

# **The association between the metabolic syndrome and bone mineral density in pre- and post-menopausal farm workers**

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## DECLARATION

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## ABSTRACT

**Introduction and aims:** The prevalence of the metabolic syndrome (MetS) is increasing, both globally and in South Africa. Albeit so, limited data have been published in the South African context. One of the factors that appear to influence the prevalence of the MetS is menopausal status, with both the MetS, and menopausal status influencing bone mineral density (BMD); however, the reported results are inconsistent. Therefore, the aim of this study was to determine the prevalence of the metabolic syndrome, investigating bone health as well as the interactions between the MetS, menopausal status and bone health, in a farm working female population in the Western Cape.

**Methods:** A total of n=80 females were recruited and classified with the MetS, using the International Diabetes Federations' definition. The data collected included basic anthropometric measurements, blood pressure, BMD, and several questionnaires to obtain information regarding physical activity, demographic information, menstrual-, diet- and family health- history. The blood parameters that were measured included alkaline phosphatase (ALP), vitamin D, parathyroid hormone (PTH), oestradiol (E<sub>2</sub>), fasting insulin (FI), fasting glucose (FG) and a lipid profile.

**Results:** A relatively high prevalence of the MetS (55.0%) was reported in the current study. When investigating the separate MetS risk factors, most of the study participants had three risk factors (32.5%), with increased BP being the most prevalent MetS risk factor (72.5%). Factors that differed between MetS and Non-MetS sub-groups (according to menopausal status and age) included waist circumference (WC), high-density lipoprotein-cholesterol (HDL-c), systolic blood pressure (SBP) and diastolic blood pressure (DBP). Significant associations between body mass (BM) and E<sub>2</sub>, and body mass index (BMI) and E<sub>2</sub>, were limited to the PreM (20-39 years) age group with the MetS (r=0.58, p=0.03, and r=0.60, p=0.02). A total of 78.8% of the study participant had normal BMD. When correlating BM and speed of sound (SOS), significant associations were limited to the PreM (≥40 years) group (MetS: r=0.56, p=0.04, Non-MetS: r=0.76, p=0.00), and significant associations between BMI and SOS were noted in both PreM groups (MetS PreM 20-39 years: r=0.53, p=0.05, Non-MetS PreM ≥40 years: r=0.73, p=0.00). The significant correlations between FI and ALP (r=0.72, p=0.00), FG and ALP (r=0.89, p=0.00), and triglycerides with ALP (r=0.82, p=0.00) were limited to the PreM (≥40 years) group.

**Conclusion:** The prevalence of the MetS was higher than that reported by previous South African studies. Irrespective of metabolic and menopausal status, most of the participants of the current study population had normal BMD.

**Key words:** Metabolic syndrome, bone mineral density, menopause

## OPSOMMING

**Inleiding en doelwit:** Die voorkoms van die metaboliese sindroom (MetS) is beide globaal en in Suid Afrika toenemend. Daarbenewens is daar beperkte data in die Suid-Afrikaanse konteks. Een van die faktore wat die voorkoms van die MetS beïnvloed is die menopousale status, waar beide die MetS en menopousale status beenmineraaldigtheid (BMD) beïnvloed. Die resultate wat hier rapporteer word varieer egter. Die doelstelling van hierdie studie was om die voorkoms van die MetS in 'n plaaswerker vroue populasie in die Wes-Kaap te ondersoek, hulle been-gesondheid te bepaal asook te ondersoek of daar korrelasies is tussen die metaboliese sindroom, menopousale status en been-gesondheid te korreleer.

**Metodes:** 'n Totaal van n=80 vroue is gewerf en geklassifiseer deur gebruik te maak van die Internasionale Diabetes Federasie se kriteria vir die MetS. Die data wat versamel was sluit onder andere in basiese antropometriese metings, bloeddruk, BMD, asook verskeie vraelyste om inligting oor fisiese aktiwiteit, demografiese inligting, menstruele-, dieet- en familie gesondheidsgeskiedenis te versamel. Die bloedparameters wat bepaal was sluit in alkaliese fosfatase (ALP), vitamien D, paratiroïed hormoon (PTH), estradiol ( $E_2$ ), vastende insulien (VI), vastende glukose (FG), en 'n lipiedprofiel.

**Resultate:** 'n Relatiewe hoë voorkoms van die MetS (55.0%) is in hierdie studie waargeneem. Indien die individuele MetS risiko faktore in ag geneem word, het die meerderheid deelnemers drie risiko faktore gehad (32.5%), met verhoogde bloeddruk wat die mees algemene MetS risiko faktor (72.5%) was. Sommige van die faktore wat tussen die MetS en Nie-MetS verskil het (volgens menopousale status en ouderdom) sluit in; middel-omtrek (MO), hoë digtheidslipoproteïen (HDL-c), sistoliese bloeddruk (SBD) en diastoliese bloeddruk (DBD). Betekenisvolle verwantskappe is waargeneem tussen liggaamsmassa indeks (LMI) en  $E_2$ , LMI en  $E_2$  is beperk tot die PreM (20-39 jaar) ouderdoms- en die MetS groepe ( $r=0.58$ ,  $p=0.03$  en  $r=0.60$ ,  $p=0.02$ ). 'n Totaal van 78.8% van die studie populasie het normale BMD gehad. Wanneer liggaamsmassa (LM) en die spoed van klank (SvK) teenoor mekaar gekorreleer is, is betekenisvolle verwantskappe beperk gewees tot die PreM ( $\geq 40$  jaar) groep (MetS:  $r=0.56$ ,  $p=0.04$ , Nie-MetS:  $r=0.76$ ,  $p=0.00$ ), en betekenisvolle verwantskappe tussen LMI en SvK in beide PreM groepe (MetS PreM 20-39 jaar:  $r=0.53$ ,  $p=0.05$ , Nie-MetS PreM  $\geq 40$  jaar:  $r=0.73$ ,  $p=0.00$ ). Die betekenisvolle korrelasies tussen VI en ALP ( $r=0.72$ ,  $p=0.00$ ), FG en ALP ( $r=0.89$ ,  $p=0.00$ ), en trigliseriede met ALP ( $r=0.82$ ,  $p=0.00$ ) is beperk tot die PreM ( $\geq 40$  jaar) groep.

**Gevolgtrekking:** Die voorkoms van die MetS was hoër as voorheen gerapporteerde Suid Afrikaanse studies. Ongeag die metaboliese en menopousale status het meeste deelnemers normale BMD gehad.

**Sleutelwoorde:** Metaboliese sindroom, beenmineraaldigtheid, menopouse

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## TABLE OF CONTENTS

<b>DECLARATION</b> .....	xx
<b>ABSTRACT</b> .....	xxi
<b>OPSOMMING</b> .....	xxii
<b>ACKNOWLEDGEMENTS</b> .....	xxiii
<b>TABLE OF CONTENTS</b> .....	xxiv
<b>LIST OF ABBREVIATIONS</b> .....	xxx
<b>STANDARD UNITS</b> .....	xxxiii
<b>LIST OF FIGURES</b> .....	xxxiv
<b>LIST OF TABLES</b> .....	xxxvi
<b>LIST OF EQUATIONS</b> .....	xxxvii
<b>Chapter 1 Literature Review</b> .....	1
<b>1.1 Introduction</b> .....	1
<b>1.2 The metabolic syndrome</b> .....	2
<b>1.2.1 Definitions and classifications of the metabolic syndrome</b> .....	2
<b>1.2.2 Incidence and prevalence of the metabolic syndrome</b> .....	5
<b>1.2.3 Pathophysiology of the metabolic syndrome</b> .....	7
<b>1.2.3.1 Obesity</b> .....	7
<b>1.2.3.2 Insulin resistance</b> .....	8
<b>1.2.3.3 Glucose intolerance</b> .....	9
<b>1.2.3.4 Dyslipidaemia</b> .....	10
<b>1.2.3.5 Hypertension</b> .....	11
<b>1.2.4 The metabolic syndrome and menopause</b> .....	12
<b>1.3 Bone</b> .....	12
<b>1.3.1 Basic bone physiology</b> .....	13
<b>1.3.1.1 Composition of bone</b> .....	13
<b>1.3.1.2 Bone mineral density</b> .....	14
<b>1.3.1.3 Bone growth</b> .....	14
<b>1.3.1.4 Bone turnover markers</b> .....	15
<b>Bone formation markers</b> .....	15

<b>Bone resorption markers</b> .....	16
<b>1.3.2 Bone pathophysiology</b> .....	18
<b>1.3.2.1 Osteopenia</b> .....	19
<b>1.3.2.2 Osteoporosis</b> .....	19
<b>1.3.3 Physical bone mineral density assessment methods</b> .....	19
<b>1.3.3.1 Dual-energy X-ray absorptiometry (DEXA)</b> .....	20
<b>1.3.3.2 Quantitative computed tomography (QCT)</b> .....	21
<b>1.3.3.3 Magnetic resonance imaging (MRI)</b> .....	22
<b>1.3.3.4 Quantitative ultrasound method</b> .....	23
<b>1.3.4 Complimentary biochemical and nutritional assessment bone markers</b> ....	26
<b>1.3.4.1 Calcium</b> .....	26
<i>Basic calcium physiology</i> .....	26
<i>Circulating calcium and the metabolic syndrome</i> .....	27
<i>Calcium, ageing and menopause</i> .....	28
<b>1.3.4.2 Vitamin D</b> .....	28
<i>Basic physiology</i> .....	28
<i>Vitamin D and the metabolic syndrome risk factors</i> .....	30
<i>Vitamin D, ageing and menopause</i> .....	32
<b>1.3.4.3 Parathyroid hormone</b> .....	33
<i>Basic physiology</i> .....	33
<i>Parathyroid hormone and the metabolic syndrome</i> .....	33
<i>Parathyroid hormone, ageing and menopause</i> .....	34
<b>1.3.5 Risk factors associated with bone mineral density</b> .....	34
<b>1.3.5.1 Blood pressure - hypertension</b> .....	35
<b>1.3.5.2 Dyslipidaemia</b> .....	36
<b>1.3.5.3 Fasting plasma glucose and insulin concentrations</b> .....	37
<b>1.3.5.4 Obesity</b> .....	38
<b>1.3.5.5 Lifestyle risk factors affecting BMD</b> .....	39
<i>Physical activity</i> .....	39
<i>Breastfeeding</i> .....	39
<i>Smoking</i> .....	40
<i>Alcohol consumption</i> .....	41
<i>Medications</i> .....	41
<b>1.3.5.6 Non-modifiable risk factors</b> .....	43

<b>Genetics (Familial bone health and disease history)</b> .....	43
<b>Ethnicity</b> .....	43
<b>Hormonal status (menopause)</b> .....	44
<b>1.4 Summary</b> .....	46
<b>1.4.1 Problem statement</b> .....	46
<b>1.4.2 Hypothesis</b> .....	46
<b>1.4.3 Aims</b> .....	46
<b>1.4.4 Objectives</b> .....	47
<b>Chapter 2 Materials and Methods</b> .....	48
<b>2.1 Ethical considerations</b> .....	48
<b>2.2 Study design and sample population characteristics</b> .....	48
<b>2.2.1 Inclusion and exclusion criteria</b> .....	49
<b>2.2.2 Definition of the metabolic syndrome</b> .....	50
<b>2.3 Data collection</b> .....	50
<b>2.3.1 Blood pressure and heart rate</b> .....	50
<b>2.3.2 Haematology</b> .....	50
<b>2.3.2.1 Enzyme-linked immunosorbent assay</b> .....	51
<b>a) Parathyroid Hormone</b> .....	52
<b>b) Vitamin D</b> .....	52
<b>2.3.3 Anthropometry: base measurements</b> .....	53
<b>2.3.3.1 Body mass</b> .....	53
<b>2.3.3.2 Stretched stature</b> .....	53
<b>2.3.3.3 The body mass index</b> .....	53
<b>2.3.3.4 The waist circumference</b> .....	54
<b>2.3.3.5 The hip circumference</b> .....	54
<b>2.3.3.6 The waist to hip ratio</b> .....	54
<b>2.3.4 Ultrasound bone densitometry measurement</b> .....	55
<b>2.3.4.1 Questionnaires</b> .....	55
<b>2.4 Data handling</b> .....	57
<b>2.5 Statistical analysis</b> .....	57
<b>Chapter 3 Results</b> .....	58
<b>3.1 Basic description of the sample population</b> .....	58
<b>3.2 The prevalence of the metabolic syndrome, and descriptive characteristics of the population</b> .....	59



3.3	Prevalence of the different metabolic syndrome risk factors .....	61
3.4	Bone health status of the total sample population, and between the MetS and Non-MetS groups .....	62
3.5	Description of participants according to menopausal status.....	67
3.6	Description of participants according to menopausal and metabolic status..	69
3.7	Correlation analysis .....	75
3.7.1	Correlation analysis between BM, BMI and E <sub>2</sub> .....	75
3.7.2	Correlation analysis between BM, BMI and SOS.....	76
3.7.3	Correlation analysis between FI, FG, TG and ALP .....	78
Chapter 4 Discussion.....		79
4.1	Introduction .....	79
4.2	Basic description of the study population .....	79
4.2.1	The study population was largely overweight .....	79
4.3	The metabolic syndrome: More than half of the study participants presented with the MetS.....	79
4.3.1	There was no age difference between the MetS and Non-MetS groups.....	80
4.3.2	The MetS group had significantly higher WC, FBG, BP and TG, and significantly lower HDL-c than the Non-MetS group .....	81
4.3.3	The majority of the MetS participants had three MetS risk factors, whereas the Non-MetS group' participants had mostly two MetS risk factors .....	81
4.3.4	Increased BP was the most prevalent MetS risk factor in the total sample population .....	82
4.3.5	The clustering of increased WC, increased BP and decreased HDL-c was the most prevalent in both the MetS and Non-MetS groups .....	82
4.4	The majority of the study population had normal BMD.....	83
4.4.1	Other factors influencing BMD .....	84
4.4.1.1	Smoking.....	85
4.4.1.2	Alcohol consumption.....	85
4.4.1.3	Contraceptive use .....	85
4.4.1.4	Physical activity.....	86
4.4.1.5	Breastfeeding.....	86
4.5	Bone health and the metabolic syndrome.....	87
4.5.1	Body mass, body mass index and BMD .....	87
4.5.2	Fasting insulin and BMD .....	88
4.5.3	Anti-hypertensive and cholesterol lowering medication use and BMD .....	88

4.5.4	Physical activity, smoking, alcohol and contraceptive use.....	88
4.5.5	Vitamin D.....	89
4.6	Menopausal distribution of study population.....	89
4.7	Bone health and menopausal status.....	90
4.8	Metabolic syndrome and menopausal status.....	93
4.8.1	Neither metabolic nor menopausal status had an effect on BMD.....	93
4.8.2	Correlation analysis between BMD, BMI and SOS.....	95
4.8.3	Differences in anthropometric and metabolic parameters restricted to the PreM group.....	96
4.8.3.1	Measures of obesity.....	96
4.8.3.2	Lipid abnormalities.....	97
4.8.3.3	Glucose and insulin.....	97
4.8.3.4	Blood pressure.....	97
4.9	Further investigation: Significant correlations between ALP and FI, FG and TG limited to the PreM ( $\geq 40$ years) group.....	98
4.9.1	Alkaline phosphatase and TG.....	98
4.9.2	Alkaline phosphatase, FI and FG.....	99
Chapter 5 Conclusion.....		100
5.1	Introduction.....	100
5.2	Major findings.....	100
5.3	Contributions.....	101
5.4	Limitations and recommendations.....	101
Chapter 6 References.....		104
APPENDIX A: Ethical documents.....		128
APPENDIX B: Participant information and consent form.....		131
APPENDIX C: Data collection sheet.....		136
APPENDIX D: PTH ELISA.....		137
Human Parathyroid Hormone ELISA kit (BioVendor, RIS003R).....		137
APPENDIX E: Vitamin D ELISA.....		139
Vitamin D ELISA kit (Elabscience, E-EL-0012).....		139
APPENDIX F: Procedure for the measurement of BMD with the Sonost Osteosys 3000.....		141
APPENDIX G: Demographic questionnaire.....		144

<b>APPENDIX H: GPAQ</b> .....	146
<b>APPENDIX I: Bone health questionnaire</b> .....	149
<b>APPENDIX J: Vitamin D concentrations in study population</b> .....	154
<b>APPENDIX K: Additional information on menstrual history between the MetS and Non-MetS groups</b> .....	155
<b>APPENDIX L: Additional analyses in the PreM and PostM women</b> .....	156
<b>APPENDIX M: Additional correlation analysis</b> .....	160

## LIST OF ABBREVIATIONS

1,25(OH) <sub>2</sub> D	1, 25-dihydroxycholecalciferol, calcitriol
25(OH)D	25-hydroxyvitamin D, calcifediol; 25-hydroxycholecalciferol, calcidiol
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AT	Adipose tissue
BAP	Bone-specific alkaline phosphatase
BB	Beta-blockers
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
BUA	Broadband ultrasound attenuation
BQI	Bone quality index
Ca <sup>2+</sup>	Calcium
Ca <sub>10</sub> (PO <sub>4</sub> ) <sub>6</sub> (OH) <sub>2</sub>	Hydroxyapatite
CCB	Calcium channel blockers
COC	Combined oral contraceptives
CTx	C-terminal telopeptide/ carboxy-terminal collagen crosslinks
CVD	Cardiovascular diseases
DEXA	Dual-energy X-ray absorptiometry
DBP	Diastolic blood pressure
DMPA	Depot medroxyprogesterone acetate
E <sub>2</sub>	Oestradiol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGIR	European Group on Insulin Resistance
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FFA	Free fatty acids
FI	Fasting insulin
FM	Fat mass
FPG	Fasting plasma glucose
FBG	Fasting blood glucose
GC	Glucocorticoid
GLUT 4	Glucose transporter type 4
GPAQ	Global physical activity questionnaire
HC	Hip circumference
HDL-c	High-density lipoprotein-cholesterol
HREC	Human research ethics committee

HRP	Horseradish peroxidase
HRPQCT	High-resolution peripheral quantitative computed tomography
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IL-9	Interleukin-9
IR	Insulin resistance
ISAK	International Society for the Advancement of Kinanthropometry
ISCD	International Society for Clinical Densitometry
IQR	Interquartile range
JIS	Joint interim statement
LDL-c	Low-density lipoprotein-cholesterol
MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
NCD	Non-communicable diseases
NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel III
NTx	Amino-terminal cross-linking telopeptide
OC	Oral contraceptives
OPG	Osteoprotegerin
PBM	Peak bone mass
PINP	Procollagen type I N pro-peptide
PeriM	Peri-menopausal
PostM	Post-menopausal
PreM	Pre-menopausal
PTH	Parathyroid hormone
QCT	Quantitative computed tomography
QUI	Quantitative ultrasound index
QUS	Quantitative ultrasound
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RDA	Recommended daily allowance
RPM	Revolutions per minute
sALP	Serum Alkaline phosphatase
SAT	Subcutaneous abdominal tissue
SBP	Systolic blood pressure
SD	Standard deviations
SEM	Standard error of mean
SHBG	Sex hormone-binding globulins
SNS	Sympathetic nervous system

SOS	Speed of sound
SST	Serum separator tube
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TMB	3,3'-5,5'-tetramethylbenzidine
TNF- $\alpha$	Tumour necrosis factor alpha
TG	Triglycerides
UV	Ultraviolet
UVB	Ultraviolet-B
VDR	Vitamin D receptor
VLDL	Very low-density lipoprotein
WC	Waist circumference
W:H	Waist-to-hip ratio
WHO	World Health Organization
W:Ht	Waist-to-height ratio

## STANDARD UNITS

Cm	Centimetre
dB/Mhz	Decibels per megahertz
IU	International unit
IU/d	International unit per day
g/cm <sup>2</sup>	Grams per square centimetre
kg/m <sup>2</sup>	Kilogram per square centimetre
Mg	Milligrams
mg/g	Milligrams per gram
mg/d	Milligrams per day
mg/Dl	Milligrams per decilitre
mmHg	Millimetres mercury
mmol/L	Millimoles per litre
m/s	Minutes per second
mu/L	Milliunits per litre
ng/L	Nanograms per litre
ng/mL	Nanograms per millimetres
Nmol	Nanomoles
nmol/L	Nanomoles per litre
pg/mL	Picograms per millilitre
µg	Microgram
µL	Microlitre
µg/min	Micrograms per minute
µM/mL	Micromoles per millilitre
U/L	Units per litre

# LIST OF FIGURES

## Chapter 1

Figure 1.1: The development of metabolic complications via abdominal obesity. ....	8
Figure 1.2: The link between obesity and the development of insulin resistance. ....	9
Figure 1.3: Pathway of increased adipose tissue contributing to dyslipidaemia. ....	10
Figure 1.4: The development of hypertension via increased AT.....	11
Figure 1.5: Different phases of bone growth throughout life, including the growth phase, consolidation phase which is followed by the rapid and gradual bone loss phases. ....	15
Figure 1.6: Dual-energy X-ray absorptiometry: Schematic representation of X-rays source and detector system, with an example of the type of image obtained by this measurement.....	21
Figure 1.7: Schematic representation of a magnetic resonance imaging scanner with an example of the type of image produced by this technique. ....	23
Figure 1.8: The QUS method illustrating (A) the movement of ultrasound through a bone section, and (B) the corresponding ultrasound pulse wave generated for each type of bone (note that sound waves travel faster through the trabecular compared to cortical bone). ....	24
Figure 1.9: Different quantitative ultrasound instruments, which includes a water bath system (A), dry contact system (B) and gel-padded system (C).....	25
Figure 1.10: Physiological interaction illustrating the role of PTH in the maintenance of serum calcium levels with key target organs and the feedback interactions with calcium (A). Plasma calcium regulation via the parathyroid glands and thyroid C-cells (B).....	27
Figure 1.11: Production of 25(OH) D and 1,25(OH) <sub>2</sub> D, and conversion of vitamin D <sub>2</sub> and D <sub>3</sub> , from dietary sources and supplements. ....	29
Figure 1.12: PTH regulating calcium levels.....	33
Figure 1.13: Variation of the bone density of women at different ages.....	45
Figure 2.1: The SONOST 3000 Ultrasound Bone Densitometer (OsteoSys 3000).....	55
Figure 3.1: The prevalence of the MetS in the sample population. ....	59
Figure 3.2: The proportion of participants with zero, one, two, three, four, and five MetS risk factors in (A) the MetS group, (B) the Non-MetS group, and (C) the total sample population. ....	61
Figure 3.3 Prevalence of specific MetS risk factors in (A) the total sample population and in (B) the MetS and Non-MetS groups.....	62
Figure 3.4: Classification of the bone health of the total sample population. ....	62
Figure 3.5: Classification of the bone health status in the MetS and the Non-MetS groups.....	63
Figure 3.6: Prevalence of fractures and stress fractures in the MetS vs. Non-MetS groups.....	63
Figure 3.7: Frequency of participants who were previous, current and non-smokers in (A) the MetS group, (B) the Non-MetS group, and (C) the total population.....	64
Figure 3.8: Frequency of participants who were previous, current, non-consumers and heavy consumers of alcohol in (A) the MetS group, (B) the Non-MetS group, and (C) the total population.....	65



Figure 3.9: The frequency of previous, current and non-contraceptive users in (A) the total sample population, and (B) the MetS and Non-MetS groups. ....	66
Figure 3.10: The proportion of participants engaging in vigorous- and moderate-intensity activities at work, as well as in sport, or recreational activities in (A) the MetS group, (B) the Non-MetS group, and (C) the total sample. ....	66
Figure 3.11: The percentage of participants who walked or cycled for more than ten minutes per day for travelling purposes in (A) the MetS group, and (B) the Non-MetS group. ....	67
Figure 3.12: Frequency of occurrence of normal BMD, osteopenia and osteoporosis in PreM and PostM women. ....	67
Figure 3.13: Classification of participants in (A) the MetS, or (B) Non-Mets groups according to menopausal status. ....	69
Figure 3.14: Descriptive characteristics for (A) age, (B) W:H, (C) BM, and (D) BMI between the MetS and Non-MetS groups with respect to menopausal status (* $p < 0.05$ ; ** $p < 0.01$ ; *** $p < 0.001$ ) ....	71
Figure 3.15: Metabolic syndrome risk factors for women grouped according to their menopausal and metabolic status for (A) waist circumference, (B) HDL-c, (C) systolic blood pressure, (D) diastolic blood pressure, (E) fasting glucose, and (F) triglycerides. Solid lines represent the normal cut-off values (IDF, 2006) (* $p < 0.05$ ; ** $p < 0.01$ ; *** $p < 0.001$ ). ....	72
Figure 3.16: The fasting insulin (A), and LDL-c (B) concentrations between groups. The solid line represents the normal cut-off values (IDF, 2006; Pathcare). ....	73
Figure 3.17: Parathyroid hormone (A), alkaline phosphatase (B), and oestradiol (C) levels between groups with respect to metabolic and menopausal status. ....	73
Figure 3.18: The different measures of bone health, including (A) T-score, (B) Z-score, (C) SOS, (D) BUA, and (E) BQI between the different menopausal and metabolic syndrome groups. ....	74
Figure 3.19: The association between BM and E2, and BMI and E2 in the PreM (20-39 years) groups (A, B), the PreM ( $\geq 40$ years) groups (C, D), and the PostM groups (E, F), respectively. ....	76
Figure 3.20: The association between BM and SOS, and BMI and SOS in the PreM (20-39 years) groups (A, B), the PreM ( $\geq 40$ years) groups (C, D), and the PostM groups (E, F), respectively. ....	77
Figure 3.21: The association between FI and ALP, in the PreM (20-39 years) (A), PreM ( $\geq 40$ years) (D), and PostM (G) groups; the association between FG and ALP, in the PreM (20-39 years) (B), PreM ( $\geq 40$ years) (E) and PostM (H) groups; the association between TG and ALP in the PreM (20-39 years) (C), PreM ( $\geq 40$ years) and PostM groups (I), respectively. ....	79

## LIST OF TABLES

### Chapter 1

Table 1.1: Different classification criteria for the MetS. ....	3
Table 1.2: Ethnic-specific waist circumference cut-off values. ....	4
Table 1.3: Comparison of studies focussing on the prevalence of the MetS. ....	6
Table 1.4: Classification of T-scores: .....	18
Table 1.5: Comparison between different assessment methods used to quantify BMD. .....	20
Table 1.6: Classification of Vitamin D levels. ....	29
Table 1.7: Comparison between 25(OH)D concentrations between MetS and Non-MetS groups in different studies. ....	31

### Chapter 2

Table 2.1: The reference levels of the bone-specific markers.....	51
Table 2.2: Classification of BMI according to the WHO.....	54
Table 2.3: Reference values per gender for waist circumference.....	54

### Chapter 3

Table 3.1: Description of the characteristics of the total sample population. ....	58
Table 3.2: Descriptive characteristics of the MetS vs. Non-MetS individuals.....	60
Table 3.3: Descriptive characteristics of the PreM vs. PostM individuals. ....	68

## LIST OF EQUATIONS

### Chapter 1

<b>Equation 1:</b> Calculation of T-score.....	18
<b>Equation 2:</b> Calculation of Z-score.....	19

### Chapter 2

<b>Equation 3:</b> Calculation of BMI in $\text{kg/m}^2$ .....	53
<b>Equation 4:</b> Calculation of Waist to hip ratio.....	55

# Chapter 1

# Literature Review

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## 1.1 Introduction

The World Bank classified South Africa as a developing and upper middle-income country (World Bank, 2016). South Africa is also part of a global population migration trend of epidemiological transition, known as urbanisation, describing people that migrate from rural to urban regions (Vorster *et al.*, 2011; George *et al.*, 2013). Accompanying this transition, distinct and measurable changes in dietary intake habits, changes in levels of physical activity, as well as socio-psychological behavioural patterns also take place (Steyn *et al.*, 2012). Collectively, these changes impact adversely on public health, resulting in the increased risk of developing chronic non-communicable diseases of lifestyle (NCDs) including type 2 diabetes mellitus (T2DM), obesity and hypertension, and the development of the metabolic syndrome (MetS) (Gill *et al.*, 2009; Vorster *et al.*, 2011; Erasmus *et al.*, 2012; Steyn *et al.*, 2012; Van Zyl *et al.*, 2012).

Obesity, which is considered one of the most important MetS risk factors, was originally believed to exert positive effects on bone health (from here on referred to as BMD) by protecting against osteoporosis-related fractures (Compston, 2013). Several conflicting evidence exists where obesity actually exerts a negative effect on bone mineral density (BMD), warranting further investigation (George *et al.*, 2013; Zillikens *et al.*, 2010). Additionally, opposing results have also been reported on the effect of the individual MetS risk factors, as well as the MetS as a whole entity on BMD. Here, findings suggest obesity to be protective against bone loss, whereas the low-grade inflammation that is associated with the MetS, promotes bone loss (Zillikens *et al.*, 2010; Jeon *et al.*, 2011; Cohen *et al.*, 2013; Alissa *et al.*, 2014; Nóbrega da Silva *et al.*, 2014; Li *et al.*, 2015).

The natural transition from pre-menopausal (PreM) to post-menopausal (PostM) status is also believed to be related to an increased prevalence of the MetS in women (Ebrahimpour *et al.* 2010). Evidence further suggests that menopausal status, or oestrogen deficiency, modifies BMD, with a decrease in BMD in PostM women (Alissa *et al.*, 2014; Bączyk *et al.*, 2012; Jeon *et al.*, 2011; You *et al.*, 2014). The abovementioned findings might therefore suggest a possible association between the MetS, menopausal status and BMD in women. However, limited South African studies report on the association between the MetS, menopausal status and bone health, which necessitates further investigation (Awotedu *et al.*, 2010; Hoebel & Malan, 2011; Kengne *et al.*, 2012; Motala *et al.*, 2011; Okafor, 2012; Peer *et al.*, 2014).

## 1.2 The metabolic syndrome

The MetS is defined as a cluster of cardio-metabolic risk factors which includes elevated blood glucose levels, dyslipidaemia, high blood pressure (BP) and central obesity, which in turn increases the risk of developing cardiovascular diseases (CVD), and lifestyle-associated diseases such as T2DM (Miranda *et al.*, 2005; Hossein-Nezhad *et al.*, 2009; Huang, 2009; Kim & Halter, 2014; Alissa *et al.*, 2014; International Diabetes Federation, 2006). Furthermore, the cut-off values of the individual MetS-associated risk factors differ depending on the definition used for the diagnosis of the MetS.

### 1.2.1 Definitions and classifications of the metabolic syndrome

Several criteria and definitions aim to characterise individuals with the MetS; nevertheless, only the most widely used are summarised in Table 1.1. The World Health Organization (WHO) describes the MetS as the presence of glucose intolerance, impaired glucose tolerance (IGT) or diabetes and/or insulin resistance (IR), together with two or more of the listed factors in Table 1.1.; whereas the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) definition involves any three of the WHO risk factors (IDF, 2006). According to the International Diabetes Federation's (IDF) definition, an individual must present with central obesity (increased waist circumference (WC)), and at least two of the following risk factors: (i) raised triglycerides (TG), (ii) reduced high-density lipoprotein cholesterol (HDL-c), (iii) raised BP, or (iv) raised fasting plasma glucose (FPG). Since central obesity is the fundamental risk factor according to the IDF, gender and ethnic based cut-off values were subsequently compiled (Table 1.2). However, since no current cut-off values are available for WC measurements for sub-Saharan African populations, the IDF recommended to use WC measures of Europeans until specific data are made available for these populations (Alberti *et al.*, 2005; IDF, 2006; Akintunde *et al.*, 2011; Ramli *et al.*, 2013).

In an effort to link WC to the risk of developing T2DM and CVDs, the Joint Interim Statement (JIS) combined data from the IDF Task Force on Epidemiology and Prevention; National Heart, Lung and Blood Institute, American Heart Association, World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. It was subsequently decided to remove abdominal obesity as the key risk factor from its definition, and to include at least three risk factors for MetS classification, in order to possibly identify more MetS cases compared to all other definitions used (IDF, 2006; Alberti *et al.*, 2009).

Table 1.1: Different classification criteria for the MetS.

Classification method	World Health Organization (WHO) (1999)	European Group on Insulin Resistance (EGIR) (1999)	National Cholesterol Education Program's (Adult Treatment Panel III, ATP III) (2002)	International Diabetes Federation (IDF) (2006)	Joint Interim Statement (JIS) (2009)
<b>Risk factors</b>					
<b>Abdominal obesity/ Waist circumference</b>	BMI >30 kg/m <sup>2</sup> and/or	Males: ≥94 cm	Males: >102 cm	Ethnic-specific (Table 1.2)	Males: ≥90 cm
	W:H: Males: >0.90				Females: ≥80 cm
	W:H: Females: >0.85	Females: ≥80 cm	Females: >88 cm		
<b>Elevated TG</b>	≥1.7 mmol/L	>2.0 mmol/L	≥1.7 mmol/L	≥1.7 mmol/L or specific treatment for lipid abnormality	≥1.7 mmol/L or on treatment for elevated TG
<b>HDL-cholesterol (Reduced)</b>	Males: <0.9 mmol/L	Males & females: <1.01 mmol/L or treatment	Males: <1.04 mmol/L	Males: <1.03 mmol/L; or specific treatment for lipid abnormality	Males: <1.0 mmol/L or on treatment for elevated HDL-c
	Females: <1.0 mmol/L		Females: <1.29 mmol/L	Females: <1.29 mmol/L or specific treatment for lipid abnormality	Females: <1.3 mmol/L or on treatment for elevated HDL-c
<b>Blood pressure (Raised)</b>	≥140/90 mmHg	≥140/90 mmHg or treatment	≥130/85 mmHg	SBP ≥130 mmHg or DBP ≥85 mmHg or treatment of previously diagnosed hypertension	SBP: ≥130/85 and/or DBP ≥85 mmHg or treatment of previously diagnosed hypertension
<b>Raised FPG</b>		≥6.1 mmol/L (non-diabetic)	≥5.6 mmol/L	FPG ≥5.6 mmol/L, or previously diagnosed with T2DM If >5.6 mmol/L, oral glucose tolerance test is strongly recommended but is not necessary to define presence of the syndrome	≥5.6 mmol/L or on treatment for elevated glucose
<b>Micro-albuminuria</b>	NA	NA	Urinary albumin excretion rate ≥20 µg/min or albumin: creatinine ratio ≥30 mg/g	NA	NA

(Alberti *et al.*, 2005; Ramli *et al.*, 2013). BMI: body mass index; DBP: diastolic blood pressure; FPG: fasting plasma glucose; NA: not applicable; SBP: systolic blood pressure; TG: triglycerides; W:H: waist-to-hip ratio.

Table 1.2: Ethnic-specific waist circumference cut-off values.

Country/Ethnic group	Gender	Waist circumference
<b>Europeans</b>	Male	≥94 cm
	Female	≥80 cm
<b>United States of America (ATP III)</b>	Male	≥102 cm
	Female	≥88 cm
<b>South Asians Based on a Chinese, Malay and Asian-Indian population</b>	Male	≥90 cm
	Female	≥80 cm
<b>Chinese</b>	Male	≥90 cm
	Female	≥80 cm
<b>Japanese</b>	Male	≥90 cm
	Female	≥80 cm
<b>Ethnic South and Central Americans</b>	Use South Asian recommendations until more specific data are available	
<b>Sub-Saharan Africans</b>	Use European data until more specific data are available	
<b>Eastern Mediterranean and Middle East (Arab) populations</b>	Use European data until more specific data are available	

(IDF, 2006).

Additionally, several studies found that the IDF criteria identifies more individuals, especially women, with the MetS compared to the NCEP-ATP III definition (Kelliny *et al.*, 2008; Kengne *et al.*, 2012; Tran *et al.*, 2011). One particular study compared three different definitions (WHO, NCEP-ATP III and IDF) and reported that the higher prevalence according to the IDF definition was likely to be attributed to the low cut-off values of WC (Akintunde *et al.*, 2011) (Table 1.1). A South African and Malaysian study compared the MetS prevalence using three different definitions. Here, they reported that the JIS definition (SA - 26.5%, Malay - 43.3%) identified more individuals, followed by the IDF (SA - 23.3%, Malay - 37.4%), and then the NCEP-ATP III definition (SA - 18.5%, Malay - 26.5%) (Motala *et al.*, 2011; Ramli *et al.*, 2013). Hoebel & Malan (2011) also found that the JIS definition identified more individuals with the MetS, whereas the IDF definition identified the least number of individuals when compared to the NCEP-ATP III definition, possibly due to the exclusion of abdominal obesity in the JIS definition.

The TG values are similar in all definitions except the European Group on Insulin Resistance' (EGIR) definition, which proposed a higher cut-off value of >2.0 mmol/L, compared to >1.7 mmol/L used by other definitions (Bloomgarden, 2004; Alberti *et al.*, 2005; Huang, 2009). The HDL-c cut-off values are similar across the IDF, NCEP-ATP III and JIS definitions (<1.3 mmol/L); whereas the WHO and EGIR definitions recommend a lower concentration (<1.0 mmol/L). Likewise, the BP cut-off values are also similar across the IDF, NCEP-ATP III and JIS definitions (BP: ≥130, and ≥85 mmHg), whereas a higher cut-off value

is used for BP according to the WHO and EGIR definitions (BP:  $\geq 140$  and  $\geq 90$  mmHg). Lastly, the FPG levels ( $\geq 5.6$  mmol/L) are similar in the IDF and JIS definitions; however, the EGIR definition uses a higher cut-off value ( $\geq 6.1$  mmol/L) (Alberti *et al.*, 2005; Huang, 2009).

Globally, many studies report on the prevalence of the MetS using different definitions, mainly because of the population diversity and differences in the definition criteria, as explained above.

### **1.2.2 Incidence and prevalence of the metabolic syndrome**

The MetS is one of the most widespread chronic diseases in the world, and the fourth or fifth leading cause of death in the developed world (IDF, 2006). When comparing the prevalence of the MetS in different world regions, an American study reported a total population prevalence of 34.5%, with a prevalence of 38.1% in the female participants (IDF definition) (Ford, 2005). In an Australian and Danish population, a prevalence of 31.7% and 21.0% were reported respectively, when using the IDF definition (Cameron *et al.*, 2007; Jeppesen *et al.*, 2007).

During 2010, an Indian study (using the IDF criteria) reported a prevalence of 40.0% (Table 1.3) (Ravikiran *et al.*, 2010). When the JIS definition is used, a Nigerian population displayed an overall MetS prevalence of 86.0% (87.0% in females) (Ogbera, 2010), whilst a more recent Nigerian study reported an overall prevalence of 34.0% (62.0% in females), when the IDF definition was used (Iloh *et al.*, 2014). Other studies are summarised in Table 1.3.

When investigating differences in ethnic groups, Hoebel & Malan (2011) reported that the MetS prevalence was much higher in non-Caucasians vs. Caucasians, and Peer *et al.* (2014) reported a 74.3% prevalence rate in a Black population in Cape Town, with prevalence 43.5% in females. Furthermore, when focussing on menopausal status, Goyal *et al.* (2013) reported a significant increase in the prevalence of the MetS from PreM (10.0%) to peri-Menopausal (PeriM) (41.7%), to PostM women (46.0%) ( $p < 0.001$  in both cases). In agreement, other studies also reported an increased prevalence of the MetS in PostM women in comparison to PreM women (Maharlouei *et al.*, 2013; Jesmin *et al.*, 2013).

From these studies, it is evident that differences in prevalence rates exist in different world regions, which could possibly be attributed to ethnicity, age, menopausal status and lifestyle differences. Additionally, the use of different definitions of the MetS might also result in differences in prevalence reported (Cameron *et al.*, 2004).



Table 1.3: Comparison of studies focussing on the prevalence of the MetS.

Authors	Country	Definition	Number of participants	Conclusions
<b>Hoebel &amp; Malan, (2011)</b>	South Africa (North West Province)	JIS (2009), NCEP-ATP III (2001), IDF (2005),	Total: n=409 Africans: males: n=101; females: n=99 Caucasians: males: n=101; females: n=108	The JIS definition included more people with the MetS, whereas the IDF has the lowest prevalence of the MetS. More Africans presented with the MetS than their Caucasian counterparts.
<b>Motala <i>et al.</i> (2011)</b>	South Africa	JIS (2009), Modified NCEP-ATP III (2001), IDF (2005),	n=947 participants n=758 females	MetS: 22.1% overall, higher in females (25.0%) than males (10.5%). The optimal WC cut-off point to predict the presence of at least two other components of the MetS was 86 cm for males and 92 cm for females. MetS prevalence higher with the JIS definition (26.5%) than with the IDF (~23.3%), or the modified ATP III (18.5%) criteria.
<b>Peer <i>et al.</i> (2014)</b>	South Africa	JIS (2009)	n=1099 participants: n=392 males, n=707 females	Prevalence: significantly higher in females (43.5%) vs males (16.5%) (in a Black South African population group).
<b>Kengne <i>et al.</i> (2012)</b>	Sub-Saharan Africa	IDF (2005), NCEP-ATP III (2001)	n=308 participants with T2D: n=157 males, n=151 females	Higher prevalence with IDF definition (71.7%) than NCEP-ATP III definition (60.4%). Significantly higher rates of the MetS in females than males (independent of definition).
<b>Ogbera, 2010</b>	Nigeria (Africa)	JIS (2009)	n= 973 patients with diabetes mellitus: n= 703 males, n=260 females	MetS (n=834): prevalence rate of 86.0%. Females with the MetS 87.0%; males with the MetS 83.0%.
<b>Iloh <i>et al.</i> (2014)</b>	Nigeria (Africa)	IDF (2005)	Total: n=365 Males: n=187 Females: n=178	MetS present: n=124 (34.0%) MetS: males n=47 (37.9%); females n=77 (62.1%)
<b>Kelliny <i>et al.</i> (2008)</b>	Seychelles (Africa)	NCEP-ATP (2005), WHO (1998), IDF (2005)	n=1255 participants	According to the ATP, WHO and IDF definitions, the prevalence of the MetS was, respectively, 24.0%, 25.0%, 25.1% in males 32.2%, 24.6%, 35.4% in females.
<b>Alkerwi <i>et al.</i> (2011)</b>	Luxembourg (Europe)	JIS (2009), IDF	n=1349 participants	JIS definition diagnosed 28.0% of participants with the MetS and there was a high agreement between the definitions.
<b>Riediger &amp; Clara, 2011</b>	Canada	NCEP-ATP III (2001)	n=1800 participants	Overall MetS prevalence: 19.1% ( $\pm 1.7$ ) Males: 17.8% ( $\pm 2.0$ ) Females: 20.5% ( $\pm 2.1$ ).
<b>Lao <i>et al.</i> (2012)</b>	China	IDF	n= 6468 residents	The prevalence of the MetS in this population was 7.3% Males: 5.3% Females: 9.0%.
<b>Ramli <i>et al.</i> (2013)</b>	Malaysia	NCEP-ATP III (2005), IDF (2005), JIS (2009)	Total: n= 8836 subjects n=3766 males; n=5070 females	JIS had the highest overall prevalence (43.4%); IDF (37.4%); NCEP-ATP III (26.5%). Female participants were more likely to have the MetS compared to males according to the NCEP-ATP III and IDF definitions, but not with the JIS definition.

### 1.2.3 Pathophysiology of the metabolic syndrome

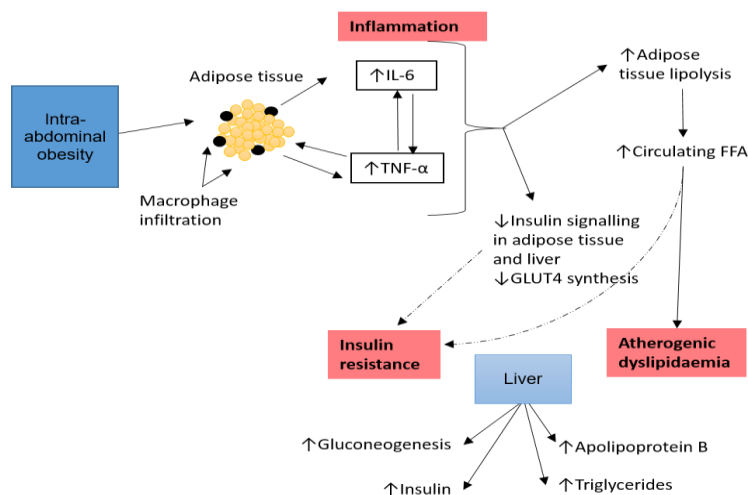
All the individual MetS risk factors exert different, as well as associated pathophysiological effects in the different physiological systems. The mechanisms and effects of the individual risk factors will be described subsequently.

#### 1.2.3.1 Obesity

Adipose tissue (AT) consists of adipocytes, stromal pre-adipocytes, immune cells and endothelium (Kaur, 2014). This metabolically active tissue can rapidly transform in response to excess caloric intake, or reduced caloric expenditure due to physical inactivity (Hardy *et al.*, 2012; Bays *et al.*, 2013; Iyengar *et al.*, 2015). Adipocytes can either undergo hypertrophy due to increased storage of TG (Blüher *et al.*, 2013) or hyperplasia (Figure 1.1) (Hardy *et al.*, 2012; Iyengar *et al.*, 2015). Excessive adipocyte hypertrophy leads to AT growth beyond vascular supply, inadequate angiogenesis, and AT hypoxia. The latter is involved in increased inflammatory marker expression in AT, as well as ectopic fat deposition in the liver, muscle, and the pancreas. In addition, excessive adipocyte hypertrophy also leads to mitochondrial and endoplasmic reticulum dysfunction, hormone dysregulation, impaired storage of fatty acids (FA) and increased circulating free fatty acids (FFAs) (Figure 1.1) (Bays, 2011; Bays *et al.*, 2013).

Both increased FFAs and altered adipokine production plays critical roles in the development of obesity-related metabolic complications (Lee *et al.*, 2013). Free fatty acids increase insulin secretion, and decrease insulin sensitivity in both skeletal muscle and the liver, leading to increased secretion of very low-density lipoprotein (VLDL), which are linked to endothelial dysfunction and atherosclerosis (Lee *et al.*, 2013).

Abdominal obesity has been linked to predictors of CVD, diabetes mellitus, and the development of conditions such as hypertension and atherosclerosis (Lu *et al.*, 2010). Furthermore, abdominal obesity has also been associated with metabolic abnormalities such as IR, hyperinsulinaemia, elevated TG, hypertension and glucose intolerance (Jennings *et al.*, 2009; Lu *et al.*, 2010). As previously mentioned, in states of obesity and adipocyte hypertrophy, blood supply to the adipocytes is reduced, causing hypoxia, tissue necrosis, as well as macrophage infiltration around the AT (Figure 1.2) (Kaur, 2014). Figure 1.1 illustrates how abdominal obesity may contribute to the development of dyslipidaemia and IR, *via* the increased release of pro-inflammatory markers and its downstream effects.



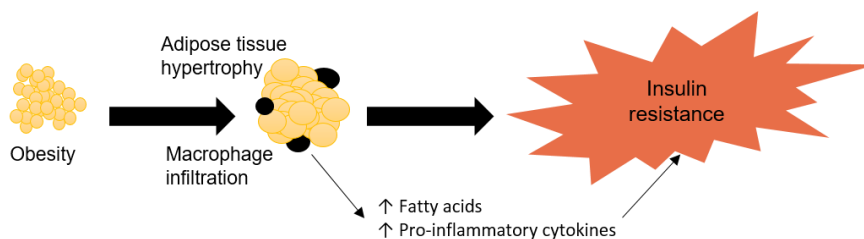
**Figure 1.1: The development of metabolic complications via abdominal obesity.**  
(Adapted from Cartier, 2010).

Evidence suggests that macrophage infiltration in subcutaneous adipose tissue (SAT) lead to a state of chronic low-grade inflammation in individuals with the MetS. This state activates pro-inflammatory markers such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- $\alpha$ ), that leads to both increased lipolysis and mobilisation of FFAs (Figure 1.1) (Cartier, 2010; Bremer *et al.*, 2011; Lee *et al.*, 2013). Other effects could be attributed to decreased insulin signalling in AT, as well as the liver, and decreased synthesis and translocation of glucose transporter type 4 (GLUT4), resulting in IR (Cartier, 2010; Lee *et al.*, 2013).

Menopausal status also contributes to increased abdominal adiposity, as stated by Sapkota *et al.*, (2015), that there is an increased in abdominal adiposity independent of the effect of age and total body adiposity. The increased WC and FM during the menopausal transition is due to decreased ovarian oestrogen secretion (Sowers *et al.*, 2007; Carr, 2003; Goyal *et al.*, 2013).

### 1.2.3.2 Insulin resistance

Insulin resistance (IR) is defined by a chronically elevated plasma insulin concentration that fails to lower blood glucose levels (Han & Lean, 2014). In the context of the MetS and obesity, the development of IR is primarily facilitated by increased abdominal obesity, which stimulates the secretion of the pro-inflammatory markers TNF- $\alpha$ , which is expressed by macrophages in the AT, and reduce both insulin secretion and sensitivity, further contributing to the development of IR (Figure 1.1 & 1.2) (Cartier, 2010).



**Figure 1.2: The link between obesity and the development of insulin resistance.**

(Adapted from Castan-Laurell *et al.*, 2012).

Tumour necrosis factor-alpha is also involved in initiating lipolysis, which increases circulating FFA levels (Cartier, 2010). Interleukin-6 on the other hand, affects both glucose and lipid metabolism, and improves insulin sensitivity, as well as glucose tolerance (Piya *et al.*, 2013).

Jeon *et al.* (2011) reported that insulin levels were higher in both MetS PreM women ( $6.10 \pm 4.87$  mU/L) compared to control PreM women ( $3.86 \pm 2.37$  mU/L;  $p=0.000$ ). Post menopausal (PostM) women with the MetS showed higher insulin concentrations ( $5.88 \pm 2.95$  mU/L) compared to Non-MetS PostM women ( $4.62 \pm 6.40$  mU/L;  $p=0.030$ ). Similarly, Alissa *et al.* (2014) reported that PostM women, serum insulin levels were significantly higher in the MetS group ( $17.68 \pm 18.15$   $\mu$ M/mL) than in Non-MetS group ( $12.15 \pm 9.38$   $\mu$ M/mL;  $p < 0.01$ ). It is thus concluded that the MetS increases insulin concentration significantly, giving rise to glucose intolerance.

In addition to the MetS altering insulin levels, the presence of menopause or oestrogen deficiency is also known to alter insulin levels (Matsui *et al.*, 2013). According to Matsui *et al.* (2013), oestrogen might have a protective effect against insulin resistance in PreM women. Matsui *et al.* (2013) reported higher levels of insulin in PreM women, although this was not significantly higher than in the PostM group.

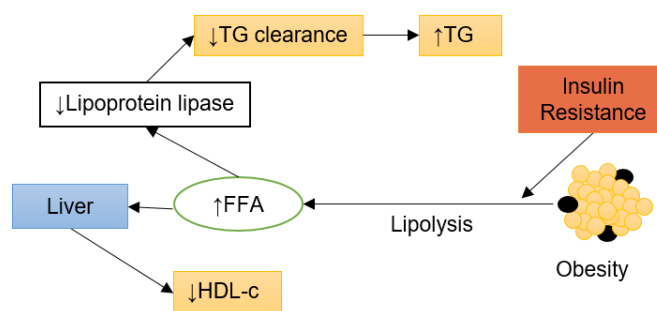
### 1.2.3.3 Glucose intolerance

Most individuals that present with the MetS also experience some level of glucose intolerance (Aganović & Dušek, 2004). Although there are many potential reasons for this, it is mostly associated with defects in the functioning of insulin and binding to the insulin receptor, i.e. failure to suppress gluconeogenesis in the liver, and the mediation of glucose uptake in insulin-sensitive tissues (muscle and AT) (Aganović & Dušek, 2004). In such cases, and because of decreased GLUT 4 synthesis and translocation to the cell membranes, there is insufficient glucose uptake into tissue, resulting in hyperglycaemia (Aganović & Dušek, 2004; Cartier, 2010).

Heianza *et al.*, (2013) reported that older age and menopausal status independently and additively influenced the high prevalence of dysglycemia in Japanese women. Jeon *et al.* (2011) reported elevated glucose levels in PreM women with the MetS ( $5.69 \pm 1.96$  mmol/L) compared to their Non-MetS ( $4.70 \pm 0.5$  mmol/L;  $p=0.000$ ) counterparts, with a similar observation for PostM MetS women ( $5.49 \pm 1.18$  mmol/L) and PostM Non-MetS women ( $4.84 \pm 0.58$  mmol/L;  $p=0.000$ ). In a study comparing only MetS and Non-MetS PostM women, fasting blood glucose (FBG) levels were reported to be significantly higher in the MetS ( $8.66 \pm 0.54$  mmol/L) than Non-MetS women ( $6.0 \pm 0.93$  mmol/L;  $p < 0.0001$ ) (Alissa *et al.*, 2014). It is therefore evident that irrespective of menopausal status, elevated glucose levels are present in individuals with the MetS.

#### 1.2.3.4 Dyslipidaemia

Dyslipidaemia describes alterations in the structure, metabolism, and biological activities of atherogenic lipoproteins and anti-atherogenic HDL-c (Kaur, 2014). Dyslipidaemia is further characterised by elevated levels of total cholesterol (TC), TG, low-density lipoprotein cholesterol (LDL-c), and low levels of HDL-c (Kaur, 2014; Mandal, 2015). These blood lipid fractions are also associated with the MetS, where elevated LDL-c levels are more common in individuals with increased abdominal AT (Han & Lean, 2014). There is also a strong correlation between IR and atherogenic dyslipidaemia (Han & Lean, 2014). Under normal metabolic states, insulin suppresses lipolysis in adipocytes, but during IR there is impaired insulin signalling which results in higher levels of lipolysis and elevated FFA levels (Figure 1.3) (Kaur, 2014; Jung & Choi, 2014).



**Figure 1.3: Pathway of increased adipose tissue contributing to dyslipidaemia.**  
(Adapted from Jung & Choi, 2014).

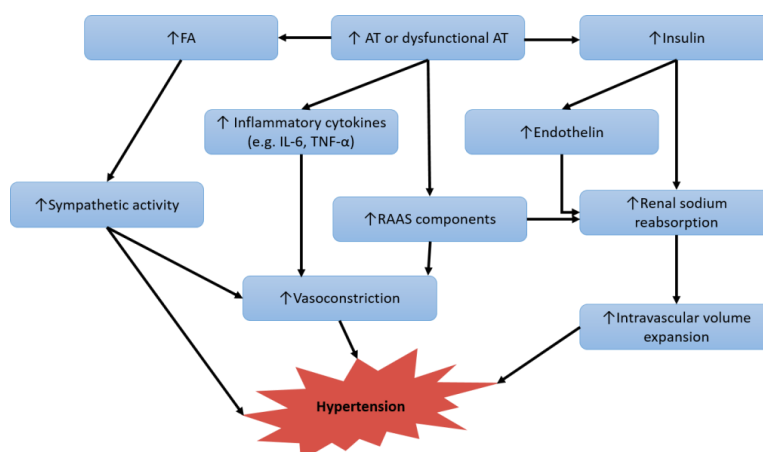
Free fatty acids serve as a substrate for TG synthesis in the liver, and further stabilises the production of apolipoprotein B, which is the major lipoprotein of VLDL particles, resulting in higher VLDL concentrations (Lee *et al.*, 2013; Kaur, 2014; Jung & Choi, 2014). The TG in VLDL is exchanged for cholesteryl esters from LDL and HDL by cholesteryl ester transport protein, resulting in the production of TG-rich LDL and HDL. Furthermore, the TGs in LDL and HDL are hydrolysed by hepatic lipases that produce small dense LDL and HDL (Jung &

Choi, 2014). Therefore, both obesity and hyperinsulinaemia contribute to the altered lipid levels present in the MetS (Gierach *et al.*, 2014; Jennings *et al.*, 2009). When considering menopausal status, Bade *et al.* (2014) reported increased levels of TG, LDL-c and decreased levels of HDL-c in PostM women in comparison to PreM women, possibly due to hormonal changes.

### 1.2.3.5 Hypertension

All of the MetS risk factors have been associated with the development of hypertension, with obesity, glucose intolerance and dyslipidaemia being the most commonly reported risk factors associated with the development hypertension (Kaur, 2014). Central obesity, measured by an increased WC, contributes to the development of hypertension *via* the secretion of adipokines (TNF- $\alpha$ , IL-6, adiponectin) and dyslipidaemia (Bodea & Popa, 2015). Additionally, endothelial dysfunction, oxidative stress and vascular inflammation further promote the development of hypertension in the context of the MetS (Figure 1.4) (Bodea & Popa, 2015).

Excess adipose tissue expresses all components of the renin-angiotensin-aldosterone systems. The latter is activated by both hyperglycaemia and hyperinsulinaemia, which may contribute to the development of hypertension in individuals with IR (Ahima & Flier, 2000; Cassis *et al.*, 2008; Kaur, 2014). Some research suggested that IR and hyperinsulinaemia lead to the activation of the sympathetic nervous system (SNS) that plays a central role in the regulation of metabolic processes in the body (Da Silva, 2009; Horita *et al.*, 2011). Here, the kidneys are stimulated to increase sodium reabsorption, the heart to increase cardiac output, and vasoconstriction of the arteries, resulting in an increased peripheral resistance and ultimately hypertension (Kaur, 2014).



**Figure 1.4: The development of hypertension *via* increased AT.**  
(Adapted from Bogaert & Linas, 2009).

Menopausal status is also known to contribute to the development of hypertension (Carr, 2003; Goyal *et al.*, 2013). During menopause the changes in the oestrogen/androgen ratio leads to endothelial dysfunction, increased endothelin secretion and decreased nitric oxide production that contributes to increased oxidative stress, renal vasoconstriction and ultimately hypertension (Coylewright & Ouyang., 2008). In addition, the changes in the oestrogen/androgen ratio also results in increased BMI that also contribute to oxidative stress that triggers renal vasoconstriction and hypertension (Coylewright & Ouyang., 2008). The increased BMI also leads to sympathetic activation, further increasing renin release and increased angiotensin II, renal vasoconstriction and the development of hypertension (Coylewright & Ouyang., 2008).

#### **1.2.4 The metabolic syndrome and menopause**

There appears to be a strong link between an increased prevalence of the MetS and ageing in women, specifically during the transition from PreM to PostM (Ebrahimpour *et al.*, 2010). The oestrogen deficiency that occurs during this period exacerbates the MetS and appears to be associated with an increased risk for the development of most of the individual MetS risk factors (Ebrahimpour *et al.*, 2010; Eshtiaghi *et al.*, 2010). Eshtiaghi *et al.* (2010) found that menopause was an independent predictor of the MetS, which may be due to oestrogen deficiency that alters lipid metabolism, causes changes in body fat distribution, and affects insulin action on the arterial wall (Eshtiaghi *et al.*, 2010). In agreement, Pandey *et al.* (2010) and Jesmin *et al.* (2013) reported a higher prevalence of the MetS in their PostM study participants (55.0% and 39.3% respectively) than in the PreM group (45.0% and 16.8%, respectively  $p < 0.0001$  and  $p < 0.001$ ). Goyal *et al.* (2013) and Maharlouei *et al.* (2013) made similar conclusions.

Apart from the association between menopause and the increased prevalence of the MetS, there also appear to be changes in bone health and BMD in women (AIDughaiter *et al.*, 2015; Al-Safi & Polotsky, 2014).

### **1.3 Bone**

Bone tissue provides mechanical support and protection to the structure of the body, as well as aiding in mineral homeostasis, and serves an important endocrine function in the body (Burr & Allen, 2013). Since one of the most important focusses of this research entailed BMD and the possible associations with the MetS and menopausal status, a brief overview of the basic bone physiology and pathophysiology will be provided.



### 1.3.1 Basic bone physiology

#### 1.3.1.1 Composition of bone

Bone tissue consists of specialised cells immersed in a mineralised extracellular matrix (ECM), which is composed of an inorganic matrix ( $\pm 65\%$ ), providing density to bone (Figure 1.5A). The inorganic matrix mainly consists of calcium and phosphate as hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ], while the organic matrix consists of a flexible type I collagen fibre framework, as well as an amorphous ground substance (Clarke, 2008; Ross *et al.*, 2011; Sharp, 2011; Johnson, 2013; Burr & Allen, 2013).

Eighty percent of bone tissue consists of a calcified cortical (compact/dense) component surrounding the marrow space, to provide both structural and protective support (Figure 1.5B) (Clarke, 2008; Kini & Nandeesh, 2012; Walsh, 2014). The remaining 20% constitutes the less calcified trabecular component that appears as a honeycomb network-like structure of trabecular plates with open cell-filled spaces (Clarke, 2008; Kini & Nandeesh, 2012; Walsh, 2014). The cortical bone has a periosteal (outer) and endosteal (inner) surface (Clarke, 2008; Kini & Nandeesh, 2012).

The periosteum consists of a fibrous connective tissue sheath, surrounding the outer cortical surface of the bone, except where joints are present. Here, the bone is covered with an articular cartilage containing a blood vessel network, nerve fibres, osteoblasts and osteoclasts, and functions to protect, nourish and aid in bone formation (Kini & Nandeesh, 2012). The endosteum consists of a membranous structure covering the inner surface of the cortical and cancellous bone, as well as the Volkmann's canals (Kini & Nandeesh, 2012).

There are mainly three cell types present in mature bone; (i) osteoblasts, (ii) osteoclasts, and (iii) osteocytes (Johnson, 2013; Kini & Nandeesh, 2012). Osteoblasts originate from the mesenchymal stem cells, or osteoprogenitor cells, of the bone marrow stroma (Kini & Nandeesh, 2012), after which they translocate to the surface of the bone tissue (Johnson, 2013). Osteoblasts are 'specialised bone-forming' cells, which produce enzymes and osteoid, a mixture of collagen and other proteins, providing a binding site for hydroxyapatite (Kini & Nandeesh, 2012; Silverthorn, 2013). Osteoblasts deposit bone matrix and produce type 1 collagen, non-collagenous proteins and regulatory factors. Osteoblasts further regulate osteoclasts, and mutually function to maintain homeostasis through continuous remodelling (Johnson, 2013). This process is particularly important for calcium ( $\text{Ca}^{2+}$ ) and phosphate homeostasis, and the adaptation to external tensional forces (Johnson, 2013; Kini & Nandeesh, 2012).

Osteoclasts are responsible for bone resorption *via* the secretion of hydrogen ions and the cathepsin K enzyme, by attaching around the periphery of a section of bone matrix



(Johnson, 2013; Kini & Nandeesh, 2012). The acid acidifies the resorption compartment, which are located beneath the osteoclast to dissolve the mineral component of bone matrix, whereas the cathepsin K digests the proteinaceous matrix (Clarke, 2008).

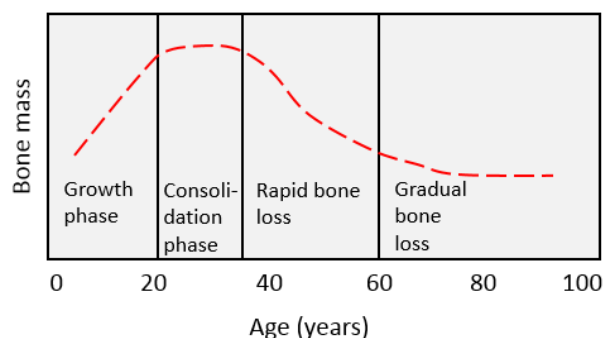
Osteocytes, the most abundant type of bone cell, develop when osteoblasts differentiate, and function in conjunction with osteoblasts to form bone matrix (Clarke, 2008; Johnson, 2013; Walsh, 2014). These cells also develop the ability to secrete bone matrix, and depending on the entrapment of some osteoblasts in the secreted bone matrix, the resultant osteoclasts will gradually stop secreting osteoid (Kini & Nandeesh, 2012).

### **1.3.1.2 Bone mineral density**

Bone mineral density describes the health of an individual's bones (National Osteoporosis Foundation, 2015; Osteogenesis Imperfecta Foundation, 2007; Office of the Surgeon General, 2004). Bone mineral density is defined as the quantity of bone mass per unit volume, or per unit area, and is a measure of the concentration of calcium and phosphorus in a specified volume of bone (Office of the Surgeon General, 2004; Osteogenesis Imperfecta Foundation, 2007; Lee *et al.*, 2010). When referring to bone strength, a combination of two main features are involved, namely: (i) bone density, and (ii) bone quality, where the latter refers to the architecture (for example trabecular, connectivity and orientation), turnover, damage accumulation and mineralisation of bone (Lee *et al.*, 2010).

### **1.3.1.3 Bone growth**

Four distinct phases of bone growth exist. The "growth phase" starts as a rapid phase lasting until late puberty (Office of the Surgeon General, 2004), followed by the consolidation phase, where the acquisition of bone mass occurs due to mechanical stresses, weight training and body weight (Office of the Surgeon General, 2004). Peak bone mass (PBM) is reached during adolescence, and during the remodelling phase, bone mass is maintained for a few years (Figure 1.5) (Mosca *et al.*, 2014; Velickovic *et al.*, 2013). Both testosterone and oestrogen are important sex hormones influencing skeletal growth, maturation and maintenance of bone (Karsenty, 2012). Oestrogens specifically exert an inhibitory effect on osteoclasts and promote osteoclast apoptosis. In contrast, male and female testosterone inhibits both osteoclast activity and osteoblast apoptosis and stimulates osteoblast proliferation and differentiation (Karsenty, 2012; Taie & Rasheed, 2014).



**Figure 1.5: Different phases of bone growth throughout life, including the growth phase, consolidation phase which is followed by the rapid and gradual bone loss phases.**

(Adapted from Online: [http://general.utpb.edu/fac/eldridge\\_j/kine3350/chapter\\_18\\_Review.htm](http://general.utpb.edu/fac/eldridge_j/kine3350/chapter_18_Review.htm)).

Bone remodelling is a homeostatic process where bone is renewed because of microdamage, and to help maintain strength and mineral homeostasis (Clarke, 2008; Walsh, 2014). In order to establish the rate and balance of bone remodelling, bone turnover markers are used (Walsh, 2014).

#### 1.3.1.4 Bone turnover markers

Early changes in bone turnover, including both bone formation and bone resorption, can predict long-term changes in BMD. The use of bone turnover markers is therefore considered a useful clinical tool for determining current, as well as future bone health (Schafer *et al.*, 2010).

#### Bone formation markers

The process of bone formation is regulated by osteoblasts (Wheater *et al.*, 2013). Bone formation markers are either by-products of active osteoblasts, expressed during the various phases of osteoblast development, or by osteoblastic enzymes (Wheater *et al.*, 2013).

#### Alkaline phosphatase

Alkaline phosphatase (ALP) is present in high concentrations in the liver, bone, kidney and intestines, although the ALP found in circulation primarily originates from either the liver or bone (Shipman *et al.*, 2013). Bone-specific alkaline phosphatase (BAP) is synthesised by osteoblasts and is involved in the calcification and mineralisation of bone matrix (Clarke, 2008; Drechsler *et al.*, 2011; Roudsari & Mahjoub, 2012).

The reference levels of ALP are both age- and gender-specific, with a gradual increase in ALP between the ages of 40 and 65 years in women (Shipman *et al.*, 2013). Gossiel *et al.* (2014) provided evidence to support this, where serum BAP was reported to be lower in younger women (35.5±2.9 years, BAP: 10.9±4.2 ng/mL) compared to older women (67.1±7.1 years, BAP: 15.1±6.4 ng/mL). Similarly, Chinese PreM women had significantly

lower BAP levels ( $17.3 \pm 6.4$  U/L) than the PostM group ( $29.9 \pm 12.1$  U/L;  $p=0.000$ ) (Wang *et al.*, 2013).

Concerning the MetS, Kim *et al.* (2013b) reported that higher ALP levels were able to predict the development of the MetS in a middle-aged female Non-MetS population. Here, it is postulated that ALP can act as a marker of visceral adiposity or hepatic steatosis, as well as low-grade inflammation. During adipogenesis, ALP activity increases in pre-adipocytes with concomitant lipid accumulation (Lowe *et al.*, 2011). It is also thought that ALP is linked to vitamin D levels, since higher BAP levels were associated with vitamin D deficiency ( $<50$  nM) (Frost *et al.*, 2010), and low vitamin D levels were reported in MetS individuals (Kim, 2015).

### **Procollagen type I N pro-peptide**

The most abundant protein in bone, procollagen type I N pro-peptide (PINP), is synthesised by osteoblasts and is deemed a precursor molecule of type I collagen (Vasikaran *et al.*, 2011; Wheeler *et al.*, 2013; Krege *et al.*, 2014; Madar *et al.*, 2015). Concentrations of PINP differed significantly between PreM ( $36.4$  ng/mL) and PostM women ( $43.3$  ng/mL;  $p<0.01$ ) (Niemann *et al.*, 2013). Furthermore, Gossiel *et al.* (2014) also indicated that older females (age  $42.5 \pm 22$  years) had higher PINP concentrations ( $67.1 \pm 7.1$  ng/mL) than younger females (age:  $35.7 \pm 18$  years; PINP:  $35.5 \pm 2.9$  ng/mL).

### **Osteocalcin**

Osteocalcin, bound to hydroxyapatite, is produced by osteoblasts, which influences osteoid mineralisation (Vasikaran *et al.*, 2011; Marrone *et al.*, 2012; Wheeler *et al.*, 2013). A significant difference was reported in osteocalcin levels in Non-MetS PostM women with osteoporosis and osteopenia (Bączyk *et al.*, 2012). When the MetS was integrated in a PostM study, osteocalcin was more strongly associated with bone mass than any of the individual MetS components (Alissa *et al.*, 2014). Confavreux *et al.* (2014) found that osteocalcin concentrations were not significantly different between MetS and Non-MetS participants. In another study comparing PreM and PostM women, osteocalcin levels differed significantly between these two groups (PreM:  $15.40 \pm 6.00$   $\mu$ g/L; PostM:  $21.69 \pm 8.58$   $\mu$ g/L;  $p<0.001$ ) (Jeong *et al.*, 2014). Therefore, menopausal, but not metabolic status seems to affect the osteocalcin levels.

### **Bone resorption markers**

Most of the bone resorption markers are products of the degradation of bone collagen (Wheater *et al.*, 2013). Some of the most frequently used bone resorption markers include carboxy-terminal telopeptide x (CTX), N-terminal cross-linking telopeptide of type I collagen

(NTx), receptor activator of nuclear factor kappa beta ligand (RANKL), and osteoprotegerin (OPG) (Vasikaran *et al.*, 2011; Wheater *et al.*, 2013).

### **Carboxy-terminal telopeptide x**

Carboxy-terminal telopeptide x (CTX) is used as the reference bone resorption marker, which is cleaved from the carboxy (C) terminal of type 1 collagen, which is then subsequently released into circulation (Vasikaran *et al.*, 2011; Wheater *et al.*, 2013). It has been shown that CTx differed significantly between osteopenic and osteoporotic women, and is also associated with an increased fracture risk (Bączyk *et al.*, 2012; Ivaska *et al.*, 2010; Lee & Vasikaran, 2012; Marrone *et al.*, 2012). Furthermore, CTx was reported to be significantly lower in PreM compared to PostM women ( $309 \pm 172 \mu\text{g/L}$  vs.  $546 \pm 256 \mu\text{g/L}$ ;  $p < 0.001$ ) (Jeong *et al.*, 2014).

### **Amino-terminal cross-linking telopeptide of type I collagen**

Amino-terminal cross-linking telopeptide of type I collagen (NTx) is cleaved from the amino (N) terminal of type 1 collagen by cathepsin-K during the process of bone resorption (Wheater *et al.*, 2013). Wang *et al.* (2013) reported a significant difference between NTx levels in PreM ( $13.70 \pm 5.40 \text{ nmoL}$ , bone collagen equivalents) compared to PostM women ( $17.29 \pm 8.1940 \text{ nmoL}$ , bone collagen equivalents;  $p = 0.000$ ).

### **Receptor activator of nuclear factor kappa beta ligand**

Receptor activator of nuclear factor kappa beta ligand (RANKL) is produced by osteoblasts and stimulates differentiation of osteoclasts by binding to receptor activator of nuclear factor kappa beta (RANK) (Azim *et al.*, 2012; Wheater *et al.*, 2013). T-cells can also express RANKL, thereby influencing osteoclastogenesis, which explains the bone loss in diseases with persistent and active inflammatory states (Nagy & Penninger, 2015).

### **Osteoprotegerin**

Osteoprotegerin (OPG) is secreted by osteoblasts and decreases bone resorption by binding to RANK, thus preventing osteoclastogenesis (Wheater *et al.*, 2013). Therefore, it is considered an entrapment substrate, competing with the binding of RANK to RANKL (Pérez de Ciriza *et al.*, 2014; Wheater *et al.*, 2013). Jabbar *et al.* (2011) reported higher OPG in PostM osteoporotic women, and it was suggested that the higher OPG concentrations lead to increased bone turnover and lower BMD. Similarly, Shinkov *et al.* (2014) reported significantly higher OPG levels in PostM vs. PreM women.

In the context of the metabolic status, OPG concentrations were reported to be significantly higher in MetS individuals compared to Non-MetS individuals ( $1255 \pm 46 \text{ pg/mL}$  vs.  $1192 \pm 47 \text{ pg/mL}$ ;  $p < 0.05$ ) (Pérez de Ciriza *et al.*, 2014). This was also confirmed by Bernardi *et al.*

(2014), where the MetS group also showed significantly higher OPG levels in comparison to controls ( $80.62 \pm 4.21$  ng/L vs.  $68.10 \pm 2.55$  ng/L,  $p < 0.0001$ ). Pérez de Ciriza *et al.* (2014) further reported an exponential increase in circulating OPG levels, with increasing numbers of MetS risk factors.

### 1.3.2 Bone pathophysiology

Scientific evidence thoroughly documents correlations between ageing and the increased prevalence of osteopenia and osteoporosis, although the prevalence of osteopenia is far greater compared to osteoporosis (Silva *et al.*, 2015). Additionally, menopause is emerging as a risk factor for the development of these conditions (Silva *et al.*, 2015). In order to describe BMD, T- and Z-scoring systems are used (Taie & Rasheed, 2014).

The T-score aims to describe an individual's BMD either as ideal or peak compared to a healthy 30-year old adult. This score is expressed as standard deviations (SD) relative to a young adult population's ideal BMD, and is both gender- and ethnic-specific (Equation 1) (Fogelman & Blake, 2000; National Institutes of Health, 2012).

#### Equation 1: Calculation of T-score.

$$\text{T-score} = \text{Measured BMD} - \frac{\text{young adult mean BMD}}{\text{young adult SD}}$$

Table 1.4 presents the classification of the T-score into the different bone conditions according to the WHO definition (McComsey *et al.*, 2010; WHO, 2015).

Table 1.4: Classification of T-scores:

Condition	T-score
Normal	Between +1 and -1 SD
Osteopenia	Between -1 and -2.5 SD
Osteoporosis	Below -2.5 SD

(WHO, 2015).

The Z-score is used in comparison with a typical, age-matched individual. It is widely recommended to use the Z-score with children, adolescents and PreM women (Equation 2) (National Osteoporosis Foundation, 2015). However, since low BMD is more common in older patients, this type of scoring can be distorted, and should therefore not be considered sensitive compared to the T-score. As a result, the Z-score is not used to diagnose osteoporosis, but rather to detect whether or not other diseases or conditions (for example kidney failure, hyperparathyroidism or diabetes), or secondary osteoporosis might prevail that could affect bone loss (National Institutes of Health, 2012; National Osteoporosis Foundation, 2015). The International Society for Clinical Densitometry (ISCD) recommends a normal Z-score to be above -2.0 (ISCD, 2015).

**Equation 2: Calculation of Z-score.**

$$\text{Z-score} = \text{Measured BMD} - \frac{\text{age-matched mean BMD}}{\text{age-matched SD}}$$

**1.3.2.1 Osteopenia**

Osteopenia is a well-known precursor of osteoporosis and is often described as a mild form of osteoporosis (Weissman, 2004). It is characterised by a below normal BMD, with a T-score ranging between -1 and -2.5 (WHO, 1994 & 2004). Although numerous physiological factors contribute to the development of osteopenia, ageing and hormone deficiencies (oestrogen deficiency during menopause) are thought to be the main cause of the development of primary osteopenia and osteoporosis (Khosla *et al.*, 2012). Some lifestyle factors which influences BMD include long-term glucocorticoid use, diabetes mellitus, eating disorders (low dietary intake of Ca<sup>2+</sup>, as well as a vitamin D deficiency), smoking, and alcohol abuse (Taie & Rasheed, 2014). Bolton *et al.* (2012) reported that exercise interventions targeting individuals with osteopenia might lower, delay or even prevent the risk for the development of osteoporosis.

**1.3.2.2 Osteoporosis**

Osteoporosis is characterised by decreased BMD, as well as decline of the skeletal microarchitecture, resulting in fragile bones and an increased fracture risk (WHO, 1994 & 2004; Eastell, 2013). There is a significant imbalance in bone turnover, which results in the inability of trabecular bone to remodel after constant micro-trauma, thereby resulting in the development of osteoporosis (Johnson, 2013).

Osteoporosis can be classified as primary or secondary osteoporosis. Primary osteoporosis usually develops as a result of ageing and/or menopause; whereas, hyperparathyroidism, hyperthyroidism, diabetes, vitamin D deficiency and/or the presence of osteoporosis in association with another chronic disease, are classified as secondary osteoporosis (WHO, 1994 & 2004; McComsey *et al.*, 2010).

**1.3.3 Physical bone mineral density assessment methods**

The assessment of BMD is a non-invasive, easy to use method that evaluates bone health status. More advanced bone assessment methods rely on low dose radiation that can either be used to evaluate the whole-body, or a specific body section, i.e. the spine, hips, legs and arms (Osteogenesis Imperfecta Foundation, 2015). Table 1.5 presents a summary and comparison of the different assessment methods used to quantify BMD.

**Table 1.5: Comparison between different assessment methods used to quantify BMD.**

	Positive characteristics	Negative characteristics
<b>DEXA</b>	<ul style="list-style-type: none"> <li>✓ Highly accurate</li> <li>✓ Assessment of reference sites (spine and hip)</li> </ul>	<ul style="list-style-type: none"> <li>- Uses X-rays (radiation exposure)</li> <li>- Expensive</li> <li>- Cannot distinguish between cortical and trabecular bone</li> <li>- Does not discriminate bone microarchitecture</li> <li>- Not useful as a screening tool</li> </ul>
<b>QCT</b>	<ul style="list-style-type: none"> <li>✓ Provides true density values</li> <li>✓ Discriminates between cortical and trabecular bone</li> <li>✓ High-resolution</li> </ul>	<ul style="list-style-type: none"> <li>- Higher radiation dose than DEXA</li> <li>- Limited accessibility</li> <li>- Not useful as a screening tool</li> </ul>
<b>QUS</b>	<ul style="list-style-type: none"> <li>✓ Rapid</li> <li>✓ Inexpensive</li> <li>✓ Radiation free</li> <li>✓ Portable</li> <li>✓ Shorter analysis time than DEXA</li> <li>✓ Useful to investigate bone structural properties</li> <li>✓ Useful screening tool</li> </ul>	<ul style="list-style-type: none"> <li>- Different guidelines for definition of osteoporosis</li> <li>- Measurements only possible at peripheral skeletal sites</li> <li>- Not directly comparable to DEXA</li> </ul>
<b>MRI</b>	<ul style="list-style-type: none"> <li>✓ Volumetric BMD calculations, without losing soft tissue signals</li> <li>✓ Ability to assess trabecular bone tissue</li> <li>✓ No exposure to ionising radiation</li> </ul>	<ul style="list-style-type: none"> <li>- Expensive</li> <li>- Long duration to acquire the scan</li> <li>- Images tend to be less detailed</li> </ul>

(Adapted from Pisani *et al.*, 2013). DEXA: dual-energy X-ray absorptiometry, QCT: quantitative computed tomography, QUS: quantitative ultrasound, MRI: magnetic resonance imaging.

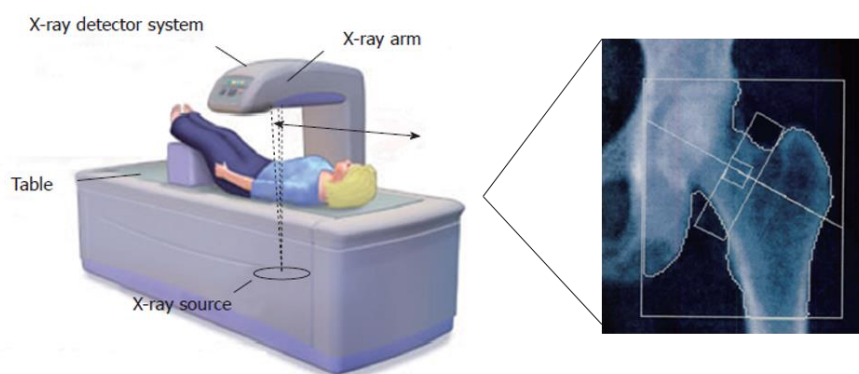
The major differences observed here are the radiation dose exposure, the discrimination between cortical and trabecular bone tissue, as well as the time and cost of each method. While dual-energy X-ray absorptiometry (DEXA) is considered the gold standard for BMD measurement and is currently the most sensitive tool for the prediction of future bone fractures, there is no clinical guarantee that it is 100% accurate (Heiss *et al.*, 2012; The National Institutes of Health, 2012; Sohl *et al.*, 2015). At both a cross-sectional and longitudinal study level, a seven-year cohort study reported a strong positive correlation between quantitative ultrasound (QUS) and DEXA measurements (Trimpou *et al.*, 2010). Another study also found a strong, and positive correlation between T-scores measured by DEXA and those measured using QUS (Beerhorst *et al.*, 2013). Furthermore, QUS was highly accurate and precise compared to DEXA, which deemed this method suitable for use in epidemiological studies (Fogelman & Blake, 2010; Beerhorst *et al.*, 2013).

### 1.3.3.1 Dual-energy X-ray absorptiometry (DEXA)

Dual-energy X-ray absorptiometry (DEXA) is a two-dimensional scanning technique in which two X-ray energies are used to estimate the area of mineralised tissue at any skeletal site; however, it is mostly used at the lumbar spine or hip regions (Fogelman & Blake, 2010; Lorente-Ramos *et al.*, 2013; Pisani *et al.*, 2013; Suman *et al.*, 2013). In short, the X-ray



beam used in DEXA uses two different photon energies (constant and pulsed), selected to compensate for the different attenuation coefficients of the mineralised bone, as well as soft tissue of the bone area being analysed (Fogelman & Blake, 2010; Pisani *et al.*, 2013). The intensities of low-energy and high-energy photons passing through the bone and soft tissue are analysed separately, where after, the attenuation values of the soft tissue are then subtracted by an algorithm, providing only the attenuation values of bone mineral (hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)(\text{OH})_2$ ) (Fogelman & Blake, 2010; Pisani *et al.*, 2013). Since the mineral content is divided by the area, it only partially corrects for body size, and, therefore, cannot assess the depth or posterior-anterior width of the bone, and can also not distinguish between cortical and trabecular bone (Pisani *et al.*, 2013; Suman *et al.*, 2013). Figure 1.6 illustrates how the X-ray source (beneath the examination table) moves simultaneously with the X-ray detector system, scanning the patient's body (Pisani *et al.*, 2013).



**Figure 1.6: Dual-energy X-ray absorptiometry: Schematic representation of X-rays source and detector system, with an example of the type of image obtained by this measurement.**

(Adapted from Pisani *et al.*, 2013).

Some of the disadvantages of the DEXA include; a long time commitment during the scan; exposure to ionising radiation; costly, and being an immobile system. These factors contribute negatively to the use thereof in developing countries (Lee *et al.*, 2010; Heiss *et al.*, 2012; Louis *et al.*, 2015; Silva *et al.*, 2015; Sohl *et al.*, 2015). Additionally, a qualified radiographer is needed to operate the DEXA, since several factors including incorrect positioning of the patient, scan analysis or mistakes in interpretation, will lead to incorrect results and diagnosis (Pisani *et al.*, 2013). Irrespective of this, DEXA assessment is still regarded as highly accurate (Suman *et al.*, 2013).

### 1.3.3.2 Quantitative computed tomography (QCT)

Quantitative computed tomography (QCT) is a specific type of bone scanning technique that measures the volumetric density of bones (Stewart & Sutton, 2012). Both cortical and trabecular bone can be distinguished from one another, which is beneficial since the high



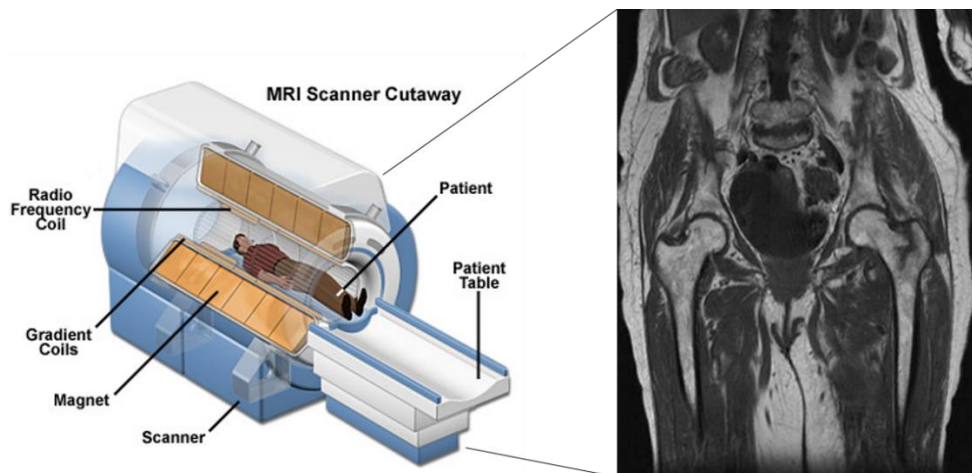
rates of bone turnover in trabecular bone can be detected. If abnormalities are present in bone formation, early detection may prevent bone disease progression, and it is therefore highly sensitive to any change in bone composition (Stewart & Sutton, 2012).

In QCT, the X-ray absorption profiles are normally obtained when the source and the detectors rotate around the bone of interest. The absorption projections are then processed at different angles to construct a three-dimensional illustration of the imaged bone (Pisani *et al.*, 2013). Although this method seems advantageous, soft tissues exhibit little signal on QCT, which makes this type of measurement quite challenging when imaging or assessing bone and soft tissue (Ho *et al.*, 2013; Louis *et al.*, 2015). Another disadvantage of using this technique is that it uses X-rays, and it is also more expensive compared to DEXA assessment (Stewart & Sutton, 2012; Pisani *et al.*, 2013; Louis *et al.*, 2015). An alternative form of computed tomography is high-resolution peripheral quantitative computed tomography (HRPQCT), which provides additional information on the quality of bone microarchitecture (Madeira *et al.*, 2014).

### **1.3.3.3 Magnetic resonance imaging (MRI)**

This imaging technique uses the body's natural magnetic properties to produce detailed images of the anatomical area of interest. Body water, as well as AT contains hydrogen with a positive charge (Berger, 2002; Lindquist & Wager, 2014). Under normal conditions, these protons are randomly orientated with respect to each other (Lindquist & Wager, 2014).

The immense magnetic field generated by the magnetic resonance-magnets align the protons in cells in one direction (Figure 1.7). Gradient coils produce radio waves that knock these protons out of its alignment. After removal of these radio waves, the protons realign or return to equilibrium, thereby sending radio signals to the receivers (radio frequency coils), which can provide the exact location of the protons in the body. Since protons in different tissues realign at different speeds, the signal can then distinguish between different tissue types in the body (Berger, 2002; Lindquist & Wager, 2014).



**Figure 1.7: Schematic representation of a magnetic resonance imaging scanner with an example of the type of image produced by this technique.**

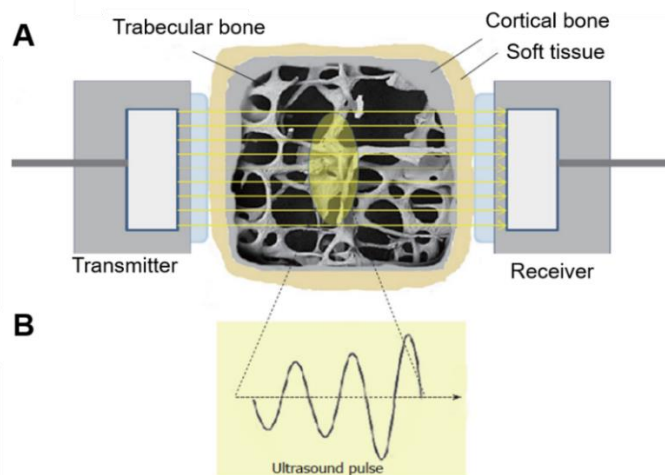
(Adapted from Alam, Willet & Ostlere, 2005 and <http://io9.com/5495712/six-ways-science-can-see-into-your-brain>).

The fact that BMD assessments can be made based on the volume of the bone, the absence of ionising radiation, and the ability to assess trabecular bone tissue, makes MRI an useful alternative to QCT and DEXA (Stewart & Sutton, 2012; Ho *et al.*, 2013; Louis *et al.*, 2015). Disadvantages of this technique include its costly nature and its lengthy scan procedure (Stewart & Sutton, 2012).

#### 1.3.3.4 Quantitative ultrasound method

An alternative measurement of BMD is QUS, usually measured at the calcaneus bone, or other peripheral skeletal sites (Sohl *et al.*, 2015). This method is widely used due to the relatively low cost, simplicity of performance, mobility, non-invasive nature, no exposure to ionising radiation, and its provision of additional information on the quality of bone (Mergler *et al.*, 2010; Trimpou *et al.*, 2010; Chin *et al.*, 2012; Sohl *et al.*, 2015).

The calcaneal bone is the only skeletal site recommended by the ISCD for BMD measurements with QUS. The calcaneal bone is one of the weight-bearing bones of the body and therefore sensitive to changes in BMD due to mechanical loading (Pisani *et al.*, 2013; Chin *et al.*, 2015; International Society for Clinical Densitometry, 2015). Ultrasound uses high-frequency sound waves to measure the alteration of travelling vibrations when passing through a solid medium, as for example bone (Trimpou *et al.*, 2010; Beerhorst *et al.*, 2013). The generated sound wave released from the transmitter, passes through the calcaneal bone, and is received by the other transducer (Chin *et al.*, 2012). Figure 1.8A illustrates the principle of sound waves passing through different types of bone tissue, whereas Figure 1.8B illustrates the speed at which sound waves pass through cortical and trabecular bone.



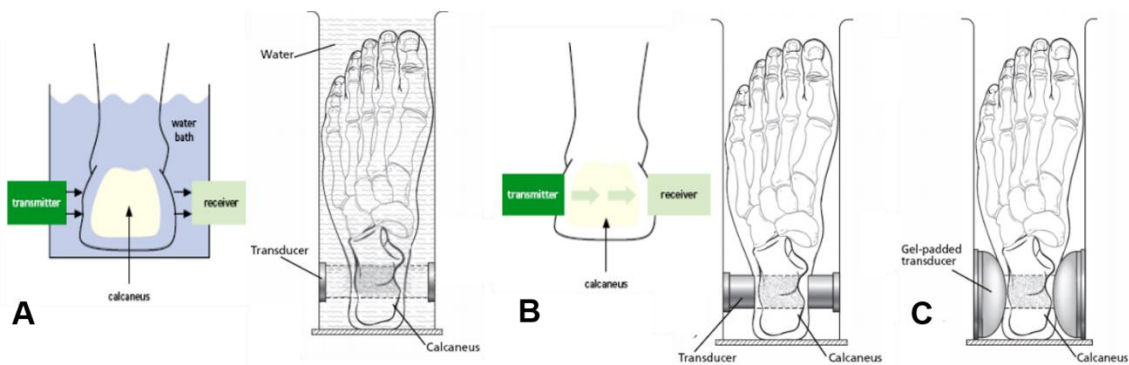
**Figure 1.8: The QUS method illustrating (A) the movement of ultrasound through a bone section, and (B) the corresponding ultrasound pulse wave generated for each type of bone (note that sound waves travel faster through the trabecular compared to cortical bone).**

(Adapted from Pisani *et al.*, 2013).

There are several reasons why the calcaneal bone is considered the most suitable site for QUS measurement. Firstly, it has very little soft tissue (makes it easy to measure the bone), secondly, it has relatively flat surfaces to ensure good contact between the calcaneus and transducer, and thirdly, it is similar in composition to the main fracture sites (90% trabecular bone). Furthermore, it is easily accessible and requires very little patient preparation (National Osteoporosis Society, 2015).

The right calcaneal bone is mostly used to assess BMD (Kim *et al.*, 2013a). Although QUS could vary between the right and left calcaneus, the right foot is dominant in most people and might therefore have a higher BMD than the left calcaneus. However, some studies prefer to use the left heel to measure the lowest BMD (Mergler *et al.*, 2010).

Some QUS instruments are water-based (Figure 1.9A), where the heel is placed in a water bath (37°C) between two ultrasonic transducers, while other equipment uses a dry contact system (Figure 1.9B), where the transmitter and receiver are in direct contact with the calcaneus (National Osteoporosis Society, 2015). Another method includes the use of “gel-padded transducers” (Figure 1.9C) (National Osteoporosis Society, Australia and New Zealand Horizon Scanning Network).



**Figure 1.9: Different quantitative ultrasound instruments, which includes a water bath system (A), dry contact system (B) and gel-padded system (C).**

(Adapted from National Osteoporosis Society and Australia and New Zealand Horizon Scanning Network).

One of the key features of the QUS system include its ability to measure bone quality index (BQI), by combining speed of sound (SOS) and broadband ultrasound attenuation (BUA). The SOS refers to the speed (m/s) at which ultrasound waves travel through the calcaneal bone (Beerhorst *et al.*, 2013). Speed of sound is directly associated with elasticity, quality and density of bone (Beerhorst *et al.*, 2013). The presence of soft tissue decreases the SOS transmitted through bone, and thus influences bone quality (Beerhorst *et al.*, 2013).

In participants with a history of fractures, Hernández *et al.* (2004) reported a lower SOS ( $1516.5 \pm 1.13$  m/s) than in those with no history of fractures ( $1527.7 \pm 0.57$  m/s;  $p < 0.0001$ ). When investigating both menopausal status and metabolic status, Sun *et al.* (2014) reported a lower SOS in PreM women with the MetS ( $1547.2 \pm 26.3$ ) than those without ( $1549.8 \pm 28.6$  m/s;  $p = 0.0198$ ), and a similar observation was made in the PostM women (MetS:  $1531.7 \pm 28.0$  vs. Non-MetS:  $1533.7 \pm 28.0$  m/s;  $p = 0.030$ ) (Sun *et al.*, 2014).

Ino *et al.* (1997) described BUA as the rate at which ultrasound waves are attenuated as they pass through bone. Broadband ultrasound attenuation measures the pattern of attenuation of ultrasonic waves in bone tissue and is influenced by connectivity and trabecular separation (Lee *et al.*, 2010; Mergler *et al.*, 2010; Scheffler *et al.*, 2014). An osteoporotic bone will yield a BUA of 50 dB/Mhz compared to normal bone (100 dB/Mhz) (Burr & Allen, 2013). In participants with a history of fractures, Hernández *et al.* (2004) reported a lower BUA ( $60.2 \pm 0.55$  dB/Mhz) than in those with no history of fracture ( $66.4 \pm 0.28$  dB/Mhz;  $p < 0.0001$ ). Even though it was insignificant, PreM women with the MetS had a lower BUA than those without the MetS (MetS:  $77.52 \pm 15.50$  vs. Non-MetS:  $79.4 \pm 15.3$  dB/Mhz;  $p = 0.110$ ) and a similar observation was noted in the PostM group (MetS:  $70.55 \pm 16.88$  vs. Non-MetS:  $71.38 \pm 16.85$  dB/Mhz;  $p = 0.137$ ) (Sun *et al.*, 2014).

Bone quality index (BQI), or the stiffness index, is used to describe the overall bone quality derived from a linear combination of BUA and SOS (Mergler *et al.*, 2010; Trimpou *et al.*, 2010). Women in the PreM MetS group showed lower BQI, or quantitative ultrasound index (QUI) (MetS:  $95.15 \pm 16.57$  vs. Non-MetS:  $96.86 \pm 17.48$ ;  $p=0.184$ ). Similarly, PostM women with the MetS also had a lower BQI than those without the MetS ( $85.95 \pm 17.74$  vs.  $87.00 \pm 17.60$ ;  $p=0.072$ ) (Sun *et al.*, 2014).

Although the abovementioned BMD assessment methods, as well as bone turnover markers, are widely used, and provide valuable information regarding bone health and fracture risk, additional information to contextualise these findings are needed. Various other biochemical and nutritional blood markers that influence bone architecture must therefore be taken into consideration.

### **1.3.4 Complimentary biochemical and nutritional assessment bone markers**

The three calcitropic hormones,  $Ca^{2+}$ , vitamin D (calcidiol; calcifediol; 25-hydroxycholecalciferol; 25(OH)D), and parathyroid hormone (PTH) are considered crucial in the regulation of BMD (Starup-Linde, 2013). These elements affect several biochemical processes in bone, including alteration of bone structure, the rate of bone metabolism, the endocrine and/or paracrine system, as well as homeostasis of  $Ca^{2+}$  and other bone-active minerals (Cashman, 2007). The following sections will discuss these calcitropic hormones including basic physiology, its correlation to the MetS, as well as the effect of ageing and menopause.

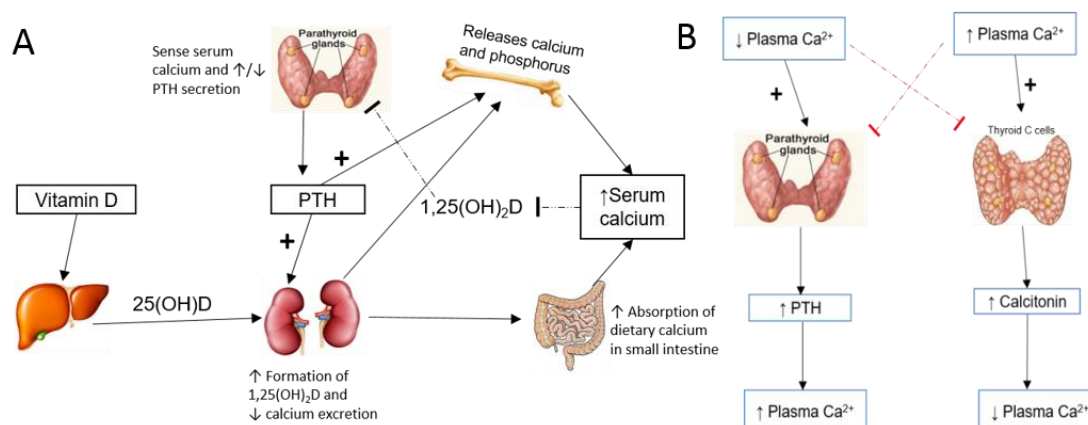
#### **1.3.4.1 Calcium**

##### ***Basic calcium physiology***

Calcium, which is either present in the form of phosphate complexes, albumin-bound, or in the free ionised form ( $Ca^{2+}$ ), plays a crucial role in assuring sufficient growth and development of the skeleton (Moe, 2008; Ross *et al.*, 2011; Campbell, 2014). Although 99% of the body's total calcium exists in bone, it has several additional physiological functions (Ross *et al.*, 2011; Campbell, 2014). Since calcium plays such an important role in the body, serum calcium concentrations are one of the most rigorously controlled parameters in the body (Campbell, 2014).

During hypocalcaemia, PTH stimulates maturation of osteoclasts and activity of osteoclasts to mobilise calcium from bone, with a simultaneous increase in gastrointestinal calcium absorption *via* the production of calcitriol (1,25-dihydroxycholecalciferol;  $1,25(OH)_2D$ ) (Figure 1.10A) (Campbell, 2014). Conversely, during hypercalcaemia, the thyroid C-cells are activated to produce calcitonin, increasing the deposition of calcium in bone by inhibiting

osteoclast activity, which in turn, decrease serum  $\text{Ca}^{2+}$  levels (Figure 1.10B) (Davey & Findlay, 2013; Campbell, 2014).



**Figure 1.10: Physiological interaction illustrating the role of PTH in the maintenance of serum calcium levels with key target organs and the feedback interactions with calcium (A). Plasma calcium regulation via the parathyroid glands and thyroid C-cells (B). (Adapted from Wimalawansa, 2012; Silverthorn, 2013 & Sherwood, 2010).**

Dietary  $\text{Ca}^{2+}$  is mainly present as a salt or associated with other dietary constituents in the form of calcium ions (Cashman, 2007; Pavone *et al.*, 2015). The recommended daily allowance (RDA) for women range between 1000 mg (19-50 years) and 1200 mg (51 – 70 years), indicating that dietary  $\text{Ca}^{2+}$  intake should increase with ageing (Ross *et al.*, 2011; Martini *et al.*, 2013; National Institutes of Health, 2012). Ageing, vitamin D deficiency and vitamin D resistance all affect the calcium homeostasis negatively (Oudshoorn *et al.*, 2009).

### ***Circulating calcium and the metabolic syndrome***

Kim *et al.* (2010) reported a strong association between serum calcium levels and the prevalence of the MetS in participants with an age of  $\geq 40$  years (independent of age, BMI and gender). Furthermore, in older study populations, both Cho *et al.* (2011) and Saltevo *et al.* (2011) reported that higher serum calcium (although still in the normal range) was associated with an increased risk of developing the MetS in healthy elderly women (47 to 75 and 60 to 85 years respectively). Both Kim *et al.* (2010) and Cho *et al.* (2011) reported significant associations between serum calcium levels and FBG, TC, HDL-c and TG, whereas Saltevo *et al.* (2011) reported associations between WC, BP, TG and FG and serum  $\text{Ca}^{2+}$  levels, but not HDL-c and serum calcium levels.

Two possible mechanisms were proposed linking calcium and the MetS: Firstly, intracellular calcium is a regulator of lipid metabolism, and increased concentrations will stimulate the expression and activity of lipogenic enzymes, reducing lipolysis and increasing the accumulation of adipocytes (resulting in obesity and the associated pathological



mechanisms described earlier) (Huang *et al.*, 2011). Lower dietary calcium intake will result in increased 1,25(OH)<sub>2</sub>D, which in turn will increase Ca<sup>2+</sup> absorption and lipogenesis. Huang *et al.* (2011) also reported a significantly decreased risk of abdominal obesity with each quartile increase in dietary calcium intake. Secondly, high circulating calcium levels can reduce the absorption of dietary fat in the small intestine, since calcium increases the excretion of fats, by forming insoluble calcium FA soaps, or by the binding of bile acids (Huang *et al.*, 2011). Calcium is also essential for insulin-mediated intracellular processes in insulin-responsive tissues (Shin *et al.*, 2015). Therefore, abnormal regulation of calcium levels can affect both insulin sensitivity and insulin release, since insulin secretion is a calcium-dependent process (Kim *et al.*, 2010).

### **Calcium, ageing and menopause**

The majority of PostM women experience oestrogen deficiency, which can lead to calcium loss *via* the indirect effects on circulating calcium homeostasis (Qureshi *et al.*, 2010; Drake *et al.*, 2015). During ageing, there is increased bone resorption, resulting in an increased loss of calcium from bone into the extracellular fluid (Drake *et al.*, 2015). To compensate for this loss, several mechanisms are at play: (i) the kidneys decrease renal calcium reabsorption, (ii) there is a decrease in intestinal calcium absorption, as well as (iii) a reduction in PTH secretion (Qureshi *et al.*, 2010; Drake *et al.*, 2015). The combination of these physiological responses results in a net negative whole-body calcium concentration, ultimately leading to skeletal demineralization.

Furthermore, menopausal women show a decrease in calcium absorption; however, the bone loss that occurs here cannot be prevented by increasing the consumption of dietary calcium, although it may help to decrease the rate of bone loss. The use of hormone replacement therapy (oestrogen and progesterone) helps to increase calcium levels and prevent osteoporosis and fractures (National Institutes of Health, 2012). Post-menopausal women showed significantly lower serum calcium levels compared to PreM women, which corroborates previous evidence (Qureshi *et al.*, 2010; Bhattarai *et al.*, 2013).

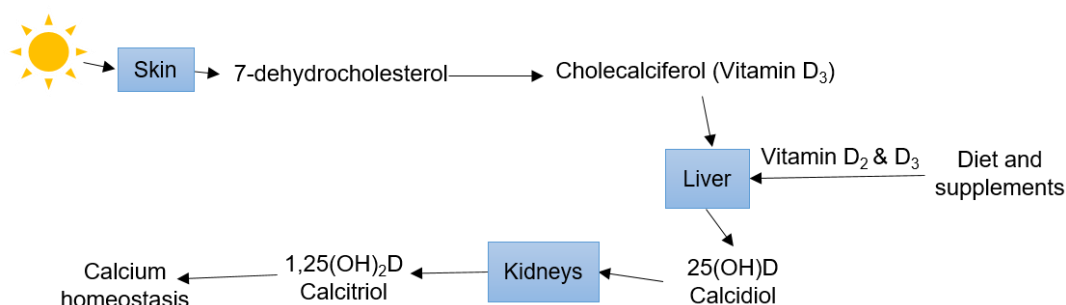
### **1.3.4.2 Vitamin D**

#### **Basic physiology**

Vitamin D, important in the development and maintenance of bone, is mainly found in two forms, (i) ergocalciferol (vitamin D<sub>2</sub>), and (ii) cholecalciferol (vitamin D<sub>3</sub>) (Pérez-López *et al.*, 2011; Campbell, 2014; International Osteoporosis Foundation, 2015; Pavone *et al.*, 2015). Vitamin D<sub>2</sub> is synthesised in plants or yeasts when exposed to ultraviolet (UV) light, and therefore needs to be included in the diet (Oudshoorn *et al.*, 2009; Chen *et al.*, 2010; Jäpelt & Jakobsen, 2013; Campbell, 2014).

Vitamin D<sub>3</sub> (cholecalciferol) is synthesised in the epidermis of skin after the precursor of vitamin D, 7-dehydrocholesterol, is exposed to ultraviolet-B (UVB) light (Figure 1.11) (Wimalawansa, 2012; Campbell, 2014; International Osteoporosis Foundation, 2015; Pavone *et al.*, 2015). An average daily sunlight exposure ranging between ten to fifteen minutes to the face or arms is recommended sufficient for synthesis of vitamin D<sub>3</sub> in most individuals (International Osteoporosis Foundation, 2015).

The liver is responsible for the conversion of cholecalciferol into calcidiol (25(OH)D), which is further converted to the active form, calcitriol (1,25(OH)<sub>2</sub>D) by the kidneys (Pérez-López *et al.*, 2011; Wimalawansa, 2012) (Figure 1.11). Due to the longer half-life (15 days) of 25(OH)D, it is considered the best marker to determine vitamin D status (Saliba *et al.*, 2011; Pérez-López *et al.*, 2011).



**Figure 1.11: Production of 25(OH) D and 1,25(OH)<sub>2</sub>D, and conversion of vitamin D<sub>2</sub> and D<sub>3</sub>, from dietary sources and supplements.**

(Adapted from Wimalawansa, 2012; Silverthorn, 2013; Sherwood, 2010).

Small deficiencies in 25(OH)D cause an increase in PTH that will result in increased bone turnover. This might subsequently lead to the development of osteopenia, osteoporosis and an increased fracture risk (Chen *et al.*, 2010; Saliba *et al.*, 2011). Depending on the concentration of 25(OH)D, individuals can be categorised as either having deficient, insufficient, sufficient or an overload of vitamin D; however, the cut-off values used differs between studies (Table 1.6).

**Table 1.6: Classification of Vitamin D levels.**

Vitamin D levels classification	Gutiérrez <i>et al.</i> (2011)	Holick <i>et al.</i> (2011)	Sharp (2011)	Park & Lee (2012)
Deficient	≤20 ng/mL	<20 ng/mL	21-29 ng/mL	20-29 ng/mL
Insufficient	21-30 ng/mL	21-29 ng/mL		
Sufficient	-	-	≥30 ng/mL	>30 ng/mL
Excess	-	-	-	-

Vitamin D deficiency is considered a global epidemic in which individuals are at a higher risk of developing osteoporosis (Conesa-Botella *et al.*, 2010). The International Osteoporosis Foundation reported that several countries, including South Africa, Russia, China, Italy, and



Germany had 25(OH)D levels between 10-20 ng/mL, whereas France, the United Kingdom, Saudi Arabia and Brazil had 25(OH)D levels between 20-29 ng/mL. Furthermore, Sweden, Thailand, Vietnam and Taiwan were the only countries to have 25(OH)D levels above 30 ng/mL (International Osteoporosis Foundation, 2016).

In a study investigating 25(OH)D status in PreM and PostM women in relation to obesity and BMD, it was reported that the main risk factors for 25(OH)D deficiency was obesity, poor sunlight exposure, inadequate vitamin D supplementation, high W:H and age (Ardawi *et al.*, 2011). Some other factors that are associated with altered vitamin D synthesis include latitude, the season of the year, air pollution, clothing, skin pigmentation, and use of sunscreen (Martini *et al.*, 2013).

The RDA for vitamin D is 15 µg (600 IU) for women between the ages of one and 70 years, and 20 µg (800 IU) for individuals older than 71 years (Ross *et al.*, 2011; Martini *et al.*, 2013; Pavone *et al.*, 2015). Older women require higher doses of vitamin D, and a study on supplementation in PostM women indicated that supplementing with both 800 IU cholecalciferol and 1000 mg calcium carbonate, exerted positive effects on BMD (Kärkkäinen *et al.*, 2010). In agreement, in a majority female study population, Bischoff-Ferrari *et al.* (2012) reported similar results where ≥800 IU/d vitamin D supplementation showed positive effects in preventing hip and non-vertebral fractures in individuals older than 65 years.

It was also suggested that optimal levels of 25(OH)D differ by race and ethnicity (Van Ballegooijen *et al.*, 2014). Melanin absorbs radiation from 290 – 700 nm including the portion that is responsible for vitamin D<sub>3</sub> synthesis, which takes place at 290 – 313 nm. Therefore, increased melanin pigmentation decreases the production of vitamin D<sub>3</sub> and requires longer exposure to sunlight to produce the same amount of vitamin D<sub>3</sub> than lighter-skinned individuals (Holick *et al.*, 2004; Chen *et al.*, 2010). Despite similar serum Ca<sup>2+</sup> levels, Gutiérrez *et al.* (2011) reported differences between Caucasian and Black individuals for both serum 25(OH)D (25.6±0.4 ng/mL vs. 14.8±0.4 ng/mL), as well as PTH levels (39.9±0.4 pg/mL vs. 46.5±0.7 pg/mL). Furthermore, in Caucasians, higher levels of 25(OH)D were associated with higher BMD, but this was not true in Afro-American Black and Hispanic individuals (Van Ballegooijen *et al.*, 2014).

### ***Vitamin D and the metabolic syndrome risk factors***

Vitamin D deficiency has been associated with some of the individual MetS risk factors (Alkharfy *et al.*, 2013; Saedisomeolia *et al.*, 2014). Several studies, as listed in Table 1.7, reported significantly lower or inadequate levels of 25(OH)D in MetS individuals in comparison to Non-MetS individuals.

**Table 1.7: Comparison between 25(OH)D concentrations between MetS and Non-MetS groups in different studies.**

	MetS	Non-MetS	p-value
<b>Hernández et al. (2011)</b>	21.4±7.8 ng/mL	24.1±8.9 ng/mL	p<0.0001
<b>Makariou et al. (2012)</b>	11.8 ng/mL	17.2 ng/mL	p=0.027
<b>Gagnon et al. (2012)</b>	25±9 ng/mL	27±10 ng/mL	p<0.001
<b>Barchetta et al. (2013)</b>	13.5 ng/mL	17.4 ng/mL	p<0.007.

Gagnon *et al.* (2011) reported that the relationship between 25(OH)D and the MetS is mediated by IR in individuals with a BMI of  $\geq 25$  kg/m<sup>2</sup>. Both Saedismeolia *et al.* (2014) and Bea *et al.* (2015) found increased FBG levels in 25(OH)D-deficient individuals, which was also corroborated by Alissa *et al.* (2015) and Kim *et al.* (2015) where an inverse correlation was made between 25(OH)D and FBG. Here, it was shown that 25(OH)D supplementation can improve insulin sensitivity through stimulation of insulin receptor expression in peripheral tissues, and that low 25(OH)D concentration was associated with beta-cell dysfunction (Al-Daghri *et al.*, 2012; Baker *et al.*, 2012; Alissa *et al.*, 2015).

Obesity has been linked to lower serum 25(OH)D levels, and/or decreased 25(OH)D bioavailability. This is due to the sequestering effect of the high quantity of SAT on circulating 25(OH)D (Martini *et al.*, 2013; Bea *et al.*, 2015). Ardawi *et al.* (2011) and George *et al.* (2013) reported an inverse association between serum 25(OH)D and BMI, suggesting that inadequate 25(OH)D levels might be a potential contributor to the development of the MetS. It was also proposed that increased adiposity is associated with increased catabolism of 25(OH)D by the 24-hydroxylase enzyme, or that adiposity is responsible for a decreased hepatic synthesis of 25(OH)D (Martini *et al.*, 2013).

When investigating the association between WC and 25(OH)D levels, Martini *et al.* (2013) reported a positive association, with an increase of 0.20 nmol/L in 25(OH)D for each one centimeter increase in WC. Gradillas Garcia *et al.* (2015) reported a negative and significant association between 25(OH)D and WC ( $r=-0.19$ ,  $p=0.0014$ ), which is in agreement with the statement made by Bea *et al.* (2015) who reported a significant association between low levels of 25(OH)D and a high WC.

Inconsistent results have been published regarding the association of 25(OH)D and BP. Forman *et al.* (2013) found that supplementing individuals with 25(OH)D for three months, significantly and exponentially lowered SBP. Vitamin D might reduce BP *via* improvement of endothelial function (Sugden *et al.*, 2008; Tarcin *et al.*, 2009) or reduce the activity of the renin-angiotensin-aldosterone system (Forman *et al.*, 2010). This is corroborated by Bea *et al.* (2015) who reported that 25(OH)D-deficient individuals tend to have higher BP. Gradillas Garcia *et al.* (2015) reported contrasting results, with a negative association between 25(OH)D and mean BP ( $r=-0.19$ ,  $p=0.002$ ).

Makariou *et al.* (2012), Baker *et al.* (2012), Alissa *et al.* (2015), Bea *et al.* (2015) and Kim *et al.* (2015) found a significant inverse association between 25(OH)D and TG. Evidence suggests a relationship between 25(OH)D deficiency and dyslipidemia, possibly due to the effects of 25(OH)D on hepatic lipid metabolism (Baker *et al.*, 2012). It is proposed that vitamin D increases intestinal  $\text{Ca}^{2+}$  absorption, with the latter binding to FA, forming insoluble complexes that inhibit lipid absorption. Therefore, 25(OH)D deficiency may lead to abnormal lipid processing *via* alterations in  $\text{Ca}^{2+}$  availability (Baker *et al.*, 2012).

Kim *et al.* (2015) also reported a negative and significant association between 25(OH)D and TC, whereas, Saedisomeolia *et al.* (2014) reported only weak no-significant associations between these two measures. Kim *et al.* (2015) reported that participants with 25(OH)D levels below 30 ng/mL have significantly higher levels of TC ( $186.5 \pm 38.1$  mg/dL) than those with 25(OH)D levels of  $\geq 30$  ng/mL ( $174.9 \pm 30.3$  mg/dL;  $p=0.021$ ). In contrast, a study by Saedisomeolia *et al.* (2014) reported no significant difference between TC levels in participants with 25(OH)D levels of  $\geq 50$  nmol/L and  $< 50$  nmol/L ( $p=0.44$ ).

### ***Vitamin D, ageing and menopause***

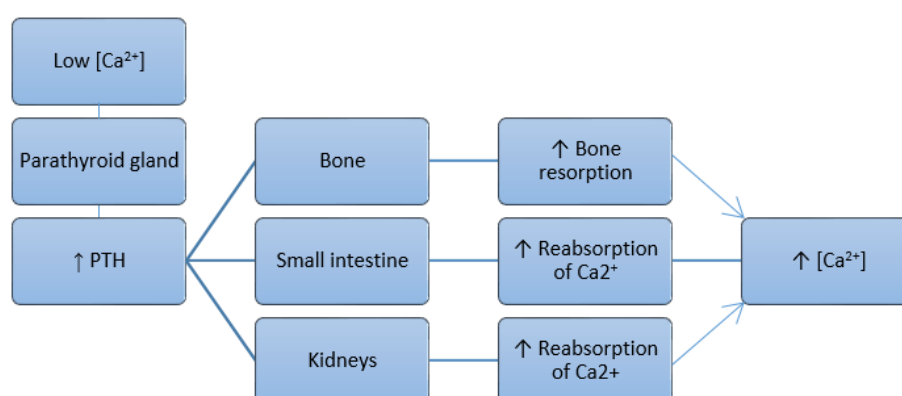
A strong association exists between vitamin D<sub>3</sub> deficiency, ageing and menopausal status, since the generation of vitamin D<sub>3</sub> after sunlight exposure is less effective in the elderly due to a decline in the levels of cutaneous 7-dehydrocholesterol (Oudshoorn *et al.*, 2009; Chen *et al.*, 2010). Supporting this evidence, Arabi *et al.* (2010) reported that 25(OH)D levels were significantly lower in older individuals ( $10.9 \pm 5.0$  ng/mL) than in younger ones ( $14.2 \pm 8.1$  ng/mL;  $p < 0.05$ ). A simultaneous decrease in sunlight exposure is also noted in the elderly, as these populations tend to be immobile and socially isolate themselves (Oudshoorn *et al.*, 2009). Another factor contributing to 25(OH)D<sub>3</sub> deficiency in the elderly is the increase in body fat with ageing, since this leads to a larger distribution volume for the fat-soluble 25(OH)D<sub>3</sub>, thereby decreasing the bioavailability of 25(OH)D<sub>3</sub> (Oudshoorn *et al.*, 2009).

Ageing is also associated with a decrease in the expression of the vitamin D receptors (VDR) in bone, gastro-intestinal tract, as well as muscle tissue (Oudshoorn *et al.*, 2009). Oestrogen, growth hormone and 1,25(OH)<sub>2</sub>D are stimulators of VDR expression, and it is established that serum levels of these stimulators decrease with ageing (Oudshoorn *et al.*, 2009). Ageing might also result in decreased binding of 1,25(OH)<sub>2</sub>D to the VDR receptor and cause VDR alterations leading to vitamin D resistance (Oudshoorn *et al.*, 2009). Two studies reported significant differences between 25(OH)D levels in PreM and PostM women, with PreM having significantly higher levels than the PostM women ( $17.22 \pm 12.21$  ng/mL vs.  $13.34 \pm 9.96$  ng/mL;  $p=0.000$  and  $30.21 \pm 4.20$  ng/mL vs.  $25.07 \pm 4.30$  ng/mL;  $p=0.000$  respectively) (Ardawi *et al.*, 2011; Wang *et al.*, 2013).

### 1.3.4.3 Parathyroid hormone

#### **Basic physiology**

The four parathyroid glands, located posterior to the thyroid gland secrete parathyroid hormone (PTH) (Silverthorn, 2013). This hormone functions as an efficient regulator of plasma  $\text{Ca}^{2+}$  levels, and maintaining bone health (Sharp, 2011), *via* the parathyroid endocrine cells, and the G-protein-coupled  $\text{Ca}^{2+}$  receptors on the membrane of the parathyroid endocrine cells (Silverthorn, 2013). A decrease in plasma  $\text{Ca}^{2+}$  stimulates PTH secretion to release  $\text{Ca}^{2+}$  into the bloodstream *via* increased bone resorption, as well as increased reabsorption of  $\text{Ca}^{2+}$  in the small intestine and kidneys (Figure 1.12) (Walsh, 2014; Campbell, 2014).



**Figure 1.12: PTH regulating calcium levels.**

(Adapted from Silverthorn, 2013).

#### **Parathyroid hormone and the metabolic syndrome**

Parathyroid hormone can stimulate the development of hypertension in both a  $\text{Ca}^{2+}$ -dependent and -independent manner (Yagi *et al.*, 2014). The  $\text{Ca}^{2+}$ -dependent manner is *via* receptors (calcium-sensing receptors) located in the parathyroid glands, the brain and kidneys that result in decreased sodium reabsorption, as well as a secondary decrease in  $\text{Ca}^{2+}$  reabsorption (Yagi *et al.*, 2014).

Parathyroid hormone receptors are also expressed in the endothelium and vascular smooth muscles of blood vessels (Garcia *et al.*, 2013; Yagi *et al.*, 2014). Circulating PTH might affect intracellular signalling, resulting in intracellular  $\text{Ca}^{2+}$  overload. This can lead to cell injury and cell death *via* promoting structural and functional modification to these cells, altering the mobility of the arteries, thereby altering BP (Garcia *et al.*, 2013; Yagi *et al.*, 2014; Van Ballegooijen *et al.*, 2014).

High serum PTH and  $\text{Ca}^{2+}$  levels are suggested to be risk factors for hypertension (Yagi *et al.*, 2014; Van Ballegooijen *et al.*, 2014), since PTH was positively correlated with BP (increased SBP and DBP) (Garcia *et al.*, 2013). Parathyroid hormone receptors are also

expressed in the myocardium and can therefore act on cardiomyocytes to promote left ventricular hypertrophy, vascular stiffness and chronotropic effects on the pacemaker cells of the heart, thereby producing an immediate and continuous increase in heart rate (Garcia *et al.*, 2013; Yagi *et al.*, 2014). In addition, two separate studies reported significantly higher PTH in hypertensive participants, where hypertensive individuals had a higher mean PTH concentration compared to normotensive individuals ( $44.3 \pm 18.6$  pg/mL vs.  $37.7 \pm 17.7$  pg/mL,  $p=0.006$ ) (Garcia *et al.*, 2013); and  $109.3 \pm 81.4$  pg/mL vs.  $56.1 \pm 58.8$  pg/mL,  $p<0.001$ ) (Yagi *et al.*, 2014).

A statistically significant difference was also noted in the intact PTH levels between MetS and Non-MetS women ( $57.8 \pm 28.8$  pg/mL vs.  $51.2 \pm 18.0$  pg/mL,  $p=0.001$ ) (Hernández *et al.*, 2011). In contrast, Guasch *et al.* (2012) found no association between PTH and the MetS and Alissa *et al.* (2014) reported no significant difference in PTH levels between MetS and Non-MetS, PostM women ( $54.52 \pm 2.82$  pg/mL vs.  $54.52 \pm 3.76$  pg/mL).

Parathyroid hormone was also positively associated with BMI, WC and percentage fat mass (% FM) (Garcia *et al.*, 2013). Higher serum PTH levels were associated with older age, Black and Hispanic race, higher BMI, SBP, lower 25(OH)D levels and phosphate concentrations (Van Ballegooijen *et al.*, 2014).

### ***Parathyroid hormone, ageing and menopause***

Supporting evidence has shown a link between ageing, and an increase in PTH concentrations, due to vitamin D deficiency, renal insufficiency and low dietary calcium intake (Oudshoorn *et al.*, 2009; Arabi *et al.*, 2010; Kruger *et al.*, 2011; Valcour *et al.*, 2012). Joo *et al.* (2013), reported a significant difference in individuals between the age ranges 50-59 years ( $64.4$  pg/mL), and 60-69 years ( $69.1$  pg/mL), and >70 years ( $78.1$  pg/mL;  $p<0.001$ ), and similarly Van Ballegooijen *et al.* (2014) found a positive association between PTH and older age.

Studies by both Ardawi *et al.* (2011) and Wang *et al.* (2012) reported significant differences in the PTH concentrations between PreM and PostM women ( $47.10 \pm 27.55$  pg/mL vs.  $54.64 \pm 31.55$  pg/mL;  $p=0.000$  and  $35.59 \pm 23.25$  pg/mL vs.  $40.94 \pm 24.67$  pg/mL;  $p=0.000$ , respectively) (Ardawi *et al.*, 2011; Wang *et al.*, 2012).

### **1.3.5 Risk factors associated with bone mineral density**

In addition to the bone-specific, biochemical and nutritional markers, other factors also affect BMD. These factors can either be modifiable or lifestyle-associated risk factors and non-modifiable risk factors. The presence of the MetS is another risk factor that can affect BMD that is of particular interest in this study.

There exists an unusual paradox between the MetS and BMD (Xue *et al.*, 2012). Studies by Hwang & Choi (2010) and Alissa *et al.* (2014) suggested that the MetS influences BMD negatively since women with the MetS had significantly lower BMD compared to Non-MetS women. Jeon *et al.* (2011) also found that the MetS was negatively correlated with BMD of the lumbar spine and femoral neck in PostM women, as well as the lumbar spine of PreM women (Finkelstein *et al.*, 2008; Jeon *et al.*, 2011). This difference observed here might be due to BMD loss that occurs at a more gradual rate at the femoral neck than the lumbar spine (Finkelstein *et al.*, 2008; Jeon *et al.*, 2011). Additionally, both Hwang & Choi (2010) and Nóbrega da Silva *et al.* (2014) reported that the individual MetS risk factors, negatively affects BMD, with individuals having two or more risk factors showing significantly lower BMD compared to individuals with only one, or no MetS risk factors.

In individuals with the MetS, a lower prevalence of osteopenia and osteoporosis were reported, even after adjustment for body weight and age (Alissa *et al.*, 2014; El Maghraoui *et al.*, 2014). It was found that the MetS have the potential to increase BMD and reduce the risk of fractures, therefore not detrimental to bone health, but rather protective (Alissa *et al.*, 2014). This was corroborated in a study by Muka *et al.* (2015), which reported that females with the MetS had significantly higher BMD than Non-MetS women.

To summarise, it is apparent that there are various discrepancies of whether the MetS and its contributing risk factors have a positive or negative effect on bone health. Reasons for the differences range from ethnicity, ageing, the presence of other pathological conditions, or treatments (Hwang & Choi, 2010). Each of the individual MetS risk factors and their effects on BMD will follow.

#### **1.3.5.1 Blood pressure - hypertension**

Contrasting evidence have been reported on the association between BMD and hypertension (Javed *et al.*, 2010; Alissa *et al.*, 2014; Kaplan *et al.*, 2010). Although not significant, a weak positive correlation ( $r=0.150$ ) was observed between DBP and BMD at the femoral neck in PostM women (Alissa *et al.*, 2014). Another study also reported DBP was associated with BMD at both the femoral neck and lumbar spine in PostM women (Jeon *et al.*, 2011).

In PreM women, SBP was significantly associated with BMD at the lumbar spine and DBP was significantly associated with BMD at the femoral neck (Jeon *et al.*, 2011). It is speculated that hypertension might be related to low BMD due an increase in the serum intact PTH concentration (Jeon *et al.*, 2011). The  $Ca^{2+}$  homeostasis abnormalities, present in hypertensive individuals, result in increased urinary excretion of  $Ca^{2+}$  (Nóbrega da Silva *et al.*, 2014; Jeon *et al.*, 2011).



### *Hypertension treatment*

There are many classes of anti-hypertensive treatment options, including; thiazide diuretics, beta-blockers, loop diuretics and Ca<sup>2+</sup> channel blockers (CCB's) (Ilić *et al.*, 2013; Ghosh & Majumbar, 2014). In a hypertensive, PostM female study population, those treated with thiazides had higher BMD and lower bone turnover marker (PINP and β-CTx) levels than the controls (Olmos *et al.*, 2010). It is hypothesised that the effects of thiazide on BMD is mediated *via* reduced urinary Ca<sup>2+</sup> excretion, increasing serum Ca<sup>2+</sup> levels, that should decrease PTH secretion and decrease bone turnover (Olmos *et al.*, 2010; Ruths *et al.*, 2015). Furthermore, thiazide diuretics also possibly stimulate osteoblasts directly and thereby increases bone formation (Ghosh & Majumbar, 2014; Ruths *et al.*, 2015).

Beta-adrenergic blockers (BB's) are a class of anti-hypertensive medications that act as an antagonist to the beta-adrenergic receptors in the heart and blood vessels (Hwang *et al.*, 2015). In addition, beta-adrenergic receptors are also found on osteoclasts and osteoblasts and BB might possibly increase BMD *via* inhibition of the beta-adrenergic receptors in bone (Ghosh & Majumbar, 2014; Hwang *et al.*, 2015). Both Yang *et al.* (2011) and Song *et al.* (2012) confirmed this where they reported a decrease in hip fractures because of treatment with BB's. In contrast, Butt *et al.* (2012) reported an increased risk of fractures after initiation of BB treatment, but this might be due to the study population or differences in the adjustment of confounding factors.

Loop diuretics increase urinary Ca<sup>2+</sup> loss, thus negatively affecting BMD (Ilic *et al.*, 2013; Ghosh & Majumbar, 2014). Here, it was hypothesised that there is an increased renal Ca<sup>2+</sup> leak, as well as increased PTH and 1,25(OH)<sub>2</sub>D concentrations in the plasma (Ilic *et al.*, 2013). Calcium channel blockers decrease arterial stiffness, which is common during hypertension, specifically in the elderly (Hwang *et al.*, 2015). Calcium channel blockers might affect BMD *via* the inhibition of voltage-gated calcium channels, but ultimately seem to have no detrimental or beneficial effect on BMD (Ghosh & Majumbar, 2014).

#### **1.3.5.2 Dyslipidaemia**

Several studies have investigated the association between lipids and BMD; however, results have been inconsistent. For example, Jeon *et al.* (2011) reported a positive correlation between HDL-c and BMD in the lumbar spine of PostM women, but Li *et al.* (2015) reported that higher levels of HDL-c were positively associated with a higher probability of having osteoporosis in a PostM Chinese population.

Jeong *et al.* (2014) reported that the pathogenesis of osteoporosis might be associated with altered lipid metabolism. Disorders of lipid metabolism may result in high levels of oxidised lipids, which is also considered to be the common trigger between these two conditions.

Oxidised lipids stimulates adipocyte differentiations and suppress osteoblast differentiation. (Wong *et al.*, 2016; Jeong *et al.*, 2014). Oxidised lipids stimulate arterial wall mineralisation, which facilitates the development of atherosclerosis (Jeong *et al.*, 2014). It was suggested that HDL-c inhibits the osteogenic activity of vascular cells *via* the induction of inflammatory cytokines (IL-6 and IL- $\beta$ ) (Nóbrega da Silva *et al.*, 2014).

Joeng *et al.* (2014) reported no differences in HDL-c and TG between women with normal and low BMD, suggesting that HDL-c and TG might not be directly related to bone metabolism (Joeng *et al.*, 2014). In agreement, in Chinese PostM women, no association were evident between TC, TG and LDL-c and osteoporosis (Li *et al.*, 2015).

In a PostM study population, TC was however negatively associated with BMD at the femoral neck (Kim *et al.*, 2010) and Joeng *et al.* (2014) reported a similar result in PreM women, but interestingly the opposite was true in PostM women, where higher TC was associated with higher BMD.

#### *Cholesterol lowering agents*

The use of statins has been associated with an increase in BMD and a decrease in fracture risk, indicating a positive effect on BMD (Jeong *et al.*, 2014). For example, a study investigating the effects of atorvastatin on BMD reported significantly higher total hip BMD (Chuengsamarn *et al.*, 2010). Similarly, daily treatment with fluvastatin for three years, resulted in moderately increased bone formation, and stability in PostM hypercholesterolaemic women (Gotoh *et al.*, 2011). The mechanism by which statins might increase bone formation and BMD is possibly *via* increased ALP activity and osteoblast differentiation or inhibition of osteoclast differentiation (Mandal, 2015).

Some statins exert a similar effect as amino-bisphosphonates, a commonly used agent for the treatment of osteoporosis, that decreases the rate of bone turnover (Chuengsamarn *et al.*, 2010; Appelman-Dijkstra & Papapoulos, 2014). Although another study reported that statin treatment did not change bone turnover makers (CTx, BAP, osteocalcin), it did significantly reduce the osteocalcin: CTx ratio (Jiang *et al.*, 2007).

#### **1.3.5.3 Fasting plasma glucose and insulin concentrations**

Kim *et al.* (2010) found no significant association between FBG and BMD at the femoral neck ( $r=-0.037$ ,  $p=0.220$ ) and similarly, Alissa *et al.* (2014) also reported non-significant associations between FBG and BMD at the lumbar spine and femoral neck of PostM women. However, in the same PostM study population, FBG was significantly associated with total body BMD ( $r=0.193$ ,  $p<0.01$ ) (Alissa *et al.*, 2014).



Furthermore, Alissa *et al.* (2014) reported that circulating insulin concentrations were the main determinant of BMD at the femoral neck and lumbar spine; whereas Muka *et al.* (2015) reported that, the association between the MetS and higher BMD was due to elevated glucose levels. A possible mechanism associated with the negative correlation between abnormal glucose levels and BMD, might be increased urinary  $\text{Ca}^{2+}$  excretion (hypercalciuria) and increases in inflammatory markers and cytokine levels (Nóbrega Da Silva *et al.*, 2014).

The low-grade inflammatory state present in IR individuals was proposed to be one of the common pathogenic mechanisms increasing fracture risk in these individuals (Jeon *et al.*, 2011; Alissa *et al.*, 2014; El Maghraoui *et al.*, 2014). During this state there is increased secretion of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 and IL-6), inducing bone resorption with a resultant lower BMD. It is further suggested that these pro-inflammatory cytokines can stimulate osteoclast activity through regulation of the RANKL-RANK system (Cao, 2011).

Furthermore, in obese individuals, increased levels of plasma insulin result in the overproduction of ovarian androgen and oestrogens with an insufficient production of sex hormone-binding globulin (SHBG) by the liver (Taie & Rasheed, 2014). These hormonal changes might result in elevated sex hormone levels and increased bone mass, due to reduced osteoclast activity and stimulation of osteoblasts (Taie & Rasheed, 2014).

#### **1.3.5.4 Obesity**

Studies investigating the relationship between BMD and fat distribution reported conflicting results (Zillikens *et al.*, 2010; Cohen *et al.*, 2013; Alissa *et al.*, 2014). Although obesity could be considered a protective factor against excessive bone loss during ageing, FM positively influenced BMD, possibly due to the increased conversion of androgens to oestrogens in AT (Zillikens *et al.*, 2010; Jeon *et al.*, 2011; Chantler *et al.*, 2012; Alissa *et al.*, 2014). In addition, obesity might also lead to an increased BMD *via* the higher mechanical loading associated with obesity (Kruger *et al.*, 2011; Xue *et al.*, 2012; El Maghraoui *et al.*, 2014).

Several studies reported abdominal obesity might reduce BMD *via* the effect of elevated pro-inflammatory cytokines in obese individuals (for example IL-6, interleukin-9 (IL-9) and TNF- $\alpha$ ), activating osteoclastic bone resorption (Kim *et al.*, 2010; Migliaccio *et al.*, 2011; Chantler *et al.*, 2012; Cohen *et al.*, 2013; Liu *et al.*, 2013; El Maghraoui *et al.*, 2014; George *et al.*, 2014). Furthermore, studies investigating the effect of the MetS on BMD reported that the negative effect of the MetS on BMD was largely predicted by WC (Kim *et al.*, 2010; Jeon *et al.*, 2011), and that the only MetS risk factor that was significantly associated with BMD measurements was WC (Alissa *et al.*, 2014).

In a study by Zillikens *et al.* (2010), the positive association between android fat distribution and BMD could largely be explained by the higher BMI, but when adjusted for BMI, android fat distribution had no or a negative association with BMD. Additionally, a study by Sohl *et al.* (2015) indicated that not only BMI, but also FM influence QUS measurements, since a higher BMI was associated with higher BUA and SOS measurements, suggesting higher BMD's.

### 1.3.5.5 Lifestyle risk factors affecting BMD

#### ***Physical activity***

Physical activity directly modifies bone remodelling *via* mechanical stimuli, resulting in increased mineralisation, as well as bone geometry (increased periosteal diameter and cortical thickness), thereby protecting against bone fractures (Langsetmo *et al.*, 2012). Weight-bearing physical activities are more beneficial to bone health than non-weight-bearing physical activities (Braun *et al.*, 2015). Therefore, it is recommended that individuals participate in weight-bearing endurance exercises, three to five times a week, and jumping and/or resistance exercise, two to three times a week for about 30 to 60 minutes per day in order to maintain bone health throughout adolescence (Braun *et al.*, 2015).

In contrast to the abovementioned findings, a study on skeletal robustness and bone strength, have shown that daily total steps as a measure of physical activity in young adults, did not significantly influence BQI (Scheffler *et al.*, 2014). Similarly, Sritara *et al.* (2015) reported that different types of physical activities had no significant effect on the lumbar spine, femoral neck or hip BMD in women.

#### ***Breastfeeding***

During lactation, hyperprolactinemia and hypoestrogenemia levels promote bone resorption, as a result of increased plasma  $\text{Ca}^{2+}$  levels, allowing an increased  $\text{Ca}^{2+}$  uptake by mammary tissue, to provide  $\text{Ca}^{2+}$ -rich milk to the infant (Lovelady *et al.*, 2009; Bjørnerem *et al.*, 2011; Prentice, 2013). It is evident that there are marked changes in  $\text{Ca}^{2+}$  metabolism during lactation where significant  $\text{Ca}^{2+}$  is lost from the mother, depending on the quantity of breast milk produced, the diet of the mother, and duration of breastfeeding (Canal-Macias *et al.*, 2012). The loss of BMD seems to be the greatest in the first five months of lactation (Lovelady *et al.*, 2009).

In contrast to the abovementioned statements, a study on PostM Turkish women found no relationship between the duration of breastfeeding and BMD, and this study further concluded that longer duration (up to 220 months) of breastfeeding did not affect bone mass later in life (Yazici *et al.*, 2011). Greater losses in trabecular vs. cortical bone was also reported during lactation, although the BMD returns to normal levels once breastfeeding is

ceased and is completely restored in the medium to long-term (Canal-Macias *et al.*, 2012). A proposed mechanism for the return of BMD to normal levels is that weaning will allow oestrogen repletion that will increase osteoblast activity. This will increase  $\text{Ca}^{2+}$  uptake into bone, that will decrease serum  $\text{Ca}^{2+}$ , resulting in increased PTH and  $1,25(\text{OH})_2\text{D}$ . The resultant increase in PTH and  $1,25(\text{OH})_2\text{D}$  will increase renal  $\text{Ca}^{2+}$  reabsorption and increase intestinal  $\text{Ca}^{2+}$  absorption respectively (Prentice, 2013).

Bjørnerem *et al.* (2011) reported that breastfeeding was associated with a lower risk of hip fracture. Similarly, Canal-Macias *et al.* (2012) reported that in PreM women with a history of breastfeeding, a higher total BMD, higher BMD at the lumbar spine, as well as cortical bone were present. It is therefore evident that breastfeeding had no deleterious effects on BMD, and in fact, may provide modest protection against fractures.

### **Smoking**

Previous smokers and/or snuff users might have a higher risk of developing osteoporosis, with heavy smokers being at an even higher risk of hip fractures (Ayo-Yusuf & Olutola, 2014). Smoking might affect BMD *via* decreasing  $\text{Ca}^{2+}$  absorption and decreasing circulating levels of PTH and  $25(\text{OH})\text{D}$  (Øyen *et al.*, 2014). Smoking is a known factor that leads to increased production of free radicals, which may result in increased bone resorption. Elements present in tobacco, decrease oestrogen production, which affects the intestinal absorption of  $\text{Ca}^{2+}$  (Kruger *et al.*, 2011).

It was also suggested that smoking causes a decrease in FM, which results in decreased bone formation and an increased fracture risk (Shahab, 2012). Others reported that the lower BMD commonly seen in smoking individuals might be due to lower body weight or FM, rather than the direct effect of smoking (Øyen *et al.*, 2014; Ayo-Yusuf & Olutola, 2014).

Nicotine has also been stated to have anti-oestrogenic effects and might lead to the development of early menopause and osteoporosis in female smokers (Ayo-Yusuf & Olutola, 2014). Shahab (2012) suggested that smoking increases oestradiol clearance and SHBG, which leads to lower levels of free oestradiol, leading to increased bone resorption.

In smokers, ascorbic acid deficiency is common due to a greater turnover of this antioxidant, since it becomes depleted due to smoke-derived oxidant species (Varvadas *et al.*, 2008; Sørensen *et al.*, 2010; Shah *et al.*, 2015). This is detrimental to bone health since ascorbic acid is essential for the development of collagen, an important component of connective tissue (Peterkofsky, 1991; Sørensen *et al.*, 2010). This water-soluble vitamin increases the rate of bone healing (Bsoul & Terezhalmay, 2004), reduces bone loss, and acts as a protective factor against fractures (Shen *et al.*, 2013). It was also found that ascorbic acid is

essential for the proliferation and differentiation of mesenchymal stem cells into bone tissue (Shen *et al.*, 2013).

### **Alcohol consumption**

Several studies have shown that moderate alcohol consumption, which some studies report to be up to three drinks and other up to two drinks per day, increased BMD, possibly due to increased calcitonin levels that inhibit bone resorption (Kruger *et al.*, 2011; Maurel *et al.*, 2012; Marrone *et al.*, 2012; Sritara *et al.*, 2015). A study on women aged between 50 and 62 years found that those who consumed more than one drink per day had significantly higher BMD at the lumbar spine and femoral neck than non-drinkers (McLernon *et al.*, 2012). In agreement, Marrone *et al.* (2012) also reported that moderate alcohol consumption may reduce the rate of bone loss in PostM women by diminishing increased bone turnover (Marrone *et al.*, 2012). In a study by Sommer *et al.* (2012), consuming more than three alcoholic drinks per week, was positively associated with BMD at the femoral neck and lumbar spine.

On the contrary, chronic alcohol consumption can result in decreased serum vitamin D levels that will decrease  $\text{Ca}^{2+}$  absorption in the small intestine and cause low serum  $\text{Ca}^{2+}$  levels in circulation that will ultimately stimulate bone resorption (Maurel *et al.*, 2012). Chronic alcohol abuse was also linked to decreased BMD, decreased osteoblast number and function, possibly due to increased bone resorption and elevated PTH levels (Kruger *et al.*, 2011; Marrone *et al.*, 2012). To summarise, it is evident that moderate alcohol consumption seems to have a positive effect on BMD, but chronic alcohol intake (abuse) generally resulted in decreased BMD.

### **Medications**

#### **Contraceptives**

Hormone oral contraceptives (OC) can be classified as either combined contraceptives containing both oestrogen and progestin (synthetic progestogens), or a progestin-only contraceptive, containing only progestin (WHO, 2016). A study comparing the use of combined oral contraceptives (COC) and progestin-only contraceptives, found that women (26-36 years old) using COC, had significantly higher BMD than those using the progestin-only contraceptives (Wei *et al.*, 2011). Differences in the effect of OC on BMD have also been reported by Elkazaz & Salama (2015), where the absence or previous use of OC had a positive effect on BMD, in comparison to current OC users.

The injectable contraceptive, depot medroxyprogesterone acetate (DMPA), suppress ovarian oestrogen synthesis *via* the hypothalamic pituitary ovarian axis (Pitts *et al.*, 2012).

Using DMPA has been linked to a decrease in bone mass, although this effect was reversed once DMPA was discontinued (Chantler *et al.*, 2012; Pitts *et al.*, 2012; Biason *et al.*, 2015).

The progestin-only injectable contraceptives are mostly used up to the age of 50 years; however, it should be discontinued thereafter, since its use significantly increases the risk for osteoporosis (National Contraception Clinical Guidelines, 2012; Lambert, 2014). Treatment with OC increases BMD in both PeriM and PreM women due to hypoestrogenic conditions. This positive association might suggest that the beneficial effect of using OC's during the reproductive years may persist even after OC cessation, and continue into the PostM years. This could be due to optimising PreM bone mass and/or the prevention of bone loss in the years prior to menopause (Wei *et al.*, 2011).

### *Glucocorticoids*

Elevated levels of glucocorticoids (GC) are detrimental to bone health (Hardy & Cooper, 2010; Lane & Yao, 2010; Lau & Adachi, 2010). The main naturally occurring GC in the body, cortisol, is produced by the adrenal glands, with receptors in almost all tissue types (Hardy & Cooper, 2010; Ragnarsson *et al.*, 2015). Cortisol, for example, has a major impact on protein, fat, and carbohydrate metabolism, the skeletal, cardiovascular and immune system (Ragnarsson *et al.*, 2015). Furthermore, GC's are widely used to treat chronic inflammatory conditions since they exert potent anti-inflammatory effects (Klein, 2015).

In PostM women, bone formation and bone resorption are usually coupled and up-regulated; however, during GC treatment, these processes are uncoupled (Lems & Saag, 2015). Prolonged use of GC's, can lead to a decreased BMD, increased fracture risk, and also an increased risk to develop CVD (Ragnarsson *et al.*, 2015).

Furthermore, the increased levels of GC's in the body may result in the development of glucocorticoid-induced osteoporosis (Hardy & Cooper, 2010; Lane & Yao, 2010; Lau & Adachi, 2010). This is possibly due to GC increasing osteocyte apoptosis or increasing skeletal fragility *via* a decrease in muscle mass and decreased mechanosensing (Hardy & Cooper, 2010). The effects on osteoblasts include a decrease in their proliferation, which then leads to a decrease in bone formation and increased apoptosis of these cells (Lane & Yao, 2010; Lau & Adachi, 2010). Conversely, the effects on osteoclasts seem to be multifactorial as decreases in growth factors, sex steroids and  $\text{Ca}^{2+}$  absorption and resorption all affect the osteoclasts (increasing PTH levels). All this contributes to the increased levels of RANKL and decreased OPG (Lau & Adachi, 2010). The decreased OPG levels and increased RANKL levels result in increased osteoclast absorption and survival, as well as decreased apoptosis of osteoclasts (Lau & Adachi, 2010).

### *Supplements*

Both vitamin D<sub>2</sub> and D<sub>3</sub> can be given as supplements to increase circulating levels of 25(OH)D, although vitamin D<sub>3</sub> generally produce higher levels of circulating vitamin D due to its longer retention time in the body (Wimalawansa, 2012). A study investigating the use of a combination of calcium and vitamin D supplements found these supplements to be associated with a better maintenance of bone health when BMD was measured at several anatomical sites (Zhou *et al.*, 2013). Another report suggested that vitamin D supplementation alone, might not be as sufficient to treat osteoporosis, and it is therefore recommended to take both vitamin D<sub>3</sub> and calcium supplements (Madar *et al.*, 2015).

#### **1.3.5.6 Non-modifiable risk factors**

##### ***Genetics (Familial bone health and disease history)***

According to Sedó Sarkis *et al.* (2011), genetic factors account for 75-80% of the variation in PBM. A study indicated that parental history of bone fractures (amongst other factors) was a predictor of lower BMD in the offspring (Bączyk *et al.*, 2012). Although this relationship exist, the correlation between parental history of fractures and reduced BMD in the offspring was very weak (Bączyk *et al.*, 2012).

A South African study found that adolescents had a decreased risk of fracture in the presence of increased maternal bone mineral content (BMC) at the lumbar spine. A 24% reduction in fracture risk with every SD increase in maternal lumbar spine BMC was noted. Despite the above-mentioned finding, maternal history of fractures was not associated with fractures in the offspring (Thandrayen *et al.*, 2014).

##### ***Ethnicity***

Since high concentrations of melanin in Black or African-American individuals reduce the skin's ability to generate vitamin D<sub>3</sub> from sunlight, vitamin D deficiencies are more common in African-Americans than in Caucasians (Sharp, 2011; Johnson, 2013). Research indicated that the Black population requires larger doses of UVB exposure to achieve a maximal cutaneous synthesis of vitamin D (Ardawi *et al.*, 2011). The vitamin D deficiency in Black or African-American individuals might result in a lower BMD in these population groups than in Caucasians.

Contrasting to the above mentioned, a South African study reported higher BMD in Black African women (age according to BMI <25 kg/m<sup>2</sup>: Black: 35±9, Caucasians: 39±6; p<0.01); BMI ≥25 kg/m<sup>2</sup>: Black: 37±8, Caucasians: 37±7). In this study, it was hypothesised that the increased BMD in Black African women were likely due to the higher body mass in these participants compared to the Caucasian participants (Conradie *et al.*, 2014). This is quite likely since a study comparing African-Americans, Caucasians, Chinese and Japanese



women also reported that the differences in the rates of bone loss during the menopausal transition was largely due to the differences in body mass between the ethnic groups (Finkelstein *et al.*, 2008). In addition, Sowers *et al.* (2013) and George *et al.* (2014) also reported differences in BMD between ethnic groups due to body mass.

A South African study reported that the femoral neck and total hip BMD, but not lumbar spine BMD, was significantly higher in Black compared to Caucasian women (Chantler *et al.*, 2012). At the femoral neck and total hip, bone consists mainly of cortical bone, which is more responsive to weight-bearing and mechanical loading than trabecular bone. In this specific study, the Black women had higher body masses than the Caucasian group, and the mechanical loading effect of increased body mass might be the reason for differences between these two groups (Chantler *et al.*, 2012).

Therefore, although studies reported differences in the 25(OH)D levels between different ethnic groups (Sharp, 2011; Johnson, 2013; Ardawi *et al.*, 2011), the results still indicated that BMD was higher in Black individuals, but this was likely due to higher body masses in these groups (Conradie *et al.*, 2014; Sowers *et al.*, 2013; George *et al.*, 2014).

### ***Hormonal status (menopause)***

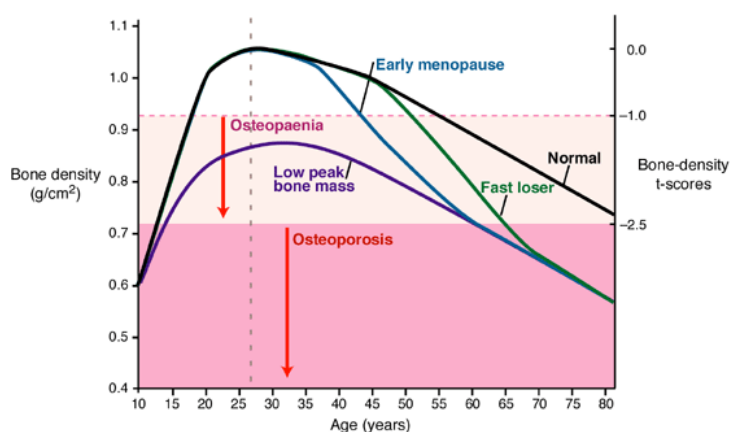
The three forms of oestrogen include 17 $\beta$ -oestradiol (E<sub>2</sub>), oestrone and oestriol, with E<sub>2</sub> being the most physiological active form (Pradhan, 2014). In PreM women, oestrogen is primarily released from the ovaries, but aromatization in AT also produces oestrogen (Pradhan, 2014). It is well known that oestrogen plays a major role in bone development and health, as well as bone mass accrual up to PBM (Khosla *et al.*, 2012; Karsenty, 2012). Decreases in oestrogen concentrations will therefore result in decreased BMD (Khosla *et al.*, 2012; Karsenty, 2012).

Age-related bone loss commences immediately after PBM is reached, although the largest portion of bone loss occurs after the age of 65 years (Silva *et al.*, 2015). Bone loss initially occurs at a rapid rate, but the rate of bone loss gradually decreases after about five to ten years into menopause. Oestrogen replacement therapy has the ability to protect against decreases in BMD during menopause, which is normally reached between the ages of 45 and 56 years (Al-Safi & Polotsky, 2014; AIDughaiter *et al.*, 2015). During menopause, the menstrual cycle stops due to the termination of ovarian hormone production, and common side effects or symptoms develop, which includes hot flushes, sleeping disturbances, joint and muscular discomfort, fatigue and joint stiffness (AIDughaiter *et al.*, 2015).

Menopause is also associated with increased FM, which has a positive effect on BMD, due to higher levels of oestrogen (Zillikens *et al.*, 2010; Migliaccio *et al.*, 2011; Al-Safi & Polotsky 2014). In PostM women, oestrogen is synthesised *via* the enzyme aromatase from

androgen precursors, situated in AT; therefore, oestrogens synthesised in AT is one of the major sources of oestrogen in PostM women (Migliaccio *et al.*, 2011). After the age of 50, weight loss increases the risk of hip fractures, whereas weight gain results in a lower risk of hip fractures (Silva *et al.*, 2015).

In PostM women, bone loss occurs as a result of increased and unbalanced bone turnover, where bone resorption dominates over bone formation (Marrone *et al.*, 2012). Loss of BMD during menopause occurs in two stages. During *Stage one*, there is an oestrogen-dependant exponential decrease in BMD continuing for approximately five to ten years (Figure 1.13). *Stage two* is considered more constant, where bone loss occurs as a function of ageing (Bączyk *et al.*, 2012) (Figure 1.13). Prior to menopause, oestrogen facilitates the maintenance of bone strength through its diminishing effect on oxidative stress in bone and bone marrow; however, this effect is lost after menopause (You *et al.*, 2014). The presence of oestrogen deficiency during menopause causes the up-regulation of RANKL in bone marrow cells, resulting in increased bone resorption (You *et al.*, 2014).



**Figure 1.13: Variation of the bone density of women at different ages. (Brown & Duncan, 1999).**

In PostM women with hypo-oestrogenism, bone loss occurs at a rate of about 0.5 - 1.5% per year, and a small percentage of women classified as “fast bone losers”, can lose up to 3-5% bone mass per year. Although these rates are dependent on hormonal, environmental and genetic factors (Silva *et al.*, 2015), bone loss during menopause is due to the increased resorption by osteoclasts, nevertheless during ageing, there is reduced bone formation due to low number and function of osteoblasts (Bermeo *et al.*, 2014). From the evidence provided here, it is clear that age and menopausal status causes a decrease in BMD.



## 1.4 Summary

From the cited literature, it can be argued that the MetS is not only a global problem, but there also seem to be some evidence linking this problem to the South African population. The literature provides sufficient reason to further explore this hypothesis. The metabolic abnormalities seem to affect BMD; however, the current literature remains controversial regarding its potential positive or negative effects. Abdominal obesity appears to be the main MetS factor affecting BMD.

However, ageing and/or menopausal status are also known risk factors that negatively affect BMD. Various assessment methods exist to determine BMD, and even though DEXA is regarded as the gold standard, this method is not easily accessible in South Africa; therefore the use of QUS might be a more practical method to assess BMD in a South African field setting. To date, no reference values are available for BMD of the South African population, more specifically for the mixed (Coloured/Black) populations of South Africa. There are also limited data on BMD in the context of the MetS and menopausal status of female farm workers in South Africa.

### 1.4.1 Problem statement

Little evidence exists investigating the combined effect of menopausal status and the MetS, on BMD. There are also limited data available on the prevalence of the MetS in South Africa, specifically the Western Cape Province.

### Research questions

We formulated the following research questions based on the problem statement:

- What is the prevalence of the MetS and osteopenia and osteoporosis in a Western Cape female farm worker population?
- Is there an association between menopausal status, MetS status, and BMD in a female farm worker community?

### 1.4.2 Hypothesis

From the second research question, this study hypothesises that there will be a difference in the BMD between PreM vs. PostM, and MetS vs. Non-MetS females.

### 1.4.3 Aims

The aims of this study were to:

- establish the prevalence of the MetS in a selected female farm worker population in the Western Cape Province

- describing the risk factors associated with BMD in this female farm worker population
- determine the interaction between BMD (bone health), menopausal status, and the MetS.

#### **1.4.4 Objectives**

- To categorise the female volunteers into groups according to their menopausal (PreM and PostM) and metabolic status (presence or absence of the MetS);
- To assess BMD using the SONOST 3000 (OsteoSys Ultrasound Bone Densitometer)
- To assess serum vitamin D, ALP, and PTH levels;
- To obtain information on the bone health history of the participants;
- To assess body composition using anthropometric measurements;
- To determine the physical activity level of the participants with the Global Physical Activity Questionnaire

## Chapter 2

## Materials and Methods

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This chapter describes the materials and methods used to collect and analyse data for this research project.

### 2.1 Ethical considerations

Ethical clearance was obtained from the Human Research Ethics Committee (HREC) of Stellenbosch University, Protocol Nr (N13/04/052) (Appendix A). Prior to the data collection, information sessions were held with potential volunteers at each of the different data collection sites, where they were informed about the study aims in an easily understood manner, and also had an opportunity to ask questions. During these sessions, individuals had the opportunity to indicate whether they would be interested to participate, after which appointments were scheduled with volunteers.

Before volunteers were recruited, the researcher made sure participants were still prepared to be part of the study. After they volunteered, each participant signed a "*Participant information and consent form*" (Appendix B) prior to data collection. Each participant received an unique study code, which was used on all questionnaires, as well as blood samples, ensuring privacy and anonymity. In addition, participants were given the autonomy to withdraw their consent and participation at any time during data collection. All hard copies of the consent forms were kept separate from the raw data, which were kept in a locked file drawer in a lockable office. The data were entered using the Microsoft Office Excel 2010 package, password protected and backed-up daily on the Stellenbosch University network server.

### 2.2 Study design and sample population characteristics

A cross-sectional study design was followed in which the association between the MetS and BMD in PreM- and PostM farm working women from the Boland wine district, Western Cape, South Africa were described. The participants were recruited from three selected farming districts, including the Villiera Wine Estate (Owethu Clinic - Stellenbosch), Neethlingshof Wine Estate (Stellenbosch), and Solms-Delta Wine Estate (Franschoek).

From the total sample population (including men), a total of n=191 participants (n=42 men, and n=149 women) between the ages of 20 and 60 years were recruited. A total of two women withdrew their consent due to time constraints, where after the remaining women

(n=147) were subsequently divided into two main groups according to their metabolic status (MetS and Non-MetS).

These women were further categorised according to age and menopausal status into (A) PreM - between the ages of 20 and 39 years, (B) PreM - older than or equal to 40 years of age, and (C) PostM. Specific to this study, menopause or PostM was defined as the absence of menstruation for at least 12 consecutive months (Alissa *et al.*, 2015; Ebrahimpour *et al.*, 2010).

A random sample of n=80 women were selected from the total sample (n=147), and subsequently categorised into the different study groups. Since there were relatively few PostM women with, and without the MetS, all the women in these specific groups were included.

The final groups used for all data analyses are described as follows:

- **Group 1:** n=14 PreM women with the MetS (between the ages of 20–39 years) (PreM 20-39 years)
- **Group 2:** n=15 PreM women with the MetS (age  $\geq$ 40 years) (PreM  $\geq$ 40 years)
- **Group 3:** n=15 PostM women with the MetS (PostM) - [All participants in this group were selected (no randomization)]
- **Group 4:** n=14 PreM women without the MetS (between the ages of 20–39 years) (PreM 20-39 years, Non-MetS)
- **Group 5:** n=14 PreM women without the MetS (age  $\geq$ 40 years) (PreM  $\geq$ 40 years, Non-MetS)
- **Group 6:** n=8 PostM women without the MetS (PostM, Non-MetS) - [All participants in this group were selected (no randomization)]

### 2.2.1 Inclusion and exclusion criteria

Before recruitment, all eligible participants were selected based on the following inclusion and exclusion criteria:

- Inclusion criteria:
  - All participants had to be women between the ages of 20-60 years;
  - All women had to be permanent South African residents, currently residing in the Western Cape Province (from the three selected districts);
- Exclusion criteria
  - Any woman younger than 20, or older than 60 years of age;
  - Not a permanent resident of South Africa, or residing in the Western Cape Province.
  - Pregnant or lactating women
  - Any known bone disease
  - Human Immunodeficiency Virus and the use of Antiretroviral Therapy

### **2.2.2 Definition of the metabolic syndrome**

The IDF definition is one of the most widely used definitions in South African studies and therefore suitable for the classification of the MetS in this study population, to allow direct comparisons between studies. This specific definition also includes a set range of optimal WC levels that are gender- and ethnic-specific. Although there are no specific WC cut-off values for South Africans, the IDF suggested that the values for Europeans be used for populations in South Africa and Sub-Saharan Africa (IDF, 2006).

Using the IDF definition, women were classified as having the MetS if they presented with an increased WC according to their ethnic group (Table 1.1), plus any two of the following criteria: increased TG ( $\geq 1.7$  mmol/L), reduced HDL cholesterol ( $< 1.29$  mmol/L for women), increased BP (SBP  $\geq 130$  mmHg, or DBP  $\geq 85$  mmHg) and elevated FPG ( $\geq 5.6$  mmol/L) (IDF, 2006).

## **2.3 Data collection**

After written informed consent was obtained, a registered HPCSA and GCP qualified medical biological scientist assessed BP and heart rate, followed by blood sampling. Basic anthropometric measurements were recorded by the researcher on a proforma sheet (Appendix C), followed by BMD assessment. Participants were then asked to complete a demographic questionnaire, a global physical activity questionnaire (GPAQ), and a bone health questionnaire.

### **2.3.1 Blood pressure and heart rate**

Prior to BP and heart rate assessments, participants were allowed to remain in a relaxed, seated position for approximately five to ten minutes. Blood pressure measurements were performed using an appropriately sized ERKA blood pressure cuff (Perfect Aneroid Clinic 48, Germany), and a Littmann 3M™ stethoscope (G13L 12829, Littmann 3M™ USA). In cases where participants presented with very high, or low BP, repeated BP measurements were taken and the results averaged. In cases where the second measurement also showed a potentially, critically elevated BP, the participant was immediately referred to medical staff. The heart rate was measured using a finger plethysmography heart rate monitor (Contec medical systems, China).

### **2.3.2 Haematology**

Blood was drawn from the cubital vein in the right arm, under aseptic conditions and collected into a plasma fluoride tube (4 ml), an ethylenediaminetetraacetic acid tube (EDTA, 4 ml) and two serum separating tubes (SST, 4 ml each). The blood tubes were immediately inverted (eight times), where after the plasma fluoride tube was placed on ice for fasting

glucose analysis by PathCare laboratory (Stellenbosch). The EDTA tube was immediately centrifuged (MRC Laboratory Centrifuge) for ten minutes at 4000 revolutions per minute (rpm), where after the plasma was aliquoted into 1.5 ml Eppendorf tubes and placed on dry ice until storage in a -80 °C bio-freezer. The two SST tubes were allowed to clot at room temperature (22 °C) for ten minutes and subsequently centrifuged. One SST tube was placed on ice for fasting insulin and lipogram analyses, performed by the local PathCare laboratory (Stellenbosch). These blood parameters, together with WC and BP measurements, were then used to classify participants into either the MetS or Non-MetS groups respectively.

Serum obtained from the remaining SST tube was aliquoted into 1.5 ml Eppendorf tubes and placed on dry ice until return to the Department of Physiological Sciences' molecular laboratory, where the samples were stored in a bio-freezer (-80°C) for later analysis. Serum samples were used to analyse vitamin D and serum alkaline phosphatase (sALP), while plasma samples were used for parathyroid hormone (PTH) analyses. With the exception of sALP, which was also determined by the local PathCare laboratory (Stellenbosch), all remaining biochemical parameters were analysed by the researcher in-house, using an enzyme-linked immunosorbent assay (ELISA). The reference levels of the different blood-specific bone markers are provided in Table 2.1.

**Table 2.1: The reference levels of the bone-specific markers.**

Biochemical parameters	Reference values	Diagnoses
Alkaline phosphatase	40 –120 units per litre (U/L)	Normal
Vitamin D	< 20 nmol/L	Deficient
	> 50 nmol/L	Sufficient
	> 75 nmol/L	No additional change in p-PTH levels
	> 125 nmol/L	Excess
Parathyroid Hormone	15-65 pg/mL	Normal
	>65 pg/mL	High

(Garcia *et al.*, 2013; Saliba *et al.*, 2011; ARUP Laboratories, 2014).

### 2.3.2.1 Enzyme-linked immunosorbent assay

The ELISAs that were used are classified as 'sandwich' ELISA assays. Briefly, the 96-well plates were pre-coated with an immobilised capture antibody that binds to the target protein and captures it from the added testing sample. Hereafter, detection antibodies were added,

allowing the formation of complexes with the captured target protein. This step catalyses the appearance of a colour reaction *via* activation of a covalently-linked enzyme. The colour produced here is then representative of the quantity of the target protein present in the sample.

#### **a) Parathyroid Hormone**

For the analysis of p-PTH, a BioVendor Human Parathyroid Hormone ELISA (Appendix D), (RIS003R) was used. All procedures were done according to the manufacturer's protocol. Samples and reagents were equilibrated at room temperature (22 °C) prior to analysis. In order to establish whether the samples needed to be diluted, literature on similar populations were consulted, which revealed that PTH levels were relatively low and that no dilution is needed (Alissa *et al.*, 2014; Ardawi *et al.*, 2011; Batchetta *et al.*, 2013; Garcia *et al.*, 2013; George *et al.*, 2013; Guasch *et al.*, 2012; Guitierrez *et al.*, 2011; Hernandez *et al.*, 2011; Joo *et al.*, 2013; Makariou *et al.*, 2012; Reis *et al.*, 2007; Van Ballegooijen *et al.*, 2014; Yagi *et al.*, 2014).

For assay procedures, the calibrators (standards) and controls were reconstituted according to the datasheet provided by the supplier. The following range of standards was supplied: 1400 pg/mL; 666 pg/mL; 224 pg/mL, 70 pg/mL, 22 pg/mL and 0 pg/mL (blank). Fifty microliters of incubation buffer was added to all wells, followed by 50 µL of the standards, controls and samples in duplicate. The plate was incubated for two hours at room temperature (22 °C), followed by a thorough wash step (4 x 400 µL wash buffer). Anti-PTH-horseradish peroxidase (HRP) was added and left for an hour at room temperature (22 °C). The plate was repeatedly washed (three times), followed by the addition of a chromogenic solution containing 3,3',5,5'-tetramethylbenzidine (TMB). The plate was incubated for 30 minutes, where after a stop solution was added. Absorbencies were recorded at 450 and 490 nm using a plate reader (EL800 Universal Microplate Reader). The quantity of substrate turnover was determined by measuring the absorbance, which was proportional to the p-PTH concentration (Biovender ELISA).

#### **b) Vitamin D**

The vitamin D ELISA kit (Elabscience, E-EL-0012) with all its constituents, as well as the serum samples, were equilibrated to room temperature (22 °C) prior to analysis (Appendix E). The standards were prepared to generate a standard curve with the following concentrations: 400 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 12.5 ng/mL, 6.25 ng/mL, and 0 ng/mL. Briefly, 50 µL of the standard solution and samples were added in duplicate to each well, followed by the addition of 50 µL of biotinylated detection antibody. The plate was then allowed to incubate for 45 minutes at 37 °C on a shaker (Orbital Shaker Incubator,

MRC), followed by a triple wash step. After this, a 100 µL HRP-conjugate was added and the plate incubated for 30 minutes at 37 °C, followed by washing the plate five times. This was followed by adding 90 µL of substrate reagent, after which the plate was incubated for 15 minutes at 37 °C until a stop solution, containing sulphuric acid, was added. The absorbencies were recorded spectrophotometrically at a wavelength of 450 nm using a plate reader (EL800 Universal Microplate Reader).

### **2.3.3 Anthropometry: base measurements**

All anthropometric assessments were done by the researcher according to the standard methodology from the International Society for the Advancement of Kinanthropometry (ISAK) and noted on the data collection sheet.

#### **2.3.3.1 Body mass**

Body mass was measured to the nearest 0.01 kg using a Seca 634 calibrated scale (Seca, United Kingdom, Birmingham, England). Participants were instructed to remove their shoes and excess clothing, and remain in a still-standing position on the scale with their weight evenly distributed.

#### **2.3.3.2 Stretched stature**

Stretched stature was measured to the nearest 0.1 cm with a portable Leicester™ stadiometer (Leicester, England). Each participant was asked to stand upright against the stadiometer, with their heels together, also ensuring that their buttocks and scapulae were in contact with the stadiometer. Participants were positioned in the “Frankfurt Plane”, and the Broca plane of the stadiometer moved downwards until it made contact with the vertex of the skull. Participants were then asked to inhale and hold their breath, where after the researcher applied a slight upward pressure from the mastoid process in order to assess the stretched stature of the participant (Stewart & Sutton, 2012).

#### **2.3.3.3 The body mass index**

The base measurements, weight and height, were used to calculate the body mass index (BMI) (Equation 3). Table 2.2 provides the different classifications for the BMI.

**Equation 3: Calculation of BMI in kg/m<sup>2</sup>**

$$\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2}$$



**Table 2.2: Classification of BMI according to the WHO.**

WHO classification of BMI	BMI (kg/m <sup>2</sup> )	Classification
	<18.50	Underweight
	18.50-24.99	Normal
	≥ 25.00	Overweight
	30-34.99	Obese (class I)
	35.00-39.99	Obese (class II)
	≥40	Obese (class III)

(WHO, 2015).

### 2.3.3.4 The waist circumference

Waist circumference was defined as the circumference of the abdomen at the narrowest point between the lower costal (10<sup>th</sup> rib) border, and the superior aspect of the iliac crest, perpendicular to the long axis of the trunk (Stewart & Sutton, 2012). The point between the iliac crest and lower rib is the generally accepted level for this measurement. Waist circumference (WC) was measured using a Lufkin<sup>®</sup> executive thin line tape measure (Lufkin W606PM) (Apex Tool Group, USA). The measuring tape is passed around the body and tightened at the required level ensuring that the level is horizontal and that the soft tissue is not compressed. The reference values for WC are given in Table 2.3.

**Table 2.3: Reference values per gender for waist circumference.**

Waist circumference	≤80 cm for women
	≤94 cm for men

(IDF, 2006).

### 2.3.3.5 The hip circumference

The hip circumference (HC) was defined as the circumference of the buttocks at the level of their greatest posterior protuberance, perpendicular to the long axis of the trunk (Stewart & Sutton, 2012). The HC measurement was recorded whilst the participant stood erect with their feet together. The tape is passed around the body and tightened at the required level, ensuring that compression of the soft tissue does not take place and the measurement is noted.

### 2.3.3.6 The waist to hip ratio

The waist to hip ratio (W:H) (Equation 4) is considered an important predictor for the development of cardiovascular diseases, the MetS, as well as altered BMD (Aghaei Meybodi *et al.*, 2011; Ardawi *et al.*, 2011; Seo *et al.*, 2008; Zillikens *et al.*, 2010).

**Equation 4: Calculation of Waist to hip ratio**

$$W:H = \frac{WC}{HC}$$

**2.3.4 Ultrasound bone densitometry measurement**

Bone mineral density was assessed using the SONOST 3000 Ultrasound Bone Densitometer (OsteoSys, Korea) (Figure 2.1). The date of birth, gender, ethnic background, foot supporter number, height and weight of each participant were recorded on the SONOST 3000 prior to BMD analysis in order to accurately estimate the T- and Z-scores (Appendix F).

The calcaneal bone is considered a validated anatomical site for the measurement of BMD and furthermore considered to be highly predictive of fracture risk (Seo *et al.*, 2008). The right calcaneus bone was used for all BMD assessments. Measuring the BMD took approximately one minute, after which an ultrasonic wave image (graph) was generated on a liquid crystal display of the bone densitometer.



Figure 2.1: The SONOST 3000 Ultrasound Bone Densitometer (OsteoSys 3000).

**2.3.4.1 Questionnaires**

Additional supplementary information was obtained from participants through standard and validated questionnaires, including demographics, physical activity level (GPAQ), overall bone health, and dietary habits.

**a) Demographic questionnaire**

To contextualise the participants' socio-demographic status, a structured basic demographic questionnaire was used focussing on age, home language, monthly or weekly income, the type of house, and specifics regarding the age and number of people in the household (Appendix G).

## **b) Physical activity questionnaire**

The Global Physical Activity Questionnaire (GPAQ), (Appendix H), developed by the WHO in 2002, formed part of the WHO STEPwise Approach to Chronic Disease Risk Factor Surveillance (STEPS). This questionnaire described physical activity during work, as well as for transport and recreational activities. The frequency and duration of the activity were recorded, and the energy required for these activities was either classified as vigorous, moderate or intense (Bull *et al.*, 2009).

## **c) Bone health questionnaires**

The bone health questionnaire was used to assess bone health in participants. This questionnaire focussed on questions such as bone health history, menstrual history, diet history, sunlight exposure, smoking and drinking habits, family health history, and physical activity (Appendix I).

## **d) Bone health history**

The bone health section of the questionnaire included questions regarding the participant's history of fractures or broken bones, stress fractures and any medications that could affect bone physiology.

## **e) Menstrual history**

Since menses play a crucial role in general bone health, questions focussed on the women's age of first menstrual cycle, date of recent menstrual cycles, and presence of oligomenorrhea, menorrhagia or amenorrhea. Participants were asked about birth control methods such as use of pharmacological or medical devices, as well as number of pregnancies and past breastfeeding practices.

## **f) Diet history**

This section of the bone health questionnaire concentrated on specific questions related to past practices of dieting, and whether participants had taken any appetite-suppressing medication. The participants were asked whether they engaged in any eating disorder behaviour such as inducing vomiting or binge eating, as well as their perception of body weight changes over the past five years.

## **g) Sunlight exposure and other contributing factors**

Assessing participant's sunlight exposure aimed to quantify the average daily sunlight exposure in minutes. Participants were also questioned about their medical history (past

and present), including medication and supplement intake that could provide any possible links between interacting pharmacological agents and vitamin D status. Smoking and alcohol consumption were also included as part of other contributing factors that could affect bone health. For the purpose of this study moderate alcohol consumption was classified here as consuming up to two drinks per day (grouped as “current” alcohol consumers), and heavy alcohol consumption was classified as having three or more drinks per day (Marrone *et al.*, 2012).

#### **h) Family health history**

The questions raised here aimed to gather information on whether close family members (maternal, paternal or siblings) had any history of specific diseases that may be related to bone health, e.g. any bone disease, osteoporosis, or kidney stones.

## **2.4 Data handling**

Each data sheet was coded with a unique study code for every participant. The hard copies were kept in a lockable filing cabinet in an office at the Department of Physiological Sciences, Stellenbosch University. Consent forms were kept in a separate folder, and locked in the office of the principle investigators since these forms contained the names and personal details of participants. Raw data were entered into an MS Office Excel 2010 spreadsheet, where after the data were independently cross-checked over separate days to ensure data capture accuracy. The MS Excel file was imported into Statistica® Version 12.0 (StatSoft Inc. 2014, USA) for statistical analyses.

## **2.5 Statistical analysis**

After the data were organised, a normality test was performed, followed by basic descriptive statistical analysis. A student t-test was employed to determine differences between two groups, provided the data were normally distributed. All results were reported as means  $\pm$  standard error of the mean (SEM), and medians and interquartile ranges (IQR) in cases where the data was not normally distributed. For multiple groups, data were analysed using a factorial analysis of variance (ANOVA) with a Bonferroni *post hoc* test. Pearson correlations were used to describe associations between selected physiological parameters. Statistical significance was set at  $p < 0.05$ .

## Chapter 3

## Results

This chapter illustrates and narrate the results obtained for this study.

### 3.1 Basic description of the sample population

The participant characteristics for the sample population are provided in Table 3.1. A total of n=80 women were recruited in this study, with a mean age of 42.2±1.1 years. The mean BMI for the sample population was 31.4±0.9 kg/m<sup>2</sup>, and the mean WC was 87.9±1.6 cm, which is indicative of an overweight/obese population. All other variables were within normal physiological ranges, with the exception of vitamin D levels which were below the detection limit in more than half the population (Appendix J), although sunlight exposure data indicated that participants were exposed to sunlight for approximately two hours (129.71 minutes) per day (data not presented). The LDL-c concentrations as well as FI concentrations were higher than normal (3.0±0.0 mmol/L; 25.6±4.4 mIU/L).

**Table 3.1: Description of the characteristics of the total sample population.**

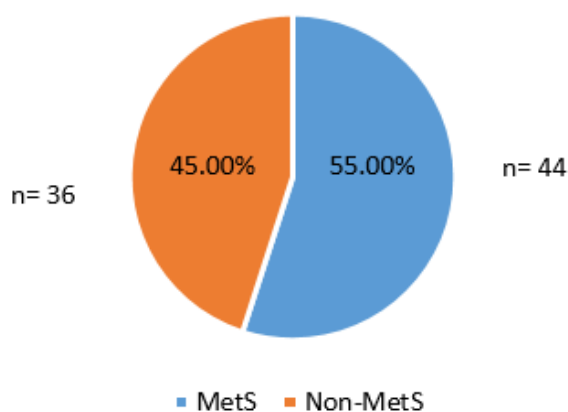
Characteristic	Mean±SEM (n=80)
Age (years)	42.18±1.12
Body mass (kg)	79.13±2.22
Height (m)	1.59±0.01
BMI (kg/m <sup>2</sup> )	31.44±0.87
<b>Blood pressure</b>	
Systolic blood pressure (mmHg)	139.18±2.48
Diastolic blood pressure (mmHg)	84.70±1.49
<b>Circumferences</b>	
Waist circumference (cm)	87.86±1.61
Hip circumference (cm)	109.78±1.83
W:H	0.80±0.01
<b>Blood parameters</b>	
Fasting glucose (mmol/L)*	5.44±0.26
Fasting Insulin (mIU/L)*	25.61±4.43
HDL-c (mmol/L)*	1.22±0.04
LDL-c (mmol/L)*	2.98±0.12
Triglycerides (mmol/L)*	1.62±0.14

Vitamin D (pg/mL)	0.74±0.11
Parathyroid hormone (pg/mL)	3.63±0.18
Alkaline phosphatase (U/L)	79.99±3.37
E2 (pmol/L)	261.70±29.81
<b>Bone status</b>	
T-score	-0.33±0.12
Z-score	0.37±0.17
SOS (m/s)	1525.42±2.30
BUA (dB/MHz)	107.58±1.99
BQI	97.41±2.21

\*Normal ranges: Fasting glucose: 3.5-5.5 mmol/L, Fasting insulin: 2.1-10.4 mmol/L, HDL-c: >1.2 mmol/L, LDL-c: <3.0 mmol/L, Triglycerides: <1.7 mmol/L

### 3.2 The prevalence of the metabolic syndrome, and descriptive characteristics of the population

Using IDF criteria (IDF, 2006), two groups from the n=80 sample were identified based on their metabolic status. More than half (55.0%; n=44) of the participants were classified with the MetS, whereas 45.0% (n=36) did not present with the MetS (Non-MetS) (Figure 3.1).



**Figure 3.1: The prevalence of the MetS in the sample population.**

Table 3.2 presents the characteristics of metabolic and bone-related variables between the MetS and Non-MetS groups. Here, it is shown that neither group had any age difference, as well as bone-related parameter differences. The following metabolic profile parameters differed significantly between the two groups: SBP ( $p<0.001$ ), DBP ( $p<0.001$ ), WC ( $p<0.001$ ), FG ( $p<0.01$ ), FI ( $p<0.01$ ), HDL-c ( $p<0.001$ ) and TG ( $p<0.01$ ). Other

anthropometric parameters which also differed significantly, included BM ( $p<0.001$ ), BMI ( $p<0.001$ ), HC ( $p<0.001$ ), and W:H ( $p<0.001$ ).

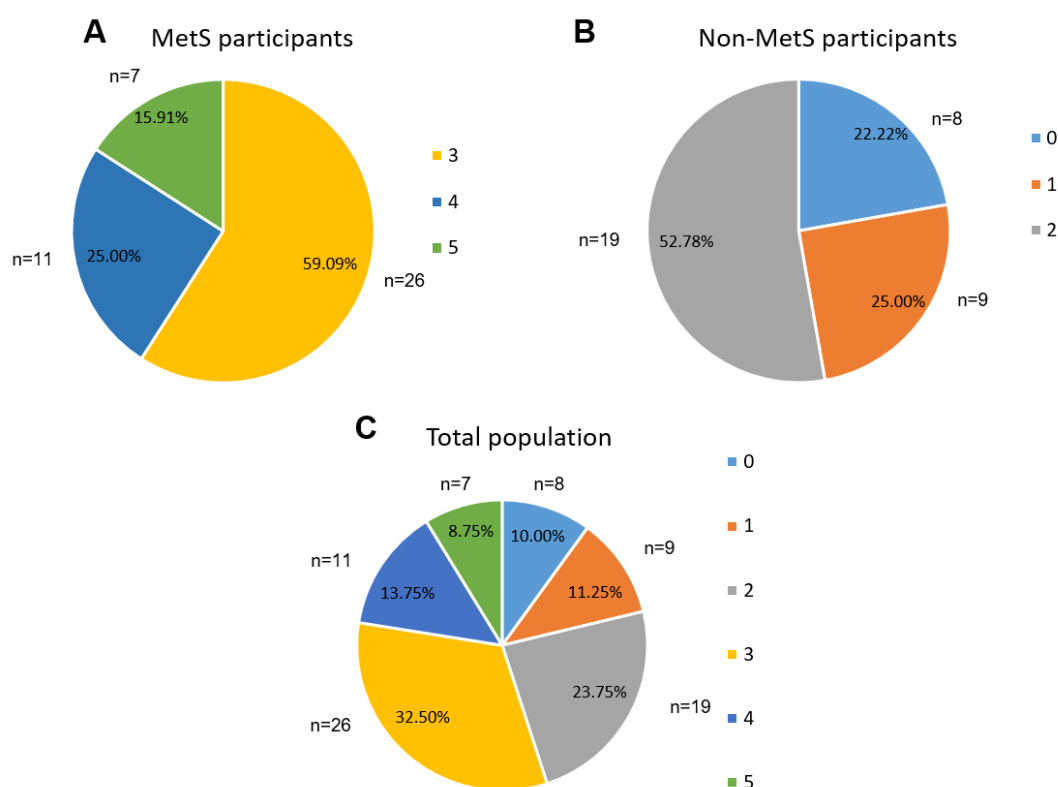
**Table 3.2: Descriptive characteristics of the MetS vs. Non-MetS individuals.**

Description	MetS (mean±SEM) n=44	Non-MetS (mean±SEM) n=36	p-value
Age (years)	43.48±1.55	40.58±1.61	NS
Body mass (kg)	87.80±2.41	68.53±3.20	$p<0.001$
Height (m)	1.58±0.01	1.59±0.01	NS
BMI (kg/m <sup>2</sup> )	35.12±0.92	26.95±1.16	$p<0.001$
<b>Blood pressure</b>			
Systolic blood pressure (mmHg)	148.55±3.14	127.72±3.05	$p<0.001$
Diastolic blood pressure (mmHg)	90.23±1.91	77.95±1.81	$p<0.001$
<b>Circumferences</b>			
Waist circumference (cm)	95.94±1.69	77.99±1.91	$p<0.001$
Hip circumference (cm)	115.74±2.01	102.48±2.83	$p<0.001$
Waist-to-hip ratio	0.83±0.01	0.77±0.01	$p<0.001$
<b>Blood parameters</b>			
Fasting glucose (mmol/L)*	6.18±0.43	4.55±0.09	$p<0.01$
Fasting insulin (mIU/L)*	35.90±6.92	13.03±2.36	$p<0.01$
HDL-c (mmol/L)*	1.06±0.03	1.42±0.07	$p<0.001$
LDL-c (mmol/L)*	3.15±0.12	2.77±0.18	NS
Triglycerides (mmol/L)*	2.01±0.22	1.15±0.12	$p<0.01$
Vitamin D (pg/mL)	0.70±0.13	0.84±0.24	NS
Parathyroid hormone (pg/mL)	3.76±0.25	3.48±0.25	NS
Alkaline phosphatase (U/L)	83.70±4.79	75.44±4.63	NS
E2 (pmol/L)	214.61±28.75	319.25±55.16	NS
<b>Bone status</b>			
T-score	-0.38±0.16	-0.31±0.18	NS
Z-score	0.43±0.26	0.26±0.26	NS
SOS (m/s)	1523.62±3.01	1526.98±3.55	NS
BUA (dB/MHz)	108.44±2.61	105.79±3.09	NS
BQI	96.46±2.86	97.79±3.49	NS

NS: Non-significant, \*Normal ranges: Fasting glucose: 3.5-5.5 mmol/L, Fasting insulin: 2.1-10.4 mmol/L, HDL-c: >1.2 mmol/L, LDL-c: <3.0 mmol/L, Triglycerides: <1.7 mmol/L

### 3.3 Prevalence of the different metabolic syndrome risk factors

In order to quantify the MetS risk factors in the sample population, each participant in their respective MetS and Non-MetS groups were listed as having zero, one, two, three, four or five risk factors (Figure 3.2). In the MetS group, 60.0% of the participants presented with three MetS risk factors, followed by 25.0% with four risk factors and 15.9% with all five MetS risk factors (Figure 3.2A). In the Non-MetS group, 52.8% of the participants had two MetS risk factors, followed by 25.0% with one risk factor, and 22.2% of the participants with zero risk factors (Figure 3.2B). From Figure 3.2C, which included the total sample population irrespective of metabolic status, it is apparent that most of the participants presented with three risk factors (32.5%), followed by two risk factors (23.8%), and four risk factors (13.8%). Only one MetS risk factor was present in 11.3% of the participants, 10.0% had zero risk factors, and 8.8% had all five MetS risk factors.

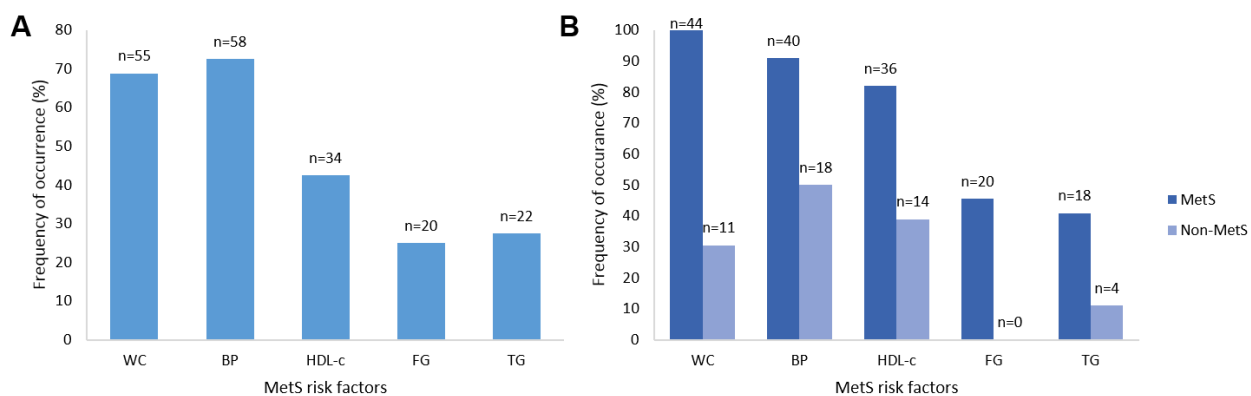


**Figure 3.2: The proportion of participants with zero, one, two, three, four, and five MetS risk factors in (A) the MetS group, (B) the Non-MetS group, and (C) the total sample population.**

In order to establish which of the MetS risk factors were more prevalent, the individual risk factors for each participant were ranked according to frequency (Figure 3.3). In the total sample population (Figure 3.3A), increased BP was the most prevalent MetS risk factor (72.5%, n=58), followed closely by increased WC (68.8%, n=55), and low levels of HDL-c (42.5%, n=34), increased TG (27.5%, n=22) and increased FG (25.0%, n=20). From Figure



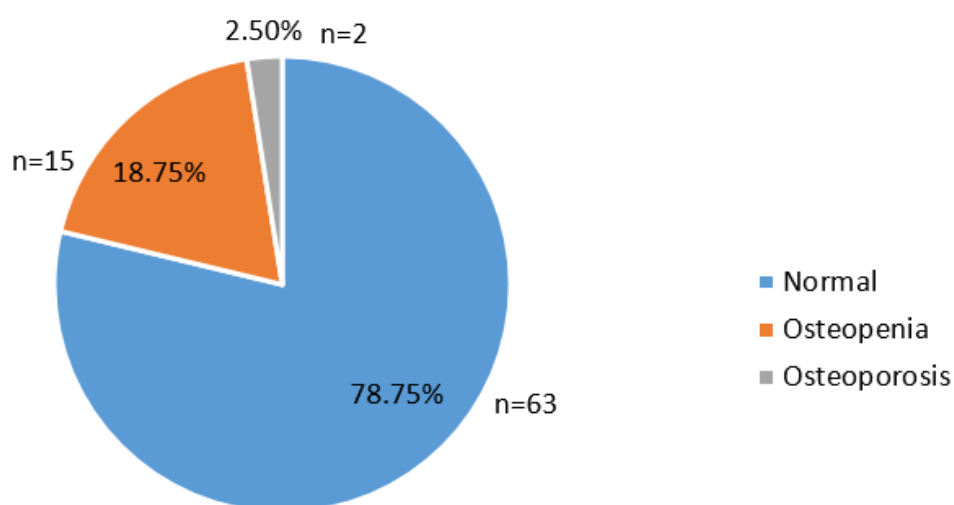
3.2B, it is evident that all of the MetS participants had higher WC's (100.0%), followed by elevated BP (90.9%), HDL-c (81.8%), FG (45.5%) and TG (40.9%). In contrast, elevated BP (50.0%) was found to be the predominant risk factor in the Non-MetS group, followed by low HDL-c (38.9%), and WC (30.6%). Although only 11.1% of the participants had elevated TG levels, none of the participants displayed elevated FG levels.



**Figure 3.3** Prevalence of specific MetS risk factors in (A) the total sample population and in (B) the MetS and Non-MetS groups.

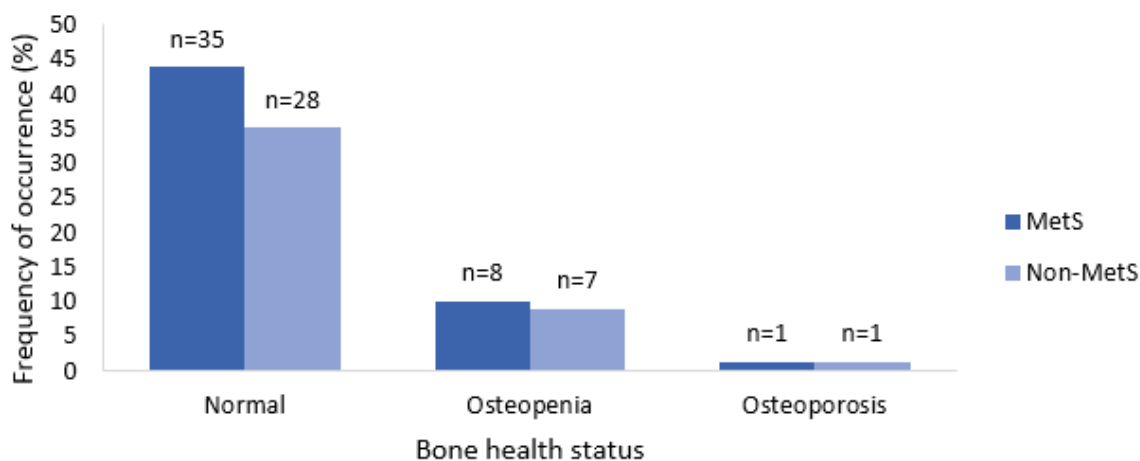
### 3.4 Bone health status of the total sample population, and between the MetS and Non-MetS groups

One of the aims was to describe the bone health status of the total sample population, irrespective of the metabolic status. In total, 78.8% (n=63) of the individuals presented with normal BMD, whereas 18.8% (n=15) presented with osteopenia, and only two and a half percent (n=2) had osteoporosis (Figure 3.4).



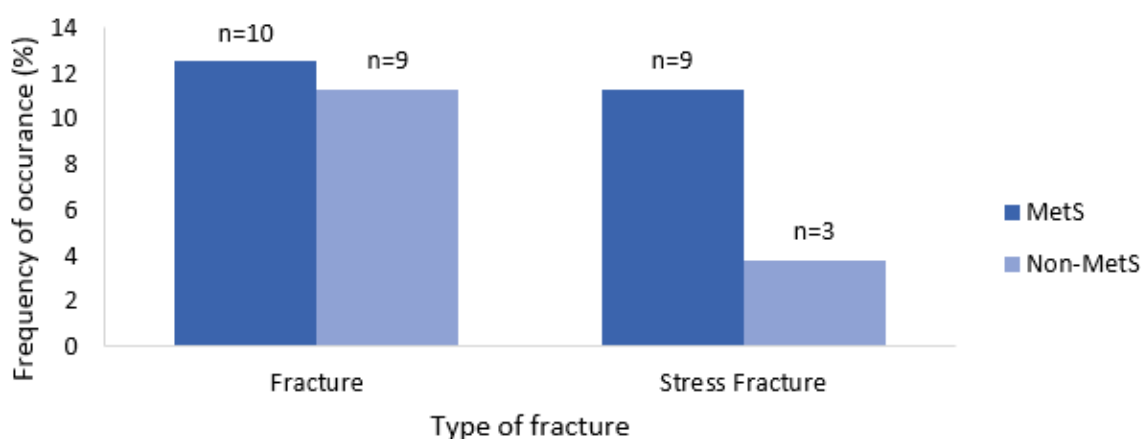
**Figure 3.4:** Classification of the bone health of the total sample population.

Considering the metabolic status, 79.6% (n=35) of the MetS participants displayed a normal BMD, whereas 18.2% (n=8) were classified as osteopenic (Figure 3.5). In the Non-MetS group, 77.8% (n=28) had a normal BMD, compared to 19.4% (n=7) who had osteopenia. Only one individual in each of the MetS and Non-MetS groups presented with osteoporosis (2.3%, and 2.8% respectively) (Figure 3.5).



**Figure 3.5: Classification of the bone health status in the MetS and the Non-MetS groups.**

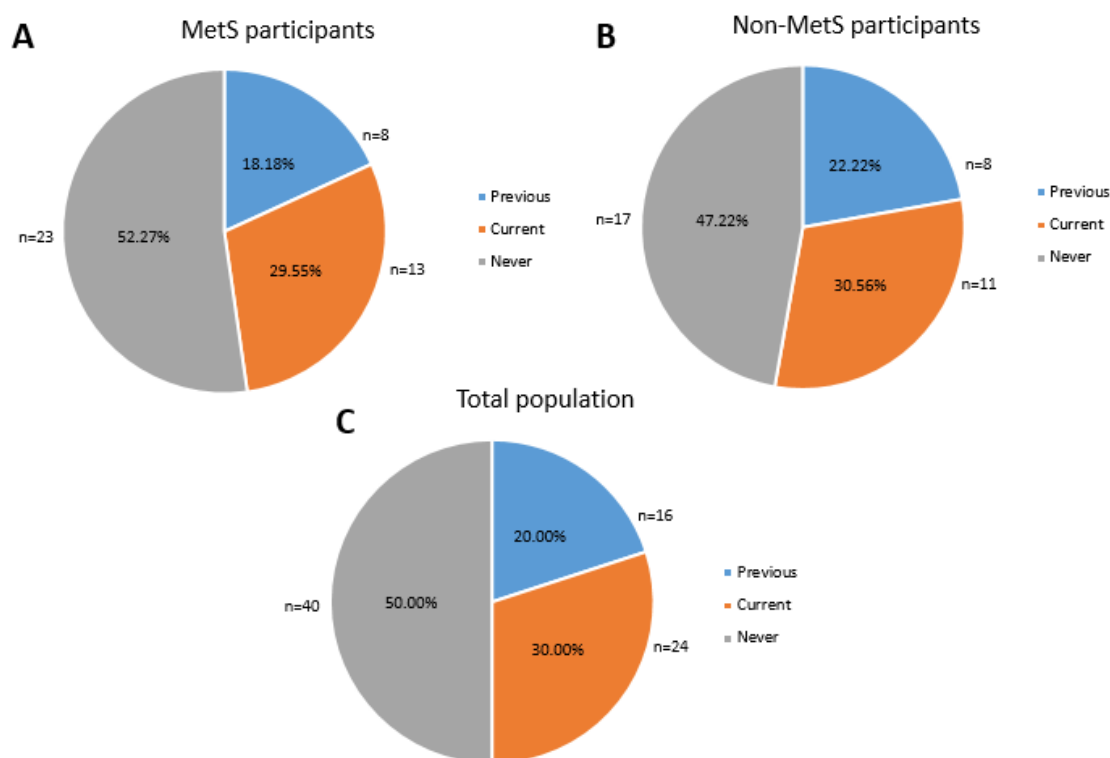
To further describe and compliment bone health status, information regarding fracture history was obtained through a questionnaire. From the total population, 23.8% of the participants reported previous fractures, compared to 15.0%, which reported previous stress fractures (not shown). When metabolic status was considered (Figure 3.6), fractures and stress fractures were found to be more prevalent in the MetS group.



**Figure 3.6: Prevalence of fractures and stress fractures in the MetS vs. Non-MetS groups.**

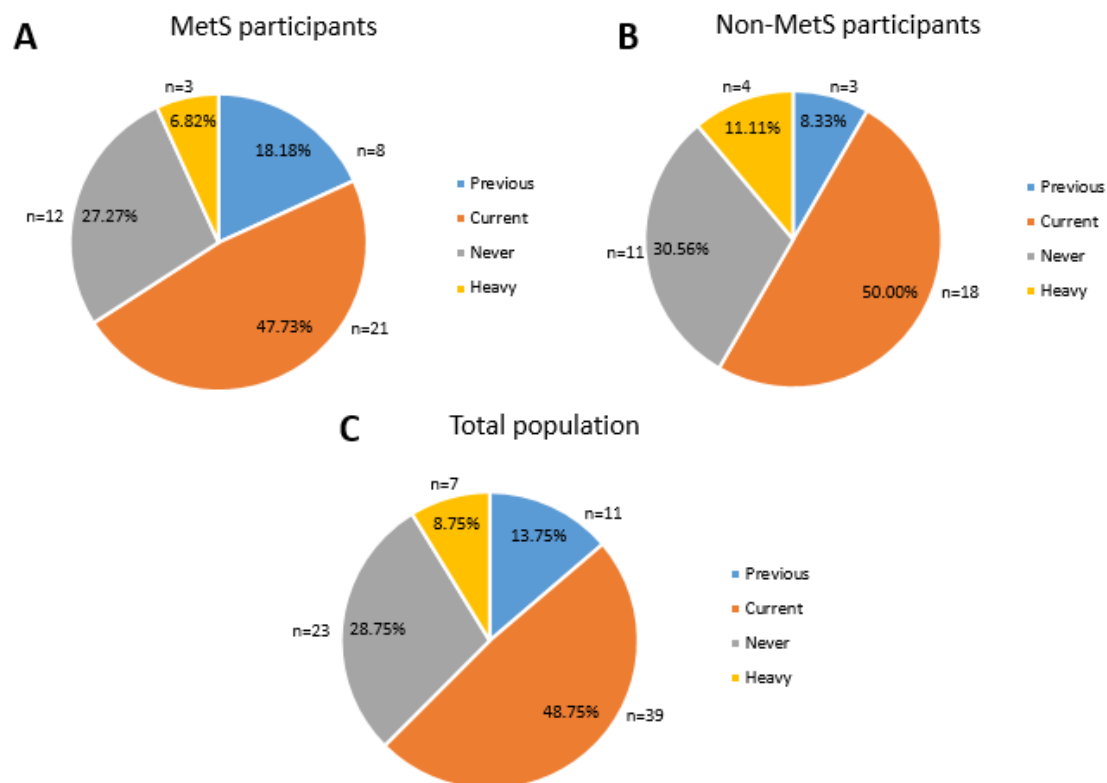
In the participants with the MetS, 52.3% of the participants indicated that they had never smoked, compared to 29.6% who were current smokers, and 22.7% who had been previous

smokers (Figure 3.7A). It is important to note that some participants were both classified as previous and current smokers, since some of them reported to have stopped for a certain time period. In the Non-MetS group, 44.4% of the participants had never smoked before, 30.6%, were current smokers, and 25.0% had been previous smokers (Figure 3.7B). Overall, almost half of the participants never smoked (48.8%), 30.0% were current smokers, and 23.8% were previous smokers (Figure 3.7C).



**Figure 3.7: Frequency of participants who were previous, current and non-smokers in (A) the MetS group, (B) the Non-MetS group, and (C) the total population.**

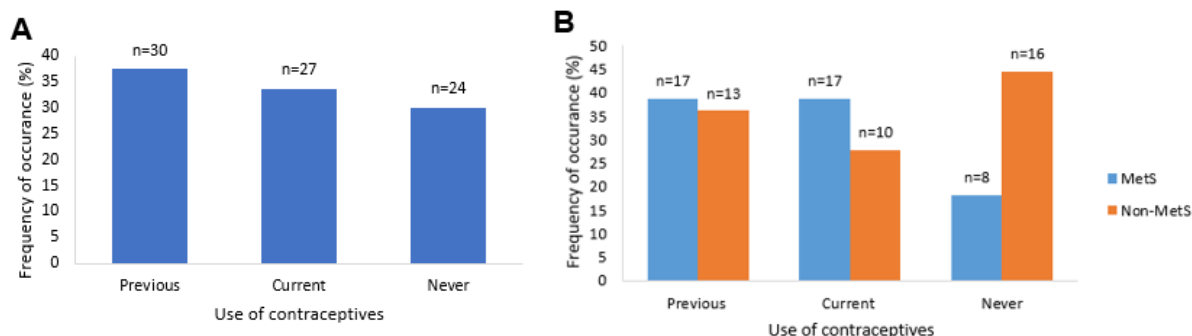
In the MetS group, 54.6% (n=24) indicated they are current alcohol consumers, 25.0% (n=11) had never consumed alcohol, 22.7% (n=10) were previous alcohol consumers, and 6.8% (n=3) were heavy alcohol consumers (Figure 3.8A). In the Non-MetS group, 61.1% (n=22) were current alcohol consumers, 30.6% (n=11) had never consumed alcohol, 19.4% (n=7) were previous alcohol consumers, and 11.1% (n=4) were heavy alcohol drinkers (Figure 3.8B). Figure 3.8C reported that more than half the participants consumed alcohol daily (57.5%, n=46), followed by 27.5% (n=22) who had never used alcohol before, 21.3% (n=17) who were previous alcohol consumers, and 8.8% (n=7) who are heavy alcohol consumers.



**Figure 3.8: Frequency of participants who were previous, current, non-consumers and heavy consumers of alcohol in (A) the MetS group, (B) the Non-MetS group, and (C) the total population.**

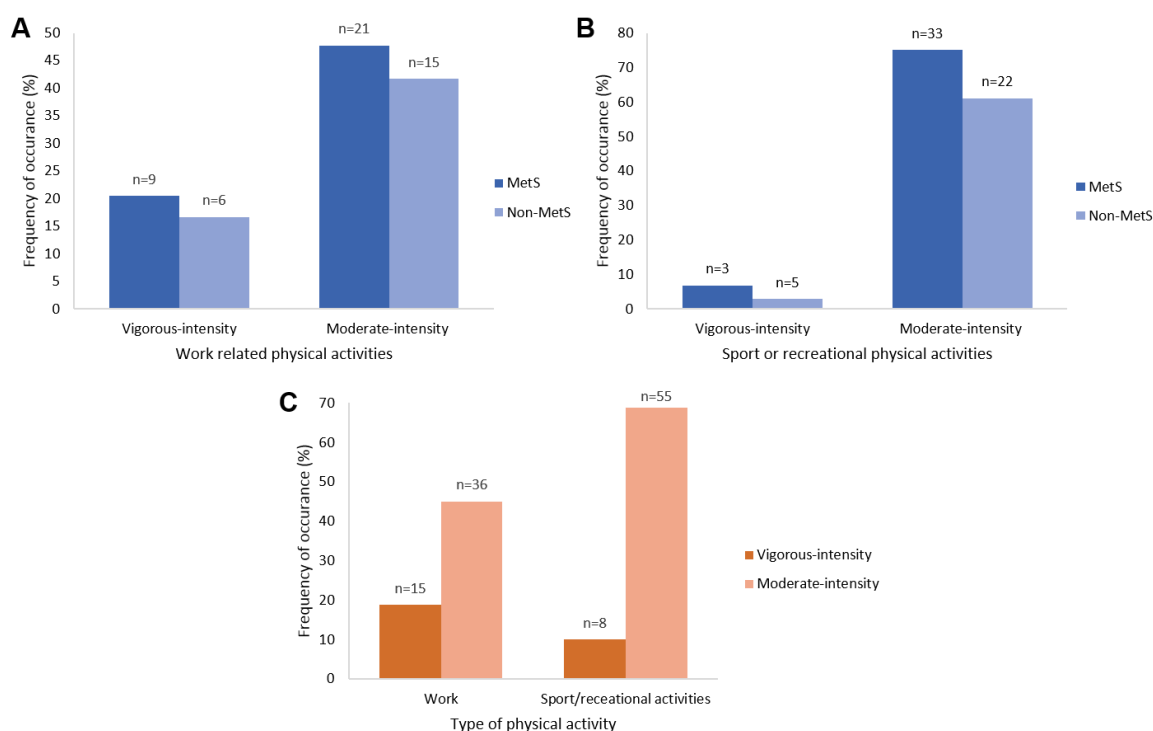
Basic information was also obtained regarding the menstrual history of the participants. The average age of the women at their first menstrual cycle was 14 years of age. A total of 86.3% (n=69) of the participants reported having previous pregnancies, and the average period of breastfeeding was 40.5 months. A total of 77.5% (n=62) of the participants also indicated that they had regular monthly menses, whereas 28.8% (n=23) reported a history of irregular menses (Appendix K). A comparison between the MetS and Non-MetS groups regarding the use of several types of medication is also shown in Appendix K.

Figure 3.9A illustrates previous, current and non-contraceptive users in the total population. A total of 37.5% (n=30) of the participants previously used contraceptives, while 33.8% (n=27) were current users of contraceptives, and 30.0% (n=24) indicated that they had never used contraceptives before. In Figure 3.9B the frequency of previous, current and non-contraceptives between the MetS and Non-MetS groups is compared. In the MetS group, 38.6% (n=17 each) were both previous, or current contraceptive users, and 18.2% (n=8) had never used contraceptives before. In the Non-MetS group, 44.4% (n=16) had never used contraceptives before, whereas 36.1% (n=13) were previous users, and 27.8% (n=10) were current users of contraceptives.



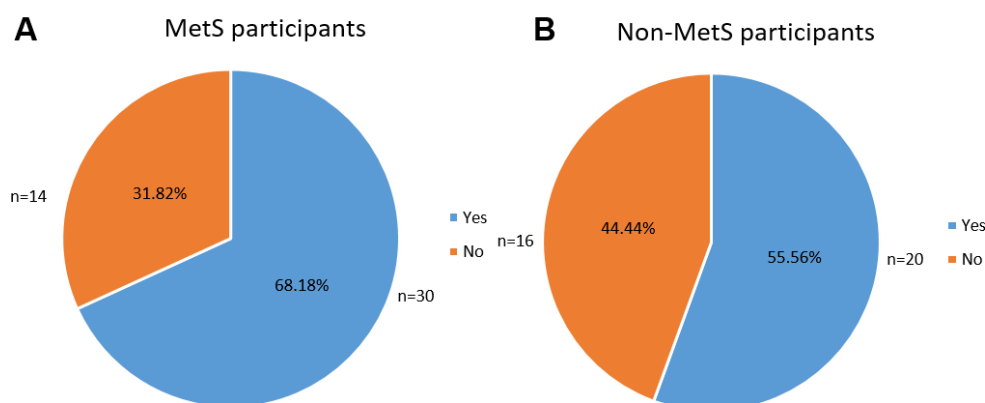
**Figure 3.9: The frequency of previous, current and non-contraceptive users in (A) the total sample population, and (B) the MetS and Non-MetS groups.**

The results obtained from the GPAQ questionnaire indicated that 47.7% of individuals with the MetS, and 41.7% of participants without the MetS performed moderate-intensity work-related activities, whereas 20.5% and 16.7% performed vigorous-intensity work-related activities respectively (Figure 3.10A). A total of 75.0% (n=33) of participants with the MetS took part in moderate-intensity recreational, or sport activities, compared to 61.1% of the participants in the Non-MetS group (n=22). Only 6.8% (n=3) and 13.9% (n=5) of the MetS and Non-MetS individuals participated in vigorous-intensity sport, or recreational activities, respectively (Figure 3.10B). In the total population, 45.0% (n=36) of the participants reported to have work that required moderate-intensity physical activity, and 68.8% (n=55) participated in moderate-intensity sport, or recreational activities (Figure 3.10C).



**Figure 3.10: The proportion of participants engaging in vigorous- and moderate-intensity activities at work, as well as in sport, or recreational activities in (A) the MetS group, (B) the Non-MetS group, and (C) the total sample.**

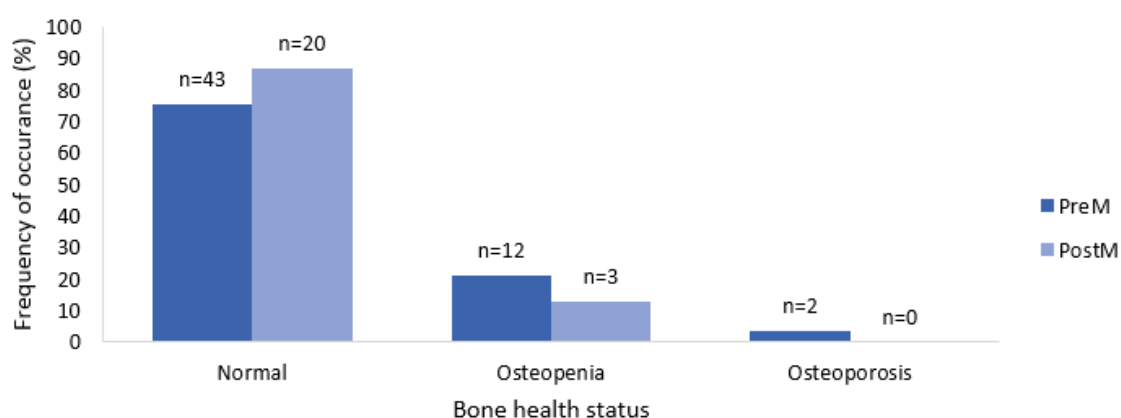
In the MetS group, 68.2% (n=30) compared to 55.6% (n=20) in the Non-MetS group engaged in walking, or cycling for more than ten minutes per day for travelling purposes (Figure 3.11A & B). In the total sample population, 62.5% (n=50) of the participants walked or cycled for travelling purposes (not shown).



**Figure 3.11:** The percentage of participants who walked or cycled for more than ten minutes per day for travelling purposes in (A) the MetS group, and (B) the Non-MetS group.

### 3.5 Description of participants according to menopausal status

Participants were further classified according to their menopausal status into either PreM, or PostM, in order to determine the association between menopausal status, and various metabolic and physiological parameters, irrespective of metabolic status (Table 3.3). Firstly, to provide a general illustration of bone health in PreM vs. PostM women, Figure 3.12 indicates that 75.4% of the PreM women had normal BMD, whereas 87.0% of the PostM women had normal BMD. Osteopenia was present in 21.1% of the PreM women and in 13.0% of the PostM women. None of the PostM women had osteoporosis, whereas 3.5% of the PreM women had osteoporosis.



**Figure 3.12:** Frequency of occurrence of normal BMD, osteopenia and osteoporosis in PreM and PostM women.

In Table 3.3 the participants in the PreM group are compared to the PostM group. Here, significant age differences were reported between the PreM and PostM groups ( $38.21 \pm 1.16$  years vs.  $52.0 \pm 1.01$  years;  $p < 0.001$ ). Furthermore, only SBP ( $135.88 \pm 2.87$  mmHg vs.  $147.35 \pm 4.56$  mmHg;  $p < 0.05$ ), PTH ( $3.29 \pm 0.16$  pg/mL vs.  $4.51 \pm 0.45$  pg/mL;  $p < 0.001$ ),  $E_2$  ( $299.30 \pm 36.40$  pmol/L vs.  $168.52 \pm 46.74$  pmol/L;  $p < 0.05$ ), and the Z-score ( $0.14 \pm 0.22$  vs.  $0.89 \pm 0.18$ ;  $p < 0.05$ ) differed significantly between the different menopausal groups. Trends towards significance were noted for height ( $p = 0.066$ ), W:H ( $p = 0.063$ ), and ALP ( $p = 0.056$ ) measurements between the PreM and PostM groups. Some additional graphs comparing PreM and PostM groups are shown in Appendix L.

**Table 3.3: Descriptive characteristics of the PreM vs. PostM individuals.**

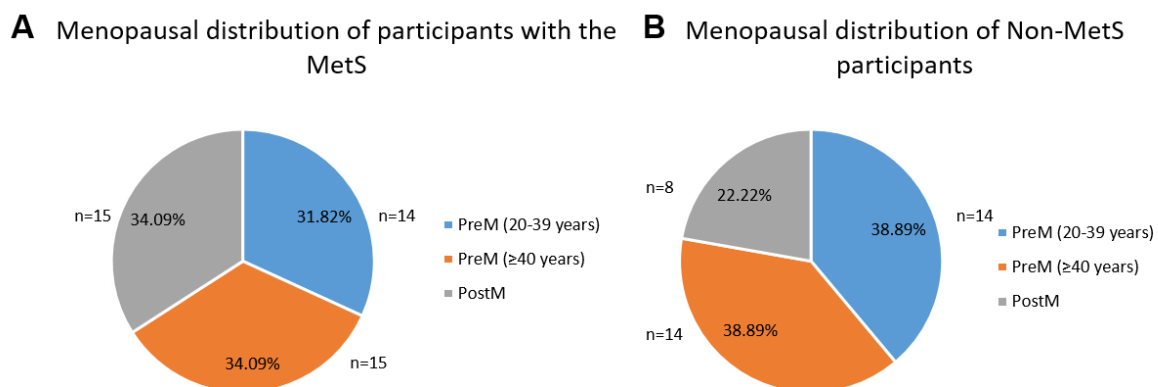
Description	Pre-Menopausal (Mean $\pm$ SEM) n=57	Menopausal (Mean $\pm$ SEM) n=23	p-value
Age	38.21 $\pm$ 1.16	52.00 $\pm$ 1.01	p<0.001
Body mass (kg)	78.86 $\pm$ 2.87	79.80 $\pm$ 3.11	NS
Height (m)	1.60 $\pm$ 0.01	1.56 $\pm$ 0.11	p=0.066
BMI (kg/m <sup>2</sup> )	30.92 $\pm$ 1.09	32.74 $\pm$ 1.27	NS
<b>Blood pressure</b>			
Systolic blood pressure (mmHg)	135.88 $\pm$ 2.87	147.35 $\pm$ 4.56	p<0.05
Diastolic blood pressure (mmHg)	84.42 $\pm$ 1.74	85.39 $\pm$ 2.91	NS
<b>Circumferences</b>			
Waist circumference (cm)	86.45 $\pm$ 2.00	91.35 $\pm$ 2.53	NS
Hip circumference (cm)	109.39 $\pm$ 2.33	110.74 $\pm$ 2.77	NS
Waist-to-hip ratio	0.79 $\pm$ 0.01	0.83 $\pm$ 0.02	p=0.063
<b>Blood parameters</b>			
Fasting glucose (mmol/L)*	5.20 $\pm$ 0.26	6.04 $\pm$ 0.60	NS
Fasting Insulin (mIU/L)*	25.98 $\pm$ 3.85	24.67 $\pm$ 10.95	NS
HDL-c (mmol/L)*	1.20 $\pm$ 0.04	1.28 $\pm$ 0.08	NS
LDL-c (mmol/L)*	3.0 $\pm$ 0.10	2.95 $\pm$ 0.27	NS
Triglycerides (mmol/L)*	1.49 $\pm$ 0.14	1.97 $\pm$ 0.34	NS
Vitamin D (pg/mL)	0.65 $\pm$ 12	0.92 $\pm$ 25	NS
Parathyroid hormone (pg/mL)	3.29 $\pm$ 0.16	4.51 $\pm$ 0.45	p<0.001
Alkaline phosphatase (U/L)	75.91 $\pm$ 4.21	90.09 $\pm$ 4.88	p=0.056

<b>E2 (pmol/L)</b>	299.30±36.40	168.52±46.74	p<0.05
<b>Bone status</b>			
<b>T-score</b>	-0.30±0.16	-0.46±0.12	NS
<b>Z-score</b>	0.14±0.22	0.89±0.18	p<0.05
<b>SOS (m/s)</b>	1525.94±3.06	1523.12±2.48	NS
<b>BUA (dB/MHz)</b>	108.15±2.51	105.00±3.07	NS
<b>BQI</b>	98.01±2.96	94.70±2.34	NS

NS: Non-significant, \*Normal ranges: Fasting glucose: 3.5-5.5 mmol/L, Fasting insulin: 2.1-10.4 mmol/L, HDL-c: >1.2 mmol/L, LDL-c: <3.0 mmol/L, Triglycerides: <1.7 mmol/L

### 3.6 Description of participants according to menopausal and metabolic status

In order to describe the association between menopausal and metabolic status, participants in their respective metabolic groups (MetS and Non-MetS) were sub-divided into either: A) PreM (between the ages of 20-39 years), B) PreM (≥40 years), and C) PostM. Figure 3.13 illustrates the distribution of participants according to both menopausal and metabolic status. An almost equal distribution of participants in all three sub-groups was noted for the MetS group (34.1%, 34.1% and 31.8% respectively) (Figure 3.13A). In the Non-MetS group, participants were equally distributed in both the PreM (20-39 years) and PreM (≥40 years) groups (39.0%, n=14) respectively, whereas only 22.0% (n=8) of participants formed part of the PostM group (Figure 3.13B).

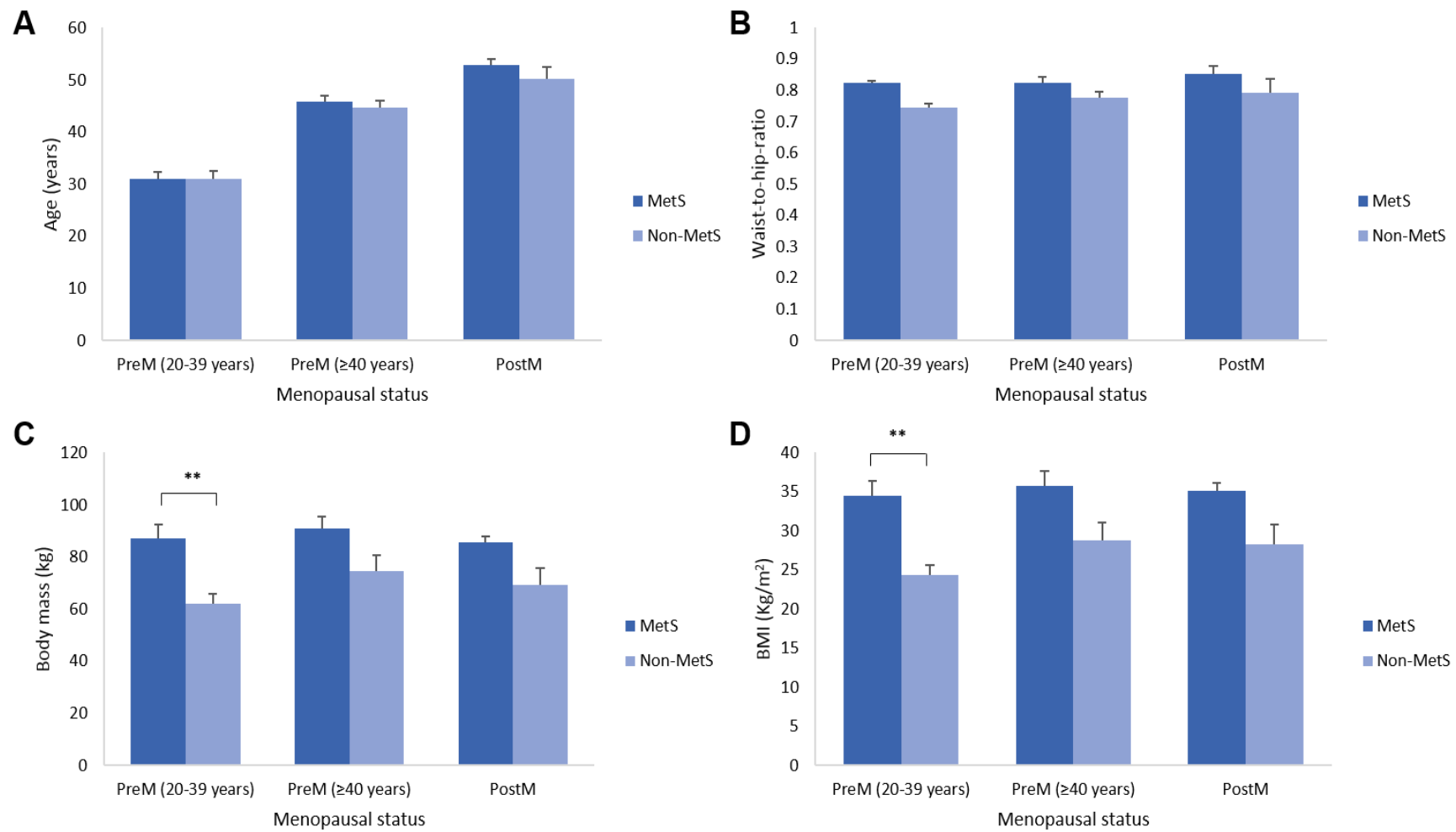


**Figure 3.13: Classification of participants in (A) the MetS, or (B) Non-Mets groups according to menopausal status.**



No significant differences were observed for age and W:H between the MetS and Non-MetS groups (Figure 3.14A & B). Women in the PreM (20-39 years) MetS group showed a significantly higher BM compared to those in the PreM (20-39 years) Non-MetS group ( $p < 0.001$ ) (Figure 3.14C). A similar pattern was observed for BMI ( $p < 0.01$ ) (Figure 3.14D), where only the PreM (20-39 years) MetS vs. Non-MetS group indicated significant differences.

The WC (Figure 3.14A) differed significantly between the PreM (20-39 years) MetS, PreM ( $\geq 40$  years) MetS, and their respective Non-MetS groups ( $p < 0.001$  and  $p < 0.01$  respectively). A similar finding was observed for HDL-c, although here, the Non-MetS groups presented with an elevated HDL-c level ( $> 1.29$  mmol/L) compared to their MetS counterparts ( $p < 0.05$  for PreM 20-39 years, and  $p < 0.01$  for PreM  $\geq 40$  years) (Figure 3.14B)



**Figure 3.14: Descriptive characteristics for (A) age, (B) W:H, (C) BM, and (D) BMI between the MetS and Non-MetS groups with respect to menopausal status (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001)**

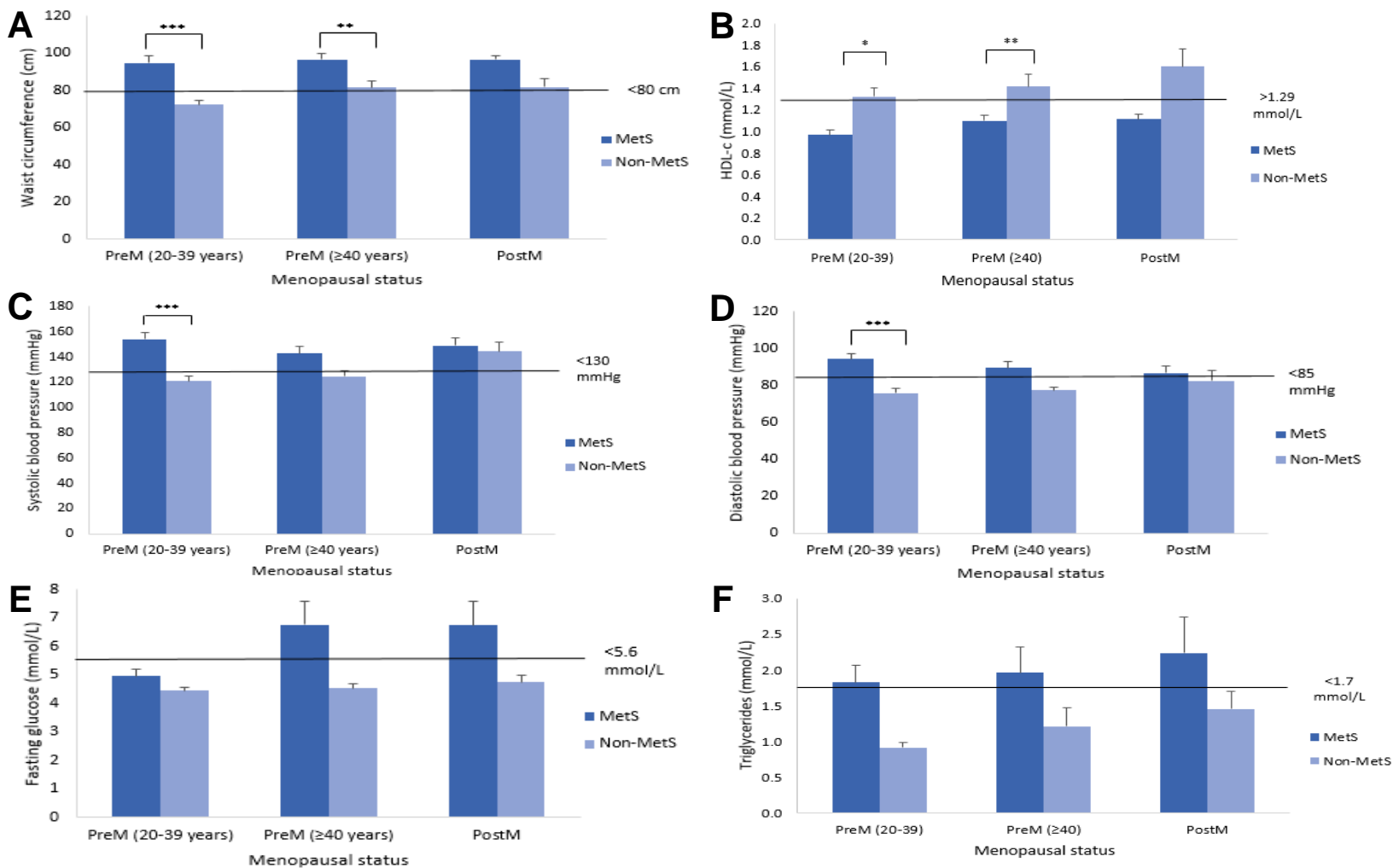
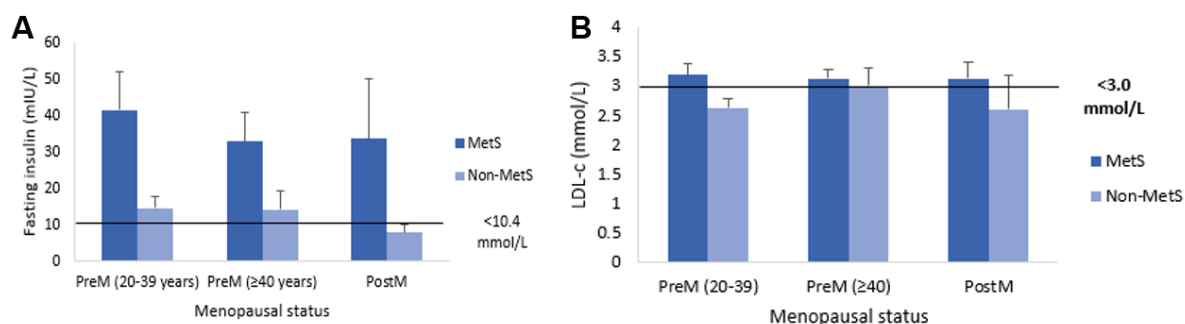


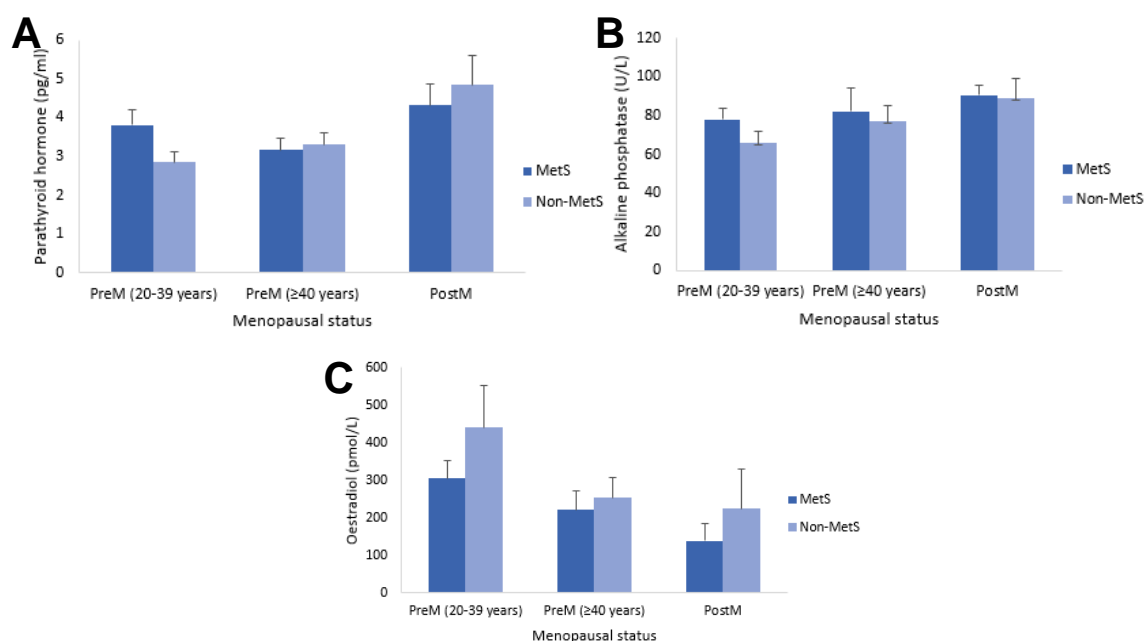
Figure 3.15: Metabolic syndrome risk factors for women grouped according to their menopausal and metabolic status for (A) waist circumference, (B) HDL-c, (C) systolic blood pressure, (D) diastolic blood pressure, (E) fasting glucose, and (F) triglycerides. Solid lines represent the normal cut-off values (IDF, 2006) (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

Similar, significant differences were observed between the MetS and Non-MetS participants in the PreM (20-39 years) groups for SBP ( $p < 0.001$ ), and DBP ( $p < 0.001$ ) (Figure 3.15C & D). No differences were observed for FG and TG between any of the subgroups (Figure 3.15E & F). Both FI and LDL-c (Figure 3.16A & B) were not significantly different between any of the groups.



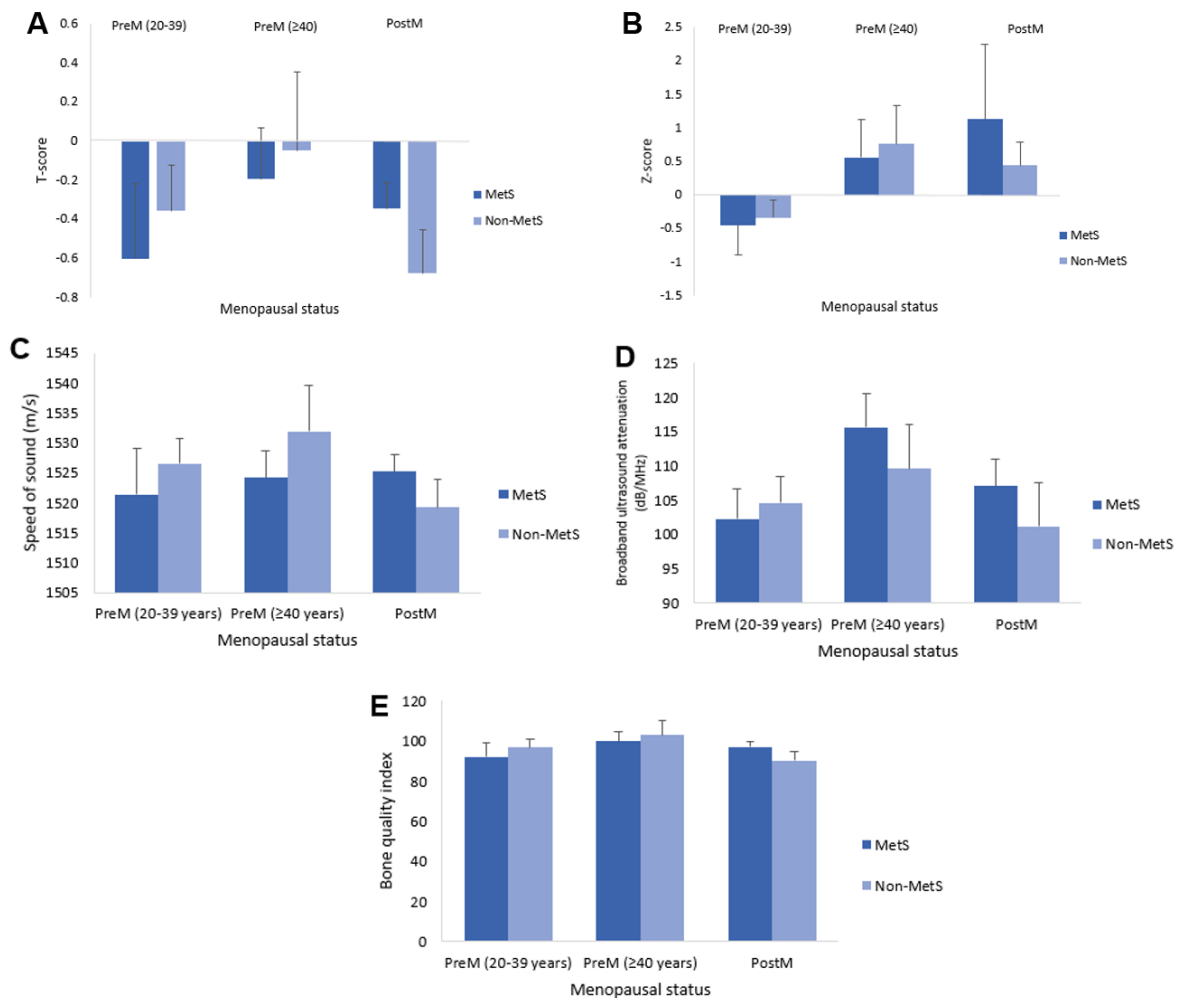
**Figure 3.16: The fasting insulin (A), and LDL-c (B) concentrations between groups. The solid line represents the normal cut-off values (IDF, 2006; Pathcare).**

For the bone-specific markers (PTH and ALP) (Figure 3.17A & B), no significant differences were noted. The female-specific hormone, oestradiol, also did not differ significantly between any of the respective groups (Figure 3.17C).



**Figure 3.17: Parathyroid hormone (A), alkaline phosphatase (B), and oestradiol (C) levels between groups with respect to metabolic and menopausal status.**

No significant differences were noted with regards to bone health status, as expressed by the T-scores (Figure 3.18A), Z-scores (Figure 3.18B), SOS (Figure 3.18C), BUA (Figure 3.18D), and BQI (Figure 3.18E)



**Figure 3.18: The different measures of bone health, including (A) T-score, (B) Z-score, (C) SOS, (D) BUA, and (E) BQI between the different menopausal and metabolic syndrome groups.**

### 3.7 Correlation analysis

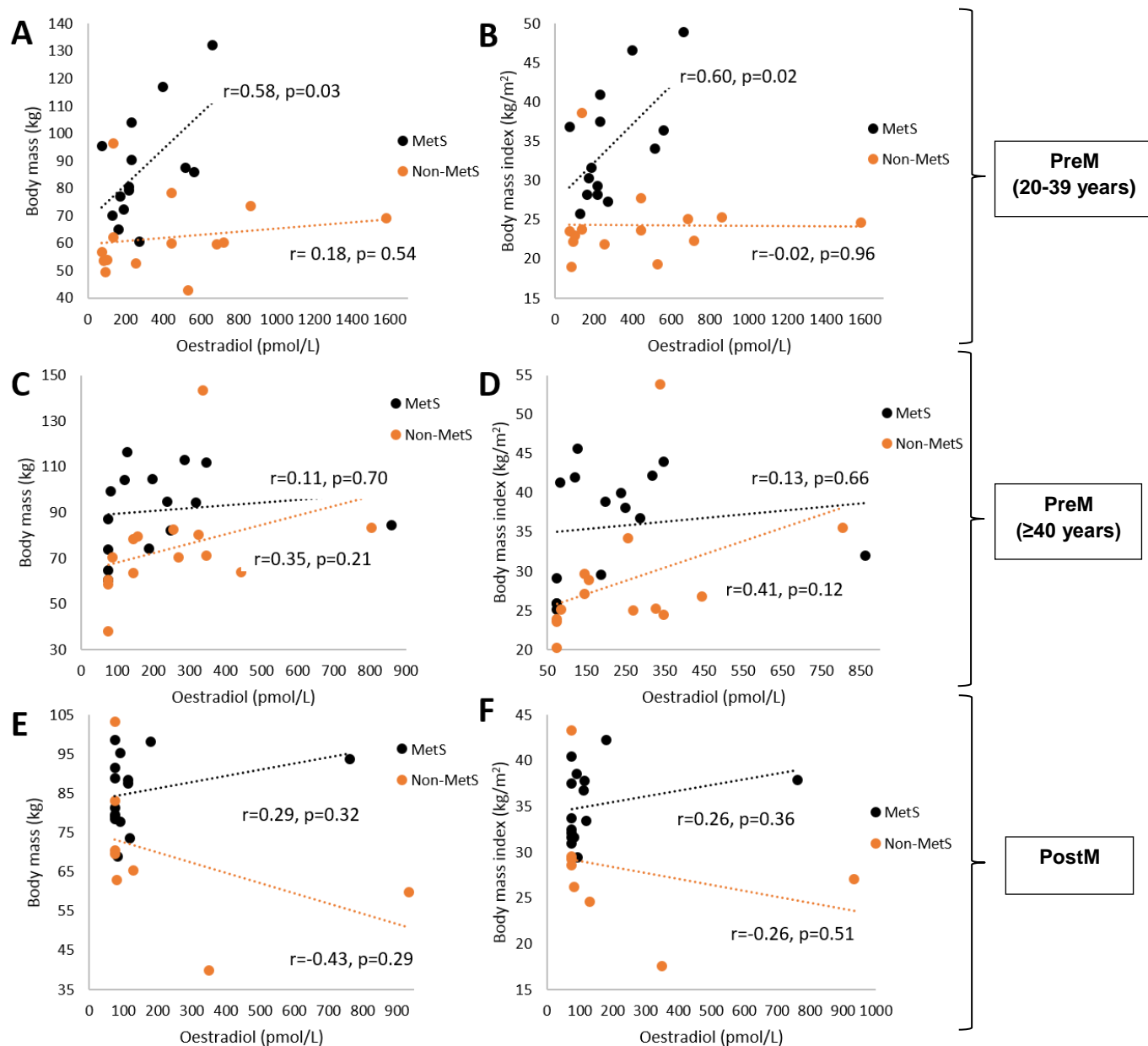
Pearson correlations were performed in order to establish possible associations between selected parameters. In order to contextualize the interpretations of these correlations, the following was considered; (i) no or negligible relationship was reported if  $r=0.01$  to  $0.19$ , (ii) correlations were classified either as positive or negative, with a weak ( $r=0.20$  to  $0.29$ ), moderate ( $r=0.30$  to  $0.39$ ), strong ( $r=0.40$  to  $0.69$ ), or very strong correlation coefficient ( $r \geq 0.7$ ) (Scally, 2014). The level of significance was accepted if  $p < 0.05$ . Furthermore, a  $p$ -value between  $0.051$  and  $0.075$  was regarded as a statistical trend.

For most of the correlations, where *more than one correlation* is discussed per group, A and B will indicate the PreM (20-39 years) groups, C and D the PreM ( $\geq 40$  years) groups, and E and F the PostM groups. In cases where *three correlations* are described, the PreM (20-39 years) groups will be indicated by the graph labelled A, B and C, the PreM ( $\geq 40$  years) groups by those labelled D, E and F, and the PostM groups by the graph labelled G, H and I.

#### 3.7.1 Correlation analysis between BM, BMI and $E_2$

Positive correlations were observed for all groups when BM and  $E_2$  were correlated with each other (Figure 3.19A, C & E), with the exception of the PostM, Non-MetS group, which showed a strong negative correlation (Figure 3.19E). The only strong, significant and positive correlation between BM and  $E_2$  was evident for the PreM (20-39 years) MetS group ( $r=0.58$ ,  $p=0.03$ ) (Figure 3.19A). All other correlations between BM and  $E_2$  were weak to moderate (Figure 3.19A, C & F).

The correlation between BMI and  $E_2$  (Figure 3.19B, D & F) also indicated a positive strong and significant correlation in the PreM (20-39 years) MetS group ( $r=0.60$ ,  $p=0.02$ ) (Figure 3.19B). All other correlations between BMI and  $E_2$  were weak and moderate, and some negative, but insignificant.

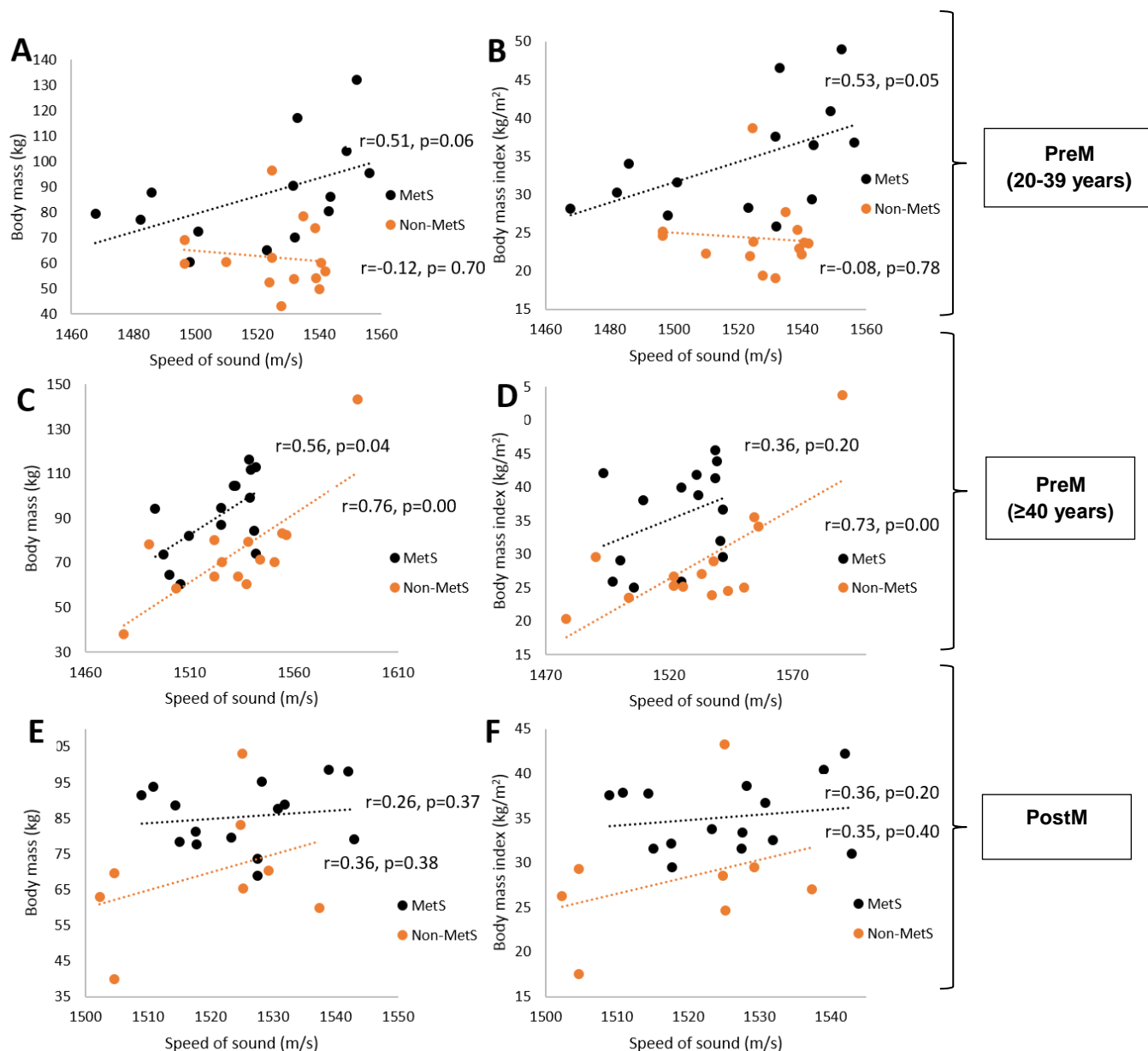


**Figure 3.19: The association between BM and E2, and BMI and E2 in the PreM (20-39 years) groups (A, B), the PreM (≥40 years) groups (C, D), and the PostM groups (E, F), respectively.**

### 3.7.2 Correlation analysis between BM, BMI and SOS

When BM and SOS were correlated with one another (Figure 3.20A, C & E), the PreM (20-39 years) MetS group revealed a strong, positive association with a trend towards significance ( $r=0.51$ ,  $p=0.06$ ) (Figure 3.19A). Significant and positive correlations were present in the PreM (≥40 years) participants in both the MetS (strong,  $r=0.56$ ,  $p=0.04$ ) and Non-MetS (very strong,  $r=0.76$ ,  $p=0.00$ ) groups (Figure 3.20C). In the PostM, MetS and Non-MetS groups, weak and moderate positive correlations were noted, respectively (Figure 3.20E).

The correlations between BMI and SOS (Figure 3.20B, D & E) indicated a significant and strong positive correlation in the PreM (20-39 years) MetS group ( $r=0.53$ ,  $p=0.05$ ), whereas the Non-MetS group indicated no association ( $r=-0.08$ ,  $p=0.78$ ) (Figure 3.20B). In the PreM ( $\geq 40$  years) Non-MetS group, a significant, and very strong positive correlation was present ( $r=0.73$ ,  $p=0.00$ ) (Figure 3.20D). The other correlations between BMI and SOS were moderate and positive (Figure 3.20D & F).



**Figure 3.20:** The association between BM and SOS, and BMI and SOS in the PreM (20-39 years) groups (A, B), the PreM ( $\geq 40$  years) groups (C, D), and the PostM groups (E, F), respectively.

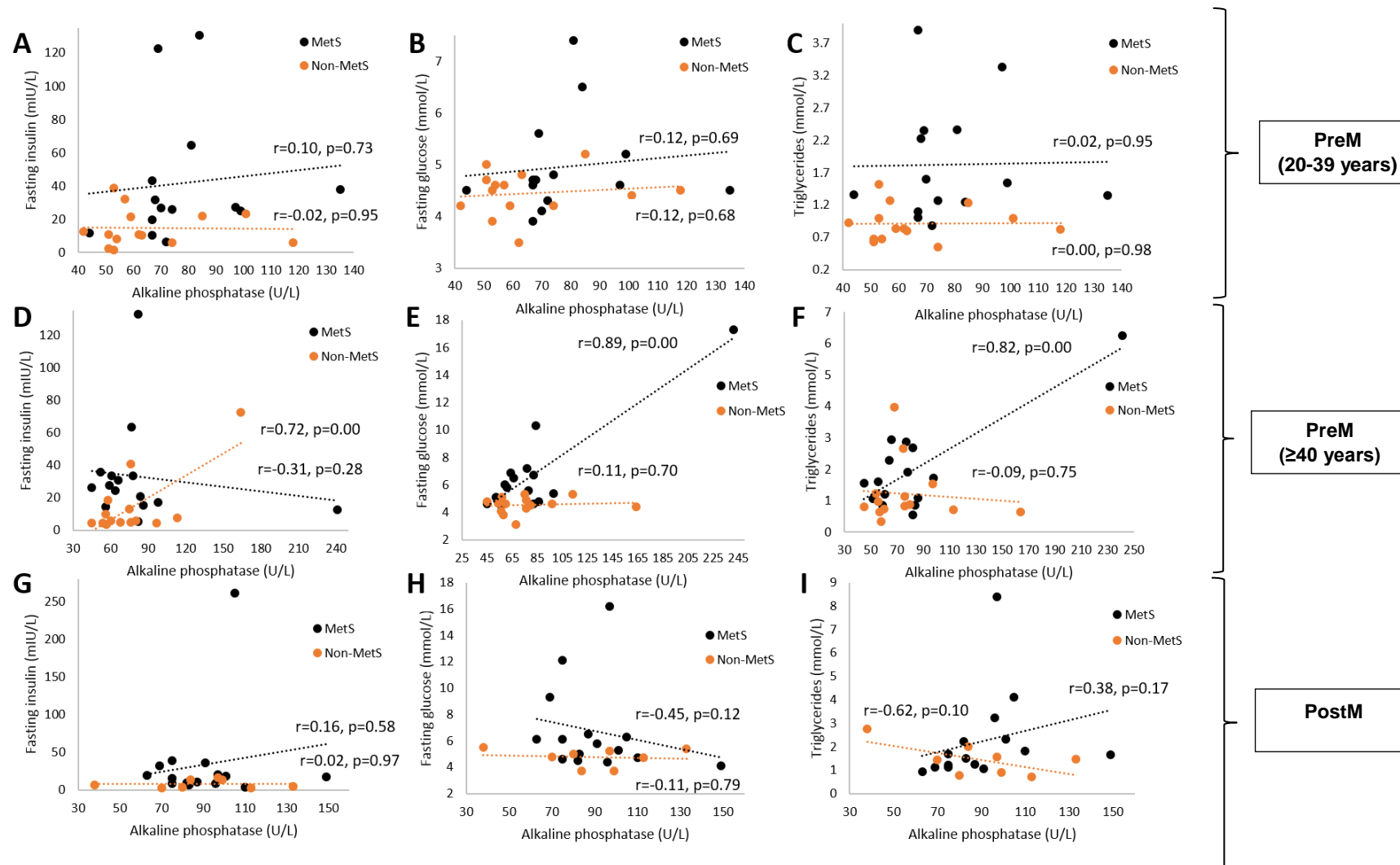


### 3.7.3 Correlation analysis between FI, FG, TG and ALP

The association between FI and ALP (Figure 3.21 A, C & E) indicated only one significant, and very strong correlation in the PreM ( $\geq 40$  years) MetS group ( $r=0.72$ ,  $p=0.00$ ) (Figure 3.21C). All other correlations were noted to be negligible to moderate.

Correlation analysis between FG and ALP (Figure 3.21B, E & H) also showed a significant and very strong association in the PreM ( $\geq 40$  years) MetS group ( $r=0.89$ ,  $p=0.00$ ) (Figure 3.20E). All other correlations between FG and ALP were non-significant.

Correlations between TG and ALP (Figure 3.21C, F & I) revealed only that the PreM ( $\geq 40$  years) MetS group displayed a very strong, significant (positive) correlation ( $r=0.82$ ,  $p=0.00$ ), (Figure 3.21F). In the PostM MetS group, a moderate positive correlation was noted ( $r=0.38$ ,  $p=0.17$ ), whereas the PostM Non-MetS group displayed a strong negative association ( $r=-0.62$ ,  $p=0.10$ ) (Figure 3.21I). No associations were evident in the PreM (20-39 years) Non-MetS and MetS groups (Figure 3.21C), as well as the PreM ( $\geq 40$  years) Non-MetS groups (Figure 3.21F). Some additional correlation analyses are shown in appendix M.



**Figure 3.21:** The association between FI and ALP, in the PreM (20-39 years) (A), PreM (≥40 years) (D), and PostM (G) groups; the association between FG and ALP, in the PreM (20-39 years) (B), PreM (≥40 years) (E) and PostM (H) groups; the association between TG and ALP in the PreM (20-39 years) (C), PreM (≥40 years) and PostM groups (I), respectively.

## Chapter 4

## Discussion

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### 4.1 Introduction

This chapter will discuss the major findings of the study and integrate these findings with relevant literature. The aim of the first part of this study was to report on the prevalence of the MetS in a female farm worker population in the Western Cape of South Africa, while the second aim was to describe bone health and possible associations between bone markers, metabolic- and menopausal status in the selected population.

### 4.2 Basic description of the study population

#### 4.2.1 The study population was largely overweight

The participants used in the current study were predominantly overweight, or obese, since both the mean BMI ( $31.4 \pm 0.9 \text{ kg/m}^2$ ) and WC ( $87.9 \pm 1.6 \text{ cm}$ ) were higher (Table 3.1) than the normal ranges reported by the WHO for BMI ( $18.5\text{-}24.99 \text{ kg/m}^2$ ) and the IDF for WC ( $\leq 80.0 \text{ cm}$ ). With the exception of FI ( $25.6 \pm 4.4 \text{ mmol/L}$ ) and LDL-c ( $3.0 \pm 0.1 \text{ mmol/L}$ ), none of the clinical markers describing metabolic and cardiovascular health, including BP, FG, HDL-c and TG were abnormally elevated or decreased.

The increased FI levels might be due to the increased abdominal AT and IR, which is commonly reported in obese individuals due to the increased levels of TNF- $\alpha$  and IL-6 as a consequence of low grade inflammation (Cartier, 2010; Han & Lean, 2014). Furthermore, the increased LDL-c observed here, is part of the dyslipidemia or lipid abnormalities noted in obese individuals and it is commonly seen in individuals with increased abdominal adipose tissue (Franssen *et al.*, 2011). During obesity there is remodeling of these lipoproteins by enzymes such as cholesteryl ester transfer protein and hepatic triglyceride lipases (Tchernof & Despres, 2013). Furthermore, it is stated that the increased LDL-c in obesity is driven by the increased concentrations of TG (Klop *et al.*, 2013). Although TG were not abnormally elevated, it was borderline normal, thus TG might help explain the elevation in LDL-c as observed here. The presence of IR also worsens the clearance of LDL by reducing the ability of insulin to stimulate the expression of the LDL receptors (Tchernof & Despres, 2013).

#### 4.3 The metabolic syndrome: More than half of the study participants presented with the MetS

South Africa, as well as smaller African countries, reported varied results regarding the prevalence of the MetS. Some of the South African studies include the study by Motala *et al.* (2011) who reported a MetS prevalence of 21.2% in women (age-adjusted), using the IDF definition. A study by Erasmus *et al.* (2012), which also used the IDF, reported a

significantly higher prevalence of 67.8% in a female mixed-race population in the Cape Town Metropole. Two more recent studies used the JIS definition, and reported an overall (men and women) prevalence of 29.0% (George *et al.*, 2013), and 43.5% in women (Peer *et al.*, 2014).

A higher prevalence of the MetS was observed in smaller African countries. For example, Kengne *et al.* (2012) described a prevalence of 86.1% in Cameroon (IDF), and a more recent study conducted in Nigeria reported a prevalence of 62.1% (Iloh *et al.*, 2014). Both these studies made use of the IDF definition and only included female participants. However, Kengne *et al.* (2012) also included T2DM individuals, which could help contextualise the high prevalence observed in their study.

Data from the current study indicated that the farm working women, from mixed ancestry, have a comparatively high prevalence of the MetS (55.0%; Figure 3.1). This prevalence is relatively higher than that reported by Motala *et al.* (2011), George *et al.* (2013) and Peer *et al.* (2014), even though the last two studies made use of the JIS definition. Although the JIS makes use of similar cut-off values as the IDF definition, it does not consider WC as an obligatory MetS risk factor as compared to the IDF definition.

The study specific population, being from a variety of ethnic backgrounds, sample size, age distribution, as well as level of urbanisation could account for the differences observed with regards to the MetS prevalence. All of the studies mentioned thus far had much larger sample sizes, as well as participants from different age groups compared to the current study. Motala *et al.* (2011) included a total of  $n=758$  women (age of  $46.6\pm 18.2$ ), compared to  $n=454$  women over the age of 51 years in the study by Erasmus *et al.* (2012). George *et al.* (2013) used a female population of  $n=375$  and Peer *et al.* (2014) included  $n=707$  women between the ages of 25 and 74 years. Irrespective of the small sample size in the current study ( $n=80$ ), and the average age of the females which were  $42.2\pm 1.1$  years, the MetS prevalence was still one of the highest reported thus far for a South African population.

#### **4.3.1 There was no age difference between the MetS and Non-MetS groups**

In several studies, the prevalence of the MetS was correlated with age (Hernández *et al.*, 2011; El Maghraoui *et al.*, 2014; Peer *et al.*, 2014). Two different studies indicated that participants characterised as having the MetS were significantly older than those without the MetS ( $66\pm 9$  vs.  $61\pm 9$ ,  $p<0.0001$ , and  $63.3\pm 8.4$  vs.  $60.1\pm 7.5$ ;  $p=0.013$  respectively) (Hernández *et al.*, 2011; El Maghraoui *et al.*, 2014). It was proposed that ageing and the accompanying physical inactivity might play a predominant role in this occurrence (Hernández *et al.*, 2011; El Maghraoui *et al.*, 2014). A study on a Black urban population from the Cape Town Metropole also concurred, but further reported a peak in the MetS

prevalence (74.3%) between the ages of 55-64 years in women (Peer *et al.*, 2014). Despite these findings, the same conclusion could not be made in the current study population, as there was no significant difference in the mean age between the MetS and Non-MetS groups (43.5±1.6 years vs. 40.6±1.6 years) (Table 3.2). Although a larger sample size might have confirmed the results of Hernández *et al.* (2011); El Maghraoui *et al.* (2014); Peer *et al.* (2014), the MetS-specific risk factors may possibly be better predictors of the MetS prevalence as observed in the current study.

#### **4.3.2 The MetS group had significantly higher WC, FBG, BP and TG, and significantly lower HDL-c than the Non-MetS group**

All five of the MetS risk factors (WC, FBG, BP, low HDL-c, and TG) differed significantly between the MetS and Non-MetS groups (Table 3.2). These results were expected, since three or more of these risk factors (using IDF criteria) needed to be present in order to classify a participant as having the MetS, and several other studies have also reported similar findings (Motala *et al.*, 2011; Alissa *et al.*, 2014). The increased WC, as a result of increased AT, is hypothesised to be responsible for the development of IR, which in turn, decrease HDL-c and increase TG levels, leading to the development of arterial hypertension (Jennings *et al.*, 2009; Gierach *et al.*, 2014). Since increased WC is considered the key contributing MetS risk factor, the abovementioned statement regarding increased AT might explain the increased FBG, BP, TG and low HDL-c observed in the MetS participants compared to the Non-MetS participants in the current study.

#### **4.3.3 The majority of the MetS participants had three MetS risk factors, whereas the Non-MetS group' participants had mostly two MetS risk factors**

The clustering of zero, one, two, three, four and five MetS risk factors was clearly distinct between the MetS and Non-MetS groups. The Non-MetS participants displayed zero, one and two risk factors, whilst the MetS group displayed three, four and five risk factors (Figure 3.2A & B). In the current study population, just over half the population in the Non-MetS group had two risk factors (52.8%), whilst the majority of the MetS group displayed three risk factors (59.1%) (Figure 3.2A & B). Bihn *et al.* (2014) reported that 39.1% of their participants had one MetS risk factor, followed by 25.8% with two risk factors and 15.9% having zero risk factors. Furthermore, 14.9% had three risk factors and 3.9% four risk factors and only 0.8% had five MetS risk factors. Although this clustering of risk factors is important to predict possible future cases of the MetS and allow early detection and treatment, the prevalence of the specific MetS risk factors are more beneficial to determine specific treatment options (Bihn *et al.*, 2014).

#### **4.3.4 Increased BP was the most prevalent MetS risk factor in the total sample population**

The prevalence of hypertension was the most predominant risk factor in the total population (72.5%; Figure 3.3). This was also the most frequent MetS risk factor according to O'Neill & O'Driscoll (2014), which increases the risk for the development of kidney damage and heart failure. It is thought that hypertension may develop *via* hyperinsulinemia causing an increased retention of sodium and increase SNS stimulation, which will both lead to the development of hypertension (Fujita, 2007; Horita *et al.*, 2011). Insulin induces a vasodilatory action *via* the stimulation of vascular endothelial cells to release nitric oxide, which in turn increase blood flow to enhance glucose uptake in skeletal musculature; however, this vasodilatory effect is attenuated in insulin-resistant states (Muniyappa *et al.*, 2008; Tziomalos *et al.*, 2010; Emanuela *et al.*, 2012; Bodea & Popa, 2015). It is therefore suggested that the high prevalence of increased BP might be partially due to the increased insulin concentration, which was also observed in this study population ( $25.6 \pm 4.4$  mIU/L) (Table 3.1).

The second, and third most prevalent MetS risk factor in this sample population was increased WC (68.8%) and low HDL-c (42.5%) respectively (Figure 3.3). An increase in abdominal obesity, as noted in the current study, is associated with dyslipidaemia, including increased TG, VLDL, LDL-c, and decreased HDL-c levels (Franssen *et al.*, 2011; Bodea & Popa, 2015). The obese participants of the current study presented with increased LDL-c in the total population ( $3.0 \pm 0.1$  mmol/L) (Table 3.1), as well as in the specific MetS ( $3.2 \pm 0.1$  mmol/L) and Non-MetS ( $2.8 \pm 0.2$  mmol/L) groups, whilst HDL-c levels were decreased in the MetS group only ( $1.1 \pm 0.0$  mmol/L) (Table 3.2). Another South African study by Peer *et al.* (2014) also reported a high prevalence of increased WC (86.0%), decreased HDL-c (75.0%) and increased BP (47.6%), however this was not in the same order as observed here for the total study sample population.

#### **4.3.5 The clustering of increased WC, increased BP and decreased HDL-c was the most prevalent in both the MetS and Non-MetS groups**

Considering the metabolic status, the current study found a high prevalence of increased WC (100.0%) in the MetS group (Figure 3.3B), which is not unexpected, since it is a compulsory risk factor according to the IDF (IDF, 2006). The increased WC is suggested to contribute to the development of hypertension *via* the secretion of adipokines and lipid abnormalities (Bodea & Popa, 2015). This might explain why increased BP (90.9%) and low levels of HDL-c (81.8%) was the second and third most prevalent MetS risk factors (Figure 3.3). A South African study by Motala *et al.* (2011) also found a high prevalence of WC (96.9%), HDL-c (90.4%) and BP (89.5%) in female participants with the MetS, albeit not in

the same order as in the current study (WC, BP and HDL-c). Although Motala *et al.* (2011) used the JIS definition, the only difference in the cut-off values between the JIS and IDF definition is the reduced HDL-c levels of <1.29 mmol/L according to the IDF definition and <1.30 mmol/L according to the JIS definition (Alberti *et al.*, 2005; Ramli *et al.*, 2013). In addition, the average HDL-c levels of the female participants were  $1.2\pm 0.4$  mmol/L in the study by Motala *et al.* (2011) in comparison to the slightly lower  $1.2\pm 0.0$  mmol/L in the current study.

Similar to the clustering of the MetS risk factors in the MetS group (WC, BP and HDL-c), BP (50.0%), HDL-c (38.9%), and WC (30.6%) was also found to be more prevalent in the Non-MetS group, but not in the same order as in the MetS group (Figure 3.3B). Even though Motala *et al.* (2011) used the JIS definition, the clustering of low HDL-c, increased WC and increased BP was also the most prevalent three risk factors in their Non-MetS group.

Our study found that 45.5% of the individuals with the MetS presented with increased FG (Figure 3.3B), which might be indicative of IR. Peripheral target tissues, such as skeletal muscle, AT and the liver can also become insulin resistant, resulting in a decreased peripheral uptake of glucose, leading to hyperglycaemia (Bodea & Popa, 2015). Fasting insulin concentrations (Table 3.2) was significantly higher in the MetS group compared to the Non-MetS group ( $35.9\pm 6.9$  vs.  $13.0\pm 2.4$ ;  $p<0.01$ ) (Table 3.2) of the current study, which could explain the increased prevalence of increased FG levels in the MetS group vs. Non-MetS group (Figure 3.3B).

Insulin resistance is also frequently associated with abdominal obesity and is considered a consequence of increased production of lipoproteins by the liver and skeletal muscle, and the inability of the AT to store the excess produced lipoproteins (Bodea & Popa, 2015). Therefore, the increased insulin levels in the MetS group might also explain the increased TG levels (MetS:  $2.0\pm 0.2$  mmol/L vs. Non-MetS:  $1.2\pm 0.1$  mmol/L) (Table 3.2) observed, and consequently the increased prevalence of increased TG levels (40.9%) in the MetS group (Figure 3.3B).

#### **4.4 The majority of the study population had normal BMD**

Bone health results in the total sample population revealed that the majority (78.8%) of the participants presented with normal BMD (Figure 3.4), followed by 18.8% with osteopenia, and only 2.5% had osteoporosis. This finding accounts for the normal levels found in all five measures of bone status (T-score, Z-score, BQI, SOS, and BUA), as well as the bone-related markers, PTH and ALP (Table 3.1). The only biochemical marker that was not within the normal ranges was the relatively low levels reported for 25(OH)D ( $0.7\pm 0.1$  pg/mL), despite participants reporting sufficient time exposed to sunlight necessary for vitamin D<sub>3</sub>



synthesis. Vitamin D is known to be an important regulator of bone health (Pérez-Lopez *et al.*, 2011), and according to the International Osteoporosis Foundation (2015), an average of ten to fifteen minutes of exposure to sunlight to the face or arms daily, is considered adequate for the synthesis of vitamin D<sub>3</sub> in most individuals. However, even though the participants in the current study reported a daily average of two hours of sunlight exposure, more than half of the sample population still had serum 25(OH)D levels lower than the detectable limit. This result was anticipated, since most of the participants in the current study were of African descent, and studies have shown that black individuals tended to be 25(OH)D deficient (Gutiérrez *et al.*, 2011; Van Ballegooijen *et al.*, 2014). For example, the study by Gutiérrez *et al.* (2011) reported a mean concentration of 14.8±0.4 pg/mL in Black individuals and stated that 81.0% of their Black participants were vitamin D deficient, whereas Van Ballegooijen *et al.* (2014) reported that 43.0% of the Black individuals had vitamin D levels below 20 pg/mL and 14.0% had levels between 20-30 pg/mL. It has also been reported that much longer sunlight exposure (about six times longer) is necessary to reach sufficient conversion of pre-vitamin D to vitamin D in these individuals (Hall *et al.*, 2010; Ardawi *et al.*, 2011).

In a study comparing 25(OH)D supplementation levels, Black individuals required a mean intake of 3916 IU/d in comparison to the 3040 IU/d in the Caucasians to achieve a serum 25(OH)D concentration of 75 nmol/L (Aloia, 2008). A study by Hall *et al.* (2010) reported a daily vitamin D intake of between 2100 and 3100 IU/d for African ancestry individuals with low sunlight exposure, and between 1000 and 2500 IU/d for European ancestry individuals to reach 75 nmol/L serum 25(OH)D, depending on the season. It is therefore evident that Black individuals require higher concentrations of vitamin D supplementation. Since only 6.3% (n=5) of the current study population reported on the use of calcium or vitamin D supplements, and considering the low vitamin D levels even after sufficient sunlight exposure time, supplementing the diet of all the study participants with vitamin D might be beneficial.

#### **4.4.1 Other factors influencing BMD**

Modifiable (physical activity, alcohol consumption, smoking, contraceptive use and breastfeeding), or non-modifiable factors (genetic susceptibility to fractures) can also influence BMD (Abrahamsen *et al.*, 2014; Mgodi *et al.*, 2015). Participants in the current study reported a relatively low history of stress fractures (15.0%), and bone fractures (23.8%).



#### 4.4.1.1 Smoking

Smoking causes an increase in fractures and/or decrease in bone mass (Shabab, 2012; Ayo-Yusuf & Olutola, 2014). Three possible mechanisms could link smoking and decreased BMD. Firstly, smoking decreases PTH, thereby decreasing  $\text{Ca}^{2+}$  absorption and oestrogen levels. It also concurrently increases cortisol and adrenal androgen levels, thus contributing to an increased risk for the development of osteoporosis. Since PTH and oestradiol concentrations were within the normal ranges in the current study population, and only 30.0% of the population were current smokers and 23.8% were previous smokers (Figure 3.7C), this could partly explain the normal BMD overall. Secondly, smoking reduces vitamin D levels, which is a prerequisite for high BMD and healthy bones. However, vitamin D levels were very low in the current study population, which is probably because of the ethnicity of the participants (mentioned earlier), rather than smoking *per se*. Lastly, smoking is a major contributor of increased free radicals and oxidative stress, leading to increases in bone resorption (Shahab, 2012). However, since free radicals and oxidative stress were not measured in any of the participants, it is still debatable whether this mechanism could potentially have influenced the results obtained. A follow-up study is needed in order to make a full conclusion.

#### 4.4.1.2 Alcohol consumption

Heavy alcohol consumption is deleterious to bone health, since it was previously linked to increased bone resorption (Kruger *et al.*, 2011; Marrone *et al.*, 2012). Fortunately, only a small proportion of participants were grouped as heavy alcohol consumers (8.8%), compared to 57.5% who were current, but moderate alcohol consumers (Figure 3.8C). The classification used here to determine heavy and moderate alcohol consumption was adopted from a study by Marrone *et al.* (2012), which stated that having up to two drinks per day were regarded as moderate alcohol consumption, whereas having more than two drinks per day were considered heavy alcohol consumption. Alcohol can directly affect BMD by (i) impairing proliferation and functioning of osteoblasts, (ii) causing malabsorption, (iii) increasing renal excretion, and (iv) disrupting the  $\text{Ca}^{2+}$ -regulating hormones, PTH, calcitonin and vitamin D metabolites (Sommer *et al.*, 2012). In the current study, PTH levels were within the normal physiological range ( $3.6 \pm 0.2$  pg/mL), therefore corroborating the results of a normal BMD overall, even though 25(OH)D levels were very low (Table 3.1).

#### 4.4.1.3 Contraceptive use

Apart from physical activity, alcohol consumption and smoking, the use of contraceptives also affects BMD (Wei *et al.*, 2011; Elkazaz & Salama, 2015). Oestrogens, or the oestrogenic effects of oral contraceptives, have beneficial effects on BMD *via* remodelling of

the bone in several ways: (i) it promotes osteoclast apoptosis, (ii) it decreases the RANKL, and (iii) it reduces cytokine activity, thereby reducing osteoclast functioning (Scholes *et al.*, 2011). Approximately 37.5% of the population were previous users of contraceptives, whereas 33.5% were current contraceptive users (Figure 3.9A). This, in combination with the normal E<sub>2</sub> levels (26.7±29.8 pmol/L) could potentially have contributed to bone health overall. Irrespective, it is not known whether the women who were either current or previous contraceptive users started using these treatments to regulate abnormal hormone profiles. This is of particular importance, since contraceptives have also been shown to exert negative effects by increasing hepatic production of SHBG, and thereby decreasing the availability of oestrogens (Scholes *et al.*, 2011).

Wei *et al.* (2010) reported an association between the duration of OC use and total body and spine BMD, most notable in women using OC for more than ten years. The participants of the current study indicated an average previous use of contraceptive of seven years and current use of about five years, suggesting that OC use would potentially not have contributed to BMD in these study participants.

#### **4.4.1.4 Physical activity**

The low prevalence of osteoporosis and fractures in our study population could be attributed to their physical activity levels, either at work or during sport or recreational activities. Approximately 45.0% of the participants indicated that their daily work included moderate-intensity physical activity, whereas 68.8% participated in moderate-intensity sport or other recreational activities (Figure 3.10). Furthermore, more than half of the total study population (62.5%) indicated that they commute by either cycling or walking for longer than ten minutes daily (Figure 3.11). Since physical activity is known to increase BMD (Janssen & Ross, 2012; Langsetmo *et al.*, 2012; Braun *et al.*, 2015), and the participants in the current study were physically active overall, a normal BMD should be anticipated. Although the exact mechanism linking physical activity and increased BMD is yet largely undiscovered, it was hypothesised that osteocytes detect the mechanical stimuli and direct it to osteoclasts and osteoblasts for bone resorption and remodelling (Kelley *et al.*, 2013). This may explain the link between the high levels of physical activity and subsequent normal BMD overall.

#### **4.4.1.5 Breastfeeding**

Mgodi *et al.* (2015) reported that breastfeeding for longer than five years was associated with lower BMD, whilst another study reported that longer duration (for up to 220 months) did not affect BMD later in life (Yazici *et al.*, 2011). An average duration of only three years of previous breastfeeding was recorded in the current study, which could also have contributed towards a normal BMD, since BMD levels were shown to return to normal after cessation of

breastfeeding (Canal-Macias *et al.*, 2012). Although Åkesson *et al.* (2004) reported that the speed of recovery of BMD may vary by skeletal site, Karlsson *et al.* (2001) reported that BMD was not fully restored within five months, and therefore, a longer period is needed for recovery of BMD after breastfeeding. Unfortunately, the time since cessation of breastfeeding was not recorded in the current study and a definite conclusion cannot be made regarding the return of BMD to normal levels.

## 4.5 Bone health and the metabolic syndrome

Studies investigating the relationship between the MetS and BMD reported inconsistent results, since some studies suggest that BMD increases due to the MetS and is therefore protective to bone health (El Maghraoui *et al.*, 2014; Muka *et al.*, 2015), whilst others report contrasting evidence (Hwang & Choi, 2010; Jeon *et al.*, 2011; Nóbrega da Silva *et al.*, 2014). In the current study, almost 80.0% of the participants with the MetS presented with normal BMD whereas 18.2% had osteopenia (Figure 3.5). Literature suggests that osteoporosis is less prevalent in individuals with the MetS, and in the current study only 2.3% were identified with osteoporosis (El Maghraoui *et al.*, 2014; Alissa *et al.*, 2014). However, we cannot rule out the possibility that a larger sample size may have yielded different results.

### 4.5.1 Body mass, body mass index and BMD

The inconsistencies in the association between the MetS and BMD revolve around abdominal obesity. Increased BM and BMI is associated with higher BMD due to increased mechanical loading in these individuals (Silva *et al.*, 2015; Sohl *et al.*, 2015). Osteocytes detect mechanical loading to the skeleton, and it is possible that in response to greater mechanical loading, increased signals are sent to osteoblasts to increase bone formation, and/or neighbouring osteoclasts to reduce bone resorption (Finkelstein *et al.*, 2008). Interestingly, Cvijetic *et al.* (2010) reported that increased BMD was present in individuals with the MetS, but this positive effect disappeared after adjustment for BMI. However, Zillikens *et al.* (2010) reported contrasting results and attributed the positive effect of android fat distribution on BMD to increased BMI.

Although both the BM and BMI of the MetS group was significantly higher (BM: 87.8±2.4 kg; BMI: 35.1±0.9 kg/m<sup>2</sup>) than the Non-MetS group (BM: 68.5±3.2 kg; BMI: 27.0±1.2 kg/m<sup>2</sup>; p<0.001 in both BM and BMI), (Table 3.2), both groups presented with normal BMD (Table 3.2; Figure 3.5). Therefore, the current study cannot corroborate or disagree with the results found by Cvijetic *et al.* (2010) and Zillikens *et al.* (2010). Despite this, and even though no differences were observed for any of the BMD measures (T-score, Z-score, BUA, SOS & BQI) between the MetS and Non-MetS groups, the majority of individuals with the MetS still had normal BMD (MetS: 79.6%, Non-MetS: 77.8%; Figure 3.5). This result can however

possibly be attributed to the difference in sample distribution between the MetS (n=44) and Non-MetS group (n=36), (Figure 3.1). Although the MetS group predominantly had normal BMD, this group had more participants with a history of stress fractures (Figure 3.5). This might be due to other contributing factors, but this was not clearly investigated in this study.

#### **4.5.2 Fasting insulin and BMD**

Not only does mechanical loading increase BMD, but hyperinsulinaemia may also increase bone formation. Hyperinsulinaemia decreases SHBG levels, thereby increasing oestrogen bioavailability in obese females and oestrogen is known to increase bone formation (Kinjo, 2007; Braun *et al.*, 2011; Karsenty, 2012). Insulin might also regulate bone growth *via* its interaction with the insulin receptors on osteoblasts to stimulate the proliferation of the osteoblasts (Reid, 2008). The MetS group in the current study had elevated FI levels ( $35.9 \pm 6.9$  mIU/L) (Table 3.2), but none of the bone-related markers differed significantly between these two groups, which might explain why there was no significant difference in BMD between the MetS and Non-MetS group. Alternatively, hyperinsulinemic states can also stimulate increase androgen production, resulting in decreased synthesis of oestrogens (Nestler, 2000).

#### **4.5.3 Anti-hypertensive and cholesterol lowering medication use and BMD**

The use of certain medications might also alter BMD, and previous studies have reported that the use of anti-hypertensive medications (BB's, diuretics and CCB's) resulted in increased BMD (Ghosh & Majumbar, 2014; Hwang *et al.*, 2015). A total of 34.1% of the MetS participants compared to only 13.9% of the Non-MetS participants reported the use of anti-hypertensive medications (Appendix K). This could potentially have contributed to the high percentage of normal BMD observed in both the MetS and Non-MetS groups in this study (Figure 3.5). The use of cholesterol-lowering agents have also been reported to increase BMD (Chuengsamarn *et al.*, 2010; Jeong *et al.*, 2014); however, only a limited number of participants in both the MetS and Non-MetS groups reported on its use (MetS: 4.5%; Non-MetS: 5.6%) (Appendix K).

#### **4.5.4 Physical activity, smoking, alcohol and contraceptive use**

Other confounding factors affecting bone health include physical inactivity, smoking, and alcohol consumption (Tamaki *et al.*, 2010; Bolton *et al.*, 2012; Marrone *et al.*, 2012). Interestingly, for all the measures of physical activity (work, sport and recreational activities, and travelling), the MetS groups appeared to be more physically active than their Non-MetS counterparts (Figure 3.10 & 11). The majority of the participants in the MetS group also never smoked before (52.3%) (Figure 3.7), and were not heavy alcohol consumers (8.8%) (Figure 3.8). Smoking has been shown to negatively affect bone health (Shabab, 2012; Ayo-

Yusuf & Olutoa, 2014), as have heavy alcohol consumption (Kruger *et al.*, 2011; Marrone *et al.*, 2012). However, considering that all confounding factors mentioned here were less pronounced in the MetS compared to the Non-MetS group, these factors could have blunted the MetS' effect, as well as that of the individual risk factors on the bone health status, and therefore contributed to the high prevalence of normal BMD observed in the MetS group.

The use of contraceptives, which have been linked to improved bone health (Elkazaz & Salama, 2015; Scholes *et al.*, 2011), was also more prevalent in the MetS group (Figure 3.9). Although the effect of an increased physical activity status, lower number of smokers, less heavy alcohol consumption and increased contraceptive use in the MetS group could indicate increased bone health status, no differences were observed between the MetS and Non-MetS groups in any of the measures of bone health. Apart from the negative effect of the MetS itself on BMD, a possible reason for this might include the fact that no differences were observed in any of the bone markers, as well as no difference in E<sub>2</sub> between these two groups (Table 3.2). Although oestrogen is known to decrease osteoclast formation and activity and increase osteoclast apoptosis *via* decreasing the production of cytokines (for example IL-1, IL-6 and TNF- $\alpha$ ) that will ultimately increase BMD, its role in the regulation of osteoblast is less clear (Karsenty, 2012).

#### **4.5.5 Vitamin D**

Vitamin D deficiency is detrimental to bone health (Pérez-Lopez *et al.*, 2011; Cauley *et al.*, 2015) and was present in both the MetS and Non-MetS groups. Brenner *et al.* (2011), Baker *et al.* (2012) and Barchetta *et al.* (2013) reported an association between low 25(OH)D levels and the occurrence of the MetS. In obese individuals, sequestering of 25(OH)D may decrease the bioavailability of this vitamin (Wortsman *et al.*, 2000), which can account for the vitamin D deficiency in the current study.

In contrast to this, several studies reported no association between 25(OH)D levels and the prevalence of the MetS (Kim *et al.*, 2012; George *et al.*, 2013; Chon *et al.*, 2014; Gradillas-García *et al.*, 2015). Navarro *et al.* (2013) also reported similar results, with no association noticeable between vitamin D, PTH and the MetS. The differences in results obtained from these studies may be due to differences in ethnicity, the levels of obesity, BMI and WC in these populations, as well as the levels of vitamin D used to classify an individual with vitamin D deficiency.

#### **4.6 Menopausal distribution of study population**

The menopausal transition is accompanied by a variety of physiological changes, the most common being the changes in body fat distribution and hormonal imbalances (Sowers *et al.*, 2007; Al-Safi & Polotsky, 2015; Sapkota *et al.*, 2015). In the current study, a significant

decrease in  $E_2$  was observed in the PostM group compared to the PreM group ( $168.5 \pm 46.7$  pmol/L vs.  $299.3 \pm 36.4$  pmol/L;  $p < 0.05$ ) (Table 3.3), which is not unexpected since less oestrogen is secreted during menopause. In PreM women, fat deposition occurs in the gluteo-femoral region due to oestrogen secretion, whereas the loss of ovarian oestrogen secretion during menopause is associated with increased abdominal fat deposition (Carr, 2003; Goyal *et al.*, 2013). The current study failed to show any significant differences in BMI and WC between the PreM and PostM groups to suggest increased abdominal fat in PostM women; however, a trend towards significance was noted for the W:H ( $p = 0.063$ ). This difference might have been significant if the samples size was larger, or if the distribution between PreM and PostM was more equal, since  $n = 57$  participants were included in the PreM group and only  $n = 23$  in the PostM group (Table 3.3).

Increased abdominal fat is usually associated with a higher risk of diabetes, hypertriglyceridemia, small dense LDL particles, hypertension and CVD (Carr, 2003; Goyal *et al.*, 2013). A study by Bade *et al.*, (2014) reported increases in TC, TG, LDL-c and a decrease in HDL-c levels between PreM and PostM women, independent of BMI, but stated that these differences was possibly due to hormonal differences. Although the current study indicated hormonal differences between PreM and PostM women (mentioned above), none of the other makers except for SBP differed significantly between the PreM and PostM groups. This can possibly be explained by the lack of significant differences in any of the measures of obesity (BMI, WC & W:H) between these two groups (Figure 3.14 & 3.15; Table 3.3). In agreement, a study by Sapkota *et al.* (2015) also found SBP, but not BDP, to differ between PreM and PostM females, and further reported that the decline in the oestrogen: androgen ratio dilutes the vasorelaxant effects of oestrogens on endothelium. This promotes the production of vasoconstrictive factors such as endothelin that ultimately increases BP (Sapkota *et al.*, 2015).

#### **4.7 Bone health and menopausal status**

The menopausal transition is associated with a decrease in BMD (Al-Safi & Polotsky, 2015; AlDughaiter *et al.*, 2015). Parathyroid hormone and the primary female sex hormone,  $E_2$ , both play crucial roles in bone health and are the most important predictors of BMD after menopause (Lu *et al.*, 2009; Karsenty, 2012; Starup-Linde, 2013; Taie & Rasheed, 2014). Parathyroid hormone was significantly higher in the PostM group than in the PreM group ( $4.5 \pm 0.5$  pg/mL vs.  $3.3 \pm 0.2$  pg/mL;  $p < 0.001$ ) (Table 3.3), which is comparable to evidence reported by Ardawi *et al.* (2011). Here, it is suggested that PTH levels increase with age (Van Ballegooijen *et al.*, 2014), and since the females in the PostM group were significantly older, it could explain the increase in PTH. Studies have also reported increased PTH levels



in hypertensive participants (Garcia *et al.*, 2013; Yagi *et al.*, 2014), which might also have contributed here, since the PostM group was indeed hypertensive (Table 3.3). Calcium is known to influence PTH levels, with PostM women usually presenting with low circulating  $\text{Ca}^{2+}$  levels due to decreased intestinal  $\text{Ca}^{2+}$  absorption that will stimulate increased secretion of PTH (Nordin *et al.*, 2004; Drake *et al.*, 2015). Calcium concentrations were however not assessed in this study population to support these statements.

Sowers *et al.* (2006) reported that the annual  $\text{E}_2$  concentration of women in the menopausal transition was not predictive of BMD loss; however, if  $\text{E}_2$  levels were below 128.5 pmol/L, it might be associated with BMD loss. Considering that both the PreM and PostM groups in the current study population had higher  $\text{E}_2$  levels than mentioned above (299.3±36.4 pmol/L, and 168.5±46.7 pmol/L respectively, Table 3.3), it suggests overall normal bone health status, as observed here. In contrast to the observation by Sowers *et al.* (2006), studies have reported that oestrogens influence BMD *via* inhibition of osteoclasts and promotion of apoptosis (Karsenty, 2012; Taie & Rasheed, 2014; Biazon *et al.*, 2015). Since oestradiol was significantly lower in the PostM group (168.5±46.7 pmol/L vs. 299.3±36.4 pmol/L;  $p < 0.05$ ), it may suggest that the inhibiting effect of oestradiol on osteoclasts will be diminished, allowing osteoclast survival and increased bone resorption, which was not evident in the current study. Here, more women in the PostM group had normal BMD (87.0%) than in the PreM group (75.4%) (Figure 3.12), an observation which is more in line with the results reported by Sowers *et al.* (2006). However, these results still warrant further investigation since other risk factors are also known to affect BMD.

Studies have shown that during menopause, increased levels of ALP are also evident due to increased bone turnover (Menezes *et al.*, 2015; Mukaiyama *et al.*, 2015). In agreement, Bhattarai *et al.* (2015), reported ALP levels to be significantly lower in PreM women (82.4±78.5 U/L) compared to PostM women (111.9±66.6 U/L;  $p = 0.046$ ). Although the current study indicated no significant difference in ALP between the PreM (75.9±4.2 U/L) and PostM groups (90.1±4.9 U/L), a trend was noted ( $p = 0.056$ ) (Table 3.3), which would probably have been significant if the sample sizes were larger, and would therefore be able to corroborate the findings of Bhattarai *et al.* (2015). Furthermore, Menezes *et al.* (2015) reported higher levels of ALP in PostM women who had fractures, than those who did not have any fractures. Despite the aforementioned fact, fractures and stress fractures were more prevalent in the PreM group than the PostM group of the current study population (Appendix L), which could have contributed to the lack of a significant difference in ALP between these two groups.

The BMD data (Table 3.3) indicated a significant difference in only the Z-score between the PreM ( $0.1\pm 0.2$ ) and the PostM group ( $0.9\pm 0.2$ ;  $p < 0.05$ ). Since this is an age-matched measure of BMD, the Z-score is not considered useful to detect low BMD and osteoporosis (National Osteoporosis Foundation, 2015). However, it is rather thought to be indicative of other conditions instead, such as hyperparathyroidism or diabetes affecting BMD (NIH, 2012; National Osteoporosis Foundation, 2015). Thus, considering that PTH was indeed higher in the PostM females ( $4.5\pm 0.5$  pg/mL) than the PreM females ( $3.3\pm 0.2$  pg/mL;  $p < 0.001$ ) (Table 3.3), it could suggest that the increased levels thereof might have affected BMD.

In the current study population, 75.4% of PreM woman had normal BMD, whereas Mishra *et al.* (2015) and Silva *et al.* (2015) reported that lower percentages (12.5% and 71.0%) of PreM women had normal BMD (Figure 3.12). In the PostM group, 87.0% of the current study population had normal BMD in comparison to the findings by Mishra *et al.* (2015) and Silva *et al.* (2015), which reported 0.0% and 29.0% respectively. Although we did not observe any of the PostM women to have osteoporosis, Mishra *et al.* (2015) and Silva *et al.* (2015) reported that 80.0% and 92.5% of their PostM women respectively, were classified as osteoporotic. It is important to note that neither of the compared studies investigated physical activity levels in their study participants, whereas the current study indicated that PostM women were more physically active than the PreM group (Appendix L). This could have possibly contributed to the high percentage of normal BMD in the PostM group of the current study. In addition, the small number of study participants in the PostM group might have contributed to the different results ( $n=23$ ).

Differences in smoking habits (Kruger *et al.*, 2011; Øyen *et al.*, 2014, Ayo-Yusuf & Otula, 2014) and alcohol consumption (Kruger *et al.*, 2011; Maurel *et al.*, 2012; Marrone *et al.*, 2012; Sritara *et al.*, 2015) might also help explain the differences in bone health between the PreM and PostM groups. For example, a total of 26.3% of the PreM women indicated that they were current smokers, whereas more females in the PostM group were current smokers (39.1%) (Appendix L). In the current study, more PreM women were current (63.2%) and heavy alcohol consumers (12.3%) compared to the women in the PostM group (current: 43.5%; heavy: 0.0%) (Appendix L). Therefore, the 63.2% of the PreM women who were current alcohol consumers might put them at a lower risk of having osteopenia or osteoporosis. Despite these differences in alcohol consumption between the PreM and PostM women, more of the PreM group' women had osteopenia (21.1% vs. 13.0%) and osteoporosis (3.5% vs. none) and less of them had normal BMD (75.4% vs. 87.0%) (Figure 3.12).



The use of certain medications might also affect the status of BMD in the PreM and PostM groups, since almost half (47.8%) of the PostM women were on anti-hypertensive treatment (Appendix L) compared to 15.8% of PreM women. The use of cholesterol-lowering agents was also more common in the PostM (8.7%) than in the PreM group (3.5%). Both these medications are known to increase BMD (Chuengsamarn *et al.*, 2010; Ghosh & Majumbar, 2014; Jeong *et al.*, 2014; Hwang *et al.*, 2015), which could account for the BMD results observed in the PostM group.

Although menopausal status can play a significant role in the overall health and bone health of individuals, one should consider the fact that the MetS and its individuals risk factors, in combination with menopausal status, might also be important to consider.

## **4.8 Metabolic syndrome and menopausal status**

The changing metabolic status and fat pattern distribution that accompanies the menopausal transition is one of the theories which might explain the increased prevalence of the MetS in PostM women (Carr, 2003; Goyal *et al.*, 2013; Seif *et al.*, 2015). In agreement, the current study also found that more PostM women were classified with the MetS (n=15) compared to their Non-MetS (n=8) counterparts (Figure 3.13). Figueiredo-Neto *et al.* (2010) reported that the increased prevalence of the MetS in PostM women was due to age. Although it is not unexpected for PostM women to be older than PreM women, as seen in the current study (Table 3.3), no significant age differences were observed between the PostM MetS and Non-MetS groups (Figure 3.14A). This might possibly be due to the smaller number of PostM participants, since only 28.8% of the study participants were classified as being PostM (Table 3.3).

### **4.8.1 Neither metabolic nor menopausal status had an effect on BMD**

Studies have shown that the presence of the MetS, as well as menopause, both affect BMD (Jeon *et al.*, 2011; Nóbrega da Silva *et al.*, 2014; Al-Safi & Polotsky, 2015). None of the BMD measures yielded any significant differences between any of the sub-groups in the current study (Figure 3.18). Since none of these markers differed significantly when the MetS and Non-MetS groups were compared (Table 3.2), or when the PreM and PostM groups were compared (except for the Z-score, Table 3.3), the division of the PreM group into two separate PreM age groups could potentially further have contributed to the “no effect” observed. Furthermore, none of the bone-specific markers (PTH & ALP) differed significantly between any of the subgroups (Figure 3.17). In addition, the primary female sex hormone E<sub>2</sub> also did not differ significantly between any of the subgroups (Figure 3.17A & B). Since none of these aforementioned biochemical markers differed significantly between the MetS and Non-MetS groups, while differences were observed in all three between the

PreM and PostM groups, the same explanation as that used for BMD could be used here. Furthermore, five of the six sub-groups had a BMI higher than 25 kg/m<sup>2</sup> with only a significant difference between the PreM (20-39 years) MetS and Non-MetS groups (Figure 3.14). This could have further contributed to the lack of significant differences in ALP and E<sub>2</sub> (Figure 3.17B & C), since obesity is known to alter both E<sub>2</sub> and ALP concentrations (Green *et al.*, 2012; Jones *et al.*, 2013; Pradhan, 2014; Lowe *et al.*, 2011).

When using BMI as a measure of obesity, the association between BMD and BM can be validated by the increased AT, which results in increased conversion of androgens to oestrogens (Green *et al.*, 2012; Jones *et al.*, 2013). Since the current study only revealed significant differences in BM and BMI between the PreM (20-39 years) MetS and Non-MetS groups, without differences in E<sub>2</sub> levels, we tried to explain our results by correlating BM, BMI and E<sub>2</sub> in the different subgroups. In the current study, when BM and BMI were correlated with E<sub>2</sub>, a strong and significant association was only observed in the PreM (20-39 years) MetS group (Figure 3.19A & B), which suggests that the increased BM and BMI, increased the oestrogen concentration. Furthermore, studies by Puntus *et al.* (2011) and Bredella *et al.* (2011) on PreM women (25-59 years old) and PostM women (40-72 years old), and obese PreM women (18-45 years old) respectively, reported no association between BMI and E<sub>2</sub>. Ali & Al-Zaidi (2011) reported an inverse (negative) association between serum E<sub>2</sub> levels and BMI in PreM diabetic women (20-45 years old, mean 29.7±4.3 years). The inconsistent results found by the different studies, might be due to the presence or absence of the MetS in the respective groups, since none of the studies used to corroborate or disprove our findings, considered the MetS.

When investigating the factors affecting BMD in a PostM study populations, Gourlay *et al.* (2014) noted that BM is the most important modifiable determinant of BMD in younger PostM (<65 years) women, since it determines the rate of bone loss during the menopausal transition (Finkelstein *et al.*, 2008). In PostM women, AT is the primary source of oestrogens and therefore an increased BMI will result in increased levels of oestrogen, *via* the increased conversion of androgens to oestrogens in (Braun *et al.*, 2011; Migliaccio *et al.*, 2011; Chan *et al.*, 2014). Al safi & Polotsky (2015) and Randolph *et al.* (2011) further explained that differences in E<sub>2</sub> levels across the menopausal transitions was less pronounced in obese compared to non-obese women. This change in the source of E<sub>2</sub> across the menopausal transition provides obese PostM women with a non-ovarian reservoir of oestrogen, which is not the case in normal weight women, which may blunt the increase in E<sub>2</sub>, and diminish ovarian oestrogen loss with menopause (Randolph *et al.*, 2011). However, in the current study the correlations between BM and E<sub>2</sub>, and BMI and E<sub>2</sub> in the PostM group were insignificant in both the MetS and Non-MetS groups, suggesting that in the PostM groups of

the current study, BM and BMI may not have influenced the E<sub>2</sub> concentrations. This can possibly be due to all participants being obese, since Al safi & Polotsky (2015) and Randolph *et al.* (2011) found that changes in E<sub>2</sub> levels are less pronounced in obese women. In the PostM Non-MetS group, a negative but insignificant correlation was noted between both BM and BMI with E<sub>2</sub> (Figure 3.19E & F). This can be explained by Jones *et al.* (2013) who reported that in PostM women, a decreased BMI would result in a decrease in oestrogen levels.

#### **4.8.2 Correlation analysis between BMD, BMI and SOS**

Further analyses were conducted to investigate the associations between BMD, BMI and SOS. Strong positive associations were noted in the PreM (20-39 years) MetS groups (Figure 3.20A & B), suggesting that as BM and BMI increase, SOS increases, which indicates denser bone tissue or increased BMD. The presence of obesity might increase BMD *via* the increased mechanical loading (Kruger *et al.*, 2011; Xue *et al.*, 2012; El Maghraoui *et al.*, 2014). However, Steinschneider *et al.* (2003) and Beerhorst *et al.* (2013) explained that SOS travels slower through soft tissue, therefore, increased AT around the site of measurements will result in a higher SOS and is not necessarily indicative of increased BMD. The use of the SOS might therefore not be that predictive of increased BMD in overweight individuals, as reported in all of the groups except the PreM (20-39 years) Non-MetS group in the current study that had a BMI below 25 kg/m<sup>2</sup> indicating normal weight. In the aforementioned group, there was no correlation between BM and SOS, and BMI and SOS (Figure 3.20A & B), possibly due to the normal weight of this group.

Bullo *et al.* (2011) reported that BM was the strongest predictor of BMD, which is in agreement with the strong and very strong, significant associations that were noted in the PreM (≥40 years) MetS and Non-MetS groups between BM and SOS, respectively (Figure 3.20C & D). Another study reported that the relationship between BMD and BM was stronger than the relationship between BMD and BMI (Salamat *et al.*, 2013). This may help explain why BMI and SOS correlated strongly and significantly in only the Non-MetS, PreM (≥40 years) group, and in both MetS and Non-MetS groups between BM and SOS in this age group (Figure 3.20C & D).

Furthermore, studies in PostM women reported significant, positive correlations between BM, BMI, and SOS (Brunner *et al.*, 2011; Sun *et al.*, 2014; Sohl *et al.*, 2015). Although positive correlations were also noted in the PostM groups of the current study, all correlations were weak and moderate (Figure 3.20E & F). Here, the effect of ageing and the accompanying decrease in height (also noted in the current study) might alter BMI, and might be responsible for the results (Canning *et al.*, 2013). In a study by Yoldemir & Erenus

(2012), a similar BMD in PostM MetS and Non-MetS women was reported, and it was proposed that the MetS had no effect on BMD in the PostM years. Some studies even suggest that obesity could be considered a protective factor against excessive bone loss during ageing (Zillikens *et al.*, 2010; Jeon *et al.*, 2011; Chantler *et al.*, 2012; Alissa *et al.*, 2014).

Although the findings of the current study are similar to those previously reported, these results raise the question of whether BM and/or BMI really affect BMD in a positive way. It was suggested by Brunner *et al.* (2011) that the changes in SOS was mainly due to age. Migliaccio *et al.* (2011) made an important statement suggesting that when using BMI as a measure of obesity, it appears to act as a protective factor in bone health. In contrast, when increased WC (central obesity) is used to define obesity, it is regarded as a risk factor for bone loss due to the secretion of inflammatory cytokines (Migliaccio *et al.*, 2011). Thus, care should be taken when interpreting results using either BMI or WC.

#### **4.8.3 Differences in anthropometric and metabolic parameters restricted to the PreM group**

##### **4.8.3.1 Measures of obesity**

When investigating both metabolic and menopausal status in the separate PreM (20-39 years), PreM ( $\geq 40$  years) and PostM groups, several observations were made. The PreM (20-39 years) MetS group showed increased BM and BMI compared to their Non-MetS counterparts (Figure 3.14C & D), possibly due to the PreM (20-39 years) Non-MetS group being the only group with a BMI below  $25\text{kg/m}^2$ , indicating a normal BMI. Jeon *et al.* (2011) also found significant differences between the BMI of the PreM MetS and Non-MetS women, but their study population were slightly older (PreM MetS:  $49.3 \pm 8.5$ ; PreM Non-MetS:  $55.4 \pm 8.3$ ), compared to this study's PreM (20-39 years) group. The findings by Jeon *et al.* (2011) could be more comparable to the PreM ( $\geq 40$  years) group of the current study; however, no significant difference was noted between the MetS and Non-MetS individuals in this age group.

The timing of the age of menopause depends on several factors, such as smoking and BMI (AIDughaiter *et al.*, 2015); however, only 26.3% of the PreM participants were current smokers, but more of them have never smoked before (54.4%) (Figure L.4), and the BMI also did not differ significantly between the PreM and PostM groups (Table 3.3). Al-Safi & Polotsky (2015) stated that women undergo physiological changes associated with menopause in the three to five years prior to the final menstrual period. According to this statement by Al-Safi & Polotsky (2015), participants in the PreM ( $\geq 40$  years) group in the current study, might already be experiencing some age-related metabolic changes, although this was not evident here, except for the difference in WC and HDL-c.

Waist circumference, which is a better predictor of fat deposition in the abdominal extremities, was significantly different between both the PreM MetS and Non-MetS groups (Figure 3.15A). In individuals with the MetS, increased WC is usually accompanied by lipid abnormalities and in addition, the presence of menopause also results in changes in lipid metabolism (Derby *et al.*, 2009; Bodea & Popa, 2015).

#### **4.8.3.2 Lipid abnormalities**

In the current study only HDL-c differed significantly between the MetS and Non-MetS groups of both PreM age groups (Figure 3.15B), whereas the other two markers indicating dyslipidaemia measured here, TG and LDL-c, did not differ significantly between any of the groups (Figure 3.15F & 3.16B).

Although increased LDL-c is not one of the MetS-associated risk factors, increased levels thereof are commonly reported in individuals with increased abdominal AT (Han & Lean, 2014) as noted in all the groups except the PreM (20-39 years) Non-MetS group (Figure 3.15A). Han & Lean (2014) explained that although elevated LDL-c is common in individuals with increased abdominal fat, it is mainly related to saturated fat consumption and not as strongly to weight gain and obesity, although these factors were not investigated in this study. Even though TG's concentrations did not differ significantly between any groups, the increased levels thereof in the MetS groups might be due to increased hepatic production of TG's due to hyperglycaemia as reported by Miranda *et al.* (2005). Furthermore, insulin levels also affect the synthesis of TG's (Bodea & Popa, 2015).

#### **4.8.3.3 Glucose and insulin**

Although insulin concentrations were elevated in five of the six groups (Figure 3.16A), only two of the six subgroups presented with increased FG concentrations (>5.6 mmol/L; Figure 3.15E). However, no significant differences were noted between any of the subgroups for FG and FI in the current study (Figure 3.15E & 3.16A). The fact that this study population were largely obese and overweight, might be one of the major contributors to elevated insulin concentrations *via* increased production of lipoproteins by the liver and the inability of the AT to store this excess lipoproteins (Han & Lean, 2014; Bodea & Popa, 2015).

#### **4.8.3.4 Blood pressure**

During ageing, there is a natural increase in BP, mostly due to changes in arterial stiffness and increased peripheral vascular resistance, and the radical decrease in oestrogen levels during menopause is also suggested to contribute to alternations in the control of BP (Pinto, 2006; Cannoletta & Cagnacci, 2014). A study by Mungreiphy *et al.* (2011) also reported that increased BP was associated with increased age, and also that a higher BMI was associated

with increases in both SBP and DBP. Although clear age groups were set aside for the study, it was expected that BP would increase with increased age; however, since none of the sub-groups' ages differed significantly (Figure 3.14A), the significant difference in both SBP and DBP between the MetS and Non-MetS PreM (20-39 years) group (Figure 3.15C & D) could be explained by the significant differences in BMI observed here (Figure 3.14D).

According to the abovementioned statement by Mungreiphy *et al.* (2011) it is expected that BP will not differ between the MetS and Non-MetS groups in the older participants of the current study, since no difference in age was observed. Canning *et al.* (2013) also explained that the increase in abdominal tissue is not entirely captured by BMI and may explain why there is a weakened association between BMI and health complications with age, since the age-related losses in height will also increase the BMI. The higher WC present in the MetS PreM (20-39 years) group (Figure 3.15A) might be partially responsible for the significant differences in the BP (Figure 3.15C & D) since an increased WC was previously suggested to contribute to the development of hypertension *via* the secretion of adipokines and alterations in lipid levels (Bodea & Popa, 2015).

#### **4.9 Further investigation: Significant correlations between ALP and FI, FG and TG limited to the PreM ( $\geq 40$ years) group**

To further investigate the relationship between some of the metabolic markers and bone health markers, correlation analyses were performed between FI, FG, TG and ALP. In addition to being a marker of bone formation, a study by Webber *et al.* (2010) reported that ALP is an indicator of cardiometabolic risk and ALP was also previously classified as a marker of visceral adiposity (Lowe *et al.*, 2011). Furthermore, Krishnamurthy *et al.* (2011) reported associations between ALP and the increased prevalence of the MetS.

Although inconsistent results regarding these parameters have been published, correlation analysis of the current study yielded interesting results. Significant associations were limited to the PreM ( $\geq 40$  years) group (Figure 3.21D, E & F) and this might be due to significant differences in the WC and HDL-c levels between the MetS and Non-MetS groups in this age group. Studies reported that abdominal obesity is correlated with changes in lipid levels (increased TG's, LDL-c and decreased HDL-c) and that increased WC is commonly associated with IR due to increased visceral AT (Robinsin *et al.*, 2013; Taverne *et al.*, 2013; Bodea & Popa, 2015).

##### **4.9.1 Alkaline phosphatase and TG**

Serum ALP levels are known to be higher in obese individuals and may play a role in the process of adipogenesis (Ali *et al.*, 2015). In agreement, a very strong correlation was noted between TG's and ALP in the PreM ( $\geq 40$  years) MetS group ( $r=0.82$ ,  $p=0.00$ ) (Figure 3.21F),



which was classified as an obese group. The hypothesis by Kim *et al.* (2013b) that intracellular lipid accumulation and ALP activity increase simultaneously during adipogenesis, might explain this result in the current study. This is in agreement with what Krishnamurthy *et al.* (2011) reported, where the levels of TG increased with increased ALP levels. Hernández-Mosquera *et al.* (2015) hypothesised that changes in the activity of ALP might correlate with changes in lipid metabolism. However, in the Non-MetS groups of the current study, no significant associations were noted between TG and ALP (Figure 3.21C, F & I), whereas Webber *et al.* (2010) and Kim *et al.* (2013b) reported positive, but weak correlation between ALP and TG ( $r=0.17$ ,  $p<0.001$  and  $r=0.068$ ,  $p<0.001$  respectively).

#### **4.9.2 Alkaline phosphatase, FI and FG**

Krishnamurthy *et al.* (2011) reported that the levels of both FG and FI increased with increasing levels of ALP. It is hypothesised that ALP present in adipocytes might regulate systemic signalling, related to glucose metabolism and insulin sensitivity, *via* adipokine synthesis and secretion (Hernández-Mosquera *et al.*, 2015). In agreement with this hypothesis, a very strong and significant positive association was noted between FI and ALP, but only in the PreM ( $\geq 40$  years) Non-MetS group (Figure 3.21D). Contrasting results have also been published, where Kim *et al.* (2013b) correlated HOMA-IR and ALP and found a weak, but significant association between these two parameters ( $r=0.045$ ,  $p<0.0001$ ).

Furthermore, when correlating FG and ALP, a study by Webber *et al.* (2010) and Kim *et al.* (2013) reported a weak positive correlation between ALP and FG in Non-MetS females and a mixed gender population, irrespective of MetS status, respectively ( $r=0.14$ ,  $p<0.001$  and  $r=0.039$ ,  $p<0.001$  respectively). Cheung *et al.* (2013) reported no association between FG and BAP. The current study however found a very strong correlation between FG and ALP in the PreM participants ( $\geq 40$  years) with the MetS ( $r=0.89$ ,  $p=0.00$ ) (Figure 3.21E). Although the abovementioned studies correlating FG and ALP, investigated the MetS, none of these studies except for Webber *et al.* (2010) performed their correlation analysis in the specific MetS and Non-MetS groups (Webber *et al.*, 2010; Kim *et al.*, 2013b, Cheung *et al.*, 2013), which complicates comparison between different studies.

## Chapter 5

## Conclusion

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### 5.1 Introduction

The final chapter of this thesis summarise the major findings, highlight study limitations and provide future recommendations.

### 5.2 Major findings

One of the major findings of this study was the relatively high prevalence of the MetS (55.0%), which was higher than that reported in previous South African studies. The prevalence of the different MetS risk factors was similar to that noted by other researchers, with increased BP being the most prevalent, followed closely by increased WC and low HDL-c levels. Although this specific combination was also evident when the sample population was divided into the MetS and Non-MetS groups, the order in which the risk factors were ranked differed. When comparing the ages of the MetS and Non-MetS groups, no significant difference was evident, although literature suggests that participants with the MetS are normally older (Hernández *et al.*, 2011; El Maghraoui *et al.*, 2014). Body mass, BMI, SBP and DBP differed significantly between the MetS and Non-MetS groups, but only for the PreM (20-39 years) age group, whereas WC and HDL-c differed in both PreM age groups. Furthermore, previous studies suggested an increased prevalence of the MetS in PostM women in comparison to PreM women (Carr, 2003; Goyal *et al.*, 2013; Seif *et al.*, 2015), which could not be confirmed in the current study population.

Another major finding was that, irrespective of metabolic and menopausal status, the majority of the participants in the current study displayed normal BMD. Although the presence of the MetS seems to be protective against bone loss, this is possibly as a result of the increased BMI noted in five of the six subgroups, which resulted in increased mechanical loading. The MetS participants were generally more physically active, have never smoked before, whilst fewer of them were heavy alcohol consumers. All of these factors could have contributed to a healthier bone status. In addition, more of the MetS participants used contraceptives, which also increase BMD.

The low 25(OH)D levels observed in the current study population might be due to the ethnicity of the study population, since similar results were found by studies using dark-skinned participants (Gutiérrez *et al.*, 2011; Van Ballegooijen *et al.*, 2014). Albeit so, the low 25(OH)D observed here did not appear to result in low BMD. The other markers related to BMD, PTH and ALP, also did not differ significantly between the MetS and Non-MetS groups; however, these two markers are rarely mentioned in the literature, possibly due to the fact that no association was evident between these parameters. When menopausal



status was taken into consideration, the increased  $E_2$  levels in the PreM groups and decreased PTH levels in the PostM groups were in agreement with that found by other studies. Some factors that might also have influenced BMD in the separate PreM and PostM groups include smoking status, alcohol consumption and medication use. More of the PostM women were current smokers, whereas more of the PreM were heavy alcohol consumers. Furthermore, both anti-hypertensive and cholesterol lowering treatment were more common in the PostM women, which are known to increase BMD.

Although both BM and BMI were associated with  $E_2$ , this correlation was only significant in the PreM (20-39 years) MetS group, possibly as a result of the increased abdominal fat (increased WC) causing increased conversion of androgens to oestrogens. The correlations between BM, BMI, and SOS were significant and positive in only the PreM (20-39 years) group with the MetS and the PreM ( $\geq 40$  years) Non-MetS group. Interestingly, significant correlations between FI, FG and ALP were restricted to the PreM ( $\geq 40$  years) groups.

We are aware that some parameters yielded contradicting and inconclusive results, for example, the effect of BMI and WC on BMD, and the effect of menopausal stage or age on the prevalence of MetS. This warrants further investigation in order to determine the exact mechanisms or effects of the MetS and menopausal status on BMD.

### **5.3 Contributions**

This study is one of the first to investigate BMD and the MetS in a farm working community in the Western Cape. The high prevalence of the MetS among these farm working women raises reason for concern and further investigation to improve the health of this community. Since no BMD database exists for the South African population, and the Sonost 3000 is not routinely used in a clinical setting to assess BMD, this study proved useful in starting such a database that can be used as a reference framework for future studies where the use of DEXA is not possible.

### **5.4 Limitations and recommendations**

The use of a larger sample size will result in a more evenly distribution of participants between the different subgroups, and might allow stronger statistical power. The MetS definition used in this study is not specific to the South African population, since ethnic-specific WC cut-off values are non-existent for South Africans, irrespective of the suggestions made by some researchers (91.5 cm) (Crowther & Norris, 2012). These suggested cut-off values need to be further studied as well as validated in a much larger sample to prove useful.

Since most of the information were gathered by means of questionnaires, it is possible that reporting bias could have influenced the results. The researcher also assumed that all participants were truthful in their responses when interviewed. It was unknown whether the women who used contraceptives, used it due to abnormal hormone profiles, but in the future, this should be included in the questionnaires. A further limitation is that BMD was only measured using the dominant foot, and since differences in BMD can occur between the right and left foot (Mergler *et al.*, 2010), it is suggested that both feet be measured in future studies. Furthermore, no reference values exists for BMD measurements for people from African and Mixed ancestry decent.

To complement our BMD data further, a more specific isoenzyme of ALP can be used, namely bone-specific ALP (BAP), since the ALP measured in the current study could possibly be from other sources including the liver or intestinal tissue. It would also be beneficial to measure both a bone formation and bone resorption marker, rather than just a single bone formation marker. For example, osteocalcin and CTx, used as reference bone resorption marker. Furthermore, the measurement of free radicals and oxidative stress can help to contextualise the effects of smoking on BMD.

Although nutritional assessments were initially included, the low response rate required us to excluded dietary data from statistical analysis. These data might have been beneficial since the measurement of macro- and micronutrients can also impact on bone health. Even though the physical activity questionnaire, the GPAQ were reported to be validated in the South African population, it is quite possible that there might be either an over, or under reporting (Bull *et al.*, 2009). In order to minimize this, it is suggested for future studies to validate physical activity against using a pedometer or accelerometer.

Although the high prevalence of the MetS did not appear to negatively affect BMD, an exercise intervention could prove beneficial to lower the high rates of overweight and obesity and thus contribute to lower the prevalence of the MetS, and concurrently positively affect the general bone health of these women. Although the use of a more sensitive vitamin D ELISA would be more beneficial, this was not true for this population, since dark-skinned people have low vitamin D concentrations (Sharp, 2011; Johnson, 2013).

Another, and probably the most important limitation is the use of a cross-sectional study design, as it is difficult to determine cause and effect of the MetS, and menopausal status with respect to BMD. The use of a longitudinal study design rather than a cross-sectional study design will allow the researcher to investigate changes over time and more accurate conclusions can be made. Furthermore, with the use of a longitudinal study design, an exercise and/or supplementation intervention can be applied. The very low levels of vitamin

D in this study population could also then be improved by a nutritional and/or a supplementation intervention.

Collectively, this study provides evidence of the relatively high prevalence of the MetS in a female farmworker population. Participants should be encouraged to maintain a healthier lifestyle, including a healthier diet and increased physical activity levels. Although the study population had generally normal BMD, the very low levels of vitamin D must be further investigated and care must be taken to ensure proper supplementation with vitamin D.

## Chapter 6

## References

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## Chapter 7

## Appendices

## APPENDIX A: Ethical documents



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 JOUW KENNISVERMOEEN • JOUW WISSENSKAP • JOUW TOEKOMST

### Approval Notice New Application

02-Sep-2013  
 Nell, Theodore TA

**Ethics Reference #:** N13/04/052

**Title:** Cancer risk during urbanisation: metabolic syndrome and cancer

Dear Doctor Theodore Nell,

The New Application received on 19-Apr-2013, was reviewed by members of Health Research Ethics Committee 1 via Minimal Risk Review procedures on 30-Aug-2013 and was approved.

Please note the following information about your approved research protocol:

**Protocol Approval Period:** 30-Aug-2013 - 30-Aug-2014

Please remember to use your protocol number (N13/04/052) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

**After Ethical Review:**

Please note a template of the progress report is obtainable on [www.sun.ac.za/rds](http://www.sun.ac.za/rds) and should be submitted to the Committee before the year has expired.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No 61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

**Provincial and City of Cape Town Approval**

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health ([healthres@pgwc.gov.za](mailto:healthres@pgwc.gov.za) Tel: +27 21 483 9907) and Dr Helene Visser at City Health ([Helene.Visser@capetown.gov.za](mailto:Helene.Visser@capetown.gov.za) Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HRBC forms and documents please visit: [www.sun.ac.za/rds](http://www.sun.ac.za/rds)

If you have any questions or need further assistance, please contact the HREC office at 0219389657.

**Included Documents:**

DEC CV LOMBARD

IC FORM

DEC CV ESSOP

DEC CV NELL

DEC CV NIEWHOUDT

DEC CV OPPERMAN

APPLIC FORM

SYNOPSIS

DEC CV OLIVIER

CHECKLIST



PROTOCOL

Sincerely,



Franklin Weber  
HREC Coordinator  
Health Research Ethics Committee 1

## Investigator Responsibilities

### Protection of Human Research Participants

Some of the responsibilities investigators have when conducting research involving human participants are listed below:

**1. Conducting the Research.** You are responsible for making sure that the research is conducted according to the HREC approved research protocol. You are also responsible for the actions of all your co-investigators and research staff involved with this research.

**2. Participant Enrolment.** You may not recruit or enrol participants prior to the HREC approval date or after the expiration date of HREC approval. All recruitment materials for any form of media must be approved by the HREC prior to their use. If you need to recruit more participants than was noted in your HREC approval letter, you must submit an amendment requesting an increase in the number of participants.

**3. Informed Consent.** You are responsible for obtaining and documenting effective informed consent using only the HREC-approved consent documents, and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Please give all participants copies of the signed informed consent documents. Keep the originals in your secured research files for at least fifteen (15) years.

**4. Continuing Review.** The HREC must review and approve all HREC-approved research protocols at intervals appropriate to the degree of risk but not less than once per year. There is no grace period. Prior to the date on which the HREC approval of the research expires, it is your responsibility to submit the continuing review report in a timely fashion to ensure a lapse in HREC approval does not occur. If HREC approval of your research lapses, you must stop new participant enrolment, and contact the HREC office immediately.

**5. Amendments and Changes.** If you wish to amend or change any aspect of your research (such as research design, interventions or procedures, number of participants, participant population, informed consent document, instruments, surveys or recruiting material), you must submit the amendment to the HREC for review using the current Amendment Form. You may not initiate any amendments or changes to your research without first obtaining written HREC review and approval. The only exception is when it is necessary to eliminate apparent immediate hazards to participants and the HREC should be immediately informed of this necessity.

**6. Adverse or Unanticipated Events.** Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research-related injuries, occurring at this institution or at other performance sites must be reported to the HREC within five (5) days of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the HREC's requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported in accordance with the Stellenbosch University Health Research Ethics Committee Standard Operating Procedures [www.sun025.sun.ac.za/portal/page/portal/Health\\_Sciences/English/Centres%20and%20Institutions/Research\\_Development\\_Support/Ethics/Application\\_package](http://www.sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package) All reportable events should be submitted to the HREC using the Serious Adverse Event Report Form.

**6. Adverse or Unanticipated Events.** Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research-related injuries, occurring at this institution or at other performance sites must be reported to the HREC within five (5) days of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the HREC's requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported in accordance with the Stellenbosch University Health Research Ethics Committee Standard Operating Procedures [www.sun025.sun.ac.za/portal/page/portal/Health\\_Sciences/English/Centres%20and%20Institutions/Research\\_Development\\_Support/Ethics/Application\\_package](http://www.sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package) All reportable events should be submitted to the HREC using the Serious Adverse Event Report Form.

**7. Research Record Keeping.** You must keep the following research-related records, at a minimum, in a secure location for a minimum of fifteen years: the HREC approved research protocol and all amendments; all informed consent documents; recruiting materials; continuing review reports; adverse or unanticipated events; and all correspondence from the HREC

**8. Reports to the MCC and Sponsor.** When you submit the required annual report to the MCC or you submit required reports to your sponsor, you must provide a copy of that report to the HREC. You may submit the report at the time of continuing HREC review.

**9. Provision of Emergency Medical Care.** When a physician provides emergency medical care to a participant without prior HREC review and approval, to the extent permitted by law, such activities will not be recognised as research nor will the data obtained by any such activities should it be used in support of research.

**10. Final reports.** When you have completed (no further participant enrolment, interactions, interventions or data analysis) or stopped work on your research, you must submit a Final Report to the HREC.

**11. On-Site Evaluations, MCC Inspections, or Audits.** If you are notified that your research will be reviewed or audited by the MCC, the sponsor, any other external agency or any internal group, you must inform the HREC immediately of the impending audit/evaluation.



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## **Ethics Letter**

16-Oct-2014

**Ethics Reference #:** N13/04/052

**Clinical Trial Reference #:** N13/04/052

**Title:** Cancer risk during urbanisation: metabolic syndrome and cancer

Dear Doctor Theodore Nell,

The HREC approved your progress report dated 14 July 2014. The approval of this project has been extended for a further year.

Approval date: 03 September 2014

Expiry date: 03 September 2015

If you have any queries or need further assistance, please contact the HREC Office 0219389657.

Sincerely,

REC Coordinator

Franklin Weber

Health Research Ethics Committee 1



**APPENDIX B: Participant information and consent form****PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM****TITLE OF THE RESEARCH PROJECT:***Cancer risk during urbanisation: metabolic syndrome and cancer***REFERENCE NUMBER:****PRINCIPAL INVESTIGATOR: Dr Theo A Nell****ADDRESS:**

Department of Physiological Sciences  
 Mike de Vries Building  
 Room 2007  
 Stellenbosch University

**CONTACT NUMBER:** 021 8083147

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

**What is this research study all about?**

- *This study will only be done in Western Cape Health districts. We will need approximately 1000 patients. We are trying to gather information on laboratory tests, body composition and patient questionnaire profiles of people undergoing migration. By getting this information we would be able to assess the prevalence of the metabolic syndrome in different regions in the Western Cape and how this might increase risk of developing cancer.*
- *More people are relocating to larger cities and with this their traditional habits change. One factor that plays an important role is nutritional changes. There is also changes in your body where some might become overweight.*

*Your participation will help us look at the markers that tell researchers what factors are important to look at. Blood will be taken by a registered medical nurse. It will then be sent away to Pathcare (Stellenbosch) where metabolic-associated parameters will be measured. Other biochemical tests that will be*

*done by Pathcare include insulin, glucose, lipids, and hormone measurements. The remainder of your blood samples will be used to analyse omega-3 fats.*

*The blood pressure and anthropometric evaluation, and life style questionnaire will be done at the clinic.*

#### **Why have you been invited to participate?**

- *We are trying to gather information on people that live in certain areas in the Western Cape provincial health districts. Your participation will help us understand what factors could lead to the development of certain cancers if people migrate from rural to urban areas. There will also be questions asked about your diet at home and how active you are during the week.*
- *By donating blood to our study you will be helping us to determine these profiles and how we can relate them to the current diagnostic tests to investigate the metabolic syndrome and development of cancer.*

#### **What will your responsibilities be?**

- *We will need to examine you as one of the selected patients. A blood sample will then be taken for laboratory tests. There will be a lifestyle questionnaire that you need to complete with the help of the researcher. A registered anthropometrist, Dr Theo Nell, will also perform anthropometric measurements to measure your waist circumference, hip circumference, arm circumference, the back of your arm's skin fold, height and weight. You will also be asked to lay on the examination bed where Dr. Nell will use a special machine that will tell us how much fat is in your body. This will only take a few minutes and does not hurt you.*

#### **Will you benefit from taking part in this research?**

- *Although there may not be any direct benefits to me/the participant by participating at this stage, future generations may benefit if the researchers succeed in finding out more about how migration could lead to increased number of people developing cancer. If you choose to know the results of your blood tests we will make these available. However, you would have to discuss this information with your usual/personal doctor, in order to assess your medical status.*

#### **Are there any risks involved in your taking part in this research?**

There are no more than minimal medical or physiological risks associated with this study.

- *If the participant may feel some pain associated with having blood drawn from a vein in my arm, and may experience some discomfort, bruising and/or slight bleeding at the site. The anthropometrical test will require you to take some of your clothes and shoes off, but there is no pain involved during this procedure. All measurements will be done in private and confidentiality is very important.*
- *The machine that will be used to determine the fat in the body uses a very small electrical current that you will not feel.*

**If you do not agree to take part, what alternatives do you have?**

- *It is your decision to participate or not, and nothing will be done from the researchers' part or medical staff at the clinic/hospital to in any way to persuade you to take part.*

**Who will have access to your medical records?**

- *Only the principal researcher (Dr Theo Nell and other collaborators) will have access to your data and records. All information will be treated with respect and utmost confidentiality. Under no circumstances will your name or any form of identification be used in any publication, poster, lecture or thesis that results from this study. Dr. Theo Nell will be the only authorised personnel who will have access to all your results from this study as well as the lifestyle questionnaire and anthropometric measurements.*

**What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?**

- *There are no risks involved that could lead to injury. Not applicable here.*

**Will you be paid to take part in this study and are there any costs involved?**

- *No, you will not be paid to take part in the study. There will be no costs involved for you, if you do take part.*

**Would you like to know the results of your blood tests?**

- Please indicate by marking the correct box with an X
- YES
- NO

**Is there anything else that you should know or do?**

- You can contact **Dr Theo Nell** on **021 808 3147** if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee** at **021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

**Declaration by participant**

By signing below, I ..... agree to take part in a research study entitled (**Cancer risk during urbanisation: metabolic syndrome and cancer**).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ..... on (*date*) ..... 2015.

.....  
Signature of participant

.....  
Signature of witness

**Declaration by investigator**

I (*name*) ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*)

Signed at (*place*) ..... on (*date*) ..... 2015.

.....  
Signature of investigator

.....  
Signature of witness

## Declaration by interpreter

I (*name*) ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of participant*) ..... using the language medium of Afrikaans/Xhosa/Zulu/English.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... on (*date*) .....2015

Id Code: \_\_\_\_\_

## APPENDIX C: Data collection sheet

**CANSA Study: Data Collection Sheet**

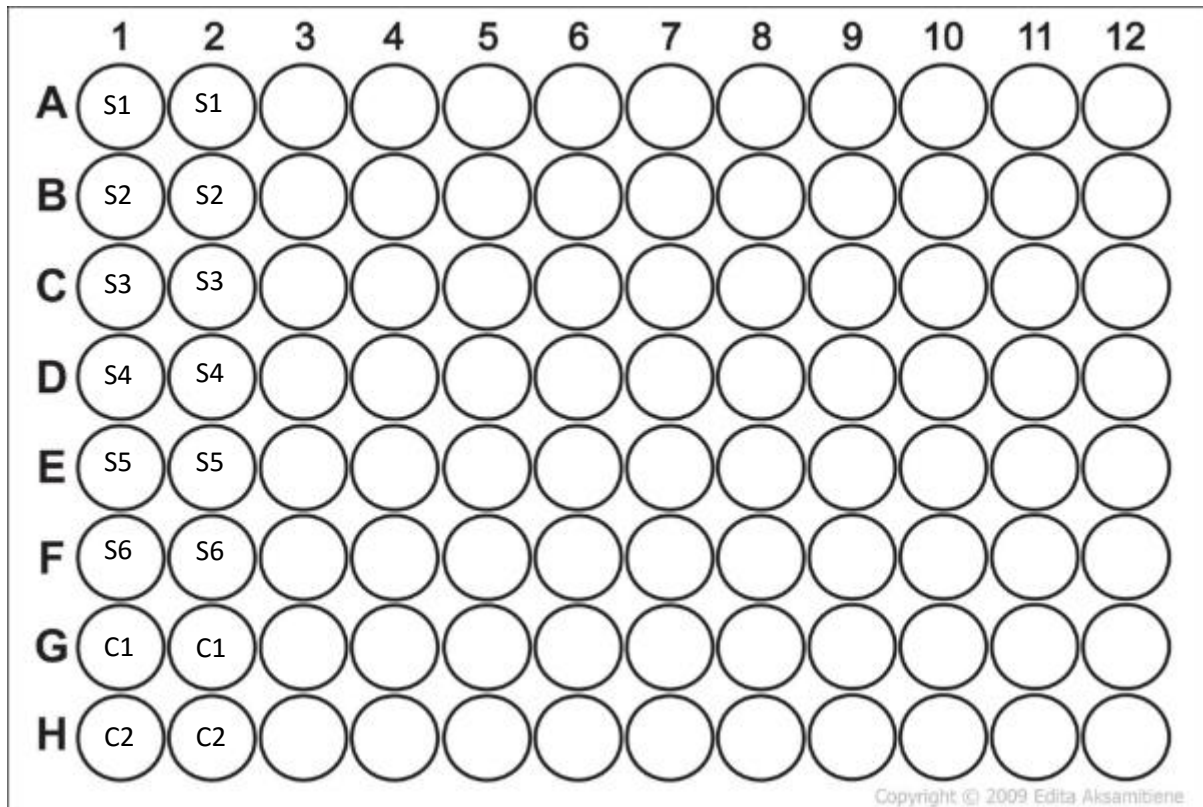
Gender	MALE		FEMALE	
Age				
Body Mass (kg)				
Blood Pressure (mmHg)			Heart Rate	
Height (m)				
BMI (kg/m <sup>2</sup> )				
Waist Circumference (cm)				
Hip Circumference (cm)				
SAD (cm)				
VAT				
Impedance (Ohms)				
Phase Angle				
SAT				
Impedance (Ohms)				
Phase Angle				
Body Shape				



## APPENDIX D: PTH ELISA

### Human Parathyroid Hormone ELISA kit (BioVendor, RIS003R)

Before the ELISA was performed, the plate layout was planned as indicated in the example, 96-well plate below.



The steps that were followed to perform this ELISA are listed below:

#### Preparation of samples:

- Samples were transferred from the  $-80^{\circ}\text{C}$  to the  $-20^{\circ}\text{C}$  bio-freezer one day prior to the analyses.
- All samples were allowed to equilibrate at room temperature ( $22^{\circ}\text{C}$ ), and mixed by a vortex mixer prior to the assay procedure.

#### Preparation of reagents:

- The zero calibrator was reconstituted with three mL of distilled water, whereas all other calibrators, as well as the controls were reconstituted with one mL of distilled water.
- The wash solution was prepared by adding one volume of wash solution (20x) to 19 of distilled water.



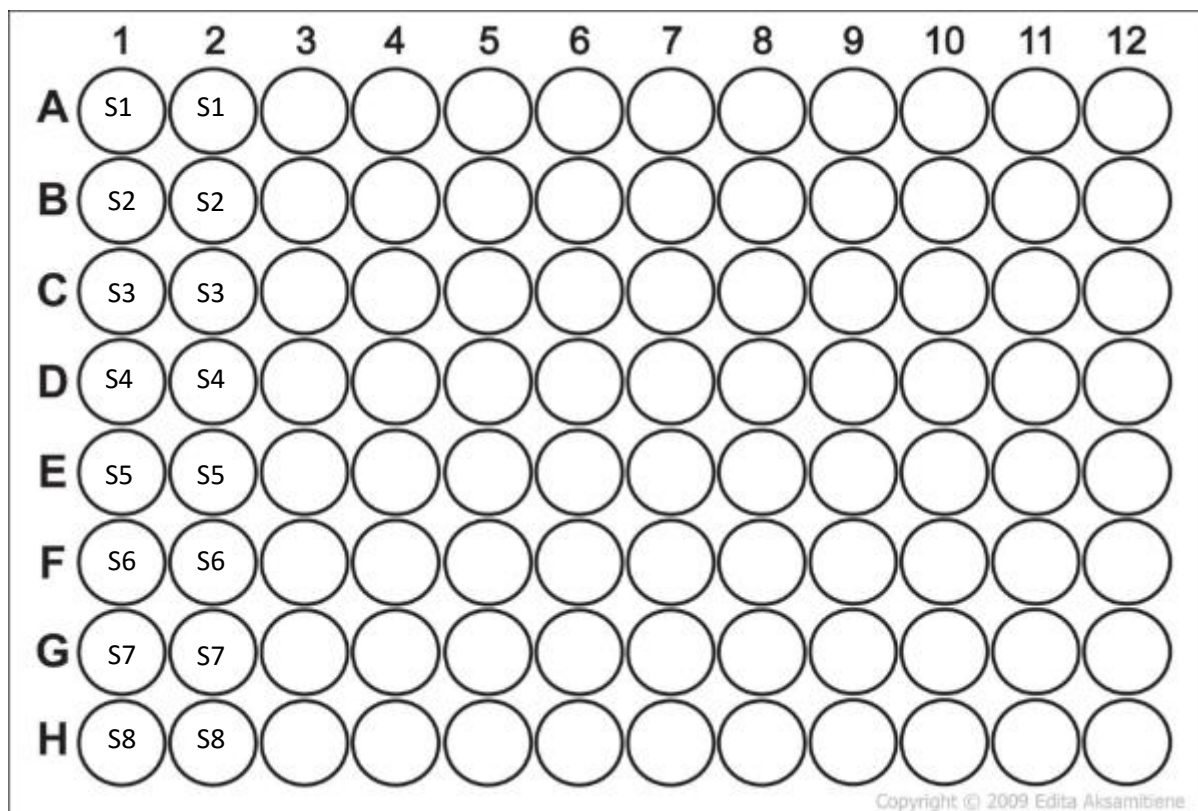
**Assay procedure:**

1. 50  $\mu$ l of incubation buffer were added to all wells.
2. The calibrators (standard one to six, S1-S6), controls (two, C1 and C2) and samples were then added in duplicate to the appropriate wells as indicated in the plate layout above (200  $\mu$ L).
3. The plate, with the aforementioned added contents, were allowed to incubate for two hours at room temperature (22 °C) on a horizontal shaker (The Belly Dancer, Stovall Life Science Incorporated, Greensboro NC USA) set at 700  $\pm$  100 rpm.
4. The liquid was aspirated from the wells and tapped on a paper towel to ensure that all contents were removed.
5. The plate was then washed four times by adding 0.4 mL of wash solution to each well and aspirating the solution from the wells.
6. 100  $\mu$ l of anti-PTH-HRP was then added to each well.
7. After this addition, the plate was incubated for another hour at room temperature on a horizontal shaker set at 700  $\pm$  100 rpm.
8. The liquid was aspirated from the wells, inverted and patted on absorbent paper to ensure that all contents were removed.
9. Within 15 minutes from the last wash step, 100  $\mu$ L of Chromogenic solution was added to all wells.
10. The plate was incubated for 30 minutes at room temperature on a horizontal shaker set at 700  $\pm$  100 rpm, this time avoiding direct sunlight.
11. Lastly, 200  $\mu$ L of Stop Solution was added to all wells.
12. The absorbencies were read at 450 and 490 nm, within one hour after the stop solution was added with a Universal Microplate Reader (EL800).

## APPENDIX E: Vitamin D ELISA

### Vitamin D ELISA kit (Elabscience, E-EL-0012)

Before the ELISA was performed, the plate layout was planned as indicated in the example 96-well plate below.



The steps that were followed to perform this ELISA are listed below:

#### Preparation of samples:

- Samples were moved from the -80 °C to the -20 °C bio-freezer one day prior to the analyses.
- Samples were kept at room temperature prior to the assay procedure.

#### Preparation of reagents:

- The wash buffer was made by diluting 30 mL of Concentrated (25X) Wash Buffer with 750 mL of distilled water.
- The standard was prepared 15 minutes before the assay procedure. The Reference Standard was centrifuged at 10 000xg for one minute and reconstituted with one millilitre of Reference Standard and Sample Diluent. After standing for ten minutes, it was mixed thoroughly by turning it upside down several times and mixing with a

pipette. This produced a stock solution of 400 ng/mL, used to make serial dilutions to render the following concentrations: 200, 100, 50, 25, 12.5, 6.25 and 0 ng/mL.

- The biotinylated detection Ab was made by using the Concentrated Biotinylated Detection Ab and Biotinylated Detection Ab Diluent. Similarly the HRP Conjugate was made by using the Concentrated HRP Conjugate and HRP Conjugate Diluent.

#### **Assay procedure:**

1. 50  $\mu$ L of Standard, Blank or Sample was added to each well (in duplicate). The Blank well contained Reference Standard & Sample Diluent. Immediately thereafter, 50  $\mu$ L of Biotinylated Detection Ab was also added to each well. The plate was covered with a Plate sealer and tapped to ensure thorough mixing, where after the plate was incubated for 45 minutes at 37 °C (Orbital Shaker Incubator, MRC).
2. The contents was aspirated from the wells and washed three times with 350  $\mu$ L of the Wash Buffer with the use of a multi-channel pipette. The solution was then removed by inverting the plate and patted on absorbent paper to ensure that all contents were removed.
3. Next, 100  $\mu$ L of HRP Conjugate working solution was added to each well, the plate covered with the plate sealer and incubated for 30 minutes at 37 °C (Orbital Shaker Incubator, MRC).
4. The plate was then washed five times with the washing buffer as described in step 2.
5. Then, 90  $\mu$ L of the Substrate Solution was added, covered with a plate sealer and incubated for 15 minutes at 37 °C (Orbital Shaker Incubator, MRC). This step required protection from light. The reaction was followed and checked to see when colour gradients appeared in the standard wells, but this time was limited to 30 minutes.
6. Thereafter, 50  $\mu$ L of the Stop solution was added to each well, which allowed for a colour change of blue to yellow.
7. The optical density of each well was then read with a microplate reader at 450 nm (EL800 Universal Microplate Reader).

## APPENDIX F: Procedure for the measurement of BMD with the Sonost Osteosys 3000

### *Pre-test procedure*

For quality control purposes the bone densitometer was calibrated each day. This was done by adding ultrasound gel to the calibrator and adding it to the heel bath in the same orientation as a participant's heel in the bath. The surfaces of the coupling pad or probe were cleaned with alcohol between each participant.

[To note: The ultrasound gel aids in the progression of the ultrasound wave between the probe and the participant's heel, as illustrated in Figure G1: A, B.]



Figure G: A: Cleaning of probes with alcohol and application of ultrasound gel, and B: Application of ultrasound gel to the ankle.

### *Testing procedure*

- 1) The participants were asked to remove all footwear and socks.
- 2) Alcohol pads (70% v/v) were used to clean both sides of the participant's calcaneus bones, followed by the application of ultrasound gel (Figure G2: B, G2: A and B). [To note: It is important to apply enough gel, as well as to the correct area, since this allows for a proper reading – see Figure G2: A, B.]
- 3) The participant was asked to place their foot between the probes, with their calf resting on the calf support. The heel, toes and calf of the individual had to be flat against the surface, as indicated in Figures G3: A and B.

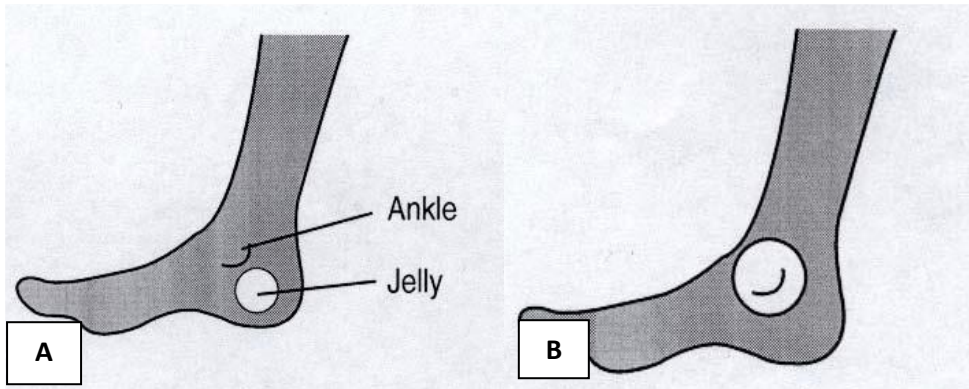


Figure G2: A: Correct position to apply the ultrasound gel, B: incorrect position of the ultrasound gel.

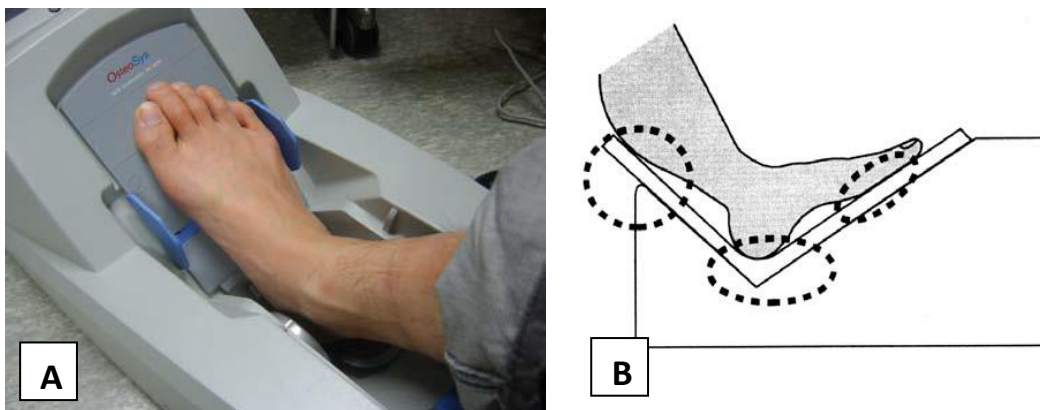


Figure G3: A: Placement of the foot between the two probes and B: Calf, heel and toes should be in contact with the surface.

4) The participant's body should also be correctly positioned, with the body and foot in line with one another. The incorrect and correct alignment is shown in Figure G4: A and B.

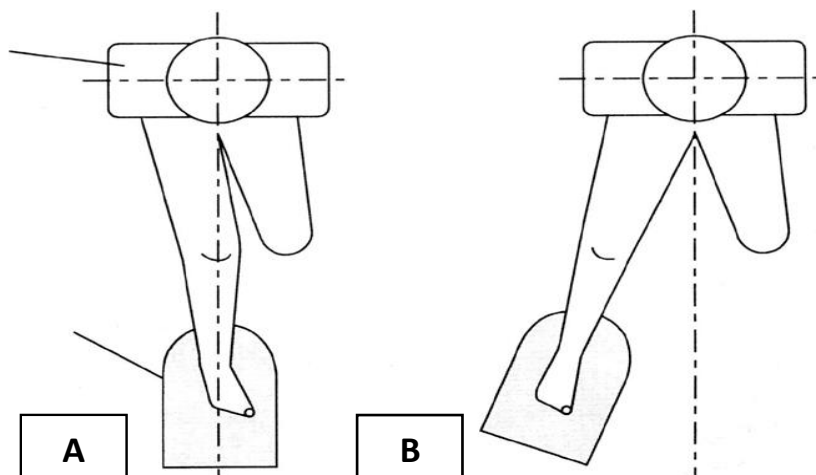


Figure G4: The participant's body and the OsteoSys should be in a straight line. A: Correct, B: Incorrect.

- 5) The year of birth, gender, ethnicity, foot support level, weight and height were recorded in the QUS machine, where after the scan was performed.
- 6) After the scan was completed, a detailed on-screen result appeared, indicating the T-score, Z-score, bone quality index (BQI), speed of sound (SOS) and broadband ultrasound attenuation (BUA). This information was printed out and attached to the data collection sheets.

An example of a detailed QUS report is shown in Figure G5, with A from a person with osteopenia and B from a person with a normal BMD.

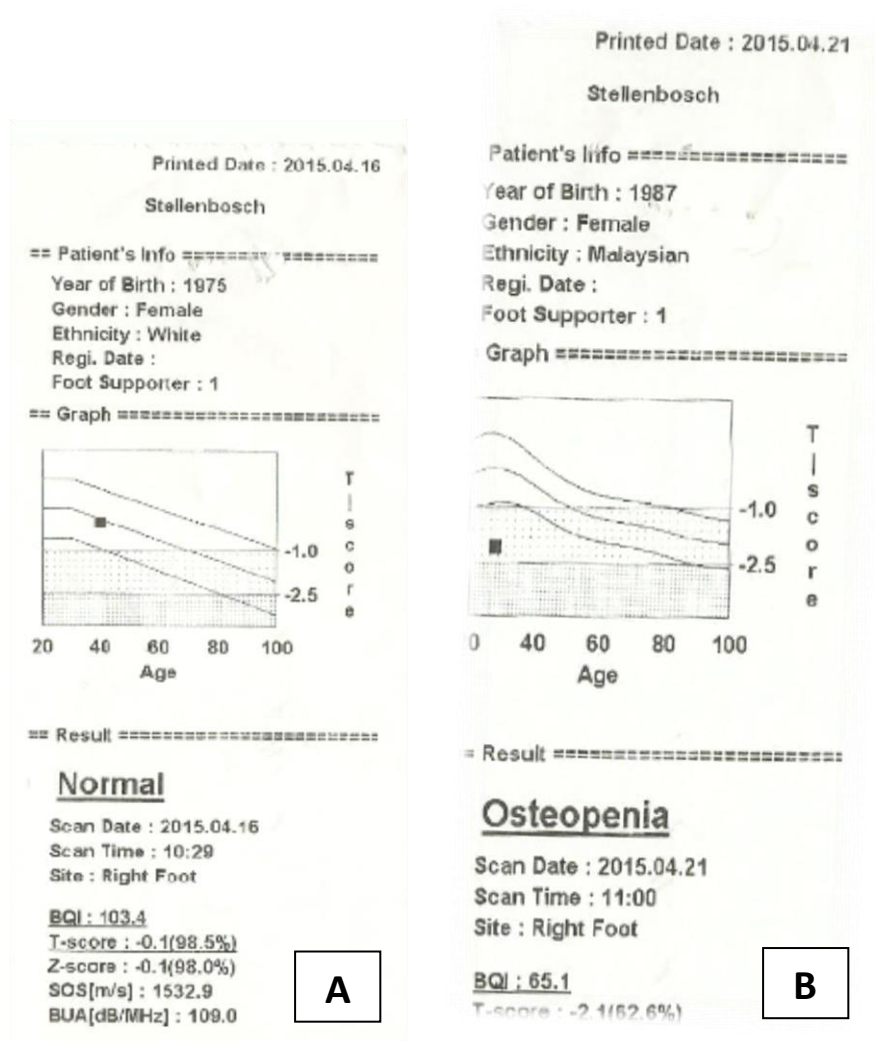


Figure G5: Example of an osteopenic (A) and normal (B) participant printout.



Id Code: \_\_\_\_\_

## APPENDIX G: Demographic questionnaire

### DEMOGRAPHIC QUESTIONNAIRE

Study title: *Cancer risk during urbanisation: metabolic syndrome and cancer*

Do you have a cell phone or land line? YES \_\_\_ NO: \_\_\_

If YES, can we call you on a specific nr? \_\_\_\_\_

Participant Information							
Date of interview:	Visit 1		Visit 2		Visit 3		
Household (Brick house, shack other dwelling)							
Total household income/week/month							
Total in household	MALE			FEMALE			
Children 0-6 yrs							
Children 7-12 yrs							
Children 13-18 yrs							
Adults 18-30							
Adults 31-45							
Adults 46-60							
Adults 61+							
LANGUAGE AND ACCULTURATION							
Home language of respondent	Afrikaans	Xhosa	Zulu	English	Sotho	Other	
Household head speaks	Home language only		Home language + Afr/Eng		English/Afrikaans only		
Can you read and understand a newspaper in your home language easily, difficult or not at all?	Easily		1				
	With difficulty		2				
	Not at all		3				
Can you read and understand a newspaper in the English language easily, difficult or not at all?	Easily		1				
	With difficulty		2				
	Not at all		3				
NEAREST CLINIC/HOSPITAL							
Name of clinic/hospital	Walk (minutes)		Mode of transport				
			Walk = 1 ; Taxi = 2 ; Own car = 3; Bicycle = 4				



<b>FAMILY CANCER HISTORY</b>				
<b>Immediate family cancer diagnosis?</b>	<b>Mother</b>	<b>Father</b>	<b>Brother</b>	<b>Sister</b>
<b>What type of cancer:</b>				
<b>When diagnosed</b>				
<b>Current status of cancer</b>	<b>Newly diagnosed</b>	<b>Living with cancer</b>	<b>Remission</b>	<b>Deceased</b>
<b>Treatment</b>	<b>Chemotherapy</b>	<b>Radiotherapy</b>	<b>Combination</b>	<b>No treatment</b>

Id Code: \_\_\_\_\_

## APPENDIX H: GPAQ

### GPAQ

Physical Activity			
Next I am going to ask you about the time you spend doing different types of physical activity in a typical week. Please answer these questions even if you do not consider yourself to be a physically active person.			
Think first about the time you spend doing work. Think of work as the things that you have to do such as paid or unpaid work, study/training, household chores, harvesting food/crops, fishing or hunting for food, seeking employment. <i>[Insert other examples if needed]</i> . In answering the following questions 'vigorous-intensity activities' are activities that require hard physical effort and cause large increases in breathing or heart rate, 'moderate-intensity activities' are activities that require moderate physical effort and cause small increases in breathing or heart rate.			
Questions		Response	Code
<b>Activity at work</b>			
1	Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like <i>[carrying or lifting heavy loads, digging or construction work]</i> for at least 10 minutes continuously?  <i>[INSERT EXAMPLES] (USE SHOWCARD)</i>	Yes 1  No 2 <i>If No, go to P 4</i>	P1
2	In a typical week, on how many days do you do vigorous-intensity activities as part of your work?	Number of days <input type="text"/>	P2
3	How much time do you spend doing vigorous-intensity activities at work on a typical day?	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P3 (a-b)
4	Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking <i>[or carrying light loads]</i> for at least 10 minutes continuously?  <i>[INSERT EXAMPLES] (USE SHOWCARD)</i>	Yes 1  No 2 <i>If No, go to P 7</i>	P4
5	In a typical week, on how many days do you do moderate-intensity activities as part of your work?	Number of days <input type="text"/>	P5
6	How much time do you spend doing moderate-intensity activities at work on a typical day?	Hours : minutes <input type="text"/> : <input type="text"/> hrs mins	P6 (a-b)
<b>Travel to and from places</b>			

The next questions exclude the physical activities at work that you have already mentioned.

Now I would like to ask you about the usual way you travel to and from places. For example to work, for shopping, to market, to place of worship. [insert other examples if needed]

7	Do you walk or use a bicycle ( <i>pedal cycle</i> ) for at least 10 minutes continuously to get to and from places?	Yes 1  No 2 <i>If No, go to P 10</i>	P7
8	In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?	Number of days <input type="text"/>	P8
9	How much time do you spend walking or bicycling for travel on a typical day?	Hours : <input type="text"/> : <input type="text"/> minutes                      hrs                      mins	P9 (a-b)

**Recreational activities**

The next questions exclude the work and transport activities that you have already mentioned.

Now I would like to ask you about sports, fitness and recreational activities (*leisure*), [insert relevant terms].

10	Do you do any vigorous-intensity sports, fitness or recreational ( <i>leisure</i> ) activities that cause large increases in breathing or heart rate like [ <i>running or football,</i> ] for at least 10 minutes continuously?  [INSERT EXAMPLES] (USE SHOWCARD)	Yes 1  No 2 <i>If No, go to P 13</i>	P10
11	In a typical week, on how many days do you do vigorous-intensity sports, fitness or recreational ( <i>leisure</i> ) activities?	Number of days <input type="text"/>	P11
12	How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?	Hours : minutes <input type="text"/> : <input type="text"/> hrs                      mins	P12 (a-b)
13	Do you do any moderate-intensity sports, fitness or recreational ( <i>leisure</i> ) activities that causes a small increase in breathing or heart rate such as brisk walking, ( <i>cycling, swimming, volleyball</i> ) for at least 10 minutes continuously?  [INSERT EXAMPLES] (USE SHOWCARD)	Yes 1  No 2 <i>If No, go to P16</i>	P13
14	In a typical week, on how many days do you do moderate-intensity sports, fitness or recreational ( <i>leisure</i> ) activities?	Number of days <input type="text"/>	P14

15	How much time do you spend doing moderate-intensity sports, fitness or recreational ( <i>leisure</i> ) activities on a typical day?	<p style="text-align: center;"> <input type="text"/> : <input type="text"/>  Hours : minutes                                   hrs                                   mins </p>	P15  (a-b)
<b>Sedentary behaviour</b>			
<p>The following question is about sitting or reclining at work, at home, getting to and from places, or with friends including time spent [sitting at a desk, sitting with friends, travelling in car, bus, train, reading, playing cards or watching television], but do not include time spent sleeping.</p> <p><i>[INSERT EXAMPLES] (USE SHOWCARD)</i></p>			
16	How much time do you usually spend sitting or reclining on a typical day?	<p style="text-align: center;"> <input type="text"/> : <input type="text"/>  Hours : minutes                                   hrs                                   min s </p>	P16  (a-b)

Id Code: \_\_\_\_\_

## APPENDIX I: Bone health questionnaire

### BONE HEALTH QUESTIONNAIRE

**Project title:** Cancer risk during urbanisation: metabolic syndrome and cancer

IDENTIFICATION CODE:			
Date of interview:			
Interviewer:			
Health District Site:	Pebbles (Villiera)	Neethlingshoff	Solms-Delta
OWN BONE HISTORY			
Fracture/broken bone?	Yes: _____		No _____
What bone & when?	Bone: _____	When: _____	
Stress fracture?	Yes _____		No _____
What bone & when?	Bone: _____	When: _____	
Treatment for stress fracture?	Immobilisation: _____	Activity: _____	
	Medications: _____	Bone stimulator: _____	
Pain during activity:	During activity _____	After activity _____	All the time _____
Medication associated with osteoporosis:	Anticoagulants (heparin)		<input type="checkbox"/>
	Anticonvulsants		<input type="checkbox"/>
	Cyclosporine A and tacrolimus		<input type="checkbox"/>
	Cancer chemotherapy drugs		<input type="checkbox"/>
	Glucocorticoids (and adrenocorticotrophic hormone [ACTH])		<input type="checkbox"/>
	Gonadotropin-releasing hormone agonists		<input type="checkbox"/>
	Lithium		<input type="checkbox"/>
	Methotrexate		<input type="checkbox"/>
	Parenteral nutrition		<input type="checkbox"/>
	Thyroxine		<input type="checkbox"/>

<b>MENSTRUAL HISTORY</b>			
<b>Age at first menstrual cycle (years):</b>			
<b>Menses start date:</b>			
<b>Menses end date:</b>			
<b>Regular monthly menstrual cycles:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>If no, how many days btw cycles:</b>			
<b>Number of cycles in last 12 months:</b>			
<b>Birth control or IUD:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 100px; text-align: center;">Birth control</td> <td style="width: 100px; text-align: center;">IUD</td> </tr> </table>	Birth control	IUD
Birth control	IUD		
<b>How long:</b>			
<b>Irregular cycles in past:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>If yes, describe:</b>			
<b>Ever been pregnant:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>Number of live births:</b>			
<b>Breastfeeding time (years):</b>			
<b>Menopausal status:</b>			

<b>DIET HISTORY</b>					
<b>Ever dieted:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Anything you avoid eating:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No		
Yes:	No				
<b>Ever taken diet pills or laxatives:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Ever made yourself vomit:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Binge eating:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Excessive exercise to lose weight:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Weight fluctuations over last 5 yrs:</b>	<table border="1" style="display: inline-table;"> <tr> <td style="width: 50px; text-align: center;">Yes</td> <td style="width: 50px; text-align: center;">No</td> </tr> </table>	Yes	No		
Yes	No				
<b>Highest weight in last 5 yrs:</b>					
<b>Lowest weight in last 5 yrs:</b>					
<b>Average days food intake:</b>	<table border="1" style="width: 100%;"> <tr> <td style="height: 20px;">Breakfast:</td> </tr> <tr> <td style="height: 20px;">Lunch:</td> </tr> <tr> <td style="height: 20px;">Dinner:</td> </tr> <tr> <td style="height: 20px;">Snacks:</td> </tr> </table>	Breakfast:	Lunch:	Dinner:	Snacks:
Breakfast:					
Lunch:					
Dinner:					
Snacks:					



<b>MEDICAL HISTORY</b>			
<b>Celiac disease:</b>	<table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding: 5px;">Yes</td> <td style="padding: 5px;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>Do you not tolerate or avoid certain foods:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>Stomach or digestion problems:</b>	<table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding: 5px;">Yes</td> <td style="padding: 5px;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>Thyroid problems:</b>	<table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding: 5px;">Yes</td> <td style="padding: 5px;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>Kidney stones:</b>	<table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding: 5px;">Yes</td> <td style="padding: 5px;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>Prescription medications:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>Over the counter medications or vitamins or supplements, including pain medications:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>Calcium and/or vitamin D pills or chews:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>Which kind and how often:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 60%;">Type:</td> <td style="width: 40%;">How often:</td> </tr> </table>	Type:	How often:
Type:	How often:		
<b>Osteopenia or osteoporosis:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>Bone density test (DEXA):</b>	<table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding: 5px;">Yes</td> <td style="padding: 5px;">No</td> </tr> </table>	Yes	No
Yes	No		
<b>If yes, when:</b>			
<b>Smoker or heavy drinker:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 80%;">Yes:</td> <td style="width: 20%;">No</td> </tr> </table>	Yes:	No
Yes:	No		
<b>If yes, how many and for how long:</b>	<table border="1" style="width: 100%;"> <tr> <td style="width: 60%;">How many:</td> <td style="width: 40%;">How long:</td> </tr> </table>	How many:	How long:
How many:	How long:		
<b>Amount of time exposed to sunlight:</b>			

<b>FAMILY HISTORY (maternal, paternal or siblings)</b>	
<b>Osteoporosis:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Hip fracture:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Bone disease:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Kyphosis (round back/hunchback):</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Kidney stones:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Parathyroid disease:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Eating disorder:</b>	Yes: <input type="text"/> No <input type="text"/>
<b>Ethnic background:</b>	<input type="text"/>
<b>TRAINING HISTORY</b>	
<b>Typical exercise routine including duration or distance of each activity per week:</b>	<input type="text"/>
<b>Rest days per week:</b>	<input type="text"/>
<b>How long have you had your current running shoes:</b>	<input type="text"/>
<b>Do you wear orthotics/shoe inserts:</b>	<input type="text"/>
<b>How long have you had these:</b>	<input type="text"/>
<b>Ever had gait analysis:</b>	Yes <input type="text"/> No <input type="text"/>
<b>Do you experience more fatigue than normal:</b>	Yes <input type="text"/> No <input type="text"/>

## APPENDIX J: Vitamin D concentrations in study population

Since only a small number of participants ( $n=29$ ) presented with detectable vitamin D concentrations, the vitamin D data could not be compared between the six respective groups. Therefore, vitamin D concentrations were only compared between the MetS and Non-MetS (Figure J1), and the PreM and PostM groups (Figure J2). There was no significant difference between the MetS (0.70 pg/mL) and Non-MetS (0.84 pg/mL) groups ( $p=0.57$ ).

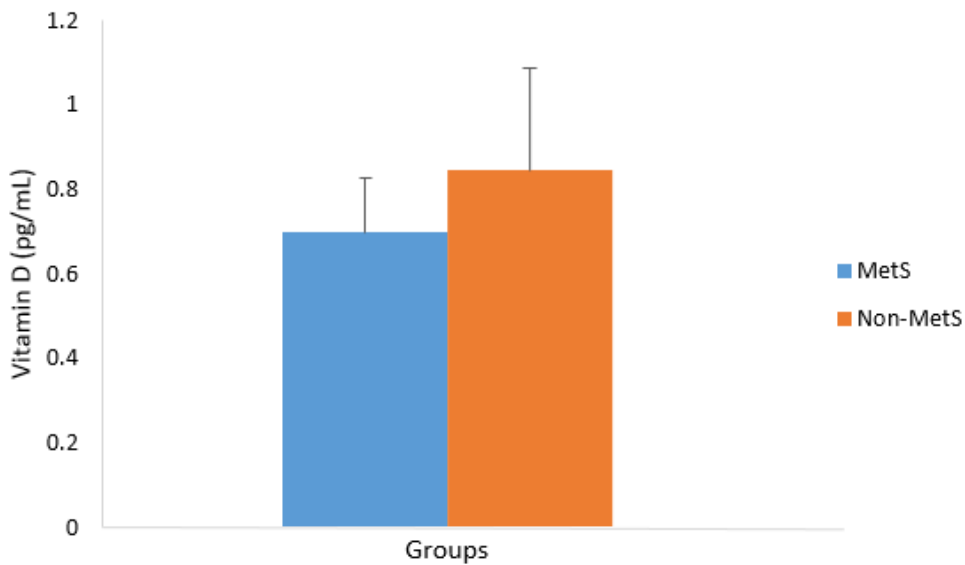


Figure J.1: Vitamin D concentrations in MetS vs. Non-MetS groups.

When comparing the vitamin D levels of PreM and PostM women, no significant difference was noted between the PreM (0.65 pg/mL) and PostM (0.92 pg/mL) women ( $p=0.28$ ).

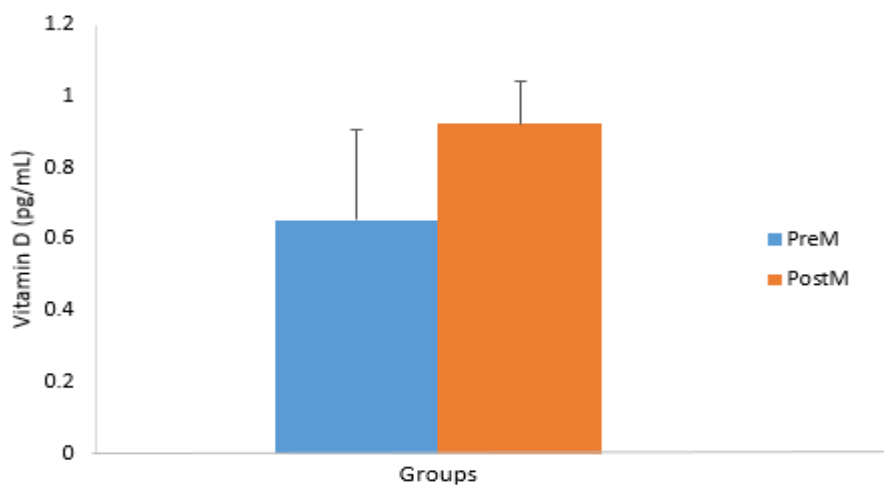


Figure J.2: Vitamin D concentrations in PreM vs. PostM groups.

## APPENDIX K: Additional information on menstrual history between the MetS and Non-MetS groups

Table K.1: Basic menstrual, pregnancy and breastfeeding history in the MetS and Non-MetS groups.

	Total (n=80)	MetS (n=44)	Non-MetS (n=36)
Age of first menstrual cycle (years)	14	14	14
History of irregular cycles	28.8% (n=23)	27.3% (n=12)	30.6% (n=11)
Regular monthly cycles	77.5% (n=62)	77.3% (n=34)	77.8% (n=28)
Previous pregnancies	86.3% (n=69)	93.2% (n=41)	77.8% (n=28)
Average time of breastfeeding (months)	40.5	48.7	34.7

Table K.2: The use of several types of medications in the MetS and Non-MetS groups.

	MetS (n=44)	Non-MetS (n=36)
Anti-hypertensive	34.1% (n=15)	13.9% (n=5)
Blood glucose medications	13.6% (n=6)	0.0% (n=0)
Cholesterol lowering agents	4.5% (n=2)	5.6% (n=2)
Thyroid medications	4.5% (n=2)	11.1% (n=4)
Anti-retroviral therapy	4.5% (n=2)	2.8% (n=1)
Hormone replacement therapy	2.3% (n=1)	0.0% (n=0)

## APPENDIX L: Additional analyses in the PreM and PostM women

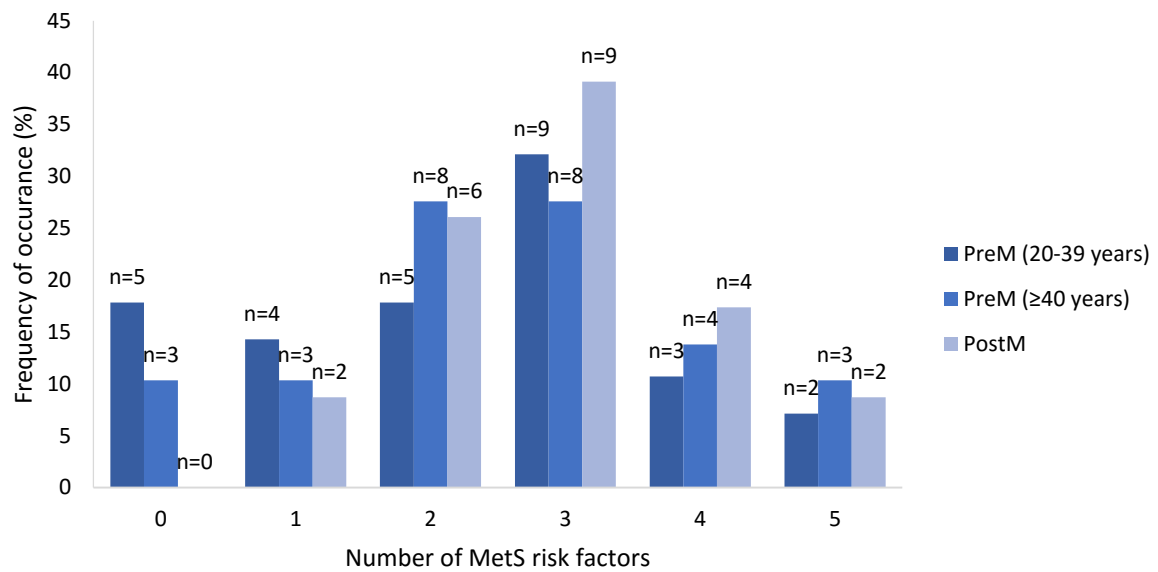


Figure L.1: Percentage of participants with zero, one and two risk factors in the PreM (20-39 years), PreM (≥40 years) and PostM groups.

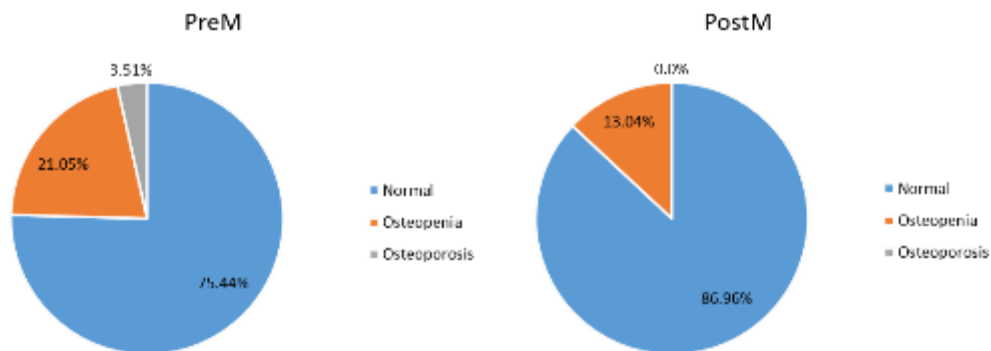


Figure L.2: Classification of participant's bone health in (A) the PreM group, (B) the PostM group.

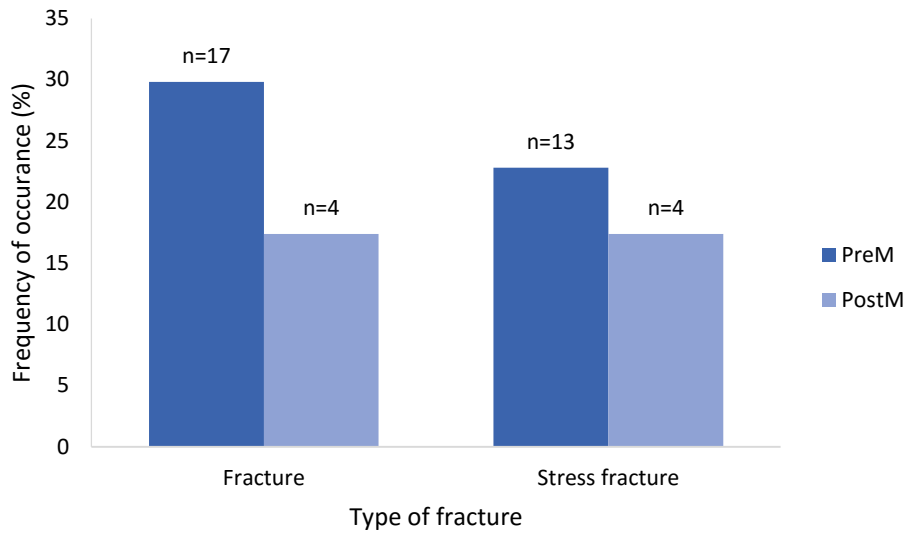


Figure L.3: Prevalence of fractures and stress fractures in the PreM vs PostM groups.

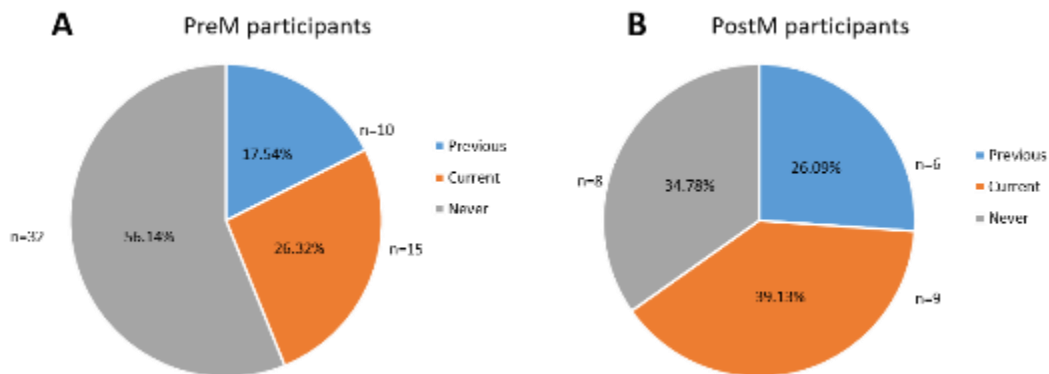


Figure L.4: Frequency of participants who were previous, current and non-smokers in (A) the PreM, (B) the PostM groups.

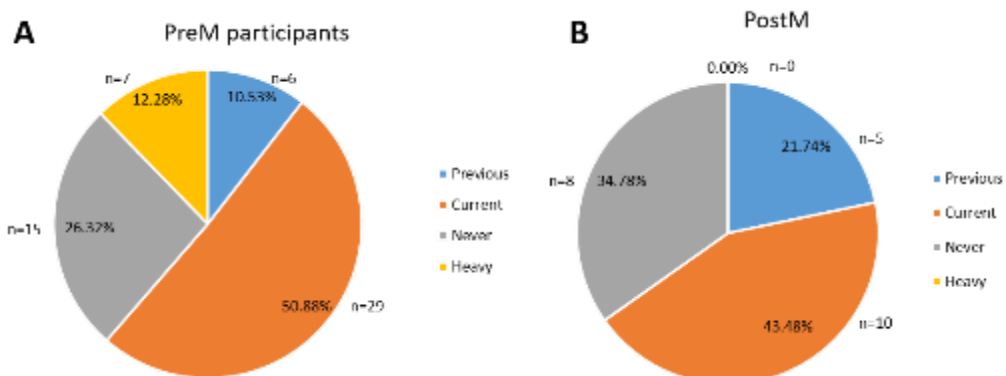


Figure L.5: Frequency of participants who were previous, current, non-consumers and heavy consumers of alcohol in (A) the PreM, (B) the PostM participants.

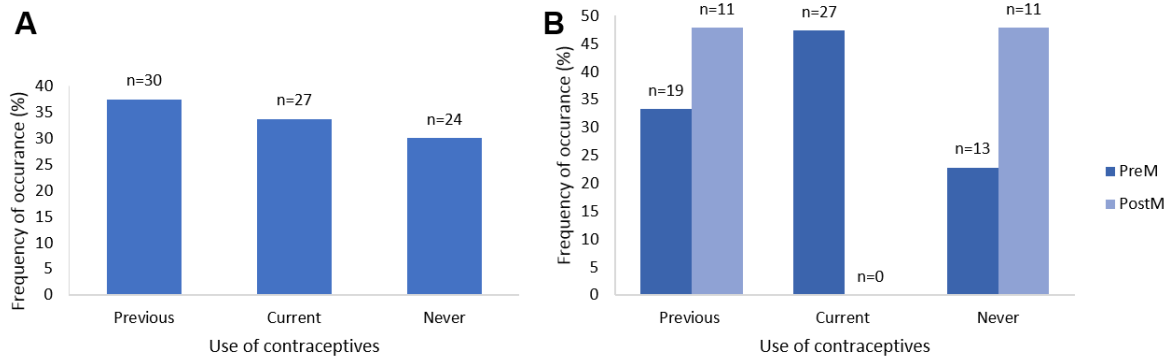


Figure L.6: The frequency of previous, current and non-contraceptive users in (A) the total sample population, and (B) the PreM vs PostM groups.

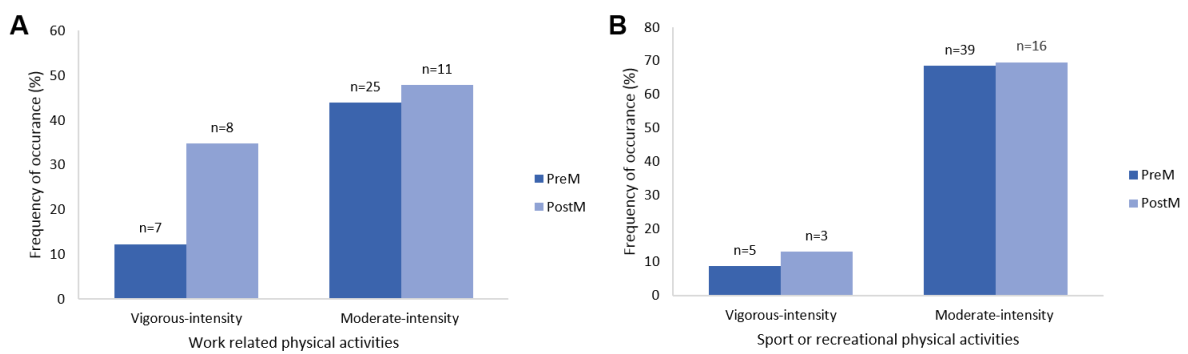


Figure L.7: The number of participants engaging in vigorous- and moderate-intensity activities at work in PreM and PostM groups (A), and in sport or recreational physical activities in PreM and PostM groups (B).

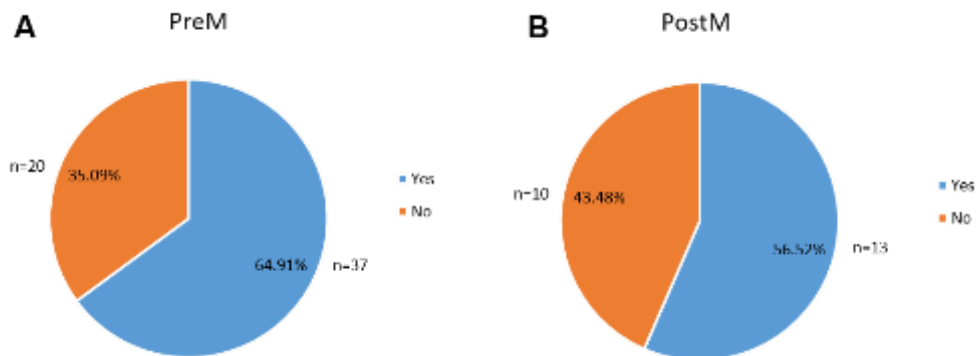


Figure L.8: The percentage of participants walking or riding a bicycle for more than ten minutes a day for travelling purposes in (A) the PreM group, and (B) the PostM group.



**Table L.1: The use of several types of medications in the PreM and PostM groups.**

	<b>PreM (n=57)</b>	<b>PostM (n=23)</b>
<b>Anti-hypertensive</b>	15.8% (n=9)	47.8% (n=11)
<b>Blood glucose medications</b>	5.3% (n=3)	13.0% (n=3)
<b>Cholesterol lowering agents</b>	3.5% (n=2)	8.7% (n=2)
<b>Thyroid medications</b>	8.8% (n=5)	4.3% (n=1)
<b>Anti-retroviral therapy</b>	3.5% (n=2)	4.3% (n=1)
<b>Hormone replacement therapy</b>	1.8% (n=1)	0.0% (n=0)

## APPENDIX M: Additional correlation analysis

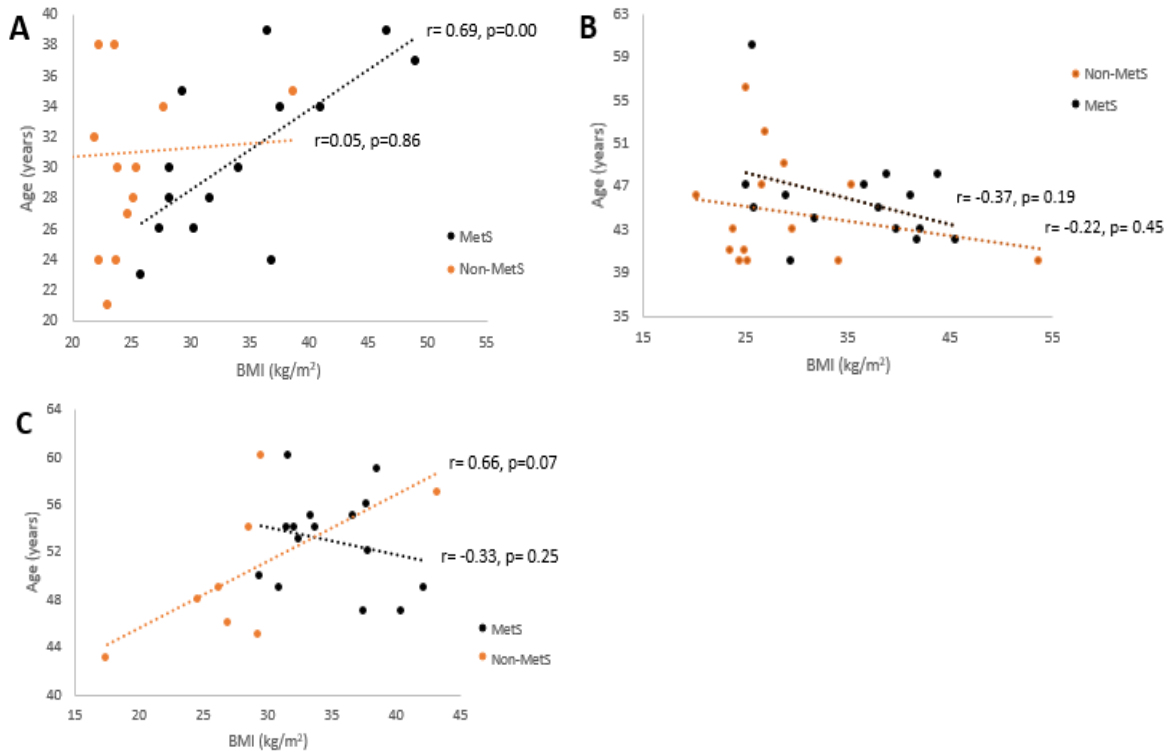


Figure M.1: Correlation between age and body mass index in (A) PreM (20-39 years), (B) PreM (≥40 years) and (C) PostM groups.

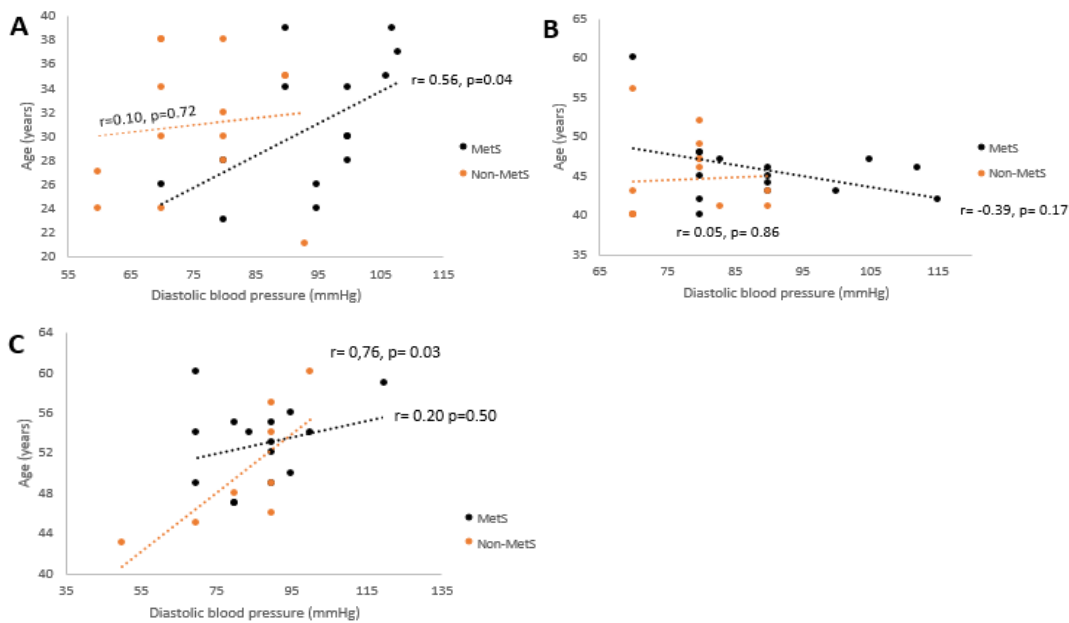


Figure M.2: Correlation between age and diastolic blood pressure in (A) the PreM (20-39 years), (B) the PreM (≥40 years), and (C) the PostM groups.

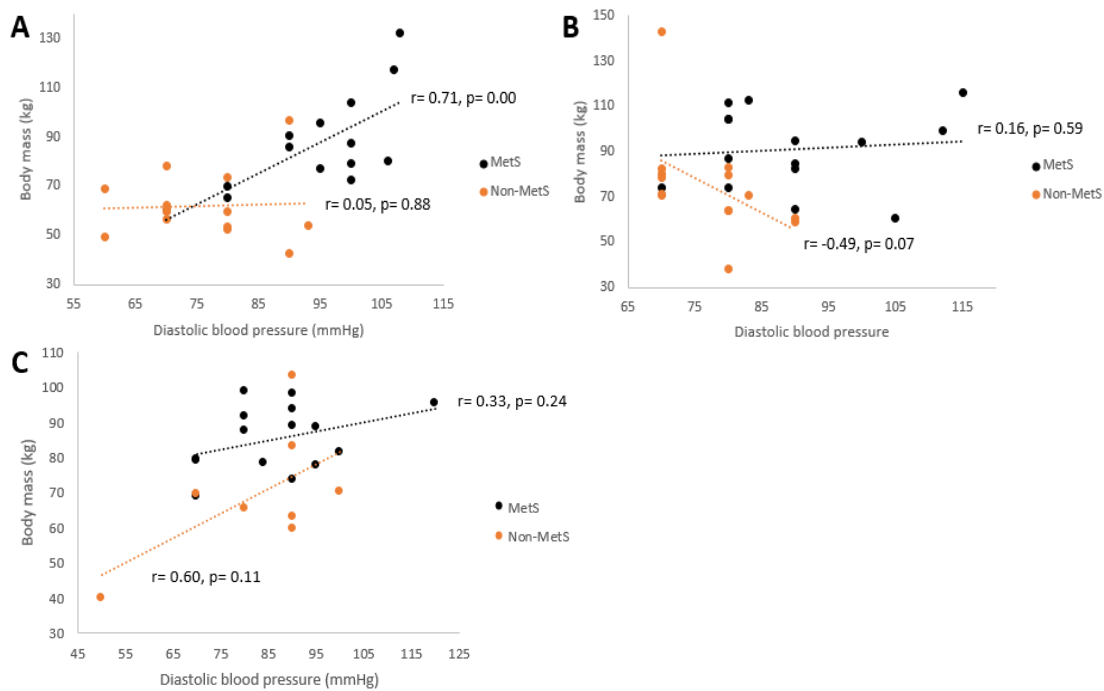


Figure M.3: Correlation between body mass and diastolic blood pressure in (A) the PreM (20-39 years), (B) the PreM (≥40 years), and (C) the PostM groups.

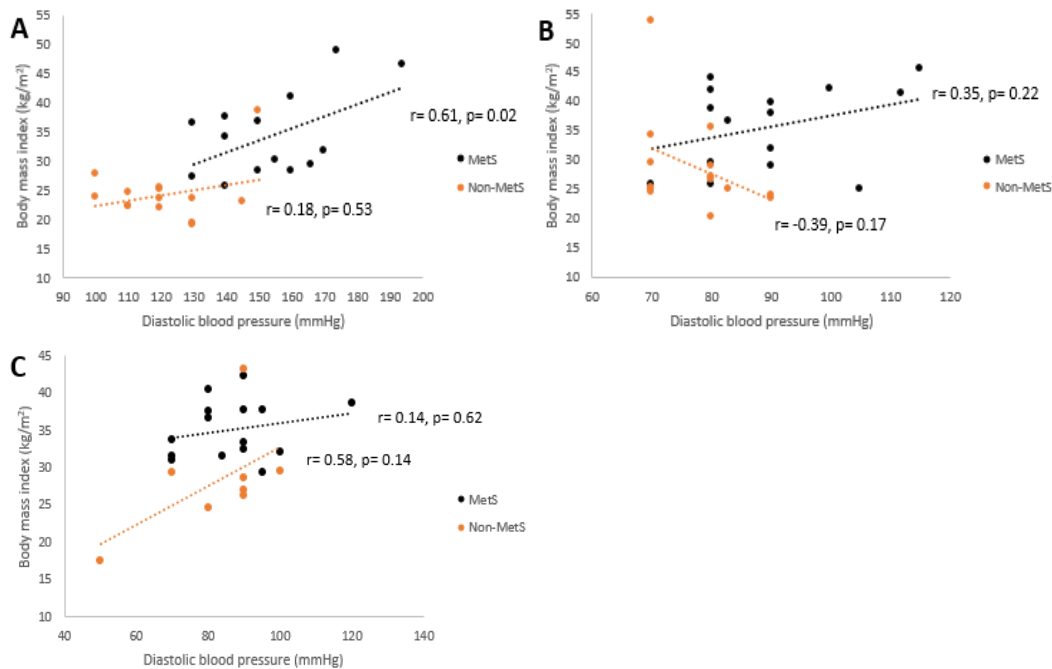
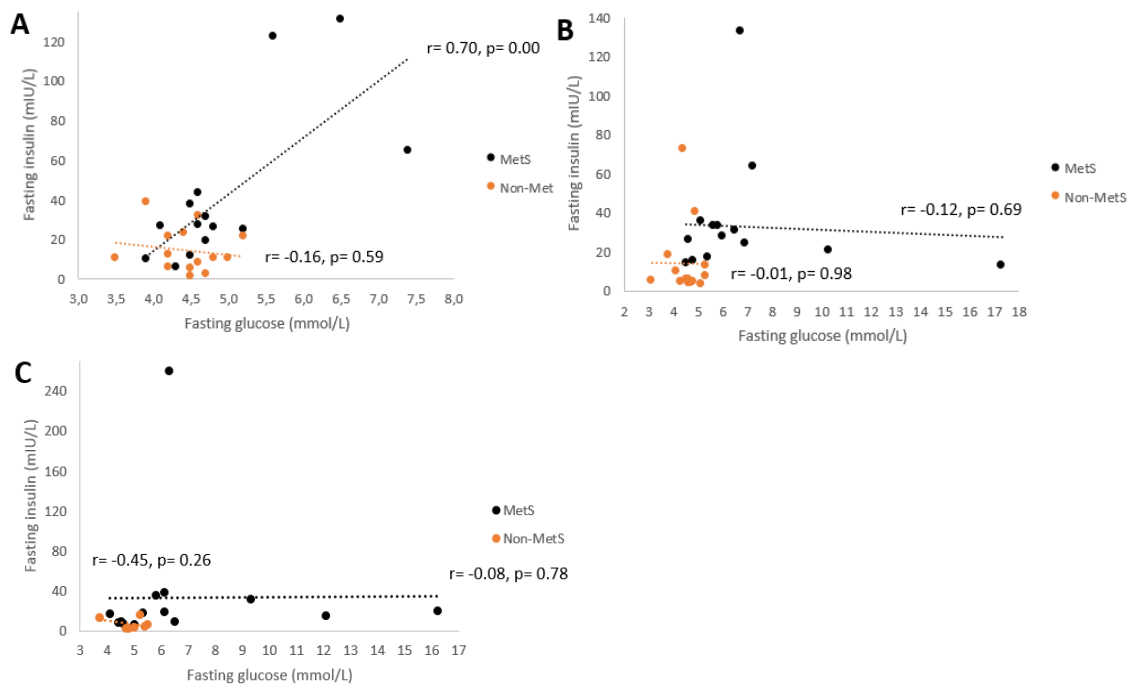


Figure M.4: Correlation between body mass index and diastolic blood pressure in (A) the PreM (20-39 years), (B) the PreM (≥40 years), and (C) the PostM group.



**Figure M.5: Correlation between fasting insulin and fasting glucose in (A) the PreM (20-39 years), (B) the PreM ( $\geq 40$  years), and (C) the PostM groups.**