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Epigenetic associations with sex hormone levels and cardiometabolic disease risk factors in pre- and post-menopausal women from West Africa

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Epigenetic associations with sex hormone levels and cardiometabolic disease risk factors in pre- and post-menopausal women from West Africa

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UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy (MDA00) in Human Genetics

Supervised by: Professor Michèle Ramsay, Professor Nigel John Crowther, and Doctor Nasrin Goolam Mahyoodeen

2025

DECLARATION

I, Merusha Naidoo, declare that this thesis is my own, unaided work.

It is being submitted for the degree of Doctor of Philosophy (PhD- MDA00) in Human Genetics at the University of the Witwatersrand, Johannesburg, South Africa.

The experimental work described in this thesis was carried out at the Sydney Brenner Institute for Molecular Bioscience (SBIMB), University of the Witwatersrand, Johannesburg, South Africa, and the National Health Laboratory Service (NHLS) – Braamfontein, Division of Human Genetics, University of the Witwatersrand, Johannesburg, South Africa.

These studies represent original work performed by the author, and it has not been submitted before for any degree or examination at any other university.

Where use has been made of the work of others, it is duly acknowledged in the text.

Candidate: Merusha Naidoo

Signed: _____ **Date:** 03/11/2025

DEDICATION

In loving memory of my grandfather, Sivalingam Pillay.

From Pre-Primary to PhD, you were always one of my greatest supporters.
Thank you for everything.

25 September 1937 – 4 December 2024

For my parents, Jayaseelan and Subashnie, whose consistent prayers, unconditional love and support, and unparalleled sacrifices are the wind beneath my wings.

All that I am, or that I hope to be, I owe to you.

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Prof. Ramsay, thank you for the encouragement, for guiding me professionally and personally, and for consistently leading by example.

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To my parents, Jay and Sue, for their unwavering love and endless sacrifices. Dad, your guidance and encouragement have been the foundation of my academic career. Mom, you have been a continual source of comfort and drive for me during the ups and downs of my PhD path.

I would also like to thank my brothers, Vishen and Shayden Naidoo, for their love, support, and commitment to ‘the three musketeers’. Your presence in my life has been a constant source of joy and strength.

To my sister in law, Tayla Amanda Frost-Naidoo, thank you for always being there, matching my sarcasm, and giving the best advice.

I am grateful to my friends Yakira Singh, Nerija Lamantha Ngundu, Caitlin Emma Kader, Callin Cole Chetty, and my cousin, Tessona Simbu, for their constant support, friendship, and endless laughter – especially when I most needed it.

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Many thanks to my extended family and friends for their encouragement throughout the journey – it really does take a village.

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To my late grandparents, Perumal and Rajma Naidoo, and Sivalingam and Dhanpathie Pillay, your love and blessings transcend time and space, and your words of wisdom will remain with me forever.

Finally, but above all, I offer my immense gratitude to Bhagawan Sri Sathya Sai Baba for His grace and guidance, and the Sri Sathya Sai Organisation for fostering an environment throughout my life that has shaped my values – we are, indeed, products of the spaces that nurture us.

ABSTRACT

Menopause is the permanent decline in ovarian function in women, signifying the discontinuation of reproductive potential. Changes in hormone levels due to the menopausal transition have been found to promote the progression to cardiometabolic diseases. Possible epigenetic mechanisms behind changes in hormone levels during the menopausal transition require further research, especially among underrepresented African populations. This cross-sectional cohort study aims to examine epigenetic mechanisms, specifically DNA methylation, to assess its role in differences in sex hormone levels and the prevalence of cardiometabolic disease in pre-and post-menopausal women from Nanoro, Burkina Faso, in West Africa. Genomic DNA derived from blood samples of 99 pre- and 98 post-menopausal participants from the 'Africa Wits-INDEPTH Partnership for Genomic Research' (AWI-Gen) was bisulfite modified in preparation for sequencing and methylation calling. Target-specific PCR products amplified with custom-designed methylation primers for androgen receptor (*AR*), follicle-stimulating hormone receptor (*FSHR*), inhibin B (*INHBB*), sex hormone binding globulin (*SHBG*) and oestrogen receptor β (*ESR2*) were purified, tagged with indexes, and pooled before next-generation sequencing. Methylation levels across five CpG islands and 86 CpG sites with high-depth sequencing coverage of ~50 000x were determined bioinformatically with the Illumina DRAGEN Methylation Pipeline through BaseSpace. Differences in demographic, behavioural, epigenetic, hormonal, cardiometabolic, anthropometric, and socioeconomic data between pre- and post-menopausal groups were assessed with the Student's T-test and Mann-Whitney U-test. Bivariate correlation analyses were conducted with Spearman's Rank Correlation analyses to inform multiple regression analyses of 19 dependent variables. Levels of diastolic blood pressure, estimated glomerular filtration rate (eGFR), LDL-c, visceral fat, oestrogen, FSH, and bioavailable testosterone differed between pre- and post-menopausal women. SHBG, oestrogen, bioavailable testosterone, DHEA-S, and FSH were significantly correlated with several cardiometabolic risk factors, anthropometric variables, and hormone levels. Gene methylation did not differ between menopause stages, and no association of gene methylation with CMD risk factors or hormone levels were noted suggesting that DNA methylation of sex hormone regulatory genes is unlikely to be a mechanism that modulates gene expression during the menopause transition in this cohort.

ABBREVIATIONS

AR – Androgen Receptor (Gene)

BGL – Blood Glucose Level

BIOT – Bioavailable Testosterone (Steroid Hormone)

CMD – Cardiometabolic Disease

CpG – Cytosine Pairing Guanine

CVD – Cardiovascular Disease

E2 – Oestrogen (Steroid Hormone)

ESR2 – Oestrogen Receptor β (Gene)

EWAS – Epigenome Wide Association Studies

FBG – Fasting Blood Glucose

FSH – Follicle Stimulating Hormone (Glycoprotein Hormone)

FSHR – Follicle Stimulating Hormone Receptor (Gene)

FT – Testosterone (Steroid Hormone)

HDL-c – High Density Lipoprotein Cholesterol

Education – Highest Level of Education

HOMA – Homeostatic Model Assessment

INHBB – Inhibin B (Gene)

INHBB – Inhibin B (Glycoprotein Hormone)

LDL-c – Low Density Lipoprotein Cholesterol

MetS – (Cardio) Metabolic Syndrome

MVPA – Moderate to Vigorous Physical Activity

NCD – Non-communicable Disease

RT-qPCR – Reverse Transcription-quantitative Polymerase Chain Reaction

SHBG – Sex Hormone Binding Globulin (Gene)

SHBG – Sex Hormone Binding Globulin (Glycoprotein)

T2DM – Type 2 Diabetes Mellitus

TChol – Total Cholesterol

TT – Testosterone (Steroid Hormone)

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CHAPTER ONE

1. Literature Review

1.1 Introduction

Menopause is the permanent ceasing of ovarian function in women, which in turn signifies the discontinuation of ovulation and reproductive potential (Sherman, 2005). Generally, the menopausal transition lasts up to 7 years, but in extreme cases may last up to 15 years (Gatenby and Simpson, 2023). Over the transition, women experience symptoms such as hot flushes, irregular menstrual cycles, sexual dysfunction, mood changes and even depression (Schmidt, 2005; Gatenby and Simpson, 2023). Symptoms of the natural decline in ovarian function begin when women are between 45 and 55 (Gatenby and Simpson, 2023). A woman is considered to be post-menopausal (having transitioned from reproductively active to inactive) following 12 consecutive months without menstruation (Sherman, 2005) - typically around the age of 51 years.

Although menopause is a natural and inevitable process that lasts only for a limited period, the effect of changes in hormone levels extends far beyond the duration of menopause (Sherman, 2005). Changes in hormone levels due to the menopausal transition have been found to promote progression of disorders related to ageing, such as cardiometabolic diseases (CMD) (cardiovascular disease (CVD), hypertension, stroke, dyslipidaemia, type 2 diabetes mellitus) (Figure 1.1) (Castro *et al*., 2003), osteoporosis and breast cancer (Gold *et al*., 2001). Early onset of menopause is linked to an increased risk of developing CVD and osteoporosis (Kok *et al*., 2005). Conversely, naturally occurring late onset of menopause is associated with a lower risk of CVD and osteoporosis (Gold *et al*., 2001). However, the late onset of menopause is also correlated with an increased risk for breast cancer in women with

pathogenic mutations in the BReast CAncer genes (*BRCA1* or *BRCA2*) (Gold *et al*., 2001), and endometrial and ovarian cancers (Ossewaarde *et al*., 2005).

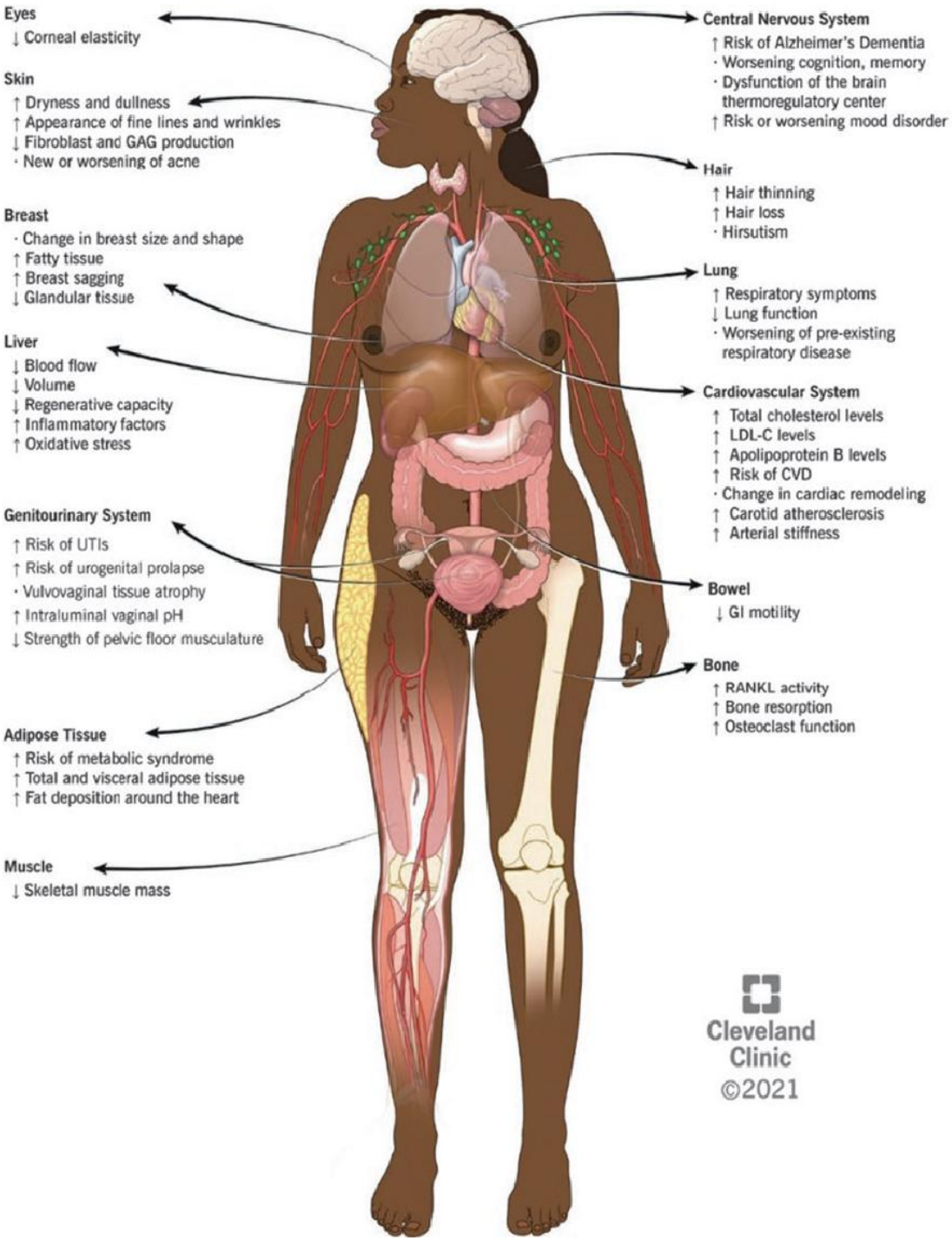


Figure 1.1 Symptoms, markers and comorbidities related to the menopausal transition (Iyer and Thacker, 2022, as depicted in Clinical Reproductive Medicine and Surgery).

Pre-menopausal women have a lower risk of developing CVD than men of the same age, however, following the transition through menopause, women become more susceptible to developing CVD (Garcia *et al*., 2016). In sub-Saharan Africa (Central, East, South and West Africa) the burden of non-communicable disease (NCD) is on the rise (Bigna and Noubiap, 2019). Disability-adjusted life years (DALYs) due to NCDs in sub-Saharan Africa was 90.6 million in 1990 with a 67% increase by 2017, and 151.3 million DALYs due to NCDs (Bigna and Noubiap, 2019). In 2017, the leading causes of disease burden due to NCDs were cardiovascular disease (22.9 million DALYs), neoplasms (16.9 million DALYs), mental disorders (13.6 million DALYs), and diabetes (10.4 million DALYs) (Bigna and Noubiap, 2019).

Women at the highest risk for developing CVD are those with metabolic syndrome, which is a cluster of factors including high blood pressure, high fasting blood glucose levels, large waist circumference, low high-density lipoprotein cholesterol (HDL-c) and high triglyceride levels (Alberti *et al*., 2009; Carr, 2003). Menopause increases the likelihood of developing the metabolic syndrome (Carr, 2003), and although the mechanism is not completely understood it is thought to be related to changes in sex hormone levels. The possibility that this occurs via epigenetic mechanisms will be investigated in this study in a cohort of menopausal women from Nanoro in Burkina Faso, West Africa.

1.2 Menopause staging and changes in sex hormone levels

The menopausal transition is essentially defined pathophysiologically as changes in hormone levels, primarily, diminished oestrogen (oestradiol – E2) levels and elevated follicle-stimulating hormone (FSH) levels (Jaff *et al*., 2014).

In women of a reproductive age, maturing ovarian follicles produce increasing levels of oestrogen (oestradiol – E2), which in turn sensitise the pituitary to gonadotropin-releasing hormone (GnRH) (Lemarchand-Béraud *et al*., 1982; Santoro *et al*., 1998; Baskind and Balen, 2016). As a result, approximately 36 hours prior to ovulation (Reed and Carr, 2018), the pituitary releases a surge of luteinising hormone (LH) and follicle

stimulating hormone (FSH) (Lemarchand-Béraud *et al*., 1982), which then triggers the rupture of a mature ovarian follicle and release of a dominant ovum (egg) (Holesh *et al*., 2019). This cyclic process is regulated by the hypothalamic-pituitary-ovarian axis (HPO) (Lemarchand-Béraud *et al*., 1982).

In post-menopausal women, the reproductive age functioning of the HPO is disrupted (Santoro *et al*., 1998). As the oestrogen-releasing ovarian reserve depletes, oestrogen (oestradiol) levels decrease (Deadmond *et al*., 2000), leading to minimal to no sensitisation of the pituitary to GnRH, and ultimately, no LH spike triggering ovulation (Santoro *et al*., 1998). In response to the decrease in oestrogen levels, the negative feedback mechanism between oestrogen and FSH causes a spike in FSH levels instead of a regulated decrease (Messinis, 2006).

Anti-Müllerian hormone (AMH), the hormone that regulates the maturation of primordial follicles to the primary follicle stage (La Marca *et al*., 2009), is fairly constant from mid-childhood to early adulthood (25 years), after which AMH starts to decline to negligible levels in post-menopausal women (Kelsey *et al*., 2011).

In women of reproductive age, inhibin B (INHBB) regulates/suppresses FSH levels via a negative feedback mechanism (Welt, 2004) and ensures that only follicle reaches maturity and is released during ovulation (Sehested *et al*., 2000). In post-menopausal women, inhibin B levels decrease, as FSH levels peak (Welt, 2004).

During the menopausal transition, testosterone levels drop, resulting in a 50% reduction of testosterone levels by the age of final menstrual period (Burger, 2002). In women, testosterone is responsible for sexual function, thus impacts fertility through facilitation (Johansen *et al*., 2020). Post-menopause, levels generally remain constant up until 65 years of age, when levels may increase and mirror those of adolescent girls (Ala-Fossi *et al*., 1998; Burger, 2002).

Both testosterone and oestrogen are regulated by sex hormone-binding globulin (SHBG), a protein that regulates levels of free oestrogens and androgens in the blood by binding to them, making them unavailable (Pasquali *et al*., 1997). In this way, SHBG impacts menstrual cycles, fertility, and as decreased SHBG levels is associated with elevated androgen levels, it is used to assess androgen status in a woman (Pasquali *et al*., 1997). It would follow that in obese women and post-menopausal women, SHBG levels have been found to decline (Tchernof *et al*., 1999).

A graphical representation of the stages, timing and hormonal changes involved in the menopausal transition was compiled by Harlow *et al*., 2012. The Staging of Reproductive Aging Workshop (STRAW) (Harlow *et al*., 2012) guided staging of the menopausal transition from pre-menopause, through peri-menopause, to post-menopause is depicted in Figure 1.2.

In pre-menopausal women, oestrogen levels are at their optimum, regulating body fat distribution by minimising abdominal visceral fat, and contributing to subcutaneous fat accumulation in the gluteofemoral and mammary gland region (Brown and Clegg, 2010). As women transition through menopause, oestrogen levels decline (Burger, 2002). With that decline comes an increase in abdominal adiposity, and as a result, an increase in cardiometabolic risk (Burger, 2002).

Studies conducted outside of Africa show that cardiometabolic risk factors are increased in post-menopausal women as compared to pre-menopausal women and that hormonal changes across the menopausal transition may play a role in modulating risk (Carr, 2003). As menopause affects every woman, and cardiometabolic disease and obesity are on the rise globally, it is essential to further investigate the correlation between the menopausal transition and the increase in CMD risk factors.

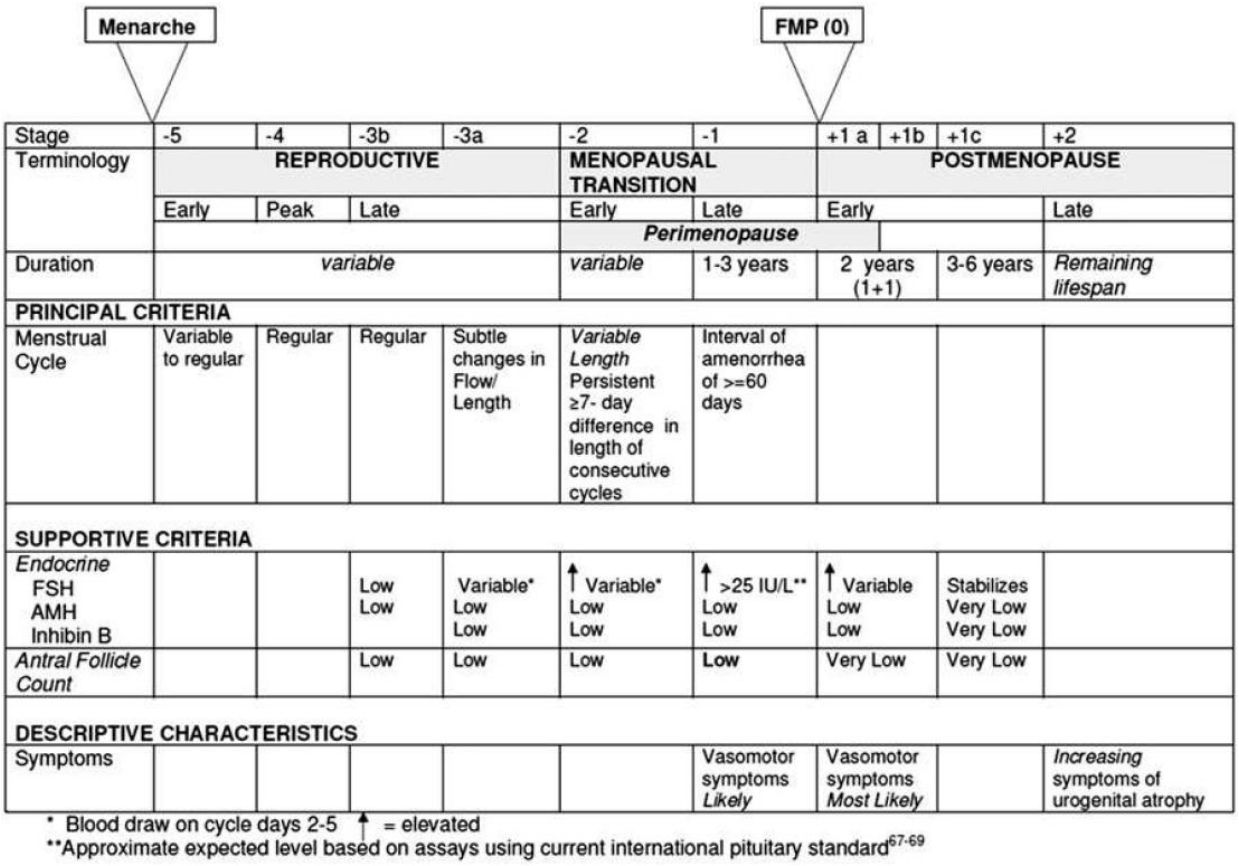


Figure 1.2 Visual depiction of menopause staging over a lifespan (Harlow *et al*., 2012).

1.3 Cardiometabolic and cardiovascular disease risk during the menopause

The risk of developing metabolic syndrome appears to be influenced by a combination of factors, including age, depression, gender, and hormonal changes during the menopausal transition. During the menopause, the decline in oestrogen leads to a change in body fat distribution as a result of an increase in pro-inflammatory visceral adipose tissue, which is inclusive of intraperitoneal/omental fat, intrahepatic fat, epicardial fat, and perivascular fat (González *et al*., 2017). This is associated with an increased risk of metabolic syndrome and CVD risk (Després, 2007; Shah *et al*., 2014). As oestrogen is heart-protective, declining levels of the hormone during the menopausal transition contribute to an increased risk for cardiovascular disease (Rosano and Panina, 1999). Menopause has been associated with elevated low density lipoprotein (LDL) cholesterol and triglyerides, along with decreased levels of high density lipoprotein (HDL) cholesterol (Matthews *et al*., 2001). This build up of high cholesterol levels then contributes to progression of arterial atherosclerotic plaque

formation (Steinberg, 1987), which in turn has the potential to develop into cardiac events and/or cardiovascular disease (CVD) (Jebari-Benslaiman *et al*., 2022).

In post-menopausal women, the decline of oestrogen levels results in vasorestriction of blood vessels as oestrogen plays an anti-inflammatory and vasodilatory role in pre-menopausal women (White, 2002). The inflexibility of the post-menopausal blood vessels puts post-menopausal women at a greater risk for developing hypertension (high blood pressure) (Maas and Franke, 2009).

High blood pressure levels facilitate the development of atherosclerotic lesions by aggravating and damaging the endothelium, furthering the progression of atherosclerosis and exacerbating the risk of a cardiac event occurring (Poznyak et al., 2022). This process leads to systemic inflammation and calcification (hardening) of the arterial endothelial layer (Poznyak *et al*., 2022).

Although there is a large body of evidence indicating increased risk for cardiometabolic and cardiovascular disease in post-menopausal women (Rosano and Panina, 1999; Matthews *et al*., 2001; White, 2002; Maas and Franke, 2009; Poznyak *et al*., 2022; Jebari-Benslaiman *et al*., 2022), there is also evidence suggesting that this risk may not be the same in all menopausal women and that women who were low birth weight babies are at an increased risk for CMD and CVD complications in their menopausal years (Alexander *et al*., 2014).

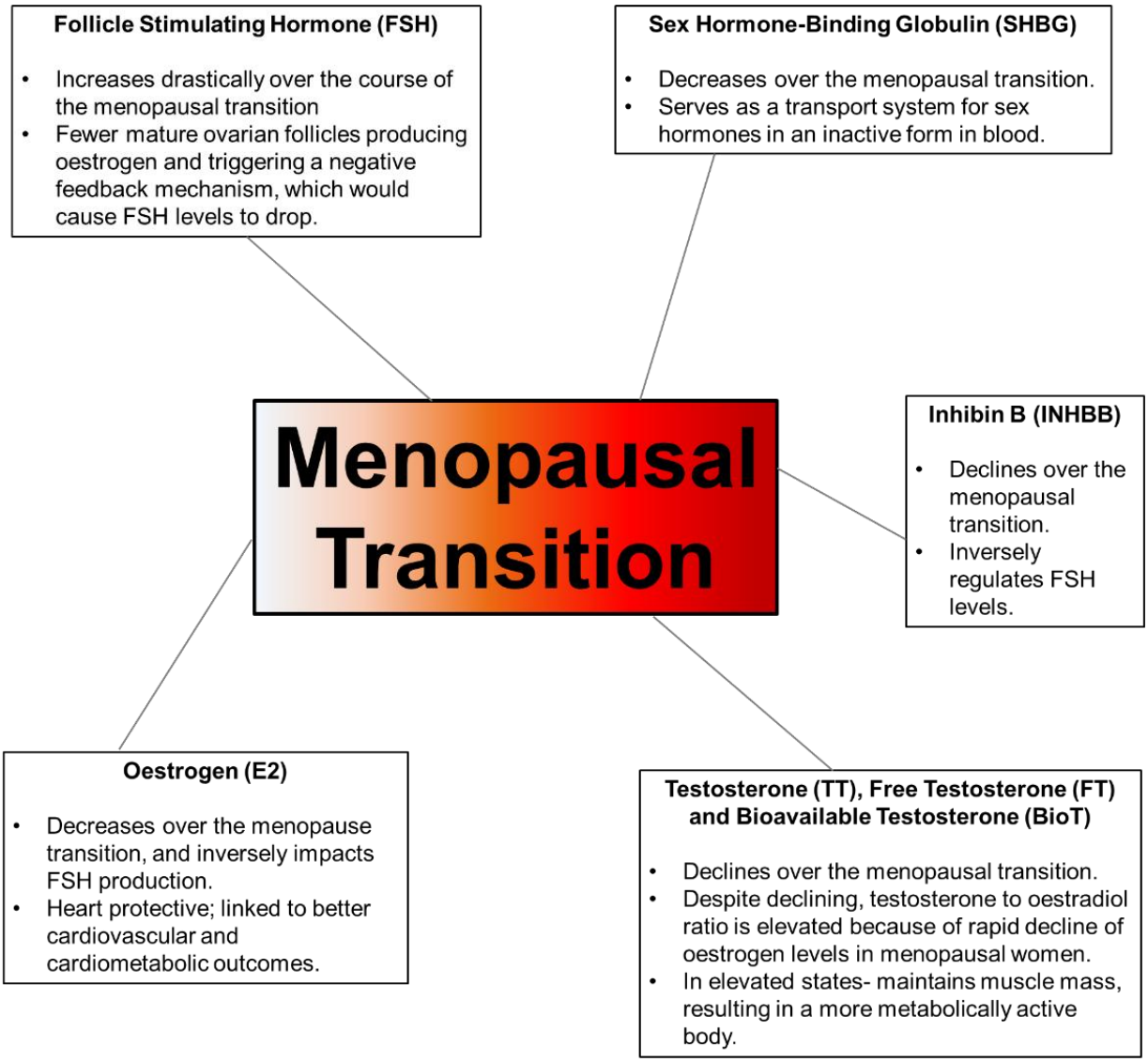


Figure 1.3 Summary of the role and relationship between the menopausal transition and follicle stimulating hormone, oestrogen, inhibin B, sex hormone-binding globulin, and testosterone.

Low birth weight, categorised as a birth weight below 2500g, has been increasingly recognised as a significant predictor of cardiometabolic risk in later life, particularly among menopausal women (WHO, 2024). The Developmental Origins of Health and Disease (DOHaD) hypothesis (Lacagnina, 2020) suggests that intrauterine growth restriction and subsequent low birth weight can impact the epigenetic programming of metabolic pathways, and in turn, lead to increased susceptibility to cardiovascular disease and metabolic disorders in adulthood. This programming effect appears to be prevalent in populations experiencing significant socioeconomic disparities, such

as Burkina Faso in West Africa, where maternal malnutrition and overall poverty contribute to high incidence of low birth weight (Ozodiegwu *et al*., 2019).

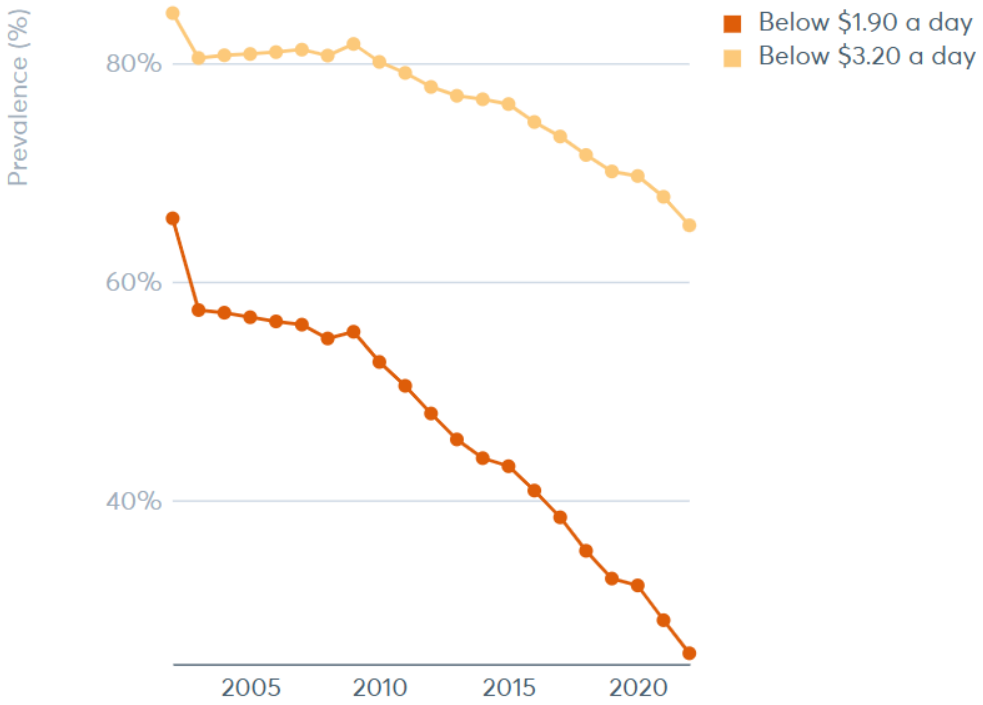


Figure 1.4 Proportion of the population of Burkina Faso living under the poverty line (Global Nutrition Report, 2022).

The prevalence of obesity (4.84% (95% CI: 3.99 to 5.86)) and overweight (13.82% (95% CI: 12.25 to 15.55)) is much lower in Burkina Faso as compared to that of other African countries (Kaboré, S. *et al*., 2020). In the latest surveys conducted since 2010 in these countries, overweight exceeded 20% in all countries except Burkina Faso and Malawi where the prevalence was around 16%. The increase over time was also not statistically significant in these two countries. In four countries, the prevalence of overweight exceeded 30% (Egypt (44%), Niger (32%), Rwanda (31%) and Ghana (30%)) (Amugsi, D.A. *et al*., 2017). According to the Global Nutrition Report on Burkina Faso, 19.6% of children under five are affected by childhood stunting, 7.7% of children

under five are affected by childhood wasting, and 52.5% of women aged 15-49 are affected by anaemia (Global Nutrition Report, 2022).

Studies conducted on post-menopausal women indicate a strong correlation between birth weight and cardiometabolic outcomes. Many have found that individuals with documented low birth weight demonstrated significantly higher incidences of hypertension and insulin resistance compared to their normal birth weight counterparts (Alexander *et al*., 2014). The relationship appears to be mediated by changes in body mass index (BMI) and menopause-linked hormonal changes, with low-birth-weight women showing a greater tendency toward insulin desensitisation related central adiposity during the menopausal transition as compared to their normal birth weight counterparts (Jornayvaz *et al*., 2016).

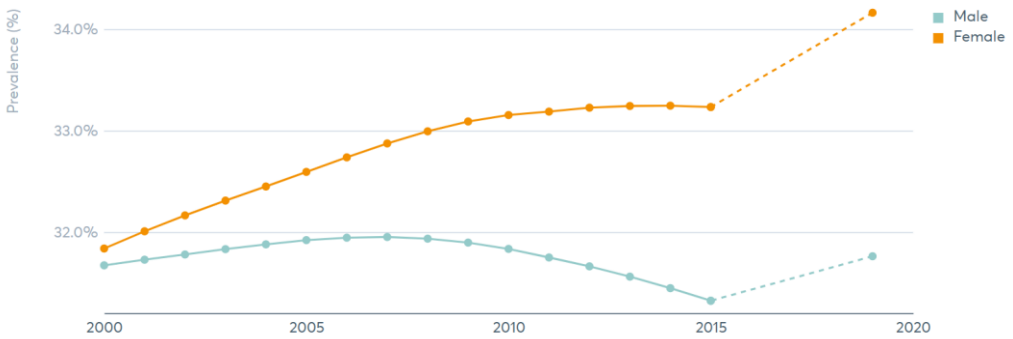


Figure 1.5 Prevalence of high blood pressure in Burkina Faso (Global Nutrition Report, 2022).

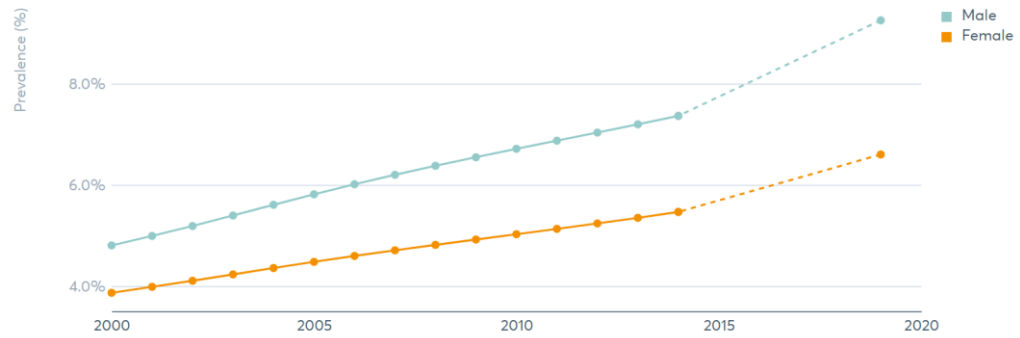


Figure 1.6 Prevalence of diabetes in Burkina Faso (Global Nutrition Report, 2022).

The impact of low birth weight on cardiometabolic risk is particularly concerning in the West African context, where poverty and limited access to healthcare contribute to early life disadvantage. Research has shown that low-birth-weight females exhibit significantly elevated levels of inflammatory markers and poor glucose metabolism by middle age, with these effects becoming more pronounced after menopause (Jornayvaz *et al*., 2016). The hormonal implications of low birth weight extend well into the menopausal transition, with evidence suggesting altered hypothalamic-pituitary-ovarian axis function (Goldsammler *et al*., 2018). These findings highlight the importance of early life interventions and suggest that targeted screening and prevention strategies may be necessary for women with a history of low birth weight, especially in resource-limited settings.

Low birth weight was also found to be associated with insulin sensitivity markers in adulthood even before clear indicators of poor glucose metabolism were detected (Ines *et al*., 2022). Another study concluded that working conditions, household income, and employment status of spouse had the greatest effects on low birth weight babies (Mahmoodi *et al*. 2013). These findings suggest that the combination of menopause-associated hormonal changes, low birth weight and socioeconomic disadvantage result in poor cardiometabolic health outcomes. A possible mechanism involved in changes in menopause-associated sex hormones and cardiometabolic risk factors may involve epigenetic processes, but this is under-studied.

1.4 Epigenetics and disease processes

Cardiometabolic and cardiovascular diseases are increasingly becoming a global burden, and cause for concern (Antoun *et al*., 2022). It is hypothesised that environment and lifestyle-linked epigenetic modulatory effects on the genome may play a role in cardiometabolic and cardiovascular disease progression (Antoun *et al*., 2022). It would follow that the associated elevated cardiometabolic and cardiovascular disease risk in menopausal women may also be linked to epigenetic regulatory effects.

Sex hormone regulatory genes which code for proteins that mediate the action or control the levels of sex hormones, have been associated with the menopausal transition and its related health outcomes (Ahmed *et al*., 2022). Research is required to fully elucidate the complex interplay between genetics, hormones, and cardiometabolic risk during the menopausal transition as the mechanisms behind the changes in hormone levels are not fully established. It is plausible that epigenetic modifications of genes involved in the regulation of hormone production modulate hormone levels.

The most researched and most common epigenetic modification is DNA methylation, which is known to be linked to the regulation of gene expression (Wagner *et al*., 2014). DNA methylation involves the DNA methyltransferase-aided (DNMT) transfer of a methyl group to position C-5 of the nucleotide, cytosine (Figure 1.7) (Robertson, 2005). The sites primed for methylation are known as CpG sites - a cytosine nucleotide connected to a guanine nucleotide by a phosphodiester bond. Several closely located CpG sites form a CpG island. The methylation of cytosine at CpG sites/islands near transcription start sites (TSS) within the promoter region of genes has been widely investigated. Methylation upstream of the promoter is often linked to the regulation of the expression of the gene (Wagner *et al*., 2014). However, the variation in DNA methylation levels across individuals, its genetic or environmental causes and its correlation with gene expression are not fully understood (Wagner *et al*., 2014). In terms of the sex hormone-associated genes described above, there is evidence that gene expression is epigenetically controlled by DNA methylation.

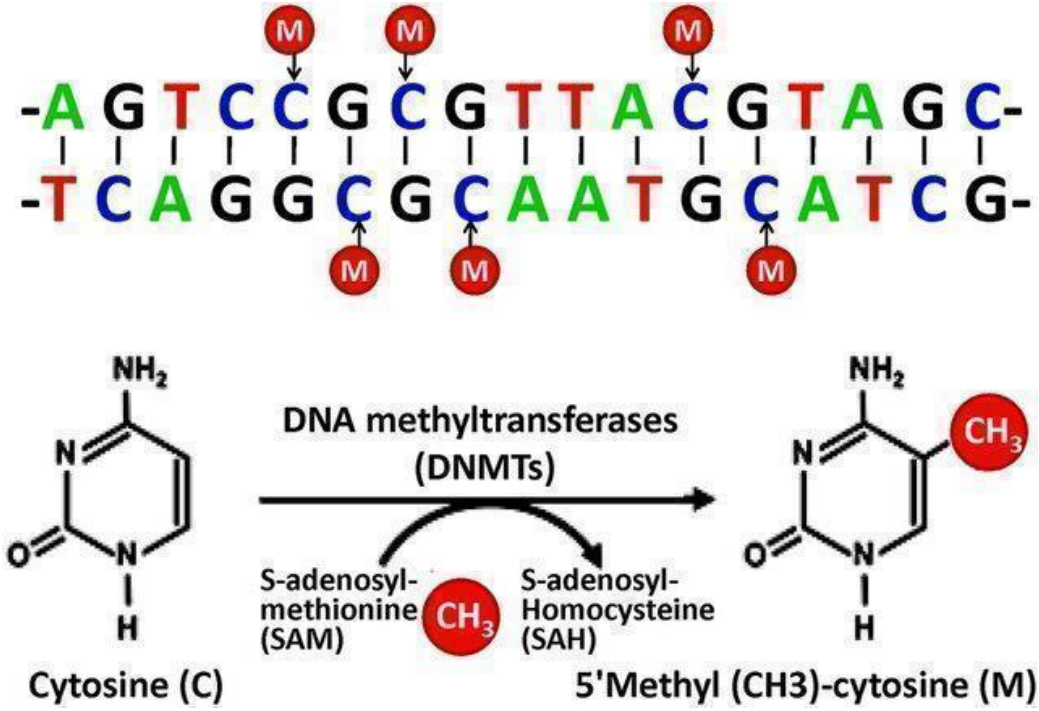


Figure 1.7 Depiction of the mechanism behind DNA methylation (Wu and Yin, 2022).

However, little is known about the possible epigenetic control of hormone levels during the menopausal transition (Table 1.1) and how this may relate to changes in cardiometabolic disease risk. It is important to undertake such studies to more clearly define the molecular processes involved in the transition. As findings may vary across different ethnicities, it is essential to generate data from Africans to ensure that there is representation of understudied populations.

Table 1.1 Summary of relevant literature related to genes selected for this study.

Gene Name:	Gene Abbreviation:	Action of the Protein:	Pre- vs Post-Menopausal Methylation Status in Existing Literature:	Population-specific Methylation Status in Existing Literature:
Oestradiol Receptor β	*ESR2*	Binds to oestradiol which regulates gene expresssion	No existing literature	Downregulation of methylation in precancerous stages of breast cancer of Chinese Han women (Gao *et al*., 2016)
Androgen Receptor	*AR*	Binds to androgen hormones which causes conformational changes and regulation of gene expression	No existing literature	Hypomethylation of *AR* in bone metastases as compared to primary prostate cancer in men (Ylitalo *et al*., 2021) Hypermethylation is correlated with impaired fasting glucose and elevated testosterone levels in women (Liu *et al*., 2020)
Follicle Stimulating Hormone Receptor	*FSHR*	Binds follicle stimulating hormone and activates intracellular signalling pathways	No existing literature	Hypermethylation of *FSHR* in postmenopausal women associated with hot flushes (Kalkan *et al*., 2020 - very small study sample size; not very well powered)
Inhibin B	*INHBB*	Suppresses pituitary follicle stimulating hormone through negative feedback mechanism	No existing literature	No existing literature
Sex Hormone Binding Globulin	*SHBG*	Binds to sex hormones in the blood to regulate availability for active use by tissues	No existing literature	No existing literature

Thus, this study aims to focus on the genes involved in the regulation of sex hormone levels and functions, including key genes encoding enzymes that catalyse steps in the sex hormone metabolic pathway and receptors that mediate hormone action. The genes being investigated are oestradiol receptor β (*ESR2*), androgen receptor (AR), follicle-stimulating hormone receptor (*FSHR*), inhibin B (*INHBB*) and sex hormone binding globulin (*SHBG*). ESR1 and ESR2 both bind oestrogen but have different affinities for the different compounds/forms of oestrogen (Pettersson and Gustafsson, 2001). Both receptors control key physiological functions, but in different tissues (Paterni *et al*., 2011). ESR1 controls physiological functions in the mammary gland, uterus, ovary (thecal cells), bone tissue, liver and adipose tissue, whilst ESR2 controls functions in the bladder, ovary (granulosa cells), colon, adipose tissue and the immune system. Both receptors are expressed at elevated levels in the tissues of the cardiovascular and central nervous systems (Paterni *et al*., 2011).

There are four forms of circulating oestrogens: the most abundant form, oestradiol (17β-oestradiol/oestrogen/E2); oestrone (E1); oestriol (E3); and oestretrol (E4) which is produced only during pregnancy (Iorga et al., 2017). E2 binds to ESR1 and ESR2, as well as the recently discovered GPER1 (Bopassa *et al*., 2010). Another receptor being studied is the androgen (testosterone) receptor (AR) that binds to testosterone, which along with dehydroepiandrosterone sulfate (DHEA-S), is decreased in post-menopausal women (Yasui *et al*., 2012). Testosterone is also a precursor substrate for aromatase enzyme (oestrogen synthetase) mediated oestrogen production. In some women who undergo menopause later in life, aromatase inhibitors are used to decrease the production of oestrogen to decrease the risk of developing breast cancer that is associated with the late-life onset of menopause (Warsy *et al*., 2017). Aside from aromatase, hydroxysteroid 17β dehydrogenase 1 (HSD17B1) is another enzyme involved in catalysing the last step in the biosynthesis of oestrogen, however instead of testosterone, oestrone is the precursor molecule used (Ronde and Jong, 2011). HSD17B1 promotes the onset of breast cancer by means of dihydrotestosterone (DHT) inactivation in conjunction with oestradiol/oestrogen/E2 production (Aka *et al*., 2010; Hilborn *et al*., 2017).

Another gene being studied encodes the sex hormone-binding globulin (SHBG) which binds to major oestrogens and androgens for transportation in the blood. Changes in SHBG protein levels can affect the amount of hormone freely available in the blood to be used by different tissues in the body. In women, 66 to 78% of testosterone is bound to SHBG, which has a higher affinity for androgens (testosterone and DHT) as opposed to oestrogens (Hammond, 2011; Hammond, 2016).

The remaining two genes encode follicle-stimulating hormone receptor (*FSHR*) and inhibin B (*INHBB*) proteins. Inhibin B inhibits FSH secretion. The normal range of FSH in pre-menopausal women is between 3 and 12mIU/ml (Odiba *et al*., 2014), whilst in post-menopausal women, FSH is consistently elevated to 30mIU/ml or higher as inhibin B levels decrease during menopause. As a result, there is insufficient inhibin B to suppress FSH levels (Klein *et al*., 1996).

1.4.1 DNA methylation of sex hormone regulatory genes

There have been very few studies conducted on epigenetic regulation of sex hormone regulatory genes, but none in sub-Saharan populations, and not many that aim to determine the direct association between hormone level and gene methylation. One of the studies conducted by Kirn *et al*. sought to determine the methylation status of oestrogen receptor α (*ESR1*) in primary breast cancer and its corresponding metastases. It was found that out of the 25 tissue specimens collected, 22 primary breast cancer samples showed positive expression of oestradiol receptor (*ER*). Of the 25 metastases samples, only 16 were positive for *ER* expression (Kirn *et al*., 2018). Furthermore, positive *ESR1* methylation was observed in 19 out of the 25 primary breast cancer tissue samples. In the metastases tissue samples, 24 out of the 25 were methylated. This implies that hypomethylation of *ESR1* in primary breast cancer tissues is correlated with increased expression of *ER*. The reverse is true for metastases tissue samples- hypermethylation of *ESR1* is correlated with decreased expression of *ER* (Kirn *et al*., 2018).

Another study aimed to determine whether DNA methylation is linked to the significantly major difference in expression of *ESR2* in endometrial and endometriotic cells. *ESR2* expression in endometriotic cells was found to be significantly higher than *ESR2* expression in endometrial cells. DNA methylation analyses showed 54.25% of methylation over 13 CpG sites in endometrial tissue and 12.69% of methylation in endometriotic tissue. The results are consistent with the notion that hypomethylation results in increased gene expression, whilst hypermethylation results in decreased gene expression (Xue *et al*., 2007), although this is not necessarily always the case. Across the menopausal transition, oestrogen production decreases, which may lead to a decrease in the expression of oestrogen receptors as a result of a lack of ligand. However, it is not known whether this is a bi-directional feedback mechanism where DNA methylation of oestrogen receptor genes influences oestrogen receptor expression (Xue *et al*., 2017), which in turn modulates the level of oestrogen hormone secreted.

These studies demonstrate that DNA methylation does occur at CpG sites in genes associated with sex hormone function in the context of cancer, but no data is available in relation to epigenetic regulation of these genes during the menopausal transition. As DNA methylation varies by tissue type and this is an exploratory study, our study will investigate the methylation levels of sex hormone regulatory genes in peripheral blood samples.

1.4.2 Relationship between DNA methylation level and gene expression

It is generally understood that increased DNA methylation levels result in downregulation of gene expression (Deng *et al*., 2019). A study conducted by Loukovaara in 1995 found that *SHBG* was more methylated in *SHBG*-negative MCF-7 cells than in *SHBG*-positive HepG2 cells, suggesting that DNA methylation of *SHBG* may be inversely correlated with SHBG expression. In another study conducted in 2006, hypermethylation of the *Herpud1* gene encoding homocysteine-induced endoplasmic reticulum protein induced downregulation of *Herpud1* mRNA (Bleich *et al*., 2006). However, there are several studies that depict a positive relationship between DNA methylation and gene expression. A recent study has highlighted the

potentially dual nature of DNA methylation in gene regulation by reporting the upregulation of a subset of Polycomb-targeted transcription factor genes upon hypermethylation in a Korean colorectal cancer cohort (Kwon *et al*., 2025).

1.4.3 Degree of DNA methylation proportional to gene expression

While major differences in the level of DNA methylation between groups relates directly to the level of gene expression, there are also instances where small, non-significant differences in methylation have been associated with variation in the levels of gene expression.

The phenomenon of statistically non-significant methylation differences affecting gene expression can be explained by the context-dependent nature of epigenetic regulation. Certain regions of the genome where DNA methylation is sparse in comparison to methylation across the gene body, such as promoters and enhancers, are more sensitive to small changes in methylation, and even minor alterations may possibly disrupt the binding of transcription factors or other regulatory proteins, leading to significant changes in gene expression (Jjingo *et al*., 2012).

The relationship between DNA methylation and gene expression is complex and can be affected by several factors, such as the tissue or cell type, the specific genomic region, and the cooperative interplay with other epigenetic mechanisms (e.g. histone modifications) (Nawaz *et al*., 2022). It should be noted that in both tissue-specific and non-malignant disorders, small changes in methylation, often at single CpG sites, frequently result in differential gene expression (Leenen *et al*., 2016). Therefore, large statistically significant differences in methylation is not necessarily the only indication of differential gene expression.

1.5 Mechanisms involved in sex hormone changes

1.5.1 Exploring the connections between the *ESR2* gene and sex hormones

The *ESR2* gene, also known as the oestrogen receptor beta (Erβ), plays a crucial role in the regulation of various hormones and their interactions (Dalal *et al*., 2022). Oestrogen, a vital sex hormone, binds to the oestrogen receptor beta. This binding interaction activates the receptor, allowing it to regulate the transcription of target genes involved in numerous physiological processes (Kuiper *et al*., 1999). The oestrogen receptor beta also plays a crucial role in regulating oestrogen's effects on reproductive tissues, bone density, and cardiovascular health. It has been found to have a higher affinity for the biologically active form of oestrogen, 17β-oestradiol, compared to the other oestrogen receptor, oestrogen receptor alpha (Erα) (Kuiper *et al*., 1997).

Follicle-stimulating hormone is a pituitary hormone that plays a crucial role in the regulation of reproductive function. The *ESR2* gene has been linked to the modulation of FSH levels and its actions, meaning that the ESR2 gene also plays a role in influencing of ovarian function and the timing of menopause. This is achieved by oestrogen and ESR2 regulating the FSH receptor expression in ovarian granulosa cells, which, in turn, modulate FSH's effects. Oestrogen receptor beta has been found to interact with and influence the expression of genes involved in FSH signalling pathways (Das and Kumar, 2018).

Bioavailable testosterone, the fraction of testosterone that is not bound to SHBG and is available for use by target tissues, is another hormone influenced by the *ESR2* gene. Genetic variations in the *ESR2* gene have been associated with changes in the levels of bioavailable testosterone (Peter *et al*., 2008). The ESR2 gene also provides a synergistic effect on testosterone levels in men (Sakka *et al*., 2010) and modulates testosterone's effects on bone density, muscle mass, and metabolic health by means of the delicate oestrogen : testosterone ratio in women.

Dehydroepiandrosterone sulfate (DHEA-S) is a precursor to other sex hormones, including testosterone and oestrogen. The *ESR2* gene has been implicated in the regulation of DHEA-S levels and has been found to interact with and modulate the enzymes involved in the metabolism (sulfation and desulfation) of DHEA-S, influencing its bioavailability (Michael Miller *et al*., 2013).

1.5.2 Androgen receptor gene and sex hormone interactions

The androgen receptor (*AR*) gene, located on the X chromosome, plays a crucial role in the regulation of various sex hormones, including oestrogen, SHBG, FSH, testosterone, free testosterone, bioavailable testosterone, and dehydroepiandrosterone-sulfate (DHEA-S) (Davey and Grossmann, 2016). When an androgen binds to the AR, the receptor undergoes a conformational change, translocates to the nucleus, and binds to specific DNA sequences called androgen response elements, thereby regulating the transcription of target genes (Quigley *et al*., 1995).

Oestrogen has been shown to interact with the AR signalling pathway. Oestrogen can bind to the AR, modulating its transcriptional activity and influencing the expression of AR-regulated genes (Weihua *et al*., 2002). This crosstalk between the oestrogen and androgen signaling pathways is important for the regulation of sexual differentiation, reproductive function, and other physiological processes (Weihua *et al*., 2002).

SHBG is a plasma glycoprotein that binds to testosterone and other sex hormones, regulating their bioavailability (Petra, 1991; Hammond, 2011). The expression of the *SHBG* gene is influenced by numerous factors, including androgens and oestrogens, and the AR has been shown to play a role in this regulation (Petra, 1991; Hammond, 2011).

FSH is a gonadotropic hormone produced by the pituitary gland that plays a crucial role in the regulation of reproductive function (Huhtaniemi and Aittomäki, 1998). The

AR has been implicated in the modulation of FSH production and secretion, as well as in the regulation of FSH receptor expression in target tissues (Huhtaniemi and Aittomäki, 1998). The *AR* gene influences FSH levels, modulating ovarian function by means of binding to androgens, influencing the level of androgens for conversion by aromatase into oestradiol (Orlowski and Sarao, 2023).

DHEA-S is a precursor of testosterone as well as other androgens, and its levels in circulation are also affected by the androgen receptor (Ye *et al*., 2020). The AR can influence the expression and activity of enzymes involved in the metabolism of DHEA-S, thereby modulating its conversion to more potent androgens through sulfation and desulfation (Ye *et al*., 2020).

1.5.3 Relationship between *SHBG* gene and sex hormones

The product of the SHBG gene plays an essential role in the regulation of sex steroid hormones in the body (Hammond, 2011). SHBG is primarily synthesized in the liver and functions as a transport protein, binding to testosterone, dihydrotestosterone, and oestradiol, to a lesser affinity than androgens (Le *et al*., 2012). The binding action serves to assist in regulation of the bioavailability and activity of these sex steroid hormones.

Research has demonstrated that SHBG variants are associated with altered oestrogen levels (Anderson, 1974). Oestrogen receptor alpha and beta interact with SHBG, modulating oestrogen's effects. In addition to this, SHBG also binds to estradiol, the primary oestrogen in the body (Anderson, 1974). The binding of SHBG to estradiol regulates the availability of this hormone as well, helping to maintain the delicate balance between sex steroid hormones (Hammond, 2011).

Variations in the *SHBG* gene can influence the expression and function of the SHBG protein, which in turn can impact the levels and actions of sex steroid hormones (Coviello *et al*., 2012). For example, certain genetic polymorphisms in the SHBG gene

have been associated with altered SHBG concentrations, which can affect the balance between bound and free (unbound) forms of testosterone and estradiol (Xita & Tsatsoulis, 2010).

The relationship between SHBG and testosterone is particularly important. SHBG binds to testosterone (androgens) with high affinity, and the majority of testosterone (44- 65% in men, and 66- 78% in women) in the bloodstream is bound to SHBG (de Ronde *et al*., 2005). Only a small fraction of testosterone remains unbound or "free," which is the biologically active form that can exert physiological effects (Meikle, 2007). Variations in SHBG levels can therefore influence the amount of free, bioavailable testosterone, impacting men in particular as compared to women.

Follicle-stimulating hormone is another hormone that can be influenced by SHBG. FSH is produced by the pituitary gland and plays a crucial role in the regulation of gonadal function, including the stimulation of follicular development and steroidogenesis (Qin, 2023). Interestingly, SHBG has been shown to positively correlate with FSH levels in men and post-menopausal women, each serving as indicators of the other, and SHBG as a potential marker of menopausal status (Wang *et al*., 2017).

The relationship between SHBG and dehydroepiandrosterone-sulfate (DHEA-S) is also worth noting. DHEA-S is a precursor to both testosterone and oestrogen, and its levels are often used as a marker of adrenal function (Sherman, 1999; Soria *et al*., 2018). The relationship between SHBG and DHEA-S in normally menstruating individuals is a negative one (Wathen et al, 1987). In post-menopausal women, DHEA-S levels decline (Wild *et al*., 1987).

1.5.4 Exploring the *INHBB* gene's role in sex hormone regulation

The INHBB gene encodes the beta B subunit of inhibin, a dimeric glycoprotein hormone that regulates the secretion of follicle-stimulating hormone (FSH) from the

anterior pituitary (Rabban *et al*., 2015). Inhibin acts to suppress FSH production, thereby playing a key role in the regulation of the hypothalamic-pituitary-gonadal axis (Burger & Igarashi, 1988).

Both inhibin B and oestrogen are secreted by the granulosa cells in response to FSH levels (Namwanje and Brown, 2016). As FSH and oestrogen and/or receptor levels influence each other, oestrogen potentially inadvertently influences inhibin B expression, and vice versa.

Inhibin B is negatively correlated with serum testosterone levels in men at around mid-puberty (Chada *et al*., 2003), and its levels remain mostly consistent throughout the male lifespan, unlike in women (Andersson *et al*., 2003).

In female infertility and reproductive aging, inhibin B is needed to upregulate DHEA production in theca cells. The DHEA plays a vital, direct role in theca androgen synthesis, implying that inhibin B likely plays a major role in the regulation of age-related reproductive potential (Ford, 2013).

1.5.5 Relationship between *FSHR* gene and sex hormones

The *FSHR* gene encodes the follicle-stimulating hormone receptor, which plays a crucial role in the regulation of reproductive processes (Segaloff and Ascoli, 2013). This receptor is expressed in the granulosa cells of ovarian follicles in females and Sertoli cells of the testes in males, where it binds to follicle-stimulating hormone (FSH) and mediates its biological effects (Segaloff and Ascoli, 2013).

The binding of FSH to the FSHR receptor activates a cascade of signaling pathways that ultimately influence the production and secretion of various sex hormones, including oestrogen, testosterone, and dehydroepiandrosterone sulfate (DHEA-S)

(Themmen and Huhtaniemi, 2000). The *FSHR* gene directly influences follicle-stimulating hormone levels and activity (Bhartiya and Patel, 2021).

Research has shown that certain *FSHR* gene polymorphisms are associated with differences in follicular development and oestrogen levels in response to ovarian hyperstimulation (Lyangasova, *et al*., 2024).

1.6 Menopause studies in sub-Saharan Africa (SSA)

There is little written about menopause and CVD risk in African women, with the exception of several studies in sub-Saharan Africa, in Cameroon, and Nigeria (Moor *et al*., 2016; Lewechi-Uke *et al*., 2023; Chikwati *et al*., 2023; Chikwati *et al*., 2024). One of these studies by Chikwati *et al*. (2024) from the AWI-Gen cohort described the relationship between menopause and cardiometabolic risk in women living with and without HIV. The authors found that menopause in women from Nairobi (Kenya), DIMAMO/Dikgale (South Africa) and Soweto (South Africa) was associated with higher LDL-c levels regardless of HIV status (Chikwati *et al*., 2024). In another publication by Chikwati *et al*. (2023), the authors concluded that menopause-related differences in risk factors for cardiometabolic disease were evident within the West African population, but not the East or South African study sites. These descriptions of the data in the AWI-Gen cohort repository by Chikwati *et al*. (2023) informed the selection of participants for this study. Considering that only the West African population reflected menopause-related differences in cardiometabolic risk factors, participants were randomly selected from the West African dataset. To maintain homogeneity of the study population, only one West African study site was selected – Nanoro, Burkina Faso.

At present, there is no literature that examines the relationship between epigenetic mechanisms of gene regulation in relation to menopause-linked hormone changes and the associated cardiometabolic risk in sub-Saharan African women, or even on a global scale. This further supports the need for such research to be conducted and illustrates the originality and novelty of this study.

1.7 Study Design

In this study, the liquid chromatography-mass spectrometry (LC-MS) measurements of sex hormone levels (oestrogen, follicle stimulating hormone, sex hormone binding globulin, dehydroepiandrosterone sulfate, total testosterone, bioavailable testosterone, and free testosterone) were performed by Dr Raylton P. Chikwati made available for comparison with DNA methylation levels in CpG islands of genes involved in sex hormone regulation. Along with DNA methylation and liquid chromatography-mass spectrometry data, cardiometabolic, anthropometric, demographic, behavioural and socioeconomic data were collected from each study participant at their research study visit. These data include:

Demographic Variables – age, sex

Behavioural Variables – alcohol consumption, and MVPA

Socioeconomic Variables – education

Anthropometric Variables – BMI, hip circumference, waist circumference, waist-to-hip ratio, visceral fat, and subcutaneous fat

Cardiometabolic Variables – systolic blood pressure, diastolic blood pressure, eGFR, fasting blood glucose, fasting insulin, HOMA, total cholesterol, HDL-c, triglycerides, LDL-c, and cIMT

1.7.1 Hypothesis

It is hypothesized that an increased cardiometabolic disease risk in post-menopausal compared to pre-menopausal West African women is associated with differences in sex hormone levels that are linked to significant differences in the epigenetic modification of genes associated with sex hormone levels and function.

1.7.2 Aim

This study aims to explore a potential epigenetic mechanism (DNA methylation) in the modulation of sex hormone levels and associated risk for cardiometabolic diseases in post-menopausal women from West Africa.

1.7.3 Objectives

The following objectives are related to genes involved in hormone regulation across the menopausal transition in West African women from Nanoro, Burkina Faso.

Genes investigated: *ESR2*; *AR*; *FSHR*; *INHBB*; and *SHBG.*

1) To determine whether there is a difference in serum levels of sex hormones, anthropometric, socioeconomic, behavioural, and cardiometabolic disease risk factors between pre- and post-menopausal women from West Africa.

2) To determine whether there is a difference in the average gene methylation (AGM) of promoter-linked regions and/or individual CpG sites associated with sex hormone regulatory genes between pre- and post-menopausal women from West Africa.

3) To assess whether the average gene methylation (AGM) of promoter-linked regions of sex hormone regulatory genes is associated with differences in serum levels of sex hormones, anthropometric, and cardiometabolic risk factors between pre- and post-menopausal women from West Africa.

CHAPTER TWO

2. Materials and Methods

This study is a sub-study of the Africa Wits-INDEPTH partnership for Genomic Studies (AWI-Gen) Project funded by the US National Institutes of Health under the umbrella of the Human Heredity & Health in Africa Consortium (H3Africa). AWI-Gen is a population cross-sectional study of men and women between the ages of 40 and 60 years. The first wave of data and sample collection occurred over 3 years between August 2013 and August 2016 (Ali *et al.,* 2018; George *et al*., 2019). Participants were randomly selected black African men and women in six study sites across Africa: South Africa (Agincourt, Dikgale/DIMAMO, Soweto); East Africa (Nairobi, Kenya); West Africa (Nanoro, Burkina Faso and Navrongo, Ghana). In this study on menopause, only samples from female participants of the Nanoro, Burkina Faso study site were included. Figure 2.1 details the aim and types of data that were collected for this study. Figure 2.2 summarises the flow of the research protocol from the processing of DNA stage, through to the data analysis stage.

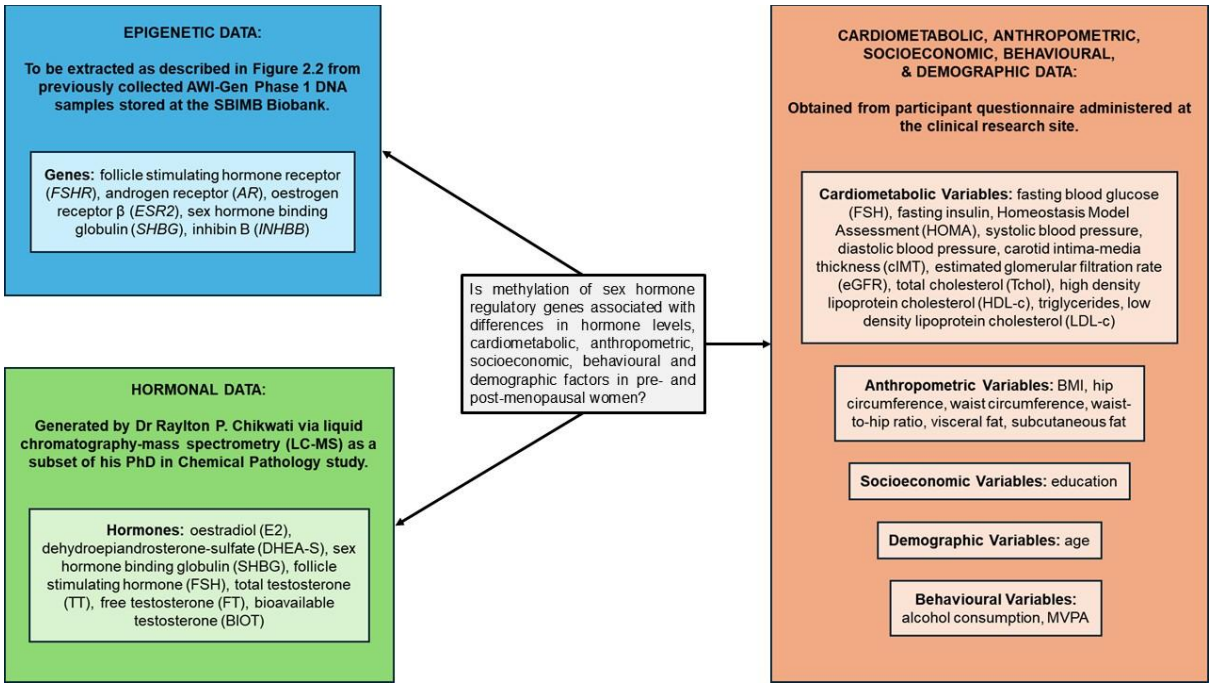


Figure 2.1 Aim and data collected to achieve the objectives of the study.

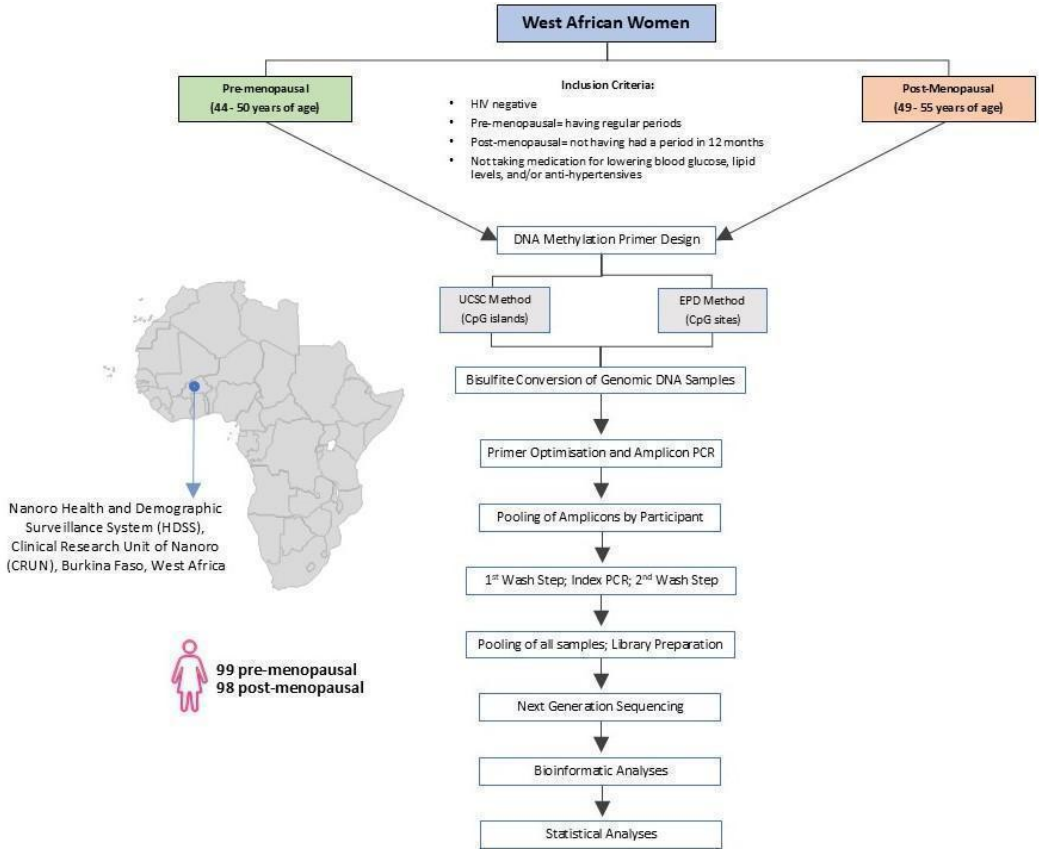


Figure 2.2 Schematic of methods carried out in this study.

2.1 Study participants

Of the 12 067 AWI-Gen participants, 6 569 were women. Their information was captured using questionnaires to collect extensive data (Ali *et al*., 2018), including data to determine their menopausal status based on frequency of menstrual periods, age, and date of last period. Menopausal state was defined as follows: pre-menopausal – having regular menstrual periods; post-menopausal – not having had a menstrual period in the last 12 months. Menopausal status was self-reported and determined by two questions within the AWI-Gen questionnaire:

1) Do you have regular (28-35 day) periods? (Yes/No)

2) Date of last period (month and year)

For this study, 100 pre-menopausal and 100 post-menopausal women were selected for downstream analyses, however, one pre-menopausal and two post-menopausal previously biobanked DNA samples were empty and no matched samples were available to replace them. The final participant count for downstream analyses was 99 pre-menopausal and 98 post-menopausal women. The age range of the women in the pre-menopausal group was between 44 and 50 years, whilst the women in the post-menopausal group were between 49 and 55 years old. While we did try to keep the ages of the women in each group as close as possible, we did not have enough samples to match age exactly. Age was included as covariate in all analyses.

2.1.1 Selection criteria

Inclusion and exclusion criteria were applied as follows:

Women taking anti-diabetic medication, statins or anti-hypertensive therapy were excluded from the study as these drugs may affect DNA methylation (Schrader *et al*., 2021). Only HIV negative women were selected for both groups (pre- and post-menopausal) to minimise confounding. As reported before, HIV infection occurs at very low rates in Burkina Faso.

2.1.2 Demography, anthropometric variables and cardiometabolic risk factors included in the analyses

Data on age (years), height (m), weight (kg), waist circumference (cm), hip circumference (cm), abdominal visceral (mm) and subcutaneous fat (mm), carotid intima-media thickness (cIMT) (micrometres – μm), blood pressure (millimetres of mercury – mmHg), fasting blood glucose (mmol/L), fasting plasma insulin (micro-international units per litre – μIU/L), total cholesterol (mmol/L), high density lipoprotein cholesterol (HDL-c) (mmol/L), low density lipoprotein cholesterol (LDL-c) (mmol/L) estimated with the Friedewald equation (Friedewald *et al*., 1972) and triglyceride (mmol/L) levels, socioeconomic status such as education level (0=no formal education; 1=primary or secondary education), alcohol intake (0=non-drinker (no alcohol intake at all); 1=drinker (any extent of alcohol intake)) and self-reported physical activity (minutes of moderate to vigorous physical activity per week-

min/week) were collected from each participant and used in statistical analyses. All data are available from the AWI-Gen wave 1 study and the variables and methods used to collect data are described in detail in Ali *et al*. 2018.

2.1.3 Hormone levels

To assess whether DNA methylation levels of CpG islands in the promoter regions of the genes of interest are linked to levels of sex hormones, hormone levels were measured using liquid chromatography-mass spectrometry (LC-MS). The following compounds were measured in each of study participants using LC-MS by a fellow PhD student on the AWI-Gen cohort study (Dr Raylton P. Chikwati) oestradiol (E2), dehydroepiandroesterone sulfate (DHEA-S), sex hormone binding globulin (SHBG), follicle stimulating hormone (FSH) and testosterone (TT) (Chikwati *et al*., 2023; Chikwati *et al*., 2024). These data have been made available for analysis for my study. From the SHBG, TT and additional serum albumin data that was made accessible for this purpose, free testosterone (FT) and bioavailable testosterone (BIOT) were calculated with the 'Free & Bioavailable Testosterone Calculator' developed at the Hormonology Department at the University Hospital of Ghent in Belgium and can be accessed at https://www.issam.ch/freetesto.htm.

2.1.4 Sample collection

Genomic DNA (gDNA) derived from the buffy coat of participant peripheral blood samples (collected into EDTA) was obtained from the Sydney Brenner Institute for Molecular Bioscience Biobank. The gDNA was extracted by means of automated DNA extraction protocols. Genomic DNA was purified from buffy coats using the automated QIAsymphony SP (Qiagen, Germany) sample purification robot and the Qiagen magnetic bead-based DNA mini-prep kit (catalogue number 931,255) running Qiagen protocol DNA_BC_400_V6_DSP and the genomic DNA eluted in a final volume of 400µl (Ali *et al*., 2018). DNA was stored at -80°C in the biobank freezers until ready for analysis.

Genes of interest were selected based on their relation to sex hormones involved in the menopausal transition and whether they possessed CpG sites upstream or occupying the promoter region of the gene. The genes selected included oestrogen receptor α (*ESR1*), oestrogen receptor β (*ESR2*), follicle stimulating hormone receptor (*FSHR*), inhibin B (*INHBB*), androgen receptor (*AR*), and sex hormone binding globulin (*SHBG*). The promoter region of follicle stimulating hormone beta subunit (*FSHB*) has no CpG islands or individual CpG sites nearby or that span across the promoter so methylation primers could not be designed for the gene. During the primer optimisation stage, a non-specific annealing temperature could not be optimised for *ESR1*. As we were exploring promoter region methylation in relation to modulation of gene expression, we did not redesign a primer for a region within the gene body. This lead to the gene being omitted from the study. The primers for the remaining five genes were designed using two methods described below.

2.2 Primer design for PCR to assess DNA methylation

Initially, the coordinates for all genes were located on the UCSC Genome Browser, and their CpG islands upstream or spanning across the promoter region were identified. However, this approach for deriving the sequence of the CpG islands could not be used for genes with no CpG island. The second approach involved the use of the Eukaryotic Promoter Database (EPD) to obtain the promoter sequence of the gene, and MethPrimer to locate individual CpG sites upstream or spanning the promoter region of the gene.

2.2.1 UCSC method for identifying CpG islands

CpG islands spanning or upstream of the promoter regions of *ESR2*, *INHBB*, *AR* and *SHBG* were located using the UCSC Genome Browser (https://genome.ucsc.edu/). More than one CpG island was identified for some genes, however, only the island most likely to affect gene expression was targeted. Typically, the island most likely to affect gene expression is found approximately 2kb upstream to 7kb downstream of the transcription start site (TSS) of the promoter region (Hong and Rhee, 2022) (Figure 2.3), so the CpG island had to meet this criterion to be selected.

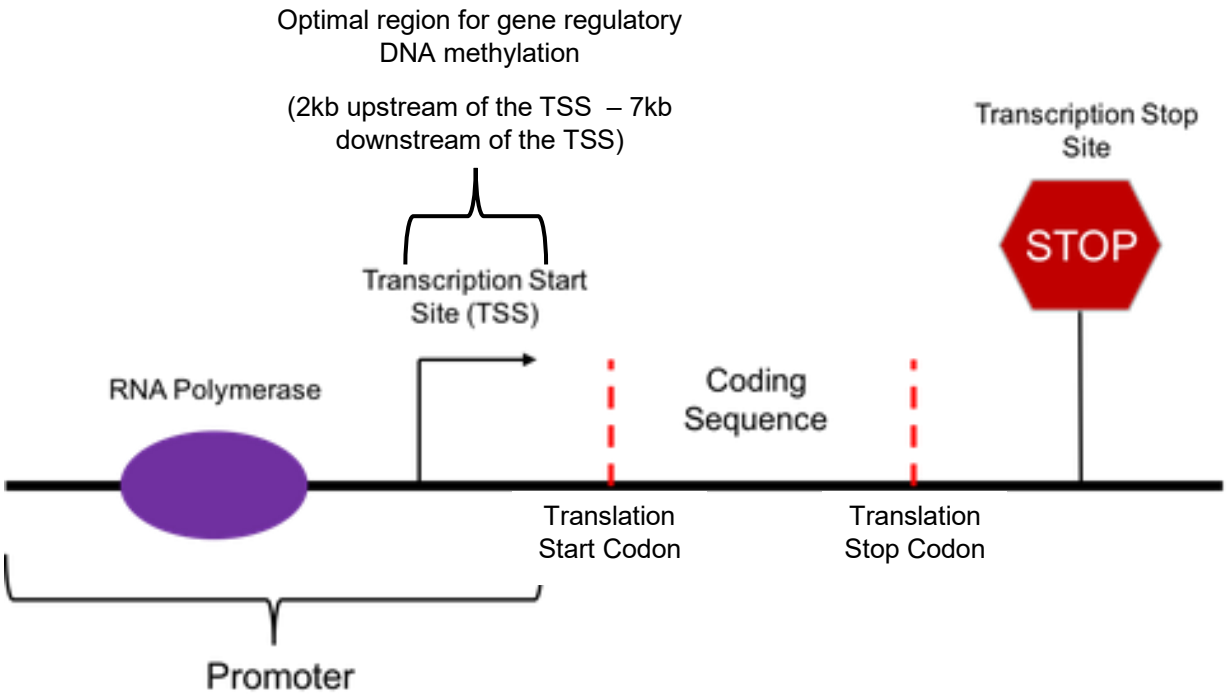


Figure 2.3 Location of the optimal region for gene regulatory methylation to occur.

Once selected, CpG island sequences were obtained from the UCSC Genome Browser and input into MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). MethPrimer then generated several primer pairs according to guidelines for designing PCR primers on DNA that has been subjected to bisulfite modification (https://www.zymoresearch.com/pages/bisulfite-beginner-guide#bisulfite_pcr_and_primer_design). The primer pair covering the largest portion of the CpG island was then selected. This ensured maximum sequencing coverage of the region of interest. Only one primer pair per gene was selected, which meant that the entire CpG island could not be sequenced, as bisulfite primers are designed optimally (26 – 30bp) to amplify relatively small regions of between 150 and 300bp. The primer pairs should also, ideally, not contain any CpG sites. This makes for the most optimal bisulfite primers, but also the most restrictive selection process.

Figures 2.4 to 2.7 highlight the targeted CpG island (in a green box) for *ESR2*, *INHBB*, *AR* and *SHBG* relative to the promoter region (in a blue box). The sequences of each of these islands were obtained from the UCSC Genome Browser, and input into MethPrimer to generate bisulfite primer pairs.

In Figure 2.4, CpG island 67 is located upstream of promoters *ESR2*_2, *ESR2*_3 and *ESR2*_4. CpG island 45 spans across the *ESR2*_1 promoter region, which may still impact transcription, however only one CpG island per gene was targeted. CpG island 67 methylation potentially influences the functionality of three *ESR2* promoters, which makes it an ideal target region for the objectives of this study.

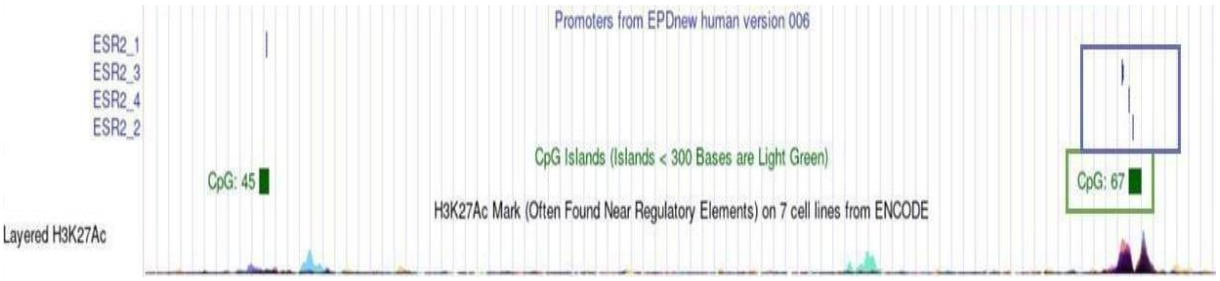


Figure 2.4 Target CpG island (CpG: 67; chr14:64288227-64342982) located upstream of promoters, ESR2_1, ESR2_3, and ESR2_4, and spanning across promoter ESR2_2 of the *ESR2* gene.

In Figure 2.5, CpG island 300 is located upstream and spans the INHBB_1 promoter region. CpG island 36 can also be found upstream of INHBB_1, however, it is more likely that methylation of the CpG island closer to the promoter region would influence gene expression. CpG islands 17 and 42 are located downstream of the promoter region and are unlikely to impact the transcription of *INHBB*.

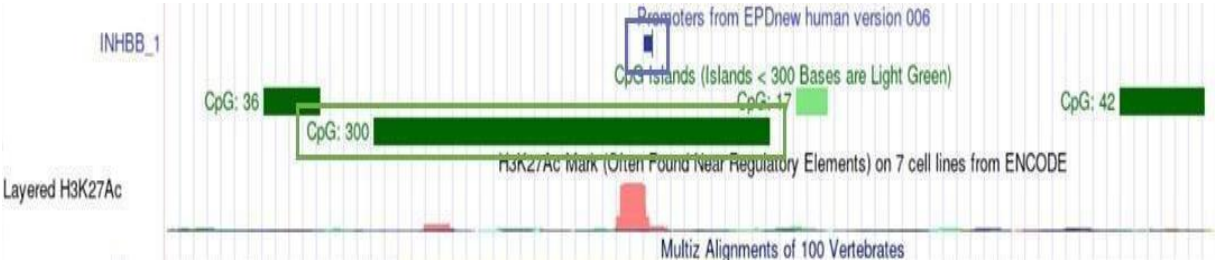


Figure 2.5 Target CpG island (CpG: 300; chr2:120342802-120351303) located upstream and spanning across promoter INHBB_1 of the *INHBB* gene.

Two CpG islands were detected by UCSC Genome Browser for the androgen receptor. In Figure 2.6, CpG island 27 (indicated by the green box) is located upstream and spanning across the androgen receptor promoter, AR_1. The second CpG island 27 is situated downstream, occupying the gene body, of promoter AR_1 and is less likely to influence transcription of *AR*.

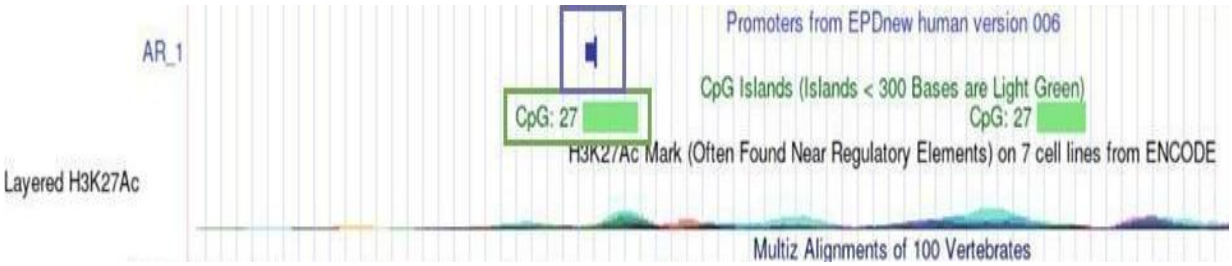


Figure 2.6 Target CpG island (CpG: 27; chrX:67542033-67548943) located upstream and spanning across promoter AR_1 of the *AR* gene.

In Figure 2.7, CpG island 43 is located upstream of promoters SHBG_1 and SHBG_3. CpG island 78 is situated downstream, embedded within the gene body of promoter SHBG_2 and is less likely to influence transcription of *SHBG*.



Figure 2.7 Target CpG island (CpG: 43; chr17:7612975-7634831) located upstream of promoters SHBG_1 and SHBG_3 of the *SHBG* gene.

Figures 2.8 to 2.11 visually depicts the location of several potential primer pairs designed on MethPrimer for each gene (*ESR2*, *INHBB*, *AR*, and *SHBG*) and the CpG sites (denoted by short red lines) clustered within the region that would be amplified.

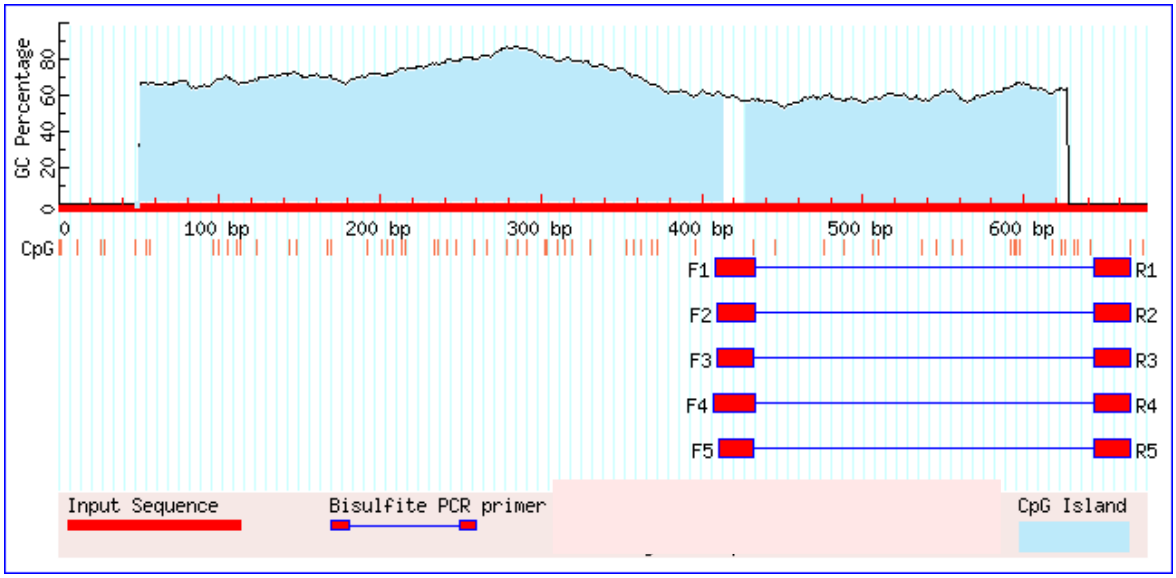


Figure 2.8 The selected bisulfite primer pair, F1 and R1, amplifying a region located upstream and spanning across the *ESR2* gene between chr14:64338799-64339057.

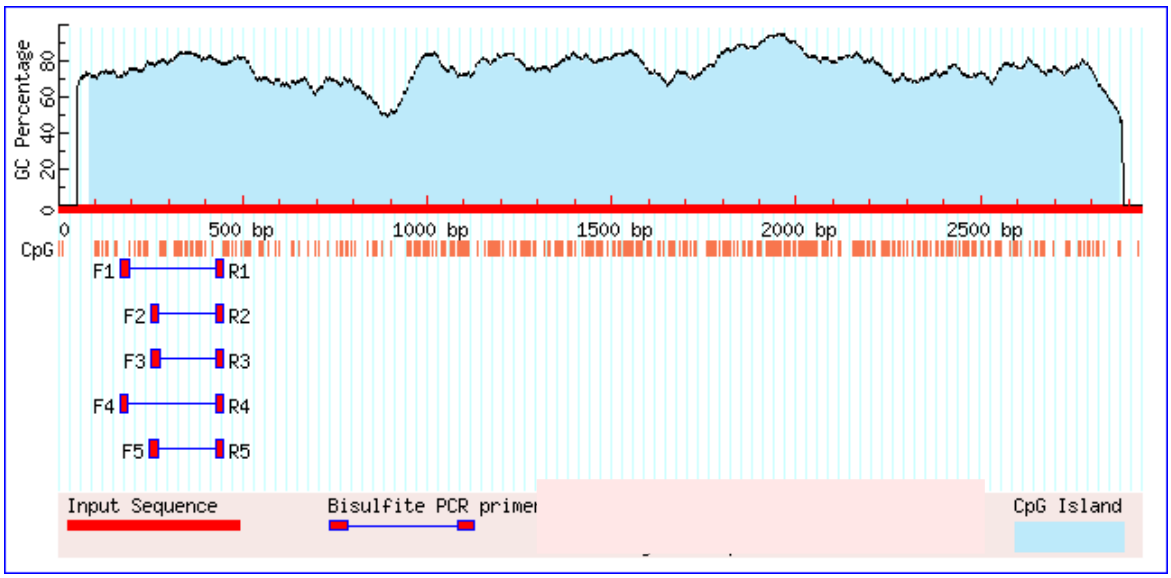


Figure 2.9 The selected bisulfite primer pair, F1 and R1, amplifying a region located upstream and spanning across the *INHBB* gene between chr2:120344294-120344570.

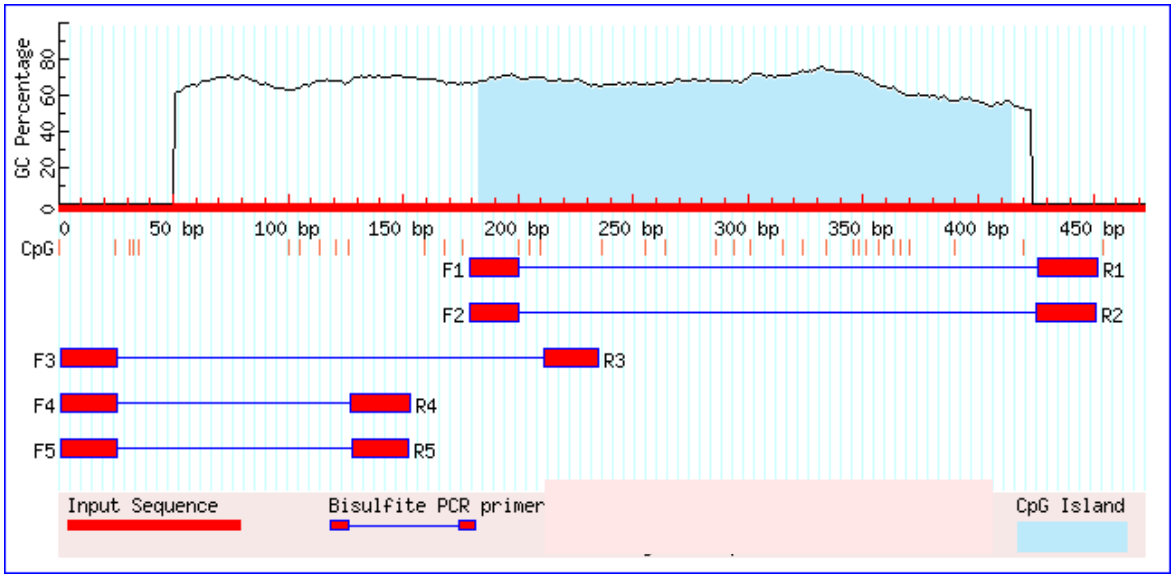


Figure 2.10 The selected bisulfite primer pair, F3 and R3, amplifying a region located upstream and spanning the *AR* gene between chrX:67543864-67544098.

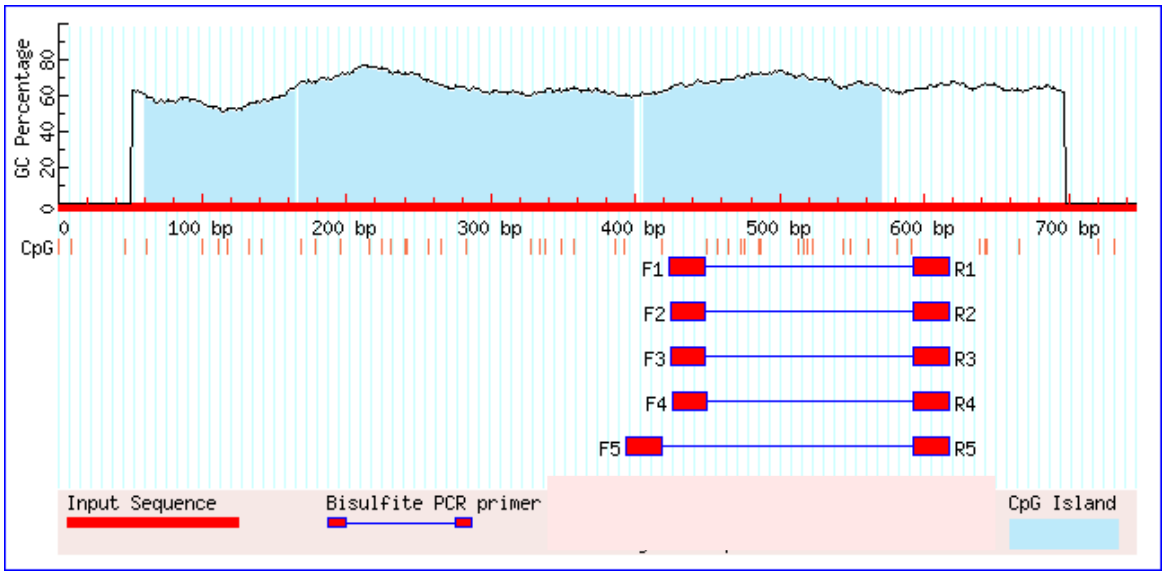


Figure 2.11 The selected bisulfite primer pair, F5 and R5, amplifying a region located upstream of the *SHBG* gene between chr17:7627681-7627904.

2.2.2 EPD method for identifying CpG sites in the absence of a CpG island

No CpG islands upstream or occupying the promoter region of *FSHR* were detected (Fig. 2.12) using the UCSC Genome Browser tool (https://genome.ucsc.edu/). Considering that follicle stimulating hormone levels are directly linked to menopausal

status, it was important to include *FSHR* in the study. Therefore, selected CpG sites were targeted. The genomic sequence of the *FSHR* promoter region was obtained from the Eukaryotic Promoter Database (https://epd.epfl.ch//index.php) and inputted into MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). MethPrimer then generated several primer pairs that amplified only a few CpG sites. The primer pair amplifying the most CpG sites was then selected.

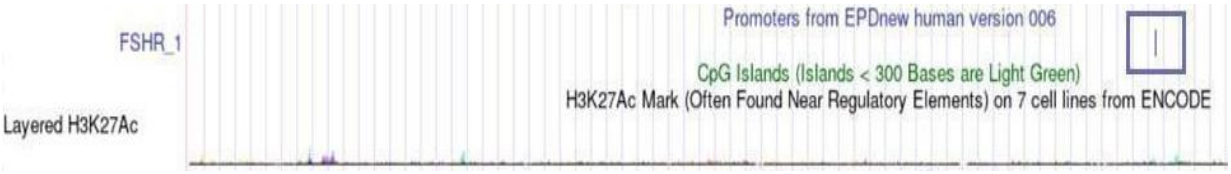


Figure 2.12 No CpG islands detected by UCSC Genome Browser upstream or spanning the *FSHR* promoter region (chr2:48800855-49313812).



Figure 2.13 *FSHR* promoter sequence obtained from the Eukaryotic Promoter Database.

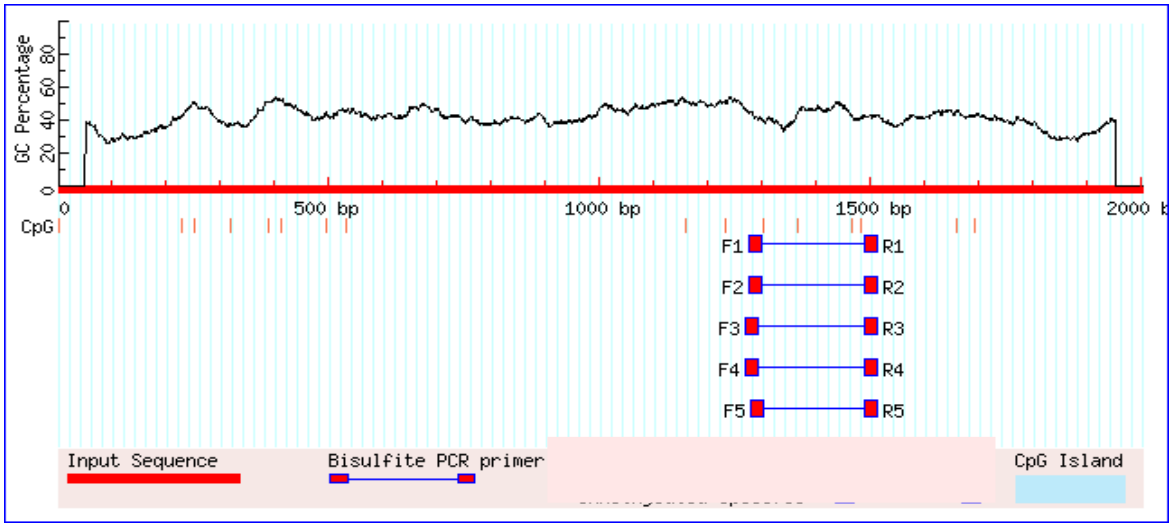


Figure 2.14 The selected bisulfite primer pair, F1 and R1, amplifying a region on the *FSHR* gene between chr2:49154790-49155027.

Table 2.1 is a collation of the location, size of amplicon, number of CpG sites, and the primer sequences for each of the five genes: *ESR2*, *INHBB*, *AR*, *SHBG*, and *FSHR*. Within each CpG island fragment being amplified (promoter-linked region) there are 20 *ESR2* CpG sites, 30 *INHBB* CpG sites, 15 *AR* CpG sites, and 17 *SHBG* CpG sites. Across the *FSHR* gene, only 4 individual (no CpG island) CpG sites were detected for amplification.

Table 2.1 Gene information detailing location of CpG island, location and size of amplicon, number of CpGs in each target region, and the forward and reverse primers designed to amplify the target regions.

Gene	Location of CpG island	Location of Amplicon	Size of Amplicon (bp)	Number of CpGs	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
ESR2	chr14:64338392-64339067	chr14:64338799-64339057	259	20	TTATATGTAAAGGTGTTTTTTGGTT	CAACCTAATACTTACAACAACTC
INHBB	chr2:120344225-120346958	chr2:120344294-120344570	277	30	AGGTTTTTAGTGGTTATTGTTTTTT	AAACCCTACCTCTATCCCAACTAC
AR	chrX:67543964-67544235	chrX:67543864-67544098	235	15	TGTTTTTTTAAAGTTATTAGGTAGG	CTCCTCTACCTCTAAACTTACTCC
SHBG	chr17:7627388-7627933	chr17:7627681-7627904	224	17	GGTAATTAGATTTTTTAAAGGGTTTA	AACTAACAATTATACCCCCTACTAC
FSHR	No CpG island	chr2:49154790-49155027	238	4	TTGTATGGTTGGTATTTGTGTATTG	AACAAAACTTTTCCCTTTACCTTTAA

2.3 Library generation

Once all bisulfite PCR primers were designed, and NGS-compatible adapter sequences ligated (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG to the 5' end of the forward primer, and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG to the 5' end of the reverse primer), the primers were ordered from Integrated DNA Technologies (IDT), Inc (Coralville, Iowa, United States).

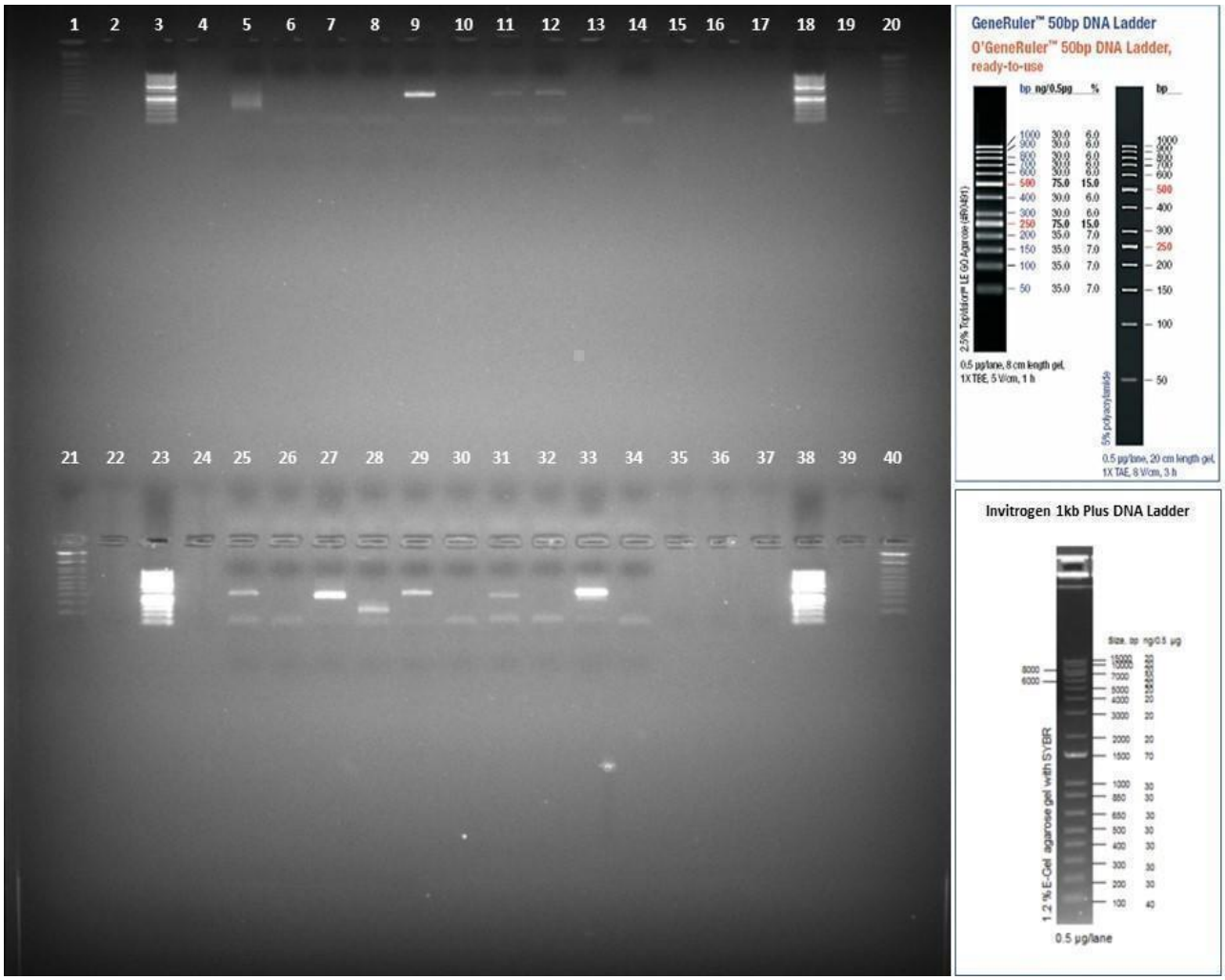
2.3.1 Bisulfite conversion of genomic DNA of study participants

Each of 99 pre-menopausal and 98 post-menopausal genomic DNA samples, as well as 4 test DNA samples of fellow postgraduate students for primer optimisation purposes, were quantified using the ThermoFisher Scientific Nanodrop One (Waltham, Massachusetts, United States). The samples were then diluted to 20ng/µl with nuclease-free water to obtain a yield of 400ng in 20µl for optimal bisulfite conversion, which was achieved using the EZ-96 DNA Methylation-Lightning MagPrep Kit by Zymo Research (Irvine, California, United States). There were no deviations from the protocol supplied with the kit, which can be found in Appendix 2.

2.3.2 Primer optimisation for PCR to generate amplicons

Amplicon PCR for each of *ESR2*, *INHBB*, *AR*, *SHBG* and *FSHR* were first carried out with 4 bisulfite modified test DNA samples to determine the optimal annealing temperature. When working with bisulfite modified DNA, it is recommended that hot-start polymerases be used, to decrease chances of non-specific binding. The odds of non-specific binding occurring are increased with bisulfite modified DNA, as the AT content is higher, due to the unmethylated cytosines (the majority of cytosines) being converted to thymine bases. Primer optimisation PCR (Appendix 2) was carried out using the Invitrogen Platinum II Hot-Start Green PCR Master Mix (2X) (Waltham, Massachusetts, United States). Although the master mix contains a buffer that enables universal annealing temperature (60°C), not all primers annealed at 60°C. No target specific annealing temperature could be achieved for oestrogen receptor α (*ESR1*), so the gene was omitted from the study.

Once the optimal annealing temperatures of *ESR2*, *INHBB*, *AR*, *SHBG* and *FSHR* were determined and confirmed with 2% agarose gels (Fig. 2.15), amplicon PCR for each of the five genes and each DNA sample was carried out. It was not possible to amplify every single gene in every participant sample, even after repeating the failed reactions, however most amplicons were obtained.



Lanes 1, 20, 21, and 40: Invitrogen 1kb Plus DNA Ladder. Lanes 3, 18, 23, and 38: GeneRuler 50bp DNA Ladder. Lane 9: *FSHR* amplicon PCR where T_a= 60°C. Lanes 11, 12 and 25: *ESR2* amplicon PCR where T_a= 61°C and 61.5°C respectively. Lane 27: *AR* amplicon PCR where T_a= 59°C. Lanes 29 and 33: *INHBB* amplicon PCR where T_a= 60°C and 61°C respectively. Lane 31: *SHBG* amplicon PCR where T_a= 61°C

Figure 2.15 Primer optimisation of *ESR2*, *INHBB*, *AR*, *SHBG* and *FSHR*.

Upon assessing the clarity and size of the bands in Figure 2.15 to determine the most optimal and specific annealing temperature, the following were finalised: Androgen Receptor (*AR*)= 59°C; Follicle Stimulating Hormone Receptor (*FSHR*)= 60°C; Inhibin B (*INHBB*)= 60°C; Oestrogen Receptor β (*ESR2*)= 61°C; and Sex Hormone-Binding Globulin (*SHBG*)= 60.5°C.

2.3.3 Pooling of amplicons by participant

Amplicons were quantified using the Nanodrop One by ThermoFisher Scientific (Waltham, Massachusetts, United States) prior to pooling, to avoid sequence read bias due to dominance of a participant sample by a specific gene(s). Following this, approximately equivalent concentrations of each amplicon (*ESR2*, *INHBB*, *AR*, *SHBG*, and *FSHR*) were pooled per participant. It is important to note that some amplicon PCR reactions did not produce amplicons, so some participant samples had to be pooled in batches of four or less, instead of the intended five amplicons (*ESR2*, *INHBB*, *AR*, *SHBG*, and *FSHR*) per participant. It was not feasible to exclude these individuals from the study altogether, as the power of the study would decrease. It is understood that this way of pooling will create a certain level of bias, as each participant does not necessarily have a full set of five amplicons.

2.3.4 Sample clean-up 1

Once pooled, the participant samples underwent a size selecting wash step to ensure that primer dimers and/or non-specific amplicons were removed prior to sequencing. The NucleoMag kit for clean-up and size selection by Macherey-Nagel (Düren, Germany) (Appendix C) was used for the above purposes.

2.3.5 Index PCR

Following clean-up of pooled participant samples, each sample was tagged with IDT Illumina Nextera DNA UD Indexes (Set C) (San Diego, California, United States), so that each participant and their respective amplicon sequences (for each of *SHBG*, *AR*, *ESR2*, *INHBB*, and *FSHR*) may be identified by their individual tag sequence for

further downstream analyses. The indexes were attached to the amplicons by means of index PCR (Appendix C(iv)).

2.3.6 Sample clean-up 2

The samples were washed once more following addition of index sequences to participant amplicons. The protocol was the same as the first wash step with the NucleoMag kit by Macherey-Nagel (Düren, Germany), except for a smaller quantity of magnetic beads being used for the second wash step to get rid of any residual undesirable amplicons that may not have been discarded in the first wash step (Appendix 2).

2.3.7 Combining of pooled and indexed samples for sequencing

After each participant sample had been indexed and washed, they had to be pooled once more to generate a single library prior to sequencing. In total, there were 99 pre-menopausal participants, and 98 post-menopausal participants in the study. The sequencing was carried out in three batches, with the size of the batch increasing as we became more familiar with our library preparation protocol. The first batch, which was conducted with an applications specialist, included 16 pre-menopausal samples, and 16 post-menopausal samples. The second batch, which was conducted without supervision, included 24 pre-menopausal and 24 post-menopausal women, and finally, the third batch contained the remaining samples - 59 pre-menopausal and 58 post-menopausal samples. As there were so few samples sequenced each time, sequencing depth was very high.

2.3.8 Assessment of library quality and quantity

Before pooling all participant samples together, each indexed and washed sample was quantified using the Qubit 4 by Invitrogen and the Qubit dsDNA broad-range assay kit (Waltham, Massachusetts, United States).

The Qubit 4 quantifies nucleic acids in nanograms per microlitre, however, DNA sequencers require the number of molecules in each sample to be known and normalised before pooling together to form the final library.

The equation used to convert ng/µl to nM is:

$$\frac{\text{Concentration in ng/µl}}{(660\ \text{g/mol} \times \text{average library size}) \times 10^6} = \text{Concentration in nM}$$

This equation has been incorporated into an online 'Nanomolar Conversion' tool that allows one to convert between ng/µl and nM for multiple samples at once and was used instead of manual calculations. It can be found on the Illumina website (https://support.illumina.com/help/nanomolar-conversion/nanomolar-conversion.htm).

Each participant sample was then diluted to 1nM, pooled, and then diluted to 50pM. A 50pM solution of the PhiX Control Kit v3 by Illumina was prepared and spiked into the library sample in a 20:80 ratio to serve as a housekeeping gene, which could verify a successful sequencing reaction, but also not take up too much of the coverage capacity intended for the target genes.

2.4 Next Generation Sequencing (NGS)

The sequencing was undertaken in three batches in order to keep the amount of laboratory preparation reasonable to avoid errors, and to maintain a high sequencing depth. For each of three sequencing reactions, an iSeq100 i1 Reagent Cartridge v2 by Illumina (San Diego, California, United States) was removed from -20°C and left to thaw at room temperature for 9 hours. Once completely thawed, an iSeq100 i1 Flow Cell by Illumina (San Diego, California, United States) was inserted into the cartridge and 20µl of the prepared library and PhiX sample was then pipetted into the cartridge. The cartridge and flow cell were then loaded into the Illumina iSeq100 (San Diego,

California, United States). Finally, the reaction was set up on the run manager, saved, and the 16-hour sequencing run commenced.

2.5 Bioinformatic analyses

The fastq.gz sequences obtained from each of the three sequencing reactions were uploaded to Illumina's BaseSpace (San Diego, California, United States), where the DRAGEN Methylation Pipeline was used to obtain methylation calling data. Once all methylation data was collected, the data was collated onto a single spreadsheet with a 'merge' script (Appendix C(i)) executed on R v 4.2.1 (Vienna, Austria), and % methylation was calculated as follows from the number of methylated CpG sites in comparison with the total CpG sites:

$$\frac{\text{Methylated Count at X}}{(\text{Methylated Count at X} + \text{Unmethylated Count at X})} \times 100\% = \%\ \text{Methylation}$$

X= individual CpG site or average gene methylation (AGM) across promoter-linked region of gene

After the calculated percentage methylation data was tabulated, it was statistically analysed as described below.

2.6 Statistical analyses

All statistical analyses were performed using TIBCO Statistica v 14.0.1.25 (Hamburg, Germany). Cardiometabolic, anthropometric, socioeconomic and hormonal continuous variables between pre- and post-menopausal groups were presented as means ± standard deviations (SD) or medians and interquartile ranges (IQR) (calculated by subtracting Q1 from Q3) for variables with normal or skewed distribution, respectively. These variables were compared between the two groups using the Student's t-test for normally distributed variables and the Mann-Whitney U test for variables with a skewed distribution. Categorical data were presented as numbers with percentages and compared between groups using the chi-squared test. Average gene methylation of CpG promoter-linked regions (fragments of CpG islands)

and individual CpG sites in pre- and post-menopausal groups were presented as medians and interquartile ranges (calculated by subtracting Q1 from Q3) and compared using the Mann-Whitney U test.

The dependent variables for 19 multiple regression models were selected according to the objectives of this study.

The 19 dependent/outcome variables (models) were:

Anthropometric variables:

Measures of weight distribution – body mass index (BMI) (model 1), hip circumference (model 2), waist circumference (model 3), visceral fat (model 4), and subcutaneous fat (model 5)

Cardiometabolic variables:

Markers of metabolic function and glycaemic regulation – fasting blood glucose (FBG) (model 6), and Homeostasis Model Assessment (HOMA) (model 7) calculated with the equation from Matthews *et al*., 1985:

$$\frac{\text{Fasting Plasma Glucose (mmol/L) x Fasting Serum Insulin (µU/ml)}}{22.5} = \text{HOMA}$$

Blood pressure-linked variables – systolic blood pressure (model 8), diastolic blood pressure (model 9), and carotid intima-media thickness (cIMT) (model 10)

Kidney function – estimated glomerular filtration rate (eGFR) (model 11)

Indicators of cardiovascular health – total cholesterol (TChol) (model 12), high density lipoprotein cholesterol (HDL-c) (model 13), triglycerides (model 14), and low density lipoprotein cholesterol (LDL-c) (model 15).

Hormonal variables:

Menopause-associated hormones – oestrogen (E2) (model 16), follicle stimulating hormone (FSH) (model 17), bioavailable testosterone (BIOT) (model 18), and sex hormone binding globulin (SHBG) (model 19)

It is well documented in literature that menopause has been associated with each of the dependent variables and it is important to know whether its association with these output variables is independent of other possible confounders, such as age. To account for this, cardiometabolic, anthropometric, socioeconomic, and hormonal independent variables for each model were then selected based on scientific plausibility and a coding variable for menopausal status i.e. pre- or post-menopausal, was included in each model to account for menopause as a confounder, as both pre- and post-menopausal women were included in each model as the sample size was too small (99 pre-menopausal; 98 post-menopausal) for separate pre- and post-menopausal models for each regressor. To select which average gene methylation (AGM) sites to include in each of the 19 models, bivariate linear correlation analyses were performed on each of the 19 variables listed above with each of the 5 AGMs (*FSHR* AGM = 4 CpGs; *SHBG* AGM = 17 CpGs; *AR* AGM = 15 CpGs; *ESR2* AGM = 20 CpGs; and *INHBB* AGM = 30 CpGs). The p-values of the bivariate analysis were then multiplied by 19 (Bonferroni correction) to adjust for multiple testing. Similar analyses were performed for each of the individual 86 CpG sites. Independent (predictor) variables with a variance inflation factor (VIF) of over 5 (Kim, 2019) were excluded to prevent collinearity. This process of multivariable regression model building was used to limit the number of independent variables to reduce overfitting.

Thereafter, we ran the models, and models with a large number of predictors (>12 variables) were reduced before being run a second time for the final version of these models. In order to reduce the number of predictors, cardiometabolic, anthropometric, socioeconomic and hormonal variables with the highest p-values were removed from the models before second run. The number of predictor variables included in each model was kept to a maximum of 12 to avoid overfitting, as the sample size was quite small (N= 99 pre-menopausal + 98 post-menopausal = 197) and only allowed for

between 10 and 15 predictor variables (Babyak, 2004). These regression models were then used to determine if there were correlations between any of the CpG sites and the selected dependent variables that were independent of any of the other possible modulating (or confounding) variables.

2.7 Funding

The data collection was funded by the National Institutes of Health (NIH) under the umbrella of the H3Africa Consortium (NIH grant number U54HG006938 and its supplements), blood and urine marker assays were co-funded by additional funding from the South African Department of Science and Technology (now Department of Science and Innovation) (award number DST/CON 0056/2014), and MN received a scholarship from the Deutscher Akademischer Austauschdienst-National Research Foundation (DAAD-NRF).

2.8 Medical ethics approval

Ethical clearance for the greater AWI-Gen umbrella study was obtained before the study commenced. Following approval of the PhD protocol by the Faculty of Health Sciences (FHS) at the University of the Witwatersrand, completion of a Research Methodology Course (FAHS1595) hosted by the FHS, and an online Introduction to Research Ethics Course facilitated by TRREE, ethical clearance for this sub-study was also obtained. The sub-study was approved by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand (clearance certificate number M2010127). All samples were retrieved from the SBIMB Biobank, which was granted clearance by the Wits Biobank Ethics Committee (approval identification number BEC20140302). The research site (the Health and Demographic Surveillance System (HDSS) at the Clinical Research Unit of Nanoro (CRUN), hosted by the Institut de Recherche en Sciences de la Santé) also obtained approval from their local ethics review board before sample and data collection.

CHAPTER THREE

3. Results

3.1 Hormone levels, demographic, behavioural, cardiometabolic, anthropometric, and socioeconomic factors

A comparison of cardiometabolic, anthropometric, socioeconomic, and hormone level data between pre- and post-menopausal groups was conducted for both normally distributed data, and non-parametric data (Table 3.1). Lipid levels of post-menopausal women were statistically significantly higher than those of pre-menopausal women. The same was true of blood pressure and FSH. However, BMI, waist circumference and visceral fat was lower in post-menopausal women as compared to pre-menopausal women, as was oestrogen and eGFR. There were no smokers in the cohort, so this variable was omitted from the study. Only three participants of the 197 indicated that they were on anti-hypertensives, so this variable was also excluded.

Table 3.1 Comparison of hormone levels, cardiometabolic, anthropometric, demographic, behavioural, and socioeconomic factors in pre- and post-menopausal groups.

Variable	Pre-menopausal Group (n= 99)	Post-menopausal Group (n= 98)	p-value
Demographic Variables:			
Age (years)	46.0 (3.0)	52.0 (3.0)	<0.001
Behavioural Variables:			
Alcohol Consumption (%)			
Non-drinker	30 (30.6)	29 (29.9)	1.000
Drinker	68 (69.4)	68 (70.1)	
MVPA (minutes/week)	2940.0 (2880.0)	3240.0 (1440.0)	0.545
Socioeconomic Variables:			
Education (%)			
No Formal Education	86 (87.8)	91 (92.9)	0.240
Primary/Secondary Education	12 (12.2)	7 (7.1)	
Anthropometric Variables:			
BMI (kg/m^2)	20.1 (4.0)	19.2 (3.4)	0.034
Hip Circumference (cm)	88.4 (8.4)	86.4 (8.7)	0.032
Waist Circumference (cm)	73.9 (10.2)	73.1 (9.3)	0.864
Waist-to-Hip Ratio	0.8 (0.1)	0.9 (0.1)	0.009
Visceral Fat (mm)	4.4 (1.6)	4.1 (1.3)	0.015
Subcutaneous Fat (mm)	0.9 (0.6)	0.9 (0.5)	0.083
Cardiometabolic Variables:			
Systolic Blood Pressure (mmHg)	103.5 (18.5)	114.3 (25.0)	<0.001
Diastolic Blood Pressure (mmHg)	68.0 (11.0)	72.8 (14.0)	<0.001
eGFR (ml/min/1.73m^2)	115.6 (8.0)	112.3 (9.6)	0.012
Fasting Blood Glucose (mmol/L)	4.9 (0.7)	5.0 (0.9)	0.370
Fasting Insulin (mIU/L)	4.0 (10.9)	4.3 (13.5)	0.575
HOMA (mmol.mU/L^2)	0.8 (2.1)	0.9 (3.1)	0.527
Total Cholesterol (mmol/L)	3.0 ± 0.8	3.4 ± 0.8	<0.001
HDL-c (mmol/L)	1.0 (0.3)	1.1 (0.5)	0.030
Triglycerides (mmol/L)	0.6 (0.3)	0.7 (0.4)	0.003
LDL-c (mmol/L)	1.7 ± 0.6	1.9 ± 0.6	0.007
cIMT (μm)	625.0 (0.1)	645.0 (0.1)	0.004
Hormonal Variables:			
E2 (pmol/L)	324.1 (524.6)	42.1 (35.0)	<0.001
FSH (mIU/mL)	15.6 (35.2)	60.4 (36.9)	<0.001
DHEA-S (μmol/L)	1.8 (1.0)	1.6 (1.2)	0.138
SHBG (nmol/L)	114.6 (60.5)	98.2 (45.6)	0.049
TT (pmol/L)	404.6 (320.0)	493.9 (530.0)	0.481
FT (pmol/L)	3.1 (3.0)	4.2 (5.0)	0.195
BIOT (pmol/L)	67.9 (52.0)	96.4 (122.0)	0.113

Results represented as 'mean ± SD' and 'median (interquartile range = Q3 - Q1)'.
Abbreviations: BMI= body mass index; MVPA= moderate to vigorous physical activity; eGFR= estimated glomerular filtration rate; HOMA= Homeostasis Model Assessment; HDL-c= high density lipoprotein cholesterol; LDL-c= low density lipoprotein cholesterol; cIMT= carotid intima media thickness; E2= oestrogen; FSH= follicle stimulating hormone; DHEA-S= dehydroepiandrosterone-sulfate; TT= total testosterone; FT= free testosterone; BIOT= bioavailable testosterone.

3.2 Individual CpG site methylation

Genomic DNA extracted from peripheral blood leukocytes was highly concentrated and of good quality with optimal A_{260}/A_{280} and A_{260}/A_{230} measurements. Amplicons were sequenced on the Illumina iSeq100 at a high depth of ~50 000x for each of the three sequencing runs, ensuring the reliability of sequencing data, and results of this study.

Percentage methylation across each of the 86 CpG sites (4 *FSHR* CpGs, 30 *INHBB* CpGs, 20 *ESR2* CpGs, 17 *SHBG* CpGs, and 15 *AR* CpGs) in pre- and post-menopausal groups was calculated (Table 3.2). The numbers after the gene symbols relate to the CpG sites that were examined in the order of 5' to 3' relative to the gene and as described in the methods. Note that in some cases, the level of methylation can differ significantly from site to site for a specific gene. For example, when examining the four CpG sites for *FSHR*, three were highly methylated (sites 1, 2 and 4), whereas CpG site 3 was only 50% methylated. There were relatively low levels of CpG site methylation for all 30 CpG sites in the promoter region of the *INHBB* gene, ranging from less than one to about 15% methylation. All 20 CpG sites upstream of the *ESR2* promoter and the 17 *SHBG* CpG sites had less than 1% methylation. With the AR gene, methylation ranged between 28 and 46% across all 15 CpG sites. However, despite the variation in methylation between CpG sites within each gene, methylation is consistent between groups, as there were no statistically significant differences in methylation at individual CpG sites between pre- and post-menopausal groups.

Table 3.2 Percentage methylation of all 86 individual CpG sites in pre- and post-menopausal groups.

Variable	% Methylation of Pre-menopausal Group (n= 99)	% Methylation of Post-menopausal Group (n= 98)	p-value
FSHR_1	85.9 (1.6)	85.7 (1.8)	0.464
FSHR_2	94.4 (1.1)	94.6 (1.3)	0.250
FSHR_3	50.1 (2.1)	50.3 (2.3)	0.504
FSHR_4	96.9 (0.7)	96.9 (0.8)	0.671
INHBB_1	8.1 (8.6)	9.0 (9.2)	0.529
INHBB_2	2.2 (5.2)	2.9 (5.4)	0.261
INHBB_3	4.4 (7.3)	2.9 (4.8)	0.074
INHBB_4	1.2 (3.4)	0.7 (2.6)	0.773
INHBB_5	3.3 (6.2)	2.0 (5.7)	0.168
INHBB_6	1.3 (4.7)	1.3 (5.7)	0.413
INHBB_7	1.6 (3.5)	1.8 (5.0)	0.317
INHBB_8	1.6 (4.0)	1.9 (6.3)	0.474
INHBB_9	4.0 (4.9)	4.0 (7.0)	0.147
INHBB_10	2.7 (5.6)	2.4 (6.0)	0.921
INHBB_11	4.3 (6.1)	3.0 (6.7)	0.417
INHBB_12	6.7 (6.1)	5.7 (7.8)	0.532
INHBB_13	6.8 (10.0)	5.9 (10.6)	0.733
INHBB_14	8.1 (8.7)	7.5 (9.7)	0.898
INHBB_15	13.0 (7.4)	12.2 (12.2)	0.796
INHBB_16	15.0 (10.5)	14.4 (12.9)	0.538
INHBB_17	7.0 (7.1)	6.6 (10.4)	0.696
INHBB_18	4.3 (7.2)	2.7 (6.5)	0.221
INHBB_19	10.0 (2.9)	1.0 (1.9)	0.209
INHBB_20	3.9 (5.4)	3.1 (6.5)	0.441
INHBB_21	1.5 (3.2)	1.5 (3.4)	0.905
INHBB_22	1.4 (2.2)	0.9 (2.3)	0.383
INHBB_23	1.8 (4.1)	1.2 (5.2)	0.899
INHBB_24	1.8 (4.9)	2.7 (5.7)	0.430
INHBB_25	3.6 (6.0)	2.2 (6.0)	0.149
INHBB_26	0.5 (1.6)	0.5 (1.7)	0.581
INHBB_27	1.2 (3.4)	0.9 (3.4)	0.736
INHBB_28	0.6 (0.9)	0.6 (0.8)	0.248
INHBB_29	0.7 (1.5)	0.6 (1.5)	0.940
INHBB_30	1.1 (2.8)	1.1 (2.8)	0.972
ESR2_1	0.3 (0.7)	0.2 (0.7)	0.745
ESR2_2	0.3 (0.6)	0.4 (0.6)	0.564
ESR2_3	0.4 (1.0)	0.3 (0.6)	0.321
ESR2_4	0.2 (0.5)	0.2 (0.5)	0.589
ESR2_5	0.2 (0.4)	0.2 (0.4)	0.675
ESR2_6	0.2 (0.4)	0.0 (0.5)	0.860
ESR2_7	0.3 (0.5)	0.3 (0.5)	0.841
ESR2_8	0.3 (0.8)	0.3 (0.7)	0.786
ESR2_9	0.3 (0.5)	0.2 (0.5)	0.930
ESR2_10	0.3 (0.8)	0.4 (0.6)	0.707
ESR2_11	0.5 (0.9)	0.3 (0.6)	0.260
ESR2_12	0.5 (0.7)	0.3 (0.6)	0.317
ESR2_13	0.4 (0.8)	0.4 (0.6)	0.678
ESR2_14	0.4 (0.7)	0.4 (0.8)	0.444
ESR2_15	0.6 (1.0)	0.5 (0.9)	0.675
ESR2_16	0.5 (1.1)	0.5 (1.0)	0.434
ESR2_17	0.3 (0.6)	0.2 (0.4)	0.231
ESR2_18	0.4 (0.8)	0.4 (0.6)	0.753
ESR2_19	0.3 (0.8)	0.3 (0.6)	0.922
ESR2_20	0.2 (0.6)	0.4 (0.7)	0.192
SHBG_1	0.3 (0.3)	0.3 (0.5)	0.437
SHBG_2	0.3 (0.3)	0.3 (0.3)	0.291

SHBG_3	0.3 (0.3)	0.3 (0.3)	0.608
SHBG_4	0.3 (0.4)	0.3 (0.4)	0.548
SHBG_5	0.3 (0.3)	0.3 (0.5)	0.936
SHBG_6	0.3 (0.4)	0.3 (0.4)	0.592
SHBG_7	0.6 (4.2)	0.6 (1.4)	0.733
SHBG_8	0.3 (0.3)	0.3 (0.3)	0.870
SHBG_9	0.3 (0.2)	0.4 (0.3)	0.364
SHBG_10	0.5 (0.4)	0.5 (0.4)	0.698
SHBG_11	0.6 (0.6)	0.6 (0.6)	0.967
SHBG_12	0.3 (0.3)	0.2 (0.3)	0.120
SHBG_13	0.4 (0.3)	0.4 (0.4)	0.627
SHBG_14	0.4 (0.3)	0.4 (0.4)	0.098
SHBG_15	0.3 (0.3)	0.3 (0.3)	0.973
SHBG_16	0.5 (0.4)	0.4 (0.4)	0.378
SHBG_17	0.5 (0.6)	0.6 (0.5)	0.959
AR_1	37.2 (2.7)	37.7 (3.4)	0.372
AR_2	38.4 (2.8)	38.2 (3.6)	0.819
AR_3	26.3 (2.8)	26.4 (2.9)	0.365
AR_4	31.4 (3.0)	31.3 (2.9)	0.575
AR_5	22.4 (3.8)	22.4 (3.7)	0.563
AR_6	22.9 (3.3)	22.9 (2.6)	0.918
AR_7	20.8 (5.5)	20.6 (4.3)	0.972
AR_8	38.2 (3.7)	38.7 (3.0)	0.352
AR_9	28.8 (2.9)	29.3 (3.3)	0.209
AR_10	32.0 (3.1)	32.4 (2.9)	0.620
AR_11	45.1 (4.4)	45.0 (4.1)	0.546
AR_12	32.1 (3.5)	32.0 (3.4)	0.935
AR_13	44.0 (4.3)	44.1 (4.5)	0.758
AR_14	41.3 (4.2)	40.8 (3.0)	0.765
AR_15	46.6 (4.3)	46.9 (4.7)	0.593

Results represented as 'median (interquartile range = Q3 - Q1)'.
Abbreviations: *FSHR*= follicle Stimulating Hormone Receptor; *INHBB*= inhibin B; *ESR2*= oestrogen receptor β; *SHBG*= sex hormone binding globulin; *AR*= androgen receptor.

3.3 Promoter-linked average gene methylation (AGM)

Methylation levels, when averaged across all the CpG sites per gene across each of the five promoter-linked regions in pre- and post-menopausal groups were compared using the Mann-Whitney U-test (Table 3.3). No statistically significant differences in average methylation between the groups were observed.

Table 3.3 Percentage average methylation of promoter-linked regions for each gene in pre- and post-menopausal groups.

Variable	% Methylation of Pre-menopausal Group (n= 99)	% Methylation of Post-menopausal Group (n=98)	p-value
FSHR (% methylation)	92.6 (22.5)	92.5 (17.5)	0.874
INHBB (% methylation)	2.7 (6.5)	2.3 (7.0)	0.377
ESR2 (% methylation)	0.3 (0.7)	0.3 (0.6)	0.119
SHBG (% methylation)	0.4 (0.4)	0.4 (0.4)	0.454
AR (% methylation)	34.3 (14.1)	34.1 (14.0)	0.880

Results represented as 'median (interquartile range = Q3 - Q1)'.
Abbreviations: FSHR= follicle Stimulating Hormone Receptor; INHBB= inhibin B; ESR2= oestrogen receptor β; SHBG= sex hormone binding globulin; AR= androgen receptor.

3.4 Multiple regression models

To determine which CpG sites would be included in each model, bivariate correlation analyses were carried out between the dependent variable (i.e. the model), the average gene methylation (AGM) of each of the five genes (Appendix B(xxii) – Appendix B(xxvi)). The AGMs with a p-value of less than 0.05 would be multiplied by 19 to adjust for multiple testing (Bonferroni correction). If the p-value remained below 0.05 that AGM would then be included in a multiple regression model with the same dependent variable along with the other cardiometabolic, anthropometric, socioeconomic, and hormone predictor variables. None of the p-values of the bivariate correlations were below 0.05, which meant that no average gene methylation (AGM) data was included in any of the 19 models. Bivariate correlations were also conducted between the 86 CpG sites (Appendix B(iii) – Appendix B(xxi)) and the dependent variables to determine possible significant correlations, however, after correcting for multiple testing (multiplying by 19), there were no p-values less than 0.05. Due to the lack of any significant correlations of AGMs or individual CpG sites with the dependent variables, we were not able to perform tests of mediation to determine whether methylation acted as an intermediate between menopause status and any of the 19 dependent variables.

The 19 models studied were:

Anthropometric models:

BMI (model 1), hip circumference (model 2), waist circumference (model 3), visceral fat (model 4), and subcutaneous fat (model 5).

Cardiometabolic models:

Metabolic function and glycaemic regulation – fasting blood glucose (model 6), and HOMA (model 7)

Blood pressure – systolic blood pressure (model 8), and diastolic blood pressure (model 9)

Kidney function – eGFR (model 10)

Cardiovascular health – total cholesterol (model 11), HDL-c (model 12), triglycerides (model 13), LDL-c (model 14), and cIMT (model 15)

Menopause sex hormone models:

E2 (model 16), FSH (model 17), BIOT (model 18), and SHBG (model 19)

Table 3.4 lists all the predictor variables included in each model. The same 9 predictors were input into each model – menopausal status, oestrogen, DHEA-S, SHBG, BIOT, age, MVPA, alcohol status, and education, as they were relevant to most, if not all, anthropometric, cardiometabolic, and hormonal models that were designed. Where any of the standard 9 variables were not relevant, they were omitted. The rest of the variables – HOMA, visceral fat, subcutaneous fat, hip circumference, systolic blood pressure, eGFR, BMI, LDL-c, FSH, and TT were included in models where they were of significance. The final number of variables included in each model was reduced to 12 or less by excluding variables with the highest p-values to avoid overfitting the models.

Table 3.4 Summary of predictor variables included in each model.

Models	Meno. Status	E2	DHEA-S	SHBG	BIOT	Age	MVPA	Alcohol Status	Education	HOMA	Visceral Fat	Subcut. Fat	Hip Circ.	Systolic BP	eGFR	BMI	LDL-c	FSH
BMI	X	X	X	X	X	X	X	X	X									
Hip Circ.	X	X	X	X	X	X	X	X	X							X		
Waist Circ.	X	X	X	X	X	X	X	X	X							X		
Visceral Fat	X	X	X	X	X	X	X	X	X							X		
Subcut. Fat	X	X	X	X	X	X	X	X	X							X		
FBG	X	X	X	X	X	X	X	X	X		X		X					
HOMA	X	X		X		X	X	X	X		X	X	X					
Systolic BP	X	X		X	X				X	X	X		X		X		X	
Diastolic BP	X	X		X	X				X	X	X		X		X		X	
cIMT	X	X	X		X	X	X	X	X	X	X	X	X					
eGFR	X	X	X	X		X	X	X	X	X	X			X		X		
TChol	X	X	X			X	X	X			X	X			X			X
HDL-c	X		X	X	X		X	X	X	X	X				X			X
Triglycerides	X	X	X	X	X				X	X	X	X						X
LDL-c	X	X	X	X			X	X		X	X		X					
Oestrogen	X		X	X	X	X	X	X	X									
FSH	X	X	X	X	X	X	X	X	X									
BIOT	X	X	X	X		X	X	X	X									
SHBG	X	X	X		X	X	X	X	X									X

The 19 regression models follow below where β is the standardised coefficient and b is the unstandardised coefficient.

3.4.1 Regression models for measures of anthropometric measurement distribution: BMI, hip circumference, waist circumference, visceral fat, and subcutaneous fat

Models were designed for five of the six anthropometric variables related to weight distribution, considering that weight gain is generally associated with the menopause. Waist-to-hip ratio was excluded, as waist and hip circumference were modelled, rendering modelling of waist-to-hip ratio redundant.

BMI as a dependent variable was significantly correlated negatively with both SHBG ($p<0.001$) and MVPA ($p=0.004$) levels but positively with education level ($p=0.001$) (Table 3.5). No correlation was found with any of the CpG sites in bivariate analyses. In the hip circumference model, only BMI ($p<0.001$) was significantly correlated (Table 3.6). For the waist circumference model, BMI was also significantly correlated ($p<0.001$), whilst SHBG was negatively correlated ($p=0.013$) (Table 3.7). Visceral fat was lower in post- than pre-menopausal groups ($p=0.042$) but correlated positively with BMI ($p<0.001$) (Table 3.8). Like visceral fat, the subcutaneous fat multiple regression model highlighted a relationship between the dependent variable and BMI ($p<0.001$) (Table 3.9).

Table 3.5 Multiple regression model for body mass index (BMI).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.091	0.136	0.559	0.839	0.506
Oestrogen (pmol/L)	0.066	0.082	0.000	0.001	0.423
DHEA-S (µmol/L)	-0.125	0.076	-0.348	0.212	0.103
SHBG (nmol/L)	-0.294	0.076	-0.022	0.006	<0.001*
BIOT (nmol/L)	0.042	0.080	1.939	3.702	0.601
Age (years)	-0.207	0.129	-0.183	0.114	0.110
MVPA (minutes/week)	-0.209	0.071	0.000	0.000	0.004*
Alcohol Status (%)	-0.115	0.070	-0.762	0.464	0.103
Education (%)	0.230	0.071	2.327	0.713	0.001*

Adjusted R^2= 0.167; $p<0.001$

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; MVPA – moderate to vigorous physical activity

Table 3.6 Multiple regression model for hip circumference.

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Oestrogen (pmol/L)	0.030	0.044	0.005	0.007	0.492
DHEA-S (µmol/L)	0.026	0.041	1.635	2.592	0.529
SHBG (nmol/L)	-0.052	0.042	-0.087	0.071	0.221
Menopausal Status (%)	-0.055	0.072	-7.723	10.194	0.450
BIOT (nmol/L)	0.031	0.043	32.639	44.977	0.469
Age (years)	0.034	0.069	0.698	1.395	0.617
MVPA (minutes/week)	-0.025	0.039	-0.001	0.002	0.527
Alcohol Status (%)	-0.013	0.037	-1.975	5.680	0.729
Education (%)	0.028	0.039	6.458	8.934	0.471
BMI (kg/m^2)	0.849	0.041	19.409	0.945	<0.001*

Adjusted R^2= 0.765; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; MVPA – moderate to vigorous physical activity; BMI – body mass index

Table 3.7 Multiple regression model for waist circumference.

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.032	0.070	5.120	11.343	0.652
Oestrogen (pmol/L)	0.002	0.042	0.001	0.008	0.955
DHEA-S (µmol/L)	-0.082	0.040	-5.988	2.884	0.040*
SHBG (nmol/L)	-0.103	0.041	-0.197	0.079	0.013*
BIOT (nmol/L)	0.063	0.041	75.654	50.043	0.133
BMI (kg/m^2)	0.841	0.040	22.031	1.051	<0.001*
MVPA (minutes/week)	-0.063	0.038	-0.003	0.002	0.099
Age (years)	0.077	0.067	1.791	1.552	0.250
Alcohol Status (%)	0.051	0.036	8.968	6.320	0.158
Education (%)	-0.007	0.038	-1.751	9.940	0.860

Adjusted R^2= 0.791; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; BMI – body mass index; MVPA – moderate to vigorous physical activity

Table 3.8 Multiple regression model for visceral fat.

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.281	0.141	-0.658	0.331	0.049*
Oestrogen (pmol/L)	-0.089	0.085	-0.000	0.000	0.295
DHEA-S (µmol/L)	-0.117	0.080	-0.124	0.084	0.144
SHBG (nmol/L)	0.005	0.083	0.000	0.002	0.950
BIOT (nmol/L)	0.131	0.083	2.304	1.462	0.117
BMI (kg/m^2)	0.303	0.081	0.116	0.031	<0.001*
MVPA (minutes/week)	0.149	0.076	0.000	0.000	0.052
Age (years)	0.075	0.134	0.026	0.045	0.574
Alcohol Status (%)	0.017	0.073	0.043	0.185	0.815
Education (%)	-0.063	0.075	-0.243	0.290	0.403

Adjusted R^2= 0.158; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; MVPA – moderate to vigorous physical activity

Table 3.9 Multiple regression model for subcutaneous fat.

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.030	0.098	0.029	0.094	0.760
Oestrogen (pmol/L)	0.004	0.059	0.000	0.000	0.947
DHEA-S (µmol/L)	-0.003	0.055	-0.001	0.024	0.951
SHBG (nmol/L)	-0.041	0.057	-0.000	0.000	0.477
BIOT (nmol/L)	0.083	0.058	0.598	0.416	0.153
BMI (kg/m^2)	0.742	0.056	0.116	0.009	<0.001*
MVPA (minutes/week)	0.022	0.053	0.000	0.000	0.674
Age (years)	0.201	0.093	0.028	0.013	0.033*
Alcohol Status (%)	0.080	0.051	0.083	0.053	0.118
Education (%)	0.005	0.052	0.008	0.083	0.921

Adjusted R^2= 0.594; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; BMI – body mass index; MVPA – moderate to vigorous physical activity

Amongst all five anthropometric models, only visceral fat was significantly positively correlated with menopausal status (p=0.049). This is likely because the majority population of Nanoro are poverty stricken and malnourished, which explains the lower body weight distribution measurements. However, accumulation of visceral fat in underweight and normal weight individuals like the Nanoro population is still possible (metabolically obese, normal weight (MONW)) (Pluta *et al*., 2022). Four of the remaining anthropometric models (excluding the BMI model) were all significantly positively correlated with BMI. These results are expected, as all measurements of weight distribution (hip circumference, waist circumference, visceral fat, and subcutaneous fat) are linked to BMI.

3.4.2 Regression models for glycaemia traits: fasting blood glucose and insulin resistance (HOMA)

Key traits for diabetes mellitus, one of the most common conditions that post-menopausal women are at risk for (Heianza *et al*., 2013), were studied through modelling of fasting blood glucose and insulin resistance (HOMA). Table 3.10 shows negative correlations of fasting blood glucose with education (p=0.043) and positive correlations with visceral fat (p=0.002) and age (p=0.006). There were no statistically significant predictors in the HOMA model (Table 3.11).

Table 3.10 Multiple regression model for fasting blood glucose (FBG).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.123	0.139	-0.166	0.186	0.375
Oestrogen (pmol/L)	0.021	0.083	0.000	0.000	0.801
DHEA-S (µmol/L)	-0.085	0.078	-0.051	0.047	0.277
SHBG (nmol/L)	-0.140	0.081	-0.002	0.001	0.084
BIOT (nmol/L)	-0.125	0.082	-1.256	0.821	0.128
MVPA (minutes/week)	0.022	0.074	0.000	0.000	0.762
Age (years)	0.361	0.130	0.070	0.025	0.006*
Alcohol Status (%)	-0.137	0.071	-0.199	0.103	0.054
Education (%)	-0.150	0.073	-0.330	0.162	0.043*
Hip Circumference (cm)	0.064	0.080	0.001	0.001	0.430
Visceral Fat (mm)	0.236	0.075	0.135	0.043	0.002*

Adjusted R^2= 0.206; p<0.001

Abbreviations: SHBG – sex hormone binding globulin; MVPA – moderate to vigorous physical activity; DHEA-S – dehydroepiandrosterone-sulfate; BIOT – bioavailable testosterone

Table 3.11 Multiple regression model for Homeostasis Model assessment (HOMA).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.259	0.149	-3.242	1.861	0.083
Oestrogen (pmol/L)	-0.036	0.090	-0.001	0.001	0.694
SHBG (nmol/L)	0.065	0.085	0.010	0.013	0.445
Hip Circumference (cm)	-0.002	0.124	-0.000	0.011	0.987
Visceral Fat (mm)	-0.002	0.082	-0.013	0.438	0.976
Subcutaneous Fat (mm)	0.041	0.121	0.533	1.579	0.736
MVPA (minutes/week)	-0.053	0.080	-0.000	0.000	0.506
Age (years)	0.282	0.143	0.509	0.258	0.050
Alcohol Status (%)	-0.082	0.078	-1.117	1.058	0.293
Education (%)	-0.077	0.080	-1.588	1.652	0.338

Adjusted R^2= 0.041; p<0.728

Abbreviations: SHBG – sex hormone binding globulin; MVPA – moderate to vigorous physical activity; DHEA-S – dehydroepiandrosterone-sulfate; BIOT – bioavailable testosterone

The fasting blood glucose model suggests that the more formally educated you are, the lower your glucose levels are. These findings are in line with current literature (Mathisen *et al*., 2020b) that associate higher levels of comprehension with better diabetes education, leading to healthier food and lifestyle choices, and metabolic longevity (Kitada *et al*., 2019) to potentially avoid/delay onset of age-related diseases.

3.4.3 Regression models for vascular-related measures: systolic blood pressure, diastolic blood pressure and cIMT

Vascular-related conditions and their contributing factors were studied by modelling systolic blood pressure, diastolic blood pressure and carotid intima-media thickness. Systolic blood pressure exhibited a significant negative correlation with E2 levels (p=0.033) and positive correlations with HOMA (p=0.003) and hip circumference (p=0.027) (Table 3.12). Diastolic blood pressure, like systolic blood pressure, was correlated with hip circumference (p=0.005), as well as menopausal status (p=0.023), and bioavailable testosterone (p=0.049) (Table 3.13). Table 3.14 depicts the relationship between cIMT and regressors. Hip circumference (p=0.034) and age (p=0.048) were the only two statistically significant independent variables in the model.

Table 3.12 Multiple regression model for systolic blood pressure.

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.150	0.086	5.360	3.070	0.083
Oestrogen (pmol/L)	-0.182	0.085	-0.008	0.004	0.033*
SHBG (nmol/L)	0.071	0.081	0.031	0.035	0.380
BIOT (nmol/L)	0.065	0.077	17.536	20.642	0.397
HOMA (mmol.mU/L^2)	0.215	0.071	0.614	0.205	0.003*
eGFR (ml/min/1.73m^2)	0.018	0.072	0.029	0.121	0.808
LDL-c (mmol/L)	0.032	0.075	0.886	2.091	0.672
Hip Circumference (cm)	0.181	0.081	0.046	0.021	0.027*
Visceral Fat (mm)	-0.033	0.075	-0.498	1.150	0.665
Education (%)	0.109	0.074	6.440	4.368	0.142

Adjusted R^2= 0.166; p<0.001

Abbreviations: SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; eGFR – estimated glomerular filtration rate

Table 3.13 Multiple regression model for diastolic blood pressure.

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.195	0.085	4.005	1.751	0.023*
Oestrogen (pmol/L)	-0.083	0.084	-0.002	0.002	0.322
SHBG (nmol/L)	0.050	0.080	0.012	0.020	0.535
BIOT (nmol/L)	0.151	0.076	23.366	11.774	0.049*
Hip Circumference (cm)	0.232	0.081	0.034	0.012	0.005*
Visceral Fat (cm)	-0.047	0.075	-0.415	0.656	0.527
HOMA (mmol.mU/L^2)	0.131	0.071	0.216	0.117	0.066
LDL-c (mmol/L)	0.036	0.074	0.577	1.193	0.629
eGFR (ml/min/1.73m^2)	0.096	0.072	0.092	0.069	0.184
Education (%)	0.106	0.074	3.603	2.492	0.150

Adjusted R^2= 0.177; p<0.001

Abbreviations: SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; eGFR – estimated glomerular filtration rate

Table 3.14 Multiple regression model for carotid intima-media thickness (cIMT).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Education (%)	0.040	0.077	0.015	0.028	0.603
HOMA (mmol.mU/L^2)	0.025	0.075	0.000	0.001	0.738
BIOT (nmol/L)	0.057	0.083	0.093	0.136	0.494
DHEA-S (µmol/L)	-0.063	0.082	-0.006	0.008	0.441
Menopausal Status (%)	0.064	0.147	0.014	0.032	0.665
Oestrogen (pmol/L)	0.094	0.084	0.000	0.000	0.264
Hip Circumference (cm)	0.251	0.117	0.000	0.000	0.034*
Subcutaneous Fat (mm)	-0.210	0.116	-0.048	0.027	0.074
Visceral Fat (mm)	-0.088	0.079	-0.008	0.007	0.267
MVPA (minutes/week)	-0.112	0.078	0.000	0.000	0.153
Age (years)	0.276	0.139	0.009	0.004	0.048*
Alcohol Status (%)	0.032	0.075	0.007	0.018	0.676

Adjusted R^2= 0.063; p<0.029

Abbreviations: BIOT – bioavailable testosterone; DHEA-S – dehydroepiandrosterone-sulfate; MVPA – moderate to vigorous physical activity

Between the vascular-related models (systolic blood pressure, diastolic blood pressure, and carotid intima-media thickness), menopausal status was only correlated (positively) with diastolic blood pressure. In a recent study by Bae *et al*., abnormal diastolic function in women with suspected angina was linked to early menopause (Bae *et al*., 2024). In the systolic and diastolic blood pressure models, HOMA and hip circumference were positively correlated with the dependent variables. cIMT was also positively correlated with hip circumference.

3.4.4 Regression model for kidney function

To assess kidney function in the AWI-Gen Nanoro subset of women, estimated glomerular filtration rate was modelled. Table 3.15 depicts the correlations between eGFR and its regressors, of which, menopausal status (p=0.027) and age (p<0.001) were statistically significantly correlated with eGFR.

Table 3.15 Multiple regression model for estimated glomerular filtration rate (eGFR).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Visceral Fat (mm)	-0.089	0.081	-0.815	0.740	0.272
Education (%)	0.153	0.079	5.423	2.796	0.054
HOMA (mmol.mU/L^2)	0.038	0.078	0.065	0.134	0.630
Oestrogen (pmol/L)	-0.058	0.089	-0.002	0.002	0.513
DHEA-S (µmol/L)	0.022	0.077	0.209	0.750	0.780
SHBG (nmol/L)	-0.068	0.082	-0.017	0.021	0.414
Menopausal Status (%)	0.326	0.146	7.030	3.150	0.027*
BMI (kg/m^2)	-0.082	0.088	-0.285	0.309	0.358
Systolic Blood Pressure (mmHg)	0.015	0.081	0.009	0.049	0.850
MVPA (minutes/week)	-0.038	0.080	0.000	0.001	0.630
Age (years)	-0.500	0.140	-1.553	0.436	<0.001*
Alcohol Status (%)	0.052	0.076	1.220	1.767	0.491

Adjusted R^2= 0.035; p<0.118

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BMI – body mass index; MVPA – moderate to vigorous physical activity

Menopausal status was positively correlated with eGFR, which is generally not the case, as kidney function tends to decline in post-menopausal women when compared with their pre-menopausal counterparts (Kim *et al*., 2020). However, eGFR was negatively correlated with age. These findings are similar to the findings in the study by Kim *et al*., where it was suggested that decline in kidney function is age-related, and not menopause-related, although aging and the menopausal transition occur simultaneously (Kim *et al*., 2020). Bivariate correlation analyses showed that menopausal status and eGFR were negatively correlated (R= -0.181; p=0.011). In the eGFR multivariate model, in the presence of several predictors, menopausal status was no longer negatively correlated with eGFR, instead, age was negatively correlated with eGFR. This further supports the findings from Kim *et al*., 2020.

3.4.5 Lipid-related indicators of cardiovascular health: total cholesterol, HDL-c, triglycerides, and LDL-c

As heart-protective oestrogen decreases throughout the menopausal transition, the risk of developing cardiovascular disease increases to greater than that of male counterparts of a similar age (Adach *et al*., 2022). To study this effect in the Nanoro population, total cholesterol, HDL-c, triglycerides and LDL-c were modelled. Total cholesterol was correlated with DHEA-S (p=0.046) and subcutaneous fat (p<0.001) (Table 3.16). Correlations with FSH (p=0.032), DHEA-S (p=0.019), BIOT (p=0.020), and eGFR (p=0.029) were significant in the high density lipoprotein cholesterol model (Table 3.17). The triglycerides multiple regression model revealed correlations with subcutaneous fat (p=0.004) (Table 3.18). Low density lipoprotein cholesterol depicted correlations with menopausal status (p=0.020) and hip circumference (p=0.012) (Table 3.19).

Table 3.16 Multiple regression model for total cholesterol (TChol).

N= 176	β	Standard Error of β	B	Standard Error of b	p-value
Menopausal Status (%)	0.194	0.143	0.306	0.225	0.175
Oestrogen (pmol/L)	-0.048	0.085	-0.000	0.000	0.570
FSH (mIU/mL)	0.139	0.095	0.003	0.002	0.144
DHEA-S (µmol/L)	-0.145	0.072	-0.104	0.051	0.046*
eGFR (ml/min/1.73m^2)	-0.001	0.074	-0.000	0.005	0.987
Visceral Fat (mm)	-0.140	0.076	-0.093	0.050	0.066
Subcutaneous Fat (mm)	0.294	0.074	0.482	0.122	<0.001*
MVPA (minutes/week)	-0.049	0.073	-0.000	0.000	0.498
Age (years)	-0.121	0.130	-0.028	0.030	0.353
Alcohol Status (%)	0.028	0.071	0.047	0.121	0.698

Adjusted R^2= 0.194; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; eGFR – estimated glomerular filtration rate; MVPA – moderate to vigorous physical activity

Table 3.17 Multiple regression model for high density lipoprotein cholesterol (HDL-c).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.033	0.096	-0.021	0.061	0.732
FSH (mIU/mL)	0.203	0.094	0.002	0.001	0.032*
DHEA-S (µmol/L)	-0.192	0.081	-0.055	0.023	0.019*
SHBG (nmol/L)	0.080	0.078	0.001	0.001	0.309
BIOT (nmol/L)	0.196	0.084	0.938	0.400	0.020*
HOMA (mmol.mU/L^2)	0.120	0.073	0.006	0.004	0.103
eGFR (ml/min/1.73m^2)	0.163	0.074	0.005	0.002	0.029*
Alcohol Status (%)	0.060	0.074	0.041	0.051	0.421
MVPA (minutes/week)	0.086	0.075	0.000	0.000	0.256
Education (%)	0.073	0.074	0.077	0.077	0.322
Visceral Fat (mm)	-0.081	0.076	-0.022	0.021	0.290

Adjusted R^2= 0.085; p<0.007

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; eGFR – estimated glomerular filtration rate; MVPA – moderate to vigorous physical activity

Table 3.18 Multiple regression model for triglycerides.

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
BIOT (nmol/L)	-0.088	0.084	-0.441	0.418	0.293
Subcutaneous Fat (mm)	0.236	0.080	0.164	0.056	0.004*
SHBG (nmol/L)	-0.136	0.084	-0.001	0.001	0.106
DHEA-S (µmol/L)	0.074	0.078	0.022	0.024	0.346
FSH (mIU/mL)	-0.136	0.099	-0.001	0.001	0.173
Oestrogen (pmol/L)	-0.081	0.092	0.000	0.000	0.382
Menopausal Status (%)	0.186	0.097	0.124	0.064	0.056
HOMA (mmol.mU/L^2)	-0.066	0.072	-0.004	0.004	0.359
Education (%)	-0.143	0.074	-0.156	0.081	0.056
Visceral Fat (mm)	-0.115	0.077	-0.033	0.022	0.138

Adjusted R^2= 0.100; p<0.002

Abbreviations: BIOT – bioavailable testosterone; SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate

Table 3.19 Multiple regression model for low density lipoprotein cholesterol (LDL-c).

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.200	0.085	0.256	0.109	0.020*
Oestrogen (pmol/L)	-0.066	0.086	-0.000	0.000	0.443
DHEA-S (µmol/L)	-0.127	0.075	-0.073	0.044	0.094
SHBG (nmol/L)	0.020	0.081	0.000	0.001	0.809
HOMA (mmol.mU/L^2)	-0.034	0.074	-0.003	0.007	0.645
Hip Circumference (cm)	0.205	0.081	0.002	0.001	0.012*
MVPA (minutes/week)	-0.097	0.078	-0.000	0.000	0.215
Alcohol Status (%)	0.026	0.074	0.037	0.103	0.723
Visceral Fat (mm)	-0.072	0.078	-0.039	0.042	0.353

Adjusted R^2= 0.113; p<0.016

Abbreviations: SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate; MVPA – moderate to vigorous physical activity

LDL-c was positively correlated with menopausal status, which is in line with current literature (Inaraja *et al*., 2020). Total cholesterol and HDL-c are negatively related to DHEA-s, which was also found in a 2009 study by Srinivasan *et al.* However, the relationship between DHEA-s and lipid profiles is complex, and studies tend to report conflicting findings (Srinivasan *et al*., 2009).

3.4.6 Menopause-associated hormones: oestrogen, follicle stimulating hormone, bioavailable testosterone and sex hormone binding globulin.

Four of the six menopause-related hormones measured were modelled to understand the link between sex hormones, epigenetic regulation, cardiometabolic disease, anthropometry, and socioeconomic and lifestyle factors. Only bioavailable testosterone was modelled as a dependent variable as it represents the entirety of testosterone that is readily available for biological interaction, whether loosely bound to albumin or completely unbound (Yang *et al*., 2019). Free testosterone only accounts for the testosterone not bound by any protein, whilst total testosterone also includes testosterone bound to SHBG, which is not bioavailable (Yang *et al*., 2019). Hence, total testosterone and free testosterone were not modelled as dependent variables, and were not included as predictors in other models, except for the SHBG model, which included total testosterone, as it consists of SHBG-bound testosterone amongst other forms of testosterone (Yang *et al*., 2019). The first hormone modelled, oestrogen, was correlated with menopausal status ($p<0.001$), and SHBG ($p=0.001$) (Table 3.20). In the follicle stimulating hormone model, the regressors, menopausal status ($p<0.001$), oestrogen ($p<0.001$), and SHBG ($p= 0.002$) were the most correlated with the predictor (Table 3.21). Bioavailable testosterone was correlated with menopausal status ($p=0.004$), and SHBG ($p<0.001$), as well as DHEA-S ($p<0.001$) (Table 3.22). In the SHBG model (Table 3.23), the outcome variable was significantly correlated with oestrogen ($p<0.001$), FSH ($p=0.002$), and bioavailable testosterone ($p<0.001$).

Table 3.20 Multiple regression model for oestrogen (E2).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.470	0.124	-386.424	102.056	<0.001*
DHEA-S (µmol/L)	-0.061	0.072	-22.829	26.810	0.396
SHBG (nmol/L)	0.247	0.070	2.418	0.685	0.001*
BIOT (nmol/L)	0.131	0.075	809.298	465.324	0.084
Age (years)	0.014	0.122	1.630	14.460	0.910
Alcohol Status (%)	-0.040	0.066	-35.941	58.802	0.542
MVPA (minutes/week)	-0.010	0.068	-0.003	0.019	0.888
Education (%)	0.047	0.067	63.187	90.340	0.485

Adjusted R^2= 0.284; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; MVPA – moderate to vigorous physical activity

Table 3.21 Multiple regression model for follicle stimulating hormone (FSH).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.466	0.111	32.717	7.796	<0.001*
Oestrogen (pmol/L)	-0.346	0.067	-0.030	0.006	<0.001*
Alcohol Status (%)	0.065	0.057	4.940	4.314	0.254
MVPA (minutes/week)	-0.002	0.058	-0.000	0.001	0.968
SHBG (nmol/L)	0.195	0.062	0.163	0.052	0.002*
DHEA-S (µmol/L)	0.049	0.062	1.560	1.969	0.429
BIOT (nmol/L)	0.024	0.065	12.880	34.411	0.709
Age (years)	-0.013	0.105	-0.127	1.060	0.905
Education (%)	-0.062	0.057	-7.157	6.630	0.282

Adjusted R^2= 0.476; p<0.001

Abbreviations: MVPA – moderate to vigorous physical activity; SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate; BIOT – bioavailable testosterone

Table 3.22 Multiple regression model for bioavailable testosterone (BIOT).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.376	0.129	0.050	0.017	0.004*
Oestrogen (pmol/L)	0.136	0.078	0.000	0.000	0.084
DHEA-S (µmol/L)	0.352	0.068	0.021	0.004	<0.001*
SHBG (nmol/L)	-0.293	0.070	-0.000	0.000	<0.001*
MVPA (minutes/week)	0.042	0.069	0.000	0.000	0.548
Age (years)	-0.176	0.124	-0.003	0.002	0.157
Alcohol Status (%)	-0.012	0.068	-0.002	0.010	0.864
Education (%)	-0.049	0.068	-0.011	0.015	0.477

Adjusted R^2= 0.256; p<0.001

Abbreviations: MVPA – moderate to vigorous physical activity; SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate

Table 3.23 Multiple regression model for sex hormone binding globulin (SHBG).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.011	0.142	0.963	11.918	0.936
Oestrogen (pmol/L)	0.367	0.083	0.037	0.008	<0.001*
FSH (mIU/mL)	0.288	0.092	0.344	0.110	0.002*
DHEA-s (µmol/L)	0.070	0.076	2.667	2.860	0.352
BIOT (nmol/L)	-0.313	0.076	-196.577	47.631	<0.001*
MVPA (minutes/week)	-0.053	0.071	-0.002	0.002	0.450
Age (years)	-0.117	0.127	-1.409	1.536	0.360
Education (%)	-0.030	0.070	-4.113	9.664	0.671
Alcohol Status (%)	0.026	0.069	2.379	6.292	0.706

Adjusted R^2= 0.182; p<0.001

Abbreviations: MVPA – moderate to vigorous physical activity; FSH – follicle stimulating hormone; DHEA-S – dehydroepiandrosterone-sulfate; TT – total testosterone; BIOT – bioavailable testosterone

Of the four hormone models, only three were statistically significantly correlated with menopausal status – oestrogen (negative correlation), FSH (positive correlation), and BIOT (positive correlation). It is well known that oestrogen levels decrease and FSH levels increase over the menopausal transition. The increase in bioavailable testosterone in post-menopausal women is potentially due to the expected decrease in SHBG, resulting in greater levels of free testosterone (Janssen *et al*., 2009). In the SHBG model, BIOT is negatively correlated with SHBG, further supporting the decrease in SHBG levels in post-menopausal women. SHBG is positively correlated with oestrogen, total testosterone and FSH. The positive correlation between SHBG and FSH is not well understood, and potentially contradicts the positive correlation with oestrogen in post-menopausal women, but it has been reported previously in a study by Wang *et al*. in 2017. As all variables studied are highly interconnected, it is best to analyse statistically significant results holistically, as represented in Table 3.24, which summarises all significant correlations from each of the 19 models.

Table 3.24 Summary of statistically significant predictors in each model.

Model	Statistically Significant Predictors (p<0.05)	Direction of Correlation
BMI	SHBG (p<0.001)	Negative
	MVPA (p=0.004)	Negative
	Education (p=0.001)	Positive
Hip circumference	BMI (p<0.001)	Positive
Waist circumference	DHEA-S (p=0.040)	Negative
	SHBG (p=0.013)	Negative
	BMI (p<0.001)	Positive
Visceral fat	Menopausal status (p=0.049)	Negative
	BMI (p<0.001)	Positive
Subcutaneous fat	BMI (p<0.001)	Positive
	Age (p=0.033)	Positive
Fasting blood glucose	Education (p=0.043)	Negative
	Visceral fat (p=0.002)	Positive
	Age (p=0.006)	Positive
HOMA	-	-
Systolic blood pressure	Oestrogen (p=0.033)	Negative
	HOMA (p=0.003)	Positive
	Hip circumference (p=0.027)	Positive
Diastolic blood pressure	Oestrogen (p=0.029)	Negative
	BIOT (p=0.013)	Positive
	Hip circumference (p=0.010)	Positive
cIMT	Hip circumference (p=0.034)	Positive
	Age (p=0.048)	Positive
eGFR	Education (p=0.054)	Positive
	Menopausal status (p=0.027)	Positive
	Age (p<0.001)	Negative
Total cholesterol	DHEA-s (p=0.046)	Negative
	Subcutaneous fat (p=0.000)	Positive
HDL-c	FSH (p=0.032)	Positive
	DHEA-s (p=0.019)	Negative
	BIOT (p=0.020)	Positive
	eGFR (p=0.029)	Positive
Triglycerides	Subcutaneous fat (p=0.004)	Positive
LDL-c	Menopausal status (p=0.020)	Positive
	Hip circumference (p=0.012)	Positive
Oestrogen	Menopausal status (p<0.001)	Negative
	SHBG (p=0.001)	Positive
FSH	Menopausal status (p<0.001)	Positive
	Oestrogen (p<0.001)	Negative
	SHBG (p=0.002)	Positive
BIOT	Menopausal status (p=0.004)	Positive
	SHBG (p<0.001)	Negative
	DHEA-s (p<0.001)	Positive
SHBG	Oestrogen (p<0.001)	Positive
	FSH (p=0.002)	Positive
	BIOT (p<0.001)	Negative

CHAPTER FOUR

4. Discussion

Ovulation and reproductive potential cease when a woman experiences menopause, which is the permanent loss of ovarian function (Sherman, 2005). The average duration of the transition is seven years but could last over ten years (Santoro *et al*., 2020). Women may have symptoms like mood swings, sadness, sexual dysfunction, hot flushes, and irregular menstrual cycles at this time (Schmidt, 2005; Santoro *et al*., 2020). Between the ages of 45 and 55, symptoms start to appear (Santoro *et al*., 2020). After 12 months without menstruation, usually around an average age of 51, women enter the post-menopause phase (Sherman, 2005). Menopause-related hormone changes contribute to the development of age-related conditions such as breast cancer, osteoporosis, and cardiometabolic diseases, namely, type 2 diabetes, obesity, cardiovascular disease, stroke, and hypertension (Gold *et al*., 2001). In women within the menopausal age range (45-55 years), the risk of developing these conditions increases in comparison to their pre-menopausal selves and age-matched male counterparts (Kamińska *et al*., 2023). There is a vast body of literature (El Khoudary *et al*., 2020; Maas *et al*., 2021; Kamińska *et al*., 2023) documenting these observations in non-African women, but not in African populations – specifically West Africa, where transition along the health epidemiological transition has not yet progressed. In this study, we focused on women from Nanoro, Burkina Faso in West Africa in order to understand the relationship between epigenetics, sex hormones and cardiometabolic risk in menopausal women. The findings of this study will mark the beginning of addressing the gap in literature around this topic and will contribute to understanding the impact of possible lifestyle and cultural differences across different African regions. There is little written about menopause and CVD risk in African women, with the exception of several studies in sub-Saharan Africa, in Cameroon, and Nigeria (Moor *et al*., 2016; Lewechi-Uke *et al*., 2023; Chikwati *et al*., 2023; Chikwati *et al*., 2024).

The most outwardly visible predictor of CVD risk and metabolic-related conditions is obesity (Powell-Wiley *et al*., 2021). Compared to other African nations, Burkina Faso

has a lower rate of overweight and obesity (Boua *et al*., 2018; Kaboré, S. *et al.*, 2020; Chikwati *et al*., 2023; Chikwati *et al*., 2024; Nonterah *et al*., 2025). According to surveys from 2010, the prevalence of overweight (BMI: 25.0-29.9 kg/m2) and obese (≥30.0 kg/m2) exceeded 20% in 13 countries selected across Africa with the exception of Burkina Faso and Malawi, where it was only about 16% (Amugsi *et al*., 2017). Unfortunately, there have been no more overweight and obese surveys conducted in Burkina Faso since 2010, making it difficult to track weight trends and compare them with the rest of Africa and the world. However, more recent data has been reported on the nutrition of children in Burkina Faso. According to a 2022 Global Nutrition Report, 52.5% of children under five from Burkina Faso suffer from anaemia, 7.7% from childhood wasting, and 19.6% from childhood stunting (Global Nutrition Report, 2022). The malnourishment of babies and young children extends into adulthood, leading to poor nutrition during pregnancy (intrauterine growth restrictions), resulting in low birth weight babies (Cutland *et al*., 2017). Birth weight and cardiometabolic outcomes are strongly correlated, according to studies on post-menopausal women (Alexander *et al*., 2014). Low birth weight, in particular, has been associated with an increased risk of developing hypertension, type 2 diabetes, and metabolic syndrome in adulthood in various population groups across the world, including Africa (Taha *et al*., 2012; Kebede *et al*., 2013; Alexander *et al*., 2014; Smith *et al*., 2016; Cutland *et al*., 2017; Costello and Bradley, 2021; Raisi-Estabragh *et al*., 2022; Ardissino *et al*., 2023).

Although cardiometabolic disease risk is strongly correlated with the menopause-associated change in sex hormone levels (Jaff *et al*., 2014) and is well documented, the mechanisms driving the relationship are not clearly understood. Based on previous studies on DNA methylation of sex hormone regulatory genes (e.g. *ESR1*, *ESR2*, *SHBG*, *AR*, *FSHR*, and *INHBB*), it is proposed that the changes in hormone levels and concurrent development of metabolic syndrome in menopausal women may be influenced by epigenetic factors (Wagner *et al*., 2014; Arathimos *et al*., 2018; Maekawa *et al*., 2019; Bos *et al*., 2022; Ahmed *et al*., 2022; Cagsin *et al*., 2023). As menopause occurs when women are older, it is difficult to ascertain whether cardiometabolic risk increases as a result of hormone changes (menopause), increase in chronological age, or a combination of both menopause and age. The findings of this study will potentially provide some answers to this complex question, contributing

to more targeted preventative treatment options for women. At present, the triggers and processes underlying the changes in hormone levels are not well understood and more research is required to better understand the intricate interactions among genetics, epigenetic changes, hormones, and cardiometabolic risk throughout the menopausal transition. Therefore, this study aimed to explore the role of a potential epigenetic mechanism (DNA methylation) in the modulation of sex hormone levels and associated risk for cardiometabolic diseases by comparing DNA methylation levels in pre-menopausal and post-menopausal women from Nanoro, Burkina Faso in West Africa.

For the purposes of this study, target specific primer pairs were designed for bisulfite modified DNA to amplify promoter-linked regions of the sex hormone regulatory genes, oestrogen receptor β (*ESR2*), follicle stimulating hormone receptor (*FSHR*), inhibin B (*INHBB*), androgen receptor (*AR*), and sex hormone binding globulin (*SHBG*). The amplicons were sequenced to high depth (~50 000x) with next generation sequencing technologies, and the percentage of cytosine methylation at each target CpG site was calculated using the Illumina DRAGEN Methylation Pipeline. There are many studies (Sergeeva *et al*., 2024; Stefansson *et al*., 2024; Jang *et al*., 2017; Kuroda *et al*., 2009; Milani *et al*., 2008) showing that individual CpG sites associate with gene expression levels, therefore it made sense to treat each CpG site individually. Thus, percentage average gene methylation (AGM) was calculated for each of the five CpG promoter-linked regions, as well as for each of 86 individual CpG sites occupying the promoter-linked regions.

The demographic, behavioural, socioeconomic, anthropometric, cardiometabolic, and sex hormone variables included in this study were as follows:

Demographic Variables – age, sex

Behavioural Variables – alcohol consumption, and MVPA

Socioeconomic Variables – education

Anthropometric Variables – BMI, hip circumference, waist circumference, waist-to-hip ratio, visceral fat, and subcutaneous fat

Cardiometabolic Variables – systolic blood pressure, diastolic blood pressure, eGFR, fasting blood glucose, fasting insulin, HOMA, total cholesterol, HDL-c, triglycerides, LDL-c, and cIMT.

Sex Hormone Variables – oestrogen (E2), follicle stimulating hormone (FSH), sex hormone binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEA-s), total testosterone (TT), bioavailable testosterone (BIOT), and free testosterone (FT)

The study aimed to fulfil three objectives:

Objective 1 – To determine whether there is a difference in serum levels of sex hormones, anthropometric, socioeconomic, behavioural, and cardiometabolic disease risk factors between pre- and post-menopausal women from West Africa.

Objective 2 – To determine whether there is a difference in the average gene methylation (AGM) of promoter-linked regions and/or individual CpG sites associated with sex hormone regulatory genes between pre- and post-menopausal women from West Africa.

Objective 3 – To assess whether the average gene methylation (AGM) of promoter-linked regions of sex hormone regulatory genes is associated with differences in serum levels of sex hormones, anthropometric, and cardiometabolic risk factors between pre- and post-menopausal women from West Africa.

4.1 Demographic, behavioural, socioeconomic, anthropometric, cardiometabolic, and hormone level differences in pre- and post-menopausal groups of West African women

The first of the three objectives was to determine whether there is a difference in serum levels of sex hormones, anthropometric, socioeconomic, behavioural, and cardiometabolic disease risk factors between pre- and post-menopausal women from West Africa (Table 3.1). In order to determine whether differential methylation plays a role in cardiometabolic risk, anthropometric measures, and sex hormone levels over the menopausal transition, significant differences between the pre- and post-menopausal groups must first be identified.

4.1.1 Demographic, behavioural, and socioeconomic variables

A deliberate effort to select participants of a relatively close age range (pre-menopausal: 44-50 years; post-menopausal: 49-55 years) was made to diminish the confounding effects of age on menopause status. Despite this, age of pre- and post-menopausal groups was still statistically significantly different ($p<0.001$). Age therefore remains a potential confounding factor; however, we diminished the confounding effects through a robust study design. Analysis approaches and models have been used to adjust for age and to tease out effects related to menopausal changes.

There were no statistically significant differences observed between socioeconomic factors of pre- and post-menopausal groups. There was no change in alcohol consumption ($p=1.000$), education ($p=0.240$), and moderate to vigorous physical activity ($p=0.545$) when women transitioned from pre-menopausal to post-menopausal. Most women in Burkina Faso do not smoke, irrespective of menopausal status, therefore smoking data was omitted from the analyses.

4.1.2 Anthropometric variables

Studies from developed nations have demonstrated increased inflammation, cardiometabolic risk, and increase in appetite during the menopausal transition (Hunter *et al*., 2011). In contrast, we have observed a decline in BMI ($p=0.034$) and

visceral fat (p=0.015) in our West African cohort of post-menopausal women. The reason for this decline in visceral adipose tissue and BMI in post-menopausal women could be related to the presence of a non-obesogenic environment in this community. Thus, the study population are rural and obtain most of their food from subsistence farming. Obesity is rare. Although hormone changes during menopause may stimulate fat accumulation, this may not be possible in an environment with low caloric intake. Most of the studies that show weight gain during menopause were undertaken in high income countries where calorie-dense foods are readily available, where such an obesogenic environment may fuel the increased adiposity related to hormonal changes during menopause. It is interesting to note that waist-to-hip ratio was higher in post- than pre-menopausal women in this study. This may reflect greater loss of subcutaneous relative to visceral fat. Such changes in body fat distribution have been observed in other studies and are likely related to falls in oestrogen levels during the menopause transition (Burger, 2002; Brown and Clegg, 2010).

4.1.3 Cardiometabolic variables

Lipid profiles of the West African women followed the same trend as described in literature, where average cholesterol levels are higher in post-menopausal women as compared to pre-menopausal women (total cholesterol – $p<0.001$; HDL-c – $p=0.030$; triglycerides – $p=0.003$; and LDL-c – $p=0.007$). It is common for total cholesterol, triglycerides and LDL-c to increase as a woman transitions through the menopause (Currie and Williams, 2008) and is related to the fall in oestrogen levels (Xiang *et al*., 2021).

The increase in HDL-c levels, however, did not follow expected trends where HDL-c levels decrease as women transition through the menopause (Kim *et al*., 2000; El Khoudary *et al*., 2021). A few other studies have reported mixed results regarding menopause-related HDL-c trends (Fernandez and Murillo, 2016) and an initial decrease in HDL-c levels followed by an increase from 53 to 69 years has been suggested in a 1986 Framingham study article and an unrelated 2021 study (Castelli *et al*., 1986; El Khoudary *et al*., 2021). However, further studies on larger cohorts are

needed to better understand HDL-c levels in post-menopausal women, specifically in a West African context.

Glucose metabolism-related factors (e.g. fasting blood glucose – p=0.370; fasting insulin – p=0.575; and HOMA – p=0.527) were not significantly different between pre- and post-menopausal groups. This is potentially a result of the restrictive diet that plagues the impoverished country of Burkina Faso, where more than 40% of its population lives below the poverty line (World Food Programme, 2019). Malnutrition leads to hypoglycaemia once glycogen stores in the liver have been metabolised (Ledger *et al*., 2020; Rao, 1984).

Both systolic ($p<0.001$) and diastolic blood pressure ($p<0.001$) was significantly higher in post-menopausal women as compared to pre-menopausal women. This is expected, and in line with other studies that indicate that low oestrogen levels impact blood flow by increasing both blood pressure and the risk of atherosclerotic plaque build-up (Zanchetti *et al*., 2005; Maas and Franke, 2009; Jain *et al*., 2022; Li *et al*., 2024).

One of the most widely utilised and accurate imaging techniques for detecting atherosclerosis and predicting cardiovascular disease risk is the measurement of the carotid intima-media thickness (Lonn, 1999). In our post-menopausal group, atherosclerotic plaque build-up may explain the larger carotid intima-media thickness (cIMT) (p=0.004). The increase in cIMT measurements, suspected atherosclerosis, and elevated blood pressure may help explain the findings of our study that show a lower estimated glomerular filtration rate (eGFR) in post-menopausal women (p=0.012). High blood pressure in particular is known to lead to a decline in renal function (Velusamy, 2024).

4.1.4 Sex hormone variables

The hormones measured in this study are oestrogen (E2), follicle stimulating hormone (FSH), sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEA-S), testosterone (TT), free testosterone (FT), and bioavailable testosterone (BIOT).

As expected, oestrogen levels in post-menopausal women were much lower in comparison to the pre-menopausal group ($p<0.001$), which aligns with literature (Yang *et al*., 2024). Furthermore, the post-menopausal levels of FSH were higher than those of pre-menopausal women ($p<0.001$) in response to the oestrogen decrease (Taneja *et al*., 2019).

Upon comparative statistical analyses, no notable differences between pre- and post-menopausal levels of DHEA-s ($p=0.138$), TT ($p=0.481$), FT ($p=0.195$), BIOT ($p=0.113$) were detected. According to previous studies, DHEA-s levels decline with age (Panjari and Davis *et al*., 2010) and are associated with disease and bad health outcomes (Baird *et al*., 2018). The same decline is also expected in total testosterone, bioavailable testosterone, and free testosterone levels of post-menopausal women (Scott and Newson, 2020). Unlike oestrogen and follicle stimulating hormone, which sharply decline and increase, respectively, after menopause, testosterone and DHEA-s both decline steadily as a woman ages (Johansen *et al*., 2020). Therefore, the significant difference in testosterone and DHEA-s levels may not be evident in a cohort with a small age range, such as ours.

SHBG levels are typically downregulated in post-menopausal women in response to the decline in oestrogen levels (Hoskin *et al*., 2004), as SHBG is a carrier protein (Victor *et al*., 1976) that binds to and transports oestrogen, testosterone and dihydrotestosterone (DHT) (Anderson, 1974). In this study, SHBG levels were also lower in post-menopausal women compared to the pre-menopausal group ($p=0.049$), however, there have been mixed results relating to SHBG levels in the literature (Maggio *et al*., 2008), which is because SHBG levels are potentially influenced by

several factors, such as age, health conditions, medication use, diet and lifestyle and genetics and such factors may vary across studies. Interestingly, a study conducted in South Africa also showed lower SHBG levels in post- than pre-menopausal African women (Jaff et al., 2015).

4.2 DNA methylation % differences in pre- and post-menopausal groups of West African women

The second of the three objectives was to determine whether there is a difference in the average gene methylation (AGM) of promoter-linked regions and/or individual CpG sites associated with sex hormone regulatory genes between pre- and post-menopausal women from West Africa.

4.2.1 Comparison of individual CpG site methylation in pre- and post-menopausal groups

When comparing individual CpG sites *ESR2*_1 to *ESR2*_20, *FSHR*_1 to *FSHR*_4, *SHBG*_1 to *SHBG*_17, *INHBB*_1 to *INHBB*_30, and *AR*_1 to *AR*_15, no statistically significant differences between pre- and post-menopausal groups were found (Table 3.2).

4.2.2 Comparison of promoter-linked average gene methylation (AGM) in pre- and post-menopausal groups

When comparing average gene % methylation of promoter-linked regions of *ESR2*, *FSHR*, *SHBG*, *INHBB*, and *AR* in pre- and post-menopausal groups, there were also no statistically significant differences observed, as with individual CpG site methylation (Table 3.3).

Although no significant differences in individual CpG site or promoter-linked regions were observed, it has been reported previously that some changes in methylation between groups are not significant enough to be reflected with a statistical test

comparing means or the spread of data (Chen *et al*., 2014). However, expression of the methylated gene may still be influenced by the small change in methylation (Chen *et al*., 2014). It is important to note that there is vast literature describing the epigenetic modulatory effects of DNA methylation acting in conjunction with histone methylation, histone acetylation and chromatin modelling (Geiman and Robertson, 2002; Phillips, 2008; Hashimoto *et al*., 2010) to regulate gene expression. Therefore, although increased methylation is 'generally' associated with lower gene expression, we were not able to explore the complex relationship and influence of other epigenetic mechanisms. It is also not possible to determine causality of epigenetic modulation without performing gene expression and post-expression (transcription and protein levels) functional studies.

No previous studies have been conducted on pre- and post-menopausal women comparing methylation of the *ESR2* gene. One study, however, did note differential methylation of a CpG island in the promoter region of *ESR2* in endometrial cells as compared to endometriotic cells (Xue *et al*., 2007). Methylation in endometrial cells was significantly higher than that of endometriotic cells (Xue *et al*., 2007). The authors also performed *in vitro* studies to determine whether methylation of the CpG island occupying the promoter region of *ESR2* inhibited expression of oestrogen receptor β (Xue *et al*., 2007). It was found that methylation of the aforementioned region does inhibit gene expression significantly in endometriosis and endometrium (Xue *et al*., 2007).

In one study on *SHBG*, no differentially methylated regions (DMRs) were identified in the adult female population, however in the adolescent female child, DMRs were identified (Arathimos *et al*., 2018). Again, like *ESR2*, there are no studies conducted on DNA methylation of *SHBG* in pre- and post-menopausal women. Current literature relating to SHBG levels in pre- and post-menopausal women vary and in the current study we observed slightly lower SHBG levels in the postmenopausal group. Larger longitudinal studies in African populations would be required to confirm these results.

AR methylation has primarily been studied in males. One such study associated *AR* promoter methylation with a decrease in expression of the androgen receptor in prostate cancer cells (Jarrard *et al*., 1998). In women with polycystic ovarian syndrome (PCOS), the *AR* promoter region was reported to be hypomethylated, resulting in an increased expression of androgen receptor (Desmawati *et al*., 2018; Salinas *et al*., 2021) – likely in response to the increased testosterone levels in women with PCOS (Bushell and Crespi, 2024). In post-menopausal women, methylation studies have not yet been conducted.

Little methylation research has been conducted on *FSHR*. The few publications identified have focused on male animal models instead of human participants. To our knowledge, this is the first study that investigates *FSHR* methylation in pre- and post-menopausal women, and the first in human participants. Since no CpG island upstream of the *FSHR* promoter region has been identified, this region has not been studied previously. In order to include *FSHR* in our menopause study, we designed bisulfite primers amplifying 4 individual CpG sites occupying the gene body of *FSHR*.

In current literature, there are a few studies discussing the methylation of *INHBB*, however, there are no studies that have been conducted on a pre- and post-menopausal cohort with a cardiometabolic risk focus. One study found that increased methylation of *INHBB* in gastric cancer patients was negatively correlated with INHBB expression (Yu *et al*., 2022), which indicates that DNA methylation influences gene expression of inhibin B.

As there is very little literature available on differential methylation and gene expression of *ESR2*, *SHBG*, *AR*, and *INHBB* between pre- and post-menopausal groups, and no information on *FSHR* in any cohort, our study is novel, and we had no preconceived expectations of the outcomes. As this is an exploratory study, these findings are important and will hopefully trigger further research, addressing gaps in the literature. The lack of association of CpG methylation with any of the outcome variables combined with no observable difference in the levels of methylation between

the pre- and post-menopausal groups highlights that gene methylation may not modulate hormone levels or CMD risk factors in this population, but more studies are required using larger sample sizes and in different populations. As gene methylation of sex hormone regulatory genes during the menopausal transition has not been studied before, it is important to carry out longitudinal studies to clarify the findings of our exploratory study. At this stage, it is biased and unsubstantiated to draw definitive conclusions as there is little to no literature available.

4.3 Central themes derived from multiple regression models of anthropometric variables, cardiometabolic risk factors, and sex hormones

The third, and last, of three objectives, was to assess whether the average gene methylation (AGM) of promoter-linked regions of sex hormone regulatory genes is associated with differences in serum levels of sex hormones, anthropometric measurements, and cardiometabolic risk factors between pre- and post-menopausal women from West Africa (Tables 3.5 – 3.23).

A composite CMD-risk index was considered but decided against because of the large number of CMD risk factors and the lack of data on what cut points would be used in an African population to denote the presence of such a risk factor. There is also much variability across studies in terms of which risk factors are enhanced during menopause. It was therefore considered best to include each individual CMD factor in the regression models to avoid masking individual effects.

Most of the related outcome variable groupings of the multiple regression models shared one or two statistically significant predictor variables. The first group of outcome variables, anthropometric measures (Tables 3.5 – 3.9) which consisted of the BMI, hip circumference, waist circumference, visceral fat, and subcutaneous fat models shared the statistically significant predictor, BMI, in common, with the exception of the BMI model.

The next group, glycaemic markers (Tables 3.10 – 3.11), which included fasting blood glucose and HOMA, varied. Fasting blood glucose was associated with education level, visceral fat, and age, whilst HOMA was not significantly associated with any independent variable.

The third grouping, vascular related measures (Tables 3.12 – 3.14), which included the systolic and diastolic blood pressure models, as well as the cIMT model, showed that blood pressure variables had oestrogen and hip circumference in common, and the cIMT model had hip circumference and age as significant predictors.

The only model that was statistically significantly correlated with both menopause and age was the eGFR model for kidney function (Table 3.15).

The fifth grouping, lipid indicators of cardiovascular health, consisted of total cholesterol, HDL-c, triglycerides, and LDL-c (Tables 3.16 – 3.19). Total cholesterol and HDL-c were correlated with DHEA-s, total cholesterol and triglycerides were correlated with subcutaneous fat, and LDL-c with hip circumference.

The final group, menopause-related hormones, which included oestrogen, FSH, BIOT, and SHBG (Tables 3.20 – 3.23), were correlated primarily with menopause, and SHBG, with the exception of SHBG, which was correlated with oestrogen, FSH and BIOT.

The following subsections will focus on:

1) Sex hormones as statistically significant predictors of cardiometabolic risk factors and hormone level outcome variables – chapter 4.3.1 (SHBG), 4.3.2 (DHEA-s), 4.3.3 (oestrogen), 4.3.4 (BIOT), and 4.3.5 (FSH).
2) Menopause vs age as a predictor variable of cardiometabolic risk factors and sex-hormone levels – chapter 4.3.6.

3) Anthropometric variables as predictors of cardiometabolic risk factors – chapter 4.3.7.

4.3.1 SHBG as a predictor of weight distribution and sex hormones

In several multiple regression models, SHBG levels were found to be a significant predictor of the outcome variable. The models included BMI (Table 3.5) (Adjusted R=0.167; β=-0.294; b=-0.022; p<0.001), waist circumference (Table 3.7) (Adjusted R=0.791; β=-0.103; b=-0.197; p=0.013), oestrogen (Table 3.20) (Adjusted R=0.284; β=0.247; b=2.418; p=0.001), follicle stimulating hormone (Table 3.21) (Adjusted R=0.476; β=0.195; b=0.163; p=0.002), and bioavailable testosterone (Table 3.22) (Adjusted R=0.256; β=-0.293; b=-0.000; p<0.001). A study by Akin *et al*. found that in pre-menopausal obese women, SHBG correlated negatively with BMI and waist circumference (Akin *et al*., 2009), which is in line with our findings, although the same study did not find any correlations of SHBG with BMI and waist circumference in obese post-menopausal women (Akin *et al*., 2009). The correlation of oestrogen, follicle stimulating hormone, and bioavailable testosterone with SHBG varies in the literature (Pasquali *et al*., 1997). SHBG in menopausal women is expected to decline as oestrogen levels fall (Pasquali *et al*., 1997), which is in line with our findings. Bioavailable testosterone levels are reported to increase as SHBG levels decrease during the menopause transition (Jassal *et al*., 2009), however in our study, BIOT levels are positively correlated with SHBG levels. We also found that FSH levels in post-menopausal women increased drastically, which is expected (Costa *et al*., 2022). SHBG levels were positively correlated with FSH levels in our cohort of both pre- and post-menopausal women.

4.3.2 DHEA-s and its associations with hyperlipidaemia and testosterone

Three multiple regression models indicated correlations between DHEA-s and predictor outcomes. Two of the models, total cholesterol (Table 3.16) (Adjusted R=0.194; β=-0.145; b=-0.104; p=0.046) and HDL-c (Table 3.17) (Adjusted R=0.085; β=-0.192; b=-0.055; p=0.019) appear to be negatively associated with DHEA-s levels. These findings seem to be in line with the results from a study conducted on

hypoadrenal women that were treated with DHEA, resulting in decreased triglycerides, HDL-c, LDL-c and total cholesterol levels (Srinivasan *et al*., 2009).

The fourth model, bioavailable testosterone (Table 3.22) (Adjusted R=0.256; β=-0.352; b=0.021; p<0.001) is indicative of a negative correlation between DHEA-s and bioavailable testosterone levels, which is the opposite of what current literature has reported (Bloch *et al*., 2013). The reason for this discrepancy is not known and would require confirmation in a larger sample size.

4.3.3 The correlations between oestrogen, systolic blood pressure and follicle stimulating hormone levels

Oestrogen appears to be negatively correlated with systolic blood pressure (Table 3.12) (Adjusted R=0.166; β=-0.182; b=-0.008; p=0.033), and a more pronounced negative relationship with follicle stimulating hormone (Table 3.21) (Adjusted R=0.476; β=-0.346; b=-0.030; p<0.001).

In menopausal women, oestrogen levels decline, which generally result in an increase in vascular stiffness of the great arteries, as well as atherosclerotic plaques in the vessel wall (Maas and Franke, 2009). As the oestrogen-androgen ratio declines, the vasorelaxant effects of oestrogens on the vessel wall are weakened, which then promotes the production of vasoconstrictive factors instead (Maas and Franke, 2009). This in turn leads to increased blood pressure in menopausal women (Maas and Franke, 2009). Our study found a negative relationship between oestrogen and systolic blood pressure, as expected.

The relationship between oestrogen and follicle stimulating hormone over the menopausal transition is well documented. As oestrogen levels decline in response to the decrease in the number of mature follicles producing oestrogen, FSH increases in an attempt to counteract this (Reed and Carr, 2018). Our participants followed the same relationship.

4.3.4 Associations of bioavailable testosterone with diastolic blood pressure, HDL-c, and SHBG

Bioavailable testosterone is positively correlated with the outcome variables of the diastolic blood pressure model (Table 3.13) (Adjusted R=0.177; β=0.151; b=23.366; p=0.049), the HDL-c model (Table 3.17) (Adjusted R=0.085; β=0.196; b=0.938; p=0.020), and negatively associated in the SHBG model (Table 3.23) (Adjusted R=0.182; β=-0.313; b=-196.577; p<0.001).

Conversely, Yang *et al*. showed that bioavailable testosterone was negatively correlated with diastolic blood pressure in a study conducted on men (Yang *et al*., 2019). They and others suggest that low bioavailable testosterone levels disrupt cardiac relaxation, leading to elevated diastolic blood pressure levels (Banga *et al*., 2021). Our study did not support this hypothesis.

An increase in bioavailable testosterone influences HDL-c levels positively according to a study conducted on men with type 2 diabetes (Stanworth *et al*., 2007). Although the findings of this study correlate with these findings, the study was conducted on males in diseased states, so this may not be a true reflection of the same relationship in women.

Bioavailable testosterone and SHBG are known to have a negative relationship (Goldman *et al*., 2017), as SHBG binds to free testosterone, rendering them unavailable. Thus, high levels of SHBG decrease bioavailable testosterone (Goldman *et al*., 2017). The findings of this study are supported by the abovementioned literature.

4.3.5 Follicle stimulating hormone as a predictor of HDL-c

One of the significant predictors of the HDL-c model was FSH (Table 3.17) (Adjusted R=0.085; β=0.203; b=0.002; p=0.032), which depicted a positive correlation between the two variables.

According to a 2019 study by Serviente and collaborators, a weak positive correlation between FSH levels and HDL-c was also found in post-menopausal women (Serviente *et al*., 2019), providing literature support for the findings of this study.

4.3.6 Comparison between menopause and age as predictors of outcome variables

There are two factors that create the distinct differentiation between our two study groups – menopausal status and age. The average age of the women in the post-menopausal group is, as expected, older than that of the pre-menopausal group. Increasing levels of cardiometabolic risk factors can be attributed to age, not necessarily menopausal status, and vice versa (Nappi *et al*., 2022), with women experiencing the onset of menopause at an earlier age (<45) demonstrating an elevated risk for CVD (Uddenberg *et al*., 2024). There is little information available that sheds light on the 'menopause or age' conundrum, hence our study sought to differentiate between menopause and age as predictors of cardiometabolic, anthropometric and sex hormone outcome variables in a West African cohort. To our knowledge, this information is the first of its nature in the specified cohort.

Out of our 19 models, six models were significantly correlated with menopausal status, whilst four models were significantly correlated with age. Of these 10 models, one model was significantly correlated with both menopause and age. The remaining 9 models were not associated with either menopause or age.

The models (outcome variables) that were correlated with menopausal status (pre- or post-menopausal) included diastolic blood pressure (Table 3.13) (Adjusted R=0.177; β=0.195; b=4.005; p=0.023), eGFR (Table 3.15) (Adjusted R=0.035; β=0.326; b=7.030; p=0.027), LDL-c (Table 3.19) (Adjusted R=0.113; β=0.200; b=0.256; p=0.020), follicle stimulating hormone (Table 3.21) (Adjusted R=0.476; β=0.466; b=32.717; p<0.001), and bioavailable testosterone (Table 3.22) (Adjusted R=0.256; β=0.376; b=0.050; p=0.004) which were positively associated with menopausal status, and visceral fat (Table 3.8) (Adjusted R=0.158; β=-0.281; b=-0.658; p=0.049), and

oestrogen (Table 3.20) (Adjusted R=0.284; β=-0.470; b=-386.424; p<0.001) which were negatively correlated with menopausal status

The outcome variables associated with age were cIMT – positive (Table 3.14) (Adjusted R=0.063; β=0.276; b=0.009; p=0.048), fasting blood glucose – positive (Table 3.10) (Adjusted R=0.206; β=0.361; b=0.070; p=0.006), and eGFR – negative (Table 3.15) (Adjusted R=0.035; β=-0.500; b=-1.553; p<0.001).

The model with both menopausal status and age as significant predictors was eGFR (Table 3.15), with a positive relationship with menopausal status and a negative relationship with age.

These findings complement the analyses in chapter 3.1 (Table 3.1) comparing pre- and post-menopausal groups. In this particular group of women from Nanoro, Burkina Faso in West Africa, increases in diastolic blood pressure, eGFR, LDL-c, FSH and bioavailable testosterone, along with a decrease in visceral fat and oestrogen are associated with menopausal status. When comparing pre- and post-menopausal groups in Table 3.1, statistically significant differences were found in six of the above outcome variables, with the exception of bioavailable testosterone.

We also found that a larger carotid intima-media thickness, higher fasting blood glucose levels and lower estimated glomerular filtration rates in our cohort were significantly correlated with increasing age. These findings are also well documented in literature. cIMT has previously been strongly associated with age in a 2018 study (van den Munckhof *et al*., 2018). It is also generally accepted that glomerular filtration rate decreases as we age (Glassock and Winearls, 2024). Interestingly, eGFR was also positively correlated with menopausal status as well, which does not follow the general trend of decreasing eGFR in menopausal women. However, it must be noted that the association of eGFR with age was far stronger than that with menopausal status.

These data clearly show that changes in cardiometabolic risk factors over time are due to the effects of aging and in some instances by menopause, and such findings are well documented in the literature.

4.3.7 Cardiometabolic associations with measures of anthropometry

Within the group of cardiometabolic risk factors, fasting blood glucose, systolic blood pressure, diastolic blood pressure, carotid intima media thickness, total cholesterol and triglycerides all correlated positively and significantly with anthropometric variables in a multiple regression model. These associations are not unexpected and show that although the prevalence of obesity is very low in this population, adiposity is still an important modulator of cardiometabolic disease risk and that any future increase in the prevalence of obesity in this population may lead to higher levels of these diseases.

4.4 Limitations of the study

Epigenetic studies are complex, and epigenetic signals are developmental stage and cell type specific. Although blood is often used and is an appropriate and useful tissue for studying many conditions, it has limitations. These limitations also depend on the biological processes being studied. Ideally researchers should examine multiple epigenetic mechanisms (DNA methylation, histone modifications, chromatin conformation and microRNAs) and their impact on both gene expression (RNA levels) and the quantity of resulting protein. This is however seldom possible, especially when leveraging existing studies that did not collect all the relevant samples. In this study we had access only to blood derived genomic DNA and serum and plasma for measuring selected biomarkers, we did not have access to RNA and could therefore not associate DNA methylation of gene targets with modulation of gene expression.

Other functional studies, that are primarily conducted in animal models, such as cellular models, organoids and potentially gene editing, could also have been carried out to determine causality of differential gene expression by quantifying mRNA of

ESR2, *AR*, *FSHR*, *SHBG*, and *INHBB* genes with real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to determine whether differential methylation influenced gene expression.

Furthermore, the potential association of *ESR2* genetic variants (SNPs) and bioavailable testosterone levels was reported in a study published in 2008 by Peter *et al*. This may have added an interesting parameter to our study, however, we did not collect any genetic variant (SNP) data aside from DNA methylation data.

The small sample size for the study, with just 99 pre-menopausal and 98 post-menopausal participants, affected the power of the study to detect modest differences between groups. However, it is a unique dataset and the decision to only include women from one geographic location meant that the effect of several potential confounders was minimised, such as diet, behavioural and cultural practices and background genetic variation.

In order to avoid overfitting our models, the number of predictors was limited to 12. For each model, the same 9 predictors were modelled, with the possible addition of three methylation variables. However, after adjusting for multiple testing, none of the methylation variables were included in any of the models. Although this rules out our potential bias issue of decreasing the number of input variables based on p-values, instead of penalised regression approaches, there was no representation of methylation data in the data models despite it being the primary focus of this study.

As low birth weight is a well documented predictor of cardiometabolic risk in later life, it would have greatly supplemented this study had birth weight data been collected. Unfortunately, this is often not the case in low and middle income countries, such as our study site.

The women in our study were classified as pre-menopausal and post-menopausal based on self-report, which is a less precise way of determining menopausal status. However, based on oestrogen (high in pre-menopausal and low in post-menopausal women) and follicle stimulating hormone (low in pre-menopausal and high in post-menopausal women) levels, it seems the women were classified correctly.

In terms of study design, a longitudinal study instead of a cross-sectional cohort study would have added much value. A longitudinal study would also have provided information on the development of CMD in post-menopausal women to further inform the hypothesis of a link between epigenetic mechanisms of hormonal changes and how these affect CMD risk.

4.5 Recommendations for implementation and future work

Designing the initial bioinformatics stage, the laboratory protocol, and even the secondary bioinformatics analyses, as well as the method for selection of CpG sites proved to be less straightforward than initially anticipated. Current literature does not provide clear guidelines for performing targeted DNA methylation research, making it less attractive for researchers to undertake such studies. To successfully carry out the laboratory and secondary bioinformatics portion of the study, we sought assistance from various specialists in South Africa, Dubai and the United States of America. Publication of works arising from this study will potentially encourage other researchers to pursue targeted methylation research as a result of the clear description of methodology, high quality of data generated (~50 000x sequencing coverage) in this study, and the affordability of targeted methylation studies in comparison with epigenome wide association studies (EWAS).

From a research perspective, more epigenetic data originating from underrepresented African populations is necessary. To complete the picture and better our understanding of the role of differential methylation in menopausal African women, more genes need to be studied to complement our study. A different set of bisulfite primers should be designed and optimised for oestrogen receptor α (*ESR1*) that was

excluded from our study, along with primers for G protein-coupled oestradiol receptor (*GPER1*), aromatase (*CYP19*), Type 1 17-β-hydroxysteroid dehydrogenase (*HSD17B1*), and anti-Müllerian hormone (*AMH*) to further epigenetics research in CMD risk in menopausal African women. These studies should also be repeated on a larger scale in several African countries to determine whether any population-specific differential methylation patterns exist.

Other methods to study methylation are the EPIC array, however this would be non-hypothesis driven as it would investigate DNA methylation of ~850 000 CpG sites across the genome. It may highlight unanticipated methylation signals in multiple genes, which can be confirmed by targeted methylation analyses.

There is a need for targeted methylation studies with a functional component to be conducted, so that causality, if differential gene expression is modulated by DNA methylation, can be confirmed. As compared to epigenome wide association studies (EWAS), targeted methylation approaches are far less costly to execute, especially is lower income countries. Developing a sound targeted methylation approach ensures that, down the line, genomic medicine is accessible to more people.

To ensure that the body of research being generated is robust, associations with other epigenetic mechanisms such as histone modifications, chromatin remodelling and noncoding RNAs (Mohana Devi *et al*., 2020), as well as additional cardiometabolic and sex hormone regulatory genes should be investigated. Such studies may have the potential to inform epigenome editing technologies much like the Coupled Histone tail for Autoinhibition Release of Methyltransferase (CHARM) (Neumann *et al*., 2024) that can be administered alone, or in conjunction with existing human genome editing approaches (CRISPR-cas9). This will contribute new knowledge to advance our understanding of the role of epigenetics in cardiometabolic disease risk in midlife women in an African cohort.

4.6 Conclusion

To conclude, although the increase in cardiometabolic disease risk in menopausal women has been frequently documented, not many studies have been conducted specifically on African populations. There is also a need to fully elucidate the mechanisms behind sex hormone regulation during the menopausal transition and how these may relate to cardiometabolic disease risk. It is a reasonable assumption that epigenetic modifications of sex hormone regulatory genes modulate sex hormone levels, but we do not know how they influence CMD risk. Furthermore, little research has been conducted in African populations, leading to a gap in the literature.

This study sought to assess whether DNA methylation of genes involved in sex hormone regulation modulates hormone levels, leading to increased cardiometabolic disease risk in post-menopausal women from West Africa when compared with a pre-menopausal group. We found DNA methylation levels did not differ between pre- and post-menopausal women and did not associate with sex hormone levels. Thus, epigenetic mechanisms at the level of DNA methylation may not be associated with the menopause transition. We did not observe associations between gene methylation and any of the anthropometric, cardiometabolic or hormonal variables suggesting that epigenetics may not play a role in the modulation of cardiometabolic risk factors in midlife women.

More studies are required on other genes that control sex hormone levels in order to definitively show that epigenetic mechanisms do not associate with the menopause transition. Functional studies would also be required to confirm causality. The present study represents the first step toward linking epigenetics with the aetiology of menopause-related increases in cardiometabolic disease risk factors and we hope that this study will encourage others to move the field further.

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APPENDICES

Appendix A

Appendix A(i) Human and Animal Research Ethics Declaration.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

University of the Witwatersrand Student Ethics Declaration Form

(To be completed during the protocol assessor meeting)

Background

All Research conducted by a University of the Witwatersrand student, with human subjects or animals, requires approval by the Wits Human Research Ethics Committee (Medical) or Animal Research Ethics Committee, respectively.

If research has been undertaken without the necessary ethics approvals, this is considered an ethics violation. This will be reported to the relevant structures, the data will have to be discarded, and in the case of students, they cannot use the data towards their degree.

To prevent any ethics violations, the ethics requirements for the proposed project will be discussed with you at the protocol assessment.

Declaration

Based on the current protocol assessment (and any proposed changes suggested by the assessor committee), we, the undersigned, understand that the proposed research requires:

1. Human Research Ethics clearance certificate	(Yes)	No
a. Covered under existing supervisor ethics	Yes	(No)
b. Requires a new HREC (Medical) application	(Yes)	No
2. Animal Research Ethics clearance certificate	Yes	(No)
3. No Human or Animal Ethics Clearance	Yes	(No)
4. Unclear, will seek appropriate guidance from the HREC/AREC committees (whichever applicable)	Yes	(No)

Signatures

Supervisor/s: _____ Student: _____

Date: 17 April 2020

11 March 2019/MP

Appendix A(ii) Human Research Ethics Committee (Medical) Clearance Certificate No. M2010127 for AWI-Gen Phase 1 sub-study.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms M Naidoo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) CLEARANCE CERTIFICATE NO. M2010127

NAME: (Principal Investigator)	Ms M Naidoo
DEPARTMENT:	School of Pathology Department of Human Genetics Sydney Brenner Institute for Molecular Bioscience Medical School University
PROJECT TITLE:	*Epigenetic associations with sex hormone levels and cardiometabolic disease risk factors in pre- and post-menopausal women from West Africa* Change in study title noted on 2023/06/30
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M 170880 and M 180535
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Profs M Ramsay & NJ Crowther; Dr NG Mahyoodeen
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/11/19 **EXPIRY DATE:** 2025/11/18

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **October** and therefore reports and re-certification will be due in the month of **October** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator — Date: 30/06/2023

Appendix A(iii) Human Research Ethics Committee (Medical) Clearance Certificate No. M121029 for AWI Gen Phase 1 Study.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Professor Michele Ramsay

CLEARANCE CERTIFICATE	**M121029**
PROJECT	WITS-INDEPTH Partnership: Genomic and Environment Risk Factors for Cardiometabolic Disease in Africans
INVESTIGATORS	Professor Michele Ramsay.
DEPARTMENT	School of Pathology/Division of Human Genetics
DATE CONSIDERED	26/10/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 21/11/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/~~We~~ fully understand the conditions under which I am/~~we~~ are authorized to carry out the abovementioned research and I/~~we~~ guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/~~we~~ undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

M. Ramsay 11 April 2013

Appendix A(iv) Human Research Ethics Committee (Medical) Clearance Certificate No. M170880 for renewal of AWI-Gen Phase 1 Study.

R14/49 Prof Michele Ramsay

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170880

NAME: (Principal Investigator)	Prof Michele Ramsay
DEPARTMENT:	School of Pathology/Division of Human Genetics National Health Laboratory Services
PROJECT TITLE:	Wits: INDEPTH Partnership - Genomic and Enviromental Risk Factors for Cardiometabolic Disease in African Populations
DATE CONSIDERED:	03/10/2014(Initial Approval 24/08/2017)
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for 5 Years Valid for the Period 01 September 2017 - 30 September 2022 (Previously M121029)
SUPERVISOR:	
APPROVED BY:	CB Penny Professor CB. Penny Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	12/09/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially review September and will therefore be due in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

M. Ramsay
Principal Investigator Signature

Date 18 September 2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix A(v) Amendment to title and bioinformatics approach of protocol no. M2010127.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2023/06/30

Ms M Naidoo
School of Pathology
Department of Human Genetics
Sydney Brenner Institute for Molecular Bioscience
Medical School
University

Sent by e-mail to: merusha.naidoo@yahoo.com

Dear Ms Naidoo

Re: **Protocol Ref No:** M2010127
Protocol Title: *Epigenetic associations with sex hormone levels and cardiometabolic disease risk factors in pre- and post- menopausal women from West Africa Change in study title noted on 2023/06/30*
Principal Investigators: Ms M Naidoo

Thank you for your e-mail of 2023/03/15 and the more recent copy of the Faculty's approval of a change of protocol title, dated 2023/05/18.

We note and approve of your proposal to use the *Illumina DRAGEN Methylation Pipeline* as your sequencing tool of choice.

We attach an updated Clearance Certificate, to refet the new study title.

Thank you for keeping us informed.

Yours Sincerely

J. Burns
.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

C.B Penny
.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

Appendix A(vi) AWI-Gen Data Access and Use Agreement for AWI-Gen Affiliated users.

This agreement stipulates the conditions of data access and use of the AWI-Gen data (The data), including participant-associated traits that will be examined: e.g. demographic, phenotype, behaviour, biomarker and genomic data.

Project Title: Epigenetic associations with sex hormone levels and cardiometabolic disease risk factors in pre- and post-menopausal women from West Africa

SPECIFIC PROJECT

Introduction and Justification for Study

Menopause is the permanent ceasing of ovarian function in women, which in turn signifies the discontinuation of ovulation and reproductive potential. Although menopause is an inevitable process that lasts only for a limited period, the effect of changes in hormone levels extends far beyond that of the duration of menopause. Changes in hormone levels due to the menopausal transition have been found to promote the progression of disorders related to ageing, such as cardiometabolic diseases (CMD) (cardiovascular disease (CVD), hypertension, stroke, dyslipidaemia, type 2 diabetes mellitus), osteoporosis and breast cancer. The genetic mechanism behind changes in hormone levels is not known. Across the menopausal transition, oestrogen production decreases, which may lead to a decrease in the expression of oestrogen receptors as a result of a lack of substrate to bind to. Expression of ESR1 and ESR1 may be regulated by DNA methylation of the *ESR1* and *ESR2* genes. For this study, liquid chromatography-mass spectrometry (LC-MS) analyses of sex hormone levels will be available for comparison with DNA methylation of genes involved in sex hormone regulation. Along with DNA methylation and liquid chromatography-mass spectrometry data, cardiometabolic and anthropometric data have also been collected from each study participant.

Aim(s)

This study aims to explore a potential epigenetic mechanism (DNA methylation) in the modulation of sex hormone levels and associated risk for cardiometabolic diseases in post-menopausal women from West Africa.

Proposed Methodology and Expected Outcomes

Buffy coat-derived DNA will undergo bisulfite modification for NGS, which will be performed using the Iseq100. Data will be analysed with DNA methylation tools, and statistical analyses will be carried out, comparing % CpG methylation with CMD risk factors and hormone levels obtained from a fellow PhD student. DNA hypermethylation is expected to correlate with decreased hormone levels and hypomethylation with increased hormone levels. CMD risk factors are expected to increase in line with differential methylation across the menopausal transition.

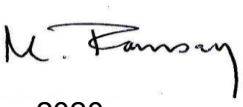
By signing this document, I agree to the following conditions:

1. I will use The Data and analyses only for the agreed purpose
2. I will only use the data for analysis on the Wits cluster and I will not copy the data to another location without prior written consent
3. I will not post the data publicly
4. I will not distribute the data to any third party
5. I will make no attempt to connect the data back to personal identifying information from any participants in the study
6. I agree that I will not publish any analysis or work based on the data without written permission from the AWI-Gen PI
7. I will not write and describe the study outcomes in such a way that will cause damage or distress, or that could be potentially stigmatizing to any data participants
8. I will appropriately acknowledge all the funders in publications resulting from these data as instructed by the PI

9. I agree to provide an update on my progress to the supervisory group as requested
10. Unless otherwise approved, I will delete data and analyses that may be stored on my personal computer on the occasion that I leave the AWI-Gen study
11. In accordance with the Wits IP policy, I understand that I have no claim to intellectual property emanating from the work I perform on these data

I understand and acknowledge that the data are experimental in nature, and that access to the data is provided without any representations or warranties of any kind in relation to the database or the data.

Should I have any questions or concerns I will contact the AWI-Gen PI or project supervisors.

Data Recipient	**AWI-Gen PI**
Name: Ms Merusha Naidoo	Name: Professor Michèle Ramsay
Email: merusha.naidoo@yahoo.com	Email: michele.ramsay@wits.ac.za
Signature: 	Signature: 
Date: 25 June 2020	Date: 26 June 2020

v. 5 August 2019

Appendix A(vii) AWI-Gen Phase 1 Participant Questionnaire for the Nanoro site at the Clinical Research Unit of Nanoro (CRUN), Nanoro, Burkina Faso, West Africa (SSA).

	AWI-Gen H3Africa Genomic and Environmental Risk Factors for Cardiometabolic Disease in Africans Nanoro

AWI-Gen Study Number: |__|__|__|__||__|	**Unique Site Identifier :** |__|__|__|__||__|__|__|

BARCODE STICKER
Please stick the barcode sticker here

1. GENERAL INFORMATION		
1.1	Data collection date	|d|d||m|m|m||y|y|y|y|
1.2	Interviewer code	_____
1.3	Start time	|h|h|:|m|m| *[Based on a 24 hour clock eg. 15:30]*

2. DEMOGRAPHIC INFORMATION		
2.1	Date of birth known? *[If no, skip to Q.2.3]*	☐ Yes ☐ No
2.2	Date of Birth *[eg. 27 SEP 1957]*	|d|d||m|m|m||y|y|y|y|
2.3	Approximate year of birth	|1|5||J|U|N||y|y|y|y|
2.4	Gender	☐Female ☐Male *[please tick the appropriate box]*
2.5	Country	**Burkina Faso**
2.6	Home language *[please tick the appropriate box]*	☐ Mooré ☐ Bissa ☐ Dyula ☐ Fula ☐ English ☐ French ☐ Unknown ☐ Other (specify)_____
2.7	Ethnic/tribal affiliation *[please tick the appropriate box]*	☐ Mossi ☐ Fula ☐ Gurma ☐ Bobo ☐ Gurunsi ☐ Senufo ☐ Lobi ☐ Tuareg ☐ Dyula ☐ Unknown ☐ Other (specify)_____

2.8. Family Ethnicity ***This question relates to the ethnicity and home languages of your parents and grandparents.***

Please tick the applicable columns

	Father		Paternal Grandfather		Paternal Grandmother		Mother		Maternal Grandfather		Maternal Grandmother	
Ethnicity/Language	E	L	E	L	E	L	E	L	E	L	E	L
Afrikaans												
English												
Mossi												
Fula												
Gurma												
Bobo												
Gurunsi												
Senufo												
Lobi												
Tuareg												
Dyula												
Unknown												
Other												

If other, please specify

Father __________ **PG\father** __________ **PG\mother** __________
Ethnicity Language *Ethnicity Language* *Ethnicity Language*

Mother __________ **MG\father** __________ **MG\mother** __________
Ethnicity Language *Ethnicity Language* *Ethnicity Language*

3. FAMILY COMPOSITION		
3.1	Do you have any siblings* with which you share at least one parent? ***half siblings and those that have passed away are included***	☐ Yes ☐ No ***[If no, skip to Q.3.4]***
3.2	How many brothers do you have?	____
3.3	How many sisters do you have?	____

3.4	Do you have any biological children?	☐ Yes ☐ No *[If no, skip to the next section- Phenotypic Data Collection]*
3.5	How many biological sons do you have?	____
3.6	How many biological daughters do you have?	____

PHENOTYPIC COLLECTION DATA

4. PREGNANCY [If male, please skip to the next section - Marital Status]		
4.1	Are you pregnant?	☐ Yes ☐ No
4.2	How many pregnancies have you had? *[If none, please skip to Q.4.4]*	____
4.3	How many live births have you had?	____
4.4	Do you have regular (28-35 day) periods?	☐ Yes ☐ No ☐ NA
4.5	Date of last period	_m_m__y_y_y_y_

5. MARITAL STATUS		
5.1	Marital Status *[please tick the appropriate box]*	☐ Married ☐ Living together ☐ Never married or co-habited ☐ Divorced with a living partner ☐ Separated with a living partner ☐ Partner deceased

6. EDUCATION		
6.1	Highest level of Education *[please tick the appropriate box]* *[If "no formal education" please skip to Q.7]*	☐ No formal education ☐ Primary ☐ Secondary ☐ Tertiary* **tertiary education includes qualifications such as certificates, diplomas or degrees*
6.2	Total number of successfully completed years at highest level of education	____ years

7. EMPLOYMENT		
7.1	Employment	☐ Self employed ☐ Formal full-time employment by someone else ☐ Part-time employment by someone else ☐ Informal employment (dependent on availability of work) ☐ Unemployed

8. HOUSEHOLD ATTRIBUTES

8.1. How many people besides you are in your household? |__|__| **people**

This applies to people who sleep in separate buildings on the property but share their main meal in the same house as you. If none, please skip to Q.8.3.

8.2.	Name	Sex	Age (years)	Relationship to you (eg. father, husband, daughter, etc.)
	1.	☐ Male ☐ Female	|__|__|__|	
	2.	☐ Male ☐ Female	|__|__|__|	
	3.	☐ Male ☐ Female	|__|__|__|	
	4.	☐ Male ☐ Female	|__|__|__|	
	5.	☐ Male ☐ Female	|__|__|__|	
	6.	☐ Male ☐ Female	|__|__|__|	
	7.	☐ Male ☐ Female	|__|__|__|	
	8.	☐ Male ☐ Female	|__|__|__|	
	9.	☐ Male ☐ Female	|__|__|__|	
	10.	☐ Male ☐ Female	|__|__|__|	
	11.	☐ Male ☐ Female	|__|__|__|	
8.2.	**Name**	**Sex**	**Age (years)**	**Relationship to you (eg. father, husband, daughter, etc.)**
	12.	☐ Male ☐ Female	|__|__|__|	

8.3	How many rooms are there in the house and outside structures used by household members?	____ rooms
8.4	How many rooms are used for sleeping in?	____ rooms

8.5	Which of the following items in working order, do you have in your household at the present time?			
1	Electricity	☐ Yes	☐ No	☐ Don't know
2	Solar energy	☐ Yes	☐ No	☐ Don't know
3	Power generator	☐ Yes	☐ No	☐ Don't know
4	Alternative power source	☐ Yes	☐ No	☐ Don't know
5	Television	☐ Yes	☐ No	☐ Don't know
6	Radio	☐ Yes	☐ No	☐ Don't know
7	Motor vehicle	☐ Yes	☐ No	☐ Don't know
8	Motorcycle	☐ Yes	☐ No	☐ Don't know
9	Bicycle	☐ Yes	☐ No	☐ Don't know
10	Refrigerator	☐ Yes	☐ No	☐ Don't know
11	Washing machine	☐ Yes	☐ No	☐ Don't know
12	Sewing machine	☐ Yes	☐ No	☐ Don't know
13	Telephone	☐ Yes	☐ No	☐ Don't know
14	Mobile phone	☐ Yes	☐ No	☐ Don't know
15	Microwave	☐ Yes	☐ No	☐ Don't know
16	DVD player	☐ Yes	☐ No	☐ Don't know
17	Satellite TV or DSTV	☐ Yes	☐ No	☐ Don't know
18	Computer or laptop	☐ Yes	☐ No	☐ Don't know
19	Internet by computer	☐ Yes	☐ No	☐ Don't know
20	Internet by mobile phone	☐ Yes	☐ No	☐ Don't know
21	Electric or gas stove	☐ Yes	☐ No	☐ Don't know
22	Toilet facilities	☐ Yes	☐ No	☐ Don't know
23	Cattle	☐ Yes	☐ No	☐ Don't know
24	Other livestock *	☐ Yes	☐ No	☐ Don't know
25	Poultry **	☐ Yes	☐ No	☐ Don't know
8.5	Which of the following items in working order, do you have in your household at the present time?			
26	Tractor	☐ Yes	☐ No	☐ Don't know
27	Plough	☐ Yes	☐ No	☐ Don't know

*** other livestock includes donkeys, goats, sheep and pigs**

** poultry includes ducks, chickens, geese and other fowl

9. SUBSTANCE USE

9.1. Tobacco use		
9.1.1	Have you ever smoked any tobacco products such as cigarettes, cigars or pipes? *[If no, skip to Q.9.1.8]*	☐ Yes ☐ No
9.1.2	Do you *currently smoke any tobacco products, such as cigarettes, cigars or pipes? **We are asking if they smoke when they have the opportunity to do so.* *[If no, skip to Q.9.1.6]* *[If yes, please do not answer Q.9.1.7]*	☐ Yes ☐ No
9.1.3	What do you smoke? *[please tick the appropriate boxes]*	☐ Cigarettes ☐ Pipe ☐ Hand rolled ☐ Cigars ☐ E-cigarettes
9.1.4	How often do you smoke tobacco products?	☐ Daily ☐ 5-6 days per week ☐ 1-4 days per week ☐ 1-3 days per month ☐ Less than once per month
9.1.5	On the days that you smoke, how many tobacco products do you smoke?	|__|__|__|
9.1.6	How old were you when you first started smoking?	|__|__| years old
9.1.7	When did you stop smoking completely?	|y|y|y|y|
9.1.8	Have you ever used any smokeless tobacco such as snuff, snus, betel with tobacco or chewing tobacco? *[If no, skip to Q.9.2]*	☐ Yes ☐ No
9.1.9	Do you use snuff? *[If no, skip to Q.9.1.13]*	☐ Yes ☐ No
9.1.10	How do you take snuff?	☐ Through your nose ☐ Through your mouth/on your lip
9.1.11	How often do you use snuff?	☐ Daily ☐ 5-6 days per week ☐ 1-4 days per week ☐ 1-3 days per month ☐ Less than once per month
9.1.12	On the days that you use snuff, how many times a day do you use it?	☐ Once a day ☐ Twice a day ☐ Three times a day ☐ More than three times a day

9.1.13	Do you use chewing tobacco? *[If no, skip to Q.9.2]*	☐ Yes ☐ No
9.1.14	How often do you use chewing tobacco?	☐ Daily ☐ 5-6 days per week ☐ 1-4 days per week ☐ 1-3 days per month ☐ Less than once per month
9.1.15	On the days that you use chewing tobacco, how many times a day do you use it?	☐ Once a day ☐ Twice a day ☐ Three times a day ☐ More than three times a day

9.2. Alcohol use		
9.2.1	Have you ever consumed an alcoholic drink such as beer, wine, spirits, or dôlo? *[If "no" or "don't know", skip to Q.9.3]*	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.2	Do you currently (in the last 30 days) consume any alcoholic drink such as beer, wine, spirits, or dôlo? *[If no, skip to Q.9.2.10]*	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.3	How often do you have at least one alcoholic drink?	☐ Daily ☐ 5-6 days per week ☐ 1-4 days per week ☐ 1-3 days per month ☐ Less than once per month
9.2.4	On the days that you drink alcoholic drinks, how many alcoholic drinks do you have? USE CARDS TO SHOW STANDARD DRINKS	__|__|
9.2.5	Have you ever felt that you should cut down on your drinking?	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.6	Have people annoyed you by criticising your drinking?	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.7	Have you ever felt bad or guilty about your drinking?	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer

9.2.8	Have you ever had an alcoholic drink first thing in the morning to steady your nerves or get rid of a hangover?	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.9	In the past year, did you ever take 6 or more alcoholic drinks in a single morning, afternoon, or night? I understand that you may share drinks and that some drinks have different sizes, but please do your best to answer. USE CARDS TO SHOW STANDARD DRINKS.	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer
9.2.10	What type of alcoholic beverage do you, or did you, usually drink?	☐ Beer ☐ Wine ☐ Spirits ☐ Home brew ☐ Other (specify) _____

9.3. Drug use		
9.3.1	Do you, or have you ever taken marijuana, heroin, methamphetamines, cocaine or any other drugs (dagga, tik, glue, rocks, crack, buttons, mandrax, acid)?	☐ Yes ☐ No ☐ Don't know ☐ Refuse to answer

10. GENERAL HEALTH

10.1. Please indicate whether you have, or have had, any of the following illnesses

[Please tick the appropriate boxes] [If male, please proceed to question 10.1.3] [If female, please skip question 10.1.3]

10.1.1	Breast cancer ***[for females only]***	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.1.2	Cervical cancer ***[for females only]***	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.1.3	Prostate cancer ***[for males only]***	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.1.4	Other cancers	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.1.5	Asthma or reactive air diseases	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.1.6	Have you ever received treatment prescribed by a doctor to treat any of the above illnesses? ***[If no, skip to Q.10.1.9]***	☐ Yes	☐ No	☐ Don't know	
10.1.7	Are you currently on treatment prescribed by a doctor to treat any of the above illnesses? ***[If no, skip to Q.10.1.9]***	☐ Yes	☐ No	☐ Don't know	

10.1.8	What medication has been prescribed? Please list names if possible.	_____ _____
10.1.9	Are you currently taking any herbal or traditional remedy for any of the above illnesses?	☐ Yes ☐ No ☐ Don't know

10.2. Please indicate if your <u>mother</u> has, or has had, any of the following illnesses

[Please tick the appropriate boxes]

10.2.1	Weight problem/obesity	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.2	High blood pressure	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.3	High cholesterol	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.4	Breast cancer	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.5	Cervical cancer	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.6	Other cancers	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.2.7	Asthma or reactive air diseases	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer

10.3. Please indicate if your <u>father</u> has, or has had any of the following illnesses

[Please tick the appropriate boxes]

10.3.1	Weight problem/obesity	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.3.2	High blood pressure	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.3.3	High cholesterol	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.3.4	Prostate cancer	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.3.5	Other cancers	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer
10.3.6	Asthma or reactive air diseases	☐ Yes	☐ No	☐ Don't know	☐ Refuse to answer

10.4. Diet ***The following questions ask about what you eat and drink.***		
10.4.1	In a typical week, on how many days do you eat fruit?	__| days
10.4.2	How many servings of fruit do you eat on a typical day? USE CARDS TO SHOW STANDARD SERVINGS	__|__| servings
10.4.3	In a typical week, on how many days do you eat vegetables?	__| days
10.4.4	How many servings of vegetables do you eat on a typical day? USE CARDS TO SHOW STANDARD SERVINGS	__|__| servings
10.4.5	How many meals per week do you buy from a *vendor or takeaway or restaurant? By meal, I mean breakfast, lunch or dinner. ***A vendor is any person or place that sells food***	__|__| meals
10.4.6	In a typical week, on how many days do you eat bread bought from a shop?	__| days
10.4.7	How many slices of bread bought from a shop, do you eat on a typical day? USE CARDS TO SHOW STANDARD SLICES	__|__| slices
10.4.8	How many cans, bottles, or cups of sugary cold drinks (eg. Coke, Pepsi, Fanta, Sprite, etc.), do you drink in a week?	__|__| drinks
10.4.9	How many cans or bottles or cups of fruit juice do you drink in a week? (eg. Liquifruit, Tropicana, Oros, etc.)	__|__| drinks

10.4.10	Has a doctor, nurse, or other healthcare worker ever told you to change your diet (eg. To eat less sugar)?	☐ Yes ☐ No ☐ Don't know
10.4.11	Has a doctor, nurse, or other healthcare worker ever advised you to lose weight?	☐ Yes ☐ No ☐ Don't know

10.5. Exposure to Pesticides		
10.5.1	Do you work with insecticides or pesticides? ***[If no, please skip to Q.10.5.3.]***	☐ Yes ☐ No ☐ Don't know
10.5.2	How long have you been working with insecticides or pesticides?	__|__| years
10.5.3	Do you live close to a farm or region where insecticides or pesticides are used? ***[If no, please skip to Q.11]***	☐ Yes ☐ No ☐ Don't know
10.5.4	Do you know what type of pesticides or insecticides are used, either by you, or within your area? ***[If not yes, please skip to Q.11]***	☐ Yes ☐ No ☐ Don't know
10.5.5	Please list them if possible :	

11. INFECTION HISTORY

11.1. Malaria				
1	Have you ever had Malaria? ***[If no, skip to Q.11.2]***	☐ Yes	☐ No	☐ Don't know
2	Have you had malaria fever in the last month? ***[If no, skip to Q.11.2]***	☐ Yes	☐ No	☐ Not sure
3	Have you travelled to an area with a *high incidence of malaria, in the last 2 months? ****Some areas with a high incidence of malaria include; Angola, Burkina Faso, Cameroon, Cote d'Ivoire, DRC, Ghana, Guinea-Bissau, Liberia, Malawi, Sierra Leone, Togo, Coastal or Western Kenya, Uganda and Zambia***	☐ Yes	☐ No	☐ Not sure

11.2. TB		
11.2.1	Have you ever been told by a doctor, nurse or other healthcare worker that you have TB? *[If no, skip to Q.11.2.7]*	☐ Yes ☐ No ☐ Don't know
11.2.2	Have you been newly diagnosed with TB in the last 12 months?	☐ Yes ☐ No ☐ Don't know
11.2.3	When was it diagnosed?	|y|y|y|y|
11.2.4	Have you ever received treatment for TB prescribed by a doctor, nurse, or other healthcare worker? *[If no, skip to Q.11.2.7]*	☐ Yes ☐ No ☐ Don't know
11.2.5	Are you currently receiving treatment for TB prescribed by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
11.2.6	Have you ever been counselled by a doctor, nurse or other healthcare worker, on how you can avoid passing TB onto others ?	☐ Yes ☐ No ☐ Don't know
11.2.7	Are you currently taking any herbal or traditional remedy for TB?	☐ Yes ☐ No ☐ Don't know

12. CARDIOMETABOLIC RISK FACTORS

12.1. Diabetes		
12.1.1	Has a doctor, nurse, or other healthcare worker ever measured your blood or urine for diabetes (sugar in the blood)?	☐ Yes ☐ No ☐ Don't know
12.1.2	Have you ever been told by a doctor or healthcare worker, that you have diabetes or high blood sugar (outside of pregnancy)? *[The brackets apply only to females]* *[If "no" or "don't know", skip to Q. 12.1.7]*	☐ Yes ☐ No ☐ Don't know
12.1.3	Have you been newly diagnosed with diabetes in the last 12 months?	☐ Yes ☐ No
12.1.4	Have you ever received treatment for diabetes prescribed by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.1.5	Are you currently receiving treatment for diabetes prescribed by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know

12.1.6	Are you doing anything to treat your diabetes (sugar in the blood)? *Note: Insulin injections and pills can't be taken at the same time!*	☐ Insulin injection ☐ Pills (that you swallow) ☐ Special Diet ☐ Weight Loss ☐ Other (specify) _____
12.1.7	Are you currently taking any herbal or traditional remedy for diabetes (sugar in the blood)?	☐ Yes ☐ No ☐ Don't know
12.1.8	Do you have a family history of diabetes? ***[If "no" or "don't know", skip to Q.12.2]***	☐ Yes ☐ No ☐ Don't know

12.1.9. Which of your family members have, or have had diabetes (sugar in the blood)?

	Mother	☐ Yes	☐ No	☐ Don't know
	Father	☐ Yes	☐ No	☐ Don't know
	Brother 1	☐ Yes	☐ No	☐ Don't know
	Brother 2	☐ Yes	☐ No	☐ Don't know
	Brother 3	☐ Yes	☐ No	☐ Don't know
	Brother 4	☐ Yes	☐ No	☐ Don't know
	Sister 1	☐ Yes	☐ No	☐ Don't know
	Sister 2	☐ Yes	☐ No	☐ Don't know
	Sister 3	☐ Yes	☐ No	☐ Don't know
	Sister 4	☐ Yes	☐ No	☐ Don't know
	Son 1	☐ Yes	☐ No	☐ Don't know
	Son 2	☐ Yes	☐ No	☐ Don't know
	Son 3	☐ Yes	☐ No	☐ Don't know
	Son4	☐ Yes	☐ No	☐ Don't know
	Daughter 1	☐ Yes	☐ No	☐ Don't know
	Daughter 2	☐ Yes	☐ No	☐ Don't know
	Daughter 3	☐ Yes	☐ No	☐ Don't know
	Daughter 4	☐ Yes	☐ No	☐ Don't know
	Other, specify :	☐ Yes	☐ No	☐ Don't know

12.2. Stroke*		
**Jones, W.J., Willams, L. S. and Meschia, J. F. (2001) Validating the Questionnaire for Verifying Stroke-Free Status (QVSFS) by neurological history and examination. Stroke, 32(10): 22232-6. Please note that the word, "physician" has been replaced with the phrase, "doctor, nurse or other healthcare worker" to accommodate the AWI-Gen study.*		
12.2.1	Have you ever been told by a doctor, nurse, or other healthcare worker that you have had a stroke? *[If "no" or "don't know", skip to Q. 12.2.3]*	☐ Yes ☐ No ☐ Don't know
12.2.2	When was it first diagnosed? *[Please indicate in which year your stroke was diagnosed]*	|_|_|_|_|
12.2.3	Have you ever been told by a doctor, nurse, or other healthcare worker that you have had a ministroke, or transient ischemic attack (TIA)?	☐ Yes ☐ No ☐ Don't know
12.2.4	Have you ever had sudden painless weakness on one side of your body?	☐ Yes ☐ No ☐ Don't know
12.2.5	Have you ever had sudden numbness or a dead feeling on one side of your body?	☐ Yes ☐ No ☐ Don't know
12.2.6	Have you ever had sudden painless loss of vision in one or both eyes?	☐ Yes ☐ No ☐ Don't know
12.2.7	Have you ever suddenly lost one half of your vision?	☐ Yes ☐ No ☐ Don't know
12.2.8	Have you ever suddenly lost the ability to understand what people are saying?	☐ Yes ☐ No ☐ Don't know
12.2.9	Have you ever suddenly lost the ability to express yourself verbally, or in writing?	☐ Yes ☐ No ☐ Don't know

12.3. Hypertension		
12.3.1	Has a doctor, nurse, or other healthcare worker ever measured your blood pressure?	☐ Yes ☐ No ☐ Don't know

12.3.2	Have you ever been told by a doctor, nurse, or other healthcare worker that you have hypertension (high blood pressure)? *[If "no" or "don't know", skip to Q. 12.3.7]*	☐ Yes ☐ No ☐ Don't know
12.3.3	Have you been newly diagnosed with hypertension in the last 12 months?	☐ Yes ☐ No ☐ Don't know
12.3.4	Have you ever received treatment for hypertension prescribed by a doctor, nurse or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.3.5	Are you currently on treatment for hypertension prescribed by a doctor, nurse or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.3.6	What medicine are you taking for this? Please list if possible.	_____
12.3.7	Are you currently taking any herbal or traditional remedy for hypertension?	☐ Yes ☐ No ☐ Don't know

12.4. Angina*

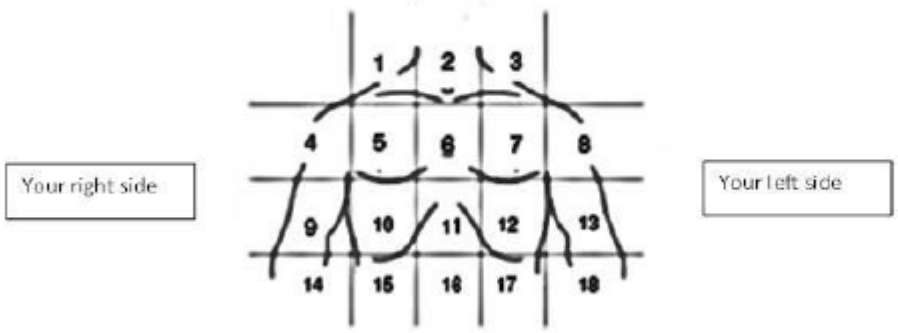
****Angina is characterised as an intense chest pain or discomfort. This pain may occur in the shoulders, arms, neck, back and jaw as well. It may also feel like indigestion. It is considered to be the main symptom of Coronary Heart Disease (CHD).***

12.4.1	Have you ever been told by a doctor, nurse, or other healthcare worker that you have angina (chest pain due to heart disease)? *[If "no" or "don't know", skip to Q. 12.4.5]*	☐ Yes ☐ No ☐ Don't know
12.4.2	Have you ever received treatment for chest pain due to heart disease prescribed by a doctor, nurse or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.4.3	Are you currently taking any medication for angina prescribed by a doctor or other healthcare worker for this?	☐ Yes ☐ No ☐ Don't know
12.4.4	What medicine are you taking for this? Please list if possible.	_____
12.4.5	Are you currently taking any herbal or traditional remedy for angina?	☐ Yes ☐ No ☐ Don't know

12.4.6	During the last 12 months, have you experienced any pain or discomfort in your chest, or pain going to the left arm or neck, when you **walk uphill or hurry**?	☐ Yes ☐ No ☐ Don't know
12.4.7	During the last 12 months, have you experienced any pain or discomfort in your chest, or pain going to the left arm or neck, when you **walk at an ordinary pace on level ground**? ***[If no to both 12.4.6 and 12.4.7, please skip to Q. 12.5]***	☐ Yes ☐ No ☐ Don't know
12.4.8	What do you do if you get the pain or discomfort when you are walking?	☐ Stop or slow down ☐ Rest for a while then carry on ☐ Carry on after taking a pain relief medicine that dissolves in your mouth (a nitro spray or tablet) ☐ Carry on walking
12.4.9	Is the pain or discomfort relieved if you stand still?	☐ Yes ☐ No ☐ Don't know

12.4.10. Will you show me where you usually experience the pain or discomfort?

Please circle the numbers in the box in the area of the body shown by the participant



12.5. Heart Attack		
12.5.1	Have you ever been told by a doctor, nurse, or other healthcare worker that you have had a heart attack? ***[If "no" or "don't know", skip to Q. 12.5.3]***	☐ Yes ☐ No ☐ Don't know
12.5.2	Did you ever receive medical treatment for your heart attack?	☐ Yes ☐ No ☐ Don't know

12.5.3	Are you currently taking any herbal or traditional remedy for your heart attack?	☐ Yes ☐ No ☐ Don't know

12.6. Congestive Heart Failure		
12.6.1	Have you ever been told by a doctor, nurse, or other healthcare worker that you have had heart failure? *[If "no" or "don't know", skip to Q. 12.6.5]*	☐ Yes ☐ No ☐ Don't know
12.6.2	Have you ever received medical treatment for heart failure prescribed by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.6.3	Are you currently on treatment for heart failure prescribed by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.6.4	What medicine are you taking for this? Please list if possible.	_____
12.6.5	Are you currently taking any herbal or traditional remedy for heart failure?	☐ Yes ☐ No ☐ Don't know

12.7. High Cholesterol		
12.7.1	Has a doctor, nurse or other healthcare worker ever measured your cholesterol?	☐ Yes ☐ No ☐ Don't know
12.7.2	Have you ever been told by your doctor or other healthcare worker told you that you have high cholesterol? *[If "no" or "don't know", skip to Q. 12.7.5]*	☐ Yes ☐ No ☐ Don't know
12.7.3	Have you ever been treated for high cholesterol by a doctor, nurse, or other healthcare worker?	☐ Yes ☐ No ☐ Don't know
12.7.4	Are you currently using any of the following to treat your high cholesterol, as prescribed by a doctor, nurse, or other healthcare worker? *[Please tick the appropriate boxes, more than one may be selected]*	☐ Special diet ☐ Weight loss ☐ Medicine ☐ Other
12.7.5	Are you currently taking any herbal or traditional remedy for high cholesterol?	☐ Yes ☐ No ☐ Don't know

13. THYROID DISEASE* **This includes underactive thyroid, overactive thyroid, thyroid nodule enlargement and thyroid cancer*		
13.1	Has a doctor ever told you that you have thyroid disease? *[If no, skip to Q. 13.5]*	☐ Yes ☐ No ☐ Don't know
13.2	Do you know what type of thyroid disease you were diagnosed with? If yes, please specify	☐ Yes ☐ No Specify _____
13.3	Have you ever been treated for it? *[If no, skip to Q. 13.5]*	☐ Yes ☐ No ☐ Don't know
13.4	What treatment did you use?	☐ Thyroid hormone ☐ Surgery ☐ Radioactive iodine ☐ Antithyroid drugs
13.5	Do either of your parents have, or have they had, thyroid disease? If yes, please specify. *[If yes please indicate which parent eg. mother, father or both]*	☐ Yes ☐ No Specify _____

14. KIDNEY DISEASE *The following questions are aligned with the H3Africa Kidney Disease Study*		
14.1	Has a doctor ever told you that you have kidney disease? *[If no, skip to Q. 14.3]*	☐ Yes ☐ No ☐ Don't know

14.2	Do you know what type of kidney disease? If yes please specify.	☐ Yes ☐ No Specify _____
14.3	Has a doctor ever told you that your kidneys have low function?	☐ Yes ☐ No ☐ Don't know
14.4	Has anyone in your family either had kidney disease, or died from it? ***[If no, please skip to Q.15]***	☐ Yes ☐ No ☐ Don't know
14.5	If yes, who? ***["Other" refers to any other blood relatives eg. Sister, aunt etc]***	☐ Mother ☐ Father ☐ Other (specify) _____
14.6	Do you know what kind of kidney disease he or she had? If yes, please specify.	☐ Yes ☐ No Specify _____

15. PHYSICAL ACTIVITY

The following questions are about the time you spend doing different types of physical activities. This includes activities you do at home, at work, travelling from place to place and during your spare time. Work can be paid or unpaid. You are requested to answer the questions even if you don't consider yourself an active person.

15.1.1	How many days do you work per week?	__ days
15.1.2	Do you work over the weekend?	☐ Yes ☐ No

15.2. Occupation-related Physical Activity (paid or unpaid work)

The following questions have been aligned with the validated GPAQ for Physical Activity. Respondents should consider their activity during a usual week. 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. In 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. PLEASE USE THE SHOWCARDS FOR PHYSICAL ACTIVITY TO ANSWER THESE QUESTIONS

15.2.1	Does your work involve mostly sitting or standing still, or walking for very short periods (less than 10 minutes)? ***[If yes, skip to Q. 15.2.8]***	☐ Yes ☐ No

15.2.2	Does your work involve **vigorous** activities (heavy lifting, digging, manual labour or construction) for at least 10 minutes at a time? *[If no, skip to Q.15.2.5]*	☐ Yes ☐ No
15.2.3	In a usual week, how many days are spent doing **vigorous** activities as part of your work?	__ days
15.2.4	On a usual day of **vigorous** work, how many hours are spent doing these activities?	___ hrs ___ mins
15.2.5	Does your work involve **moderate-intensity** activities (brisk walking or carrying light loads) for at least 10 minutes at a time? *[If no, skip to Q.15.3]*	☐ Yes ☐ No
15.2.6	In a usual week, how many days are spent doing **moderate-intensity** activities at work?	__ days
15.2.7	On a usual work day, how many hours are spent doing **moderate-intensity** activities?	___ hrs ___ mins
15.2.8	How long is your usual work day? *[Please indicate how many hours you work per day on average]*	___ hrs ___ mins

15.3. Travel-related physical activity

The following questions have been aligned with the validated GPAQ for Physical Activity. Respondents should consider their activity during a usual week. These questions exclude the physical activities at work that you have already mentioned. These questions are about the usual way you travel to and from places. For example, to work, for shopping, to market, to place of worship. PLEASE USE THE SHOWCARDS FOR PHYSICAL ACTIVITY TO ANSWER THESE QUESTIONS

15.3.1	Do you walk or use a bicycle (for at least 10 minutes at a time) to get to and from places? *[If no, please skip to Q.15.4.]*	☐ Yes ☐ No
15.3.2	In a usual week, how many days do you walk or cycle for at least 10 minutes to get to and from places?	__ days
15.3.3	On a usual day, how many hours do you spend walking or cycling for travel?	___hrs ___ mins

15.4. Non-work related and leisure time Physical Activity

The following questions have been aligned with the validated GPAQ for Physical Activity. Respondents should consider their activity during a usual week. The next questions, exclude the work and transport activities that you have already mentioned, they are about sports, fitness and recreational activities (leisure). PLEASE USE THE SHOWCARDS FOR PHYSICAL ACTIVITY TO ANSWER THESE QUESTIONS

15.4.1	In your spare time, do you engage in any **vigorous** or **moderate-intensity** physical activities lasting more than 10 minutes at a time? *[If no, skip to Q. 15.5]*	☐ Yes ☐ No

15.4.2	In your spare time do you do any **vigorous** activities like running, strenuous sport or exercise for at least 10 minutes at a time? ***[If no, skip to Q.15.4.5]***	☐ Yes ☐ No
15.4.3	In a usual week, how many days do you engage in **vigorous** activities as part of your leisure time?	|__| days
15.4.4	In a normal day, how many leisure hours are spent doing **vigorous** activities?	|__|__| hrs |__|__| mins
15.4.5	In your spare time, do you engage in any **moderately intense** physical activities like walking or swimming for at least 10 minutes at a time? ***[If no, skip to Q.15.5]***	☐ Yes ☐ No
15.4.6	In a normal week, how many days are spent engaging in **moderately intense** physical activity as part of your leisure time?	|__| days
15.4.7	How many leisure hours are spent doing **moderate-intensity** activities in a normal day?	|__|__| hrs |__|__| mins

15.5. Sitting/Resting Activity

The first question has been aligned with the validated GPAQ for Physical Activity. Respondents should consider their activity during a usual week. A usual day is defined as a weekday.

15.5.1	Over the past 7 days, how many hours did you spend sitting or reclining on a usual day (excluding sleep)? This may include time sitting at a desk, visiting friends, reading, or sitting down to watch television during working hours and leisure or spare time.	|__|__| hrs |__|__|mins
The following questions provide a better understanding of sedentary behaviour.		
15.5.2	How many hours per day do you spend **sitting**, while you are at work?	|__|__| hrs |__|__|mins
15.5.3	How many hours do you spend sitting **watching TV** per day, during the **week**?	|__|__| hrs |__|__|mins
15.5.4	How many hours per day, do you spend **watching TV** during the **weekend**?	|__|__| hrs |__|__|mins
15.5.5	How many hours per day, are spent sitting while using a **computer** outside of your normal working hours during the **week**?	|__|__| hrs |__|__|mins
15.5.6	How many hours per day are spent using a **computer** during the **weekend**?	|__|__| hrs |__|__|mins
15.5.7	How many hours per day do you spend sitting (eg. in car, bus, train) while **travelling** from place to place during the **week**?	|__|__| hrs |__|__|mins
15.5.8	How many hours per day are spent sitting while **travelling** from place to place (eg. in car, bus, train) during the **weekend**?	|__|__| hrs |__|__|mins

15.5.9	How many hours per day do you spend sitting while **socialising** during the **week**?	____ hrs ____mins
15.5.10	How many hours per day do you spend sitting while **socialising** over the **weekend**?	____ hrs ____mins
15.5.11	How many hours per day do you spend sitting while **relaxing** during the **week**?	____ hrs ____mins
15.5.12	How many hours per day do you spend sitting while **relaxing** during the **weekend**?	____ hrs ____mins
15.5.13	How many hours per day are spent sitting while at church during the **week**?	____ hrs ____mins
15.5.14	How many hours per day are spent sitting while at church during the **weekend** ?	____ hrs ____mins

16. SLEEP

The following questions relate to how much time is spent asleep per day

[Based on a 24 hour clock eg.15:30]

16.1	What time do you go to sleep during the week?	_h_h_:_m_m_
16.2	What time do you wake up during the week?	_h_h_:_m_m_
16.3	What time do you go to sleep during the weekend?	_h_h_:_m_m_
16.4	What time do you wake up during the weekend?	_h_h_:_m_m_

17.	Time at completion of questionnnaire	_h_h_:_m_m_ ***[Based on a 24 hour clock eg.15:30]***

Please place barcode sticker here	Unique Site Identifier : |__|__|__|__|__|__|__|__|

SAMPLE COLLECTION DATA

1. ANTHROPOMETRIC MEASUREMENTS		
1.1	Standing height	|__|__|__|__| mm
1.2	Weight	|__|__|__|.|__| kg
1.3	Waist circumference	|__|__|__|__| mm
1.4	Hip circumference	|__|__|__|__| mm

2. BLOOD PRESSURE		
2.1	Systolic 1	|__|__|__| mmHg
2.2	Systolic 2	|__|__|__| mmHg
2.3	Systolic 3	|__|__|__| mmHg
2.4	Diastolic 1	|__|__|__| mmHg
2.5	Diastolic 2	|__|__|__| mmHg
2.6	Diastolic 3	|__|__|__| mmHg
2.7	Time blood pressure taken in the first instance *[Based on a 24 hour clock eg.15:30]*	|h|h|:|m|m|

3. PULSE		
3.1	Pulse 1	|__|__|__| beats/min
3.2	Pulse 2	|__|__|__| beats/min
3.3	Pulse 3	|__|__|__| beats/min
3.4	Person performing measurements	_____

3.5	Notes or comments related to measurements	

4. ULTRASOUND MEASUREMENTS		
4.1	Ultrasound	☐ Yes ☐ No
4.2	Visceral (medial) fat	|__|__|.|__|__| cm
4.3	Subcutaneous (transverse) fat	|__|__|.|__|__| cm
4.4	Ultrasound operator name	_____
4.5	Date ultrasound taken *[eg. 27 SEP 1957]*	|d|d| |m|m|m| |y|y|y|y|
4.6	cIMT	☐ Yes ☐ No
4.7	Minimum cIMT on the right	|__|.|__|__| mm
4.8	Maximum cIMT on the right	|__|.|__|__| mm
4.9	Mean cIMT on the right	|__|.|__|__| mm
4.10	Minimum cIMT on the left	|__|.|__|__| mm
4.11	Maximum cIMT on the left	|__|.|__|__| mm
4.12	Mean cIMT on the left	|__|.|__|__| mm

4.13	Notes or comments related to ultrasound measurements	

Please place barcode sticker here	**Unique Site Identifier :** |__|__|__|__|__|__|__|__|

1. BLOOD COLLECTION		
1.1	Fasting blood? *[If YES, skip to Q1.3]*	☐ Yes ☐ No
1.2	At what time did you last eat? *[Based on a 24 hour clock eg.15:30]*	|h|h|:|m|m|
1.3	Fasting confirmed	☐ Yes ☐ No

1.4. *Please complete the following table*

4ml or 6ml Tubes	Number		Reason	Notes
Red	2	☐ Yes ☐ No	lipid	
Purple	2	☐ Yes ☐ No	DNA extraction	
Grey	1	☐ Yes ☐ No	glucose	

1.5	Phlebotomist Name	_____
1.6	Date blood taken *[eg. 27 SEP 1957]*	|d|d||m|m|m||y|y|y|y|
1.7	Time blood taken	|h|h|:|m|m|

2. HIV			
2.1	Have you ever been tested for HIV? *[If "no" or "don't know", skip to Q 2.5]*	☐ Yes ☐ No	☐ Don't know ☐ Refuse to answer
2.2	Do you know your status? *[If "no" or "don't know", skip to Q.2.5.]*	☐ Yes ☐ No	☐ Don't know ☐ Refuse to answer
2.3	Have you ever tested HIV positive? *[If "no" or "don't know", skip to Q.2.5.]*	☐ Yes ☐ No	☐ Don't know ☐ Refuse to answer

2.4	Do you use medication prescribed by a doctor, nurse or healthcare worker to treat it?	☐ Yes ☐ No	☐ Don't know ☐ Refuse to answer
2.5	Are you currently taking any herbal or traditional remedy for HIV?	☐ Yes ☐ No	☐ Don't know ☐ Refuse to answer
2.6	Do you agree to have your blood sample tested for HIV? *[If no, please mark Q. 2.6 as NA]*	☐ Yes	☐ No

2.6	Result	☐ Positive ☐ Negative ☐ NA

3. Test Results *Please complete the table below*		
Test	**Result**	**Notes**
Fasting Plasma Glucose		
Fasting Insulin		
HbA1c		
HDL		
LDL		
Triglycerides		
Total Cholesterol		

4. Urine Collection		
4.1	Urine sample taken	☐ Yes ☐ No

AWI-Gen H3Africa

Genomic and Environmental Risk Factors for Cardiometabolic Disease in Africans

Nanoro

Please place barcode sticker here	**Unique Site Identifier :** __|__|__|__|__|__|__|

CHECKLIST

1	Informed consent	☐ Yes ☐ No
2	Questionnaire	☐ Yes ☐ No
3	Anthropometric measurements	☐ Yes ☐ No
4	Blood pressure	☐ Yes ☐ No
5	Pulse	☐ Yes ☐ No
6	Blood samples	☐ Yes ☐ No
7	Ultrasound	☐ Yes ☐ No
8	cIMT	☐ Yes ☐ No
9	HIV test	☐ Yes ☐ No
10	Urine sample	☐ Yes ☐ No
11	Quality controller ID	|_|_|_|_|_|_|_|_|_|_|_|
12	Travel Reimbursement	☐ Yes ☐ No

Appendix B

Appendix B(i): AWI-Gen deidentified participant ID list with concentrations of genomic DNA for bisulfite modification in preparation for methylation calling.

Participant Study Site ID	Concentration of gDNA (ng/µl)	Normalisation of gDNA to 20µl of 20ng/µl for Bisulfite Modification:	Menopausal Status
IFR0E	25.6	13.6µl gDNA + 14.4µl NFW	Pre-menopausal
IFT0G	42.7	9.4µl gDNA + 10.6µl NFW	Pre-menopausal
IGA0N	84.0	4.8µl gDNA + 15.2µl NFW	Pre-menopausal
IGC0Q	95.5	4.2µl gDNA + 15.8µl NFW	Pre-menopausal
IGS0H	152.5	2.6µl gDNA + 17.4µl NFW	Pre-menopausal
IHN0F	50.4	7.9µl gDNA + 12.1µl NFW	Pre-menopausal
IIJ0D	54.6	7.3µl gDNA + 12.7µl NFW	Pre-menopausal
IIN0H	101.0	4.0µl gDNA + 16.0µl NFW	Pre-menopausal
IIP0I	85.1	4.7µl gDNA + 15.3µl NFW	Pre-menopausal
IIY0R	93.6	4.3µl gDNA + 15.7µl NFW	Pre-menopausal
IJA0V	47.6	8.4µl gDNA + 11.6µl NFW	Pre-menopausal
IKK0I	98.1	4.1µl gDNA + 15.9µl NFW	Pre-menopausal
ILJ0J	113.5	3.5µl gDNA + 16.5µl NFW	Pre-menopausal
ILY0Y	124.8	3.2µl gDNA + 16.8µl NFW	Pre-menopausal
IMV0X	9.8	20.0µl gDNA + 0.0µl NFW	Pre-menopausal
INE0I	83.2	4.8µl gDNA + 15.2µl NFW	Pre-menopausal
INR0W	135.4	3.0µl gDNA + 17.0µl NFW	Pre-menopausal
IPF0L	234.0	4.7µl gDNA + 18.3µl NFW	Pre-menopausal
IPJ0Q	164.6	2.4µl gDNA + 17.6µl NFW	Pre-menopausal
IPT0B	339.3	1.2µl gDNA + 18.8µl NFW	Pre-menopausal
IPY0F	356.4	1.1µl gDNA + 18.9µl NFW	Pre-menopausal
IQF0N	177.8	2.2µl gDNA + 17.8µl NFW	Pre-menopausal
IQH0Q	149.5	2.7µl gDNA + 17.3µl NFW	Pre-menopausal
IRE0P	286.9	1.4µl gDNA + 18.6µl NFW	Pre-menopausal
IRJ0V	286.9	1.4µl gDNA + 18.6µl NFW	Pre-menopausal
IRN0A	20.2	20µl gDNA + 0.0µl NFW	Pre-menopausal
IRS0E	70.0	5.7µl gDNA + 14.3µl NFW	Pre-menopausal
IRX0I	65.7	6.1µl gDNA + 13.9µl NFW	Pre-menopausal
ISQ0E	690.7	1.0µl gDNA + 33.5µl NFW	Pre-menopausal
ITG0W	257.0	1.6µl gDNA + 18.4µl NFW	Pre-menopausal
ITH0X	284.4	1.4µl gDNA + 18.6µl NFW	Pre-menopausal
IYM0L	129.7	3.1µl gDNA + 16.9µl NFW	Pre-menopausal
IYX0W	83.1	4.8µl gDNA + 15.2µl NFW	Pre-menopausal
IZM0N	27.3	14.7µl gDNA + 5.3µl NFW	Pre-menopausal
JAE0I	45.1	8.9µl gDNA + 11.1µl NFW	Pre-menopausal

JBL0S	218.0	1.8µl gDNA + 15.2µl NFW	Pre-menopausal
JBV0C	64.2	6.2µl gDNA + 13.8µl NFW	Pre-menopausal
JBZ0G	196.5	2.0µl gDNA + 18.0µl NFW	Pre-menopausal
JCP0Y	193.6	2.1µl gDNA + 17.9µl NFW	Pre-menopausal
JDI0T	614.0	1.0µl gDNA + 29.7µl NFW	Pre-menopausal
JDZ0K	50.8	7.9µl gDNA + 12.1µl NFW	Pre-menopausal
JEC0P	131.0	3.1µl gDNA + 16.9µl NFW	Pre-menopausal
JED0Q	398.4	1.0µl gDNA + 19.0µl NFW	Pre-menopausal
JEQ0E	280.6	1.4µl gDNA + 18.6µl NFW	Pre-menopausal
JFF0V	118.0	3.4µl gDNA + 16.6µl NFW	Pre-menopausal
JGY0Q	195.5	2.0µl gDNA + 18.0µl NFW	Pre-menopausal
JHQ0K	145.5	2.7µl gDNA + 17.3µl NFW	Pre-menopausal
JIX0T	271.3	1.5µl gDNA + 18.5µl NFW	Pre-menopausal
JKK0L	153.1	2.6µl gDNA + 17.4µl NFW	Pre-menopausal
JKN0P	171.4	2.3µl gDNA + 17.7µl NFW	Pre-menopausal
JKR0S	34.5	11.6µl gDNA + 8.4µl NFW	Pre-menopausal
JKT0V	58.7	6.8µl gDNA + 13.2µl NFW	Pre-menopausal
JKW0X	195.9	2.0µl gDNA + 18.0µl NFW	Pre-menopausal
JLD0G	333.8	1.2µl gDNA + 18.8µl NFW	Pre-menopausal
JLY0C	63.0	6.3µl gDNA + 13.7µl NFW	Pre-menopausal
JNI0Q	24.2	16.5µl gDNA + 3.5µl NFW	Pre-menopausal
JNQ0Y	68.9	5.8µl gDNA + 14.2µl NFW	Pre-menopausal
JPA0J	76.5	5.2µl gDNA + 14.8µl NFW	Pre-menopausal
JPM0X	76.5	5.2µl gDNA + 14.8µl NFW	Pre-menopausal
JQQ0D	51.8	7.7µl gDNA + 12.3µl NFW	Pre-menopausal
JQV0H	80.3	15.0µl gDNA + 5.0µl NFW	Pre-menopausal
JRV0J	78.9	5.1µl gDNA + 14.9µl NFW	Pre-menopausal
JSZ0Q	21.2	18.9µl gDNA + 1.1µl NFW	Pre-menopausal
JTT0M	89.7	4.4µl gDNA + 15.6µl NFW	Pre-menopausal
JTY0R	88.4	4.5µl gDNA + 15.5µl NFW	Pre-menopausal
JVA0V	90.0	4.4µl gDNA + 15.6µl NFW	Pre-menopausal
JVI0E	113.4	3.5µl gDNA + 16.5µl NFW	Pre-menopausal
JVJ0F	166.0	2.4µl gDNA + 17.6µl NFW	Pre-menopausal
JVK0G	8.6	20.0µl gDNA + 0.0µl NFW	Pre-menopausal
JVR0M	84.8	4.7µl gDNA + 15.3µl NFW	Pre-menopausal
JWZ0X	119.5	3.3µl gDNA + 16.7µl NFW	Pre-menopausal
JXH0H	3.5	20.0µl gDNA + 0.0µl NFW	Pre-menopausal
JYF0H	70.1	5.7µl gDNA + 14.3µl NFW	Pre-menopausal
JYZ0C	56.0	7.1µl gDNA + 12.9µl NFW	Pre-menopausal
JZQ0V	44.0	8.7µl gDNA + 11.3µl NFW	Pre-menopausal
JZS0X	44.6	9.0µl gDNA + 11.0µl NFW	Pre-menopausal
KAI0Q	451.2	0.9µl gDNA + 19.1µl NFW	Pre-menopausal

KAK0S	103.9	3.8µl gDNA + 16.2µl NFW	Pre-menopausal
KAL0T	90.7	4.4µl gDNA + 15.6µl NFW	Pre-menopausal
KAX0F	119.9	3.3µl gDNA + 16.7µl NFW	Pre-menopausal
KBM0X	76.9	5.7µl gDNA + 14.3µl NFW	Pre-menopausal
KCB0M	133.0	3.0µl gDNA + 17.0µl NFW	Pre-menopausal
KCN0B	56.2	7.4µl gDNA + 12.4µl NFW	Pre-menopausal
KCY0K	98.5	4.1µl gDNA + 15.9µl NFW	Pre-menopausal
KEF0W	61.6	6.5µl gDNA + 13.3µl NFW	Pre-menopausal
KEI0A	83.0	4.5µl gDNA + 15.2µl NFW	Pre-menopausal
KFK0E	67.3	5.9µl gDNA + 14.1µl NFW	Pre-menopausal
KFY0R	48.4	8.3µl gDNA + 11.7µl NFW	Pre-menopausal
KGH0D	42.6	9.4µl gDNA + 10.6µl NFW	Pre-menopausal
KGR0M	113.0	3.5µl gDNA + 16.5µl NFW	Pre-menopausal
KHN0L	87.6	4.6µl gDNA + 15.4µl NFW	Pre-menopausal
KIB0B	37.1	10.8µl gDNA + 9.2µl NFW	Pre-menopausal
KII0I	53.3	7.5µl gDNA + 12.5µl NFW	Pre-menopausal
KIM0M	73.5	5.4µl gDNA + 14.6µl NFW	Pre-menopausal
KJA0C	74.2	5.4µl gDNA + 14.6µl NFW	Pre-menopausal
KJB0D	69.1	5.8µl gDNA + 14.2µl NFW	Pre-menopausal
KJJ0L	96.8	4.1µl gDNA + 15.9µl NFW	Pre-menopausal
KXA0D	80.0	15.0µl gDNA + 5.6µl NFW	Pre-menopausal
KXQ0T	71.7	5.6µl gDNA + 14.4µl NFW	Pre-menopausal
ICN0T	153.3	2.6µl gDNA + 17.4µl NFW	Post-menopausal
ICP0V	87.7	4.6µl gDNA + 15.4µl NFW	Post-menopausal
IDF0M	83.8	4.8µl gDNA + 15.2µl NFW	Post-menopausal
IER0C	134.5	3.0µl gDNA + 17.0µl NFW	Post-menopausal
IEZ0J	90.2	4.4µl gDNA + 16.9µl NFW	Post-menopausal
IGD0R	141.9	2.8µl gDNA + 17.2µl NFW	Post-menopausal
IGG0V	113.8	3.5µl gDNA + 16.5µl NFW	Post-menopausal
IHW0M	35.5	11.3µl gDNA + 8.7µl NFW	Post-menopausal
IIB0T	18.4	20.0µl gDNA + 0.0µl NFW	Post-menopausal
IIS0L	112.0	3.6µl gDNA + 16.4µl NFW	Post-menopausal
IIZ0S	118.9	3.4µl gDNA + 16.6µl NFW	Post-menopausal
IJC0X	117.3	3.4µl gDNA + 16.6µl NFW	Post-menopausal
IJF0B	77.5	5.2µl gDNA + 14.8µl NFW	Post-menopausal
IKD0B	94.0	4.3µl gDNA + 15.7µl NFW	Post-menopausal
IKF0D	128.0	3.1µl gDNA + 16.4µl NFW	Post-menopausal
IKG0E	92.9	4.3µl gDNA + 15.7µl NFW	Post-menopausal
ILK0K	106.4	3.8µl gDNA + 16.7µl NFW	Post-menopausal
ILX0X	52.0	7.7µl gDNA + 12.3µl NFW	Post-menopausal
IMB0D	80.9	4.9µl gDNA + 15.7µl NFW	Post-menopausal
ING0K	87.5	4.6µl gDNA + 15.4µl NFW	Post-menopausal

INH0E	70.1	3.7µl gDNA + 14.3µl NFW	Post-menopausal
INS0X	86.4	4.6µl gDNA + 5.4µl NFW	Post-menopausal
IPC0I	135.1	3.0µl gDNA + 17.9µl NFW	Post-menopausal
ISS0G	267.3	1.5µl gDNA + 18.5µl NFW	Post-menopausal
ITK0B	305.5	1.3µl gDNA + 18.7µl NFW	Post-menopausal
ITN0E	254.4	1.6µl gDNA + 18.4 µl NFW	Post-menopausal
IWJ0E	117.2	3.4µl gDNA + 16.6µl NFW	Post-menopausal
IWL0G	110.9	3.6µl gDNA + 16.4µl NFW	Post-menopausal
IWR0L	103.1	3.9µl gDNA + 16.1µl NFW	Post-menopausal
IWX0R	75.3	5.3µl gDNA + 14.7µl NFW	Post-menopausal
IXA0W	181.7	2.2µl gDNA + 9.8µl NFW	Post-menopausal
JAL0Q	93.4	4.3µl gDNA + 15.7µl NFW	Post-menopausal
JBB0H	122.8	3.3µl gDNA + 16.7µl NFW	Post-menopausal
JDW0H	38.0	10.5µl gDNA + 9.5µl NFW	Post-menopausal
JGR0J	29.7	13.5µl gDNA + 16.5µl NFW	Post-menopausal
JKB0C	201.4	2.0µl gDNA + 18.0µl NFW	Post-menopausal
JNF0X	44.3	9.0µl gDNA + 11.0µl NFW	Post-menopausal
JPC0L	105.3	3.8µl gDNA + 16.2µl NFW	Post-menopausal
JPF0P	109.1	3.7µl gDNA + 16.3µl NFW	Post-menopausal
JPH0R	47.2	8.5µl gDNA + 11.5µl NFW	Post-menopausal
JPI0S	90.8	4.4µl gDNA + 15.6µl NFW	Post-menopausal
JQC0N	33.6	11.9µl gDNA + 8.1µl NFW	Post-menopausal
JQD0P	77.8	5.1µl gDNA + 14.9µl NFW	Post-menopausal
JQJ0V	49.7	8.0µl gDNA + 12.0µl NFW	Post-menopausal
JQX0J	46.5	8.6µl gDNA + 11.4µl NFW	Post-menopausal
JRH0W	59.2	6.8µl gDNA + 13.2µl NFW	Post-menopausal
JRJ0Y	47.3	8.5µl gDNA + 11.5µl NFW	Post-menopausal
JRN0D	100.5	4.0µl gDNA + 16.0µl NFW	Post-menopausal
JSD0T	42.3	9.5µl gDNA + 10.5µl NFW	Post-menopausal
JSG0X	70.1	5.7µl gDNA + 14.3µl NFW	Post-menopausal
JWL0J	27.3	14.7µl gDNA + 5.2µl NFW	Post-menopausal
JWM0K	33.2	12.0µl gDNA + 8.0µl NFW	Post-menopausal
JWN0L	76.1	5.3µl gDNA + 14.7µl NFW	Post-menopausal
JXC0C	10.2	20.0µl gDNA + 0.0µl NFW	Post-menopausal
JXP0P	119.8	3.3µl gDNA + 16.7µl NFW	Post-menopausal
JXV0V	85.6	4.7µl gDNA + 15.3µl NFW	Post-menopausal
JYC0E	56.8	7.0µl gDNA + 13.0µl NFW	Post-menopausal
JYE0G	47.6	8.4µl gDNA + 11.6µl NFW	Post-menopausal
JYI0K	46.8	8.5µl gDNA + 11.5µl NFW	Post-menopausal
JYL0N	96.6	4.1µl gDNA + 15.9µl NFW	Post-menopausal
JYR0T	121.0	3.3µl gDNA + 16.7µl NFW	Post-menopausal
JYW0Y	99.0	4.0µl gDNA + 16.0µl NFW	Post-menopausal

JYX0A	113.0	3.5µl gDNA +16.5µl NFW	Post-menopausal
JYY0B	88.0	4.5µl gDNA + 15.5µl NFW	Post-menopausal
JZR0W	9.4	20.0µl gDNA + 0.0µl NFW	Post-menopausal
KAD0K	122.5	3.3µl gDNA + 16.7µl NFW	Post-menopausal
KAV0D	91.8	4.4µl gDNA + 15.6µl NFW	Post-menopausal
KBA0J	91.0	4.4µl gDNA + 15.6µl NFW	Post-menopausal
KBC0L	100.4	4.0µl gDNA + 16.0µl NFW	Post-menopausal
KBI0S	123.8	3.2µl gDNA + 16.8µl NFW	Post-menopausal
KBJ0T	101.9	3.9µl gDNA + 16.1µl NFW	Post-menopausal
KCA0L	80.0	5.0µl gDNA + 15.0µl NFW	Post-menopausal
KCD0P	74.6	5.4µl gDNA + 14.6µl NFW	Post-menopausal
KCF0R	70.5	5.7µl gDNA + 4.3µl NFW	Post-menopausal
KDB0P	119.5	3.3µl gDNA + 16.7µl NFW	Post-menopausal
KDY0M	64.4	6.2µl gDNA + 13.8µl NFW	Post-menopausal
KEE0V	82.4	4.9µl gDNA + 15.1µl NFW	Post-menopausal
KEH0Y	81.6	4.9µl gDNA + 15.1µl NFW	Post-menopausal
KES0J	71.1	5.6µl gDNA + 14.4µl NFW	Post-menopausal
KEZ0Q	83.2	4.8µl gDNA + 15.2µl NFW	Post-menopausal
KFN0H	128.3	3.1µl gDNA + 16.9µl NFW	Post-menopausal
KFX0Q	117.3	3.4µl gDNA + 16.6µl NFW	Post-menopausal
KGL0H	117.5	3.4µl gDNA + 16.6µl NFW	Post-menopausal
KGM0I	113.2	3.5µl gDNA + 16.5µl NFW	Post-menopausal
KGT0P	105.0	3.8µl gDNA + 16.2µl NFW	Post-menopausal
KHA0X	136.1	2.9µl gDNA + 17.1µl NFW	Post-menopausal
KIS0S	74.0	5.4µl gDNA + 14.6µl NFW	Post-menopausal
KJI0K	88.3	4.5µl gDNA + 15.5µl NFW	Post-menopausal
KTC0Y	78.9	5.1µl gDNA + 14.9µl NFW	Post-menopausal
KTH0E	81.6	4.9µl gDNA + 15.1µl NFW	Post-menopausal
KVK0J	134.4	3.0µl gDNA + 17.0µl NFW	Post-menopausal
KVM0L	38.2	10.5µl gDNA + 9.5µl NFW	Post-menopausal
KVN0M	82.4	4.9µl gDNA + 15.1µl NFW	Post-menopausal
KVV0T	148.9	2.7µl gDNA + 17.3µl NFW	Post-menopausal
KWD0E	54.0	7.4µl gDNA + 12.6µl NFW	Post-menopausal
KWL0M	118.5	3.4µl gDNA + 16.6µl NFW	Post-menopausal
KXI0L	35.5	11.3µl gDNA + 8.7µl NFW	Post-menopausal
KXS0W	73.1	5.5µl gDNA + 14.5µl NFW	Post-menopausal

Study site: Clinical Research Unit of Nanoro (CRUN), Nanoro, Burkina Faso, West Africa (SSA)

Appendix B(ii): Key to chromosomal location of CpG site variables.

CpG Site	Chromosomal Location	Strand
FSHR_1	chr2_49154033	-
FSHR_2	chr2_49154050	-
FSHR_3	chr2_49154150	-
FSHR_4	chr2_49154214	-
INHBB_1	chr2_120344319	+
INHBB_2	chr2_120344331	+
INHBB_3	chr2_120344342	+
INHBB_4	chr2_120344347	+
INHBB_5	chr2_120344356	+
INHBB_6	chr2_120344359	+
INHBB_7	chr2_120344362	+
INHBB_8	chr2_120344367	+
INHBB_9	chr2_120344401	+
INHBB_10	chr2_120344408	+
INHBB_11	chr2_120344411	+
INHBB_12	chr2_120344414	+
INHBB_13	chr2_120344440	+
INHBB_14	chr2_120344442	+
INHBB_15	chr2_120344445	+
INHBB_16	chr2_120344452	+
INHBB_17	chr2_120344456	+
INHBB_18	chr2_120344461	+
INHBB_19	chr2_120344470	+
INHBB_20	chr2_120344474	+
INHBB_21	chr2_120344476	+
INHBB_22	chr2_120344484	+
INHBB_23	chr2_120344489	+
INHBB_24	chr2_120344498	+
INHBB_25	chr2_120344504	+
INHBB_26	chr2_120344510	+
INHBB_27	chr2_120344513	+
INHBB_28	chr2_120344516	+
INHBB_29	chr2_120344523	+
INHBB_30	chr2_120344545	+

ESR2_1	chr14_64338824	+
ESR2_2	chr14_64338838	+
ESR2_3	chr14_64338868	+
ESR2_4	chr14_64338880	+
ESR2_5	chr14_64338898	+
ESR2_6	chr14_64338902	+
ESR2_7	chr14_64338929	+
ESR2_8	chr14_64338938	+
ESR2_9	chr14_64338948	+
ESR2_10	chr14_64338954	+
ESR2_11	chr14_64338984	+
ESR2_12	chr14_64338986	+
ESR2_13	chr14_64338988	+
ESR2_14	chr14_64338990	+
ESR2_15	chr14_64339010	+
ESR2_16	chr14_64339016	+
ESR2_17	chr14_64339018	+
ESR2_18	chr14_64339024	+
ESR2_19	chr14_64339026	+
ESR2_20	chr14_64339034	+
SHBG_1	chr17_7627707	+
SHBG_2	chr17_7627737	+
SHBG_3	chr17_7627745	+
SHBG_4	chr17_7627752	+
SHBG_5	chr17_7627761	+
SHBG_6	chr17_7627764	+
SHBG_7	chr17_7627773	+
SHBG_8	chr17_7627775	+
SHBG_9	chr17_7627801	+
SHBG_10	chr17_7627804	+
SHBG_11	chr17_7627807	+
SHBG_12	chr17_7627811	+
SHBG_13	chr17_7627832	+
SHBG_14	chr17_7627837	+
SHBG_15	chr17_7627849	+
SHBG_16	chr17_7627869	+

SHBG_17	chr17_7627879	+
AR_1	chrX_67543889	+
AR_2	chrX_67543895	+
AR_3	chrX_67543897	+
AR_4	chrX_67543899	+
AR_5	chrX_67543964	+
AR_6	chrX_67543969	+
AR_7	chrX_67543978	+
AR_8	chrX_67543985	+
AR_9	chrX_67543990	+
AR_10	chrX_67544023	+
AR_11	chrX_67544032	+
AR_12	chrX_67544040	+
AR_13	chrX_67544064	+
AR_14	chrX_67544069	+
AR_15	chrX_67544074	+

Appendix B(iii) Bivariate correlation analyses between body mass index (BMI) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
BMI & FSHR_1	177	0.150	0.047
BMI & FSHR_2	177	-0.025	0.738
BMI & FSHR_3	177	0.162	0.031
BMI & FSHR_4	177	-0.018	0.816
BMI & INHBB_1	176	0.042	0.579
BMI & INHBB_2	176	0.080	0.293
BMI & INHBB_3	176	0.168	0.026
BMI & INHBB_4	176	0.114	0.131
BMI & INHBB_5	176	0.109	0.149
BMI & INHBB_6	176	0.153	0.042
BMI & INHBB_7	176	0.083	0.272
BMI & INHBB_8	176	-0.014	0.855
BMI & INHBB_9	176	-0.074	0.330
BMI & INHBB_10	176	-0.027	0.717
BMI & INHBB_11	176	0.034	0.659
BMI & INHBB_12	176	0.045	0.555
BMI & INHBB_13	176	0.018	0.815
BMI & INHBB_14	176	-0.040	0.598
BMI & INHBB_15	176	0.129	0.087
BMI & INHBB_16	176	0.096	0.204
BMI & INHBB_17	176	0.226	0.003
BMI & INHBB_18	176	0.083	0.271
BMI & INHBB_19	176	0.013	0.867
BMI & INHBB_20	176	0.063	0.404
BMI & INHBB_21	176	0.064	0.396
BMI & INHBB_22	176	0.039	0.604
BMI & INHBB_23	176	0.021	0.781
BMI & INHBB_24	176	-0.010	0.894
BMI & INHBB_25	176	0.066	0.386
BMI & INHBB_26	176	0.006	0.937
BMI & INHBB_27	176	-0.013	0.867
BMI & INHBB_28	176	0.013	0.862
BMI & INHBB_29	176	0.114	0.132
BMI & INHBB_30	176	0.060	0.428
BMI & ESR2_1	106	-0.159	0.104
BMI & ESR2_2	106	0.030	0.761
BMI & ESR2_3	106	0.111	0.256
BMI & ESR2_4	106	-0.106	0.277
BMI & ESR2_5	106	0.136	0.164
BMI & ESR2_6	106	-0.033	0.738
BMI & ESR2_7	106	0.003	0.974
BMI & ESR2_8	106	-0.045	0.650
BMI & ESR2_9	106	0.101	0.305
BMI & ESR2_10	106	0.073	0.459
BMI & ESR2_11	106	0.004	0.972
BMI & ESR2_12	106	-0.053	0.587
BMI & ESR2_13	106	-0.032	0.745
BMI & ESR2_14	106	0.105	0.282
BMI & ESR2_15	106	0.093	0.342
BMI & ESR2_16	106	0.243	0.012

BMI & ESR2_17	106	0.045	0.649
BMI & ESR2_18	106	0.149	0.128
BMI & ESR2_19	106	0.074	0.448
BMI & ESR2_20	105	0.108	0.273
BMI & SHBG_1	166	-0.108	0.167
BMI & SHBG_2	166	0.076	0.332
BMI & SHBG_3	166	0.005	0.952
BMI & SHBG_4	166	-0.070	0.373
BMI & SHBG_5	166	-0.115	0.140
BMI & SHBG_6	166	-0.075	0.335
BMI & SHBG_7	166	-0.051	0.512
BMI & SHBG_8	166	-0.111	0.156
BMI & SHBG_9	166	-0.010	0.900
BMI & SHBG_10	166	<0.001	0.996
BMI & SHBG_11	166	0.158	0.042
BMI & SHBG_12	166	0.060	0.441
BMI & SHBG_13	166	-0.010	0.901
BMI & SHBG_14	166	0.069	0.376
BMI & SHBG_15	166	-0.038	0.625
BMI & SHBG_16	166	-0.090	0.251
BMI & SHBG_17	166	-0.050	0.523
BMI & AR_1	177	0.096	0.205
BMI & AR_2	177	0.096	0.203
BMI & AR_3	177	0.065	0.393
BMI & AR_4	177	0.134	0.075
BMI & AR_5	177	-0.023	0.763
BMI & AR_6	177	0.154	0.041
BMI & AR_7	177	0.084	0.266
BMI & AR_8	177	0.099	0.188
BMI & AR_9	177	0.116	0.123
BMI & AR_10	177	0.063	0.406
BMI & AR_11	177	0.060	0.426
BMI & AR_12	177	0.037	0.622
BMI & AR_13	177	0.090	0.233
BMI & AR_14	177	0.036	0.632
BMI & AR_15	177	0.066	0.386

Appendix B(iv) Bivariate correlation analyses between fasting blood glucose (FBG) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
FBG & FSHR_1	175	-0.072	0.343
FBG & FSHR_2	175	-0.116	0.126
FBG & FSHR_3	175	-0.019	0.802
FBG & FSHR_4	175	-0.071	0.351
FBG & INHBB_1	174	0.090	0.237
FBG & INHBB_2	174	0.053	0.483
FBG & INHBB_3	174	-0.012	0.878
FBG & INHBB_4	174	0.098	0.198
FBG & INHBB_5	174	0.099	0.192
FBG & INHBB_6	174	0.061	0.420
FBG & INHBB_7	174	0.034	0.653
FBG & INHBB_8	174	0.068	0.370
FBG & INHBB_9	174	0.087	0.253
FBG & INHBB_10	174	0.010	0.891
FBG & INHBB_11	174	0.031	0.689
FBG & INHBB_12	174	0.166	0.029
FBG & INHBB_13	174	0.032	0.677
FBG & INHBB_14	174	0.050	0.512
FBG & INHBB_15	174	0.070	0.361
FBG & INHBB_16	174	0.064	0.403
FBG & INHBB_17	174	0.152	0.046
FBG & INHBB_18	174	0.131	0.084
FBG & INHBB_19	174	0.135	0.075
FBG & INHBB_20	174	0.061	0.422
FBG & INHBB_21	174	0.091	0.231
FBG & INHBB_22	174	-0.075	0.326
FBG & INHBB_23	174	0.132	0.083
FBG & INHBB_24	174	-0.061	0.420
FBG & INHBB_25	174	0.108	0.154
FBG & INHBB_26	174	0.066	0.390
FBG & INHBB_27	174	0.048	0.528
FBG & INHBB_28	174	0.013	0.863
FBG & INHBB_29	174	0.031	0.680
FBG & INHBB_30	174	0.073	0.337
FBG & ESR2_1	105	0.052	0.595
FBG & ESR2_2	105	0.046	0.643
FBG & ESR2_3	105	0.024	0.804
FBG & ESR2_4	105	-0.058	0.556
FBG & ESR2_5	105	0.014	0.885
FBG & ESR2_6	105	-0.122	0.214
FBG & ESR2_7	105	-0.068	0.489
FBG & ESR2_8	105	0.034	0.734
FBG & ESR2_9	105	-0.103	0.295
FBG & ESR2_10	105	-0.121	0.220
FBG & ESR2_11	105	-0.035	0.721
FBG & ESR2_12	105	-0.025	0.799
FBG & ESR2_13	105	-0.013	0.899
FBG & ESR2_14	105	-0.083	0.397
FBG & ESR2_15	105	0.032	0.750
FBG & ESR2_16	105	-0.056	0.573

FBG & ESR2_17	105	-0.057	0.561
FBG & ESR2_18	105	0.122	0.217
FBG & ESR2_19	105	0.138	0.161
FBG & ESR2_20	104	0.007	0.947
FBG & SHBG_1	164	-0.138	0.078
FBG & SHBG_2	164	-0.040	0.612
FBG & SHBG_3	164	-0.131	0.093
FBG & SHBG_4	164	-0.100	0.205
FBG & SHBG_5	164	-0.092	0.242
FBG & SHBG_6	164	-0.061	0.434
FBG & SHBG_7	164	0.064	0.416
FBG & SHBG_8	164	-0.016	0.835
FBG & SHBG_9	164	-0.003	0.965
FBG & SHBG_10	164	0.058	0.460
FBG & SHBG_11	164	0.115	0.143
FBG & SHBG_12	164	-0.081	0.303
FBG & SHBG_13	164	-0.032	0.684
FBG & SHBG_14	164	-0.002	0.983
FBG & SHBG_15	164	-0.109	0.163
FBG & SHBG_16	164	-0.203	0.009
FBG & SHBG_17	164	-0.110	0.161
FBG & AR_1	175	-0.105	0.168
FBG & AR_2	175	-0.097	0.204
FBG & AR_3	175	-0.112	0.142
FBG & AR_4	175	-0.073	0.339
FBG & AR_5	175	-0.057	0.456
FBG & AR_6	175	-0.051	0.503
FBG & AR_7	175	-0.043	0.571
FBG & AR_8	175	0.014	0.850
FBG & AR_9	175	-0.069	0.368
FBG & AR_10	175	-0.128	0.092
FBG & AR_11	175	-0.069	0.367
FBG & AR_12	175	-0.126	0.097
FBG & AR_13	175	-0.042	0.584
FBG & AR_14	175	-0.027	0.727
FBG & AR_15	175	-0.035	0.648

Appendix B(v) Bivariate correlation analyses between homeostasis model assessment (HOMA) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
HOMA & FSHR_1	175	-0.215	0.004
HOMA & FSHR_2	175	-0.114	0.132
HOMA & FSHR_3	175	-0.002	0.981
HOMA & FSHR_4	175	0.080	0.295
HOMA & INHBB_1	174	-0.013	0.866
HOMA & INHBB_2	174	-0.080	0.297
HOMA & INHBB_3	174	-0.004	0.960
HOMA & INHBB_4	174	-0.095	0.211
HOMA & INHBB_5	174	0.140	0.066
HOMA & INHBB_6	174	-0.021	0.779
HOMA & INHBB_7	174	0.093	0.221
HOMA & INHBB_8	174	-0.055	0.471
HOMA & INHBB_9	174	-0.041	0.590
HOMA & INHBB_10	174	0.124	0.103
HOMA & INHBB_11	174	-0.151	0.047
HOMA & INHBB_12	174	-0.058	0.446
HOMA & INHBB_13	174	-0.001	0.988
HOMA & INHBB_14	174	0.144	0.058
HOMA & INHBB_15	174	-0.005	0.944
HOMA & INHBB_16	174	-0.044	0.561
HOMA & INHBB_17	174	0.058	0.450
HOMA & INHBB_18	174	-0.037	0.626
HOMA & INHBB_19	174	0.012	0.873
HOMA & INHBB_20	174	0.006	0.934
HOMA & INHBB_21	174	-0.010	0.895
HOMA & INHBB_22	174	-0.104	0.172
HOMA & INHBB_23	174	-0.057	0.452
HOMA & INHBB_24	174	0.170	0.025
HOMA & INHBB_25	174	0.013	0.868
HOMA & INHBB_26	174	0.064	0.404
HOMA & INHBB_27	174	0.015	0.840
HOMA & INHBB_28	174	-0.008	0.918
HOMA & INHBB_29	174	-0.181	0.017
HOMA & INHBB_30	174	0.052	0.497
HOMA & ESR2_1	105	-0.007	0.942
HOMA & ESR2_2	105	0.173	0.078
HOMA & ESR2_3	105	0.300	0.002
HOMA & ESR2_4	105	0.108	0.274
HOMA & ESR2_5	105	0.021	0.835
HOMA & ESR2_6	105	0.232	0.017
HOMA & ESR2_7	105	-0.084	0.397
HOMA & ESR2_8	105	-0.056	0.571
HOMA & ESR2_9	105	0.120	0.223
HOMA & ESR2_10	105	0.151	0.124
HOMA & ESR2_11	105	0.106	0.284
HOMA & ESR2_12	105	0.083	0.403
HOMA & ESR2_13	105	-0.066	0.502
HOMA & ESR2_14	105	0.079	0.426
HOMA & ESR2_15	105	0.116	0.239
HOMA & ESR2_16	105	0.045	0.651

HOMA & ESR2_17	105	-0.176	0.072
HOMA & ESR2_18	105	0.078	0.429
HOMA & ESR2_19	105	0.096	0.331
HOMA & ESR2_20	104	-0.011	0.915
HOMA & SHBG_1	164	-0.221	0.004
HOMA & SHBG_2	164	0.041	0.606
HOMA & SHBG_3	164	-0.104	0.184
HOMA & SHBG_4	164	-0.046	0.560
HOMA & SHBG_5	164	-0.028	0.725
HOMA & SHBG_6	164	<0.001	1.000
HOMA & SHBG_7	164	-0.199	0.011
HOMA & SHBG_8	164	-0.131	0.093
HOMA & SHBG_9	164	<0.001	0.998
HOMA & SHBG_10	164	-0.089	0.259
HOMA & SHBG_11	164	0.105	0.181
HOMA & SHBG_12	164	-0.109	0.166
HOMA & SHBG_13	164	0.006	0.934
HOMA & SHBG_14	164	0.004	0.958
HOMA & SHBG_15	164	-0.081	0.302
HOMA & SHBG_16	164	-0.178	0.022
HOMA & SHBG_17	164	-0.072	0.357
HOMA & AR_1	175	-0.012	0.875
HOMA & AR_2	175	-0.002	0.982
HOMA & AR_3	175	-0.066	0.386
HOMA & AR_4	175	0.011	0.883
HOMA & AR_5	175	0.008	0.916
HOMA & AR_6	175	0.028	0.716
HOMA & AR_7	175	0.036	0.633
HOMA & AR_8	175	0.130	0.087
HOMA & AR_9	175	-0.025	0.744
HOMA & AR_10	175	-0.131	0.084
HOMA & AR_11	175	0.017	0.824
HOMA & AR_12	175	0.026	0.734
HOMA & AR_13	175	0.104	0.172
HOMA & AR_14	175	0.063	0.409
HOMA & AR_15	175	-0.004	0.955

Appendix B(vi) Bivariate correlation analyses between systolic blood pressure and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Systolic BP & FSHR_1	177	-0.048	0.522
Systolic BP & FSHR_2	177	0.156	0.038
Systolic BP & FSHR_3	177	0.031	0.680
Systolic BP & FSHR_4	177	0.133	0.078
Systolic BP & INHBB_1	176	0.015	0.842
Systolic BP & INHBB_2	176	0.046	0.544
Systolic BP & INHBB_3	176	-0.091	0.230
Systolic BP & INHBB_4	176	-0.199	0.008
Systolic BP & INHBB_5	176	-0.002	0.977
Systolic BP & INHBB_6	176	-0.014	0.857
Systolic BP & INHBB_7	176	0.033	0.667
Systolic BP & INHBB_8	176	-0.096	0.207
Systolic BP & INHBB_9	176	0.092	0.226
Systolic BP & INHBB_10	176	0.067	0.375
Systolic BP & INHBB_11	176	0.077	0.309
Systolic BP & INHBB_12	176	-0.002	0.980
Systolic BP & INHBB_13	176	0.017	0.818
Systolic BP & INHBB_14	176	0.065	0.393
Systolic BP & INHBB_15	176	-0.075	0.323
Systolic BP & INHBB_16	176	-0.128	0.090
Systolic BP & INHBB_17	176	-0.093	0.218
Systolic BP & INHBB_18	176	0.001	0.988
Systolic BP & INHBB_19	176	-0.046	0.548
Systolic BP & INHBB_20	176	0.007	0.930
Systolic BP & INHBB_21	176	-0.024	0.750
Systolic BP & INHBB_22	176	-0.038	0.613
Systolic BP & INHBB_23	176	-0.029	0.706
Systolic BP & INHBB_24	176	0.020	0.788
Systolic BP & INHBB_25	176	0.006	0.935
Systolic BP & INHBB_26	176	-0.001	0.990
Systolic BP & INHBB_27	176	-0.056	0.462
Systolic BP & INHBB_28	176	0.077	0.308
Systolic BP & INHBB_29	176	-0.063	0.407
Systolic BP & INHBB_30	176	<0.001	0.999
Systolic BP & ESR2_1	106	-0.030	0.759
Systolic BP & ESR2_2	106	-0.114	0.246
Systolic BP & ESR2_3	106	-0.005	0.959
Systolic BP & ESR2_4	106	0.020	0.840
Systolic BP & ESR2_5	106	-0.001	0.996
Systolic BP & ESR2_6	106	-0.073	0.455
Systolic BP & ESR2_7	106	-0.029	0.771
Systolic BP & ESR2_8	106	0.036	0.713
Systolic BP & ESR2_9	106	0.017	0.866
Systolic BP & ESR2_10	106	0.062	0.529
Systolic BP & ESR2_11	106	0.007	0.942
Systolic BP & ESR2_12	106	-0.079	0.420
Systolic BP & ESR2_13	106	-0.049	0.617
Systolic BP & ESR2_14	106	-0.135	0.168
Systolic BP & ESR2_15	106	0.046	0.638
Systolic BP & ESR2_16	106	0.016	0.872

Systolic BP & ESR2_17	106	-0.024	0.809
Systolic BP & ESR2_18	106	-0.062	0.528
Systolic BP & ESR2_19	106	0.094	0.339
Systolic BP & ESR2_20	105	0.122	0.216
Systolic BP & SHBG_1	166	0.006	0.941
Systolic BP & SHBG_2	166	-0.011	0.893
Systolic BP & SHBG_3	166	-0.057	0.466
Systolic BP & SHBG_4	166	-0.089	0.254
Systolic BP & SHBG_5	166	0.096	0.218
Systolic BP & SHBG_6	166	-0.128	0.101
Systolic BP & SHBG_7	166	0.061	0.432
Systolic BP & SHBG_8	166	-0.085	0.278
Systolic BP & SHBG_9	166	-0.018	0.817
Systolic BP & SHBG_10	166	-0.031	0.690
Systolic BP & SHBG_11	166	0.026	0.741
Systolic BP & SHBG_12	166	0.014	0.858
Systolic BP & SHBG_13	166	0.038	0.631
Systolic BP & SHBG_14	166	0.051	0.514
Systolic BP & SHBG_15	166	-0.109	0.161
Systolic BP & SHBG_16	166	0.018	0.814
Systolic BP & SHBG_17	166	-0.023	0.769
Systolic BP & AR_1	177	0.060	0.428
Systolic BP & AR_2	177	0.100	0.186
Systolic BP & AR_3	177	0.003	0.964
Systolic BP & AR_4	177	0.027	0.724
Systolic BP & AR_5	177	0.084	0.265
Systolic BP & AR_6	177	0.007	0.921
Systolic BP & AR_7	177	0.051	0.497
Systolic BP & AR_8	177	0.090	0.234
Systolic BP & AR_9	177	0.067	0.372
Systolic BP & AR_10	177	0.058	0.445
Systolic BP & AR_11	177	0.016	0.833
Systolic BP & AR_12	177	0.027	0.718
Systolic BP & AR_13	177	0.086	0.254
Systolic BP & AR_14	177	0.051	0.498
Systolic BP & AR_15	177	0.101	0.181

Appendix B(vii) Bivariate correlation analyses between diastolic blood pressure and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Diastolic BP & FSHR_1	177	-0.009	0.908
Diastolic BP & FSHR_2	177	0.195	0.009
Diastolic BP & FSHR_3	177	0.070	0.356
Diastolic BP & FSHR_4	177	0.041	0.592
Diastolic BP & INHBB_1	176	-0.008	0.911
Diastolic BP & INHBB_2	176	0.093	0.222
Diastolic BP & INHBB_3	176	-0.042	0.579
Diastolic BP & INHBB_4	176	-0.141	0.062
Diastolic BP & INHBB_5	176	0.045	0.557
Diastolic BP & INHBB_6	176	0.007	0.930
Diastolic BP & INHBB_7	176	0.083	0.275
Diastolic BP & INHBB_8	176	-0.068	0.371
Diastolic BP & INHBB_9	176	0.075	0.322
Diastolic BP & INHBB_10	176	0.059	0.434
Diastolic BP & INHBB_11	176	0.080	0.289
Diastolic BP & INHBB_12	176	0.018	0.817
Diastolic BP & INHBB_13	176	0.026	0.733
Diastolic BP & INHBB_14	176	0.043	0.573
Diastolic BP & INHBB_15	176	-0.039	0.610
Diastolic BP & INHBB_16	176	-0.020	0.791
Diastolic BP & INHBB_17	176	-0.037	0.629
Diastolic BP & INHBB_18	176	0.051	0.499
Diastolic BP & INHBB_19	176	-0.006	0.942
Diastolic BP & INHBB_20	176	0.062	0.412
Diastolic BP & INHBB_21	176	-0.005	0.943
Diastolic BP & INHBB_22	176	0.001	0.992
Diastolic BP & INHBB_23	176	0.001	0.984
Diastolic BP & INHBB_24	176	-0.029	0.707
Diastolic BP & INHBB_25	176	0.064	0.400
Diastolic BP & INHBB_26	176	0.016	0.836
Diastolic BP & INHBB_27	176	-0.036	0.635
Diastolic BP & INHBB_28	176	0.115	0.128
Diastolic BP & INHBB_29	176	-0.002	0.980
Diastolic BP & INHBB_30	176	-0.028	0.712
Diastolic BP & ESR2_1	106	-0.014	0.887
Diastolic BP & ESR2_2	106	-0.068	0.488
Diastolic BP & ESR2_3	106	-0.019	0.848
Diastolic BP & ESR2_4	106	0.029	0.768
Diastolic BP & ESR2_5	106	0.071	0.467
Diastolic BP & ESR2_6	106	-0.101	0.302
Diastolic BP & ESR2_7	106	0.005	0.957
Diastolic BP & ESR2_8	106	0.076	0.438
Diastolic BP & ESR2_9	106	0.076	0.440
Diastolic BP & ESR2_10	106	0.039	0.689
Diastolic BP & ESR2_11	106	0.038	0.697
Diastolic BP & ESR2_12	106	-0.044	0.656
Diastolic BP & ESR2_13	106	-0.018	0.854
Diastolic BP & ESR2_14	106	-0.074	0.452
Diastolic BP & ESR2_15	106	0.010	0.922
Diastolic BP & ESR2_16	106	0.025	0.797

Diastolic BP & ESR2_17	106	0.015	0.878
Diastolic BP & ESR2_18	106	0.011	0.910
Diastolic BP & ESR2_19	106	0.082	0.406
Diastolic BP & ESR2_20	105	0.164	0.094
Diastolic BP & SHBG_1	166	-0.018	0.820
Diastolic BP & SHBG_2	166	0.011	0.883
Diastolic BP & SHBG_3	166	-0.036	0.643
Diastolic BP & SHBG_4	166	-0.087	0.266
Diastolic BP & SHBG_5	166	0.099	0.204
Diastolic BP & SHBG_6	166	-0.129	0.097
Diastolic BP & SHBG_7	166	0.077	0.322
Diastolic BP & SHBG_8	166	-0.095	0.222
Diastolic BP & SHBG_9	166	-0.030	0.705
Diastolic BP & SHBG_10	166	-0.018	0.819
Diastolic BP & SHBG_11	166	0.037	0.634
Diastolic BP & SHBG_12	166	-0.038	0.628
Diastolic BP & SHBG_13	166	0.040	0.605
Diastolic BP & SHBG_14	166	0.025	0.753
Diastolic BP & SHBG_15	166	-0.111	0.155
Diastolic BP & SHBG_16	166	0.011	0.886
Diastolic BP & SHBG_17	166	-0.006	0.939
Diastolic BP & AR_1	177	0.095	0.207
Diastolic BP & AR_2	177	0.124	0.099
Diastolic BP & AR_3	177	0.012	0.878
Diastolic BP & AR_4	177	0.104	0.169
Diastolic BP & AR_5	177	0.106	0.160
Diastolic BP & AR_6	177	0.057	0.451
Diastolic BP & AR_7	177	0.087	0.248
Diastolic BP & AR_8	177	0.086	0.253
Diastolic BP & AR_9	177	0.111	0.140
Diastolic BP & AR_10	177	0.028	0.708
Diastolic BP & AR_11	177	0.031	0.680
Diastolic BP & AR_12	177	0.016	0.836
Diastolic BP & AR_13	177	0.096	0.206
Diastolic BP & AR_14	177	0.031	0.683
Diastolic BP & AR_15	177	0.122	0.105

Appendix B(viii) Bivariate correlation analyses between estimated glomerular filtration rate (eGFR) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
eGFR & FSHR_1	176	0.079	0.299
eGFR & FSHR_2	176	-0.054	0.480
eGFR & FSHR_3	176	0.050	0.511
eGFR & FSHR_4	176	-0.066	0.385
eGFR & INHBB_1	175	0.043	0.570
eGFR & INHBB_2	175	-0.038	0.615
eGFR & INHBB_3	175	0.076	0.320
eGFR & INHBB_4	175	-0.027	0.721
eGFR & INHBB_5	175	0.041	0.594
eGFR & INHBB_6	175	0.107	0.157
eGFR & INHBB_7	175	0.157	0.038
eGFR & INHBB_8	175	-0.020	0.795
eGFR & INHBB_9	175	0.064	0.402
eGFR & INHBB_10	175	-0.029	0.706
eGFR & INHBB_11	175	-0.114	0.132
eGFR & INHBB_12	175	0.111	0.142
eGFR & INHBB_13	175	0.054	0.482
eGFR & INHBB_14	175	-0.073	0.338
eGFR & INHBB_15	175	0.038	0.622
eGFR & INHBB_16	175	0.018	0.814
eGFR & INHBB_17	175	0.011	0.881
eGFR & INHBB_18	175	0.187	0.013
eGFR & INHBB_19	175	0.121	0.110
eGFR & INHBB_20	175	0.093	0.221
eGFR & INHBB_21	175	0.072	0.342
eGFR & INHBB_22	175	0.059	0.437
eGFR & INHBB_23	175	0.012	0.876
eGFR & INHBB_24	175	-0.066	0.383
eGFR & INHBB_25	175	0.005	0.950
eGFR & INHBB_26	175	-0.020	0.790
eGFR & INHBB_27	175	-0.066	0.385
eGFR & INHBB_28	175	0.083	0.276
eGFR & INHBB_29	175	0.067	0.378
eGFR & INHBB_30	175	-0.147	0.052
eGFR & ESR2_1	105	0.063	0.525
eGFR & ESR2_2	105	0.112	0.255
eGFR & ESR2_3	105	0.153	0.118
eGFR & ESR2_4	105	0.125	0.205
eGFR & ESR2_5	105	0.047	0.632
eGFR & ESR2_6	105	0.239	0.014
eGFR & ESR2_7	105	0.052	0.600
eGFR & ESR2_8	105	-0.056	0.568
eGFR & ESR2_9	105	0.128	0.192
eGFR & ESR2_10	105	0.126	0.200
eGFR & ESR2_11	105	0.299	0.002
eGFR & ESR2_12	105	0.061	0.533
eGFR & ESR2_13	105	-0.115	0.243
eGFR & ESR2_14	105	0.209	0.033
eGFR & ESR2_15	105	0.129	0.190
eGFR & ESR2_16	105	0.149	0.129

eGFR & ESR2_17	105	-0.053	0.593
eGFR & ESR2_18	105	0.042	0.672
eGFR & ESR2_19	105	0.108	0.272
eGFR & ESR2_20	104	-0.005	0.963
eGFR & SHBG_1	165	0.022	0.783
eGFR & SHBG_2	165	0.130	0.095
eGFR & SHBG_3	165	0.103	0.189
eGFR & SHBG_4	165	0.090	0.250
eGFR & SHBG_5	165	0.131	0.094
eGFR & SHBG_6	165	0.085	0.281
eGFR & SHBG_7	165	0.089	0.257
eGFR & SHBG_8	165	0.033	0.678
eGFR & SHBG_9	165	-0.064	0.413
eGFR & SHBG_10	165	-0.038	0.624
eGFR & SHBG_11	165	0.040	0.606
eGFR & SHBG_12	165	-0.093	0.234
eGFR & SHBG_13	165	-0.117	0.134
eGFR & SHBG_14	165	0.080	0.307
eGFR & SHBG_15	165	0.042	0.596
eGFR & SHBG_16	165	0.076	0.331
eGFR & SHBG_17	165	0.018	0.815
eGFR & AR_1	176	0.079	0.298
eGFR & AR_2	176	0.002	0.982
eGFR & AR_3	176	0.044	0.566
eGFR & AR_4	176	0.094	0.217
eGFR & AR_5	176	-0.103	0.175
eGFR & AR_6	176	0.077	0.310
eGFR & AR_7	176	-0.019	0.803
eGFR & AR_8	176	0.063	0.403
eGFR & AR_9	176	0.073	0.334
eGFR & AR_10	176	-0.046	0.540
eGFR & AR_11	176	0.060	0.428
eGFR & AR_12	176	0.089	0.241
eGFR & AR_13	176	-0.034	0.656
eGFR & AR_14	176	-0.050	0.511
eGFR & AR_15	176	-0.022	0.767

Appendix B(ix) Bivariate correlation analyses between total cholesterol (TChol) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
TChol & FSHR_1	176	0.091	0.232
TChol & FSHR_2	176	0.085	0.265
TChol & FSHR_3	176	0.054	0.475
TChol & FSHR_4	176	-0.001	0.991
TChol & INHBB_1	175	-0.161	0.033
TChol & INHBB_2	175	-0.011	0.882
TChol & INHBB_3	175	-0.085	0.264
TChol & INHBB_4	175	-0.101	0.186
TChol & INHBB_5	175	-0.177	0.019
TChol & INHBB_6	175	-0.001	0.987
TChol & INHBB_7	175	0.080	0.291
TChol & INHBB_8	175	-0.069	0.366
TChol & INHBB_9	175	-0.039	0.610
TChol & INHBB_10	175	-0.033	0.660
TChol & INHBB_11	175	-0.006	0.940
TChol & INHBB_12	175	-0.111	0.142
TChol & INHBB_13	175	-0.138	0.069
TChol & INHBB_14	175	-0.166	0.028
TChol & INHBB_15	175	-0.057	0.451
TChol & INHBB_16	175	0.107	0.158
TChol & INHBB_17	175	0.020	0.795
TChol & INHBB_18	175	-0.034	0.656
TChol & INHBB_19	175	-0.081	0.286
TChol & INHBB_20	175	-0.059	0.440
TChol & INHBB_21	175	0.001	0.990
TChol & INHBB_22	175	-0.096	0.208
TChol & INHBB_23	175	0.083	0.277
TChol & INHBB_24	175	0.174	0.021
TChol & INHBB_25	175	0.034	0.659
TChol & INHBB_26	175	0.036	0.633
TChol & INHBB_27	175	-0.027	0.723
TChol & INHBB_28	175	-0.185	0.014
TChol & INHBB_29	175	-0.023	0.766
TChol & INHBB_30	175	0.001	0.993
TChol & ESR2_1	105	-0.120	0.224
TChol & ESR2_2	105	0.097	0.326
TChol & ESR2_3	105	-0.003	0.972
TChol & ESR2_4	105	0.007	0.941
TChol & ESR2_5	105	-0.032	0.747
TChol & ESR2_6	105	-0.130	0.188
TChol & ESR2_7	105	-0.004	0.965
TChol & ESR2_8	105	-0.006	0.949
TChol & ESR2_9	105	-0.014	0.891
TChol & ESR2_10	105	0.109	0.270
TChol & ESR2_11	105	0.010	0.918
TChol & ESR2_12	105	0.004	0.966
TChol & ESR2_13	105	0.133	0.177
TChol & ESR2_14	105	0.124	0.207
TChol & ESR2_15	105	0.164	0.095
TChol & ESR2_16	105	0.130	0.188

TChol & ESR2_17	105	0.029	0.772
TChol & ESR2_18	105	0.156	0.113
TChol & ESR2_19	105	0.164	0.094
TChol & ESR2_20	104	0.038	0.699
TChol & SHBG_1	165	-0.037	0.640
TChol & SHBG_2	165	-0.005	0.951
TChol & SHBG_3	165	0.074	0.342
TChol & SHBG_4	165	0.038	0.627
TChol & SHBG_5	165	-0.103	0.186
TChol & SHBG_6	165	-0.045	0.569
TChol & SHBG_7	165	0.024	0.760
TChol & SHBG_8	165	-0.057	0.470
TChol & SHBG_9	165	0.038	0.627
TChol & SHBG_10	165	0.016	0.842
TChol & SHBG_11	165	-0.017	0.832
TChol & SHBG_12	165	-0.089	0.254
TChol & SHBG_13	165	0.025	0.749
TChol & SHBG_14	165	-0.010	0.895
TChol & SHBG_15	165	-0.029	0.714
TChol & SHBG_16	165	0.043	0.583
TChol & SHBG_17	165	-0.003	0.971
TChol & AR_1	176	0.096	0.204
TChol & AR_2	176	-0.055	0.467
TChol & AR_3	176	0.118	0.120
TChol & AR_4	176	-0.023	0.766
TChol & AR_5	176	-0.084	0.266
TChol & AR_6	176	-0.032	0.675
TChol & AR_7	176	0.001	0.986
TChol & AR_8	176	-0.046	0.543
TChol & AR_9	176	0.128	0.090
TChol & AR_10	176	0.051	0.502
TChol & AR_11	176	0.082	0.282
TChol & AR_12	176	0.070	0.358
TChol & AR_13	176	0.041	0.585
TChol & AR_14	176	-0.032	0.676
TChol & AR_15	176	0.141	0.062

Appendix B(x) Bivariate correlation analyses between high density lipoprotein cholesterol (HDL-c) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
HDL-c & FSHR_1	176	-0.005	0.951
HDL-c & FSHR_2	176	0.154	0.041
HDL-c & FSHR_3	176	-0.071	0.348
HDL-c & FSHR_4	176	0.053	0.486
HDL-c & INHBB_1	175	-0.163	0.031
HDL-c & INHBB_2	175	-0.045	0.553
HDL-c & INHBB_3	175	-0.035	0.646
HDL-c & INHBB_4	175	-0.124	0.101
HDL-c & INHBB_5	175	-0.005	0.944
HDL-c & INHBB_6	175	0.008	0.917
HDL-c & INHBB_7	175	0.063	0.406
HDL-c & INHBB_8	175	-0.056	0.460
HDL-c & INHBB_9	175	-0.027	0.722
HDL-c & INHBB_10	175	-0.015	0.842
HDL-c & INHBB_11	175	-0.033	0.667
HDL-c & INHBB_12	175	-0.037	0.629
HDL-c & INHBB_13	175	-0.077	0.313
HDL-c & INHBB_14	175	-0.043	0.571
HDL-c & INHBB_15	175	0.041	0.590
HDL-c & INHBB_16	175	0.073	0.337
HDL-c & INHBB_17	175	0.005	0.946
HDL-c & INHBB_18	175	-0.109	0.152
HDL-c & INHBB_19	175	-0.049	0.520
HDL-c & INHBB_20	175	0.040	0.597
HDL-c & INHBB_21	175	-0.030	0.693
HDL-c & INHBB_22	175	-0.106	0.161
HDL-c & INHBB_23	175	0.074	0.328
HDL-c & INHBB_24	175	0.097	0.200
HDL-c & INHBB_25	175	0.063	0.411
HDL-c & INHBB_26	175	0.007	0.925
HDL-c & INHBB_27	175	-0.031	0.683
HDL-c & INHBB_28	175	-0.129	0.090
HDL-c & INHBB_29	175	-0.152	0.045
HDL-c & INHBB_30	175	-0.118	0.121
HDL-c & ESR2_1	105	-0.149	0.130
HDL-c & ESR2_2	105	-0.044	0.656
HDL-c & ESR2_3	105	0.130	0.186
HDL-c & ESR2_4	105	-0.118	0.230
HDL-c & ESR2_5	105	-0.279	0.004
HDL-c & ESR2_6	105	-0.219	0.025
HDL-c & ESR2_7	105	-0.208	0.033
HDL-c & ESR2_8	105	-0.072	0.462
HDL-c & ESR2_9	105	-0.172	0.079
HDL-c & ESR2_10	105	0.072	0.465
HDL-c & ESR2_11	105	-0.055	0.574
HDL-c & ESR2_12	105	-0.091	0.354
HDL-c & ESR2_13	105	-0.167	0.089
HDL-c & ESR2_14	105	-0.066	0.500
HDL-c & ESR2_15	105	0.098	0.320
HDL-c & ESR2_16	105	-0.142	0.150

HDL-c & ESR2_17	105	-0.170	0.082
HDL-c & ESR2_18	105	-0.094	0.340
HDL-c & ESR2_19	105	-0.057	0.562
HDL-c & ESR2_20	104	-0.072	0.470
HDL-c & SHBG_1	165	-0.005	0.953
HDL-c & SHBG_2	165	0.091	0.248
HDL-c & SHBG_3	165	0.042	0.590
HDL-c & SHBG_4	165	0.063	0.424
HDL-c & SHBG_5	165	0.033	0.671
HDL-c & SHBG_6	165	-0.147	0.060
HDL-c & SHBG_7	165	-0.015	0.846
HDL-c & SHBG_8	165	-0.054	0.488
HDL-c & SHBG_9	165	0.108	0.169
HDL-c & SHBG_10	165	-0.100	0.200
HDL-c & SHBG_11	165	0.011	0.886
HDL-c & SHBG_12	165	-0.081	0.304
HDL-c & SHBG_13	165	-0.084	0.281
HDL-c & SHBG_14	165	-0.043	0.587
HDL-c & SHBG_15	165	-0.003	0.970
HDL-c & SHBG_16	165	0.150	0.055
HDL-c & SHBG_17	165	-0.078	0.320
HDL-c & AR_1	176	0.119	0.116
HDL-c & AR_2	176	0.062	0.413
HDL-c & AR_3	176	0.095	0.209
HDL-c & AR_4	176	0.050	0.510
HDL-c & AR_5	176	-0.038	0.620
HDL-c & AR_6	176	-0.004	0.959
HDL-c & AR_7	176	0.024	0.756
HDL-c & AR_8	176	0.049	0.515
HDL-c & AR_9	176	0.147	0.052
HDL-c & AR_10	176	0.030	0.695
HDL-c & AR_11	176	0.106	0.162
HDL-c & AR_12	176	0.146	0.053
HDL-c & AR_13	176	0.044	0.559
HDL-c & AR_14	176	0.006	0.941
HDL-c & AR_15	176	0.096	0.207

Appendix B(xi) Bivariate correlation analyses between triglycerides and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Triglycerides & FSHR_1	176	0.088	0.244
Triglycerides & FSHR_2	176	-0.056	0.463
Triglycerides & FSHR_3	176	-0.020	0.787
Triglycerides & FSHR_4	176	0.012	0.870
Triglycerides & INHBB_1	175	-0.095	0.212
Triglycerides & INHBB_2	175	0.073	0.339
Triglycerides & INHBB_3	175	-0.023	0.760
Triglycerides & INHBB_4	175	0.059	0.438
Triglycerides & INHBB_5	175	-0.074	0.333
Triglycerides & INHBB_6	175	0.084	0.268
Triglycerides & INHBB_7	175	0.072	0.344
Triglycerides & INHBB_8	175	-0.042	0.579
Triglycerides & INHBB_9	175	0.020	0.794
Triglycerides & INHBB_10	175	-0.095	0.213
Triglycerides & INHBB_11	175	0.070	0.356
Triglycerides & INHBB_12	175	0.040	0.596
Triglycerides & INHBB_13	175	-0.011	0.881
Triglycerides & INHBB_14	175	-0.063	0.409
Triglycerides & INHBB_15	175	-0.014	0.850
Triglycerides & INHBB_16	175	0.020	0.790
Triglycerides & INHBB_17	175	-0.039	0.608
Triglycerides & INHBB_18	175	-0.029	0.707
Triglycerides & INHBB_19	175	-0.026	0.733
Triglycerides & INHBB_20	175	0.005	0.946
Triglycerides & INHBB_21	175	0.066	0.383
Triglycerides & INHBB_22	175	-0.045	0.550
Triglycerides & INHBB_23	175	0.034	0.651
Triglycerides & INHBB_24	175	-0.009	0.903
Triglycerides & INHBB_25	175	0.002	0.981
Triglycerides & INHBB_26	175	-0.072	0.345
Triglycerides & INHBB_27	175	-0.014	0.857
Triglycerides & INHBB_28	175	-0.125	0.098
Triglycerides & INHBB_29	175	0.059	0.441
Triglycerides & INHBB_30	175	0.051	0.501
Triglycerides & ESR2_1	105	0.048	0.628
Triglycerides & ESR2_2	105	-0.096	0.331
Triglycerides & ESR2_3	105	-0.087	0.376
Triglycerides & ESR2_4	105	0.074	0.454
Triglycerides & ESR2_5	105	0.176	0.072
Triglycerides & ESR2_6	105	-0.096	0.329
Triglycerides & ESR2_7	105	-0.004	0.969
Triglycerides & ESR2_8	105	0.127	0.196
Triglycerides & ESR2_9	105	0.071	0.475
Triglycerides & ESR2_10	105	0.052	0.600
Triglycerides & ESR2_11	105	0.102	0.300
Triglycerides & ESR2_12	105	-0.009	0.931
Triglycerides & ESR2_13	105	0.043	0.665
Triglycerides & ESR2_14	105	0.177	0.071
Triglycerides & ESR2_15	105	0.090	0.364
Triglycerides & ESR2_16	105	0.391	<0.001

Triglycerides & ESR2_17	105	0.027	0.788
Triglycerides & ESR2_18	105	0.096	0.332
Triglycerides & ESR2_19	105	0.045	0.650
Triglycerides & ESR2_20	104	0.030	0.760
Triglycerides & SHBG_1	165	-0.057	0.469
Triglycerides & SHBG_2	165	0.028	0.724
Triglycerides & SHBG_3	165	0.147	0.059
Triglycerides & SHBG_4	165	-0.013	0.873
Triglycerides & SHBG_5	165	-0.012	0.883
Triglycerides & SHBG_6	165	-0.061	0.433
Triglycerides & SHBG_7	165	0.030	0.699
Triglycerides & SHBG_8	165	-0.068	0.388
Triglycerides & SHBG_9	165	-0.073	0.353
Triglycerides & SHBG_10	165	-0.024	0.760
Triglycerides & SHBG_11	165	0.033	0.677
Triglycerides & SHBG_12	165	-0.016	0.842
Triglycerides & SHBG_13	165	0.032	0.680
Triglycerides & SHBG_14	165	-0.007	0.927
Triglycerides & SHBG_15	165	-0.033	0.677
Triglycerides & SHBG_16	165	-0.001	0.987
Triglycerides & SHBG_17	165	0.001	0.992
Triglycerides & AR_1	176	-0.062	0.410
Triglycerides & AR_2	176	-0.098	0.195
Triglycerides & AR_3	176	-0.102	0.177
Triglycerides & AR_4	176	-0.083	0.273
Triglycerides & AR_5	176	-0.113	0.135
Triglycerides & AR_6	176	-0.022	0.769
Triglycerides & AR_7	176	-0.046	0.548
Triglycerides & AR_8	176	-0.031	0.687
Triglycerides & AR_9	176	0.159	0.035
Triglycerides & AR_10	176	0.049	0.519
Triglycerides & AR_11	176	0.034	0.656
Triglycerides & AR_12	176	0.029	0.703
Triglycerides & AR_13	176	-0.038	0.618
Triglycerides & AR_14	176	-0.037	0.628
Triglycerides & AR_15	176	0.044	0.558

Appendix B(xii) Bivariate correlation analyses between low density lipoprotein cholesterol (LDL-c) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
LDL-c & FSHR_1	176	0.120	0.114
LDL-c & FSHR_2	176	0.040	0.597
LDL-c & FSHR_3	176	0.111	0.142
LDL-c & FSHR_4	176	-0.023	0.762
LDL-c & INHBB_1	175	-0.076	0.316
LDL-c & INHBB_2	175	<0.001	0.998
LDL-c & INHBB_3	175	-0.041	0.586
LDL-c & INHBB_4	175	-0.068	0.369
LDL-c & INHBB_5	175	-0.205	0.006
LDL-c & INHBB_6	175	-0.013	0.861
LDL-c & INHBB_7	175	0.039	0.611
LDL-c & INHBB_8	175	-0.047	0.534
LDL-c & INHBB_9	175	-0.049	0.515
LDL-c & INHBB_10	175	-0.025	0.745
LDL-c & INHBB_11	175	0.020	0.788
LDL-c & INHBB_12	175	-0.079	0.301
LDL-c & INHBB_13	175	-0.115	0.128
LDL-c & INHBB_14	175	-0.181	0.017
LDL-c & INHBB_15	175	-0.088	0.245
LDL-c & INHBB_16	175	0.083	0.272
LDL-c & INHBB_17	175	0.020	0.793
LDL-c & INHBB_18	175	0.053	0.485
LDL-c & INHBB_19	175	-0.039	0.612
LDL-c & INHBB_20	175	-0.063	0.408
LDL-c & INHBB_21	175	0.026	0.737
LDL-c & INHBB_22	175	-0.040	0.597
LDL-c & INHBB_23	175	0.036	0.639
LDL-c & INHBB_24	175	0.116	0.127
LDL-c & INHBB_25	175	-0.008	0.921
LDL-c & INHBB_26	175	0.046	0.547
LDL-c & INHBB_27	175	-0.021	0.784
LDL-c & INHBB_28	175	-0.121	0.110
LDL-c & INHBB_29	175	0.070	0.359
LDL-c & INHBB_30	175	0.027	0.720
LDL-c & ESR2_1	105	-0.047	0.636
LDL-c & ESR2_2	105	0.148	0.132
LDL-c & ESR2_3	105	-0.059	0.548
LDL-c & ESR2_4	105	0.023	0.816
LDL-c & ESR2_5	105	0.027	0.782
LDL-c & ESR2_6	105	-0.090	0.359
LDL-c & ESR2_7	105	0.044	0.659
LDL-c & ESR2_8	105	-0.033	0.740
LDL-c & ESR2_9	105	0.081	0.410
LDL-c & ESR2_10	105	0.114	0.249
LDL-c & ESR2_11	105	0.031	0.757
LDL-c & ESR2_12	105	0.074	0.450
LDL-c & ESR2_13	105	0.243	0.012
LDL-c & ESR2_14	105	0.138	0.159
LDL-c & ESR2_15	105	0.128	0.194
LDL-c & ESR2_16	105	0.094	0.340

LDL-c & ESR2_17	105	0.136	0.167
LDL-c & ESR2_18	105	0.146	0.136
LDL-c & ESR2_19	105	0.192	0.049
LDL-c & ESR2_20	104	0.053	0.594
LDL-c & SHBG_1	165	-0.026	0.743
LDL-c & SHBG_2	165	-0.028	0.718
LDL-c & SHBG_3	165	0.062	0.432
LDL-c & SHBG_4	165	0.060	0.442
LDL-c & SHBG_5	165	-0.112	0.152
LDL-c & SHBG_6	165	0.041	0.599
LDL-c & SHBG_7	165	0.030	0.699
LDL-c & SHBG_8	165	-0.064	0.414
LDL-c & SHBG_9	165	-0.023	0.773
LDL-c & SHBG_10	165	0.033	0.677
LDL-c & SHBG_11	165	0.009	0.907
LDL-c & SHBG_12	165	-0.015	0.852
LDL-c & SHBG_13	165	0.062	0.432
LDL-c & SHBG_14	165	0.038	0.631
LDL-c & SHBG_15	165	0.029	0.710
LDL-c & SHBG_16	165	0.012	0.879
LDL-c & SHBG_17	165	0.043	0.582
LDL-c & AR_1	176	0.105	0.167
LDL-c & AR_2	176	-0.023	0.766
LDL-c & AR_3	176	0.137	0.070
LDL-c & AR_4	176	-0.009	0.907
LDL-c & AR_5	176	-0.009	0.901
LDL-c & AR_6	176	-0.015	0.842
LDL-c & AR_7	176	0.043	0.573
LDL-c & AR_8	176	-0.050	0.514
LDL-c & AR_9	176	0.083	0.272
LDL-c & AR_10	176	0.092	0.225
LDL-c & AR_11	176	0.097	0.202
LDL-c & AR_12	176	0.051	0.505
LDL-c & AR_13	176	0.091	0.230
LDL-c & AR_14	176	-0.001	0.987
LDL-c & AR_15	176	0.158	0.036

Appendix B(xiii) Bivariate correlation analyses between carotid intima-media thickness (cIMT) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
cIMT & FSHR_1	177	-0.037	0.624
cIMT & FSHR_2	177	-0.011	0.884
cIMT & FSHR_3	177	-0.054	0.472
cIMT & FSHR_4	177	0.023	0.762
cIMT & INHBB_1	176	-0.070	0.357
cIMT & INHBB_2	176	-0.057	0.449
cIMT & INHBB_3	176	-0.133	0.079
cIMT & INHBB_4	176	-0.085	0.262
cIMT & INHBB_5	176	-0.046	0.547
cIMT & INHBB_6	176	-0.055	0.465
cIMT & INHBB_7	176	-0.147	0.052
cIMT & INHBB_8	176	-0.038	0.615
cIMT & INHBB_9	176	0.037	0.623
cIMT & INHBB_10	176	-0.116	0.124
cIMT & INHBB_11	176	-0.029	0.699
cIMT & INHBB_12	176	0.004	0.958
cIMT & INHBB_13	176	-0.050	0.506
cIMT & INHBB_14	176	-0.124	0.100
cIMT & INHBB_15	176	-0.035	0.647
cIMT & INHBB_16	176	-0.035	0.649
cIMT & INHBB_17	176	-0.037	0.626
cIMT & INHBB_18	176	-0.154	0.041
cIMT & INHBB_19	176	-0.187	0.013
cIMT & INHBB_20	176	-0.075	0.322
cIMT & INHBB_21	176	-0.212	0.005
cIMT & INHBB_22	176	-0.186	0.013
cIMT & INHBB_23	176	-0.109	0.149
cIMT & INHBB_24	176	0.141	0.062
cIMT & INHBB_25	176	0.003	0.964
cIMT & INHBB_26	176	-0.036	0.638
cIMT & INHBB_27	176	0.023	0.766
cIMT & INHBB_28	176	-0.115	0.127
cIMT & INHBB_29	176	-0.039	0.609
cIMT & INHBB_30	176	-0.031	0.680
cIMT & ESR2_1	106	-0.094	0.340
cIMT & ESR2_2	106	0.056	0.571
cIMT & ESR2_3	106	-0.224	0.021
cIMT & ESR2_4	106	-0.055	0.576
cIMT & ESR2_5	106	0.113	0.249
cIMT & ESR2_6	106	0.056	0.568
cIMT & ESR2_7	106	0.061	0.532
cIMT & ESR2_8	106	0.019	0.850
cIMT & ESR2_9	106	0.011	0.910
cIMT & ESR2_10	106	0.180	0.065
cIMT & ESR2_11	106	0.092	0.347
cIMT & ESR2_12	106	-0.133	0.175
cIMT & ESR2_13	106	0.008	0.931
cIMT & ESR2_14	106	-0.078	0.429
cIMT & ESR2_15	106	-0.066	0.504
cIMT & ESR2_16	106	-0.025	0.799

cIMT & ESR2_17	106	-0.064	0.512
cIMT & ESR2_18	106	-0.032	0.744
cIMT & ESR2_19	106	-0.019	0.844
cIMT & ESR2_20	105	0.048	0.629
cIMT & SHBG_1	166	-0.014	0.859
cIMT & SHBG_2	166	0.021	0.792
cIMT & SHBG_3	166	-0.017	0.830
cIMT & SHBG_4	166	0.086	0.268
cIMT & SHBG_5	166	-0.105	0.176
cIMT & SHBG_6	166	0.011	0.892
cIMT & SHBG_7	166	-0.120	0.122
cIMT & SHBG_8	166	-0.043	0.583
cIMT & SHBG_9	166	-0.188	0.015
cIMT & SHBG_10	166	-0.123	0.115
cIMT & SHBG_11	166	-0.104	0.184
cIMT & SHBG_12	166	-0.100	0.200
cIMT & SHBG_13	166	0.077	0.323
cIMT & SHBG_14	166	-0.087	0.267
cIMT & SHBG_15	166	-0.125	0.109
cIMT & SHBG_16	166	0.014	0.854
cIMT & SHBG_17	166	-0.023	0.771
cIMT & AR_1	177	-0.056	0.456
cIMT & AR_2	177	-0.078	0.300
cIMT & AR_3	177	-0.099	0.191
cIMT & AR_4	177	-0.143	0.058
cIMT & AR_5	177	-0.077	0.307
cIMT & AR_6	177	-0.102	0.176
cIMT & AR_7	177	-0.095	0.209
cIMT & AR_8	177	-0.050	0.507
cIMT & AR_9	177	-0.056	0.455
cIMT & AR_10	177	-0.003	0.967
cIMT & AR_11	177	-0.034	0.656
cIMT & AR_12	177	-0.078	0.302
cIMT & AR_13	177	-0.029	0.704
cIMT & AR_14	177	0.009	0.907
cIMT & AR_15	177	-0.027	0.720

Appendix B(xiv) Bivariate correlation analyses between hip circumference and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Hip Circumference & FSHR_1	177	0.101	0.183
Hip Circumference & FSHR_2	177	-0.037	0.622
Hip Circumference & FSHR_3	177	0.110	0.143
Hip Circumference & FSHR_4	177	-0.019	0.803
Hip Circumference & INHBB_1	176	0.004	0.959
Hip Circumference & INHBB_2	176	0.135	0.073
Hip Circumference & INHBB_3	176	0.192	0.011
Hip Circumference & INHBB_4	176	0.167	0.027
Hip Circumference & INHBB_5	176	0.149	0.049
Hip Circumference & INHBB_6	176	0.094	0.214
Hip Circumference & INHBB_7	176	0.039	0.609
Hip Circumference & INHBB_8	176	-0.028	0.708
Hip Circumference & INHBB_9	176	-0.088	0.248
Hip Circumference & INHBB_10	176	-0.120	0.114
Hip Circumference & INHBB_11	176	-0.019	0.797
Hip Circumference & INHBB_12	176	0.023	0.759
Hip Circumference & INHBB_13	176	0.032	0.671
Hip Circumference & INHBB_14	176	-0.045	0.549
Hip Circumference & INHBB_15	176	0.070	0.354
Hip Circumference & INHBB_16	176	0.085	0.259
Hip Circumference & INHBB_17	176	0.144	0.057
Hip Circumference & INHBB_18	176	0.072	0.341
Hip Circumference & INHBB_19	176	-0.008	0.914
Hip Circumference & INHBB_20	176	0.073	0.338
Hip Circumference & INHBB_21	176	0.012	0.872
Hip Circumference & INHBB_22	176	0.017	0.826
Hip Circumference & INHBB_23	176	-0.042	0.581
Hip Circumference & INHBB_24	176	-0.055	0.469
Hip Circumference & INHBB_25	176	0.064	0.398
Hip Circumference & INHBB_26	176	-0.021	0.784
Hip Circumference & INHBB_27	176	-0.077	0.308
Hip Circumference & INHBB_28	176	-0.038	0.615
Hip Circumference & INHBB_29	176	0.081	0.286
Hip Circumference & INHBB_30	176	-0.040	0.597
Hip Circumference & ESR2_1	106	-0.205	0.035
Hip Circumference & ESR2_2	106	0.057	0.564
Hip Circumference & ESR2_3	106	0.125	0.201
Hip Circumference & ESR2_4	106	-0.077	0.430
Hip Circumference & ESR2_5	106	0.107	0.274
Hip Circumference & ESR2_6	106	-0.081	0.407
Hip Circumference & ESR2_7	106	-0.076	0.436
Hip Circumference & ESR2_8	106	-0.007	0.947
Hip Circumference & ESR2_9	106	0.038	0.698
Hip Circumference & ESR2_10	106	-0.004	0.971
Hip Circumference & ESR2_11	106	0.011	0.908
Hip Circumference & ESR2_12	106	-0.039	0.688
Hip Circumference & ESR2_13	106	-0.088	0.371
Hip Circumference & ESR2_14	106	0.050	0.609
Hip Circumference & ESR2_15	106	0.118	0.228
Hip Circumference & ESR2_16	106	0.269	0.005

Hip Circumference & ESR2_17	106	-0.022	0.822
Hip Circumference & ESR2_18	106	0.160	0.102
Hip Circumference & ESR2_19	106	0.064	0.514
Hip Circumference & ESR2_20	105	0.045	0.650
Hip Circumference & SHBG_1	166	-0.078	0.317
Hip Circumference & SHBG_2	166	0.073	0.353
Hip Circumference & SHBG_3	166	-0.033	0.675
Hip Circumference & SHBG_4	166	-0.033	0.673
Hip Circumference & SHBG_5	166	-0.086	0.271
Hip Circumference & SHBG_6	166	-0.121	0.119
Hip Circumference & SHBG_7	166	-0.016	0.840
Hip Circumference & SHBG_8	166	-0.069	0.378
Hip Circumference & SHBG_9	166	-0.001	0.985
Hip Circumference & SHBG_10	166	0.037	0.640
Hip Circumference & SHBG_11	166	0.111	0.154
Hip Circumference & SHBG_12	166	0.076	0.327
Hip Circumference & SHBG_13	166	0.027	0.726
Hip Circumference & SHBG_14	166	<0.001	0.997
Hip Circumference & SHBG_15	166	-0.060	0.442
Hip Circumference & SHBG_16	166	-0.104	0.183
Hip Circumference & SHBG_17	166	-0.063	0.423
Hip Circumference & AR_1	177	0.098	0.193
Hip Circumference & AR_2	177	0.062	0.413
Hip Circumference & AR_3	177	0.016	0.832
Hip Circumference & AR_4	177	0.150	0.047
Hip Circumference & AR_5	177	0.006	0.938
Hip Circumference & AR_6	177	0.133	0.077
Hip Circumference & AR_7	177	0.083	0.269
Hip Circumference & AR_8	177	0.064	0.399
Hip Circumference & AR_9	177	0.090	0.232
Hip Circumference & AR_10	177	0.040	0.595
Hip Circumference & AR_11	177	0.037	0.627
Hip Circumference & AR_12	177	0.023	0.759
Hip Circumference & AR_13	177	0.041	0.584
Hip Circumference & AR_14	177	0.014	0.849
Hip Circumference & AR_15	177	0.052	0.489

Appendix B(xv) Bivariate correlation analyses between waist circumference and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Waist Circumference & FSHR_1	177	0.150	0.046
Waist Circumference & FSHR_2	177	-0.004	0.957
Waist Circumference & FSHR_3	177	0.115	0.127
Waist Circumference & FSHR_4	177	-0.054	0.473
Waist Circumference & INHBB_1	176	0.048	0.525
Waist Circumference & INHBB_2	176	0.110	0.146
Waist Circumference & INHBB_3	176	0.145	0.055
Waist Circumference & INHBB_4	176	0.106	0.163
Waist Circumference & INHBB_5	176	0.078	0.306
Waist Circumference & INHBB_6	176	0.080	0.291
Waist Circumference & INHBB_7	176	0.025	0.743
Waist Circumference & INHBB_8	176	<0.001	0.999
Waist Circumference & INHBB_9	176	-0.063	0.405
Waist Circumference & INHBB_10	176	-0.116	0.124
Waist Circumference & INHBB_11	176	-0.024	0.754
Waist Circumference & INHBB_12	176	0.001	0.991
Waist Circumference & INHBB_13	176	0.046	0.548
Waist Circumference & INHBB_14	176	-0.073	0.334
Waist Circumference & INHBB_15	176	0.070	0.357
Waist Circumference & INHBB_16	176	0.047	0.533
Waist Circumference & INHBB_17	176	0.096	0.203
Waist Circumference & INHBB_18	176	0.042	0.582
Waist Circumference & INHBB_19	176	-0.026	0.730
Waist Circumference & INHBB_20	176	0.007	0.923
Waist Circumference & INHBB_21	176	0.014	0.856
Waist Circumference & INHBB_22	176	0.024	0.755
Waist Circumference & INHBB_23	176	-0.019	0.800
Waist Circumference & INHBB_24	176	0.008	0.919
Waist Circumference & INHBB_25	176	0.056	0.459
Waist Circumference & INHBB_26	176	-0.042	0.576
Waist Circumference & INHBB_27	176	-0.030	0.697
Waist Circumference & INHBB_28	176	-0.020	0.793
Waist Circumference & INHBB_29	176	0.137	0.070
Waist Circumference & INHBB_30	176	0.004	0.963
Waist Circumference & ESR2_1	106	-0.067	0.496
Waist Circumference & ESR2_2	106	0.055	0.579
Waist Circumference & ESR2_3	106	0.059	0.546
Waist Circumference & ESR2_4	106	-0.028	0.772
Waist Circumference & ESR2_5	106	0.152	0.121
Waist Circumference & ESR2_6	106	-0.082	0.405
Waist Circumference & ESR2_7	106	-0.109	0.268
Waist Circumference & ESR2_8	106	0.013	0.896
Waist Circumference & ESR2_9	106	0.102	0.299
Waist Circumference & ESR2_10	106	0.065	0.505
Waist Circumference & ESR2_11	106	0.005	0.961
Waist Circumference & ESR2_12	106	-0.102	0.299
Waist Circumference & ESR2_13	106	0.006	0.949
Waist Circumference & ESR2_14	106	0.134	0.171
Waist Circumference & ESR2_15	106	0.160	0.100
Waist Circumference & ESR2_16	106	0.243	0.012

Waist Circumference & ESR2_17	106	0.049	0.618
Waist Circumference & ESR2_18	106	0.136	0.164
Waist Circumference & ESR2_19	106	0.083	0.397
Waist Circumference & ESR2_20	105	0.091	0.354
Waist Circumference & SHBG_1	166	-0.131	0.092
Waist Circumference & SHBG_2	166	0.006	0.936
Waist Circumference & SHBG_3	166	0.023	0.768
Waist Circumference & SHBG_4	166	-0.030	0.702
Waist Circumference & SHBG_5	166	-0.052	0.505
Waist Circumference & SHBG_6	166	-0.066	0.395
Waist Circumference & SHBG_7	166	0.032	0.681
Waist Circumference & SHBG_8	166	-0.077	0.326
Waist Circumference & SHBG_9	166	0.007	0.928
Waist Circumference & SHBG_10	166	0.052	0.509
Waist Circumference & SHBG_11	166	0.131	0.091
Waist Circumference & SHBG_12	166	0.047	0.552
Waist Circumference & SHBG_13	166	0.074	0.345
Waist Circumference & SHBG_14	166	0.060	0.445
Waist Circumference & SHBG_15	166	-0.045	0.565
Waist Circumference & SHBG_16	166	-0.100	0.199
Waist Circumference & SHBG_17	166	-0.031	0.689
Waist Circumference & AR_1	177	0.134	0.076
Waist Circumference & AR_2	177	0.081	0.285
Waist Circumference & AR_3	177	0.076	0.317
Waist Circumference & AR_4	177	0.119	0.114
Waist Circumference & AR_5	177	-0.046	0.540
Waist Circumference & AR_6	177	0.143	0.057
Waist Circumference & AR_7	177	0.082	0.275
Waist Circumference & AR_8	177	0.114	0.132
Waist Circumference & AR_9	177	0.130	0.084
Waist Circumference & AR_10	177	0.083	0.272
Waist Circumference & AR_11	177	0.044	0.559
Waist Circumference & AR_12	177	0.011	0.881
Waist Circumference & AR_13	177	0.063	0.404
Waist Circumference & AR_14	177	0.010	0.893
Waist Circumference & AR_15	177	0.060	0.426

Appendix B(xvi) Bivariate correlation analyses between visceral fat and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Visceral Fat & FSHR_1	177	-0.049	0.521
Visceral Fat & FSHR_2	177	-0.004	0.954
Visceral Fat & FSHR_3	177	-0.043	0.570
Visceral Fat & FSHR_4	177	-0.119	0.115
Visceral Fat & INHBB_1	176	0.172	0.023
Visceral Fat & INHBB_2	176	-0.040	0.596
Visceral Fat & INHBB_3	176	0.085	0.261
Visceral Fat & INHBB_4	176	0.126	0.095
Visceral Fat & INHBB_5	176	0.070	0.356
Visceral Fat & INHBB_6	176	-0.004	0.962
Visceral Fat & INHBB_7	176	0.030	0.688
Visceral Fat & INHBB_8	176	0.068	0.371
Visceral Fat & INHBB_9	176	-0.079	0.297
Visceral Fat & INHBB_10	176	0.011	0.881
Visceral Fat & INHBB_11	176	0.045	0.550
Visceral Fat & INHBB_12	176	0.038	0.618
Visceral Fat & INHBB_13	176	0.078	0.301
Visceral Fat & INHBB_14	176	0.026	0.736
Visceral Fat & INHBB_15	176	-0.017	0.820
Visceral Fat & INHBB_16	176	-0.115	0.130
Visceral Fat & INHBB_17	176	-0.030	0.688
Visceral Fat & INHBB_18	176	0.061	0.420
Visceral Fat & INHBB_19	176	0.165	0.029
Visceral Fat & INHBB_20	176	0.036	0.633
Visceral Fat & INHBB_21	176	0.017	0.821
Visceral Fat & INHBB_22	176	-0.031	0.687
Visceral Fat & INHBB_23	176	0.024	0.749
Visceral Fat & INHBB_24	176	-0.018	0.809
Visceral Fat & INHBB_25	176	0.045	0.555
Visceral Fat & INHBB_26	176	-0.073	0.338
Visceral Fat & INHBB_27	176	0.076	0.316
Visceral Fat & INHBB_28	176	0.028	0.708
Visceral Fat & INHBB_29	176	0.033	0.668
Visceral Fat & INHBB_30	176	0.049	0.521
Visceral Fat & ESR2_1	106	-0.007	0.943
Visceral Fat & ESR2_2	106	0.197	0.043
Visceral Fat & ESR2_3	106	0.171	0.079
Visceral Fat & ESR2_4	106	-0.064	0.516
Visceral Fat & ESR2_5	106	-0.049	0.617
Visceral Fat & ESR2_6	106	0.030	0.760
Visceral Fat & ESR2_7	106	-0.051	0.602
Visceral Fat & ESR2_8	106	0.029	0.767
Visceral Fat & ESR2_9	106	0.055	0.578
Visceral Fat & ESR2_10	106	-0.086	0.383
Visceral Fat & ESR2_11	106	-0.042	0.672
Visceral Fat & ESR2_12	106	-0.032	0.742
Visceral Fat & ESR2_13	106	-0.037	0.706
Visceral Fat & ESR2_14	106	0.083	0.398
Visceral Fat & ESR2_15	106	0.121	0.216
Visceral Fat & ESR2_16	106	-0.113	0.248

Visceral Fat & ESR2_17	106	-0.037	0.707
Visceral Fat & ESR2_18	106	0.057	0.561
Visceral Fat & ESR2_19	106	-0.050	0.609
Visceral Fat & ESR2_20	105	0.120	0.222
Visceral Fat & SHBG_1	166	-0.032	0.681
Visceral Fat & SHBG_2	166	0.086	0.272
Visceral Fat & SHBG_3	166	0.003	0.967
Visceral Fat & SHBG_4	166	0.074	0.344
Visceral Fat & SHBG_5	166	0.032	0.686
Visceral Fat & SHBG_6	166	0.028	0.717
Visceral Fat & SHBG_7	166	0.036	0.646
Visceral Fat & SHBG_8	166	0.130	0.095
Visceral Fat & SHBG_9	166	0.019	0.805
Visceral Fat & SHBG_10	166	0.143	0.066
Visceral Fat & SHBG_11	166	0.087	0.266
Visceral Fat & SHBG_12	166	0.065	0.408
Visceral Fat & SHBG_13	166	-0.025	0.745
Visceral Fat & SHBG_14	166	0.068	0.381
Visceral Fat & SHBG_15	166	0.048	0.543
Visceral Fat & SHBG_16	166	-0.060	0.441
Visceral Fat & SHBG_17	166	-0.020	0.800
Visceral Fat & AR_1	177	-0.037	0.629
Visceral Fat & AR_2	177	-0.004	0.954
Visceral Fat & AR_3	177	-0.027	0.723
Visceral Fat & AR_4	177	-0.037	0.628
Visceral Fat & AR_5	177	-0.021	0.785
Visceral Fat & AR_6	177	0.018	0.812
Visceral Fat & AR_7	177	0.012	0.875
Visceral Fat & AR_8	177	0.016	0.835
Visceral Fat & AR_9	177	-0.094	0.212
Visceral Fat & AR_10	177	-0.064	0.399
Visceral Fat & AR_11	177	-0.069	0.362
Visceral Fat & AR_12	177	-0.135	0.072
Visceral Fat & AR_13	177	-0.009	0.909
Visceral Fat & AR_14	177	-0.054	0.473
Visceral Fat & AR_15	177	-0.068	0.369

Appendix B(xvii) Bivariate correlation analyses between subcutaneous fat and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
Subcutaneous Fat & FSHR_1	177	0.078	0.301
Subcutaneous Fat & FSHR_2	177	0.015	0.844
Subcutaneous Fat & FSHR_3	177	0.072	0.340
Subcutaneous Fat & FSHR_4	177	0.011	0.889
Subcutaneous Fat & INHBB_1	176	0.012	0.875
Subcutaneous Fat & INHBB_2	176	0.055	0.465
Subcutaneous Fat & INHBB_3	176	0.164	0.030
Subcutaneous Fat & INHBB_4	176	0.062	0.415
Subcutaneous Fat & INHBB_5	176	0.140	0.064
Subcutaneous Fat & INHBB_6	176	-0.003	0.974
Subcutaneous Fat & INHBB_7	176	0.054	0.475
Subcutaneous Fat & INHBB_8	176	-0.049	0.515
Subcutaneous Fat & INHBB_9	176	-0.082	0.278
Subcutaneous Fat & INHBB_10	176	-0.086	0.258
Subcutaneous Fat & INHBB_11	176	-0.080	0.288
Subcutaneous Fat & INHBB_12	176	-0.071	0.352
Subcutaneous Fat & INHBB_13	176	-0.003	0.972
Subcutaneous Fat & INHBB_14	176	-0.069	0.365
Subcutaneous Fat & INHBB_15	176	0.009	0.907
Subcutaneous Fat & INHBB_16	176	0.018	0.813
Subcutaneous Fat & INHBB_17	176	0.123	0.104
Subcutaneous Fat & INHBB_18	176	-0.010	0.890
Subcutaneous Fat & INHBB_19	176	-0.088	0.246
Subcutaneous Fat & INHBB_20	176	-0.015	0.841
Subcutaneous Fat & INHBB_21	176	-0.016	0.834
Subcutaneous Fat & INHBB_22	176	-0.005	0.943
Subcutaneous Fat & INHBB_23	176	-0.036	0.634
Subcutaneous Fat & INHBB_24	176	-0.050	0.507
Subcutaneous Fat & INHBB_25	176	-0.025	0.742
Subcutaneous Fat & INHBB_26	176	-0.016	0.832
Subcutaneous Fat & INHBB_27	176	-0.063	0.404
Subcutaneous Fat & INHBB_28	176	-0.134	0.076
Subcutaneous Fat & INHBB_29	176	0.032	0.678
Subcutaneous Fat & INHBB_30	176	0.023	0.764
Subcutaneous Fat & ESR2_1	106	-0.065	0.506
Subcutaneous Fat & ESR2_2	106	-0.004	0.967
Subcutaneous Fat & ESR2_3	106	0.104	0.291
Subcutaneous Fat & ESR2_4	106	0.007	0.939
Subcutaneous Fat & ESR2_5	106	0.090	0.358
Subcutaneous Fat & ESR2_6	106	0.004	0.967
Subcutaneous Fat & ESR2_7	106	-0.101	0.302
Subcutaneous Fat & ESR2_8	106	0.016	0.870
Subcutaneous Fat & ESR2_9	106	0.047	0.629
Subcutaneous Fat & ESR2_10	106	-0.092	0.348
Subcutaneous Fat & ESR2_11	106	-0.105	0.285
Subcutaneous Fat & ESR2_12	106	-0.023	0.816
Subcutaneous Fat & ESR2_13	106	-0.021	0.831
Subcutaneous Fat & ESR2_14	106	0.065	0.509
Subcutaneous Fat & ESR2_15	106	0.064	0.512
Subcutaneous Fat & ESR2_16	106	0.167	0.088

Subcutaneous Fat & ESR2_17	106	-0.044	0.654
Subcutaneous Fat & ESR2_18	106	0.025	0.797
Subcutaneous Fat & ESR2_19	106	0.106	0.279
Subcutaneous Fat & ESR2_20	105	0.118	0.231
Subcutaneous Fat & SHBG_1	166	-0.140	0.072
Subcutaneous Fat & SHBG_2	166	0.035	0.650
Subcutaneous Fat & SHBG_3	166	0.021	0.793
Subcutaneous Fat & SHBG_4	166	0.004	0.961
Subcutaneous Fat & SHBG_5	166	-0.145	0.062
Subcutaneous Fat & SHBG_6	166	-0.027	0.732
Subcutaneous Fat & SHBG_7	166	-0.053	0.500
Subcutaneous Fat & SHBG_8	166	-0.124	0.111
Subcutaneous Fat & SHBG_9	166	0.012	0.878
Subcutaneous Fat & SHBG_10	166	-0.125	0.108
Subcutaneous Fat & SHBG_11	166	0.141	0.071
Subcutaneous Fat & SHBG_12	166	-0.045	0.561
Subcutaneous Fat & SHBG_13	166	-0.004	0.964
Subcutaneous Fat & SHBG_14	166	-0.020	0.797
Subcutaneous Fat & SHBG_15	166	0.001	0.991
Subcutaneous Fat & SHBG_16	166	-0.010	0.897
Subcutaneous Fat & SHBG_17	166	-0.004	0.961
Subcutaneous Fat & AR_1	177	0.118	0.118
Subcutaneous Fat & AR_2	177	-0.003	0.965
Subcutaneous Fat & AR_3	177	0.056	0.458
Subcutaneous Fat & AR_4	177	0.067	0.376
Subcutaneous Fat & AR_5	177	-0.035	0.642
Subcutaneous Fat & AR_6	177	0.054	0.473
Subcutaneous Fat & AR_7	177	0.045	0.549
Subcutaneous Fat & AR_8	177	0.104	0.167
Subcutaneous Fat & AR_9	177	0.069	0.363
Subcutaneous Fat & AR_10	177	-0.042	0.582
Subcutaneous Fat & AR_11	177	0.014	0.850
Subcutaneous Fat & AR_12	177	0.023	0.759
Subcutaneous Fat & AR_13	177	-0.006	0.935
Subcutaneous Fat & AR_14	177	-0.024	0.751
Subcutaneous Fat & AR_15	177	0.016	0.832

Appendix B(xviii) Bivariate correlation analyses between oestrogen (E2) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
E2 & FSHR_1	158	-0.112	0.162
E2 & FSHR_2	158	-0.107	0.179
E2 & FSHR_3	158	-0.039	0.629
E2 & FSHR_4	158	0.013	0.871
E2 & INHBB_1	157	-0.204	0.010
E2 & INHBB_2	157	-0.060	0.453
E2 & INHBB_3	157	0.113	0.159
E2 & INHBB_4	157	0.084	0.297
E2 & INHBB_5	157	0.117	0.145
E2 & INHBB_6	157	0.005	0.950
E2 & INHBB_7	157	0.039	0.629
E2 & INHBB_8	157	0.067	0.401
E2 & INHBB_9	157	0.016	0.838
E2 & INHBB_10	157	-0.083	0.303
E2 & INHBB_11	157	0.065	0.422
E2 & INHBB_12	157	0.071	0.374
E2 & INHBB_13	157	-0.140	0.081
E2 & INHBB_14	157	-0.053	0.509
E2 & INHBB_15	157	0.045	0.577
E2 & INHBB_16	157	0.098	0.223
E2 & INHBB_17	157	0.040	0.622
E2 & INHBB_18	157	0.057	0.476
E2 & INHBB_19	157	0.066	0.413
E2 & INHBB_20	157	0.034	0.675
E2 & INHBB_21	157	0.096	0.230
E2 & INHBB_22	157	-0.060	0.454
E2 & INHBB_23	157	0.086	0.283
E2 & INHBB_24	157	0.065	0.420
E2 & INHBB_25	157	0.143	0.075
E2 & INHBB_26	157	-0.165	0.039
E2 & INHBB_27	157	0.042	0.605
E2 & INHBB_28	157	0.040	0.621
E2 & INHBB_29	157	0.107	0.184
E2 & INHBB_30	157	-0.047	0.563
E2 & ESR2_1	94	0.082	0.432
E2 & ESR2_2	94	-0.031	0.765
E2 & ESR2_3	94	-0.083	0.425
E2 & ESR2_4	94	0.064	0.538
E2 & ESR2_5	94	-0.025	0.810
E2 & ESR2_6	94	0.033	0.750
E2 & ESR2_7	94	0.036	0.730
E2 & ESR2_8	94	-0.027	0.798
E2 & ESR2_9	94	0.079	0.452
E2 & ESR2_10	94	-0.025	0.810
E2 & ESR2_11	94	0.069	0.512
E2 & ESR2_12	94	0.160	0.124
E2 & ESR2_13	94	-0.033	0.752
E2 & ESR2_14	94	0.110	0.290
E2 & ESR2_15	94	-0.067	0.521
E2 & ESR2_16	94	0.059	0.573

E2 & ESR2_17	94	0.017	0.871
E2 & ESR2_18	94	0.071	0.495
E2 & ESR2_19	94	-0.041	0.694
E2 & ESR2_20	93	-0.053	0.614
E2 & SHBG_1	147	0.063	0.449
E2 & SHBG_2	147	0.098	0.239
E2 & SHBG_3	147	0.017	0.834
E2 & SHBG_4	147	-0.054	0.513
E2 & SHBG_5	147	0.018	0.833
E2 & SHBG_6	147	-0.118	0.153
E2 & SHBG_7	147	-0.161	0.051
E2 & SHBG_8	147	0.032	0.701
E2 & SHBG_9	147	-0.070	0.400
E2 & SHBG_10	147	0.057	0.496
E2 & SHBG_11	147	-0.099	0.231
E2 & SHBG_12	147	0.055	0.510
E2 & SHBG_13	147	-0.026	0.754
E2 & SHBG_14	147	0.123	0.137
E2 & SHBG_15	147	-0.032	0.700
E2 & SHBG_16	147	-0.026	0.752
E2 & SHBG_17	147	-0.027	0.741
E2 & AR_1	158	-0.062	0.436
E2 & AR_2	158	0.037	0.648
E2 & AR_3	158	-0.047	0.556
E2 & AR_4	158	-0.064	0.422
E2 & AR_5	158	-0.036	0.651
E2 & AR_6	158	-0.047	0.559
E2 & AR_7	158	-0.079	0.325
E2 & AR_8	158	-0.108	0.176
E2 & AR_9	158	-0.108	0.177
E2 & AR_10	158	-0.079	0.325
E2 & AR_11	158	-0.106	0.184
E2 & AR_12	158	-0.116	0.148
E2 & AR_13	158	0.049	0.540
E2 & AR_14	158	-0.035	0.661
E2 & AR_15	158	-0.069	0.390

Appendix B(xix) Bivariate correlation analyses between follicle stimulating hormone (FSH) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
FSH & FSHR_1	158	0.046	0.565
FSH & FSHR_2	158	0.100	0.211
FSH & FSHR_3	158	-0.033	0.677
FSH & FSHR_4	158	-0.069	0.389
FSH & INHBB_1	157	0.056	0.486
FSH & INHBB_2	157	0.110	0.172
FSH & INHBB_3	157	-0.182	0.022
FSH & INHBB_4	157	-0.194	0.015
FSH & INHBB_5	157	-0.024	0.769
FSH & INHBB_6	157	-0.076	0.343
FSH & INHBB_7	157	-0.017	0.836
FSH & INHBB_8	157	-0.058	0.472
FSH & INHBB_9	157	0.009	0.915
FSH & INHBB_10	157	0.065	0.421
FSH & INHBB_11	157	-0.011	0.889
FSH & INHBB_12	157	-0.096	0.233
FSH & INHBB_13	157	0.006	0.942
FSH & INHBB_14	157	0.036	0.656
FSH & INHBB_15	157	-0.157	0.049
FSH & INHBB_16	157	-0.111	0.166
FSH & INHBB_17	157	-0.107	0.182
FSH & INHBB_18	157	0.002	0.980
FSH & INHBB_19	157	-0.034	0.675
FSH & INHBB_20	157	0.005	0.947
FSH & INHBB_21	157	-0.089	0.266
FSH & INHBB_22	157	0.053	0.510
FSH & INHBB_23	157	-0.038	0.639
FSH & INHBB_24	157	0.049	0.542
FSH & INHBB_25	157	-0.033	0.682
FSH & INHBB_26	157	0.089	0.266
FSH & INHBB_27	157	-0.135	0.091
FSH & INHBB_28	157	-0.191	0.017
FSH & INHBB_29	157	-0.104	0.193
FSH & INHBB_30	157	0.086	0.285
FSH & ESR2_1	94	-0.049	0.639
FSH & ESR2_2	94	-0.009	0.932
FSH & ESR2_3	94	-0.013	0.904
FSH & ESR2_4	94	-0.100	0.338
FSH & ESR2_5	94	0.007	0.949
FSH & ESR2_6	94	-0.042	0.686
FSH & ESR2_7	94	-0.050	0.634
FSH & ESR2_8	94	-0.042	0.690
FSH & ESR2_9	94	-0.045	0.664
FSH & ESR2_10	94	0.048	0.645
FSH & ESR2_11	94	-0.180	0.082
FSH & ESR2_12	94	-0.108	0.299
FSH & ESR2_13	94	0.048	0.647
FSH & ESR2_14	94	-0.116	0.267
FSH & ESR2_15	94	-0.059	0.572
FSH & ESR2_16	94	-0.084	0.420

FSH & ESR2_17	94	-0.123	0.239
FSH & ESR2_18	94	0.008	0.939
FSH & ESR2_19	94	-0.053	0.611
FSH & ESR2_20	93	0.035	0.736
FSH & SHBG_1	147	-0.049	0.557
FSH & SHBG_2	147	-0.156	0.059
FSH & SHBG_3	147	0.009	0.914
FSH & SHBG_4	147	0.023	0.780
FSH & SHBG_5	147	-0.047	0.571
FSH & SHBG_6	147	0.047	0.571
FSH & SHBG_7	147	0.164	0.048
FSH & SHBG_8	147	-0.018	0.830
FSH & SHBG_9	147	0.026	0.752
FSH & SHBG_10	147	-0.119	0.150
FSH & SHBG_11	147	0.014	0.866
FSH & SHBG_12	147	-0.039	0.643
FSH & SHBG_13	147	-0.006	0.939
FSH & SHBG_14	147	-0.059	0.480
FSH & SHBG_15	147	-0.074	0.372
FSH & SHBG_16	147	-0.001	0.992
FSH & SHBG_17	147	0.067	0.419
FSH & AR_1	158	0.055	0.496
FSH & AR_2	158	-0.102	0.201
FSH & AR_3	158	0.021	0.789
FSH & AR_4	158	-0.004	0.958
FSH & AR_5	158	0.021	0.796
FSH & AR_6	158	0.018	0.821
FSH & AR_7	158	0.044	0.586
FSH & AR_8	158	0.041	0.608
FSH & AR_9	158	0.047	0.559
FSH & AR_10	158	0.040	0.618
FSH & AR_11	158	0.015	0.851
FSH & AR_12	158	0.009	0.912
FSH & AR_13	158	-0.133	0.095
FSH & AR_14	158	-0.013	0.872
FSH & AR_15	158	-0.005	0.948

Appendix B(xx) Bivariate correlation analyses between bioavailable testosterone (BIOT) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
BIOT & FSHR_1	158	-0.040	0.618
BIOT & FSHR_2	158	0.095	0.234
BIOT & FSHR_3	158	0.107	0.180
BIOT & FSHR_4	158	-0.150	0.060
BIOT & INHBB_1	157	-0.061	0.447
BIOT & INHBB_2	157	0.038	0.640
BIOT & INHBB_3	157	0.130	0.103
BIOT & INHBB_4	157	-0.036	0.654
BIOT & INHBB_5	157	0.156	0.051
BIOT & INHBB_6	157	-0.026	0.747
BIOT & INHBB_7	157	0.017	0.835
BIOT & INHBB_8	157	-0.118	0.140
BIOT & INHBB_9	157	0.071	0.377
BIOT & INHBB_10	157	-0.066	0.414
BIOT & INHBB_11	157	-0.018	0.824
BIOT & INHBB_12	157	0.008	0.924
BIOT & INHBB_13	157	0.128	0.110
BIOT & INHBB_14	157	0.007	0.927
BIOT & INHBB_15	157	0.003	0.968
BIOT & INHBB_16	157	0.027	0.742
BIOT & INHBB_17	157	0.053	0.507
BIOT & INHBB_18	157	0.079	0.323
BIOT & INHBB_19	157	-0.010	0.904
BIOT & INHBB_20	157	-0.058	0.468
BIOT & INHBB_21	157	-0.038	0.635
BIOT & INHBB_22	157	-0.033	0.681
BIOT & INHBB_23	157	-0.063	0.433
BIOT & INHBB_24	157	-0.044	0.585
BIOT & INHBB_25	157	0.086	0.284
BIOT & INHBB_26	157	0.079	0.322
BIOT & INHBB_27	157	0.052	0.516
BIOT & INHBB_28	157	-0.029	0.723
BIOT & INHBB_29	157	0.050	0.535
BIOT & INHBB_30	157	-0.116	0.147
BIOT & ESR2_1	94	-0.099	0.344
BIOT & ESR2_2	94	-0.112	0.282
BIOT & ESR2_3	94	-0.083	0.426
BIOT & ESR2_4	94	0.002	0.985
BIOT & ESR2_5	94	-0.171	0.099
BIOT & ESR2_6	94	-0.083	0.427
BIOT & ESR2_7	94	0.002	0.983
BIOT & ESR2_8	94	0.041	0.698
BIOT & ESR2_9	94	-0.102	0.329
BIOT & ESR2_10	94	0.041	0.696
BIOT & ESR2_11	94	0.051	0.623
BIOT & ESR2_12	94	-0.057	0.588
BIOT & ESR2_13	94	-0.076	0.466
BIOT & ESR2_14	94	0.005	0.964
BIOT & ESR2_15	94	-0.080	0.446
BIOT & ESR2_16	94	0.015	0.888

BIOT & ESR2_17	94	-0.051	0.622
BIOT & ESR2_18	94	0.046	0.662
BIOT & ESR2_19	94	-0.152	0.144
BIOT & ESR2_20	93	0.026	0.803
BIOT & SHBG_1	147	0.009	0.916
BIOT & SHBG_2	147	-0.049	0.557
BIOT & SHBG_3	147	-0.053	0.521
BIOT & SHBG_4	147	-0.025	0.762
BIOT & SHBG_5	147	-0.034	0.686
BIOT & SHBG_6	147	-0.038	0.652
BIOT & SHBG_7	147	0.022	0.788
BIOT & SHBG_8	147	0.004	0.959
BIOT & SHBG_9	147	-0.055	0.511
BIOT & SHBG_10	147	-0.219	0.008
BIOT & SHBG_11	147	-0.107	0.196
BIOT & SHBG_12	147	-0.005	0.957
BIOT & SHBG_13	147	-0.044	0.599
BIOT & SHBG_14	147	-0.111	0.179
BIOT & SHBG_15	147	-0.180	0.029
BIOT & SHBG_16	147	0.010	0.904
BIOT & SHBG_17	147	-0.024	0.772
BIOT & AR_1	158	0.089	0.268
BIOT & AR_2	158	0.059	0.465
BIOT & AR_3	158	-0.013	0.874
BIOT & AR_4	158	0.057	0.477
BIOT & AR_5	158	0.002	0.976
BIOT & AR_6	158	0.083	0.301
BIOT & AR_7	158	0.066	0.412
BIOT & AR_8	158	0.035	0.659
BIOT & AR_9	158	0.086	0.284
BIOT & AR_10	158	0.112	0.159
BIOT & AR_11	158	0.011	0.895
BIOT & AR_12	158	-0.039	0.631
BIOT & AR_13	158	0.023	0.773
BIOT & AR_14	158	-0.004	0.958
BIOT & AR_15	158	0.032	0.688

Appendix B(xxi) Bivariate correlation analyses between sex hormone binding globulin (SHBG) and % methylation per CpG site variables.

	Valid (N)	Spearman (R)	p-value
SHBG & FSHR_1	158	-0.013	0.874
SHBG & FSHR_2	158	-0.040	0.620
SHBG & FSHR_3	158	0.109	0.172
SHBG & FSHR_4	158	-0.028	0.727
SHBG & INHBB_1	157	-0.032	0.692
SHBG & INHBB_2	157	-0.055	0.497
SHBG & INHBB_3	157	-0.064	0.423
SHBG & INHBB_4	157	-0.013	0.877
SHBG & INHBB_5	157	-0.049	0.543
SHBG & INHBB_6	157	-0.090	0.264
SHBG & INHBB_7	157	-0.053	0.509
SHBG & INHBB_8	157	0.017	0.833
SHBG & INHBB_9	157	-0.038	0.636
SHBG & INHBB_10	157	0.004	0.962
SHBG & INHBB_11	157	-0.010	0.904
SHBG & INHBB_12	157	-0.084	0.296
SHBG & INHBB_13	157	-0.120	0.135
SHBG & INHBB_14	157	-0.010	0.897
SHBG & INHBB_15	157	-0.046	0.568
SHBG & INHBB_16	157	0.092	0.254
SHBG & INHBB_17	157	-0.015	0.853
SHBG & INHBB_18	157	-0.026	0.745
SHBG & INHBB_19	157	-0.052	0.521
SHBG & INHBB_20	157	-0.081	0.312
SHBG & INHBB_21	157	-0.159	0.047
SHBG & INHBB_22	157	0.053	0.509
SHBG & INHBB_23	157	-0.061	0.449
SHBG & INHBB_24	157	-0.072	0.373
SHBG & INHBB_25	157	-0.010	0.899
SHBG & INHBB_26	157	0.033	0.686
SHBG & INHBB_27	157	-0.037	0.645
SHBG & INHBB_28	157	0.003	0.971
SHBG & INHBB_29	157	-0.125	0.119
SHBG & INHBB_30	157	0.035	0.666
SHBG & ESR2_1	94	0.314	0.002
SHBG & ESR2_2	94	0.093	0.374
SHBG & ESR2_3	94	0.0135	0.898
SHBG & ESR2_4	94	0.115	0.268
SHBG & ESR2_5	94	0.036	0.731
SHBG & ESR2_6	94	0.160	0.122
SHBG & ESR2_7	94	0.207	0.045
SHBG & ESR2_8	94	0.011	0.915
SHBG & ESR2_9	94	0.216	0.036
SHBG & ESR2_10	94	-0.052	0.620
SHBG & ESR2_11	94	0.027	0.795
SHBG & ESR2_12	94	0.202	0.050
SHBG & ESR2_13	94	0.248	0.016
SHBG & ESR2_14	94	-0.085	0.417
SHBG & ESR2_15	94	-0.071	0.498
SHBG & ESR2_16	94	-0.249	0.016

SHBG & ESR2_17	94	0.142	0.172
SHBG & ESR2_18	94	-0.033	0.755
SHBG & ESR2_19	94	0.214	0.038
SHBG & ESR2_20	93	-0.003	0.977
SHBG & SHBG_1	147	0.078	0.351
SHBG & SHBG_2	147	0.087	0.294
SHBG & SHBG_3	147	0.016	0.850
SHBG & SHBG_4	147	0.014	0.863
SHBG & SHBG_5	147	0.049	0.552
SHBG & SHBG_6	147	0.143	0.085
SHBG & SHBG_7	147	-0.158	0.057
SHBG & SHBG_8	147	0.021	0.803
SHBG & SHBG_9	147	0.063	0.449
SHBG & SHBG_10	147	-0.036	0.666
SHBG & SHBG_11	147	-0.049	0.555
SHBG & SHBG_12	147	0.061	0.465
SHBG & SHBG_13	147	-0.021	0.798
SHBG & SHBG_14	147	-0.033	0.691
SHBG & SHBG_15	147	0.042	0.611
SHBG & SHBG_16	147	0.090	0.276
SHBG & SHBG_17	147	0.065	0.436
SHBG & AR_1	158	0.053	0.506
SHBG & AR_2	158	-0.007	0.934
SHBG & AR_3	158	-0.032	0.694
SHBG & AR_4	158	0.019	0.809
SHBG & AR_5	158	0.108	0.177
SHBG & AR_6	158	-0.013	0.875
SHBG & AR_7	158	0.048	0.547
SHBG & AR_8	158	-0.006	0.944
SHBG & AR_9	158	-0.096	0.232
SHBG & AR_10	158	-0.106	0.185
SHBG & AR_11	158	0.026	0.748
SHBG & AR_12	158	0.032	0.688
SHBG & AR_13	158	0.128	0.110
SHBG & AR_14	158	0.055	0.495
SHBG & AR_15	158	0.071	0.373

Appendix B(xxii) Bivariate correlation analyses between the 19 models and average gene methylation of follicle stimulating hormone receptor (*FSHR_1-FSHR_4*).

	Valid (N)	Spearman (R)	p-value
FSHR AGM & BMI	177	0.175	0.020
FSHR AGM & Hip Circumference	177	0.098	0.196
FSHR AGM & Waist Circumference	177	0.121	0.108
FSHR AGM & Visceral Fat	177	-0.052	0.492
FSHR AGM & Subcutaneous Fat	177	0.044	0.558
FSHR AGM & FBG	175	-0.099	0.194
FSHR AGM & HOMA	175	-0.097	0.202
FSHR AGM & Systolic BP	177	0.090	0.233
FSHR AGM & Diastolic BP	177	0.128	0.090
FSHR AGM & cIMT	177	-0.007	0.926
FSHR AGM & eGFR	176	0.031	0.680
FSHR AGM & TChol	176	0.061	0.418
FSHR AGM & HDL-c	176	-0.010	0.896
FSHR AGM & Triglycerides	176	0.012	0.874
FSHR AGM & LDL-c	176	0.108	0.154
FSHR AGM & E2	158	-0.114	0.154
FSHR AGM & FSH	158	0.023	0.771
FSHR AGM & BIOT	158	0.074	0.353
FSHR AGM & SHBG	158	0.038	0.639

Appendix B(xxiii) Bivariate correlation analyses between the 19 models and average gene methylation of inhibin B (*INHBB_1-INHBB_30*).

	Valid (N)	Spearman (R)	p-value
INHBB AGM & BMI	176	0.045	0.552
INHBB AGM & Hip Circumference	176	-0.007	0.925
INHBB AGM & Waist Circumference	176	-0.034	0.653
INHBB AGM & Visceral Fat	176	-0.047	0.532
INHBB AGM & Subcutaneous Fat	176	-0.085	0.261
INHBB AGM & FBG	174	0.074	0.331
INHBB AGM & HOMA	174	0.042	0.580
INHBB AGM & Systolic BP	176	-0.027	0.725
INHBB AGM & Diastolic BP	176	-0.029	0.700
INHBB AGM & cIMT	176	-0.059	0.437
INHBB AGM & eGFR	175	0.024	0.753
INHBB AGM & TChol	175	-0.067	0.376
INHBB AGM & HDL-c	175	0.036	0.635
INHBB AGM & Triglycerides	175	-0.021	0.781
INHBB AGM & LDL-c	175	-0.102	0.179
INHBB AGM & E2	157	0.015	0.853
INHBB AGM & FSH	157	-0.034	0.677
INHBB AGM & BIOT	157	-0.043	0.590
INHBB AGM & SHBG	157	-0.075	0.350

Appendix B(xxiv) Bivariate correlation analyses between the 19 models and average gene methylation of oestrogen receptor β (*ESR2_1-ESR2_20*).

	Valid (N)	Spearman (R)	p-value
ESR2 AGM & BMI	106	-0.001	0.991
ESR2 AGM & Hip Circumference	106	0.012	0.906
ESR2 AGM & Waist Circumference	106	-0.026	0.791
ESR2 AGM & Visceral Fat	106	0.060	0.544
ESR2 AGM & Subcutaneous Fat	106	-0.108	0.270
ESR2 AGM & Fasting Glucose	105	0.029	0.768
ESR2 AGM & HOMA	105	0.046	0.638
ESR2 AGM & Systolic BP	106	0.050	0.610
ESR2 AGM & Diastolic BP	106	0.044	0.654
ESR2 AGM & cIMT	106	-0.123	0.208
ESR2 AGM & eGFR	105	-0.014	0.891
ESR2 AGM & TChol	105	0.090	0.360
ESR2 AGM & HDL-c	105	0.128	0.193
ESR2 AGM & Triglycerides	105	-0.000	0.996
ESR2 AGM & LDL-c	105	0.045	0.649
ESR2 AGM & E2	94	-0.032	0.761
ESR2 AGM & FSH	94	0.008	0.938
ESR2 AGM & BIOT	94	0.000	0.999
ESR2 AGM & SHBG	94	0.012	0.912

Appendix B(xxv) Bivariate correlation analyses between the 19 models and average gene methylation of sex hormone binding globulin (*SHBG_1-SHBG_17*).

	Valid (N)	Spearman (R)	p-value
SHBG AGM & BMI	166	0.013	0.867
SHBG AGM & Hip Circumference	166	0.070	0.369
SHBG AGM & Waist Circumference	166	0.077	0.322
SHBG AGM & Visceral Fat	166	0.077	0.321
SHBG AGM & Subcutaneous Fat	166	0.028	0.721
SHBG AGM & FBG	164	-0.080	0.306
SHBG AGM & HOMA	164	-0.138	0.079
SHBG AGM & Systolic BP	166	0.112	0.153
SHBG AGM & Diastolic BP	166	0.111	0.156
SHBG AGM & cIMT	166	-0.029	0.708
SHBG AGM & eGFR	165	0.037	0.633
SHBG AGM & TChol	165	0.037	0.640
SHBG AGM & HDL-c	165	0.001	0.988
SHBG AGM & Triglycerides	165	0.010	0.899
SHBG AGM & LDL-c	165	0.069	0.375
SHBG AGM & E2	147	-0.136	0.102
SHBG AGM & FSH	147	-0.012	0.889
SHBG AGM & BIOT	147	-0.051	0.541
SHBG AGM & SHBG	147	-0.079	0.344

Appendix B(xxvi) Bivariate correlation analyses between the 19 models and average gene methylation of androgen receptor (*AR_1-AR_15*).

	Valid (N)	Spearman (R)	p-value
AR AGM & BMI	177	0.106	0.159
AR AGM & Hip Circumference	177	0.079	0.295
AR AGM & Waist Circumference	177	0.100	0.184
AR AGM & Visceral Fat	177	-0.059	0.433
AR AGM & Subcutaneous Fat	177	0.043	0.568
AR AGM & FBG	175	-0.081	0.285
AR AGM & HOMA	175	0.024	0.756
AR AGM & Systolic BP	177	0.074	0.325
AR AGM & Diastolic BP	177	0.105	0.163
AR AGM & cIMT	177	-0.071	0.346
AR AGM & eGFR	176	0.044	0.565
AR AGM & TChol	176	0.045	0.551
AR AGM & HDL-c	176	0.104	0.171
AR AGM & Triglycerides	176	-0.002	0.977
AR AGM & LDL-c	176	0.057	0.452
AR AGM & E2	158	-0.074	0.358
AR AGM & FSH	158	-0.005	0.951
AR AGM & BIOT	158	0.054	0.504
AR AGM & SHBG	158	0.026	0.750

Appendix C

Appendix C(i) R Script for extracting methylation count data (CG) from Illumina DRAGEN 'report.txt.gz' output files.

```
#move where are data

setwd('C:/Users/merus/OneDrive/Desktop/CpG\ Methylation')

#scan sub folder to find report

listfile<-dir('.', pattern='_report.txt.gz', full.name=T,recursive=T)


#

Cmt<-1

for(File in listfile){

namesind<-strsplit(basename(File),split='.',fixed=T)[[1]][1]

dataextract<-read.table(gzfile(File),header=F)

subextract<-dataextract[dataextract$V6=="CG",]

subextract$Ind<-namesind

if(Cmt==1)DataF<-subextract

else DataF<-rbind(subextract,DataF)

Cmt<-Cmt+1

}

write.csv(DataF, row.names=F, file='merge_CG.csv',quote=F)
```

Appendix C(ii) EZ-96 DNA Methylation-Lightning MagPrep Kit by Zymo Research.

1. Add 130µl of **Lightning Conversion Reagent** to 20µl of a DNA sample in a **Conversion Plate**. Mix the samples by pipetting up and down.

2. Seal the plate with the provided film. Transfer the **Conversion Plate** to a thermal cycler and perform the following steps:

1. 98°C for 8 minutes
2. 54°C for 60 minutes
3. 4°C storage for up to 20 hours (*optional step)*

3. Pre-heat a plate heating element to 55°C.

4. Add 600µl of **M-Binding Buffer** and 10µl of **MagBinding Beads** to each well of a **Collection Plate**.

5. Transfer the samples from the **Conversion Plate** into the **Collection Plate** containing the **M-Binding Buffer** and **MagBinding Beads**. Mix by pipetting up and down 3-6 times and, if available, vortexing at 1,300-1,500 rpm for 30 seconds (e.g. Tecan - Te-Shake™).

6. Let plate stand at room temperature for 5 minutes, then transfer plate to a magnetic stand for an additional 5 minutes or until beads pellet and supernatant is cleared. With the plate on the magnetic stand remove the supernatant and discard.

7. Remove the **Collection Plate** from the magnetic stand for this and each subsequent buffer addition. Add 400µl of **M-Wash Buffer** to the beads. Re-suspend the beads by pipetting up and down or vortexing the plate at 1,300-1,500 rpm for 30 seconds. Replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant.

8. Add 200µl of **L-Desulphonation Buffer** to the beads. Re-suspend the beads by pipetting up and down or vortexing for 30 seconds. Let plate stand at room

temperature (20-30°C) for 15-20 minutes. After the incubation, replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant.

9. Add 400µl of **M-Wash Buffer** to the beads. Re-suspend the beads by pipetting up and down or vortexing for 30 seconds. Replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant. Repeat this wash step.

10. Transfer the plate to a heating element at 55°C for 20-30 minutes to dry the beads and remove residual **M-Wash Buffer**.

11. Add 25µl of **M-Elution Buffer** directly to the dried beads and pipette or vortex for 30 seconds to re-suspend. Heat the elution at 55°C for 4 minutes then transfer the plate to the magnetic stand for 1 minute or until beads pellet. Remove the supernatant and transfer to a clean **Elution Plate**.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long-term storage, store at or below -70°C. We recommend using 1-4µl of eluted DNA for each PCR, however, up to 25µl can be used if necessary. The elution volume can be > 25µl depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentrations.

Appendix C(iii) Invitrogen Platinum II Hot-Start Green PCR Master Mix (2X) (for optimisation of primers- PCR).

1. Thaw, mix, and briefly centrifuge each component before use.

2. Prepare PCR Master Mix

2(i). Add the following components to each reaction tube.

Component	Volume for 20µL rxn	Final concentration
Water, nuclease-free	to 20µL	-
Platinum™ II Hot-Start Green PCR Master Mix (2X)	10µL	1X
Platinum™ GC Enhancer	4µL	1X

2(ii). Mix, then briefly centrifuge the components.

3. Add template DNA and primers

3(i). Add your template DNA and primers to each tube for a final reaction volume of 20µL.

Component	Volume for 20µL rxn	Final concentration
10µM forward primer	0.4µL	0.2µM
10µM reverse primer	0.4µL	0.2µM
Template DNA	varies	<500 ng/rxn

3(ii). Cap each tube, mix, then briefly centrifuge the contents.

4. Incubate reactions in a thermal cycler.

		Step	Temperature	Time
		Initial Denaturation	94°C	2 min
40X		Denature	94°C	15 sec
PCR		Anneal	Optimal Annealing Temperature	30 sec
Cycles		Extend	68°C	1 min
		Hold	4°C	∞

Optimal Annealing Temperature: Androgen Receptor (*AR*)= 59°C; Follicle Stimulating Hormone Receptor (*FSHR*)= 60°C; Inhibin B (*INHBB*)= 60°C; Oestrogen Receptor β (*ESR2*)= 61°C; Sex Hormone-Binding Globulin (*SHBG*)= 60.5°C.

5. Analyse with agarose gel electrophoresis.

Appendix C(iv) NucleoMag kit for clean-up and size selection by Macherey-Nagel.

1. Remove the NucleoMag NGS Bead Suspension from the refrigerator. Let stand for approximately 30 min to bring the bead suspension to room temperature.

2. Mix 4.9µl of beads and 7µl of DNA sample in wells of 96-well plate (0.7µl of beads for every 1µl of DNA; use 0.6µl of beads for every 1µl of DNA in the 2nd wash step after index PCR).

3. Incubate the 96-well plate on a magnetic separator at room temperature for 5 minutes.

4. Remove the supernatant from each well.

5. Add 200µl 80% ethanol to each well and mix thoroughly. Incubate the 96-well plate on the magnetic separator at room temperature for 30 seconds.

6. Remove the supernatant from each well.

7. Repeat steps 5. and 6.

8. Dry the beads for 10 minutes at room temperature.

9. Mix 25µl of elution buffer with the dry beads in each well.

10. Incubate for 5 minutes on the magnetic separator at room temperature.

11. Transfer supernatant containing DNA and elution buffer into a new 96-well plate.

12. Discard the 96-well plate with the beads in it.

Appendix C(v) Index PCR.

1. Thaw, mix, and briefly centrifuge each component before use.

2. Prepare PCR Master Mix

2(i). Add the following components to each reaction tube.

Component	Volume for 50µL rxn	Final concentration
Water, nuclease-free	0µL	-
Platinum™ II Hot-Start Green PCR Master Mix (2X)	25µL	1X
Platinum™ GC Enhancer	10µL	1X

2(ii). Mix, then briefly centrifuge the components.

3. Add template DNA and primers

3(i). Add your template DNA and primers to each tube for a final reaction volume of 50µL.

Component	Volume for 50µL rxn	Final concentration
IDT for Illumina Nextera DNA UD Index Set C	10µL	-
Template DNA	5µL	-

3(ii). Cap each tube, mix, then briefly centrifuge the contents.

4. Incubate reactions in a thermal cycler.

	Step	Temperature	Time
	Initial Denaturation	95°C	3 min
8X	Denature	95°C	30 sec
PCR	Anneal	55°C	30 sec
Cycles	Extend	72°C	30 sec
	Hold	4°C	∞

Appendix C(vi) Qubit 4 by Invitrogen and the Qubit dsDNA broad-range assay kit.

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit dsDNA BR Assay requires 2 standards.

2. Label the tube lids.

3. Prepare the Qubit working solution by diluting the Qubit dsDNA BR Reagent 1:200 in Qubit dsDNA BR Buffer. Use a clean plastic tube each time you prepare Qubit working solution. Do not mix the working solution in a glass container.

4. Add 190µL of Qubit working solution to each of the tubes used for standards.

5. Add 10µL of each Qubit standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.

6. Add Qubit working solution to individual assay tubes so that the final volume in each tube after adding sample is 200µL.

7. Add each sample to the assay tubes containing the correct volume of Qubit working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200µL.

8. Allow all tubes to incubate at room temperature for 2 minutes.

9. On the Home screen of the Qubit 3.0 Fluorometer, press DNA, then select dsDNA Broad Range as the assay type. The “Read standards” screen is displayed. Press Read Standards to proceed.

10. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

11. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

12. Press Run samples.

13. On the assay screen, select the sample volume and units:

(a.) Press the + or – buttons on the wheel to select the sample volume added to the assay tube (from 1–20µL).

(b.) From the dropdown menu, select the units for the output sample concentration.

14. Insert a sample tube into the sample chamber, close the lid, then press the ‘read tube’ button. When the reading is complete (~3 seconds), remove the sample tube.

15. Repeat step 14 until all samples have been read.

Article title: Exploration of epigenetic modulation of sex hormone regulatory genes across cardiometabolic risk-associated menopausal transition

Short title for running head: DNA methylation in West African menopausal women.

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Scientific heading: Epigenetics

1. Introduction

Menopause is the permanent ceasing of ovarian function in women, which in turn signifies the discontinuation of ovulation and reproductive potential (Sherman, 2005). Generally, the menopausal transition lasts up to 7 years, but in extreme cases may last up to 15 years (Gatenby and Simpson, 2023). Over the transition, women experience symptoms such as hot flushes, irregular menstrual cycles, sexual dysfunction, mood changes and even depression (Schmidt, 2005; Gatenby and Simpson, 2023). Symptoms of the natural decline in ovarian function begin when women are between 45 and 55 (Gatenby and Simpson, 2023). A woman is considered to be post-menopausal (having transitioned from reproductively active to inactive) following 12 consecutive months without menstruation (Sherman, 2005) - typically around the age of 51 years.

Although menopause is a natural and inevitable process that lasts only for a limited period, the effect of changes in hormone levels extends far beyond the duration of menopause (Sherman, 2005). Pre-menopausal women have a lower risk of developing CVD than men of the same age, however, following the transition through menopause, women become more susceptible to developing CVD (Garcia *et al*., 2016). In sub-Saharan Africa (Central, East, South and West Africa) the burden of non-communicable disease (NCD) is on the rise (Bigna and Noubiap, 2019). Women at the highest risk for developing CVD are those with metabolic syndrome, which is a cluster of factors including high blood pressure, high fasting blood glucose levels, large waist circumference, low high-density lipoprotein cholesterol (HDL-c) and high triglyceride levels (Alberti *et al*., 2009; Carr, 2003). Menopause increases the likelihood of developing cardiometabolic syndrome (Carr, 2003) and cardiovascular disease, and although the mechanism is not completely understood it is thought to be related to changes in sex hormone levels. The possibility that this occurs via epigenetic mechanisms was investigated in this study.

1.1 Menopause and hormone changes

The menopausal transition is essentially defined pathophysiologically as changes in hormone levels, primarily, diminished oestrogen (oestradiol – E2) levels and elevated follicle-stimulating hormone (FSH) levels (Jaff *et al*., 2014).

In women of a reproductive age, maturing ovarian follicles produce increasing levels of oestrogen (oestradiol – E2), which in turn sensitise the pituitary to gonadotropin-releasing hormone (GnRH) (Lemarchand-Béraud *et al*., 1982; Santoro *et al*., 1998; Baskind and Balen, 2016). As a result, approximately 36 hours prior to ovulation (Reed and Carr, 2018), the pituitary releases a surge of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Lemarchand-Béraud *et al*., 1982), which then triggers the rupture of a mature ovarian follicle and release of a dominant ovum (egg) (Holesh *et al*., 2019). This cyclic process is regulated by the hypothalamic-pituitary-ovarian axis (HPO) (Lemarchand-Béraud *et al*., 1982).

In post-menopausal women, the reproductive age functioning of the HPO is disrupted (Santoro *et al*., 1998). As the oestrogen-releasing ovarian reserve depletes, oestrogen (oestradiol) levels decrease (Deadmond *et al*., 2000), leading to minimal to no sensitisation of the pituitary to GnRH, and ultimately, no LH spike triggering ovulation (Santoro *et al*., 1998). In response to the decrease in oestrogen levels, the negative feedback mechanism between oestrogen and FSH causes a spike in FSH levels instead of a regulated decrease (Messinis, 2006).

Anti-Müllerian hormone (AMH), the hormone that regulates the maturation of primordial follicles to the primary follicle stage (La Marca *et al*., 2009), is fairly constant from mid-childhood to early adulthood (25 years), after which AMH starts to decline to negligible levels in post-menopausal women (Kelsey *et al*., 2011).

In women of reproductive age, inhibin B (INHBB) regulates/suppresses FSH levels via a negative feedback mechanism (Welt, 2004) and ensures that only follicle reaches maturity and is released during ovulation (Sehested *et al*., 2000). In post-menopausal women, inhibin B levels decrease, as FSH levels peak (Welt, 2004).

During the menopausal transition, testosterone levels drop, resulting in a 50% reduction of testosterone levels by the age of final menstrual period (Burger, 2002). In women, testosterone is responsible for sexual function, thus impacts fertility through

facilitation (Johansen *et al*., 2020). Post-menopause, levels generally remain constant up until 65 years of age, when levels may increase and mirror those of adolescent girls (Ala-Fossi *et al*., 1998; Burger, 2002).

Both testosterone and oestrogen are regulated by sex hormone-binding globulin (SHBG), a protein that regulates levels of free oestrogens and androgens in the blood by binding to them, making them unavailable (Pasquali *et al*., 1997). In this way, SHBG impacts menstrual cycles, fertility, and as decreased SHBG levels is associated with elevated androgen levels, it is used to assess androgen status in a woman (Pasquali *et al*., 1997). It would follow that in obese women and post-menopausal women, SHBG levels have been found to decline (Tchernof *et al*., 1999).

A graphical representation of the stages, timing and hormonal changes involved in the menopausal transition was compiled by Harlow *et al*., 2012. The Staging of Reproductive Aging Workshop (STRAW) (Harlow *et al*., 2012) guided staging of the menopausal transition from pre-menopause, through peri-menopause, to post-menopause is depicted in Figure 1.1.

In pre-menopausal women, oestrogen levels are at their optimum, regulating body fat distribution by minimising abdominal visceral fat, and contributing to subcutaneous fat accumulation in the gluteofemoral and mammary gland region (Brown and Clegg, 2010). As women transition through menopause, oestrogen levels decline (Burger, 2002). With that decline comes an increase in abdominal adiposity, and as a result, an increase in cardiometabolic risk (Burger, 2002).

Studies conducted outside of Africa show that cardiometabolic risk factors are increased in post-menopausal women as compared to pre-menopausal women and that hormonal changes across the menopausal transition may play a role in modulating risk (Carr, 2003). As menopause affects every woman, and cardiometabolic disease and obesity are on the rise globally, it is essential to further investigate the correlation between the menopausal transition and the increase in CMD risk factors.

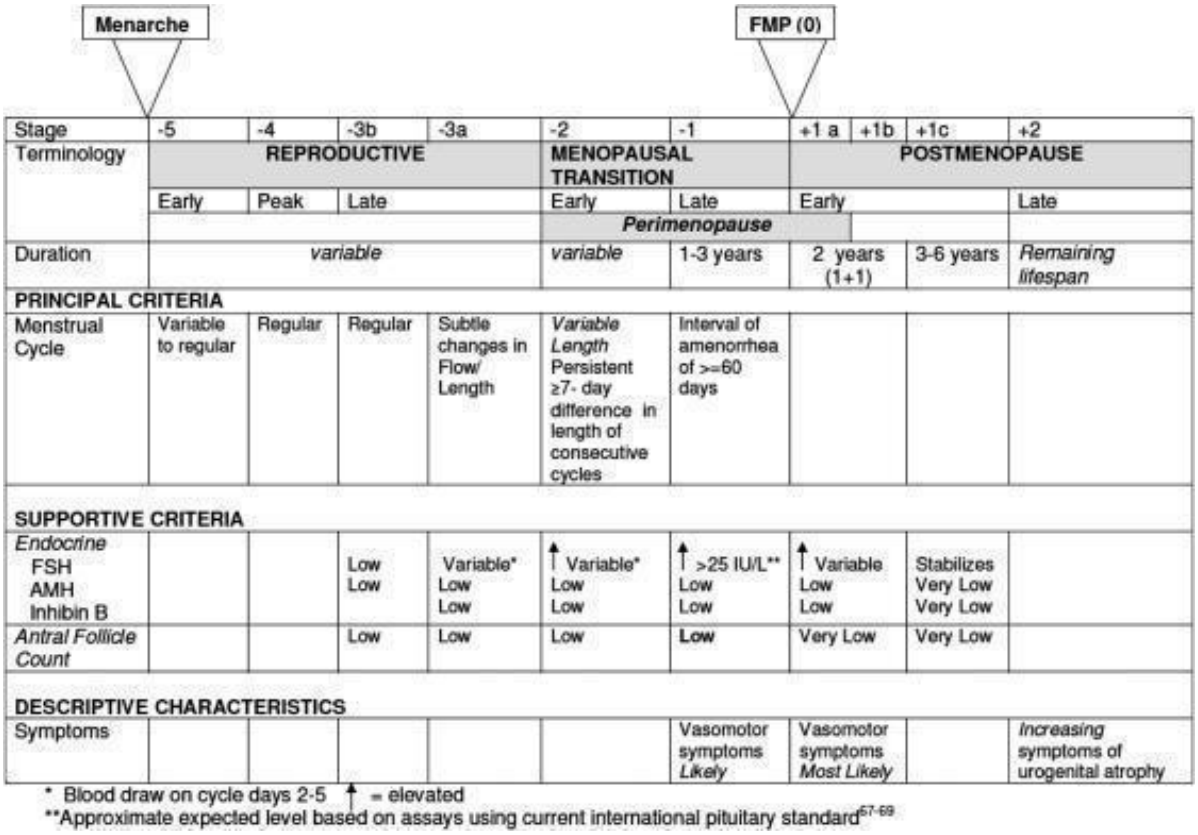


Figure 1.1 Visual depiction of menopause staging over a lifespan (Harlow *et al*., 2012).

1.2 Cardiometabolic and cardiovascular disease risk during the menopause

The risk of developing metabolic syndrome appears to be influenced by a combination of factors, including age, depression, gender, and hormonal changes during the menopausal transition. During the menopause, the decline in oestrogen leads to a change in body fat distribution as a result of an increase in pro-inflammatory visceral adipose tissue, which is inclusive of intraperitoneal/omental fat, intrahepatic fat, epicardial fat, and perivascular fat (González *et al*., 2017). This is associated with an increased risk of metabolic syndrome and CVD risk (Després, 2007; Shah *et al*., 2014). As oestrogen is heart-protective, declining levels of the hormone during the menopausal transition contribute to an increased risk for cardiovascular disease (Rosano and Panina, 1999). Menopause has been associated with elevated low density lipoprotein (LDL) cholesterol and triglyerides, along with decreased levels of high density lipoprotein (HDL) cholesterol (Matthews *et al*., 2001). This build up of high cholesterol levels then contributes to progression of arterial atherosclerotic plaque

formation (Steinberg, 1987), which in turn has the potential to develop into cardiac events and/or cardiovascular disease (CVD) (Jebari-Benslaiman *et al*., 2022).

In post-menopausal women, the decline of oestrogen levels results in vasorestriction of blood vessels as oestrogen plays an anti-inflammatory and vasodilatory role in pre-menopausal women (White, 2002). The inflexibility of the post-menopausal blood vessels puts post-menopausal women at a greater risk for developing hypertension (high blood pressure) (Maas and Franke, 2009).

High blood pressure levels facilitate the development of atherosclerotic lesions by aggravating and damaging the endothelium, furthering the progression of atherosclerosis and exacerbating the risk of a cardiac event occurring (Poznyak et al., 2022). This process leads to systemic inflammation and calcification (hardening) of the arterial endothelial layer (Poznyak *et al*., 2022).

Although there is a large body of evidence indicating increased risk for cardiometabolic and cardiovascular disease in post-menopausal women (Rosano and Panina, 1999; Matthews *et al*., 2001; White, 2002; Maas and Franke, 2009; Poznyak *et al*., 2022; Jebari-Benslaiman *et al*., 2022), there is also evidence suggesting that this risk may not be the same in all menopausal women and that women who were low birth weight babies are at an increased risk for CMD and CVD complications in their menopausal years (Alexander *et al*., 2014).

Cardiometabolic and cardiovascular diseases are increasingly becoming a global burden, and cause for concern (Antoun *et al*., 2022). It is hypothesised that environment and lifestyle-linked epigenetic modulatory effects on the genome may play a role in cardiometabolic and cardiovascular disease progression (Antoun *et al*., 2022). It would follow that the associated elevated cardiometabolic and cardiovascular disease risk in menopausal women may also be linked to epigenetic regulatory effects.

Sex hormone regulatory genes which code for proteins that mediate the action or control the levels of sex hormones, have been associated with the menopausal transition and its related health outcomes (Ahmed *et al*., 2022). Research is required to fully elucidate the complex interplay between genetics, hormones, and cardiometabolic risk during the menopausal transition as the mechanisms behind the changes in hormone levels are not fully established. It is plausible that epigenetic

modifications of genes involved in the regulation of hormone production modulate hormone levels.

The most researched and most common epigenetic modification is DNA methylation, which is known to be linked to the regulation of gene expression (Wagner *et al*., 2014). DNA methylation involves the DNA methyltransferase-aided (DNMT) transfer of a methyl group to position C-5 of the nucleotide, cytosine (Figure 1.7) (Robertson, 2005). The sites primed for methylation are known as CpG sites - a cytosine nucleotide connected to a guanine nucleotide by a phosphodiester bond. The methylation of cytosine at CpG sites near transcription start sites (TSS) within the promoter region of genes has been widely investigated. Methylation upstream of the promoter is often linked to the regulation of the expression of the gene (Wagner *et al*., 2014). However, the variation in DNA methylation levels across individuals, its genetic or environmental causes and its correlation with gene expression are not fully understood (Wagner *et al*., 2014). In terms of the sex hormone-associated genes described above, there is evidence that gene expression is epigenetically controlled by DNA methylation.

However, little is known about the possible epigenetic control of hormone levels during the menopausal transition and how this may relate to changes in cardiometabolic disease risk. It is important to undertake such studies to more clearly define the molecular processes involved in the transition. As findings may vary across different ethnicities, it is essential to generate data from Africans to ensure that there is representation of understudied populations.

Thus, this study aims to focus on the genes involved in the regulation of sex hormone levels and functions, including key genes encoding enzymes that catalyse steps in the sex hormone metabolic pathway and receptors that mediate hormone action. The genes being investigated are oestradiol receptor β (*ESR2*) and sex hormone binding globulin (*SHBG*).

2. Materials & Methods

This study is a sub-study of the Africa Wits-INDEPTH partnership for Genomic Studies (AWI-Gen) Project funded by the US National Institutes of Health under the umbrella of the Human Heredity & Health in Africa Consortium (H3Africa). AWI-Gen is a population cross-sectional study of men and women between the ages of 40 and 60 years. The first wave of data and sample collection occurred over 3 years between August 2013 and August 2016 (Ali *et al.,* 2018; George *et al*., 2019). Participants were randomly selected black African men and women in six study sites across Africa: South Africa (Agincourt, Dikgale/DIMAMO, Soweto); East Africa (Nairobi, Kenya); West Africa (Nanoro, Burkina Faso and Navrongo, Ghana). In this study on menopause, only samples from female participants of the Nanoro, Burkina Faso study site were included. Figure 2.1 summarises the flow of the research protocol from the processing of DNA stage, through to the data analysis stage.

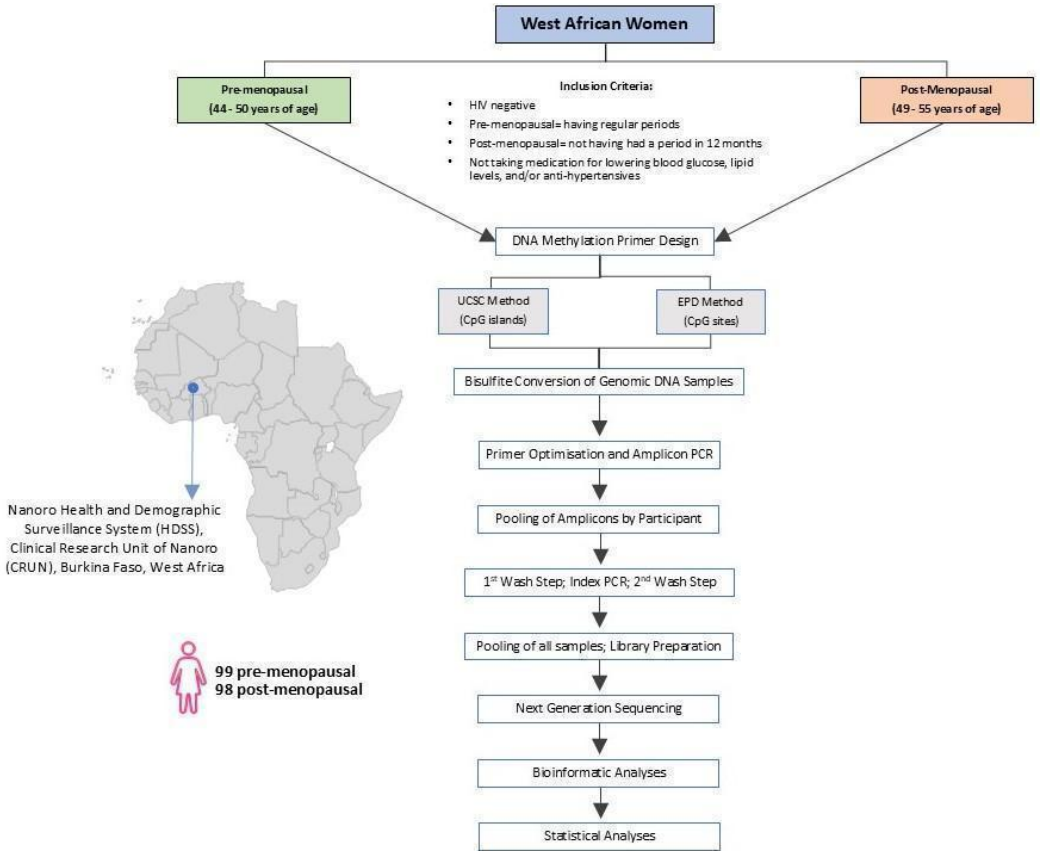


Figure 2.1 Schematic of methods carried out in this study.

2.1 Study participants

For this study, 99 pre-menopausal and 98 post-menopausal women were selected for downstream analyses. The age range of the women in the pre-menopausal group was between 44 and 50 years, whilst the women in the post-menopausal group were between 49 and 55 years old.

2.1.1 Selection criteria

Inclusion and exclusion criteria were applied as follows:

Women taking anti-diabetic medication, statins or anti-hypertensive therapy were excluded from the study as these drugs may affect DNA methylation (Schrader *et al*., 2021). Only HIV negative women were selected for both groups (pre- and post-menopausal) to minimise confounding. As reported before, HIV infection occurs at very low rates in Burkina Faso.

2.1.2 Demography, anthropometric variables and cardiometabolic risk factors included in the analyses

Data on age (years), height (m), weight (kg), waist circumference (cm), hip circumference (cm), abdominal visceral (mm) and subcutaneous fat (mm), carotid intima-media thickness (cIMT) (micrometres – μm), blood pressure (millimetres of mercury – mmHg), fasting blood glucose (mmol/L), fasting plasma insulin (micro-international units per litre – μIU/L), total cholesterol (mmol/L), high density lipoprotein cholesterol (HDL-c) (mmol/L), low density lipoprotein cholesterol (LDL-c) (mmol/L) estimated with the Friedewald equation (Friedewald *et al*., 1972) and triglyceride (mmol/L) levels, tuberculosis (TB) infection status, socioeconomic status such as education level (0=no formal education; 1=primary or secondary education), alcohol intake (0=non-drinker; 1=drinker) and physical activity (minutes of moderate to vigorous physical activity per week- min/week) were collected from each participant and used in statistical analyses. All data are available from the AWI-Gen wave 1 study and the variables and methods used to collect data are described in detail in Ali *et al*. 2018.

2.1.3 Hormone levels

To assess whether DNA methylation levels of CpG islands in the promoter regions of the genes of interest are linked to levels of sex hormones, hormone levels were measured using liquid chromatography-mass spectrometry (LC-MS). The following compounds were measured: oestradiol (E2), dehydroepiandroesterone sulfate (DHEA-S), sex hormone binding globulin (SHBG), follicle stimulating hormone (FSH) and testosterone (TT) (Chikwati *et al*., 2023; Chikwati *et al*., 2024). From the SHBG, TT and additional serum albumin data, free testosterone (FT) and bioavailable testosterone (BIOT) were calculated with the 'Free & Bioavailable Testosterone Calculator' developed at the Hormonology Department at the University Hospital of Ghent in Belgium and can be accessed at https://www.issam.ch/freetesto.htm.

2.1.4 Sample collection

Genomic DNA (gDNA) derived from the buffy coat of participant peripheral blood samples (collected into EDTA) was obtained from the Sydney Brenner Institute for Molecular Bioscience Biobank. The gDNA was extracted by means of automated DNA extraction protocols. Genomic DNA was purified from buffy coats using the automated QIAsymphony SP (Qiagen, Germany) sample purification robot and the Qiagen magnetic bead-based DNA mini-prep kit (catalogue number 931,255) running Qiagen protocol DNA_BC_400_V6_DSP and the genomic DNA eluted in a final volume of 400µl (Ali *et al*., 2018). DNA was stored at -80°C in the biobank freezers until ready for analysis.

2.2 Primer design for PCR to assess DNA methylation

CpG islands spanning or upstream of the promoter regions of *ESRR* and *SHBG* were located using the UCSC Genome Browser (https://genome.ucsc.edu/). More than one CpG island was identified for both genes, however, only the island most likely to affect gene expression was targeted. Typically, the island most likely to affect gene expression is found approximately 2kb upstream to 7kb downstream of the transcription start site (TSS) of the promoter region (Hong and Rhee, 2022), so the CpG island had to meet this criterion to be selected.

Once selected, CpG island sequences were obtained from the UCSC Genome Browser and input into MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). MethPrimer then generated several primer pairs according to guidelines for designing PCR primers on DNA that has been subjected to bisulfite modification (https://www.zymoresearch.com/pages/bisulfite-beginner-guide#bisulfite_pcr_and_primer_design). The primer pair covering the largest portion of the CpG island was then selected. This ensured maximum sequencing coverage of the region of interest. Only one primer pair per gene was selected, which meant that the entire CpG island could not be sequenced, as bisulfite primers are designed optimally (26 – 30bp) to amplify relatively small regions of between 150 and 300bp. The primer pairs should also, ideally, not contain any CpG sites. This makes for the most optimal bisulfite primers, but also the most restrictive selection process.

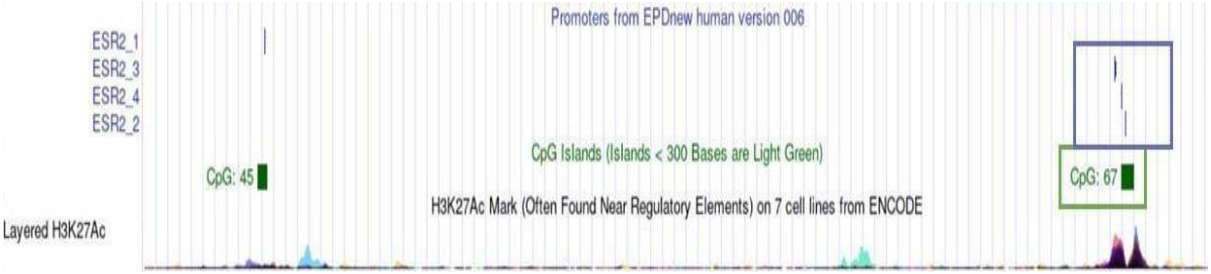


Figure 2.2 Selection of target CpG island (CpG: 67; chr14:64288227-64342982) located upstream of promoters, ESR2_1, ESR2_3, and ESR2_4, and spanning across promoter ESR2_2 of the *ESR2* gene.



Figure 2.3 Selection of target CpG island (CpG: 43; chr17:7612975-7634831) located upstream of promoters SHBG_1 and SHBG_3 of the *SHBG* gene.

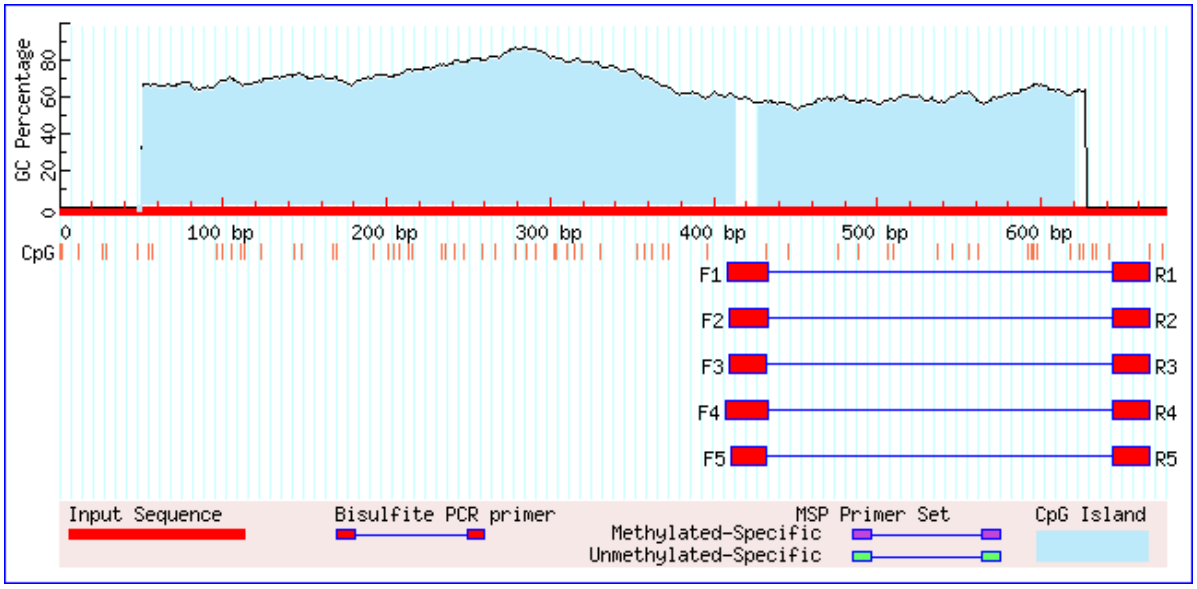


Figure 2.4 The selected bisulfite primer pair, F1 and R1, amplifying a region located upstream and spanning across the *ESR2* gene between chr14:64338799-64339057.

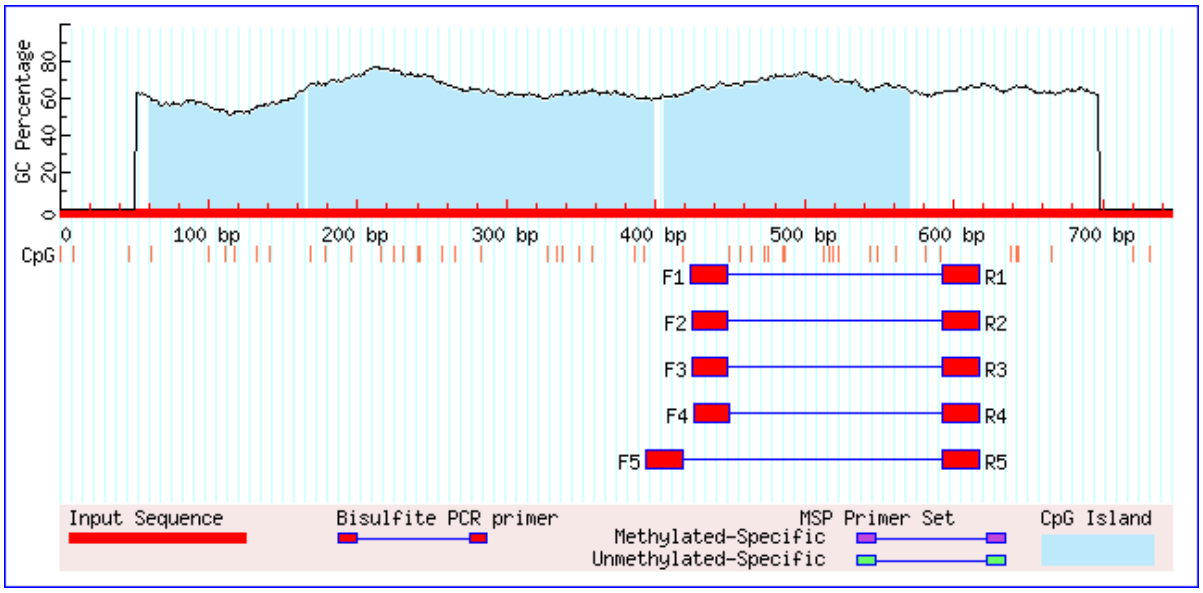


Figure 2.5 The selected bisulfite primer pair, F5 and R5, amplifying a region located upstream of the *SHBG* gene between chr17:7627681-7627904.

2.3 Library generation

Once all bisulfite PCR primers were designed, and NGS-compatible adapter sequences added on (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG to the 5' end of the forward primer, and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG to the 5' end of the reverse primer), the primers were ordered from Integrated DNA Technologies (IDT), Inc (Coralville, Iowa, United States).

2.3.1 Bisulfite conversion of genomic DNA of study participants

Each of 99 pre-menopausal and 98 post-menopausal genomic DNA samples, as well as 4 test DNA samples of fellow postgraduate students for primer optimisation purposes, were quantified using the ThermoFisher Scientific Nanodrop One (Waltham, Massachusetts, United States). The samples were then diluted to 20ng/µl with nuclease-free water to obtain a yield of 400ng in 20µl for optimal bisulfite conversion, which was achieved using the EZ-96 DNA Methylation-Lightning MagPrep Kit by Zymo Research (Irvine, California, United States).

2.3.2 Primer optimisation for PCR to generate amplicons

Amplicon PCR for each of *ESR2* and *SHBG* were first carried out with 4 bisulfite modified test DNA samples to determine the optimal annealing temperature. When working with bisulfite modified DNA, it is recommended that hot-start polymerases be used, to decrease chances of non-specific binding. The odds of non-specific binding occurring are increased with bisulfite modified DNA, as the AT content is higher, due to the unmethylated cytosines (the majority of cytosines) being converted to thymine bases. Primer optimisation PCR was carried out using the Invitrogen Platinum II Hot-Start Green PCR Master Mix (2X) (Waltham, Massachusetts, United States).

2.3.3 Pooling of amplicons by participant

Amplicons were quantified using the Nanodrop One by ThermoFisher Scientific (Waltham, Massachusetts, United States) prior to pooling, to avoid sequence read bias due to dominance of a participant sample by a specific gene(s). Following this,

approximately equivalent concentrations of each amplicon (*ESR2* and *SHBG*) were pooled per participant.

2.3.4 Sample clean-up 1

Once pooled, the participant samples underwent a size selecting wash step to ensure that primer dimers and/or non-specific amplicons were removed prior to sequencing. The NucleoMag kit for clean-up and size selection by Macherey-Nagel (Düren, Germany) was used for the above purposes.

2.3.5 Index PCR

Following clean-up of pooled participant samples, each sample was tagged with IDT Illumina Nextera DNA UD Indexes (Set C) (San Diego, California, United States), so that each participant and their respective amplicon sequences (for each of *SHBG* and *ESR2*) may be identified by their individual tag sequence for further downstream analyses. The indexes were attached to the amplicons by means of index PCR.

2.3.6 Sample clean-up 2

The samples were washed once more following addition of index sequences to participant amplicons. The protocol was the same as the first wash step with the NucleoMag kit by Macherey-Nagel (Düren, Germany), except for a smaller quantity of magnetic beads being used for the second wash step to get rid of any residual undesirable amplicons that may not have been discarded in the first wash step.

2.3.7 Combining of pooled and indexed samples for sequencing

After each participant sample had been indexed and washed, they had to be pooled once more to generate a single library prior to sequencing. In total, there were 99 pre-menopausal participants, and 98 post-menopausal participants in the study.

2.3.8 Qubit

Before pooling all participant samples together, each indexed and washed sample was quantified using the Qubit 4 by Invitrogen and the Qubit dsDNA broad-range assay kit (Waltham, Massachusetts, United States).

2.3.9 Conversion of concentration from ng/µl to nM

The Qubit 4 quantifies nucleic acids in nanograms per microlitre, however, DNA sequencers require the number of molecules in each sample to be known and normalised before pooling together to form the final library.

Each participant sample was then diluted to 1nM, pooled, and then diluted to 50pM. A 50pM solution of the PhiX Control Kit v3 by Illumina was prepared and spiked into the library sample in a 20:80 ratio.

2.4 Next Generation Sequencing (NGS)

For each sequencing reaction, an iSeq100 i1 Reagent Cartridge v2 by Illumina (San Diego, California, United States) was removed from -20°C and left to thaw at room temperature for 9 hours. Once completely thawed, an iSeq100 i1 Flow Cell by Illumina (San Diego, California, United States) was inserted into the cartridge and 20µl of the prepared library and PhiX sample was then pipetted into the cartridge. The cartridge and flow cell were then loaded into the Illumina iSeq100 (San Diego, California, United States). Finally, the reaction was set up on the run manager, saved, and the 16-hour sequencing run commenced.

2.5 Bioinformatic analyses

The fastq.gz sequences obtained from each of the three sequencing reactions were uploaded to Illumina's BaseSpace (San Diego, California, United States), where the DRAGEN Methylation Pipeline was used to obtain methylation calling data. Once all methylation data was collected, the data was collated onto a single spreadsheet with a 'merge' script executed on R v 4.2.1 (Vienna, Austria), and % methylation was

calculated as follows from the number of methylated CpG sites in comparison with the total CpG sites:

$$\frac{\text{Methylated Count at X}}{\text{(Methylated Count at X + Unmethylated Count at X)} \times 100\% = \text{\% Methylation}}$$

X= individual CpG site or across promoter-linked region of gene

After the calculated percentage methylation data was tabulated, it was statistically analysed as described below.

2.6 Statistical analyses

All statistical analyses were performed using TIBCO Statistica v 14.0.1.25 (Hamburg, Germany). Cardiometabolic, anthropometric, socioeconomic and hormonal continuous variables between pre- and post-menopausal groups were presented as means ± standard deviations (SD) or medians and interquartile ranges (IQR) (calculated by subtracting Q1 from Q3) for variables with normal or skewed distribution, respectively. These variables were compared between the two groups using the Student's t-test for normally distributed variables and the Mann-Whitney U test for variables with a skewed distribution. Categorical data were presented as numbers with percentages and compared between groups using the chi-squared test. Average gene methylation of CpG promoter-linked regions (fragments of CpG islands) and individual CpG sites in pre- and post-menopausal groups were presented as medians and interquartile ranges (calculated by subtracting Q1 from Q3) and compared using the Mann-Whitney U test.

The dependent variables for 6 multiple regression models were selected according to the objectives of this study.

The 6 dependent/outcome variables (models) were:

Markers of metabolic function and glycaemic regulation – fasting blood glucose (FBG) (model 1), and Homeostasis Model Assessment (HOMA) (model 2) calculated with the equation from Matthews *et al*., 1985:

$$\frac{\text{Fasting Plasma Glucose (mmol/L) x Fasting Serum Insulin (µU/ml)}}{22.5} = \text{HOMA}$$

Indicators of cardiovascular health – total cholesterol (TChol) (model 3), high density lipoprotein cholesterol (HDL-c) (model 4), triglycerides (model 5), and low density lipoprotein cholesterol (LDL-c) (model 6).

Cardiometabolic, anthropometric, socioeconomic, and hormonal independent variables for each model were then selected based on scientific plausibility and a coding variable for menopausal status i.e. pre- or post-menopausal, was included in each model to account for menopause as a confounder, as both pre- and post-menopausal women were included in each model as the sample size was too small (99 pre-menopausal; 98 post-menopausal) for separate pre- and post-menopausal models for each regressor. To select which average gene methylation (AGM) sites to include in each of the 6 models, bivariate linear correlation analyses were performed on each of the 6 variables listed above with each of the 5 AGMs (*FSHR* AGM = 4 CpGs; *SHBG* AGM = 17 CpGs; *AR* AGM = 15 CpGs; *ESR2* AGM = 20 CpGs; and *INHBB* AGM = 30 CpGs). The p-values of the bivariate analysis were then multiplied by 6 (Bonferroni correction) to adjust for multiple testing. Similar analyses were performed for each of the individual 86 CpG sites.

Thereafter, models were executed, and models with a large number of predictors (>12 variables) were reduced before being executed a second time for the final version of

these models. In order to reduce the number of predictors, cardiometabolic, anthropometric, socioeconomic and hormonal variables with the highest p-values were removed from the models before second execution. The number of predictor variables included in each model was kept to a maximum of 12 to avoid overfitting, as the sample size was quite small (N= 99 pre-menopausal + 98 post-menopausal = 197). These regression models were then used to determine if there were correlations between any of the CpG sites and the selected dependent variables that were independent of any of the other possible modulating (or confounding) variables.

3. Results

3.1 Hormone levels, demographic, behavioural, cardiometabolic, anthropometric, and socioeconomic factors

A comparison of cardiometabolic, anthropometric, socioeconomic, and hormone level data between pre- and post-menopausal groups was conducted for both normally distributed data, and non-parametric data (Table 3.1). Lipid levels of post-menopausal women were statistically significantly higher than those of pre-menopausal women. The same was true of blood pressure and FSH. However, BMI, waist circumference and visceral fat was lower in post-menopausal women as compared to pre-menopausal women, as was oestrogen and eGFR. There were no smokers in the cohort, so this variable was omitted from the study. Only three participants of the 197 indicated that they were on anti-hypertensives, so this variable was also excluded.

Table 3.1 Comparison of hormone levels, cardiometabolic, anthropometric, demographic, behavioural, and socioeconomic factors in pre- and post-menopausal groups.

Variable	Pre-menopausal Group (n= 99)	Post-menopausal Group (n= 98)	p-value
Demographic Variables:			
Age (years)	46.0 (3.0)	52.0 (3.0)	<0.001
Behavioural Variables:			
Alcohol Consumption (%)			
Non-drinker	30 (30.6)	29 (29.9)	1.000
Drinker	68 (69.4)	68 (70.1)	
MVPA (minutes/week)	2940.0 (2880.0)	3240.0 (1440.0)	0.545
Socioeconomic Variables:			
Education (%)			
No Formal Education	86 (87.8)	91 (92.9)	0.240
Primary/Secondary Education	12 (12.2)	7 (7.1)	
Anthropometric Variables:			
BMI (kg/m^2)	20.1 (4.0)	19.2 (3.4)	0.034
Hip Circumference (cm)	88.4 (8.4)	86.4 (8.7)	0.032
Waist Circumference (cm)	73.9 (10.2)	73.1 (9.3)	0.864
Waist-to-Hip Ratio	0.8 (0.1)	0.9 (0.1)	0.009
Visceral Fat (mm)	4.4 (1.6)	4.1 (1.3)	0.015
Subcutaneous Fat (mm)	0.9 (0.6)	0.9 (0.5)	0.083
Cardiometabolic Variables:			
Systolic Blood Pressure (mmHg)	103.5 (18.5)	114.3 (25.0)	<0.001
Diastolic Blood Pressure (mmHg)	68.0 (11.0)	72.8 (14.0)	<0.001
eGFR (ml/min/1.73m^2)	115.6 (8.0)	112.3 (9.6)	0.012
Fasting Blood Glucose (mmol/L)	4.9 (0.7)	5.0 (0.9)	0.370
Fasting Insulin (mIU/L)	4.0 (10.9)	4.3 (13.5)	0.575
HOMA (mmol.mU/L^2)	0.8 (2.1)	0.9 (3.1)	0.527
Total Cholesterol (mmol/L)	3.0 ± 0.8	3.4 ± 0.8	<0.001
HDL-c (mmol/L)	1.0 (0.3)	1.1 (0.5)	0.030
Triglycerides (mmol/L)	0.6 (0.3)	0.7 (0.4)	0.003
LDL-c (mmol/L)	1.7 ± 0.6	1.9 ± 0.6	0.007
cIMT (µm)	625.0 (0.1)	645.0 (0.1)	0.004
Hormonal Variables:			
E2 (pmol/L)	324.1 (524.6)	42.1 (35.0)	<0.001
FSH (mIU/mL)	15.6 (35.2)	60.4 (36.9)	<0.001
DHEA-S (µmol/L)	1.8 (1.0)	1.6 (1.2)	0.138
SHBG (nmol/L)	114.6 (60.5)	98.2 (45.6)	0.049
TT (pmol/L)	404.6 (320.0)	493.9 (530.0)	0.481
FT (pmol/L)	3.1 (3.0)	4.2 (5.0)	0.195
BIOT (pmol/L)	67.9 (52.0)	96.4 (122.0)	0.113

3.2 Individual CpG site methylation

Genomic DNA extracted from peripheral blood leukocytes was highly concentrated and of good quality with optimal A260/A280 and A260/A230 measurements. Amplicons were sequenced on the Illumina iSeq100 at a high depth of ~50 000x for

each of the three sequencing runs, ensuring the reliability of sequencing data, and results of this study.

Percentage methylation across each of the 37 CpG sites (20 *ESR2* CpGs and 17 *SHBG* CpGs) in pre- and post-menopausal groups was calculated. Despite the variation in methylation between CpG sites within each of the two genes, methylation was consistent between groups, as there were no statistically significant differences in methylation at individual CpG sites between pre- and post-menopausal groups.

3.3 Promoter-linked average gene methylation

Methylation levels, when averaged across all the CpG sites per gene across each of the five promoter-linked regions in pre- and post-menopausal groups were compared using the Mann-Whitney U-test. No statistically significant differences in average methylation between the groups were observed.

3.4 Multiple regression models

To determine which CpG sites would be included in each model, Bivariate correlation analyses were carried out between the dependent variable (i.e. the model) and all 37 CpG sites. The CpG sites with a p-value of less than 0.05 were then included in a multiple regression model with the same dependent variable, and those which still had p-values of less than 0.05 were then included in the final model for the dependent variable, along with the other cardiometabolic, anthropometric, socioeconomic, and hormone predictor variables.

The models investigated in this study were:

Regression models for glycaemia traits: fasting blood glucose (model 1); HOMA/insulin resistance (model 2)

Lipid-related indicators of cardiovascular health: total cholesterol (model 3); HDL-c (model 4); triglycerides (model 5); LDL-c (model 6).

3.4.1 Regression models for glycaemia traits: fasting blood glucose and insulin resistance (HOMA)

Key traits for diabetes mellitus, one of the most common conditions that post-menopausal women are at risk for (Heianza *et al*., 2013), were studied through modelling of fasting blood glucose and insulin resistance (HOMA). Table 3.2 shows negative correlations of fasting blood glucose with education (p=0.043) and positive correlations with visceral fat (p=0.002) and age (p=0.006). There were no statistically significant predictors in the HOMA model (Table 3.3).

Table 3.2 Multiple regression model for fasting blood glucose (FBG).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.123	0.139	-0.166	0.186	0.375
Oestrogen (pmol/L)	0.021	0.083	0.000	0.000	0.801
DHEA-S (µmol/L)	-0.085	0.078	-0.051	0.047	0.277
SHBG (nmol/L)	-0.140	0.081	-0.002	0.001	0.084
BIOT (nmol/L)	-0.125	0.082	-1.256	0.821	0.128
MVPA (minutes/week)	0.022	0.074	0.000	0.000	0.762
Age (years)	0.361	0.130	0.070	0.025	0.006*
Alcohol Status (%)	-0.137	0.071	-0.199	0.103	0.054
Education (%)	-0.150	0.073	-0.330	0.162	0.043*
Hip Circumference (cm)	0.064	0.080	0.001	0.001	0.430
Visceral Fat (mm)	0.236	0.075	0.135	0.043	0.002*

Adjusted R^2= 0.206; p<0.001

Abbreviations: SHBG – sex hormone binding globulin; MVPA – moderate to vigorous physical activity; DHEA-S – dehydroepiandrosterone-sulfate; BIOT – bioavailable testosterone

Table 3.3 Multiple regression model for Homeostasis Model assessment (HOMA).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.259	0.149	-3.242	1.861	0.083
Oestrogen (pmol/L)	-0.036	0.090	-0.001	0.001	0.694
SHBG (nmol/L)	0.065	0.085	0.010	0.013	0.445
Hip Circumference (cm)	-0.002	0.124	-0.000	0.011	0.987
Visceral Fat (mm)	-0.002	0.082	-0.013	0.438	0.976
Subcutaneous Fat (mm)	0.041	0.121	0.533	1.579	0.736
MVPA (minutes/week)	-0.053	0.080	-0.000	0.000	0.506
Age (years)	0.282	0.143	0.509	0.258	0.050
Alcohol Status (%)	-0.082	0.078	-1.117	1.058	0.293
Education (%)	-0.077	0.080	-1.588	1.652	0.338

Adjusted R^2= 0.041; p<0.728

Abbreviations: SHBG – sex hormone binding globulin; MVPA – moderate to vigorous physical activity; DHEA-S – dehydroepiandrosterone-sulfate; BIOT – bioavailable testosterone

The fasting blood glucose model suggests that the more formally educated you are, the lower your glucose levels are. These findings are in line with current literature (Mathisen *et al*., 2020b) that associate higher levels of comprehension with better diabetes education, leading to healthier food and lifestyle choices, and metabolic longevity (Kitada *et al*., 2019) to potentially avoid/delay onset of age-related diseases.

3.4.2 Lipid-related indicators of cardiovascular health: total cholesterol, HDL-c, triglycerides, and LDL-c

As heart-protective oestrogen decreases throughout the menopausal transition, the risk of developing cardiovascular disease increases to greater than that of male counterparts of a similar age (Adach *et al*., 2022). To study this effect in the Nanoro population, total cholesterol, HDL-c, triglycerides and LDL-c were modelled. Total cholesterol was correlated with DHEA-S (p=0.046) and subcutaneous fat (p<0.001) (Table 3.4). Correlations with FSH (p=0.032), DHEA-S (p=0.019), BIOT (p=0.020), and eGFR (p=0.029) were significant in the high density lipoprotein cholesterol model (Table 3.5). The triglycerides multiple regression model revealed correlations with subcutaneous fat (p=0.004) (Table 3.6). Low density lipoprotein cholesterol depicted correlations with menopausal status (p=0.020) and hip circumference (p=0.012) (Table 3.7).

Table 3.4 Multiple regression model for total cholesterol (TChol).

N= 176	β	Standard Error of β	B	Standard Error of b	p-value
Menopausal Status (%)	0.194	0.143	0.306	0.225	0.175
Oestrogen (pmol/L)	-0.048	0.085	-0.000	0.000	0.570
FSH (mIU/mL)	0.139	0.095	0.003	0.002	0.144
DHEA-S (µmol/L)	-0.145	0.072	-0.104	0.051	0.046*
eGFR (ml/min/1.73m^2)	-0.001	0.074	-0.000	0.005	0.987
Visceral Fat (mm)	-0.140	0.076	-0.093	0.050	0.066
Subcutaneous Fat (mm)	0.294	0.074	0.482	0.122	<0.001*
MVPA (minutes/week)	-0.049	0.073	-0.000	0.000	0.498
Age (years)	-0.121	0.130	-0.028	0.030	0.353
Alcohol Status (%)	0.028	0.071	0.047	0.121	0.698

Adjusted R^2= 0.194; p<0.001

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; eGFR – estimated glomerular filtration rate; MVPA – moderate to vigorous physical activity

Table 3.5 Multiple regression model for high density lipoprotein cholesterol (HDL-c).

N= 175	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	-0.033	0.096	-0.021	0.061	0.732
FSH (mIU/mL)	0.203	0.094	0.002	0.001	0.032*
DHEA-S (µmol/L)	-0.192	0.081	-0.055	0.023	0.019*
SHBG (nmol/L)	0.080	0.078	0.001	0.001	0.309
BIOT (nmol/L)	0.196	0.084	0.938	0.400	0.020*
HOMA (mmol.mU/L^2)	0.120	0.073	0.006	0.004	0.103
eGFR (ml/min/1.73m^2)	0.163	0.074	0.005	0.002	0.029*
Alcohol Status (%)	0.060	0.074	0.041	0.051	0.421
MVPA (minutes/week)	0.086	0.075	0.000	0.000	0.256
Education (%)	0.073	0.074	0.077	0.077	0.322
Visceral Fat (mm)	-0.081	0.076	-0.022	0.021	0.290

Adjusted R^2= 0.085; p<0.007

Abbreviations: DHEA-S – dehydroepiandrosterone-sulfate; SHBG – sex hormone binding globulin; BIOT – bioavailable testosterone; eGFR – estimated glomerular filtration rate; MVPA – moderate to vigorous physical activity

Table 3.6 Multiple regression model for triglycerides.

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
BIOT (nmol/L)	-0.088	0.084	-0.441	0.418	0.293
Subcutaneous Fat (mm)	0.236	0.080	0.164	0.056	0.004*
SHBG (nmol/L)	-0.136	0.084	-0.001	0.001	0.106
DHEA-S (µmol/L)	0.074	0.078	0.022	0.024	0.346
FSH (mIU/mL)	-0.136	0.099	-0.001	0.001	0.173
Oestrogen (pmol/L)	-0.081	0.092	0.000	0.000	0.382
Menopausal Status (%)	0.186	0.097	0.124	0.064	0.056
HOMA (mmol.mU/L^2)	-0.066	0.072	-0.004	0.004	0.359
Education (%)	-0.143	0.074	-0.156	0.081	0.056
Visceral Fat (mm)	-0.115	0.077	-0.033	0.022	0.138

Adjusted R^2= 0.100; p<0.002

Abbreviations: BIOT – bioavailable testosterone; SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate

Table 3.7 Multiple regression model for low density lipoprotein cholesterol (LDL-c).

N= 176	β	Standard Error of β	b	Standard Error of b	p-value
Menopausal Status (%)	0.200	0.085	0.256	0.109	0.020*
Oestrogen (pmol/L)	-0.066	0.086	-0.000	0.000	0.443
DHEA-S (µmol/L)	-0.127	0.075	-0.073	0.044	0.094
SHBG (nmol/L)	0.020	0.081	0.000	0.001	0.809
HOMA (mmol.mU/L^2)	-0.034	0.074	-0.003	0.007	0.645
Hip Circumference (cm)	0.205	0.081	0.002	0.001	0.012*
MVPA (minutes/week)	-0.097	0.078	-0.000	0.000	0.215
Alcohol Status (%)	0.026	0.074	0.037	0.103	0.723
Visceral Fat (mm)	-0.072	0.078	-0.039	0.042	0.353

Adjusted R^2= 0.113; p<0.016

Abbreviations: SHBG – sex hormone binding globulin; DHEA-S – dehydroepiandrosterone-sulfate; MVPA – moderate to vigorous physical activity

LDL-c was positively correlated with menopausal status, which is in line with current literature (Inaraja *et al*., 2020). Total cholesterol and HDL-c are negatively related to DHEA-s, which was also found in a 2009 study by Srinivasan *et al.* However, the relationship between DHEA-s and lipid profiles is complex, and studies tend to report conflicting findings (Srinivasan *et al*., 2009).

Discussion

Ovulation and reproductive potential cease when a woman experiences menopause, which is the permanent loss of ovarian function (Sherman, 2005). The average duration of the transition is seven years but could last over ten years (Santoro *et al*., 2020). Women may have symptoms like mood swings, sadness, sexual dysfunction, hot flushes, and irregular menstrual cycles at this time (Schmidt, 2005; Santoro *et al*., 2020). Between the ages of 45 and 55, symptoms start to appear (Santoro *et al*., 2020). After 12 months without menstruation, usually around an average age of 51, women enter the post-menopause phase (Sherman, 2005). Menopause-related hormone changes contribute to the development of age-related conditions such as breast cancer, osteoporosis, and cardiometabolic diseases, namely, type 2 diabetes, obesity, cardiovascular disease, stroke, and hypertension (Gold *et al*., 2001). In women within the menopausal age range (45-55 years), the risk of developing these conditions increases in comparison to their pre-menopausal selves and age-matched male counterparts (Kamińska *et al*., 2023). There is a vast body of literature (El Khoudary *et al*., 2020; Maas *et al*., 2021; Kamińska *et al*., 2023) documenting these observations in non-African women, but not in African populations – specifically West African women, where transition along the health epidemiological transition has not yet progressed. In this study, we focused on women from Nanoro, Burkina Faso in West Africa in order to understand the relationship between epigenetics, sex hormones and cardiometabolic risk in menopausal women. The findings of this study will mark the beginning of addressing the gap in literature around this topic and will contribute to understanding the impact of possible lifestyle and cultural differences across different African regions. There is little written about menopause and CVD risk in African women, with the exception of several studies in sub-Saharan Africa, in

Cameroon, and Nigeria (Moor *et al*., 2016; Lewechi-Uke *et al*., 2023; Chikwati *et al*., 2023; Chikwati *et al*., 2024).

Although cardiometabolic disease risk is strongly correlated with the menopause-associated change in sex hormone levels (Jaff *et al*., 2014) and is well documented, the mechanisms driving the relationship are not clearly understood. Based on previous studies on DNA methylation of sex hormone regulatory genes (e.g. *ESR2* and *SHBG*), it is proposed that the changes in hormone levels and concurrent development of metabolic syndrome in menopausal women may be influenced by epigenetic factors (Wagner *et al*., 2014; Arathimos *et al*., 2018; Maekawa *et al*., 2019; Bos *et al*., 2022; Ahmed *et al*., 2022; Cagsin *et al*., 2023). As menopause occurs when women are older, it is difficult to ascertain whether cardiometabolic risk increases as a result of hormone changes (menopause), increase in chronological age, or a combination of both menopause and age. The findings of this study will potentially provide some answers to this complex question, contributing to more targeted preventative treatment options for women. At present, the triggers and processes underlying the changes in hormone levels are not well understood and more research is required to better understand the intricate interactions among genetics, epigenetic changes, hormones, and cardiometabolic risk throughout the menopausal transition. Therefore, this study aimed to explore the role of a potential epigenetic mechanism (DNA methylation) in the modulation of sex hormone levels and associated risk for cardiometabolic diseases by comparing DNA methylation levels in pre-menopausal and post-menopausal women from Nanoro, Burkina Faso in West Africa.

4.1 Demographic, behavioural, socioeconomic, anthropometric, cardiometabolic, and hormone level differences in pre- and post-menopausal groups of West African women

In order to determine whether differential methylation plays a role in cardiometabolic risk, anthropometric measures, and sex hormone levels over the menopausal transition, significant differences between the pre- and post-menopausal groups had to first be identified.

4.1.1 Demographic, behavioural, and socioeconomic variables

A deliberate effort to select participants of a relatively close age range (pre-menopausal: 44-50 years; post-menopausal: 49-55 years) was made to diminish the confounding effects of age on menopause status. Despite this, age of pre- and post-menopausal groups was still statistically significantly different ($p<0.001$). Age therefore remains a potential confounding factor; however, we diminished the confounding effects through a robust study design. Analysis approaches and models have been used to adjust for age and to tease out effects related to menopausal changes.

There were no statistically significant differences observed between socioeconomic factors of pre- and post-menopausal groups. There was no change in alcohol consumption ($p=1.000$), education ($p=0.240$), and moderate to vigorous physical activity ($p=0.545$) when women transitioned from pre-menopausal to post-menopausal. Most women in Burkina Faso do not smoke, irrespective of menopausal status, therefore smoking data was omitted from the analyses.

4.1.2 Anthropometric variables

Studies from developed nations have demonstrated increased inflammation, cardiometabolic risk, and increase in appetite during the menopausal transition (Hunter *et al*., 2011). In contrast, we have observed a decline in BMI ($p=0.034$) and visceral fat ($p=0.015$) in our West African cohort of post-menopausal women. The reason for this decline in visceral adipose tissue and BMI in post-menopausal women could be related to the presence of a non-obesogenic environment in this community. Thus, the study population are rural and obtain most of their food from subsistence farming. Obesity is rare. Although hormone changes during menopause may stimulate fat accumulation, this may not be possible in an environment with low caloric intake. Most of the studies that show weight gain during menopause were undertaken in high income countries where calorie-dense foods are readily available, where such an obesogenic environment may fuel the increased adiposity related to hormonal changes during menopause. It is interesting to note that waist-to-hip ratio was higher in post- than pre-menopausal women in this study. This may reflect greater loss of subcutaneous relative to visceral fat. Such changes in body fat distribution have been

observed in other studies and are likely related to falls in oestrogen levels during the menopause transition (Burger, 2002; Brown and Clegg, 2010).

4.1.3 Cardiometabolic variables

Lipid profiles of the West African women followed the same trend as described in literature, where average cholesterol levels are higher in post-menopausal women as compared to pre-menopausal women (total cholesterol – $p<0.001$; HDL-c – $p=0.030$; triglycerides – $p=0.003$; and LDL-c – $p=0.007$). It is common for total cholesterol, triglycerides and LDL-c to increase as a woman transitions through the menopause (Currie and Williams, 2008) and is related to the fall in oestrogen levels (Xiang *et al*., 2021).

The increase in HDL-c levels, however, did not follow expected trends where HDL-c levels decrease as women transition through the menopause (Kim *et al*., 2000; El Khoudary *et al*., 2021). A few other studies have reported mixed results regarding menopause-related HDL-c trends (Fernandez and Murillo, 2016) and an initial decrease in HDL-c levels followed by an increase from 53 to 69 years has been suggested in a 1986 Framingham study article and an unrelated 2021 study (Castelli *et al*., 1986; El Khoudary *et al*., 2021). However, further studies on larger cohorts are needed to better understand HDL-c levels in post-menopausal women, specifically in a West African context.

Glucose metabolism-related factors (e.g. fasting blood glucose – $p=0.370$; fasting insulin – $p=0.575$; and HOMA – $p=0.527$) were not significantly different between pre- and post-menopausal groups. This is potentially a result of the restrictive diet that plagues the impoverished country of Burkina Faso, where more than 40% of its population lives below the poverty line (World Food Programme, 2019). Malnutrition leads to hypoglycaemia once glycogen stores in the liver have been metabolised (Ledger *et al*., 2020; Rao, 1984).

Both systolic ($p<0.001$) and diastolic blood pressure ($p<0.001$) was significantly higher in post-menopausal women as compared to pre-menopausal women. This is expected, and in line with other studies that indicate that low oestrogen levels impact blood flow by increasing both blood pressure and the risk of atherosclerotic plaque

build-up (Zanchetti *et al*., 2005; Maas and Franke, 2009; Jain *et al*., 2022; Li *et al*., 2024).

One of the most widely utilised and accurate imaging techniques for detecting atherosclerosis and predicting cardiovascular disease risk is the measurement of the carotid intima-media thickness (Lonn, 1999). In our post-menopausal group, atherosclerotic plaque build-up may explain the larger carotid intima-media thickness (cIMT) ($p=0.004$). The increase in cIMT measurements, suspected atherosclerosis, and elevated blood pressure may help explain the findings of our study that show a lower estimated glomerular filtration rate (eGFR) in post-menopausal women ($p=0.012$). High blood pressure in particular is known to lead to a decline in renal function (Velusamy, 2024).

4.1.4 Sex hormone variables

The hormones measured in this study are oestrogen (E2), follicle stimulating hormone (FSH), sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEA-S), testosterone (TT), free testosterone (FT), and bioavailable testosterone (BIOT).

As expected, oestrogen levels in post-menopausal women were much lower in comparison to the pre-menopausal group ($p<0.001$), which aligns with literature (Yang *et al*., 2024). Furthermore, the post-menopausal levels of FSH were higher than those of pre-menopausal women ($p<0.001$) in response to the oestrogen decrease (Taneja *et al*., 2019).

Upon comparative statistical analyses, no notable differences between pre- and post-menopausal levels of DHEA-s ($p=0.138$), TT ($p=0.481$), FT ($p=0.195$), BIOT ($p=0.113$) were detected. According to previous studies, DHEA-s levels decline with age (Panjari and Davis *et al*., 2010) and are associated with disease and bad health outcomes (Baird *et al*., 2018). The same decline is also expected in total testosterone, bioavailable testosterone, and free testosterone levels of post-menopausal women (Scott and Newson, 2020). Unlike oestrogen and follicle stimulating hormone, which sharply decline and increase, respectively, after menopause, testosterone and DHEA-s both decline steadily as a woman ages (Johansen *et al*., 2020). Therefore, the

significant difference in testosterone and DHEA-s levels may not be evident in a cohort with a small age range, such as ours.

SHBG levels are typically downregulated in post-menopausal women in response to the decline in oestrogen levels (Hoskin *et al*., 2004), as SHBG is a carrier protein (Victor *et al*., 1976) that binds to and transports oestrogen, testosterone and dihydrotestosterone (DHT) (Anderson, 1974). In this study, SHBG levels were also lower in post-menopausal women compared to the pre-menopausal group (p=0.049), however, there have been mixed results relating to SHBG levels in the literature (Maggio *et al*., 2008). Interestingly, a study conducted in South Africa also showed lower SHBG levels in post- than pre-menopausal African women (Jaff et al., 2015).

4.2 DNA methylation % differences in pre- and post-menopausal groups of West African women

4.2.1 Comparison of individual CpG site methylation in pre- and post-menopausal groups

When comparing individual CpG sites *ESR2*_1 to *ESR2*_20, and *SHBG*_1 to *SHBG*_17, no statistically significant differences between pre- and post-menopausal groups were found.

4.2.2 Comparison of promoter-linked average gene methylation in pre- and post-menopausal groups

When comparing average gene methylation (AGM) of promoter-linked regions of *ESR2*, and *SHBG* in pre- and post-menopausal groups, there were also no statistically significant differences observed, as with individual CpG site methylation.

Although no significant differences in individual CpG site or promoter-linked regions were observed, it has been reported previously that some changes in methylation between groups are not significant enough to be reflected with a statistical test comparing means or the spread of data (Chen *et al*., 2014). However, expression of the methylated gene may still be influenced by the small change in methylation (Chen *et al*., 2014). It is important to note that there is vast literature describing the epigenetic

modulatory effects of DNA methylation acting in conjunction with histone methylation, histone acetylation and chromatin modelling (Geiman and Robertson, 2002; Phillips, 2008; Hashimoto *et al*., 2010) to regulate gene expression. Therefore, although increased methylation is 'generally' associated with lower gene expression, we were not able to explore the complex relationship and influence of other epigenetic mechanisms. It is also not possible to determine causality of epigenetic modulation without performing gene expression and post-expression (transcription and protein levels) functional studies.

4.2.3 DNA methylation in the context of menopause-related cardiometabolic risk

Prior to regression modelling, univariate correlation analyses were carried out to determine which CpG sites or average gene methylation (AGM) of promoter-linked regions to include in the regression models. After adjusting for multiple testing, none of the methylation data had a p-value of less than 0.05, and therefore could not be included in any of the regression models.

It is possible that DNA methylation does not play a significant role in regulation of sex hormone regulatory genes, and is not linked to menopausal hormonal changes and associated cardiometabolic risk factors.

The sample size (197) may not have been powerful enough to detect such miniscule changes as in the case of differential methylation studies. The study may be worth repeating with a larger sample size, a different population group, and potentially the addition of other relevant genes.

5. Conclusion

To conclude, although the increase in cardiometabolic disease risk in menopausal women has been frequently documented, not many studies have been conducted specifically on African populations. There is also a need to fully elucidate the mechanisms behind sex hormone regulation during the menopausal transition as it is these hormone level changes that signal the menopausal transition. It is a reasonable assumption that epigenetic modifications of sex hormone regulatory genes modulate sex hormone levels, but we do not know how they influence CMD risk. Furthermore,

not much research has been conducted on non-Africans, let alone under-represented African populations, leading to a gap in literature.

This study sought to assess whether DNA methylation of genes involved in sex hormone regulation modulates hormone levels, leading to increased cardiometabolic disease risk in post-menopausal women from West Africa when compared with a pre-menopausal group.

Our findings suggest that it is possible that DNA methylation does not play a significant role in regulation of sex hormone regulatory genes, and is not linked to menopausal hormonal changes and associated cardiometabolic risk factors.

The sample size (197) may not have been powerful enough to detect such miniscule changes as in the case of differential methylation studies. The study may be worth repeating with a larger sample size, a different population group, and potentially the addition of other relevant genes.

To build on our study, future work aimed at furthering epigenetics research in CMD risk in menopausal African women should include more sex hormone regulatory/related genes, as well as cardiometabolic associative genes with the study repeated in several African countries to elucidate population-specific differential methylation patterns. There is also a need to carry out functional studies to determine causality, such as mRNA quantitative analyses, as they may have the potential to inform epigenome editing technologies much like the Coupled Histone tail for Autoinhibition Release of Methyltransferase (CHARM) (Neumann et al., 2024). Epigenome editing may provide a means of delaying the onset of menopause and its associated cardiometabolic risks.

The interplay between DNA methylation of sex hormone regulatory genes, sex hormone levels, cardiometabolic risk factors, demographics, anthropometric variables, and socioeconomic factors across the menopausal transition is complex. As our study was an exploratory one where very little or no information had been reported on the relationship between methylation of our genes of interest and their respective hormone level associations, we had no definitive preconceived notions regarding the outcomes of this research.

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