3	16 ± 6	0.14
<i>Body composition</i>				
Total body weight (kg)	86.6 ± 17.3	79.5 ± 19.2	63.8 ± 15.4	<0.001
BMI (kg/m ²)	34.3 ± 6.4	31.7 ± 8.4	25.8 ± 6.2	<0.001
Waist/Hip ratio	0.91 ± 0.06	0.88 ± 0.08	0.91 ± 0.14	0.48
Total body fat (%)	46.9 ± 5.0	44.9 ± 6.6	41.6 ± 5.9	0.144
FMI (kg/m ²)	15.0 ± 4.3	14.0 ± 4.9	10.5 ± 3.6	<0.001
LMI (kg/m ²)	17.6 ± 2.5	16.3 ± 2.9	14.2 ± 1.9	<0.001
Appendicular lean mass (kg/m ²)	8.1 ± 7.6	6.6 ± 1.1	5.5 ± 1.1	0.19
<i>Biochemistry</i>				
Vitamin D (ng/ml)	19.8 ± 5.8	18.9 ± 6.3	22.1 ± 8.4	0.290
• Total n	49	35	11	
• Sufficient [>30ng/ml] n (%)	3 (6%)	2 (5%)	2 (18%)	
• Insufficient [20-30ng/ml] n (%)	21 (43%)	12 (34%)	3 (27%)	
• Deficient: [< 20ng/ml] n (%)	25 (51%)	21 (60%)	6 (54%)	
PTH (pmol/L)	5.54 ± 2.87	5.10 ± 1.99	6.77 ± 4.36	0.221
• Total n	48	36	11	
• normal [1.6-6.9 pmol/L] n (%)	36 (75%)	29 (80%)	6 (54%)	
• elevated n (%)	12 (25%)	7 (20%)	5 (46%)	

Table 5.4. Body composition within DXA-BMD subcategories

All values expressed as means ± SD, unless otherwise specified. Yrs = years; BMI = body mass index; FMI = fat mass index; LMI = lean mass index; BMD = bone mineral density. BMD subcategories refers to DXA BMD T-score: normal = less than 1SD below norm, osteopenia = -1 to -2.49 SD below normal and osteoporosis = ≥-2.5 SD below norm. p-value significant at < 0.05 for continuous comparison normal BMD versus osteopenia and osteoporosis subgroups.

Fifty percent of women (7/14) with baseline OP had a low/normal BMI of ≤ 25 kg/m². The other half had either an overweight BMI (n=4) or were obese (n=3). No woman with morbid obesity

was osteoporotic. Although a low/normal BMI in our cohort indicates increased risk for OP, overweight and obese BMI did not exclude the potential of having OP.

The mean 25-OH-Vitamin D ($p = 0.290$) and PTH levels ($p = 0.221$) were similar in the BMD subcategories (Table 5.4). Twenty-one percent of women with secondary hyperparathyroidism had OP, slightly higher than documented for the entire cohort (14%). Near half (45%) of women with OP, however, had compensatory secondary hyperparathyroidism. When comparing BMD in ascending PTH tertiles, a significant adverse BMD effect could not be demonstrated ($p = 0.720$).

When looking at BMD subcategories at specific bone sites i.e. at the femur neck, total hip and lumbar spine, a similar trend was noted for clinical characteristics, body composition and biochemistry relationships compared to the composite BMD. BMD at all measured sites increased significantly with increasing BMI based on WHO subcategories as demonstrated in figure 5.1.

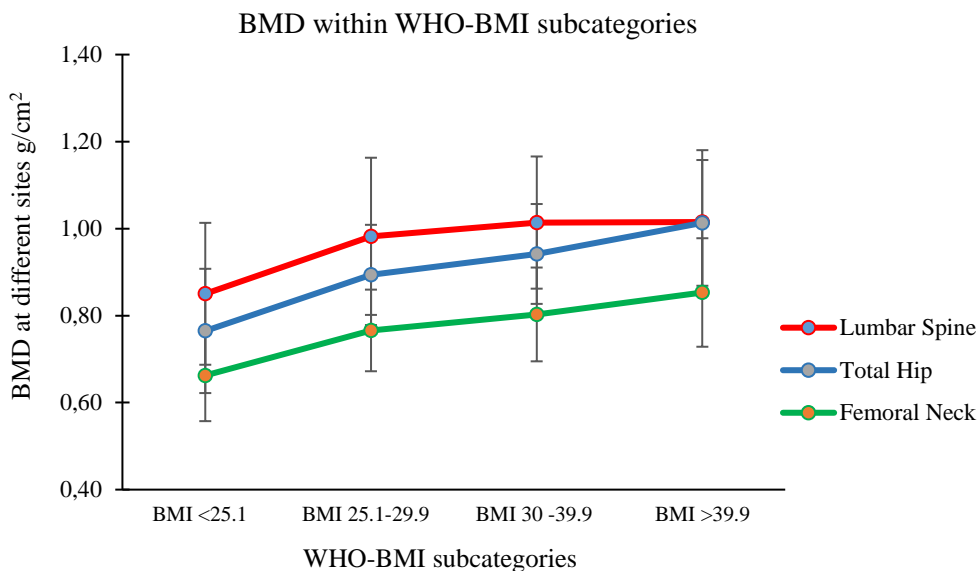


Figure 5.1. Bone mineral density within the WHO-BMI subcategories

Multinomial regression adjusting for known confounders indicated PTH (RR 1.61, 95% CI 1.15-1.25) and LMI (RR 0.3, 95% CI 0.11-0.85) were significantly associated for OP compared to normal bone status (Table 5.5).

Predictors of BMD	Crude RR		Adjusted RR	
	Osteopenia	Osteoporosis	Osteopenia	Osteoporosis
BMI	0.95 (0.83-1.01)	0.82 (0.77-0.92)	1.14 (0.90-1.44)	1.24 (0.80-1.93)
Fat mass index	0.91 (0.83-1.00)	0.74 (0.62-0.88)	0.87 (0.66-1.14)	0.73 (0.44-1.21)
Lean mass index	0.82 (0.69-0.98)	0.54 (0.35-0.75)	0.70 (0.47-1.03)	0.30* (0.11-0.85)
PTH	0.93 (0.79-1.10)	1.13 (0.92-1.38)	0.98 (0.81-1.17)	1.61* (1.15-2.25)

Table 5.5. Crude versus adjusted predictors (reporting relative risk) for BMD status at baseline. *Comparison of predictors of normal BMD. RR (95% CI), *p-value significant (adjusted only) at <0.05.*

Discussion

In this study of predominantly mixed ancestry postmenopausal women, a concerning 14% had osteoporosis at baseline and half of the cohort were osteopenic prior to any intervention. Body composition, especially lean mass, was significantly associated with bone mass at all measured sites and the risk of being osteoporotic significantly less with increasing lean mass index ($p < 0.0001$). All clinical and densitometric measures of body weight and composition were universally lowest in the women with OP ($p < 0.001$).

Only seven study subjects had sufficient 25-OH Vitamin D levels. Compensatory, secondary hyperparathyroidism was documented in more women with OP (45%) compared to the rest of the cohort (23%).

Osteoporosis is a critical public health issue, especially in ageing postmenopausal women. The addition of AIs in the endocrine treatment of breast cancer compounds the problem and could

adversely affect bone health. A Joint Position Statement by experts from the International Osteoporosis Foundation Bone and Cancer Working Group indicates that women commenced on adjuvant AI therapy for breast cancer, experience a two to four-fold increase in bone loss compared to the normal rate of bone loss with menopause (21). Clinical trials have shown an approximately 10% increase in absolute fracture risk for women on AI therapy (13, 19). The fracture incidence in women with breast cancer on AI therapy was reported to be around 18-20% after five years of follow-up (22). This indicates that about one in five women on AI's will sustain an AI related fracture. These fragility fractures result in morbidity with prolonged disability and may lead to a loss of independence and should be actively prevented.

It is thus essential to delineate BMD, body composition and clinical risk factors for bone loss and fracture at the start of treatment in these women. This will enable appropriate risk stratification and allow for appropriate preventative measures as indicated. Breast cancer treatment seeks not only to prolong survival, but also to limit side effects (13, 23).

BMD is a precise and reproducible measure of mineral content, determines up to 70% of bone strength and is viewed as the most robust indicator of fracture risk in untreated patients (3). Ethnic differences in bone mass and the risk of osteoporotic fracture have been described globally, but established data are particular to black and white populations (2, 24).

More than 80% of our study population were of mixed ancestry, a population subgroup in whom bone mineral density and the prevalence of fractures are largely unknown. In the only reported data from South Africa on BMD in women of mixed ancestry (25), BMD measurements at both the lumbar ($p=0.25$) and femoral regions ($p=0.52$) were similar to whites. BMD in SA black women are higher at the femoral regions, but similar or even lower at the lumbar spine, compared to white women (26-28). Extrapolated from densitometric studies, fracture risk is expected to be

similar in white women and those of mixed ancestry at all sites. Limited data suggest that South African black women may be protected from hip fractures, but is expected to have a vertebral fracture risk similar to the other ethnic groups (29, 30). No formal study looking at vertebral fracture prevalence in women of mixed ancestry in SA has been conducted to date and the prevalence of vertebral fractures in this ethnic group thus remains unknown. Such knowledge will facilitate prevention and management strategies for osteoporosis and consequent fragility fractures across all ethnic groups as well as in the post-menopausal woman treated for breast cancer (31).

The prevalence of low BMD in more than half of our study population at baseline is concerning. This argues for routine bone density measurements in all postmenopausal women of mixed ancestry presenting with breast cancer in whom AI therapy is considered. In the majority of our subjects, significant bone loss was confined to the axial skeleton. This is especially concerning for an increased risk to sustain vertebral fracture. Guidelines have proposed different cut-offs for intervention based on baseline assessment of bone health in women starting AI therapy for breast cancer. According to the most recent global consensus recommendation, all women with a BMD T-score ≤ -2 SD at any measured site, should be pharmacologically protected with bone-directed therapy (21). In addition, patients with a BMD T-score between -1.5 SD and -2 SD with added risk factors for bone loss, should also be considered for treatment. These risk factors include age above 65 years, smoking, a family history of hip fracture or a personal history of fragility as well as low body weight and a longer than 3-month course of glucocorticoid therapy. FRAX (fracture risk assessment score), an algorithm designed to provide long-term (10-year) fracture risk, is also used to determine the need for active intervention in women considered for AI therapy. This algorithm can, however, only be used in countries where the background prevalence of fragility fractures of the hip is known and thus at present not an option in our patient population.

Based on these recommendations, eighteen of the women in our cohort (18%) warranted bone specific therapy based on BMD criteria per se (BMD T-score \leq -2 SD). Seventeen of our participants had a BMD T-score \leq -1.5 SD, but $>$ -2 SD. The presence of one or more conventional risk factors for bone loss in fourteen of these women also dictated the need for active intervention at baseline (one risk factor, n = 6; two risk factors, n = 7; three risk factors, n =1). Near one-third (32%) of postmenopausal women at baseline in our study fulfilled global criteria for bone-specific intervention.

Obesity, based on WHO-BMI criteria, is associated with increased peak bone mineral density, with higher bone mineral density in postmenopausal women and with slower rates of bone loss at both the hip and spine. Low body weight, also specifically studied in breast cancer cohorts, represent an important risk factor for low bone mineral density and even osteoporosis (4). Eighty-five percent of our study cohort was overweight, and although associated with significant metabolic adversity, this may afford bone protection.

Fat mass has been shown to be positively associated with BMD due to increased mechanical loading and the release of osteogenic hormones from adipose tissue (32). In addition, after menopause, body fat becomes the main determinant of endogenous estrogen activity. The production of androgens is higher in obese than in normal weight women, and the excess body fat will increase adipocyte conversion of androgens to estrogen (33). In contrast, fat mass also produces inflammatory cytokines, which may negatively influence BMD tissue (34). Skeletal muscle mass or fat-free lean body mass has been consistently shown to be associated with increased BMD in all women due to the mechanical forces placed on bone during locomotion and muscle activity (4, 35).

In this study, all clinical and densitometric measures of body composition were significantly lower in women with OP ($p < 0.001$) and osteopenia ($p < 0.001$), compared to those with normal BMD as illustrated in Figure 5.1. A lower total body weight, BMI, FMI, total fat percentage and LMI, was noted in women who displayed bone loss at baseline based on a BMD T-score of $-1SD$ or lower at any site (p -value < 0.001). Similar relationships between body composition parameters and BMD at individual skeletal sites i.e. the spine and both hip regions were documented. This indicates a beneficial effect of increased fat and lean mass on BMD maintenance irrespective of site. Prior studies have suggested a more pronounced effect of body weight on BMD in the hip region as a more weight-bearing site (26, 36). Our data indicate benefit and a positive correlation irrespective of site in accordance with another local study in community dwelling, healthy black and white women (27).

Fifty percent of women with OP had a low/normal BMI of $\leq 25\text{kg/m}^2$ in contrast to our study cohort in whom 85% were obese. Low body weight is a well-established risk factor for OP in breast cancer patients and also documented in our study cohort. In our cohort, 85% of women were overweight according to WHO-BMI categories (17). This percentage exceeds the national figure of 68% for obesity in adult South African women as reported in the Department of Health, South Africa Demographic and Health Survey 2016 (37). It is noteworthy, that BMI in the overweight and obese categories did not preclude the possibility of OP in this cohort.

In continuous comparison, the relationship between fat mass indices (total body fat percentage and FMI) and lean mass indices (LMI) with BMD measurements were similar. In our relatively young postmenopausal cohort, lean mass was well maintained and above the third centile (as indicator of significant loss of muscle mass) in all but three study subjects. Likewise, appendicular lean mass, a parameter closely associated with sarcopenia and fall risk in the elderly, was normal in the vast

majority of our cohort (98%). Our findings indicate a significant role for lean mass in maintaining BMD. Measurement of this component of total body weight may be especially important in older women with breast cancer in whom accurate risk stratification of bone health is pertinent.

Other conventional clinical risk factors for bone loss and OP were not significantly associated with BMD in our study subjects, but merit discussion. Alcohol intake was minimal amongst study participants with no one exceeding the recommended maximum daily intake. The women were all active, with 81% of the cohort reporting out-of-house activities. Univariate analysis did not identify active lifestyle or prior smoking to have a significant adverse impact on BMD. Although years since menopause (YSM) increased with declining BMD categories, the increase was not statistically significant. In all the women with OP, in whom the duration of menopause was documented (n=6), YSM did exceed five years and warrants consideration in risk stratification programs.

Vitamin D insufficiency is common in the general population (13) and also reflects in our mixed race population. A marked seasonal variation in vitamin D₃ production was noted in Cape Town, with very little being formed during the winter months of April through September in a study conducted in the late 1990's (38). Increased skin pigment and obesity are well known risk factors for decreased cutaneous vitamin D production, both present in the majority of our study subjects.

Low vitamin D is a known risk factor for osteoporosis due to the associated negative calcium balance and compensatory secondary hyperparathyroidism with increased bone resorption (39, 40). The vast majority of our study participants (93%) had insufficient or deficient vitamin D levels, an extremely high and concerning figure. The mean 25-OH-Vitamin D (p=0.290) did not

differ amongst the BMD subcategories, but this may partly be due to the almost universal Vitamin D insufficiency in the study cohort.

Only a minority of women with insufficient vitamin D status had elevated PTH-levels in keeping with a diagnosis of secondary hyperparathyroidism. Of the 38 women with insufficient Vitamin D levels, eight women had elevated serum-PTH (22%). Of the 52 women with deficient Vitamin D, 15 manifested with secondary hyperparathyroidism (29%). Twenty-one percent of women with secondary hyperparathyroidism had OP, a figure slightly higher than that documented for the entire cohort (14%). When comparing BMD in ascending PTH tertiles, no significant adverse BMD effect was evident ($p = 0.720$). It is noteworthy that near half (46%) of the women with OP had compensatory secondary hyperparathyroidism.

Our study had limitations. Conclusions drawn from this study are limited by the small sample size of 101 women. Data obtained in this cohort nonetheless do contribute to the current small knowledge pool regarding the baseline bone health of South African postmenopausal women with breast cancer considered for AI therapy. The cohort furthermore consisted almost exclusively of women of mixed descent and therefore our study data cannot be extrapolated to the other ethnic groups in our country in whom knowledge regarding baseline bone health is also very limited. A strength of our study, on the other hand, is that densitometric data were obtained by making use of a single, very experienced densitometrist that positively impact on the validity of both our body composition and BMD data.

Conclusion

A substantial 32% of our cohort of postmenopausal women considered for AI therapy fulfilled criteria for bone-specific pharmacological protection. Inadequate baseline assessment of bone

health may have dire consequences when life-saving breast cancer therapy, with known potential adverse bone effects, is prescribed. This study emphasizes the absolute need for BMD and clinical risk assessment in all postmenopausal women of mixed race with breast cancer, considered for AI therapy. Ultimately, it may also inform local health policy.

The study provides valuable information regarding the relationship between body composition variables and bone health of postmenopausal women of mixed ancestry. It further highlights the importance of lower body weight as a risk factor in the assessment of bone health. The concerning high percentage of Vitamin D insufficiency noted in our study cohort requires additional investigation. Evaluation in larger cohorts may clarify the significance and magnitude of the impact of insufficient vitamin D status on bone health.

This is the first study of its kind conducted in a group of women of mixed race residing in the Western Cape Province of South Africa. Improved insights into ethnic variations of bone health, provided by studies such as ours, will enable preventive approaches to osteoporosis for postmenopausal women on breast cancer treatment (31). Further work in this same cohort will report on changes in bone health during the course of AI treatment.

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Declaration of Interest

Prof. MJ Kotze is a director and shareholder of Gknowmix (Pty) Ltd.

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CHAPTER 6

Exploring the role of genomics in postmenopausal breast cancer patients treated with aromatase inhibitors: One-year follow-up.

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Abstract

Background

Significant individual variation in bone loss associated with aromatase inhibitors (AIs) emphasizes the importance of identifying postmenopausal breast cancer patients at high risk for this adverse effect. The study explores the clinical relevance of genetic variation in the Cytochrome P450 19A1 (*CYP19A1*) gene in a subset of South African patients during the first year of taking AIs for estrogen receptor (ER)-positive breast cancer.

Methods

The study population consisted of ER-positive breast cancer patients on AIs, followed in real-life clinical practice. Body mass index (BMI) was measured and bone mineral density (BMD) determined at baseline and at month 12. *CYP19A1* genotyping was performed using real-time polymerase chain reaction analysis of rs10046, extended to Sanger sequencing and whole exome sequencing (WES) in 10 patients with more than 5% bone loss at month 12 at the lumbar spine.

Results

After 12 months of AI treatment, 72 patients had completed BMD and were successfully genotyped. Ten patients (14%) experienced more than 5% bone loss at the lumbar spine over the study period. Genotyping for *CYP19A1* rs10046 revealed that patients with two copies of the A-allele were 10.79 times more likely to have an ordinal category change of having an increased percentage of bone loss or no increase at the lumbar spine, compared to patients with the GA or GG genotypes (CI of 1.771- 65.830, $p=0.01$). None of the 34 patients without lumbar spine bone loss at month 12 were homozygous for the functional *CYP19A1* polymorphism. At the total hip region, patients with the AA genotype were 7.37 times more likely to have an ordinal category

change of having an increased percentage of bone loss or no increase (CI of 1.101- 49.336, p=0.04).

Conclusion

Homozygosity for the *CYP19A1* rs10046 A-allele may provide information, in addition to clinical and biochemical factors that may be considered in risk stratification to optimize bone health in postmenopausal breast cancer women on AIs.

Introduction

Aromatase inhibitors (AIs), the gold standard for treatment of estrogen receptor (ER)-positive postmenopausal breast cancer (1)(2), are associated with bone loss and fracture risk. There is however, significant individual variation in the bone loss induced by AIs. This is related to factors such as age, menopausal status, years since menopause and body mass index (BMI). Individual vulnerability to AI side effects is unpredictable and may also be explained by diverse genetic profiles (3).

The aromatase enzyme plays a critical role in bone health. Rare loss of function mutations in the Cytochrome P450 19A1 (*CYP19A1*) gene, may cause decreased bone mineral density (BMD) (4, 5). Common functional polymorphisms may affect enzyme activity in a context dependent manner (6). *CYP19A1* rs10046 explains 1.6% of the variance in the estradiol-testosterone ratio (7). The A allele of *CYP19A1* rs10046 is associated with raised estrogen levels, which in turn is expected to be beneficial for bone health (8).

In view of the findings quoted above and our literature curation (9), we identified rs10046 as a clinically useful single nucleotide polymorphism (SNP) for risk stratification in AI

pharmacogenetics pending validation in ethnically diverse South African breast cancer patients. However, genotype association with clinical outcome does not constitute biological significance; therefore it is important to substantiate the statistics with biological information (10).

The study is the first to report on the impact of genetic variation within metabolic pathways underlying bone health in South African breast cancer patients, during the first year on AIs.

Methods

Study population and design

We prospectively evaluated postmenopausal women with newly diagnosed, histologically confirmed endocrine sensitive early breast cancer between the ages of 50 to 80 years. The study was conducted at the Tygerberg Hospital Breast Clinic in affiliation with Stellenbosch University. The study population was derived from a larger cohort (n=101, Chapter 5) prospectively followed up on AI therapy to assess bone health outcomes and included 72 postmenopausal breast cancer patients who were successfully genotyped at baseline and in whom BMD were measured both at baseline and following 12 months of AI therapy. Patients with metabolic bone disease or on medication known to adversely affect BMD at baseline were excluded from the study. All participants were treated with Anastrozole, a non-steroidal AI according to guidelines, at the time of diagnosis.

Information on other clinical variables were recorded at enrolment, including age at recruitment, gynaecological history, weight, height, calcium and Vitamin D status, activity levels and smoking status.

Bone mineral density

Dual energy X-ray absorptiometry (DXA); Hologic Discovery-W (S/N 70215), software Version 13.1 was employed in this study. Femoral neck (FN), total hip (TH) and lumbar spine (LS) BMD were measured at baseline and month 12 on treatment. A single experienced DEXA technician (MM Conradie) completed scans on all subjects. Intra-operator variation was below 1% for all bone sites.

Genotyping

DNA was extracted from whole blood using the QIAGEN QIAamp® DNA Blood Mini Kit (Hilden, Germany). At the time of study design, candidate SNPs were identified from publications in the literature based on their role in AI-associated effects (9, 11). *CYP19A1* rs10046 was genotyped using real-time polymerase chain reaction (PCR) (TaqMan® technology), extended to Sanger sequencing and whole exome sequencing (WES) in 10 patients with more than 5% bone loss at month 12. WES was performed at the Central Analytical Facility of Stellenbosch University, using the protocol previously described (12).

Statistical analyses

Data management and analysis were conducted in STATA 14. Descriptive statistics were used to describe baseline characteristics and 12-month outcome data. BMD were expressed as percent (%) change from baseline to 12 months. Continuous data were tested for normality using descriptive statistics (e.g. histograms) where normally distributed data were presented as means and standard deviations, or as medians and interquartile ranges (IQR) for non-normally distributed data. Categorical data were presented as proportions and 95% confidence intervals. The associations between biological parameters and BMD was determined using one-way ANOVA

and χ^2 tests. To account for confounding, significant univariate predictors were included in a final ordered logistic regression model at $p < 0.2$. An alpha of 0.05 was considered statistically significant. Associations were reported as relative risks with 95% confidence intervals. The Research Electronic Data Capture (REDCap) application was used for data management (13).

Ethics approval

The research complied with the World Medical Association Declaration of Helsinki- ethical principles for medical research involving human subjects- and the study was approved by the Ethical Review Board of the Faculty of Medicine, Stellenbosch University (S13/05/103).

Results

Baseline characteristics were documented in 101 breast cancer patients (Chapter 5). Seventy-two of these women were successfully genotyped and underwent BMD testing at baseline and after 12 months of AI therapy.

No significant change in the average body weight, height or BMI of the genotyped study cohort ($n=72$) was observed over the one-year course of AI therapy. When assessing changes in individual BMI, ten women experienced a decrease in BMI of 3 kg/m^2 or more (14% of cohort), whereas the BMI of only four women increased to a similar degree (6% of cohort). A change in body weight in excess of 5 kg after 12 months of AI therapy was observed in 26 women (decrease: $n=14$, increase $n=12$).

The average absolute BMD measured at all skeletal sites for this cohort on AI therapy was significantly lower at month 12 compared to baseline (Table 6.1). Significant bone loss based on average absolute BMD was thus demonstrated for the study cohort at the lumbar spine ($p < 0.0001$).

	Baseline (n=72)	Month 12 (n=72)	<i>p value</i>
Anthropometry			
Weight (kg)	82.4 ± 19.1	81.9 ± 19.5	0.44
Height (cm)	159.6 ± 5.9	158.9 ± 6.0	0.22
BMI (kg/cm ²)	32.5 ± 7.8	31.8 ± 8.8	0.33
Bone Mineral Density (g/cm²)			
Lumbar spine	1.001 ± 0.154	0.978 ± 0.151	< 0.0001
Femoral neck	0.798 ± 0.116	0.779 ± 0.117	<0.0001
Total hip	0.939 ± 0.136	0.928 ± 0.129	0.02

Table 6.1. Comparison of clinical anthropometry and BMD at baseline and after 12 months of AI therapy.

Absolute parameters are presented as means ± standard deviation for patients who were genotyped.

Genotyping was performed in 72 patients and included 60 women of Mixed Ancestry, 10 Caucasians, one Black and one Indian patient. There was no difference in genotype distribution and allele frequency for the *CYP19A1* rs10046 polymorphism in the relatively small group of Caucasian and Mixed Ancestry patients studied. Table 6.2 shows the genotype distribution of *CYP19A1* rs10046 in relation to baseline clinical characteristics and ethnicity and to BMD measurements documented both at baseline and at month 12 of AI use. The genotype distribution of this polymorphism was in Hardy–Weinberg Equilibrium.

The three genotype groups were comparable with regard to age and BMI, determined both at baseline and at month 12. No significant weight loss, expressed as a change in BMI, were noticed from baseline to month 12 in any of the three genotype cohorts.

There was an even distribution of the *CYP19A1* rs10046 GG and GA genotypes in the predominant Mixed Ancestry group within our study population. Of the six patients with genotype AA, 67% (n=4) were Mixed Ancestry patients and the other two Caucasian.

The absolute BMD at all the measured sites, i.e. at the lumbar spine, femoral neck and total hip was similar in all three genotype groups. Although not statistically significant, a clear trend towards lower measured BMD was observed in the heterozygous and homozygous genotype groups with the *CYP19A1* rs10046 A allele, compared to GG homozygotes. The lowered trend was more pronounced at 12 months, suggestive of accentuated loss in the genotype groups with the *CYP19A1* polymorphism. This observation was noted at all skeletal sites and equates to a percentage difference in absolute average BMD between the GG homozygotes and AA homozygotes of 4.5%, 4.9% and 7.6% at the lumbar spine, femoral neck and total hip regions respectively, at baseline and a difference of 6.5%, 5.3% and 8.9% at the mentioned sites at 12 months.

<i>CYP19A1</i> rs10046 (n = 72)				
	Genotype GG 34 (47%)	Genotype GA 32 (45%)	Genotype AA 6 (8%)	<i>p</i>-value
Clinical characteristics				
Age (yrs.)	60 ± 5.8	61 ± 7.3	62 ± 9.7	0.71
BMI (kg/m ²)				
baseline	33.0 ± 7.3	32.5 ± 8.7	30.1 ± 4.2	0.70
month 12*	32.9 ± 7.7	31.0 ± 10.2	30.3 ± 6.2	0.62
Ethnicity				
MA (n = 60)	28	28	4	N/A
Caucasian (n =10)	5	3	2	N/A
Black (n = 1)	1	0	0	N/A
Indian (n = 1)	1	0	0	N/A
Bone mineral density (g/cm²)				
<i>Lumbar Spine</i>				
baseline	1.028 ± 0.154	0.976 ± 0.158	0.982 ± 0.129	0.38
month 12	1.001 ± 0.153	0.961 ± 0.154	0.936 ± 0.132	0.44
<i>Femur Neck</i>				
baseline	0.819 ± 0.123	0.781 ± 0.115	0.770 ± 0.060	0.34
month 12	0.803 ± 0.118	0.758 ± 0.119	0.750 ± 0.071	0.24
<i>Total Hip</i>				
baseline	0.961 ± 0.131	0.926 ± 0.149	0.885 ± 0.063	0.35
month 12	0.950 ± 0.132	0.917 ± 0.132	0.861 ± 0.070	0.24

6.2: Clinical characteristics, ethnicity and BMD in relation to genotype distribution of *CYP19A1* rs10046 in the total genotyped study population

BMI- body mass index; *MA*- Mixed Ancestry; *n/a*-not applicable *no significant change in *BMI* from baseline to month 12 in any of the three genotypes

A statistically significant ($p= 0.003$) decline in absolute BMD was noted for all skeletal sites in the three genotype groups from baseline to month 12. The greatest absolute loss (BMD decline of 0.046) was noted for the lumbar spine region in *CYP19A1* genotype subgroup AA. The decline in all groups were most pronounced in the lumbar and femoral neck regions known to be rich in trabecular bone. This is expected as a result of reduced tissue exposure to estrogen at this early time point of 12 months (Table 6.3).

BMD according to genotype	Baseline	Month 12	<i>p</i> value
Genotype GG			
Lumbar spine	1.028 ± 0.154	1.001 ± 0.153	0.003
Femur Neck	0.819 ± 0.123	0.803 ± 0.118	0.003
Total Hip	0.961 ± 0.131	0.950 ± 0.132	0.03
Genotype GA			
Lumbar Spine	0.976 ± 0.158	0.961 ± 0.154	0.0005
Femur Neck	0.781 ± 0.115	0.758 ± 0.119	0.0001
Total Hip	0.926 ± 0.149	0.917 ± 0.132	0.29
Genotype AA			
Lumbar Spine	0.982 ± 0.129	0.936 ± 0.132	0.0004
Femur Neck	0.770 ± 0.060	0.750 ± 0.071	0.19
Total Hip	0.885 ± 0.063	0.861 ± 0.070	0.09

Table 6.3. Bone mineral density measurements at different skeletal sites within the three genotype groups

Table 6.4 shows the change in BMD at month 12 expressed as percentage bone loss. The degree of bone loss at the lumbar spine and total hip region is tabulated into three bone loss categories i.e. no change; $\leq 5\%$, but significant bone loss or $>5\%$ bone loss for all genotyped patients. In the total study population 47% of individuals (n=34) maintained their lumbar spine bone mass with no significant change from baseline, 39% (n= 28) had bone loss of up to 5% and 14% (n= 10) had bone loss in excess of 5%. At the total hip, 72% maintained bone mass (n=52), 19% (n=14) had up to 5% bone loss, whereas 8% of women (n = 6) lost more than 5% of their bone mass. Bone loss at the trabecular rich lumbar region were more pronounced within the limited observation period of 12 months compared to the total hip region mostly comprised of cortical bone as expected and as alluded to before.

The percentage bone loss for the three genotypes were also calculated at the lumbar and hip region. All patients with the *CYP19A1* rs10046 AA genotype displayed bone loss at the lumbar spine region over the observation period of 12 months. The individual losses in the two *CYP19A1* AA homozygotes among patients with more than 5% bone loss were -5.8 and -7.6% respectively (one Caucasian, one Mixed Ancestry). The losses in the four AA homozygotes in the up to 5 % bone loss group ranged between -3.2 to -4.5% (one Caucasian, three Mixed Ancestry). Only 8% (n=6) of the study group displayed the AA genotype. Notably, this genotype group represented 20% of the cohort who suffered bone loss in excess of 5% over the 12-month period of AI therapy.

	No bone loss	≤ 5% bone loss	>5% bone loss
	n (%)	n (%)	n (%)
Lumbar Spine			
Total (n = 72)	34 (47)	28 (39)	10 (14)
GG genotype (n = 34)	17 (50)	12 (35)	5 (15)
GA genotype (n = 32)	17 (53)	12 (37)	3 (9)
AA genotype (n = 6)	0	4 (67)	2 (33)
Total Hip			
Total (n = 72)	52 (72)	14 (19)	6 (8)
GG genotype (n = 34)	27 (79)	5 (15)	2 (6)
GA genotype (n = 32)	22 (69)	7 (22)	3 (9)
AA genotype (n = 6)	3 (50)	2 (33)	1 (17)

Table 6.4: Proportional bone loss at month 12 at Lumbar Spine (LS) and Total Hip (TH).

Body composition parameters including clinically determined BMI and both Fat Mass Index and Lean Mass Index as determined by DXA were significant predictors of baseline BMD status in the larger study population, of whom 72 women underwent genotyping (Chapter 5). At the lumbar spine, *CYP19A1* rs10046 AA homozygotes were 10.79 times more likely to have an ordinal category change of having an increased percentage of bone loss or no increase, compared to patients with the GA or GG genotypes (CI of 1.771- 65.830, p=0.01). Genotyping for *CYP19A1* rs10046 revealed that patients with two copies of the A-allele are 7,37 times more likely to have an ordinal category change of having an increased percentage bone loss or no increase, at the total hip compared to those without this allele (CI of 1.101- 49.336, p=0.04). None of the 34 patients without bone loss at the lumbar spine at month 12, were homozygous for the functional *CYP19A1* polymorphism. DNA sequencing in the 10 patients with more than 5% bone loss, supported these findings and confirmed the genotype allocation of A and G alleles using real-time PCR. WES

demonstrated sufficient coverage to accurately detect this SNP within a greater pharmacogenetics and diagnostic screening context, as evidenced by the concomitant detection of a pathogenic *BRCA2* mutation (c.582G>A) in one of these patients.

Discussion

This study describes the changes in BMD measured at baseline and after one year of AI treatment and its relation to genetic variation in the *CYP19A1* gene. In our study cohort, the rs10046 genotype distribution was 46% in both the GG and GA genotype groups in the Mixed Ancestry patients compared to 50% and 30% for GG and GA, respectively, in the Caucasians. In the AA genotype group (n=6), Mixed Ancestry patients comprised 67% (n=4). The three genotype groups were similar in terms of age and BMI, as at baseline and no significant weight loss was evident over the one-year period, in any of the groups.

Recent international consensus guidelines suggest that all women starting AI therapy should have a baseline clinical risk assessment of osteoporosis for individualized bone protective intervention (13, 14). Our baseline evaluation revealed that a third of our study population already had BMD findings necessitating active bone protection. Body composition was identified as the most important clinical predictor of baseline BMD in these women (Chapter 5). Our prospective bone health evaluation at month 12 of AI therapy, revealed that the average absolute BMD of the cohort is statistically lower than that measured at baseline, but similar amongst the genotype groups. This finding is in keeping with most large clinical trials reporting accelerated loss up to 7.5% annually (16). A statistically significant decline in absolute BMD was noted in the total cohort for all skeletal sites, and also within all three genotype groups from baseline to month 12. The greatest bone loss evaluated within the three genotype groups was noted for the lumbar spine region in

genotype subgroup AA (average decline of 4.5% over 12 months). The decline in all groups was most pronounced in the lumbar and femoral neck regions known to be rich in trabecular bone. This is expected as reduced tissue exposure to estrogen at this early time point of 12 months will predominantly affect metabolically more active trabecular bone tissue.

A trend was noted towards a lower BMD (all skeletal sites) in groups with *CYP19A1* rs10046 GA/AA genotype compared to the GG genotype, but this was not statistically significant. Small patient numbers within some of the genotype groups may have limited our ability to detect significant differences in BMD amongst these groups and warrants further health outcomes studies in an extended patient cohort.

Nearly 50% of the study cohort had significant loss of lumbar BMD. Fourteen percent of these women had more than 5% bone loss at the lumbar spine. At the hip, nearly a third of women had significant bone loss at month 12 and only six patients (8%) had more than 5% bone loss. These findings support the earlier loss of trabecular bone following reduced skeletal exposure to estrogen in postmenopausal women due to AI therapy. Furthermore, the observation in our study all patients with the AA genotype had significant bone loss is noteworthy.

Although many studies support the role of functional polymorphisms in breast cancer, the mechanism underpinning the bone loss associated with AI therapy remains elusive (17). We explored the clinical relevance of *CYP19A1* rs10046 as an additional tool for risk stratification in AI-related bone outcomes. Our results reveal that women with the *CYP19A1* AA genotype are 10.79 times more likely to have an ordinal category change of having an increased percentage bone loss or no increase, at the lumbar spine and are 7,37 times more likely to have an ordinal category change of having an increased percentage bone loss or no increase, at the total hip compared to those without this allele.

CYP19A1 rs10046 AA homozygotes may represent a population of breast cancer patients who could be at increased risk for bone loss with long-term AI therapy. Early intervention and close follow-up of bone health is indicated in these patients who may comprise a subgroup who should be considered for other forms of endocrine therapy.

Our single institution study is limited by small numbers, marked by a significant degree of attrition in a real life clinical practice setting. Although the interval of BMD assessment at one year may be considered too short to experience the full impact of AIs on bone, the majority of bone loss have been noted early in the course of AI therapy in several studies (17, 18). This prospective evaluation of bone health, performed in a predominantly Mixed Ancestry population, identified the *CYP19A1* rs10046 A allele as conferring risk to bone loss. This conflicts with findings from other studies in mainly European populations.

Discrepancies reported in pharmacogenetic studies may be attributed to background genetic influences, environmental factors and prescribed therapies (20). Notably, the A allele was reported as the minor allele in African population, while the G allele is the minor allele in most other populations. Further investigation is therefore required to place the clinical effect observed for a single SNP in a genomic context, with the aim to distinguish between true linkage and association resulting from shared ancestry. The genetic structure of the Mixed Ancestry population provides a valuable tool for admixture linkage disequilibrium mapping of pharmacogenetic markers (21). This is of particular relevance to the unique genetic structure of the Mixed Ancestry population of South Africa that clusters at positions between Africans and non-Africans.

Conclusion

CYP19A1 rs10046 AA homozygotes may represent a target group most likely to benefit from translation of research into a clinical management pipeline for individualized risk stratification. WES enabled screening of the entire *CYP19A1* gene simultaneously targeting other genetic variants previously implicated in bone health, which are scattered throughout the human genome.

Conflict of interest

Prof. MJ Kotze is a director and shareholder of Gknowmix (Pty) Ltd.

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CHAPTER 7

Conclusion

The high proportion of postmenopausal women of Mixed Ancestry with estrogen receptor (ER)-positive breast cancer fulfilling international criteria for bone protective measures, was the most striking clinical finding of the study. International guidelines recommend accurate clinical and biochemical bone risk assessment in all postmenopausal breast cancer patients considered for AI therapy. Variability in side effects of breast cancer patients with similar clinical bone risk profiles can be ascribed to genetic differences, also evident in our cohort.

The underlying pathophysiology of AI related bone loss entails complex interactions between clinical, biochemical and genetic factors and none of these aspects can be interpreted in isolation. Incorporation of pharmacogenetics into the clinical scenario is a major challenge which was addressed responsively in this study by the development of adaptable reports for continued monitoring, beyond a single research objective. The significant effect of *CYP19A1* rs10046 in the homozygous state provided a glimpse into the context-dependency of enzyme activity underpinned by genetic variation. Though impressive, no direct association can be assumed for the 7-10 times increased likelihood of bone loss at the lumbar spine and hip in *CYP19A1* rs10046 AA homozygotes. This statistical association requires further investigation with robust health outcome studies to validate biological significance in different clinical scenarios in the genetically diverse

South African population. The database resource developed in parallel to patient recruitment for this study would facilitate this process.

Aligning clinical, biochemical and genetic information translated into adaptable patient reports may overcome the fragmentation between service delivery and research silos. This study merged multi-disciplinary research in a real-life clinical setting by utilising high throughput genotyping and advanced next generation sequencing technologies. Insights gained from this research, may inform policy development for implementation of a refined bone risk stratification strategy for endocrine therapy in postmenopausal breast cancer patients in South Africa.

Appendix

Declaration by the candidate: Karin Baatjes

With regard to Chapter 2 to 6, the nature and scope of my contribution were as follows:

Chapter	Nature of contribution	Extent (%)
2	The PhD candidate performed the literature review relating to the clinical and biochemical risk factors associated with endocrine treatment related bone side effects, prepared and submitted the manuscript for publication and thesis chapters.	70
3	The candidate performed the literature curation pertaining to the genetic aspects of bone loss in endocrine treatment of breast cancer, prepared and submitted the manuscript for publication and thesis chapters.	70
4	The candidate recruited and obtained consent from participants, collected samples and information data, uploaded the information into the REDcap database, followed the patients up at sequential visits. The candidate analysed the data, developed the report format, prepared the manuscript and thesis chapters. The candidate was assisted with questionnaire completion by -Dr van der Merwe, Ms Moremi and Sr P Opperman	60
5	The candidate recruited and obtained consent from participants, collected samples and information data, uploaded the information into the REDcap database, and followed the patients up at sequential visits. The candidate analysed the data, prepared the manuscript and thesis chapters. The candidate was assisted with questionnaire completion by -Dr van der Merwe, Ms Moremi and Sr P Opperman	55
6	The candidate recruited and obtained consent from participants, collected samples and information data, uploaded the information into the REDcap database, followed the patients up at sequential visits. The candidate analysed the data, prepared the manuscript and thesis chapters.	60

The candidate was assisted by

-Dr van der Merwe, Ms Moremi and Sr P Opperman

The following co-authors have contributed to Chapters 2 to 6 of the dissertation:

Name	E mail address	Nature of contribution	Extent (%)
Maritha Kotze	maritha@sun.ac.za	Chapter 2: Provided comments on chapter.	5
		Chapter 3: Provided comments on chapter.	20
		Chapter 4: Contributed to data interpretation and provided comments on chapter.	30
		Chapter 5: Provided comments on chapter.	2
		Chapter 6: Contributed to data interpretation provided comments on chapter.	15
		Chapter 2: Provided comments on chapter.	15
Magda Conradie	mc4@sun.ac.za	Chapter 3: Provided comments on chapter	5
		Chapter 4: Provided comments on chapter	5
		Chapter 5: Contributed to data interpretation and provided comments on chapter	40
		Chapter 6: Contributed to data interpretation and provided comments on chapter	15

JP Apffelstaedt	justus@apffelstaedt.com	Chapter 2: Provided comments on chapter	10
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		Chapter 6: Provided comments on chapter	1
Micheal McCaul	mmccaul@sun.ac.za	Chapter 5: Provided statistical analysis and support	3
		Chapter 6: Provided statistical analysis and support	3
Armand Peeters	avpeeters@gmail.com	Chapter 4: Provided methodological assistance and guidance. Contributed to data interpretation	3
		Chapter 6: Provided methodological assistance and guidance. Contributed to data interpretation.	3
MM Conradie	rc2@sun.ac.za	Chapter 6: Provided methodological guidance	3



Signature of candidate:

Date: ...1.8.18.....

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapters 2 to 5.
2. no other authors contributed to Chapters 2 to 5 besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 2 to 5 of this dissertation.

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