The role of biochemical markers and genetic susceptibility in the pathogenesis of hormone dependent malignancies

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To my family……..my world - my husband, my boys (Ben and Karl) as well as my mum, dad and sister who have all been very supportive. I would like to thank them for their constant love in their varied ways.

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ABSTRACT

Keywords: postmenopausal breast cancer, endometrial cancer, genetic risk score, BMI

Introduction

Multiple studies have associated the global increase of postmenopausal breast and endometrial cancer with the worldwide increase in obesity and the metabolic syndrome. The Maltese population has also been repeatedly shown to have markedly increased obesity, metabolic syndrome and insulin resistance, with increasing trends of breast and endometrial cancers.

Aims

To evaluate which markers - metabolic/hormonal and genetic markers related to the metabolic syndrome – are associated with increased risk of breast and/or endometrial cancer. Also, it aims to compare the performance of polygenic risk scores relative to anthropometric/clinical predictors in classifying cancer from control patients.

Method

A random sample of three study populations was recruited: Study Group 1- Patients with a history of endometrial carcinoma; Study Group 2 - Patients with a history of breast carcinoma; and Study Group 3: A control group including women with histologically confirmed absence of endometrial carcinoma (after hysterectomy) and no history of breast carcinoma. All the patients recruited were postmenopausal

patients of Maltese ethnicity. Each subject was interviewed and anthropometric data measured. Blood was collected for biochemical and hormonal tests. The risk factors were associated with breast/endometrial cancer risk and logistic regression was done. DNA was extracted from whole blood and genetic profiling by LP-WGS was then carried out. Association of genetic risk scores of single nucleotide polymorphisms known to be association with diabetes mellitus type II and insulin resistance were determined by logistic regression.

Results

300 patients have been recruited - 132 patients were diagnosed with breast cancer, 90 patients with endometrial cancer (four patients had both endometrial and breast cancer) and 82 patients controls.

The study observed a positive association between early menarche, nulliparity and high BMI with both breast ($p=0.02$, $p=0.049$, and $p=0.04$ respectively] and endometrial cancer risk ($p=0.01$, $p=0.017$, $p<0.01$) respectively. Family history of breast cancer and high SHBG level were also found to be associated with increased breast cancer risk ($p=0.009$ and $p=0.02$ respectively) while a positive association between history of hypertension ($p<0.01$), diabetes mellitus type 2 ($p<0.01$), presence of the metabolic syndrome ($p<0.01$), family history of hypertension ($p=0.007$), high serum triglycerides ($p<0.01$), HbA1C ($p<0.01$), HOMA-IR ($p=0.01$) were found with endometrial cancer. History of breastfeeding was observed to be negatively associated with both breast ($p<0.01$) and endometrial cancer risk ($p<0.01$). Serum FSH and LH levels were also found to be negatively associated with breast cancer ($p<0.01$ and $p<0.01$ respectively) while serum SHBG and

progesterone showed a negative association with endometrial cancer (p=0.01 and p=0.01 respectively).

The logistic regression models showed that that BMI was the best predictor of breast and endometrial cancers - for every 1 kg/m2 increase in BMI, the odds of having breast cancer increased by 3.9% (OR=1.039) while the odds of having endometrial cancer increased by 8.4% (OR=1084).

Genetic profiling showed that a greater number of alleles from genetic risk scores with loci for diabetes mellitus type 2 and insulin resistance were significantly present in the breast and endometrial cancer cohorts. After adjustment for age, fasting insulin, fasting glucose, WHR and serum triglycerides level, quintile 5 of GRS 1 was found to have an OR for cancer risk (breast/endometrial) of 21.738 ($p<0.01$) while quintile 5 of GRS 2 had OR of 43.406 ($p<0.01$).

Conclusion

This study gave better understanding on the risk significance of various factors related to breast and endometrial carcinogenesis in the Maltese population. By determining risk factors, women can be risk-stratified and individualised intervention/s can be implemented according to their risk for developing breast/endometrial cancer.

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ADCY5	adenylase cyclase 5
AI	aromatase inhibitors
AR	androgen receptor
ASTEC	A Study in the Treatment of Endometrial Cancer
BCSC	The Breast Cancer Surveillance Consortium
BMI	body mass index
BPSO	prophylactic surgical removal of ovaries and fallopian tubes
BRCA	breast cancer gene
BPM	bilateral prophylactic mastectomy
CI	confidence Interval
CIS	carcinoma in situ
DHEA	dehydroepiandrosterone
DHT	5-dihydrotestosterone
DIAGRAM	DIAbetes Genetics Replication and Meta-analysis
DM	diabetes mellitus
ECIS	European Centre Information System
EGF	epidermal growth factor
EHBCCG	Endogenous Hormones Breast Cancer Collaborative Group
EIN	Endometrial intraepithelial neoplasia
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	oestrogen receptor
FH	family history

FSH	follicle stimulating hormone
FTO	fat mass and obesity associated gene
FOS	Framingham Offspring
GPER	G-protein coupled oestrogen receptor
GRS	genotype risk score
GWAS	genome-wide association scans
HbA1c	haemoglobin A1c
HELENA	Health Lifestyle in Europe by Nutrition in Adolescence
HER	Human epidermal growth factor receptor
HOMA-IR	homeostatic model of insulin resistance
HRT	hormone replacement therapy
IARC	International Agency for Research on Cancer
IDF	International Diabetes Federation
IGF1	insulin-like growth factor 1
IRS1	insulin receptor substrate 1
LDL	low density lipoprotein
LP-WGS	Low-pass whole genome Sequencing Analysis
LH	luteinizing hormone
MAF	minor allele frequency
MC4R	melanocortin-4 receptor
MTNR1B	Melatonin Receptor 1B
OCP	combined oral contraceptive pill
PCA	Principal component analysis plot
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism

PEG	polyethylene glycol
PI3K	phosphoinositide 3-kinase
POLE	Polymerase Epsilon
POMC	pro-opiomelanocortin
PORTEC	The Postoperative Radiotherapy in Endometrial cancer
PR	progesterone receptor
ROC	receiver operating characteristics curve
SEER	Surveillance, Epidemiology, and End Results Program
SERD	selective ER downregulators
SERM	selective oestrogen receptor modular
SHBG	sex hormone binding globulin
SNPs	single nucleotide polymorphisms
StAR	Steroidogenic Acute Regulatory Protein
TDLU	terminal duct lobular unit
TMEM18	transmembrane protein 18
TRIPOD	Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis
UKCTOCS	UK Collaborative Trial of Ovarian Cancer Screening
VAT/SAT	visceral adipose tissue to subcutaneous adipose tissue ratio
WHI	Women's Health Initiative
WHR	Waist-hip ratio

Chapter 1

Introduction

1.1 The hormone dependent malignancies

The hormone dependent malignancies include malignancies that involve the reproductive tract – breast, endometrial and ovarian in women and testicular and prostate cancers in men. In women, breast cancer is the most common cancer worldwide accounting for around 2.3 million new cancer cases in 2020 and 685,000 deaths while endometrial cancer ranks the 6th most common cancer in women worldwide, responsible for over 400,000 cases in 2020 and over 97,000 deaths (IARC, 2020; WCRF, 2018). The global burden of both breast and endometrial cancer, with their associated morbidity and mortality, has been on the increase over the last decades (IARC, 2020).

The sex steroid hormones – oestrogens, androgens and progestins influence both the normal growth and function of the breast and uterus as well as being key in carcinogenesis. Multiple studies have associated obesity and its accompanying feature insulin resistance with increased oestrogen levels through peripheral conversion of testosterone and androstenedione into oestrogen (Desta et al., 2009). This hyperoestrogenic state thus produced, especially in postmenopausal women, increases the risk of breast and endometrial cancer (Savona-Ventura et al., 2009).

Worldwide, obesity and the metabolic syndrome have been on the increase; the prevalence of overweight and obesity amongst adults and children combined has more than doubled between 1980 and 2013 (Ng et al., 2014). The Maltese

population also shows a high prevalence of obesity, diabetes mellitus and insulin resistance as well as increasing incidence rates of breast and endometrial cancer.

Screening strategies for breast cancer by mammography are commonly performed to help in early detection. However, there is no evidence of any appropriate effective screening test for endometrial cancer. Transvaginal ultrasound assessment of the endometrial thickness and/or screening with endometrial biopsy (starting testing at 35 years) is only advocated for asymptomatic women who have or are at very high risk of endometrial cancer including known/suspected Lynch syndrome mutation (Smith et al., 2017; Morrison et al., 2021).

Several risk assessment tools have been developed to identify patients at risk of breast cancer, with varied accuracy rates in different populations (Nickson et al., 2018). Work is also being done to develop such tools for endometrial cancer. These models include lifestyle factors, family history, past/medical history as well as genetic factors. The role of genetics in carcinogenesis of these malignancies is increasingly being investigated. Evidence is showing the importance of identifying potential risk factors, stratification into appropriate risk groups and implementing cancer risk reducing strategies. The adoption of chemo-preventive measures and surgical intervention to reduce the risk of developing these malignancies makes it imperative that a risk prediction model is developed. The investigation of the relationship between these two gynaecological malignancies and markers of the metabolic syndrome and insulin resistance will help aid to identify individuals at risk on the Maltese Islands and thus hopefully introduce early management interventions for disease prevention.

1.2 The Global and Maltese burden of breast and endometrial cancers

1.2.1 Breast cancer

The palpability of the lumps and the visible signs of breast cancer especially at later stage has led to easy diagnosis and documentation by physicians in almost every era of recorded history. But due to the sexual connotations of the breast, breast cancer was a taboo subject and still is in some cultures. Descriptions were limited to clinical journals. Today however discussions are open to the general public through the Internet and electronic media. In 1990s, the pink symbol of breast cancer was used as the international symbol to raise breast cancer awareness (Lukong, 2017) .

The first descriptions of the clinical diagnosis of breast cancer date back to the Ancient Egyptians (around 3500BC), in two papyri: the *Edwin Smith Surgical Papyrus* and the *Ebers Papyrus* (Brawanski, 2012). Galen (168BC) noted that breast cancer occurs more common in women who had abnormal menstrual cycles or who were not menstruating (Lukong, 2017). In 1713, Bernardino Ramazzini observed that breast cancer was more common in nuns (Olson, 2002) while in 1926 Janet Elizabeth Lane-Claypon identified several breast cancer risk factors, including parity, age of menopause, duration of lactation, age of first born (Press & Pharoah, 2010). The tumour suppressor genes BRCA1 and BRCA2 were identified in 1994 as inherited mutations that increase risk of breast and ovarian cancer (Narod & Foulkes, 2004). In 1962, Robert Egan reported a case series of breast cancer detected using mammography (Kalaf, 2014).

Nowadays, diagnosis is based on (1) clinical examination – palpation of the breasts and lymph nodes (clinical diagnosis may however prove difficult in patients with high BMI), (2) imaging by mammography and ultrasound; MRI considered in high risk women (3) core needle biopsy for pathological confirmation (ESMO, 2022).

Data from GLOBOCAN 2020 shows that breast cancer is the most common cancer worldwide. It accounts for 11.7% of all cancers and 1 in 4 cases in women with an estimate of 2.3million new cases in 2020. The age standardized rate (ASR) world per 100,00 amounts to 47.8 per 100,000 worldwide. Incidence rates vary across different countries of the world, from 26.2 per 100,000 in South-Central Asia to 90.7 per 100 000 in Western Europe and 95.5 per 100,000 in Australia and New Zealand (Figure 1.1). Factors contributing to these differences include differences in population background risk, different awareness and different screening programmes (Armaroliet al., 2020).

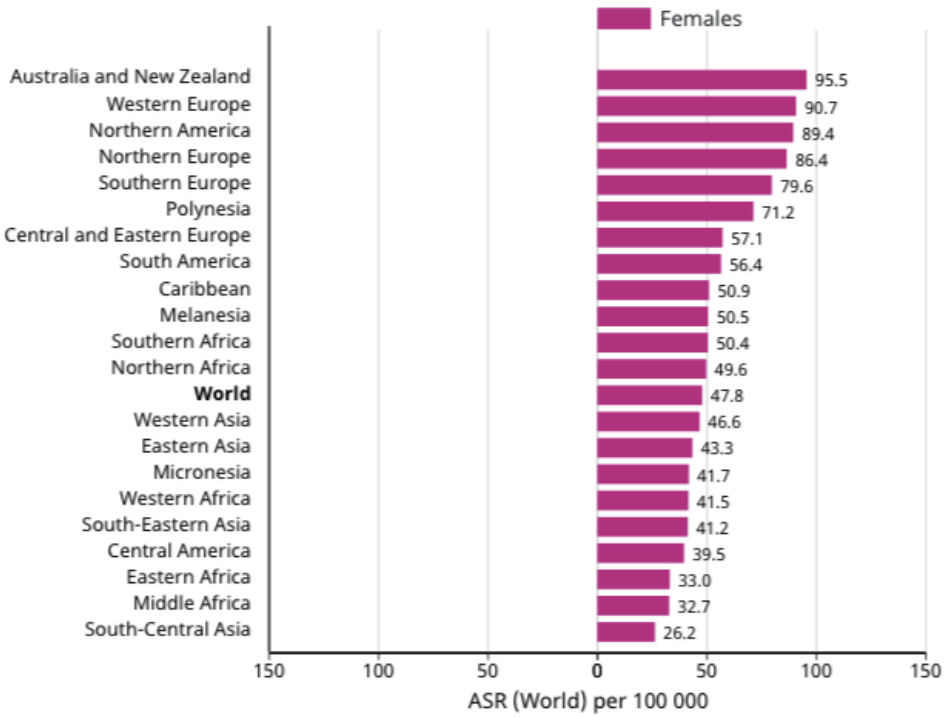


Figure 1.1 Age standardized (World) incidence rates, breast (IARC, 2020)

Breast cancer is also the most common cancer in females in Malta, accounting for 35.4% of all female cancers, with 403 new cases in 2020. The age-standardized rate (ASR) (world) of breast cancer in Malta in 2020 was 89.5 per 100 000 (IARC, 2020). Malta ranked in the 17th place with the highest rate of breast cancer worldwide (IARC, 2018).

Incidence rates of breast cancer are on the increase. Figure 1.2 is showing data from selected European Union countries and Regions – one from each European region: Central and Eastern, Northern, Southern, Western) from available incidence series of at least 20 years from the European Centre Information System (ECIS). The number of new cases of breast cancer between 2004-2015 in Malta is shown in Figure 1.3.

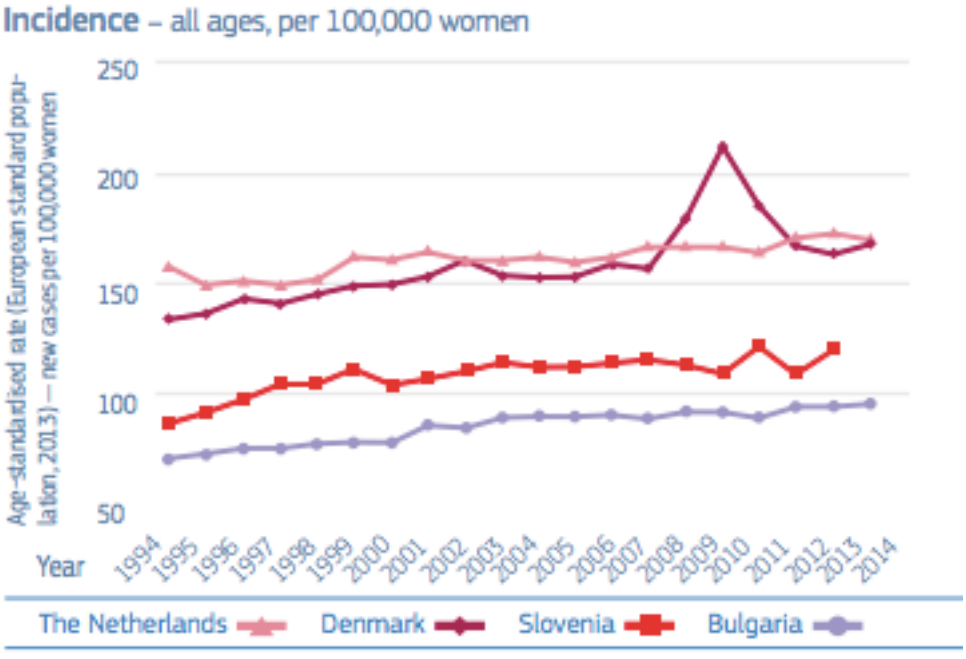


Figure 1.2 Breast cancer incidence rates 1994-2014 (ECIS – European Cancer Information System)

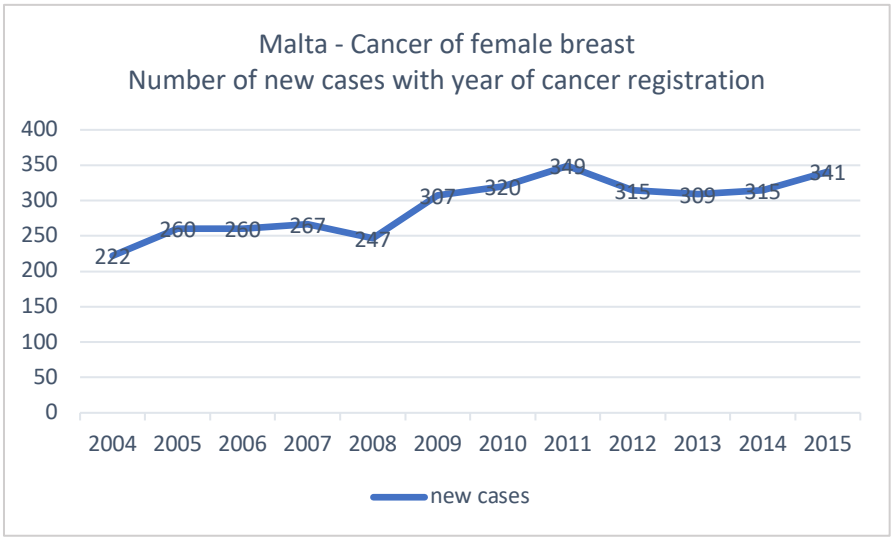


Figure 1.3 Breast cancer new cases, females, Malta, 2004-2015 (IARC, 2018)

Breast cancer ranks the fifth leading cause of cancer deaths worldwide (IARC, 2020) accounting for 6.9% of all cancer deaths, with a total of around 685 000. and the fourth cause of cancer death in the US (NCI SEER, 2017). In Malta, breast cancer is responsible for around 69 deaths annually, with an ASR (World) of 11.5 per 100,000 in 2020 (IARC, 2020) and ASR (Europe) of 27.34 per 100,000. Mortality rate of breast cancer has been on the decrease (Figure 1.4) with increasing 5-year relative survival rate – which is around 80% (Attard & England, 2016) .

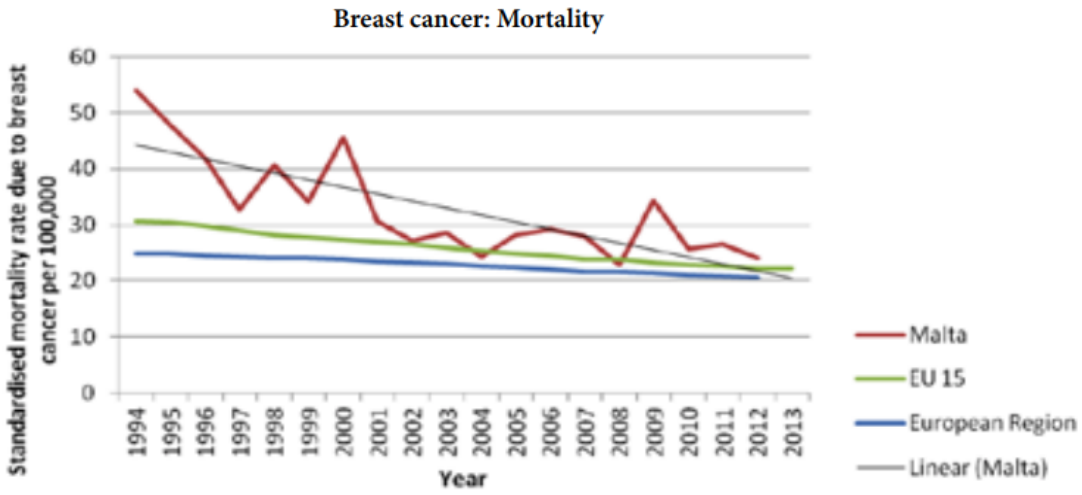


Figure 1.4 Breast cancer mortality rates 1994-2013 (Attard & England, 2016)

1.2.2 Endometrial cancer

Endometrial cancer was recognised since the Egyptian times of the mummies (Quirke, 2012). Throughout the years, authors provided descriptions about endometrial cancer – its symptomatology, clinical examination, and treatment.

The Greek physician Hippocrates recognized the clinical presentation of uterine cancer back in 460BC (Tsoucalas et al., 2015). The general symptoms included anorexia, pain, cachexia, cessation of menstruation while local symptoms included vaginal bleeding, inflammation, oedema and ulceration. Anulus Cornelius Celsus (25 BC-50 AD), a Roman encyclopaedist, devoted a whole chapter on endometrial cancer in his work 'De Medina' (Karamanou et al., 2008). He stated that "females are subject to a malignant disease of the womb".

Postmenopausal vaginal bleeding has long been recognised as the most common presentation of endometrial cancer (Sorosky, 2012). However, only 10-20% of all cases presenting with postmenopausal bleeding are diagnosed with endometrial cancer (Bennett et al., 2011). In the premenopausal age group, abnormal uterine bleeding accounts for around 20% of endometrial cases (Bennett et al., 2011). Women older than 45years with abnormal uterine bleeding should be evaluated for endometrial cancer, as well as women younger than 45 years with abnormal bleeding and a history of unopposed oestrogen exposure (ACOG, 2013).

Transvaginal ultrasonography to measure the endometrial thickness is usually the first diagnostic study, with a cut-off greater than 4-5mm in postmenopausal women

warranting endometrial biopsy (ACOG, 2009; Bennett et al., 2011). In the presence of postmenopausal bleeding, a cut-off of 5mm endometrial thickness or less had a 96% sensitivity and 51.55% specificity for endometrial cancer (Long et al., 2020). Endometrial tissue biopsy is ultimately necessary for definite diagnosis (Saso et al., 2011)

In 2020, endometrial cancer ranked the sixth most common cancer in women worldwide and the 15th most common cancer overall (IARC, 2020), with more than 400,000 cases. According to GLOBOCAN 2020, the ASR (World) of uterine cancer worldwide is 8.7 per 100,000. In Europe, the ASR (World) is higher, at 12.9 per 100,000(Western Europe)- 20.2 per 100,000 (Central and Eastern Europe) (IARC, 2020). In Malta, endometrial cancer ranks the 8th most common cancer, but the third most common cancer in women, with 82 new cases in 2018. It accounts for 7.7% of all cancers in Maltese females, with an age-standardized (world) incidence rate of 17.8 per 100 000 in year 2018. These figures are considerable higher when compared to those obtained from women worldwide (IARC, 2018).

In a study looking at endometrial cancer incidence in 43 countries from 1978-2013, it was concluded that the incidence rate of endometrial cancer varies across countries, the highest rates are found in North America, Eastern Europe and Northern Europe (Lortet-Tieulent et al., 2018). Also, the incidence rates showed an increasing trend over the last 10 data years in 26 out of the 43 populations, especially in countries showing rapid socioeconomic changes (Lortet-Tieulent et al., 2018) .

Figures 1.5, 1.6 and 1.7 depict the upward trend in incidence rates in US, UK, and Malta respectively, which corresponds with the upward trend seem in numerous other countries across the world.

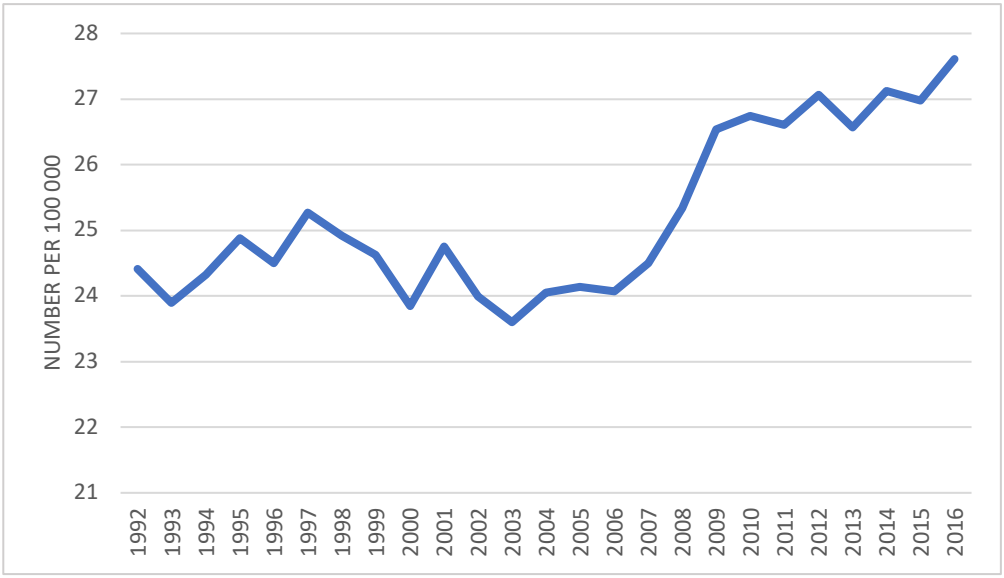


Figure 1.5 Number of age adjusted observed new cases of uterine cancer in US, per 100 000 (data from SEER-13 – Surveillance, Epidemiology, and End Results) (WCRF SEER, 2018)

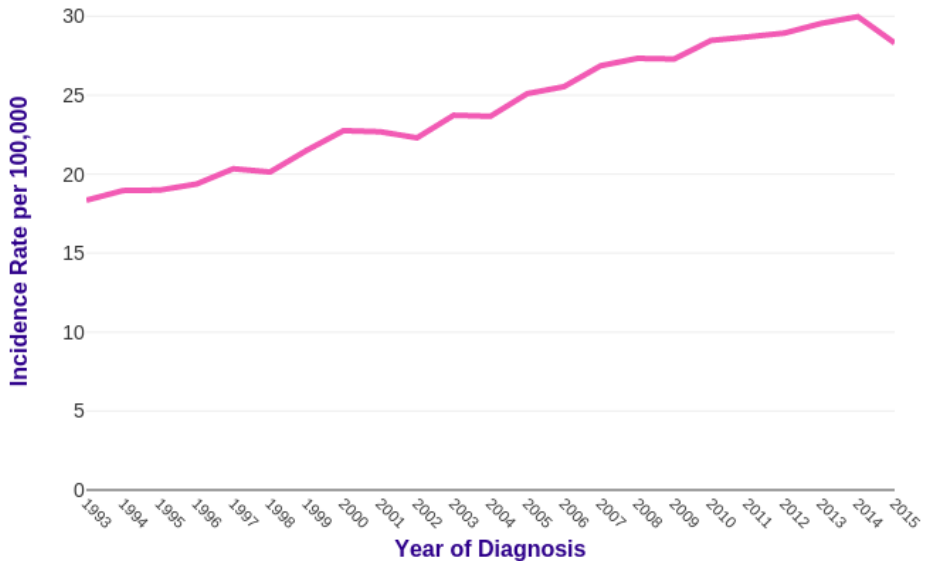


Figure 1.6 Endometrial Cancer, European Age-Standardized incidence rates, females, UK 1993-2015(Cancer Research UK, 2015)

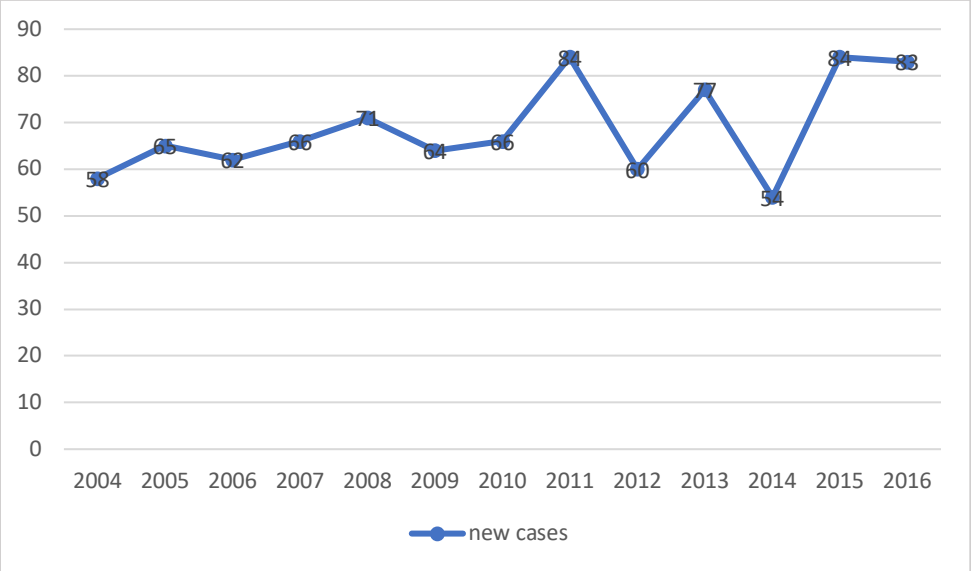


Figure 1.7 Uterine cancer, new cases, females, Malta, 2004-2014 (Azzopardi, 2017)

In Malta, endometrial cancer is responsible for around 19 deaths annually, with a mean 2007-2016 ASR (World) of 3.66 per 100 000 and ASR (Europe) of 5.47 per 100 000. Endometrial cancer has a high 5-year relative survival rate of around 70% in the US (American cancer society, 2019).

1.3 Overview of sex hormones and their action on endometrial and breast tissue

Sex hormones refer to the steroid-type hormones that interact with oestrogen or androgen receptors in tissue. They are important in the regulation of various developmental and physiological processes. Three major forms of oestrogen exist: oestrone (E1) which is the predominant oestrogen after menopause, oestradiol (E2) the main oestrogen in non-pregnant females, and oestriol (E3) mainly produced during pregnancy (Hamilton et al., 2017). The most important derivative of the androgen steroids is testosterone. Progestogens are sometimes regarded as the third class of sex steroids, with progesterone being the only naturally occurring progestogen. Oestrogen and progesterone are considered as female sex hormones; while androgens have masculinizing effects and considered as the primary male sex hormone. The pituitary hormones – luteinizing hormone, follicle stimulating hormone and gonadotrophin-releasing hormone – also have sex-related functions but these are polypeptide hormones that have that exert their effects through a stimulatory influence on gonadal steroid hormone synthesis.

All steroid hormones are synthesized from cholesterol, the primary substrate, and share a basic structure – the cyclopentanophenanthrene 4-ring structure (Figure 1.8). Each class of steroid hormone is however not based on difference/s in structure of the steroid but rather on the receptor/s that it binds to. (Miller & Auchus, 2011).

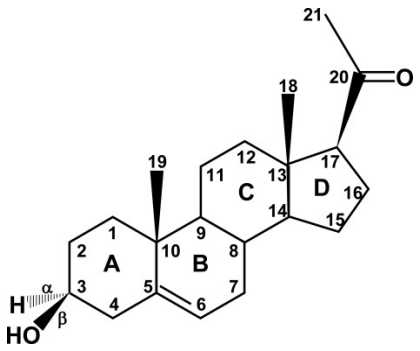


Figure 1.8 Structure of pregnenolone, showing the cycloperhydropentano-phenanthrene structure. The carbon atoms are labelled by numbers, while the carbon rings are labelled by letters. Hydrogens and substitutes are designated α and ß to show whether they are positioned behind or in front of the plane, respectively. (Miller & Auchus, 2011)

Steroidogeneses occurs mainly in the adrenal gland and the gonads. Cholesterol, mostly from circulating low-density lipoproteins (LDLs), enters the cell through receptor mediated endocytosis where cholesterol is directed to endosomes. In the endoplasmic reticulum of the adrenals, a small proportion of cholesterol is synthesised de-novo from acetate. Free cholesterol can then be stored in lipid droplets as cholesterol esters through its esterification by acyl-CoA cholesterol transferase (ACAT). Lipid droplets can form free cholesterol again by the enzyme hormone-sensitive lipase (HSL). In view that cholesterol is nearly insoluble, it needs ways to transport across the aqueous cellular membranes. Star D4 is probably the responsible protein involved in the delivery of cholesterol to the outer mitochondrial membrane (OMM) whereas steroidogenic acute regulatory protein (StAR) aids the transport of cholesterol from the OMM to the inner mitochondrial membrane (IMM) (Miller, 2007; Miller & Auchus, 2011) (Figure 1.9).

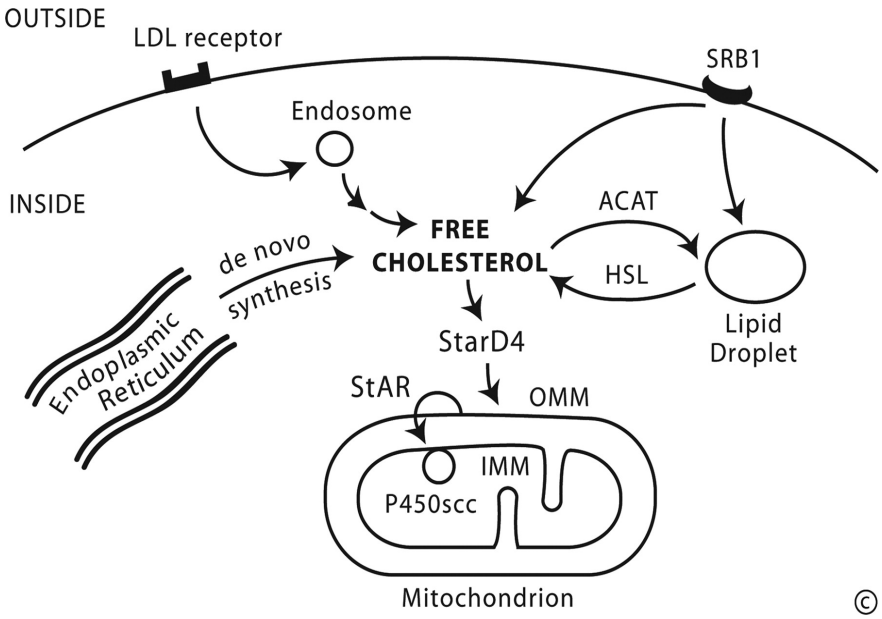


Figure 1.9 Transport of cholesterol into the cell. Adapted from (Miller, 2007)

The first reaction in steroidogenesis occurs in the mitochondria and involves the conversion of cholesterol into the first steroid hormone, pregnenolone (Stocco et al., 2017). This rate-limiting reaction is catalysed by the cytochrome P450 cholesterol side chain cleavage enzyme (P450scc, CYP11A1, also called cholesterol desmolase enzyme), which is located on the matrix side of the inner mitochondrial membrane (Farkash et al., 1986). The qualitative regulation of the respective steroid hormones is mediated by various enzymes and cofactors. The steroidogenic enzymes can be either cytochrome P450 enzymes or hydroxysteroid dehydrogenases (HSD) (Miller & Auchus, 2011).

The classes of steroids hormones produced are the 21-carbon steroids progestogen, glucocorticoids and mineralocorticoids, the 19-carbon androgen steroids and the 18-carbon oestrogen steroid hormones (Simpson & Davis, 2001).

Figure 1.10 is a diagrammatic representation of ovarian steroidogenesis, showing the precursors of oestradiol, testosterone and progesterone.

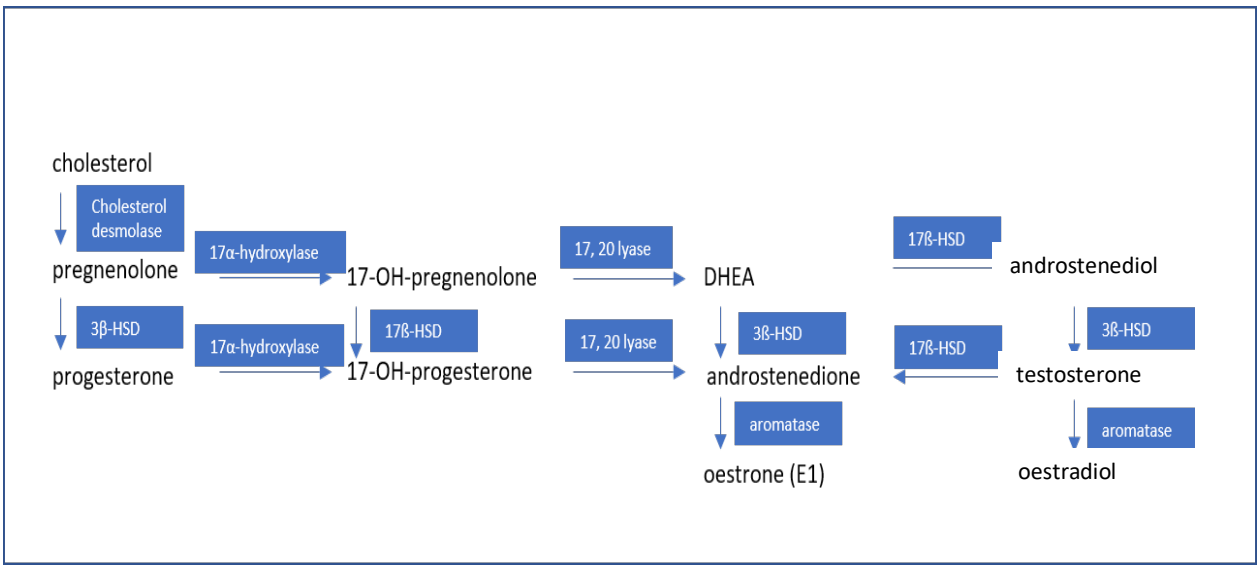


Figure 1.10 Ovarian Steroidogenesis (Brodowska et al., 2014)

Abbreviations: DHEAS, dehydroepiandrosterone sulphate; HSD, hydroxysteroid dehydrogenases

Apart from the above classical pathway of androgen biosynthesis, there are other pathways involved in androgen biosynthesis (Figure 1.11). The alternative 'backdoor' pathway and the 5α-dione pathway directly synthesises dihydrotestosterone, bypassing the production of testosterone. The other pathway involved in androgen biosynthesis is the 11-oxygenated androgen pathway.

Testosterone production occurs in the ovary, adrenal, and peripherally, through conversion of androstenedione (Chuffa et al., 2017). In adipose tissue, androstenedione is converted to testosterone by 17β-HSD5 and testosterone is then activated to dihydrotestosterone by 5α −reductase (Labrie, 2015).

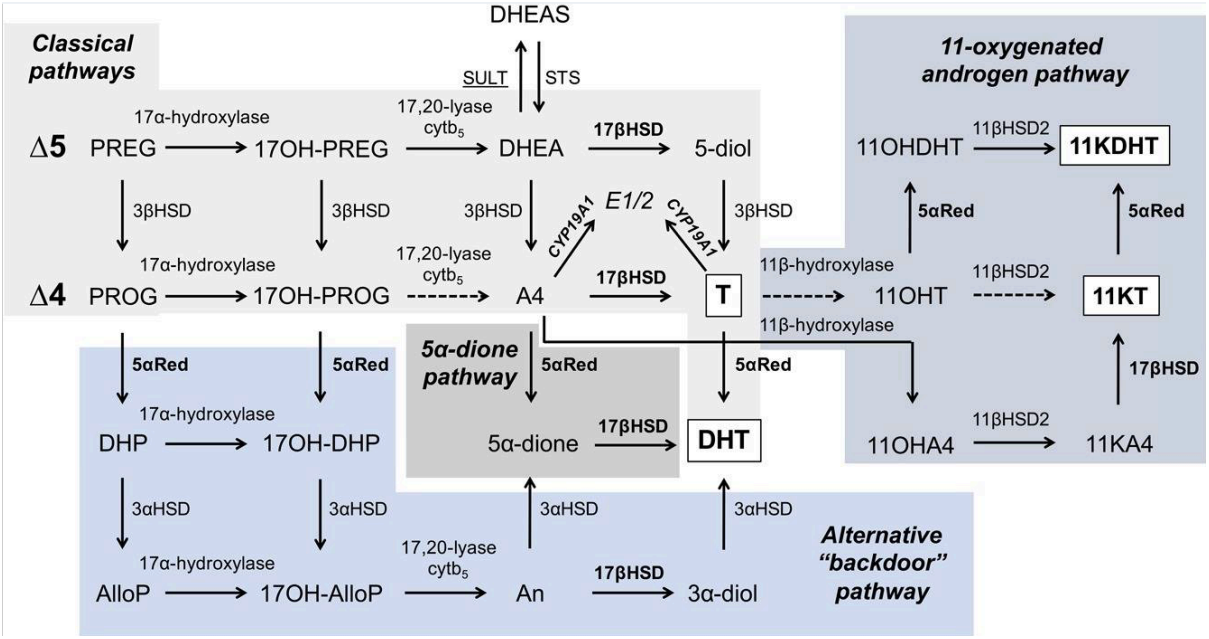


Figure 1.11 Androgen biosynthesis pathways (Schiffer et al., 2017a)

Steroid abbreviations: 3α-diol, 5α-androstanediol; 5α-dione, 5α-androstenedione; 5-diol, androstenediol; 11KA4, 11-keto-androstenedione; 11OHDHT, 11β-hydroxytestosterone; 17OH-AlloP, 17-hydroxyallopregnanolone; 17OH-DHP, 17-hydroxydihydroprogesterone; 17OH-PREG, 17-hydroxypregnenolone; 17OH-PROG, 17-hydroxyprogesterone; AlloP, allopregnanolone; An, androsterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; DHP, 5α-dihydroprogestrone; PROG, progesterone. Enzyme abbreviations: STS, steroid sulfatase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ4–5 isomerase; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; cytb5, cytochrome b5.

Androgen excess is a feature of polycystic ovarian syndrome (PCOS) which affects around 5-10% of reproductive age women (Yildiz et al., 2012). PCOS is diagnosed if two of these features are present: polycystic ovarian morphology on ultrasound, anovulation and androgen excess (Rotterdam criteria). It is associated with various metabolic dysfunctions including insulin resistance, dyslipidaemia, visceral and central adiposity as well as cardiovascular disease (O'Reilly et al., 2014). Androgen excess can be measured by evaluating serum testosterone and androstenedione. If serum dehydroepiandrosterone sulphate (DHEAS) ester, 11OH-A4 androstenedione and 11-oxygenated androgens are elevated, this is indicative of

androgen excess from adrenal origin. To contribute further to the local and systemic androgen burden, the enzymes involved in androgen biosynthesis - steroid 5α-reductase, 17β-hydroxysteroid dehydrogenase and 17βHSD are upregulated (Schiffer et al., 2017). Adipose tissue plays an important role in androgen generation as shown by Renato et al., who observed that weight loss improves features of PCOS (Renato et al., 2011).

In the premenopausal woman, oestradiol is the principal source of oestrogen produced by the granulosa cells of the ovarian follicle. Oestrogens are also produced by the action of aromatase CYP450 enzyme conversion of testosterone and androstenedione into oestradiol and oestrone respectively in peripheral tissues.

Production of the female sex hormones, oestrogen and progesterone fluctuates cyclically, throughout the menstrual cycle. Their levels are regulated by a feedback control mechanism acting on the anterior pituitary trophic hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH). These glycoproteins are under the control of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus. Regulation of sex hormones production occurs in two phases: the acute phase which occurs within minutes, and the chronic regulation which occurs within hours. The acute phase is facilitated by StAR. The importance of this protein in steroidogenesis was highlighted by Lin et al, who observed that in the autosomal recessive condition congenital lipoid adrenal hyperplasia, where the StAR gene is mutated, the new-born has almost complete inability to produce steroids (Lin et al., 1995). In chronic regulation of steroidogenesis, there is long-term expression of the

mRNAs and proteins responsible for the production of the enzymes involved in this pathway (Miller, 2007).

At the time of menopause, there is a progressive decrease and eventual complete cessation of the cyclical function of the ovaries. The number of oocytes and follicles decline with menopause leading to decreased production of oestradiol, progesterone and inhibin, and increased levels of FSH and LH. The ovarian stroma still retains some steroidogenic capacity producing small amounts of oestradiol, androstenedione and testosterone under the stimulation of LH (Brodowski et al., 2012). Brodowski et al measured ovarian and serum oestradiol, androstenedione and testosterone in women who had ovariectomy due to benign disease and found that levels decrease with time from last menstrual period (Brodowski et al., 2012). Kim et al described that although androstenedione and oestrone concentrations decrease slightly after menopause, the oestradiol concentrations level declined markedly (Kim et al., 2006).

In postmenopausal women, the adrenal gland becomes the main source of sex hormone production, mainly androgens (Havelock et al., 2006). Oestrogen is produced from testosterone in extra-gonadal sites including adipose tissue, brain, vascular smooth muscle and bone through the action of the enzyme aromatase. Testosterone is aromatized to oestradiol while dehydroepiandrosterone (DHEA) is aromatized peripherally via androstenedione to oestrone, the latter accounts for most of the circulating oestrogen in the menopause.

In the bloodstream, oestrogen and testosterone circulate loosely bound to albumin and tightly bound to sex hormone binding globulin, SHBG. The remaining 1-2% is unbound and biologically active. SHBG is a 93.4kDa glycated homo-dimeric plasma transport glycoprotein produced mainly by the liver, which inhibits the function of sex hormones (Fortunati et al., 2010). Different sex steroid hormones bind to SHBG with different affinities (having different clearance rates), with 5-dihydrotestosterone (DHT) binding to SHBG with more affinity than testosterone > androstenedione > oestradiol > oestrone (Selby, 1990). DHEA binds comparatively weakly to SHBG. In women, SHBG levels are about twice as high as in men, thus limiting bioavailability of oestrogens and androgens. It also varies between individuals, and is affected by metabolic, hormonal, growth, nutritional factors, and drugs (Allen et al., 2002).

Higher values of plasma SHBG are found in hyperthyroidism, pregnancy, liver cirrhosis and with oral contraceptive use. Low levels of SHBG are found in polycystic ovary syndrome, obesity, hypothyroidism, acromegaly, Cushing's syndrome and following the administration of anabolic steroids. This leads to higher levels of androgens which results in hirsutism, acne and virilisation (Davison et al., 2005; Selby, 1990). Since the level of SHBG plays a major role on the metabolic clearance and the bioavailability of the sex steroid hormones (Kahn et al., 2002), it directly affects the concentration of sex hormones reaching target tissues. Sex hormones then exert their action after binding to their receptors.

Sex hormone receptors, including oestrogen receptors (ERs), androgen receptor (AR) and progesterone receptors (PRs) are classified as Class 1 nuclear receptors;

the nuclear receptors being a ligand-activated nuclear receptor superfamily of transcription factors (Hewitt & Korach, 2018; Wang, L. et al., 2017). All members of the nuclear receptor superfamily share a similar structure, with multidomains carrying separate interactions and functions. They all share four distinct domains (Figure 1.12): N-terminal domain (NTD, A/B domain), DNA binding domain (DBD, C-domain), a hinge region (D-domain) and a ligand binding domain (LBD, E-domain). Among members of the ligand-activated nuclear receptor superfamily of transcription factors, the amino terminal (A/B domain) is the more variable in terms of size and amino acid sequence (Hilser & Thompson, 2011).

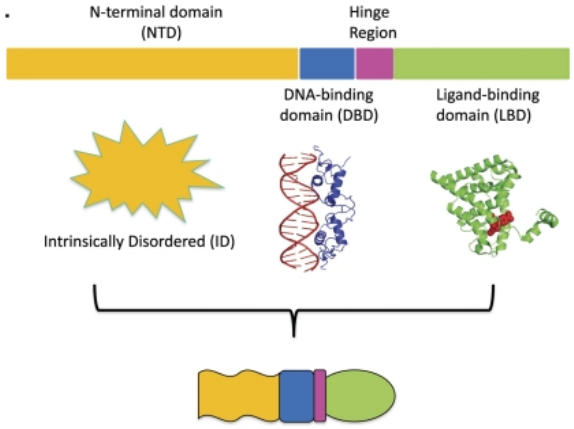


Figure 1.12 Sex hormone receptor structural architecture (Hilser & Thompson, 2011)

Oestrogen is lipophilic and passes freely through cell membranes. It binds to ER subtypes, ERα and ERβ in the nucleus and G-protein coupled oestrogen receptor (GPER) which is membrane associated. Oestogen plays key roles in the development and maintenance of the reproductive system. In addition, it exerts effects on the cardiovascular, immune, musculoskeletal, and central nervous systems. The biological actions of oestrogen vary depending on the expression of ERα and ERβ and its isoforms; their expression vary in normal and cancer tissue. ERα receptors

are found in reproductive organs, bone, white adipose tissue, and kidney, while ERβ receptors are expressed mainly in the ovary, uterus, bladder, central nervous system, breast and prostate in males (Matthews & Gustafsson, 2003). There are 5 isoforms of ERβ: the wild type isoform ERβ1, ERβ2 (identical to ERβcx), ERβ3, ERβ4 and ERβ5. Breast tissue expresses predominantly ERβ1, ERβ2 and ERβ5, but expression of ERβ4 is also observed (Tong et al., 2002). The difference in expression of ERβ isoforms also effect the Erα/ERβ expression balance. (Warner & Gustafsson, 2010). When a cell expresses both Erα and ERβ, ERβ can inhibit Erα dependent transcription (Matthews et al., 2006; Fuentes & Silveyra, 2019).

The main difference in the actions of ERα and ERβ appears to be through the non-genomic pathways. In a study by Acconcia et al, it was shown that Erα induces transduction pathways (ERK/MAPK and PI3K/AKT) related to cell cycle progression and inhibition of apoptosis while ERβ activates phosphorylation of p38/MAPK, leading to poly (ADP-ribose) polymerase activation and driving the cells into apoptosis (Acconcia et al., 2005). Abnormal ER signalling plays a role in development of malignancy (Jia et al., 2015). ERα seems to be associated with proliferation, inflammation and carcinogenesis while ERβ is involved in modulation expression of ERα regulated genes and causes antimigratory/anti-invasive/anti-proliferation responses (Thomas & Gustafsson, 2011; Omoto & Iwase, 2015). The role of ERβ in ERα-negative tumours is controversial, some studies found ERβ to have a proliferative role rather than a tumour-suppressor effect (Austin et al., 2018; Piperigkou et al., 2016).

In 1997, with the discovery of a novel seven transmembrane-domain G-protein couple receptor also grew evidence to describe the rapid action of E2 via this receptor – which was then officially named – GPER. Interestingly, the expression pattern of GPER varies according to age (developmentally regulated), species, gender and tissue type. Research into the functions of GPER, both physiological and/or pathological has been accelerating. Both tamoxifen and raloxifene are found to act as GPER agonist. The active metabolite of tamoxifen, 4-hydroxytamoxifen was shown to act on the phosphoinositide 3-kinase (PI3K) in GPER expressing cells but failed to activate ERα positive cells (Prossnitz et al, 2007), while raloxifene elicited response via GPER in ERα deficient endometrial cancer cells (Petrie et al, 2013). Upon stimulation with oestrogen, GPER can enhance cancer cell proliferation in the classical-ER-negative breast cancer cells and endometrial cancer cells (Vivacqua et al, 2006).

Several pathways have been postulated that describe the pro-oncogenic pathway of unopposed oestrogen:

i) the genetic pathway: oestrogen activates ER which in turn induces gene transcription. Factors like epidermal growth factor (EGF) or IGF-I can also induce ER gene transcription

ii) non-genomic pathways: oestrogen can activate other signalling pathways including ERK1/2 signalling pathway via activation of voltage gated calcium channels causing calcium influx (Zhang et al., 2009)

iii) epigenetics: DNA methylation of oestrogen receptor 1 and PRB promoters have been associated with endometrial cancer (Campan et al., 2006)

iv) mutagenic pathways: unopposed oestrogen is associated with increased catechol oestrogen metabolites (4-hydroxylated oestrogen) which have been shown to induce DNA damage (Hayes et al., 1996).

Progesterone exerts its effects through the progesterone receptor (PR), a member of the superfamily of transcription factors. Progesterone receptor occurs in two isoforms – isoform A (PR-A) and B (PR-B) - which affect the target genes in different manners. The ratios of the different isoforms vary with development, hormonal changes and during carcinogenesis. Female mice with a null mutation of the gene encoding both PR isoforms resulted in a lack of both PR-A and PR-B. These mice exhibited impaired gonadotrophin regulation with anovulation, impaired sexual behaviour, and hyperplasia of the uterine epithelial tissue (both luminal and glandular) due to unopposed oestrogen action. The mammary gland showed normal ductal elongation but had impaired ductal branching morphogenesis and lobuloalveolar differentiation (Chappell et al., 1999; Lydon et al., 1995).

Progesterone works via three possible mechanisms: i) inhibition of ERα activation by increasing IGF1 binding protein and thus inactivates IGF1; ii) activation of PR action causing apoptosis of endometrial epithelial cells (Kurita et al., 2001); and iii) activation of transcription factor Hand2 which suppresses the production of fibroblast growth factors, the latter promotes the mitogenic effects of oestrogen (Li et al., 2011).

The physiological role of sex hormones on breast and endometrial tissue is well established. Strong evidence indicates that when the balance between oestrogen

and progesterone is dominated by oestrogen excess or unopposed oestrogen due to progestogen insufficiency, this can result in breast and endometrial cancer.

Expression of ER and PR have also been used in the classification systems of breast cancer. Around 75% of breast cancers are ER+ (De Santis, 2019), in which active ER signalling upregulate proliferation (Vuong et al., 2014). Breast cancer tumours are classified as ER+ if ≥1% of cells stain positive by immunohistochemistry while PR+ if ≥1% cells stain positive.

The endometrium exhibits dynamic expression pattern of sex hormones and their receptors as it passes through a proliferative and secretory phase in premenopausal women. Oestrogen binds to ER and promotes mucosal proliferation during the proliferative phase. After ovulation, progesterone secretion suppresses oestrogen-induced endometrial growth, changes endometrium into a receptive state for blastocyst implantation and prevents endometrial hyperplasia. Animal models investigating the role of oestrogen and progestogens on the endometrium showed that high oestrogen levels can lead to endometrial hyperplasia and endometrial cancer (Vollner et al., 2003). The endometrium still exhibits plasticity and is responsive to steroid hormones after menopause. Administration of oestrogen alone given over an extended period of time can increase risk of endometrial cancer in postmenopausal women (Yu et al., 2022).

1.4 Obesity – a key determinant factor of serum sex hormones level and the hormone dependent malignancies

1.4.1 The obesity pandemic

When energy intake is in excess of energy expenditure or there is little energy expenditure, weight gain occurs and body fat increase, leading to obesity. Until recently, obesity was perceived as being a problem of the individual with interventions for prevention and treatment of obesity focused on the individual level (Caballero, 2007). But the perception has changed, and obesity is recognized as a global pandemic (NCD Risk Factor Collaboration, 2016).

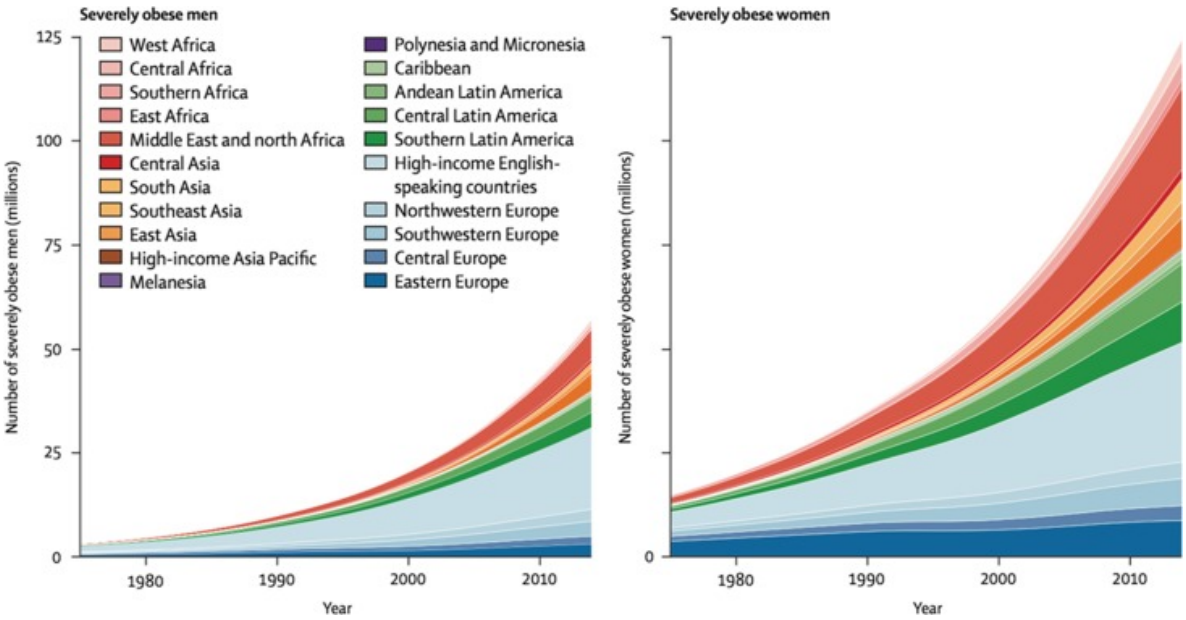


Figure 1.13 Prevalences of severe obesity in men and women in representative sample in 200 countries worldwide (NCD Risk Factor Collaboration, 2016)

Worldwide, the prevalence of overweight and obesity amongst adults and children combined has more than doubled between 1980 and 2013, from 857 million to 2.1 billion in 2013 (Figure 1.13) (Ng et al., 2014) accounting for around 30% of the world's population. Data from World Health Organization (WHO) /European

Region 2013 shows that over 50% of people are overweight or obese (WHO, 2019). It is predicted that if the global obesity trends continue with the same rising trends by 2025, 18% of men and more than 21% of women will be obese (NCD Risk Factor Collaboration, 2016).

Malta ranks at the forefront. Figure 1.14 shows how health issues in Malta compare to other EU countries. The closer the dot is to the centre, the better the country performs compared to other EU countries. The dots for obesity in adults and overweight/obesity in adolescents in Malta are furthest away from the centre, thus compared to other EU countries, Malta doesn't perform well as regards obesity. According to the WHO European Health Report 2018, using data from 2016, Malta ranks second place, with 28.9% of the adult population being overweight or obese. The situation is worse for adolescents aged 13 years - Malta has the highest percentage of overweight or obese adolescents in the EU, with a rate of around 35% (WHO, 2019).

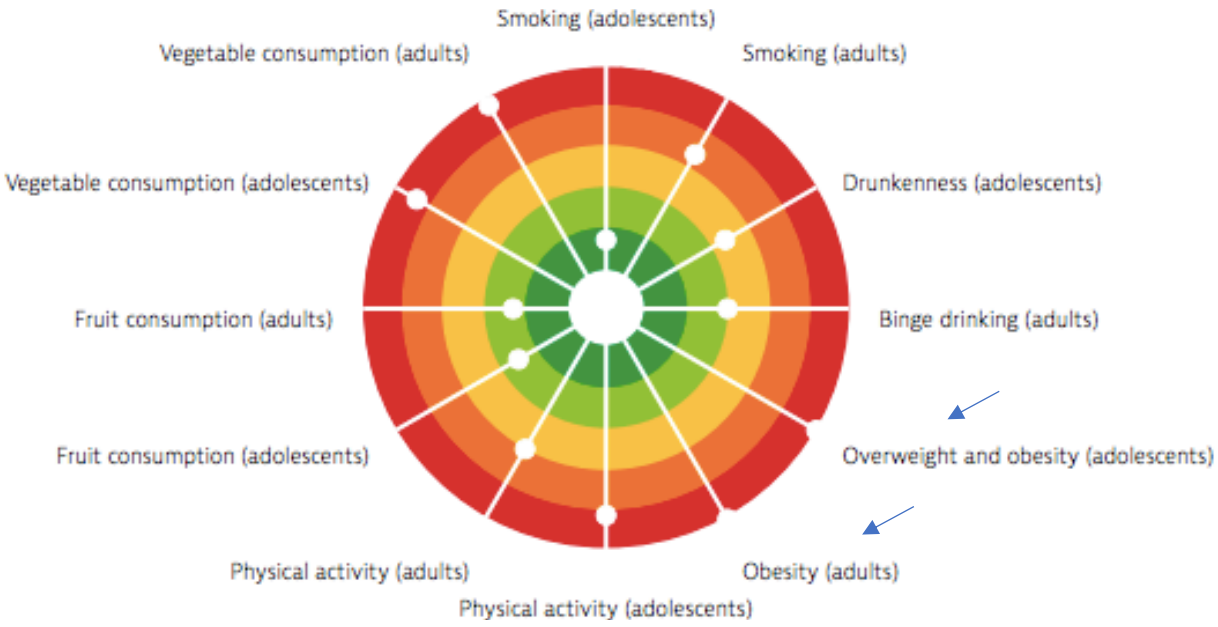


Figure 1.14. Obesity is a major health issue in Malta (State of Health in the EU · Malta · Country Health Profile 2021, OECD and WHO)

A measure of obesity is body mass index (BMI) which is weight (in kg) divided by the square of the height (in m^2) (Anderson et al., 2015). The BMI categories, as defined by the WHO, are overweight: BMI 25.00-29.99 kg/m^2 and obesity: 30.00 kg/m^2 or more. Obesity can be further classified in three levels of severity: class I, II and III (Table 1.1).

Table 1.1 The International Classification of underweight, overweight and obesity according to BMI (WHO, 2019)

Classification	BMI(kg/m^2)	
	Principal cut-off points	Additional cut-off points
Underweight	<18.50	<18.50
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	18.50 - 24.99	18.50 - 22.99
		23.00 - 24.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

Other parameters of obesity can also be measured: waist circumference to assess abdominal obesity and waist–hip ratio (waist circumference divided by hip circumference) to determine body fat distribution. The higher the waist-hip ratio, the more fat is found within the abdominal cavity and subcutaneously in the abdomen compared with the hips. The cut-off points associated with substantially increased risk of metabolic complications for waist circumference (women) is more than 88cm and waist-hip ratio (women) is ≥ 0.85 cm (WHO, 2000).

Obesity is a major risk factor for cardiovascular disease, hypertension, diabetes and certain cancers (WHO, 2018). The International Agency for Research into Cancer (IARC) and World Cancer Research Fund (WRCF) reports show that obesity is linked with increased risk of endometrial, postmenopausal breast, prostate, kidney, oesophageal and colorectal cancers. Adiposity is responsible for approximately 20% of all cancer cases (Wolin et al., 2010). A 5 kg/m^2 increase in BMI in women is associated with increased risk of endometrial cancer (relative risk 1.59, $p<0.0001$) and postmenopausal breast cancer (1.12, $p<0.0001$) (Renehan et al., 2008).

1.4.2 Genetic factors related to obesity and the insulin resistance

Although obesity is determined by environmental factors, like unhealthy diet and sedentary lifestyle, genetic predisposition also plays a role. Several studies have shown evidence that genetic factors predispose to overweight/obese phenotype. Up to the end of the last century, obesity related genes were identified through linkage studies in obese rodents caused by single gene mutation (monogenic obesity) and candidate-gene-based approaches in severely obese individuals.

In 1997 Montague et al and in 1998, Clement et al observed that homozygous mutations in adipocyte specific hormone, leptin and in human leptin receptor gene respectively resulted in morbidly obese phenotype early in life (Montague et al., 1997, Clement et al., 1998). Krude et al. showed that monogenic severe early-onset obesity was also associated with genetic defects within POMC gene (Krude et al., 1998).

Frameshift mutations in MC4R have been long implicated in monogenic forms of obesity in humans (Vaisee et al., 1998). MC4R is a G protein-coupled receptor found in regions of the nervous system (Horstmann et al., 2013) and forms part of the leptin system. When the body is in a negative energy state, leptin levels decrease leading to lower pro-opiomelanocortin (POMC) and α melanonocyte-stimulating hormone levels. This stimulates agouti-related peptide expression which results in repression of MC4R and increased food intake, which can lead to obesity (Horstmann et al., 2013). More recently, multiple SNPs near the melanocortin-4 receptor, MC4R (Park et al., 2016) (rs17782313, rs571312, rs17700144, and rs2331841) were found to be strongly associated with obesity. Mutations in MC4R are a common cause of obesity and the most common cause of monogenic obesity (Iepsen et al., 2018) .

With the advent of genome-wide association scans (GWAS), obesity susceptibility loci have been identified (Figure 1.15).

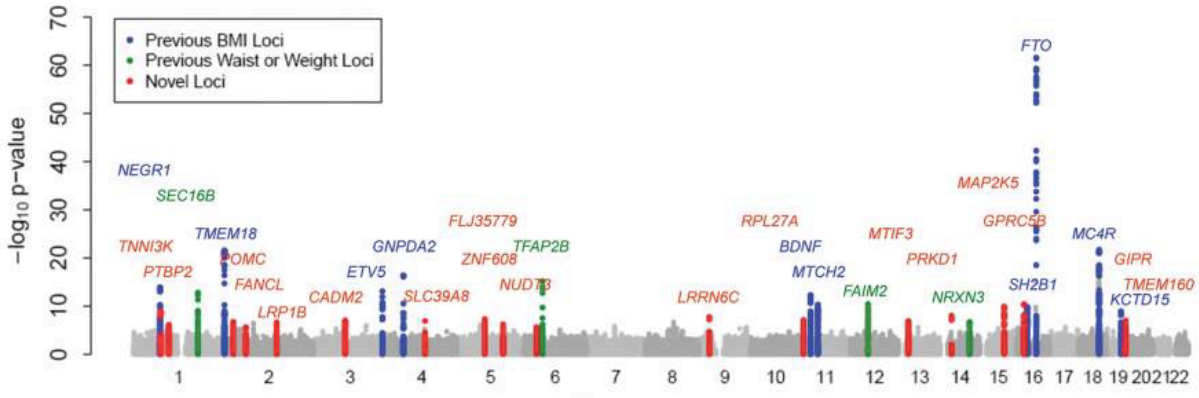


Figure 1.15 Association between SNPs and BMI meta-analysis (Speliotes et al., 2010)

In 2007, the first GWAS obesity-susceptibility gene, fat mass and obesity associated gene (FTO) was identified. It is located on chromosome 16q12.2 (Scuteri et al., 2007) and encodes a 505 amino acid protein. In its promoter region, there is a forkhead

member Foxa2 response element which is a transcription factor for genes responsible for glucose and lipid metabolism (von Meyenn et al., 2013). People carrying FTO single nucleotide polymorphisms (SNPs) have been associated with adipogenesis as well as tumorigenesis (Deng et al., 2018). A GWAS metanalysis on 249,796 adults showed a 0.39 kgm^{-2} change in BMI per FTO effected allele (P-value<10) (Speliotes et al., 2010).

Another obesity-associated gene is the transmembrane protein 18 (*TMEM18*). It comprises of an intergenic region of chromosome 2 (2p25.3) and is a four transmembrane protein that acts within the central nervous system. Germline loss of *TMEM18* expression in male mice caused an increased fat and lean mass, with the resultant increase in body weight. When *TMEM18*-deficient mice were fed a 45% fat diet, they consumed significantly more as compared to wild-type littermates. They also showed increased energy expenditure which partially compensated for the increased food intake, resulting in only a modest increase in body weight (Larder et al., 2017).

Further genetic mutations are associated with the metabolic syndrome. Withers concluded that deletion of insulin receptor substrate1 (IRS1) in mice produced a mild metabolic phenotype associated with compensated insulin resistance (Withers, 2001). IRS proteins are proteins that mediate the intracellular effects of insulin and insulin-like growth factor 1 (IGF1). IRS1 is key in glucose transport in muscle and adipose tissue as well as being key in insulin signalling and metabolism in the liver (Thirone et al., 2006). Polymorphisms of melatonin receptor 1B, a member of the G-protein-coupled receptor superfamily involved in insulin

secretion, was observed to be associated with increased risk of impaired glucose regulation and type 2 diabetes (Qing Xia et al., 2012).

Another SNP which was also found to be associated with type 2 diabetes is adenylate cyclase 5 (ADCY5) gene. It encodes adenylyl cyclase 5, which mediates G-protein receptor activity through synthesis of second messenger cyclic AMP. The latter is itself a second messenger molecule involved in insulin secretion from the pancreatic βcells (Roman et al., 2017).

Polymorphisms of genes coding for inflammatory markers have also been linked to obesity susceptibility. Ibrahim et al. (2017) showed that the interleukin-6 gene polymorphism 174G/C was found in Egyptian children with higher BMI (Ibrahim et al., 2017), while Ramiez-Lopez (2013) observed association between interleukin-6 polymorphisms and obesity, hyperglycaemia, and low HDL cholesterol in Mexican adolescents (Ramírez-López et al., 2013). Association between interleukin-1α polymorphisms and obese healthy Korean women was also demonstrated (Ellulu et al., 2017).

Pace et al (2013) looked at the genomics of type 2 diabetes in the Maltese population, claiming that GWAS-identified SNPs explain around 10% of the heritability of type 2 DM in Malta. A variant in the beta-2 adrenergic receptor showed high odds ratio for the development of Type 2 DM. Their studies also found an association between SNPs in the melatonin-receptor 1 beta gene (MTNR1β) and HOMA-IR and an association between SNPs in IGF-1 and body weight (Pace et al., 2013).

International collaborative efforts produced large meta-analyses highlighting the implicated loci for increased risk of obesity (as measure by BMI), central obesity (as measured by WHRadjBMI), body fat %, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). These genetic associations are represented in Figure 1.16.

It's important to note that the closest gene is often used to denote the locus involved in causing the effect. Examination of identified loci interestingly show common variants in or near genetic loci that were previously found to be affected in monogenic obesity studies. Severe rare mutations will result in early onset monogenic obesity, whereas common variants of these genes will have subtle effects on gene function or expression but can influence population BMI (Fall et al., 2017).

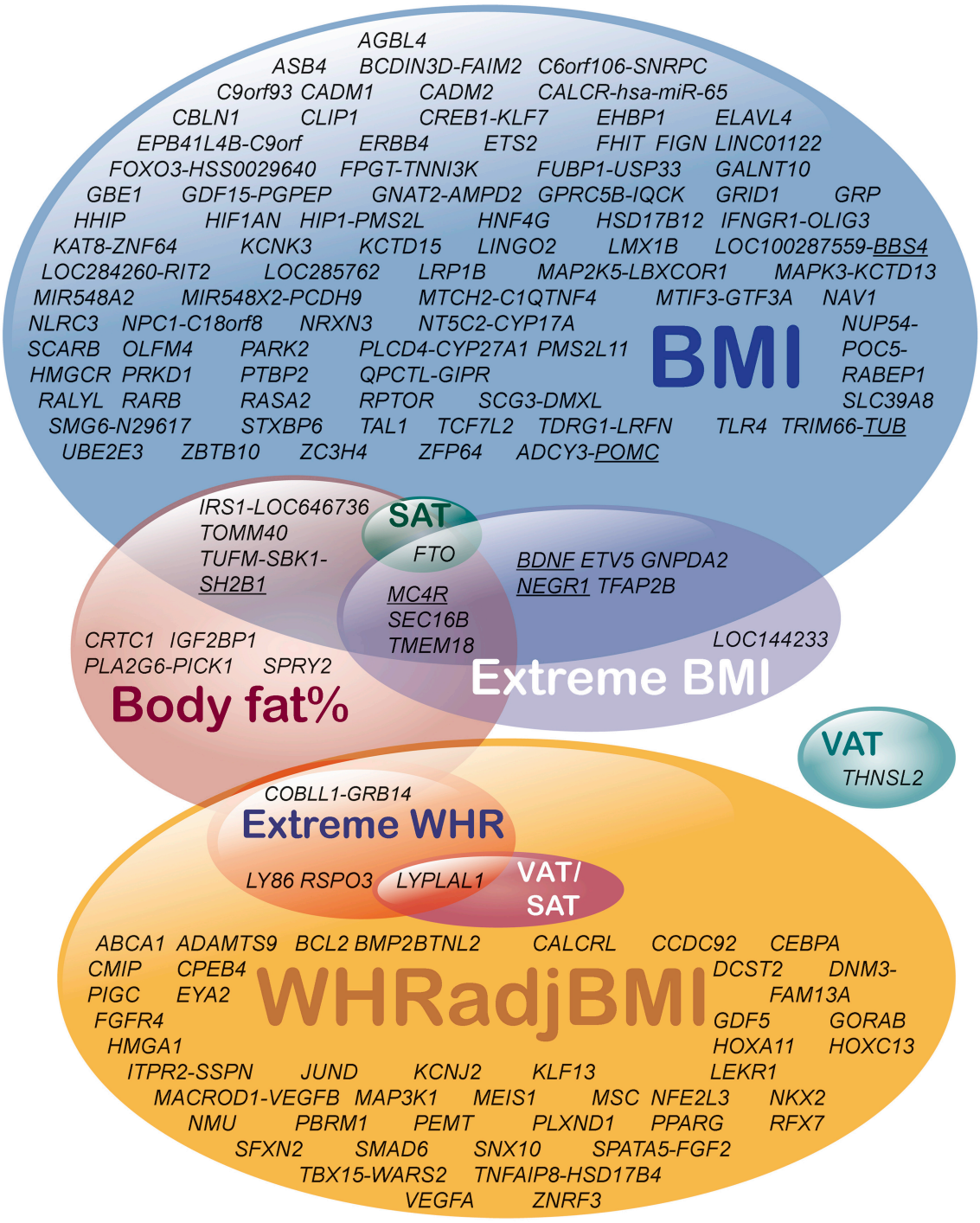


Figure 1.16 Venn diagram for loci associated with BMI – 97 loci (Locke et al., 2015), body fat percentage – 12 loci (Lu et al., 2016), waist-hip-ratio adjusted for BMI – 49 loci (Shungin et al., 2015), VAT, SAT, and their ratio (VAT/SAT)- 3 loci (Fox et al., 2012) and extremes of body mass index and waist-hip-ratio 4 loci and 7 loci (Berndt et al., 2013). Genes implicated in monogenic obesity are underlined. (Fall et al., 2017)

In a GWAS meta-analysis, the interaction between obesity and other lifestyle factors, including physical inactivity and high-fat diet were tested with genetic variants and insulin resistance phenotypes (hyperglycaemia, hyperinsulinemia and Homeostatic Model Assessment of Insulin Resistance, HOMA-IR). This GWAS meta-analysis of gene-environmental interaction included 11,794 postmenopausal women and 18,717,781 common autosomal SNPs. 58 loci reached genome-wide significance (Figure 1.17).

For hyperglycaemia, 5 SNPs in the following regions were detected: 3 were detected near G6PC2 (rs13431652) in the overall and high fat diet groups; rs117911989 in the region of MKLN1 and rs7273292 in the region of NKX2-2 in the active (metabolic equivalents $\geq$ 10) group.

For hyperinsulinemia, 39 SNPs were found, 34 of which were located in the intergenic region of MTRR/LOC729506 (rs 13188458 as index SNP) in the inactive group (metabolic equivalents <10). In relation to interaction with BMI, 4SNPs in the region of NR5A2 were identified (3 of them rs10919774 as index SNP had genome-wide significance in the obese, BMI $\geq$30 group). By interacting to high fat diet, 1 novel SNP (rs6683451) was identified within PLA2G4A in an intronic region of LINC01036 in the low-fat diet group (Jung et al., 2019).

For high level of HOMA-IR, 14 SNPs were identified having genome-wide significant association. By interacting with a high fat diet, 7 SNPs were revealed in the region of PABPC1P2 (rs7772624 as index SNP) in the overall and high-fat diet groups.

Furthermore, 5SNPs (with rs13277245 as index SNP) were identified in the region of MSC and 1SNP in the region of DOCK1 in the low-fat diet group (Jung et al., 2019).

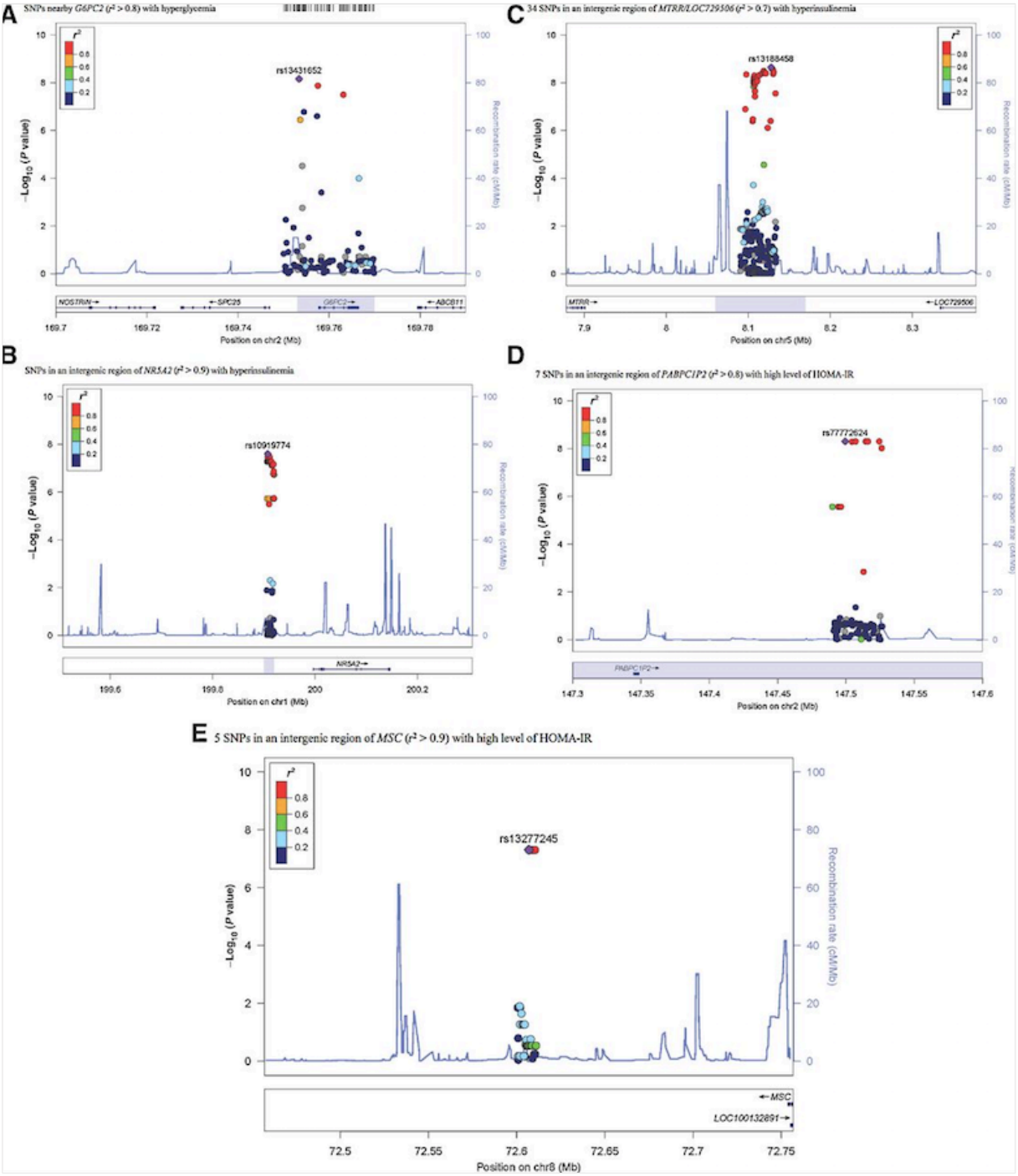
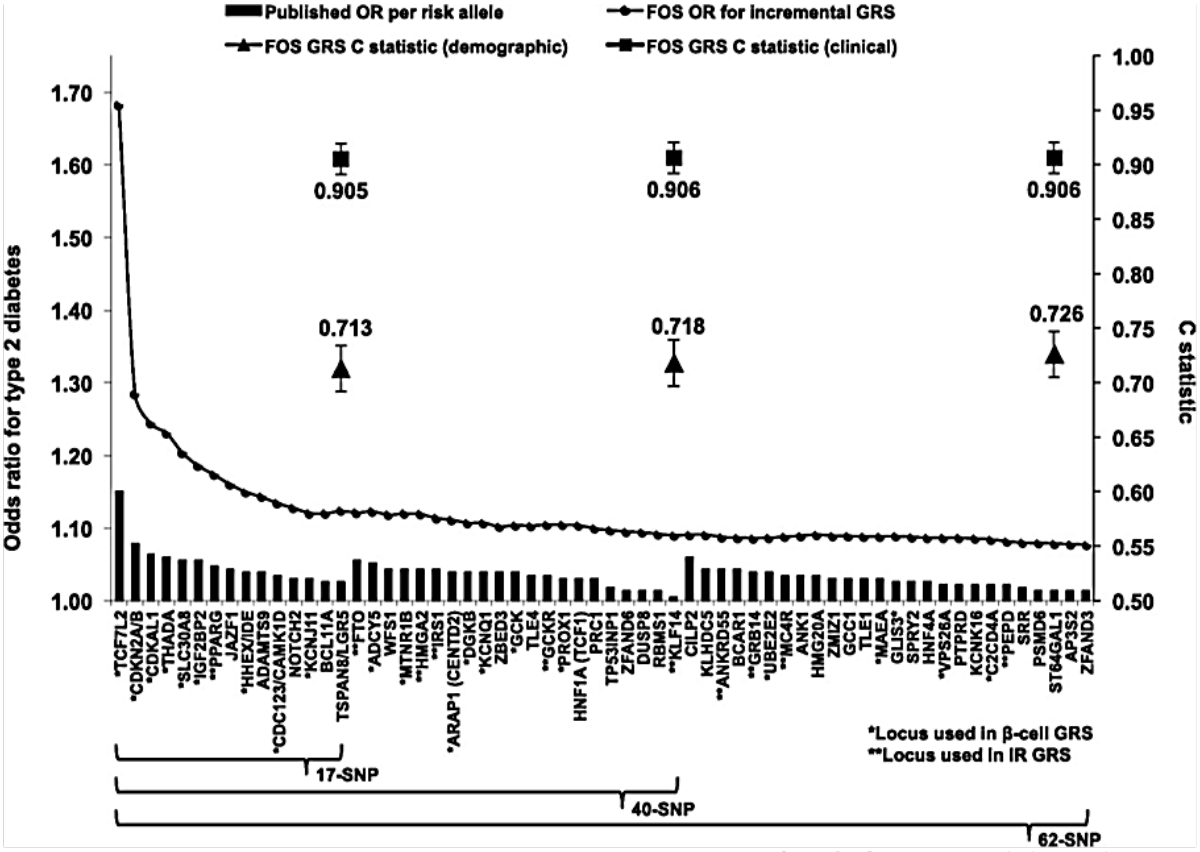


Figure 1.17 Regional SNP association plots (linkage disequilibrium, LD (r^2) is shown by colour intensity gradient) (Jung et al., 2019)

In a GWAS analysis by Lotta LA et al., which investigated a population-based sample of 188,577 individuals, 53 independent genomic regions were found in association with a triad of insulin resistance phenotypes - fasting insulin levels (adjusted for BMI), higher serum triglycerides level and lower HDL cholesterol level. These included 10 loci which had previously been implicated in insulin resistance and 25 loci which were previously associated with triglycerides or HDL cholesterol (Lotta et al., 2017).

The DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium v3 identified 65 independent type 2 diabetes loci (Morris et al., 2012). Vassy et al. used data from Framingham Offspring (FOS) to differentiate the genotype risk score (GRS) of insulin resistance and GRS for B-cell in type 2 diabetic adults (Vassy et al., 2014). Below is a summary of the identified genetic loci (Figure 1.18)



Loci on the x-axis are ordered by inclusion in published 17-, 40- and 62-SNP GRS. Black bars (left y-axis) indicate published DIAGRAMv3 odds ratio (OR) for T2D per risk allele at each locus. The black line plots the T2D OR in the FOS per allele increase in a GRS containing the loci up to that point on the x-axis. Points with error bars plot the C statistics (95% CI) from pooled logistic regression models for T2D in FOS including 17-, 40-, and 62-SNP GRS in demographic and clinical models. Loci used in separate β-cell and IR GRS in the present analyses are also indicated.

Figure 1.18 Genetic loci associated with Type2 diabetes (Vassy et al., 2014)

Recent multi-ethnic GWAS studies involved participation of multi-ethnic of subjects from European, African, Hispanic, Native Hawaiian and Asian populations in the GWAS Population Architecture using Genomins and Epidemiology (PAGE) Study and DIAGRAM Consortia. A multi-ethnic risk variant, rs13052926 in BACE2 gene was identified. They however observed significant difference in the results of a multi-ethnic GRS across populations, underscoring the importance of risk characterization and genetic-risk prediction which is population-specific (Polfus et al., 2021, Downie et al., 2022).

1.4.3 Mechanisms linking obesity to cancer

Different mechanisms have been proposed linking obesity to carcinogenesis. The proposed pathways include (Figure 1.19):

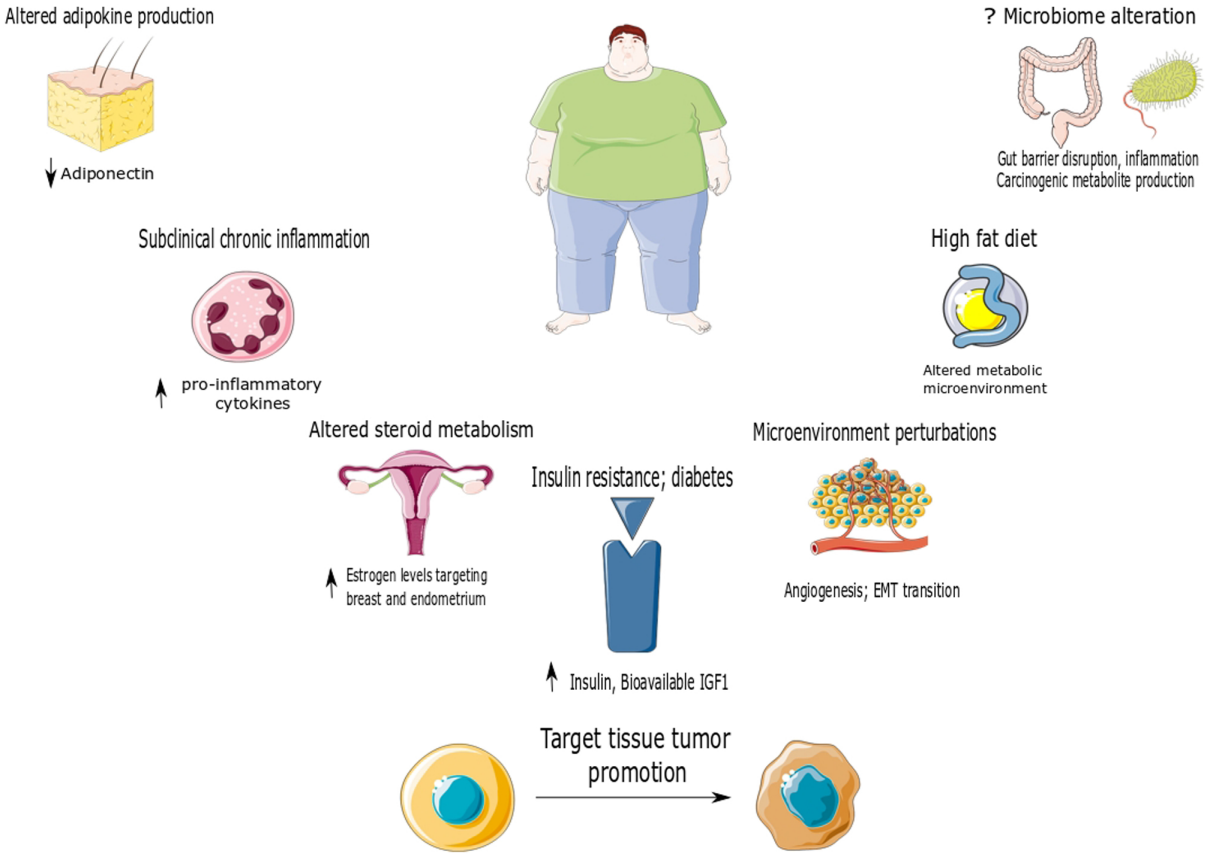


Figure 1.19 Obesity is associated with multiple systemic changes including increased insulin, insulin-like growth factor (IGF), interleukin-6 (IL-6), leptin and decreased level of adiponectin (Avgerinos et al., 2019)

A carcinogenesis mechanism that is specific to hormone dependent malignancies, including breast and endometrial cancer is through obesity effects on sex hormones biosynthesis. Obesity poses a great influence on the reproductive axis and sex hormones. Energy intake stimulates secretion of insulin from the pancreas (Templeman et al., 2017) which results in decreased SHBG production by the liver.

This in turn leads to increased testosterone production by the theca cells of the ovaries (Bergh et al., 1993), and a higher functional androgen level.

Apart from their key roles in the development and maintenance of human reproduction and libido, androgens exert effects on two metabolic tissues – the skeletal and adipose tissues. In skeletal muscle, androgens promote muscle growth, enhance insulin sensitivity, glucose utilization and lipid oxidation (Schiffer et al., 2017). In women, androgen excess inhibits preadipocyte differentiation, impairing adipose tissue hyperplasia/adipogenesis. This may result in adipocyte hypertrophy and adipose tissue dysfunction characterized by hypoxia, apoptosis and decreased insulin sensitivity (Klöting & Blüher, 2014). Excess androgens correlate with increased adiposity and visceral fat. (Schiffer et al., 2019)

Peripheral adipose tissue is also responsible for aromatization of androgens and androgen precursors to oestrogens. Increase in adipose tissue leads to increase in peripheral aromatization of androgens leading to increased circulating oestrogen concentrations. Hetemaki et al measured the oestrone and oestradiol concentrations in visceral and abdominal adipose tissue in postmenopausal women. Oestradiol is more effectively produced from oestrone in subcutaneous adipose tissue as compared to visceral adipose tissue. However, oestrone levels were higher in visceral adipose tissue rather than subcutaneous adipose tissue. Oestrone level showed a positive correlation with body mass index and its conversion to oestradiol in visceral adipose tissue increased with waist circumference (Hetemäki et al., 2017). The resultant hyperoestrogenic state in obese women increases the risk of both postmenopausal breast cancer and endometrial cancer (Figure 1.20).

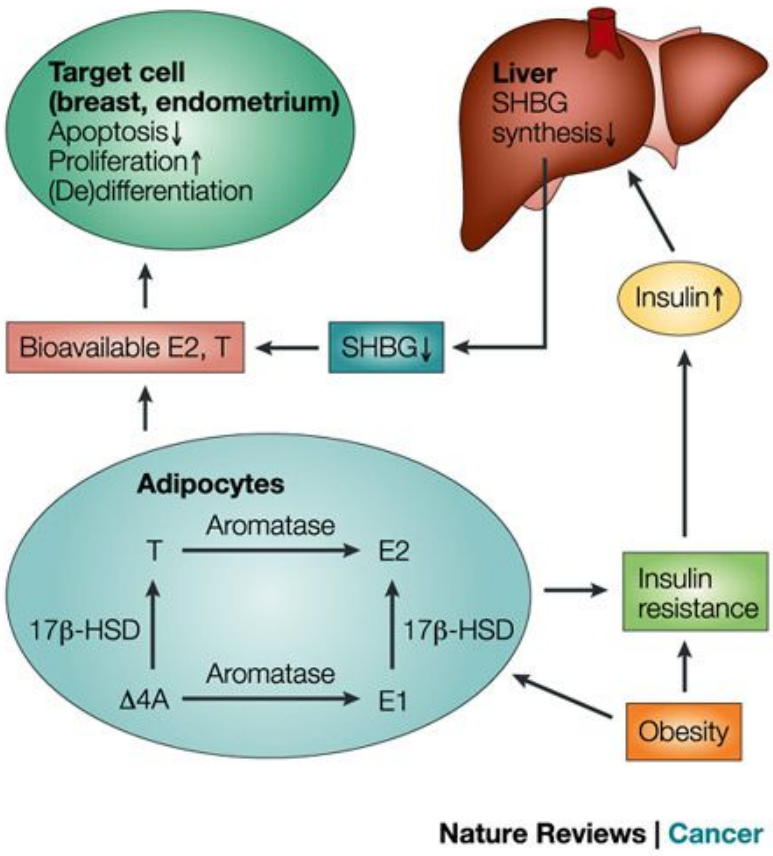


Figure 1.20 Sex hormone biosynthesis pathway linking obesity to hormone dependent malignancies, breast and endometrial cancer (Calle & Kaaks, 2004)

Other mechanisms by which obesity can induce cancer include:

1. *Hyperinsulinemia/insulin resistance and increased insulin-like growth factor 1 (IGF-1):* Obesity is associated with insulin resistance, a condition that arises when the energy storage pathways are exposed to chronic surplus. Accumulation of lipid in the liver and skeletal muscle impairs insulin signalling which leads to decreased glucose uptake by the muscle, decreased glycogen synthesis in the liver (Samuel & Shulman, 2016) and increased pancreatic secretion of insulin.

The resultant hyperinsulinemic state stimulates production of IGF-1 in the liver by increasing growth hormone receptors; growth hormone being the main hormone that is responsible for IGF-1 production in the liver. Hyperinsulinemia also decreases hepatic secretion of IGF-binding protein-1 and 2, thus increasing IGF-1 bioavailability. Insulin and IGFs promotes cell cycle progression and tissue growth while inhibiting apoptosis (Uzunlulu et al., 2016).

Acromegaly, a disorder characterized by hypersecretion of growth hormone and increased serum IGF-1, is associated with increased risk for cancer, especially colorectal cancer (Dworakowska & Grossman, 2019). Epidemiological studies also show that increased level of IGF-1 and/or alteration in their binding proteins correlate with increased risk of malignancy while patients with genetic defects linked with GH resistance/deficiency have some protection against tumour growth (Boguszewski & Boguszewski, 2019).

1) *Subclinical chronic inflammation and oxidative stress:* Adipose tissue secretes adipocytokines that are involved in energy metabolism and in inflammatory response, including leptin and adiponectin. Leptin level directly correlates with body fat mass and signals appetite inhibition and energy expenditure. Moreover, leptin stimulates the release of inflammatory factors including interleukin (IL)-1, IL-6, IL-12, tumour necrosis factor (TNF)α, leukotriene B4, cyclooxygenase 2 and reactive oxygen species (ROS) release from the neutrophils. It also promotes T cell proliferation while suppressing

regulatory T cells. Adiponectin acts synergic to leptin and its level inversely correlate with visceral obesity. It has insulin-sensitizing and anti-inflammatory functions (Ellulu et al., 2017). It secretes anti-inflammatory cytokines including IL-10, interleukin 1 receptor antagonist (IL-1RA) while suppresses release of TNFα and interferon (Carbone et al., 2012). Obesity, characterized by excess macronutrients found in adipose tissue and the associated high leptin levels and hypoadiponectinaemia is thus a pro-inflammatory state. In response to this low-grade inflammatory state, macrophages are recruited to obese white adipose tissue. Chemokines including CCL2, CCL3 and RANTES/CCL5 are secreted by adipose tissue macrophages which further recruit macrophages into adipose tissue (Kolb et al., 2016).

Obesity-associated inflammation is linked to carcinogenesis of various cancers including, breast, colon, liver and endometrial (Kolb et al., 2016; Deng et al., 2016; Iyengar et al., 2016). Metanalysis have linked low adiponectin with breast cancer development (Gu et al., 2018) while high serum adiponectin was associated with decreased endometrial cancer risk (Zeng et al., 2015). Local ectopic fat deposition including breast, intrahepatic/intrapancreatic adipose tissue is associated with an increased inflammatory response and hypoadiponectinaemia promoting tumour growth and progression (Avgerinos et al., 2019).

2) *Changes in microenvironment* – vascular, epithelial-mesenchymal transition, endoplasmic reticulum stress: The increase in adipose tissue volume in

obesity is followed by the angiogenic switch - expansion of the vascular bed through the formation of new blood vessels from existing vessels. If the angiogenesis does not happen in proportion to adipocyte tissue volume, hypoxia develops and the transcriptional complex hypoxia-inducible factor 1 (HIF-1) is upregulated. Signalling factors involved in angiogenesis are activated, including vascular endothelial growth factor (VEGF) isoforms, angiopoietins 1 and 2, fibroblast growth factor (FGF), TNFβ, IL-6 and IL-8. This microenvironment resembles that observed in tumorigenesis (Cozzo et al., 2017). The low-grade inflammation associated with obesity also promotes the release of pro-angiogenic factors from tumour-associated immune cells (Albini & Noonan, 2012).

The microenvironment changes present in obesity lead to epithelial-mesenchymal transition (EMT), whereby epithelial cells differentiate into the mesenchymal phenotype. EMT is normal in embryo development and causes loosening of cell-to-cell adherence increasing cell migration. EMT programs in cancer cells however facilitate migration, invasion, apoptosis resistance and can also stimulate further proinflammatory signals (Suarez-Carmona et al., 2017).

3) *Dietary nutrients:* Diet has been implicated in both causation of cancer and as therapy/prevention. Various food elements or using high temperatures/grilling/barbecue can be associated with increased cancer risk. High fat diets increase free fatty acids which induce ROS formation. ROS oxidize proteins, increasing the number of unfolded proteins in the

endoplasmic reticulum and eliciting an inflammatory response and a favourable metabolic environment for carcinogenesis. On the other hand, adopting a plant-based diet, low in red and processed meats, limiting simple sugars, carbohydrates and alcohol reduces cancer risk (Bail et al., 2016; Mentella et al., 2019).

4) *Alteration of intestinal microbiome:* Obesity, microbiota and cancer can be linked by two mechanisms – inflammation and production of carcinogenic substances. The intestinal microbiota in obese individuals is different from that of lean individuals. This is supported by studies that show that obese mice (both genetically obese and diet-induced) have a relative decrease of *Bacteroidetes* and an increase in *Firmicutes* compared to lean mice - a gut microbiome with a higher capacity to harvest energy from food. When germ free mice were colonized with 'obese microbiota', they showed a higher increase in body fat when compared to mice colonized with 'lean microbiota' (Turnbaugh et al., 2006; Sanmiguel et al., 2015). The difference in weight gain is due to the bacterial fermentation of indigestible carbohydrates and production of short-chain fatty acids, the main producers of which are the *Firmicutes* (Raybould, 2012).

Cani et al. further demonstrated that ingestion of high fat diet is associated with shifts in gut microbiome, intestinal mucosal inflammation, changes in gut permeability and increase in serum lipopolysaccharide. The increase in lipopolysaccharides results in 'metabolic endotoxemia' (Cani et al., 2007) with resultant upregulation of cytokines such as IL-6, TNF-a, IL-17 and IL-23,

which promotes carcinogenesis (Kolb et al., 2016) as well as activates Toll-like receptor 4 on vagal nerve endings in the lamina propria resulting in hyperphagia and obesity (Raybould, 2012).

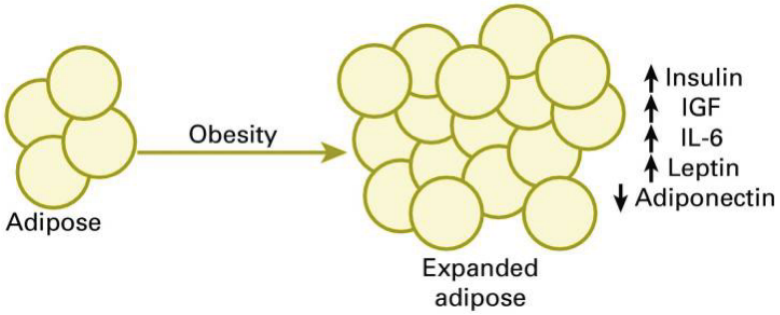


Figure 1.21 Effects of obesity on systemic haemostasis (Hopkins et al., 2016)

The metabolic changes associated with obesity (Figure 1.21) are linked with increased risk of neoplastic transformation through altered intracellular and systemic signalling pathways (Figure 1.22).

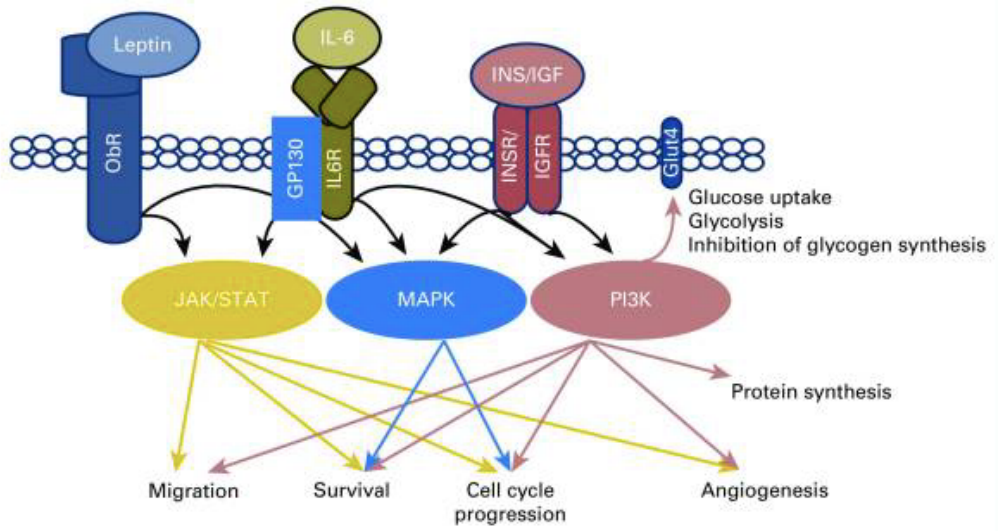


Figure 1.22 Signalling pathways altered in the obese state that drives cell growth and oncogenic transformation (Hopkins et al., 2016)

Abbreviations: insulin (INS), Janus kinase (JAK)/signal transducers and activators of transcription (STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) Glut4, glucose transporter type 4; GP130, glycoprotein 130; IGFR, insulin-like growth factor receptor; IL6R, interleukin 6 receptor; INSR, insulin receptor; ObR, leptin receptor.

Chapter 2

Literature Review

2.1 Breast Cancer

2.1.1 Breast Cancer – mammary gland development and histology

The human breast is composed of a ductolobular system with epithelial columnar cells resting on a basement membrane and surrounded by stroma. There are two types of stroma: the interlobular stroma which surrounds large ducts and terminal duct lobular units (TDLUs) and the intralobular stroma surrounding the acini within the TDLU, the latter being hormone responsive (Figures 2.1, 2.2).

Development of breast tissue starts around the 4-6 weeks of gestation from mammary-specific progenitor cells which eventually form two ridges between the foetal axilla and inguinal region – the mammary crests. The primary mammary buds arise from paired epithelial masses in the mammary crest. These differentiate further into a well-defined mammary bud which penetrates the upper dermis. From the main mammary bud develop the secondary epithelial buds which eventually coalesce to form the lactiferous ducts. In the third trimester, further branching occurs and canalization of the secondary epithelial buds. Around 15-20 lobes are formed by term which undergo maturation from birth to two years of age. Then the gland remains quiescent till puberty (Moore et al., 2013; Javed & Lteif, 2013).

Breast development then continues at puberty under the influence of the pubertal surge of oestrogen in girls. This surge is dependent on the pituitary growth hormone and its production of insulin-like growth factor-1 in the breast tissue (Christopoulos et al., 2015). This usually happens between 8 ½ and 13 ½ years and the breast

changes that eventually ensue are best described by Tanner stages (Marshall & Tanner, 1969). At a cellular level, ductal elongation and lobular development occur forming the TDLU – collection of acini with the surrounding loose intra-lobular stroma (Javed & Lteif, 2013). Surrounding the TDLU forming a thick layer is the dense collagenous interlobular stroma which separates the TDLU from the subcutaneous adipose tissue. Breast adipose tissue ranges from 7% to 56% of the adult breast volume (Vandeweyer & Hertens, 2002).

The breast tissue changes with the menstrual cycle, developing larger lobules, more basal cell proliferation and vacuolization and stromal oedema (breast fullness) in the secretory phase. The lobules increase further in size and number if pregnancy occurs, and the myoepithelial cells become attenuated in the breast lobules. After delivery of the placenta, human placental lactogen falls and the high levels of prolactin secreted lactotrophs in the anterior pituitary gland initiate milk production. Oxytocin, released by crying or sucking from the posterior pituitary, causes contraction of the myoepithelial cells and milk expulsion.

As opposed to the pregnancy state where breast tissue is fully mature and functional, the menopausal state is characterized by atrophy. With the lack of oestrogen and progesterone brought about by ovarian failure, there is progressive involution of the breast stroma and atrophy of TDLU and acini resulting in small atrophic lobules surrounded by fat (Lategan, 2019).

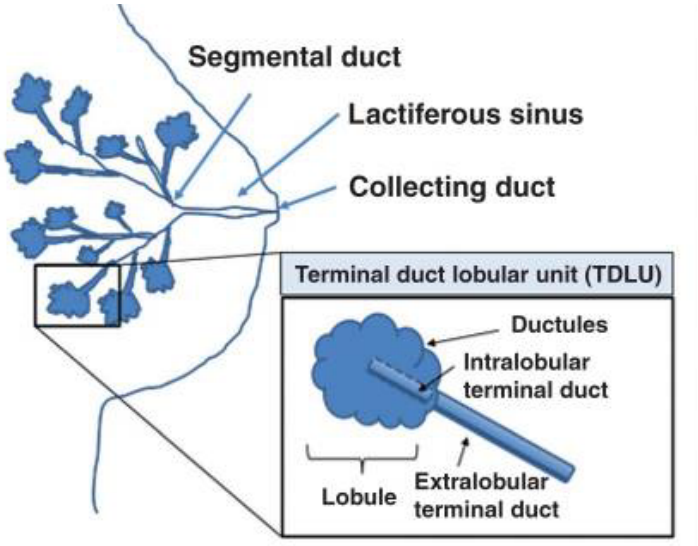


Figure 2.1 Anatomical structure of human mammary gland (Cozzo et al., 2017)

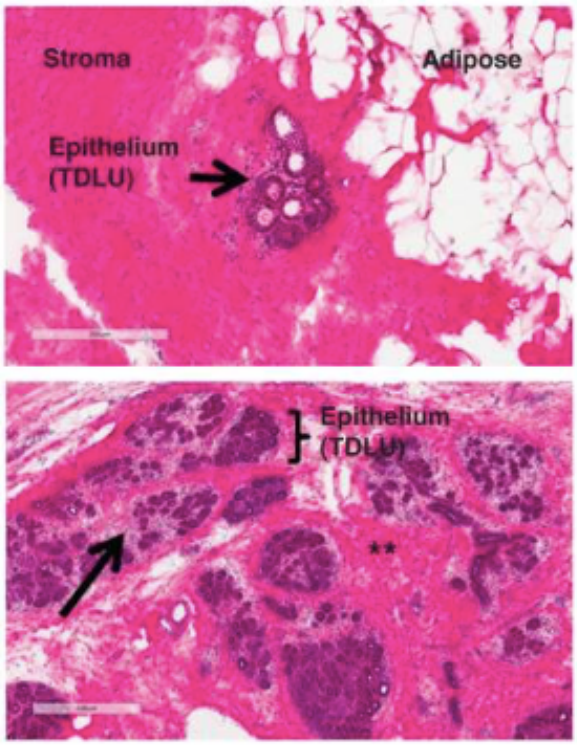


Figure 2.2 Histology of the human mammary gland (H&E stained). Arrowhead refers to loose intra-lobular stroma and asterisks refer to dense interlobular stroma. (Cozzo et al., 2017)

The most common type of breast cancer is breast carcinoma, i.e., arising from the epithelial cells that line the TDLU. Breast carcinoma most frequently arise from the

ductal epithelial cells generating a precancerous lesion called ductal carcinoma in situ which subsequently invades the mammary stroma forming invasive ductal carcinoma. Around 80% of breast carcinomas are invasive ductal carcinomas while 5-15% are invasive lobular carcinomas (Makki, 2015). The latter arise from the lobules as lobular carcinoma in-situ which progress to invasive lobular carcinoma. All invasive breast carcinomas are graded; the most widely used system is based on that developed by Bloom and Richarson (Bloom et al., 1957) and modified by Elston and Ellis. Histological grade has strong correlation with prognosis, with grade I tumours showing better survival rates than those with grade II and III ($P<0.0001$) (Elston et al., 1991). Tumour size, lymph node stage and grading aid stratification of patients for appropriate treatment.

As already discussed, breast cancer can also be subclassified according to the tissue ER and PR status. 15% - 20% breast cancer tumours secrete higher levels of human epidermal growth factor receptor 2 (HER2), a protein that drives cellular proliferation and migration. Breast cancer tumour cells which exhibit over-expression of HER2 are referred to as HER2 positive tumours. Triple positive tumours (ER+, PR+ and HER2+) are most responsive for hormonal therapy that lower oestrogen or block oestrogen receptors and treatment that targets HER2 protein.

Ki67is a nuclear antigen expressed in the active phases of the cell cycle; and thus, a marker of cell proliferation. It is overexpressed in hyperplastic breast lobular units. It can be also be used as a prognostic marker and in prediction of therapeutic response. (Zhang et al, 2021)

2.1.2 Breast cancer clinical issues and treatment

Breast cancer commonly presents with a painless lump. Other symptoms include alteration in size/shape of the breast, dimpling/redness/pitting of the skin, change in nipple appearance/in areola or abnormal nipple discharge. Advanced disease can present with ulcerations or symptoms of metastases such as bone pains/headaches. Imaging with mammography, ultrasound +/- MRI will help identify and characterise any lesion. A through cut biopsy will rule out/confirm malignancy. Once breast cancer is confirmed, imaging is further carried out to assess staging.

Surgery is usually the first treatment modality for breast cancer, as lumpectomy or mastectomy, with sentinel lymph node biopsy or axillary lymph node dissection. Surgical excision of breast lump was first proposed by Galen (Lukong, 2017). However, the first radical mastectomy for breast cancer treatment was done by William Halsted, in 1882, at John Hopkins Hospital, Baltimore USA. In 1970s the concept of the two-step approach in management of breast cancer was proposed – where a biopsy is followed by surgery later as necessary (Lerner, 2001) . In 1930s radiotherapy was introduced as an alternative to radical mastectomy (Lukong, 2017). Radiotherapy still has a role in nowadays treatment including as adjuvant after breast-conserving surgery or after mastectomy where there is a high risk of local recurrence (ESMO, 2022).

Adjuvant systemic treatment has further improved breast cancer survival rates. Treatment modalities for breast cancer have increasingly become more targeted/precise. Different treatment approaches have been used according to the oestrogen receptor/progesterone receptor or HER2 status. Endocrine treatment

modalities for ER positive breast tumours are categorized according to their mode of action (Figure 2.3).

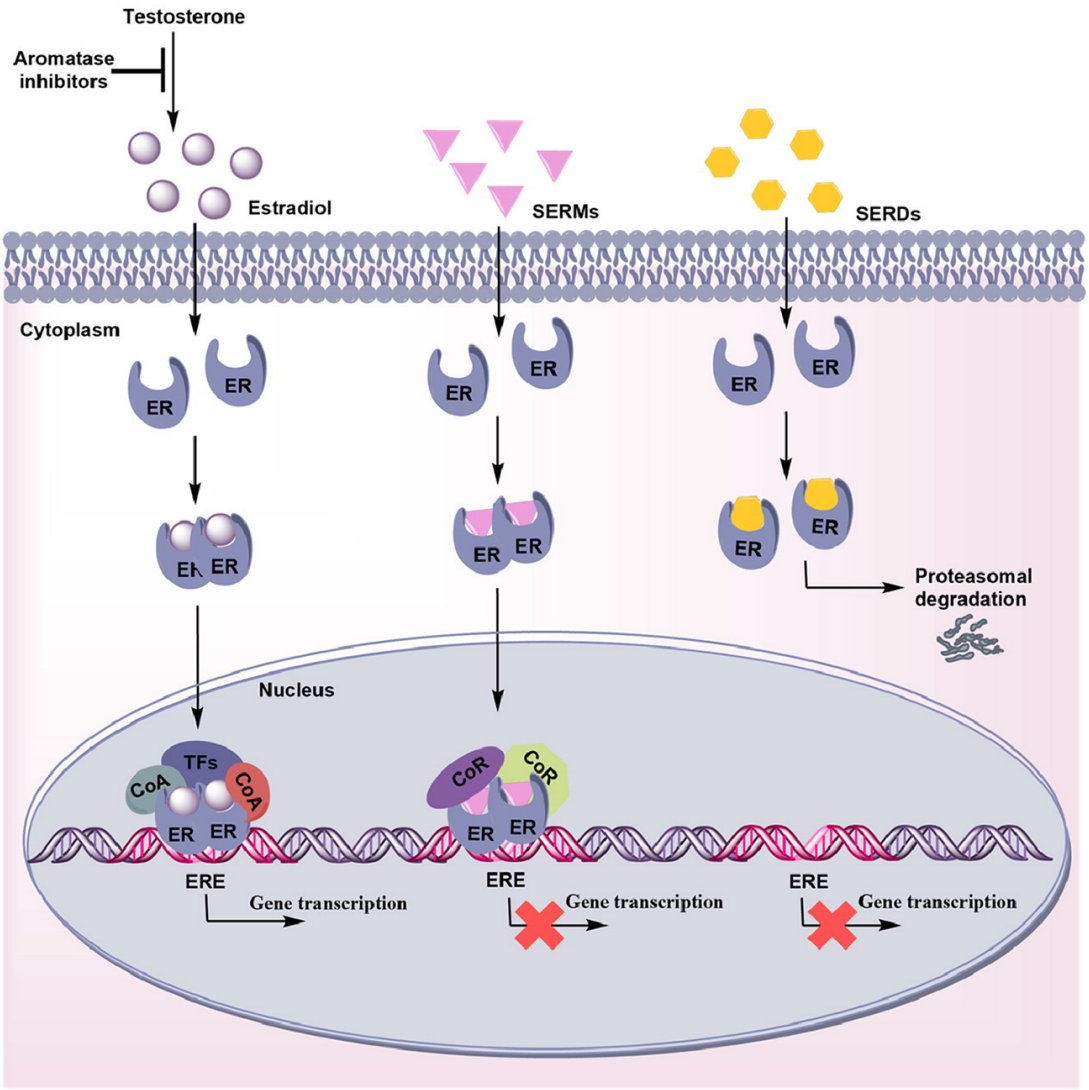


Figure 2.3 The different modes of action of endocrine therapies on the oestrogen receptor pathway. Aromatase inhibitors inhibit conversion of testosterone into oestradiol, decreasing synthesis of oestradiol and preventing ER signaling; SERMs and SERDs prevent ER signaling by binding to ER forming an inactive complex and by causing ER degradation respectively (Patel and Bihani, 2018).

Selective ER modulators (SERMs) and selective ER down-regulators (SERDs) act on ER reducing signalling pathways – SERMs compete directly with oestrogen binding on ER while SERDs bind to ER and cause ER degradation. Aromatase inhibitors and

gonadotropin-releasing hormone (GnRH) agonists decease the production of endogenous oestrogen; aromatase inhibitor inhibits conversion of testosterone to oestrogen while GnRH agonists downregulate pituitary GnRh receptors, causing suppression of LH and FSH, thereby reducing production of oestradiol in the ovaries (Patel et al., 2018).

Tamoxifen was the first SERM treatment approved in 1977. It is indicated for treatment of metastatic breast cancer in ER positive breast cancer patients, to reduce the incidence of contralateral breast cancer and in high risk patients to reduce the risk of breast cancer (Cohen et al., 2001; Fisher, Bernard et al., 1998). Fulvestrant, approved in 2002, is a SERD, used as a second-line treatment for oestrogen receptor positive metastatic breast cancer in postmenopausal women (Morris & Wakeling, 2002) . In 2005, the first aromatase inhibitor, Letrozole was approved (The Breast International Group (BIG) 1-98 Collaborative Group, 2005) for locally advanced or metastatic breast cancer in postmenopausal women (Cohen et al., 2001). GnRH agonists include goserelin (Zoladex) is considered a treatment option alone or with tamoxifen in the management of ER+ breast cancer in premenopausal patients (Tan & Wolff, 2007). Fulvestrant, a second-line treatment for oestrogen receptor positive metastatic breast cancer in postmenopausal women acts as an antioestrogen (Morris & Wakeling, 2002). It binds to oestrogen receptor monomers inhibiting receptor dimerization and translocation of ER into the nucleuswhile accelerating degradationof ER (Carson, 2005).

The identification of various breast cancer subtypes led to the development of further targeted therapy including HER2 inhibitors. The first targeted anti-breast

cancer therapy/monoclonal antibody Herceptin (trastuzumab) was approved by FDA in 1998 (Dillman, 1999). Lapatinib (Rimawi, et al., 2015), a dual tyrosine kinase inhibitor which acts on HER2 and EGRF pathways is indicated in combination for HER2 positive metastatic breast cancer and hormone positive/HER2 positive postmenopausal breast cancer. Next generation targeted antibody-drug conjugate, (neo) adjuvant treatment - trastuzumab emtansine, indicated for HER2-positive breast cancer, was approved by FDA in 2013 (Basel, 2013). It has several mechanisms of action, including those of trastuzamab and those of emtansine, the latter is a chemotherapy cytotoxic drug that becomes active once it enters cancer cells.

2.2 Endometrial cancer

2.2.1 Endometrial cancer - Uterine development and histology

Embryologically, the uterus forms from a pair of two paramesonephric or Mullerian ducts that fuse together to form the uterus and the upper vagina (DeUgarte, 2013). The inner layer of the uterine mesenchyme develops into the endometrium. The basic shape of the uterovaginal canal is seen at around 9-10 weeks of gestation. Lateral expansion of the cranial portion of the uterovaginal canal and narrowing caudally subsequently follows, which together with increase in overall size form the final adult uterine morphology (Robboy et al., 2017).

The histoarchitecture of the uterus at birth is similar to that of an adult but reaches full maturation with endometrial glands reaching through all endometrial stroma

thickness at pubertal age (Cooke et al., 2013). A cross-sectional study involving an ultrasound examination to assess the female internal genitalia demonstrated uterine growth from the age of 8 years (around 2 years before breast development) and continued even after menarche till around age 20 (Holm et al., 1995).

In the adult, the uterine endometrium consists of the transient superficial stratum functionalis layer which is sloughed off with menstruation and the permanent stratum basalis, which lies adjacent to the myometrium (Paxton et al., 2003). The endometrial layer is made up of different cell types - epithelial, stromal, endothelial and leucocyte cell types (Kamal et al., 2016) (Figure 2.4).

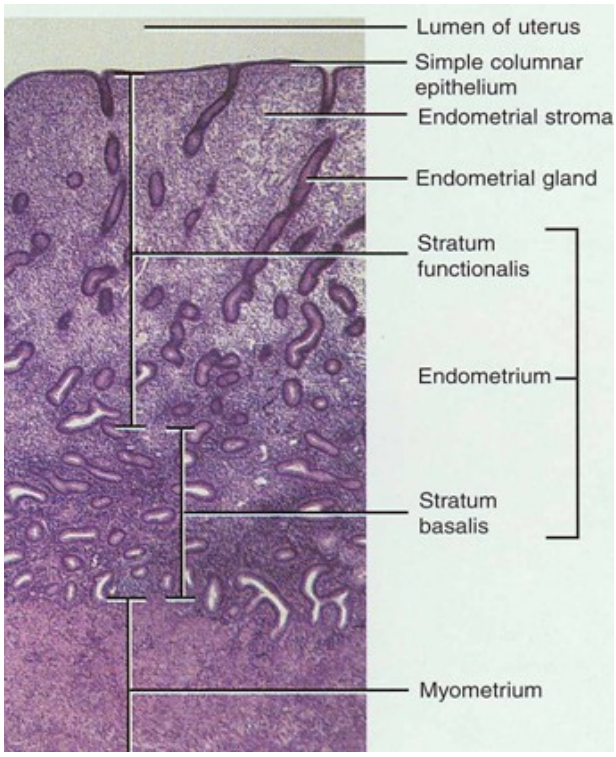


Figure 2.4 Histology of the uterine endometrium (Tortora and Grabowski, 2000)

Most endometrial cancers are carcinomas that arise from the epithelial cells. Adenocarcinoma comprises around 80% of uterine cancer. Other histological types include adenosquamous carcinoma, papillary serous carcinoma and uterine

sarcoma, the latter arising from the uterine myometrium (leiomyosarcoma) or uterine stroma (endometrial stromal sarcoma).

In the menopause, with the decline of ovarian hormone production (oestradiol and progesterone), the endometrium progressively changes into an atrophic endometrium, with the complete loss of the stratum functionalis and the retention of only the luminal and basalis cell groups. The glands of the endometrium become inactive, and the stroma becomes less cellular with no mitotic activity. Since most endometrial cancers generally occur in postmenopausal women, endometrial cancer is proposed to arise either from the luminal or basalis cells of the endometrial epithelium (Kamal et al., 2016). The uterine serous type of endometrial cancer is however suggested to arise from the fallopian tube epithelium (Tolcher et al., 2015).

Endometrial cancer precursors can be classified according to (1) the WHO94 scheme, comprising of 4 categories – simple hyperplasia, complex hyperplasia, simple hyperplasia with atypia, complex hyperplasia with atypia and (2) the endometrial intraepithelial neoplasia diagnostic criteria developed by the International Endometrial Collaborative Group (Silverberg et al, 2003) in which endometrial precancer is termed endometrial intraepithelial neoplasia (EIN).

Two types of endometrial carcinoma have been classically described - type I and type II - based on different endocrine, clinical and histopathological characteristics. The majority of Type I (endometrioid) endometrial cancer are ER+, associated with high levels of ERα and thus oestrogen-dependent adenocarcinoma, comprising 70-80% of endometrial cancers. (Akhmedkhanov et al., 2001). Type II are ER- and non-

oestrogen–dependent. This latter form is associated with a worse prognosis and includes different histological types such as the high-grade adenocarcinoma, the serous papillary carcinoma, or carcinoma with a clear cell morphology. The Cancer Genome Atlas Research Network has recently identified four molecular subtypes of endometrial cancer: Polymerase Epsilon (POLE) ultramutated, microsatellite instability hypermutated, copy umber low and copy number high. This molecular classification helps in risk stratification, provides prognostic information and drives clinical management.

2.2.2 Endometrial cancer clinical issues and treatment

Endometrial cancer typically presents with abnormal uterine bleeding (irregular/intermenstrual) premenopausal or bleeding postmenopausal. Since 75% of women with endometrial cancer are postmenopausal, the most common symptom is postmenopausal bleeding. With advanced disease, women may present with pelvic mass, dyspareunia or problems with micturition. Transvaginal ultrasound will determine the thickness of the endometrial lining (a thickness of >4-5mm with postmenopausal bleeding necessitates biopsy). An endometrial biopsy, via endometrial tissue sampler or during hysteroscopy/dilatation and curettage will confirm or exclude diagnosis. Once endometrial cancer is confirmed on histology, radiological staging is carried out.

Multiple classical authors including Soranus of Ephesus (98-138 AD), Oribasius, a Byzantine physician (c. 325-403 AD), Aetius of Amida (c. 502-575) and Cleopatra Metrodora (c. 7th century AD) had advocated surgical removal of endometrial cancer. Aristotle 384-322 BC, in his work 'History of Animals' described the first

hysterectomy and surgical removal of ovaries in animals (Karamanou, et al., 2013). Other authors, however believed that endometrial cancer is an incurable disease and treated endometrial cancer conservatively (Tsoucalas, et al., 2015; Geni, 2017).

The first abdominal hysterectomy was carried out by Charles Clay in Manchester in 1843 (and the patient did not survive the immediate post-operative period). It was in 1853 that Ellis Burnham, from Lowell, Massachusetts, performed the first successful abdominal hysterectomy. With the introduction of anaesthesia (in 1846 (Chaturvedi & Gogna, 2011)), antibiotics (in 1930s (Sneader, 2001)), blood transfusion (the first human blood transfusion was performed in 1667), antiseptic techniques (first demonstrated by Joseph Lister in 1877 (Worboys, 2013) and intravenous therapy (1800s), hysterectomy became a safer procedure (Sutton, 1997). Until the 1940s, subtotal/supracervical hysterectomy was performed, where the cervical stump was retained. This involved a simpler operation with less complications concerning the ureter as well as decreasing the risks from ascending infections (Sutton, 1995).

Dr Richardson proposed total abdominal hysterectomy with the advantage of avoiding future development of cervical carcinoma in the cervical stump in 1929, at a time where there was no cervical screening. His technique became popular in 1950s (Johns, 1997). The Pfannenstiel/transverse incision was advocated in the 1920s by Johanns Pfannenstiel (Sutton, 1997). The first laparoscopic hysterectomy was performed in Kingston, Pennsylvania in 1988 (Sutton, 1997) while FDA approved the robotic system Da Vinci Surgical System for gynaecological surgery in

2005 (Sinha et al., 2015).

Standard treatment nowadays of endometrial carcinoma (and atypical endometrial hyperplasia) is primary hysterectomy and bilateral salpingo-oophoectomy. Pelvic lymphadenectomy and/or adjuvant treatment is tailored according to grade and stage of tumour. In 2009, the ASTEC (A Study in the Treatment of Endometrial Cancer) trial showed that pelvic lymphadenectomy did not improve overall or recurrence-free survival in women with endometrial cancer confined to the uterus (early endometrial cancer) (Kitchener et al., 2009). The EN.5 trial data was pooled with the ASTEC trial data (2009) to determine the role of pelvic external beam irradiation in women with early endometrial carcinoma, with intermediate or high-risk pathological features of recurrence, post-surgical treatment. ASTEC/EN.5 concludes that external beam pelvic radiotherapy reduces the risk of pelvic recurrence but not survival (ASTEC/EN.5 writing committee, 2009).

Similar findings were obtained from 'The Postoperative Radiotherapy in Endometrial cancer' (PORTEC)-1 study, results published in 2011 (Nout. et al., 2011). PORTEC-2 study (2010) demonstrated that vaginal brachytherapy is as effective as pelvic external beam radiotherapy to reduce local recurrence but poses less adverse effects (Nout et al., 2010).

Surgery may not be an option for patients with multiple comorbidities and for young patients who wish to preserve their fertility. For this subset of patients with atypical hyperplasia or stage 1A low grade endometrial tumours, progestins hormonal treatment is an acceptable option as primary treatment. The most commonly used

progestins include medroxyprogesterone acetate (Provera) and megestrol acetate (Megace). An intrauterine device containing levonorgestrel can also be used to treat endometrial hyperplasia or early endometrial cancer, alone or in combination with medroxyprogesterone acetate or a LHRH agonist.

Tamoxifen (SERM) alone has been shown repeatedly to be associated with endometrial hyperplasia. However, it demonstrated modest remission in patients with advanced or recurrent endometrial cancer, who had not received prior systematic treatment (Tate et al., 2001). The Gynaecologic Oncology Group studied the use of LHRH agonists - Goserelin (Zoladex) as single agent for advanced and recurrent endometrial carcinoma but concluded that it has limited activity against endometrial cancer (Asbury et al., 2002). Aromatase inhibitors including letrozole (Femara), anastrazole (Arimidex) and exemastane (Aromasin) can aid in endometrial cancer treatment by inhibiting conversion of oestrogen from testosterone in fat tissue especially in the early stages (Gao et al., 2014).

The role of chemotherapy for high-risk endometrial cancer was studied in the PORTEC-3 trial. This trial showed that adjuvant chemotherapy given with and after radiotherapy for high-risk endometrial cancer did increase failure-free survival but not 5-year survival (de Boer et al., 2018). Immunotherapy or targeted therapy for endometrial cancer is also advancing. In 2017, the FDA approved pembrolizumab, a monoclonal antibody indicated for use for metastatic microsatellite instability-high tumours or mismatch repair deficient tumours, including advanced/recurrent endometrial carcinoma after being treated with chemotherapy or for patients who are not candidates for surgery or radiation. (Arora et al., 2018).

2.3 Role of patients' clinical and biochemical/hormonal characteristics in breast carcinogenesis

Multiple factors have been implicated in the aetiology of breast cancer. When describing breast cancer, Huber Campbell, in 1971, noted that "the one clear factor about the aetiology is its complexity" (Campbell, 1972). The hormonal influence on breast carcinogenesis has also been long established. Back in 1899, surgical removal of the ovaries was being considered as part of the treatment for breast cancer, since 'it is hardly possible to doubt that in some cases the removal of the ovaries does induce the disappearance of cancer tissue' (Butlin, 1902) .

2.3.1 Age

The risk of breast cancer is strongly related to age, being more common after menopause. Its risk doubles every decade until the age of menopause, around 50 years, after which the increase in risk plateaus (WCRF, 2018). Data from SEER 21 shows that in the US, female breast cancer is most commonly diagnosed in women aged between 55-64 years, with a mean age at diagnosis of 62 years (Cancer of the breast (female) - cancer stat facts). In Western Europe, the peak age of breast cancer is 65-69 age group (Globocan, 2012). Figure 2.5 shows the number of new cases of breast cancer per year and age-specific incidence rate per 100,000 females in the UK, 2016-2018, the peak also at age 65-69. The age-specific incidence rate is however at the highest rate in the 90+ age group (Figure 2.5). The peak age of breast cancer in Malta is at 60-64years (Figure 2.6).

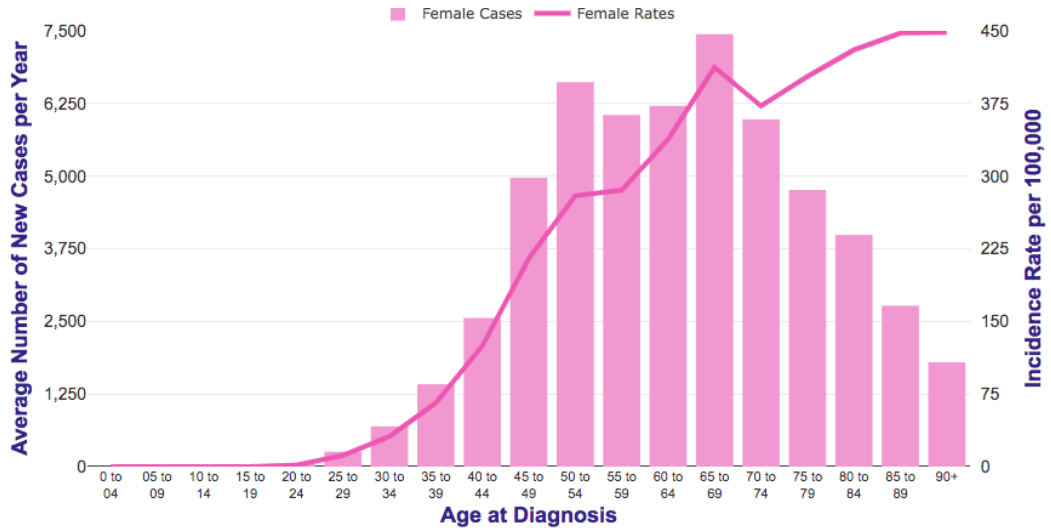
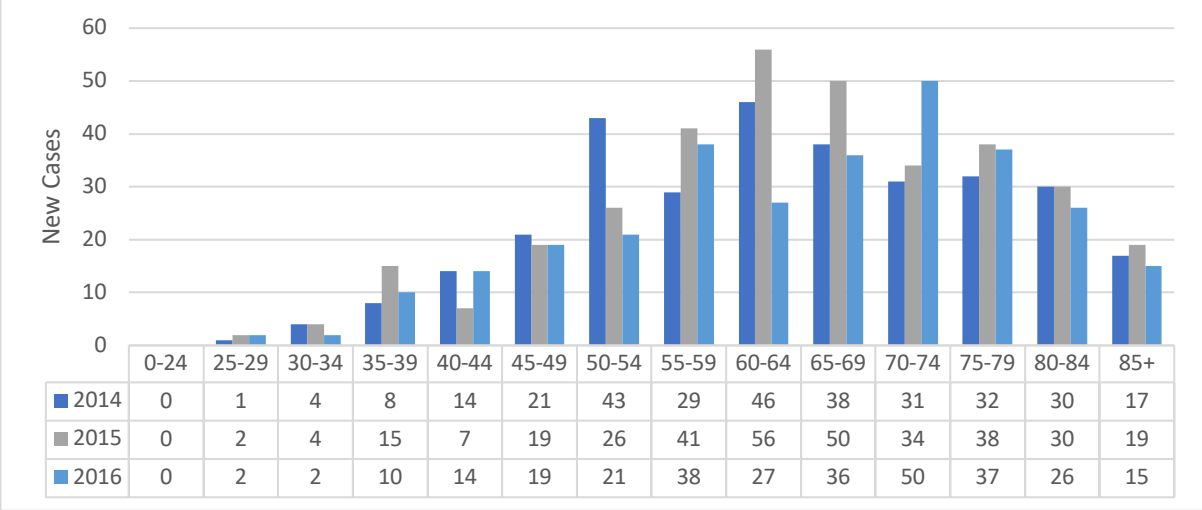


Figure 2.5 Average number of new cases of breast cancer per year and age-specific incidence rate per 100,000 in the UK female population, 2016-1018 (Cancer Research UK)



	0-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85+
2014	0	1	4	8	14	21	43	29	46	38	31	32	30	17
2015	0	2	4	15	7	19	26	41	56	50	34	38	30	19
2016	0	2	2	10	14	19	21	38	27	36	50	37	26	15

Figure 2.6 Breast Cancer new cases by age, Malta, 2014-2016 (Azzopardi, 2017)

2.3.2 Parity and breastfeeding

In the 18th century, Bernadino Ramazzini (1633-1714) observed that nuns had a higher risk for breast cancer (Olson, 2002). Janet Elizabeth Lane-Claypon (1877-

1967), in the first retrospective case control study, identified factors associated with increased breast cancer risk including low parity, late age of marriage-proxy for late age of first born and lack of breastfeeding (Press & Pharoah, 2010). Works by Wainright also concluded similar findings (Collaborative Group on Hormonal Factors in Breast Cancer, 2002). In 1970, Brian MacMahon, founder of modern epidemiology, studied the relation of breast cancer risk and age of first birth in seven areas of the world. He concluded that having a first birth before the age of 18 years is associated with about one-third the breast cancer risk when compared to having a first birth after the age of 35 years (MacMahon et al., 1970).

These associated factors were further consolidated in more recent studies. Analysis of 47epidemiological studies by the Collaborative Group on Hormonal Factors in Breast Cancer, comparing 50,302 women who had breast cancer and 96,973 women without breast cancer, showed that parity and breastfeeding are both protective factors for breast cancer. Women with a history of breast cancer were found to have a lower mean parity when compared to controls; the relative risk of breast cancer was calculated to decrease by 7.0% for each birth. Breast cancer risk was shown to be lower the younger the women were when they had their first-born child (an increased risk of 3% for each added year). Breastfeeding was less common among parous women with history of breast cancer as compared to parous controls. The average duration of breastfeeding was also shorter in parous women with the disease. Breast cancer risk was decreased by 4.3% for every 12 months of breastfeeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002) .

The Nurses' Health Study observed negative associations between parity and ER+ breast cancer (parous vs nulliparous: OR 0.82, 95% CI0.77-0.88) and breastfeeding and ER-tumours (ever breastfeeding vs never breastfed and nulliparous: OR 0.83 95%CI 0.75-0.92) (Renee et al., 2019). A meta-analysis of 15 studies by Lambertini et al (2016) looked at the effect of breastfeeding and parity on the development on different breast cancer subtypes based on the expression of hormone receptors (HR) and HER2. The HR-positive, HER2-negative or HER2 positive tumour subtypes showed a negative correlation with parity and breastfeeding. On the other hand, advanced age at first birth increased the risk of developing this subtype of breast carcinoma. The risk of developing triple negative (HR-negative, HER2-negative) breast cancer subtype was found to be reduced with breastfeeding (Lambertini et al., 2016).

Controversial findings regarding effect of breastfeeding on breast cancer risk were obtained from a systematic review including 31 studies (30 case control studies and 1 cohort study) published between 1999 and 2007. Only 11 studies (out of the 27 studies that assessed the effect of breastfeeding) found that breastfeeding causes a significant risk reduction of breast cancer when compared with never breastfeeding. When this review looked at breastfeeding duration, only 13 studies (of the 24 studies that looked at breastfeeding duration) showed a decreased breast cancer risk with extended lactation (Yang & Jacobsen, 2008).

A more recent meta-analysis including 28 papers from PubMed published between 2010 and 2016 and including 12,031 patients with breast cancer and 19,766 controls, concluded that a history of at least one pregnancy event is suggested to

decrease breast cancer risk while breastfeeding is suggested to decrease breast cancer risk, with a relative risk of 0.84 (Babalou, 2017). Akbari et al. (2011), in a study including Iranian women, found that the best results of decreased breast cancer risk were obtained with 1-3 full term pregnancies and 24months breastfeeding per child (Akbari et al., 2011). Breastfeeding and parity – full term pregnancy is thought to decrease the risk for breast cancer by causing differentiation of breast tissue and by reducing the number of ovulatory menstrual cycles. In preparation for breastfeeding, during pregnancy the breast undergoes proliferation and differentiation of the TDLU making breast tissue less susceptible to carcinogenesis (Russo et al., 2005). The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) has classified breastfeeding as protective factor for breast cancer.

The relation between breast cancer risk and early pregnancy loss is somewhat controversial (Guo et al., 2015). In a meta-analysis of 15 prospective studies, it was concluded that there is no significant association between abortion (spontaneous or induced) and breast cancer risk (Guo et al., 2015) .

2.3.3 Menarche and menopause

The link between menarche and menopausal age with breast cancer risk has long been studied. It was found that both early menarche and late menopause are associated with increased risk of breast cancer (Press & Pharoah, 2010).

The meta-analysis by the Collaborative Group on Hormonal Factors in Breast Cancer showed that the younger the age of menarche and the later the age of menopause,

the greater the risk of developing breast cancer (Figures 2.7 and 2.8). The relative increased risk associated by one-year younger age of menarche is associated with more increased risk than one-year-older age of menopause. For every year earlier, a woman had her menarche, there was an associated 5% increase of breast cancer risk. The later the menopause is also a risk factor for breast cancer, with a 3% increased risk for every year older at menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 2012).

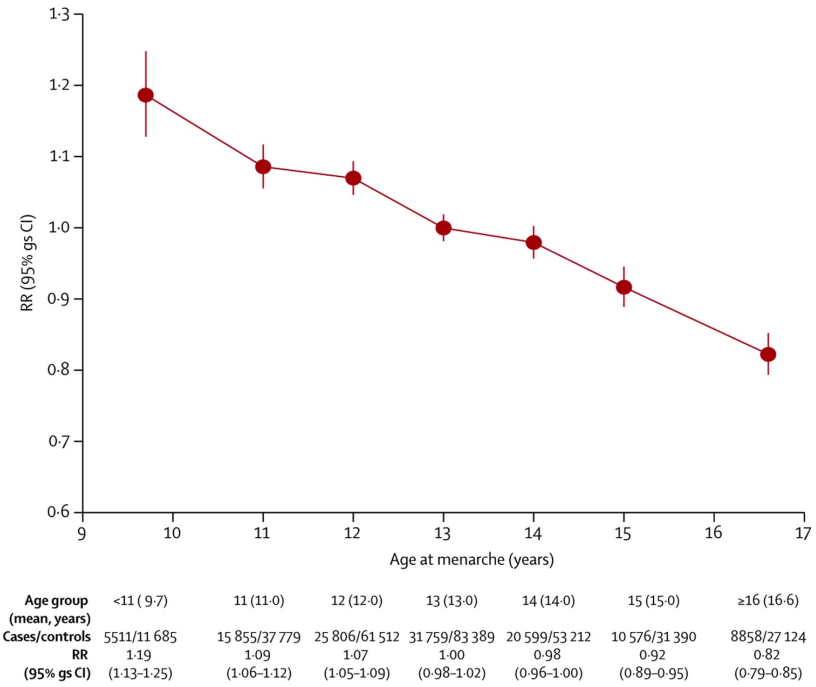
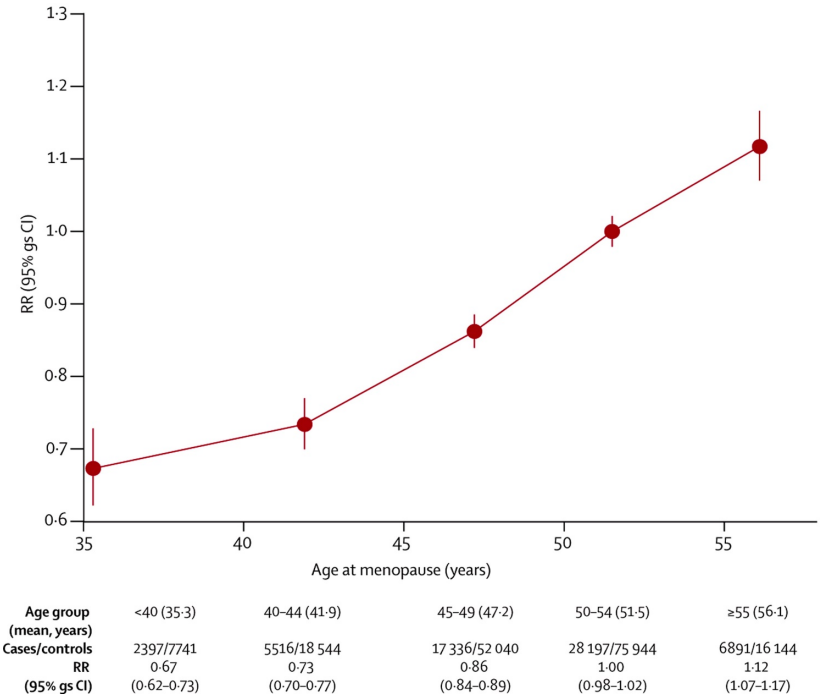


Figure 2.7 Relative risk of breast cancer with age of menarche

(Collaborative Group on Hormonal Factors in Breast Cancer, 2012)



Age group (mean, years)	<40 (35·3)	40–44 (41·9)	45–49 (47·2)	50–54 (51·5)	≥55 (56·1)
Cases/controls	2397/7741	5516/18 544	17 336/52 040	28 197/75 944	6891/16 144
RR	0·67	0·73	0·86	1·00	1·12
(95% gs CI)	(0·62–0·73)	(0·70–0·77)	(0·84–0·89)	(0·98–1·02)	(1·07–1·17)

Figure 2.8 Relative risk of breast cancer with age of menopause

(Collaborative Group on Hormonal Factors in Breast Cancer, 2012)

The effects of menarche and menopause on breast cancer risk may be attributed to lengthening of the women's reproductive years. Surgical menopause, having hysterectomy and oophorectomy before menopause was associated with a reduced risk for breast cancer. Tubal sterilization only was however not associated with a decreased breast cancer risk (Press et al., 2011).

Data from the Nurses' Health Study also showed an inverse association with breast cancer, however the association with lobular type was stronger than ductal breast carcinoma, 8% vs 2% decrease in breast cancer risk for each year increase in menarche age respectively (Kotsopoulos et al., 2020).

2.3.4 Polycystic ovarian syndrome (PCOS)

The relation between PCOS and breast cancer is not consistent. Meta-analyses of multiple studies however show no association between PCOS and breast cancer risk (Barry et al., 2014; Ding et al., 2018; Harris & Terry, 2016).

2.3.5 Medical Conditions and markers of metabolic dysfunction

In a systematic review and meta-analysis of publications from 2007 to 2013, diabetes mellitus (DM) was found to be associated with a 23% increased risk of breast cancer, when compared to those without diabetes (De Bruijn et al., 2013). However, when looking at 22 case-control studies and cohorts, the incidence of breast cancer was significantly increased with DM in cohort studies (RR=1.32 95% CI 1.06-1.65) but not in case-control studies (RR=1.46 95%CI 0.99-2.26) (Bernard et al., 2016). Although serum HbA1C was observed to be higher in breast cancer population, the association was not significant (Price TR et al., 2020).

Hyperinsulinaemia or insulin resistance has been linked with carcinogenesis of breast cancer. When comparing insulin level with breast cancer incident, the highest insulinaemia quartile had a hazard ratio of 1.46, p=0.02 when compared to the lowest insulinaemia hazard ratio. Similar results were obtained by HOMA-IR. Insulin exerts anabolic effects by binding to insulin receptor or insulin-like growth factor receptors, increasing DNA synthesis and cell proliferation. Both insulin receptors and insulin like growth factor receptors tend to be overexpressed in breast cancer (Milazzo et al., 1992) causing activation of insulin receptor substrate 2 and

upregulation of phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase pathways involved in cell proliferation (deCensi et al., 2011).

The EHBCCG also analysed circulating insulin-like growth factor1 (IGF1) concentration with breast cancer risk. High levels of IGF1 were found to be associated with increased risk of oestrogen-receptor-positive breast cancer (this positive association between IGF1 and breast cancer was not however seen in oestrogen receptor negative breast cancers) (Key et al., 2010).

The association between hypertension and breast cancer was also studied through a systematic review and meta-analysis. This involved 29 studies and 11643 cases of breast cancer and observed a significantly increased risk (RR:1.20; 95% CI 1.09-1.31) of postmenopausal breast cancer with hypertension (Han et al., 2017).

High total cholesterol is also found to be positively associated with breast cancer, with a 17% increased risk (Kitahara et al., 2011). A low high-density lipoprotein (HDL)-Cholesterol have been linked to increased breast cancer risk (Agnoli et al., 2010; Li, X. et al., 2017). The relation between serum triglycerides concentration and breast cancer risk shows inconsistent results (Agnoli et al., 2010; Li et al., 2017). Studies have shown that cholesterol exert a pro-proliferative effect on human breast cancer cell lines (Danilo & Frank, 2012) through multiple potential mechanisms:

i) It is a precursor to oestrogen, the latter increases proliferation while decreases apoptosis of breast cancer cells.

ii) Cholesterol is transported in lipoproteins, low density lipoprotein (LDL) and HDL which are also responsible for cellular proliferation as well as

activating signalling pathways associated with breast carcinogenesis (Danilo & Frank, 2012).

iii) Cholesterol is a major component of lipid rafts, microdomains of plasma membrane, which serve as a platform for signalling molecules that regulating cell cycle (George & Wu, 2012).

iv) Another proposed mechanism linking hypercholesterolaemia to breast cancer is through 27-hydroxycholeserol, a primary metabolite of cholesterol that is responsible for ER-positive breast cancer cell proliferation (Nelson et al., 2013).

In a case-control study to determine the association between the metabolic syndrome and postmenopausal breast cancer, the metabolic syndrome was found to be strongly associated with breast cancer risk, with an increased risk of 58%. This risk increases with increasing number of the components of the metabolic syndrome (high serum glucose and triglycerides, low HDL-cholesterol, high blood pressure, and abdominal obesity). The metabolic syndrome was an independent risk factor for breast cancer (Agnoli et al., 2010).

2.3.6 Obesity

Excess body weight is associated with increased risk of multiple cancers including breast cancer (Renehan et al., 2008). Body fatness is classified as a cause of postmenopausal breast cancer by the International Agency for Research on Cancer (IARC) and the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR). Around 9% of female breast cancers in the UK are associated with excess body weight (Cancer Research UK, 2015). The effects of

weight gain during life and breast cancer risk is summarized in Figure 2.9. Interestingly recent studies have shown inverse association between body fatness during childhood/adolescence and breast cancer lifetime risk (Baer et al, 2010; Xue et al, 2016).

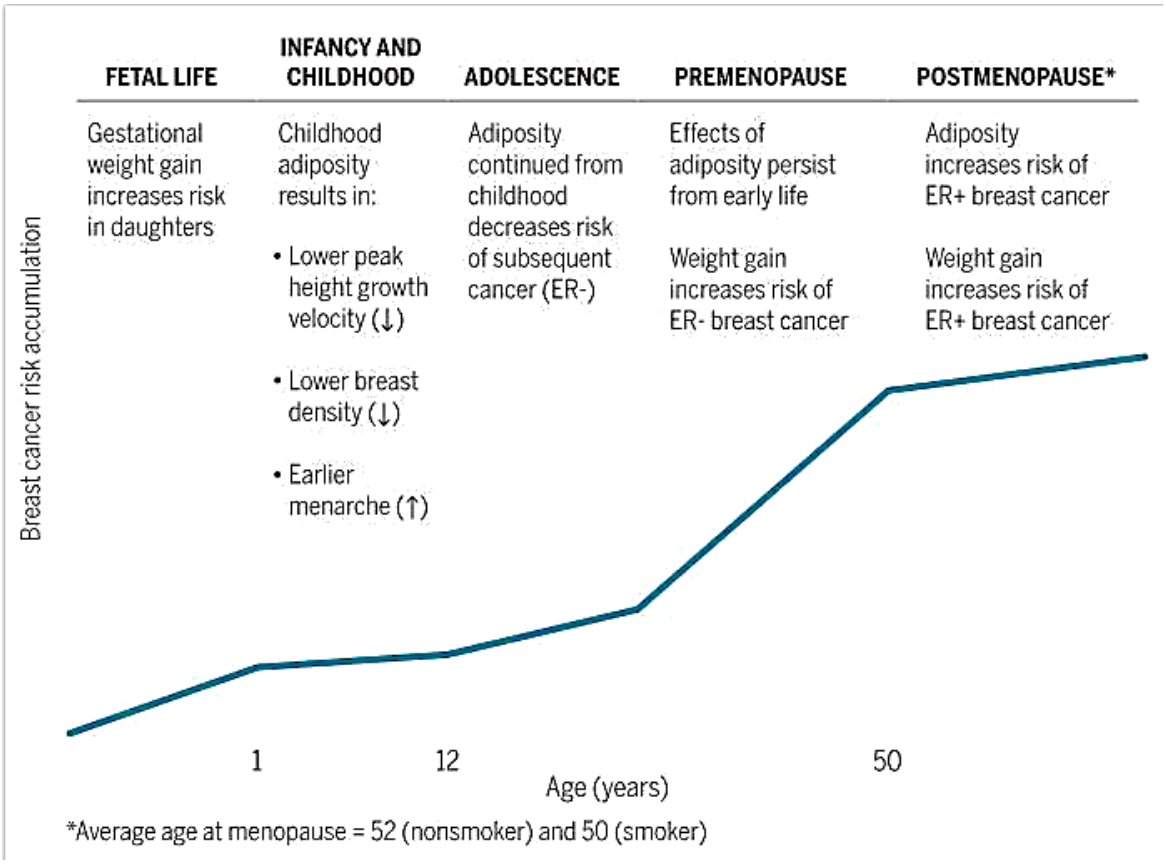


Figure 2.9 Life course relationship of adiposity with breast cancer risk relative to the breast cancer risk for women with normal BMI (Moley & Colditz, 2016)

A meta-analysis of 50 studies concluded that for each 5kg increase in weight gain there is an increased relative risk of 1.11 (95% CI=1.08 to 1.13) for postmenopausal breast cancer in non- or low-HRT users (Keum et al., 2015). The Endogenous Hormones Breast Cancer Collaborative Group (EHBCCG) analysed data for 624 cases of postmenopausal women with breast cancer and 1669 controls also had concluded positive correlation between weight gain and breast cancer risk. Results

showed that BMI increases the risk of breast cancer, with an increased relative risk of 1.19 for every 5 kg/m2 increase in BMI (Key et al., 2003).

Results from the Nurses' Health Study suggested that women who gained 25kg or more since the age of 18years or 10kg or more since menopause compared with those who maintained the same weight are at increased risk of invasive breast cancer (relative risk of 1.45, 1.18 respectively). In this population studied, of 4,393 cases, weight gain of 2 kg or more since age of 18years is suggested to be responsible for 15% of breast cancer cases, while 4.4% of breast cancer cases were found to be attributed to weight gain of 2kg or more since menopause. Further findings from the Nurses' Health Study included that weight loss (10 kg or more) in postmenopausal women who never used HRT was associated with more than 50% reduced risk of developing invasive breast cancer compared to women with steady weight after the menopause (Eliassen et al., 2006).

The Nurses' Health Study also found positive association with both ductal and lobular breast cancer subtypes. Women with BMI ≥30 had a relative risk of 1.60 (95% CI, 1.42-1.80) and 1.47 (5% CI 1.08-2.00) for ductal and lobular subtypes respectively when compared with women with BMI <21. Interestingly, BMI at age of 18years was inversely associated with both subtypes' risk (Kotsopoulos et al., 2010).

A meta-analysis by Vrieling et al., looking on the association of weight gain on oestrogen and progesterone receptor status breast cancer concluded that there is a stronger positive association between weight gain and ER(+)PR(+) postmenopausal

breast cancer than ER(-)PR(-) tumour (Vrieling et al., 2010). The association between increased BMI and breast cancer incidence was also observed to vary across different populations. A significantly stronger association was found between increased BMI and incidence of breast cancer in Asia-Pacific women compared to European-Australian and North Americans (Wang et al., 2016).

2.3.7 Tobacco and alcohol consumption

Tobacco and alcohol consumption were correlated with breast cancer risk by The Collaborative Group on Hormonal Factors in Breast cancer in their meta-analysis (Hamajima et al., 2002). Women who drank 35-44 g per day of alcohol had an increased relative risk of breast cancer of 1.32 (1.19-1.45, P<0.00001) while those who consumed ≥45g of alcohol per day were at an increased relative risk of 1.46 (1.33-1.61, P<0.00001) when compared to women who reported no alcohol intake. The relative risk of breast cancer increased for each 10g of extra alcohol intake (extra unit/drink of alcohol daily) by 7.1%. This association was not found to be modified by other factors including smoking, reproductive factors, family history of breast cancer and use of OCP or HRT. In this analysis, the association of smoking with breast cancer risk however appeared to be confounded by alcohol and current or history of smoking was not found to be associated with increased breast cancer risk compared with non-smokers (Hamajima et al., 2002).

Epidemiological evidence regarding smoking and breast cancer is inconsistent. But findings from the Generations Study cohort showed that smoking increases breast cancer risk especially if smoking started in adolescence or perimenarcheal age (Jones, et al., 2017) . Data from the Danish nurse cohort study showed similar

findings. They report an 18% increased risk of breast cancer in ever smokers and 27x increased risk in current smokers (when compared to never smokers). Smoking (>10pack-years) before first birth was associated with the highest risk for breast cancer (Andersen et al., 2017) .

Alcoholic beverages are listed as a cause of breast cancer by the International Agency for Research on Cancer (IARC) and WCRF/AICR while tobacco smoking is listed as a probable cause of breast cancer by the IARC (WCRF, 2010).

2.3.8 Role of exogenous hormones

The role of exogenous hormones (oestrogen and progesterone) was initially thought to be protective against breast cancer (Wilson, 1962). However, both current/recent use of oestrogen-progesterone contraceptives (combined) and current use of combined oestrogen-progesterone hormone replacement therapy (HRT) are classified as being a cause for breast cancer by the IARC (Cogliano et al., 2011).

The Million Women Study, Nurses' Health Study and the Women's Health Initiative (WHI) concluded that compared with placebo, oestrogen plus progestin hormone replacement therapy (HRT) is associated with increased breast cancer risk. In the Million Women Study, current HRT use was found to be associated with a 66% increased risk of breast cancer than never users. In this study including 3.6million person-years of follow-up, the increased relative risk of breast-cancer was noted to vary according to the histological type. Current users of HRT compared with never users were associated with a relative risk of 2.82 (95% CI 1.72-4.63) for lobular carcinoma in-situ, 2.66 for tubular cancers (95% CI 2.16-3.28), 2.25 (95% CI 2.00-

2.52) for lobular carcinoma and 2.13 (95% CI 1.68-2.70) for mixed ductal-lobular carcinoma. The effect of HRT on invasive ductal, lobular and tubular breast carcinoma were greater for oestrogen and progesterone therapy when compared to oestrogen only therapy (Reeves et al., 2006).

The Nurses' Health Study also showed significant increased risk of both subtypes of breast cancer with current oestrogen and progesterone HRT use, with a stronger association with lobular than ductal breast cancer. Use of oestrogen only HRT for 5-10years was also associated with both types of breast cancer, showing a stronger increase in risk of lobular cancer compared to ductal breast cancer. Of note, current use of oestrogen only HRT less than 5years was only associated with lobular breast cancer increase and not ductal subtype (Kotsopoulos et al., 2010).

Results from the WHI showed similar results for oestrogen-plus-progestin HRT but differed in oestrogen-alone HRT trial results. In the first trial intake of oestrogen and progestin resulted in increased breast cancer risk – 9 extra cases of breast cancer for every 10,000 postmenopausal women taking HRT. On the other hand, women taking oestrogen only HRT showed decreased risk for breast cancer (Manson et al., 2013).

Studies show conflicting results about the breast cancer risk with combined oral contraceptive pill (OCP) use. Three large prospective cohort studies (Nurses Health Study, Royal College of General Practitioners Research and the Oxford Family Planning Association Research show no increased breast cancer risk with past/current OCP use (Vessey et al, 2013; Hankinson et al, 1997; Hannaford et al,

2007). On the other hand, in a meta-analysis by the Collaborative Group on Hormonal Factors in Breast Cancer OCP use was related to an increased risk of BC (RR 1.24, 95% CI: 1.15–1.33), which faded after cessation (RR 1.16 after 1–4 years, RR 1.07 after 5–9 years) and ceased after 10 or more years. March et al in their study involving 1.8 million Danish women also found an increased risk of breast cancer in users compared to non-users (RR 1.20, 95% CI: 1.14–1.26), which rose with a longer duration of use (March et al, 2017).

The association between progestin only contraception and breast cancer risk is controversial. Recent or current use of depomedroxyprogesterone acetate contraception appeared to be associated with an increased risk of breast cancer (Kaunitz, 1996). A prospective study by Fabre et al identified an increased breast cancer risk among current users of ≥4.5 years of continuous use (RR= 1.44; 95% CI: 1.03–2.00). However, multiple studies found no increased risk of breast cancer with progestin-only contraception (Marchbanks et al, 2002; Kumle et al, 2002; Samson et al., 2016).

2.3.9 Family history of breast cancer

Brewer et al., identified family history of breast cancer as a strong risk factor for breast cancer, $p<0.0001$. Postmenopausal women who had a family history of breast cancer had an increased relative risk of 1.58 (95% CI 1.40-1.79); risk varied according to degree and number of relatives (Brewer et al., 2017). Positive association between family history of breast cancer with both lobular and ductal breast cancer were found in The Nurses' Health Study (Kotsopoulos et al., 2020).

Brewer et al., stated that breast cancer risk increased significantly ($p<0.0001$) with a higher family history score (Brewer et al., 2017).

Results from the Nurses' Health Study also showed no heterogeneity between the two subtypes and the following risk factors: age at menopause, parity, nulliparity, personal history of benign breast disease, alcohol consumption, adult BMI and BMI at 18years. Only age of menarche, age of first birth and postmenopausal hormonal use had stronger positive association with lobular rather than ductal breast cancer. In a second analysis where only ER-positive, PR-positive tumous were included, the above findings were maintained. (Kotsopoulos et al., 2017).

2.3.10 Endogenous serum hormonal levels

Analysis of the endogenous sex hormones level in 2,428 postmenopausal women showed that those with the highest circulating levels of sex hormones (including oestradiol, oestriol, and testosterone) had twice the breast cancer risk as compared with those with the lowest levels of hormones. SHBG was however associated with a reduction on breast cancer risk (Key et al., 2002).

The EHBCCG reported that higher levels of the steroid hormones – dehydroepiandrosterone sulphate, dehydroepiandrosterone, 4-androstenedione, testosterone, oestradiol and oestrone and decreased SHBG concentrations were found to increase postmenopausal breast cancer risk. The strongest association was found between steroid hormones and BMI. The EHBCCG suggested that the increased risk found between BMI and breast cancer was related to steroid hormone levels. Breast cancer risk was substantially reduced after adjustment for serum

oestrogens, particularly oestradiol. The relative risk was also moderately reduced after adjusting for SHBG. Adjusting for androgens however had little effect on the relative risk (Key et al., 2003).

The WHI study investigated the relationship of serum oestrogen level and its metabolites in postmenopausal women with the anthropometric measures. Their data showed that postmenopausal patients with higher BMI had higher levels of oestrogens and reduced methylation of carechol oestrogen metabolites – both factors are associated with increased risk of breast carcinoma (Hannah et al., 2017).

The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) distinguished between ER-positive and ER-negative breast cancer. In postmenopausal women with ER-positive breast cancer, the level of free oestradiol, oestradiol, SHBG and free testosterone were found to be significantly higher as compared to controls (Fourkala et al., 2016). In ER-negative breast cancer, the levels of androgens and SHBG however were not found to be raised. In postmenopausal ER-negative breast cancer, oestradiol and free oestradiol were significantly higher than controls and women with levels in the top quartile had a two-fold increased risk for ER-negative breast cancer (Key et al., 2003).

A higher serum FSH level was observed in Her-2+ postmenopausal breast cancer patients while a higher FSH and LH levels were identified in postmenopausal breast cancer patients with high Ki67 expression (Zhou et al, 2013). Higher levels of these hormones was also suggested to be involved in progression of breast cancer (Sanchez et al., 2018; Zhou, J. et al., 2013).

2.4 Role of patients' clinical and biochemical/hormonal characteristics in endometrial carcinogenesis

The endometrium is receptive to the steroid hormones: oestrogens, progesterone and androgens. As described in the introduction, oestrogen exert its action on the endometrium via the eRα and eRβ. The classical eRα activity mediates endometrial cell growth and differentiation, angiogenesis and apoptosis inhibition, while eRβ is thought to have opposing action and has an antiproliferative role (Hapangama et al., 2015).

Endometrial cancer has long been considered as an oestrogen driven malignancy. The relationship between endometrial cancer and oestrogen is described by the 'unopposed oestrogen' hypothesis (Key & Pike, 1988). It states that endogenous or exogenous oestrogen exposure, which is unopposed by progesterone/synthetic progestogen causes an increased mitotic activity of the endometrial cells leading to increased risk of endometrial carcinoma. Conditions that lead to increased unopposed oestrogen exposure include early menarche, late menopause, nulliparity, anovulatory states including PCOS and obesity.

2.4.1 Age

The peak in the number of new cases of endometrial cancer in Western Europe females is seen at age 65-69 years (Ferlay et al., 2012), that of the UK is at 65-74 years. The highest age-specific incidence rate in the UK is however in the 75-79 age group (Figure 2.10).

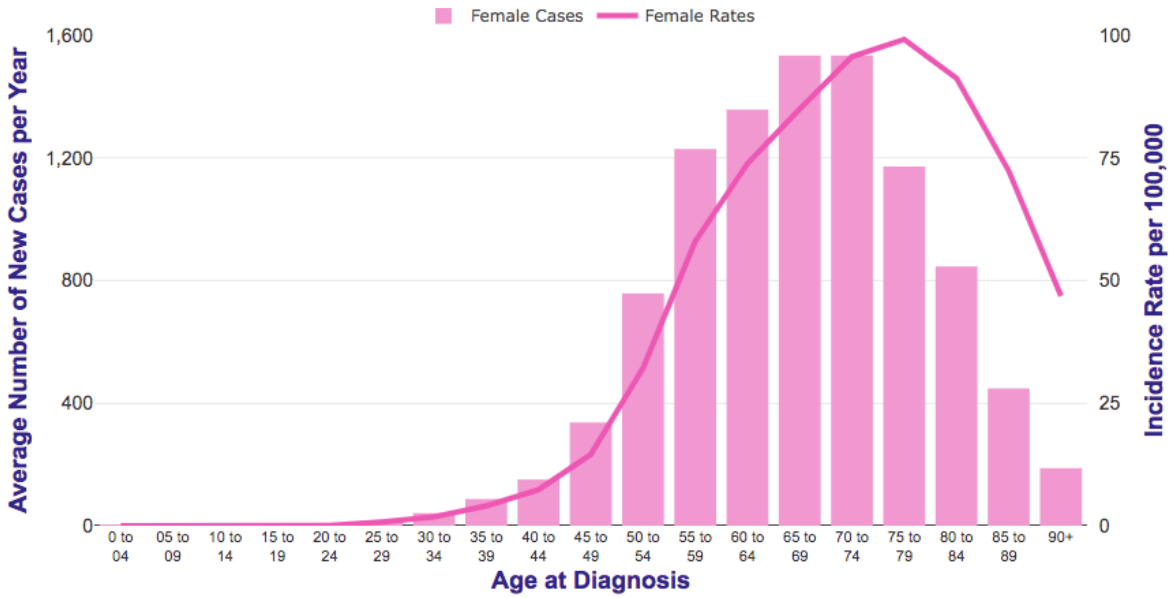
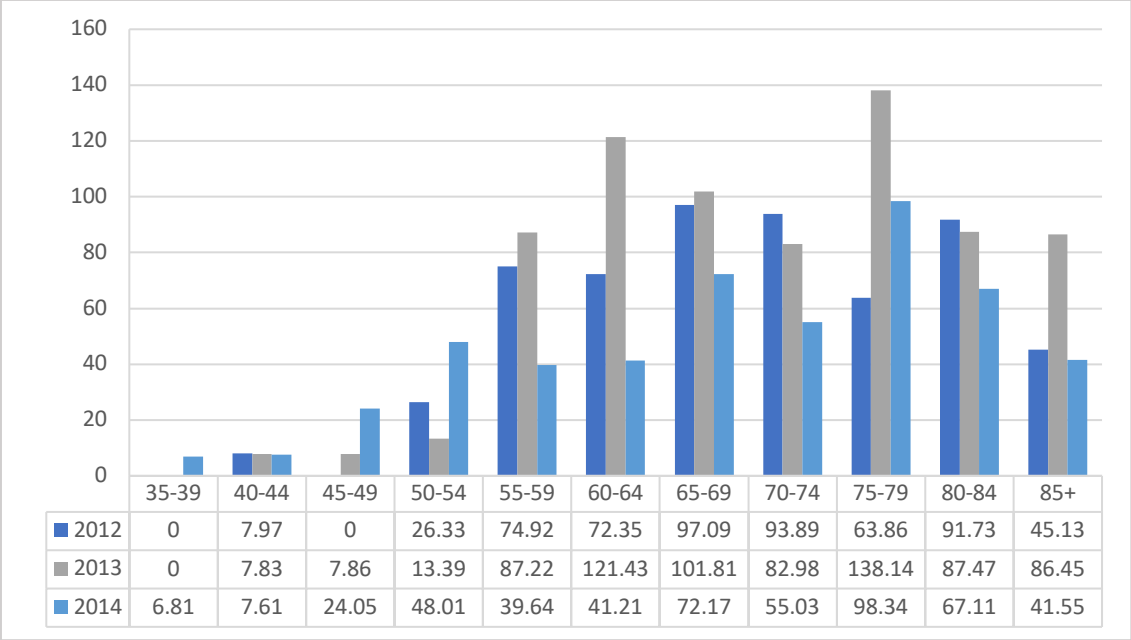


Figure 2.10 Average number of new cases of uterine cases per year and age-specific incidence rate per 100,000 in the UK female population, 2016-2018 (Cancer Research UK)

The peak incidence age of endometrial cancer in Malta between 2012 and 2014 is 75-79 age group, followed by 65-69 age group (Figure 2.11).



	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85+
2012	0	7.97	0	26.33	74.92	72.35	97.09	93.89	63.86	91.73	45.13
2013	0	7.83	7.86	13.39	87.22	121.43	101.81	82.98	138.14	87.47	86.45
2014	6.81	7.61	24.05	48.01	39.64	41.21	72.17	55.03	98.34	67.11	41.55

Figure 2.11 Uterine cancer, Estimated incidence rate (rate per 100 000) by age, females, Malta, 2012-2014 (Azzopardi, 2017)

In a study by K. Plagens-Rotman et al. (2016), the risk of developing endometrial carcinoma was found to increase with increasing age. The odds ratios of endometrial carcinoma for group of responders 50-59 years was 4.91 which increased to 25.17 for responders aged 60-69 and to 37.12 for responders aged 70-79 years. The odds ratio for group of responders aged more than 80 was 23.3 (Figure 2.12) (Plagens-Rotman et al., 2016).

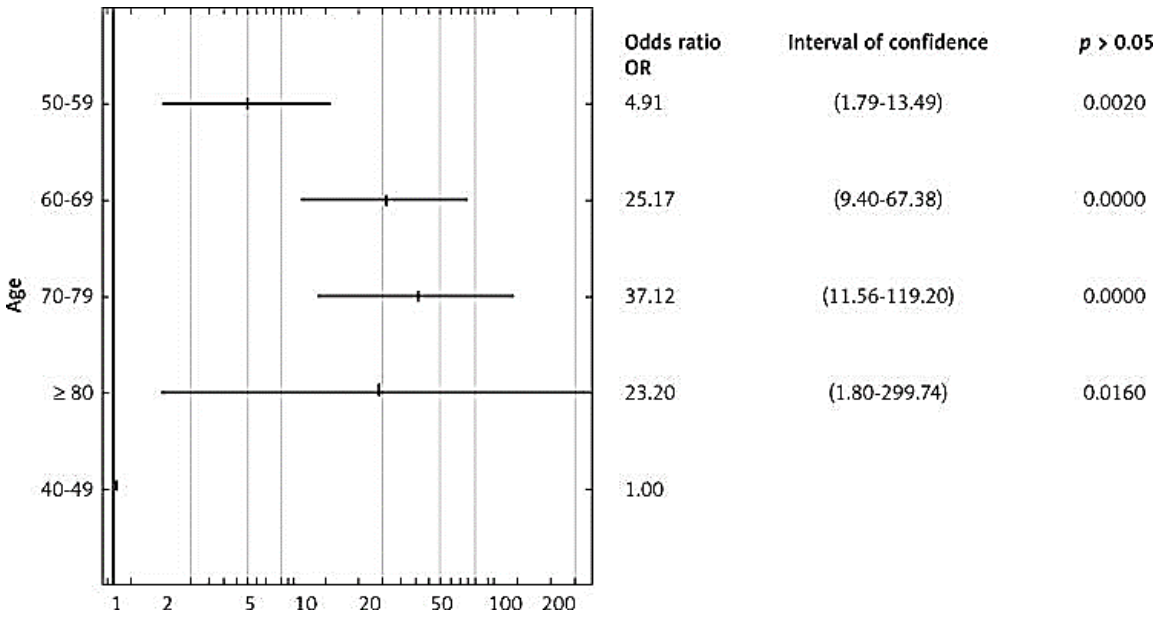


Figure 2.12 Odds ratio of endometrial carcinoma according to different age groups; with increasing age, the OR of endometrial cancer increases (Plagens-Rotman et al., 2016)

2.4.2 Parity and breastfeeding

A meta-analysis of epidemiological studies including 69,681 patients showed that parity is negatively associated with endometrial cancer risk; the relative risk decreased with increasing number of pregnancies (Wu et al., 2015). In the prospective Black Women's Health Study, parous women were found to have an incident rate ratio of 0.77 (95% CI 0.57, 1.05) when compared to nulliparous in developing endometrial cancer. A strong association with endometrial cancer was

found if the age of first birth was ≥30 years when compared to age at first birth <20years (incident rate ratio 0.26, 95% CI 0.13, 0.50) (Sponholtz et al., 2017) .

The relationship between breastfeeding and endometrial cancer was inconsistent but several recent meta-analyses concluded that breastfeeding is protective for endometrial cancer (Jordan et al., 2017; Ma et al., 2018; Zhan et al., 2015). Ever breastfeeding was found to be associated with an 11% decrease in endometrial cancer risk (Jordan et al., 2017). For every 6months increase in duration of breastfeeding, the endometrial cancer risk was decreased by 7% (relative risk 0.93; 95% CI 0.88, 0.97) (Ma et al., 2018). In a separate meta-analysis, endometrial cancer risk was found to decrease by 1.2% for every month increase in breastfeeding (Zhan et al., 2015). Although data from the meta-analysis from the Epidemiology of Endometrial cancer Consortium also confirmed that the longer the duration of breastfeeding, the lower the endometrial cancer risk, they suggested levelling of this association beyond 6-9months (Jordan et al., 2017).

2.4.3 Menarche and menopause

A meta-analysis investigating relation between age at menarche and endometrial cancer risk concluded that age of menarche is inversely associated with risk for endometrial cancer. A 4% decrease in endometrial cancer risk was noted for every two-year delay in age of menarche (Gong et al., 2015). In the Black Women's Health Study, age of menarche at less than 11 years was associated with an incident rate ratio of 1.82 (95% CI 1.31-2.52) when compared with age at menarche of 12-13years (Sponholtz et al., 2017) . In a case-control study from Iran, early menarche

(<12years) was associated with odds ratio of 2.10 (95% CI 1.17-3.75) (Ghanbari Andarieh et al., 2016).

Age of menopause was found to be negatively associated with risk of endometrial cancer – the later the menopause age, the increased endometrial cancer risk (Dunneram et al., 2019; Wernli et al., 2006). In the Korean Heart Study, a duration of ovarian hormone exposure of ≥40years was also associated with increased risk of endometrial cancer (Jung et al., 2016).

2.4.4 Polycystic ovarian syndrome in endometrial cancer

A Taiwan population-based cohort study including 40,775 participants, found a statistically increased risk of endometrial cancer in women with PCOS as compared to patients without PCOS (Ding et al., 2018). Meta-analyses also support the positive association between PCOS and endometrial cancer. However, the authors comment that confounding factors like BMI were not taken into consideration in most studies (Barry et al., 2014; Harris & Terry, 2016).

2.4.5 Medical conditions and markers of metabolic dysfunction

Friberg et al (2007), in their meta-analysis of 96,003 participants, concluded that DM, both type 1 and type 2 are positively associated with endometrial cancer risk. Diabetes type 2 was found to be associated with a relative risk of 2.2 (95% CI 1.8-2.74). For type 1 diabetes mellitus, the relative risk of endometrial cancer is 3.15 (95%CI 1.07-9.29) (Friberg et al., 2007). A study including 88,107 postmenopausal women participating in the WHI, suggest that the association of diabetes with

endometrial cancer is largely confounded by obesity. Adjusting for BMI, the association between DM and endometrial cancer became not significant (Luo et al., 2014).

Higher levels of triglycerides, C-peptide and glucose were noted in patients with endometrial cancer compared to controls (Dossus et al., 2013). However, Kho et al concluded that role of triglycerides in endometrial cancer is unsupported (Kho et al., 2021). Evidently, hyperinsulinemia increases the risk of endometrial cancer (Hursting & Berger, 2010). Higher levels of insulin were found to be associated with increased endometrial cancer risk; association was independently of BMI (Nead et al., 2015).

Results from a meta-analysis to determine the association between hypertension and endometrial cancer suggest a relative risk of 1.61 (95% CI 1.41-1.85) for case-control studies and 1.32 (95% CI 1.12-1.56) for cohort studies (Aune et al., 2017). The Swedish Apolipoprotein MOrtality RISk (AMORIS) study of 225,432 participants showed that triglycerides, total cholesterol and triglyceride/HDL ratio are positively associated with endometrial cancer (Seth et al., 2012) .

The presence of the metabolic syndrome was also associated with increased risk for endometrial cancer, with an increased risk of 61% (Esposito et al., 2012) or more than two-fold increase in endometrial cancer risk (hazard ratio 2.20; 95% CI 1.61-3.02) independent of obesity (Arthur RS et al., 2020).

2.4.6 Obesity

Several studies have linked obesity to increased risk for endometrial cancer. Obesity has been attributed to at least 40% of endometrial cancers in the UK (Bhaskaran et al., 2014). Epidemiological evidence shows that compared to normal-weight women (BMI <25 kg/m^2), women who are obese (BMI >30, <35 kg/m2) were at 2.6-fold increased risk for endometrial cancer while women who are severely obese (BMI >35kg/m2) were at 4.7-fold increased risk for endometrial cancer (Shaw et al., 2016). Results from the Million Women Study (Figure 2.13) (Yang et al., 2012) and Black Women's Health Study (Sponholtz et al., 2016) also confirms that BMI is strongly associated with increased risk of endometrial cancer.

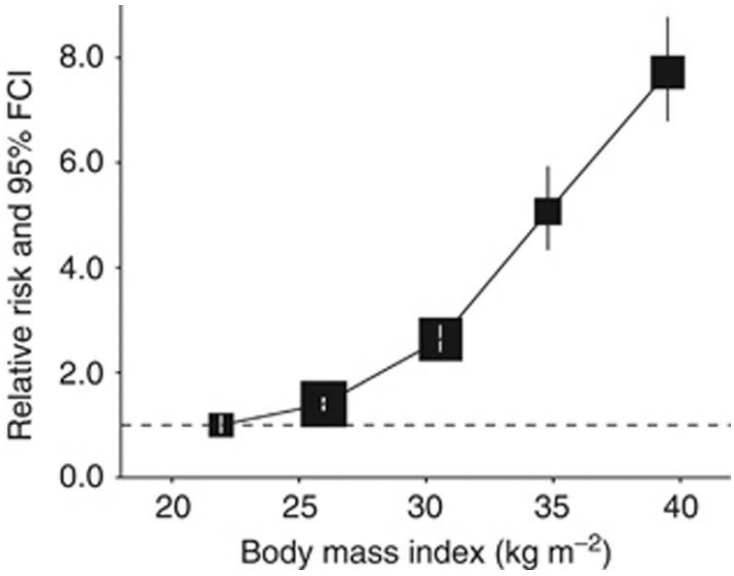


Figure 2.13 Relative risk and 95% floated confidence intervals (FCI) of endometrial cancer by BMI (Yang et al., 2012)

The Black Women's Health Study concluded that a high weight-to-height ratio was associated with an increased endometrial cancer risk (incidence rate ratio of 2.83, 95% CI 1.77-4.53). Central obesity was not found to be significantly associated with endometrial cancer after confounding for BMI (Sponholtz et al., 2016). However, in

a separate study, central obesity was found to be in itself a risk factor for endometrial cancer- associated with a 1.5-twofold increased risk (Shaw et al., 2016). The European Prospective Investigation into Cancer and Nutrition (EPIC) study, which involved approximately 370,000 women from 10 European countries, also concluded that patients with endometrial cancer higher BMI and waist circumference (Dossus et al., 2013).

BMI in early adulthood and weight gain throughout life-course were also found to be associated with increased endometrial cancer risk among women in the Cancer Prevention Study II Nutrition Cohort (Stevens et al., 2014) . Data from the Nurses' Health Study and Nurses' Health Study II showed that weight gain throughout life-course was positively associated with endometrial cancer risk; weight gain of ≥25 kg was found to be associated with 2.54 hazard ratio (95% CI 1.80-3.59) when compared to stable weight (Dougan et al., 2015). A meta-analysis of 50 studies also concluded that weight gain during adulthood is associated with an increased risk of endometrial cancer more so in the HRT nonusers –for a 5 kg increase in weight gain, the relative risk was 1.39 (95% CI 1.29-1.49) among HRT nonusers and 1.09 (95% CI 1.02-1.16) in HRT users (Keum et al., 2015).

2.4.7 Tobacco and alcohol consumption

Cigarette smoking shows a protective effect against endometrial cancer risk, through multiple anti-oestrogenic mechanisms (Kamal et al., 2016). The National Institutes of Health-AARP Diet and Health Study showed a relative risk of 0.89 (95% CI 0.80-1.00) among former smokers and a relative risk of 0.65 (95% CI 0.55-0.78) among current smokers as compared to never smokers (Felix et al., 2014).

Multiple studies have provided controversial evidence of the effect of alcohol consumption on endometrial cancer risk. Meta-analyses concluded that alcohol consumption was not significantly associated with endometrial cancer risk (Sun et al., 2011; Turati et al., 2010; Zhou et al., 2017).

2.4.8 Role of exogenous hormones

The use of the combined oral contraceptive pill (COCP) is associated with lower rates of endometrial cancer. A systematic review of 15 case-control studies and 4 cohort studies demonstrated that the use of COCP decreased risk of endometrial cancer by about 50% and this protective effect lasted for more than 20years (Mueck et al., 2010). The Collaborative Group on epidemiological studies on endometrial cancer looked at the association of oral contraception use on endometrial cancer in a meta-analysis of 27,276 participants. It also concluded that long term use of oral contraception decreases the endometrial cancer risk. Every 5 years of oral contraceptives use was associated with an odds ratio of 0.76 (95% CI 0.73-0.78; $p<0.0001$) (Chaturvedi & Gogna, 2011) .

The use of depot medroxyprogesterone contraception is also associated with risk reduction of endometrial cancer, the risk reduction is even greater than that described with the COCP (Kaunitz, 1996). Similarly, the levonorgestrel-releasing intrauterine system is associated with a protective effect against endometrial cancer. It was observed to decrease the cases of endometrial adenocarcinoma by half (incidence ratio 0.50, 95% CI 0.35-0.70) (Soini et al., 2014).

The WHI, which consisted of clinical trials and an observational study concerning generally healthy postmenopausal women, concluded that use of oestrogen plus progestin HRT is associated with a reduction in endometrial cancer (Manson et al., 2013). However, users of unopposed oestrogens including those taking cyclic/sequential combined HRT with less than 10 days of progesterone per month, had increased endometrial cancer risk (Brinton & Felix, 2014; Sjögren et al., 2016).

2.4.9 Family history of endometrial cancer

In a study to determine cancer risk according to family history of endometrial cancer in 1353 endometrial cancer patients and 628 controls, it was reported that history of endometrial cancer in one or more first/second degree relatives was associated with increased endometrial cancer risk ($p=3.8x10^{-7}$), irrespective of lifestyle factors. The endometrial cancer risk increased the closer relatedness and younger the age of endometrial cancer diagnosis in relatives (Johnatty et al., 2017).

2.4.10 Endogenous serum hormonal levels

There is a strong association between circulating sex hormones and endometrial cancer. Results from the prospective study within the New York University Women's Health Study cohort revealed that endometrial cancer risk was increased with higher levels of oestradiol, percent free oestradiol and oestrone while the risk of endometrial cancer was reduced with higher levels of SHBG. After adjusting for height and BMI, these associations persisted (Zeleniuch-Jacquotte et al., 2001).

In a study to determine whether the level of sex hormones differed between type 1 and type 2 endometrial cancer, Wann et al. concluded that the levels of oestradiol, progesterone, testosterone, FSH and LH were not different between the two histological types (Wann et al., 2016). Ashihara et al. however found that, although the levels of serum oestradiol collected from the peripheral vein did not differ between type 1 and type 2 endometrial carcinoma, women with type 1 endometrial cancer had higher level of oestradiol in the ovarian vein (Ashihara et al., 2014).

Data from the EPIC cohort study showed that the hormones oestrone, testosterone and postmenopausal oestradiol were higher in patients with endometrial cancer compared to controls (Dossus et al., 2013). Hyperandrogenism has been debated as a cause for endometrial cancer however it has not been shown to be a significant risk factor after adjusting for oestrogen (Kamal et al., 2016).

2.5 The interrelationships of various characteristics with breast and endometrial cancer

The preceding review confirms that there are similar risk factor associations between breast and endometrial carcinomas., summarised below:

Table 2.1 Clinical/biochemical risk factors identified from literature review

Risk factor	**Breast carcinoma**	**Endometrial carcinoma**
Biological Risk Factors		
Peak age (Western Europe)	65-69 years	65-69 years
Medical conditions		
DM	inconsistent	Inconsistent
High total cholesterol	↑	↑
Low HDL	↑	↑
Triglycerides	Inconsistent	↑
Hypertension	↑	↑
Metabolic syndrome	↑	↑
Family history of respective cancer	↑	↑
Tobacco consumption	↑	↓
Alcohol consumption	↑	inconsistent

Obesity	↑	↑

Table 2.2 Hormonal Risk factors identified from literature review

Risk factor	**Breast carcinoma**	**Endometrial carcinoma**
Hormonal Risk Factors		
Parity - nulliparity	↑	↑
Menstrual history		
Early menarche	↑	↑
Late menopause	↑	↑
PCOS	inconsistent	↑
Exogenous hormones		
combined HRT	↑	↓
oestrogen only HRT	inconsistent	↑
COCP	↑	↓
Progestin-only contraception	inconsistent	↓
Endogenous biochemical levels		
Oestradiol	↑	↑
Testosterone	↑ in ER positive	inconsistent
SHB	↑ in ER positive	↓

2.6 Role of genetic factors in breast carcinogenesis

Genetic factors do play a role in breast carcinogenesis. 27% of breast cancer cases were found to be attributed to hereditable factors (Lichtenstein et al., 2000) and 12% of women with breast cancer had one family member diagnosed with breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). Women who have one or more first degree relatives affected with breast cancer are at an increased risk for breast cancer than those who do not. The lifetime increased risk of breast cancer is 5.5% if one first-degree relative is affected and 13.3% if two first–degree relatives had a history of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

Inherited mutations of a BReast CAncer gene (BRCA) 1 or 2 increases the risk of female breast and ovarian cancers. These genes produce tumour suppressor proteins which are responsible for DNA repair and stability. If a woman is BRCA 1 or 2 mutation carrier, the average cumulative risk for breast cancer by age of 70 years is 65% and 45% respectively (Antoniou et al., 2003). Other gene mutations that predispose to breast cancer include: CHEK2, ATM, BRIP1, and PALB2 (rare mutations that confer an intermediate breast cancer risk) and inherited gene mutations related to Peutz-Jeghers syndrome, L-Fraumeni syndrome and Cowden syndrome (Turnbull & Rahman, 2008).

Several studies evaluated the role of genes related to obesity and the metabolic syndrome with breast cancer risk. In a study to investigate the association between

the obesity-related single nucleotide polymorphisms and breast cancer risk, it was concluded that MC4R rs17782313 did show increased breast cancer risk but FTO rs1121980 and rs9939609 did not show any correlation. However, it was observed that the interaction of FTO and MC4R polymorphisms did increase significantly breast cancer risk, having a 4.59-fold increased risk with the allele combination C/T/C (FTO rs1121980/FTO rs9939609/MC4R rs17782313) (da Cunha et al., 2013). FTP rs9939609 polymorphism was implicated in increased breast cancer risk in Iranian overweight women (Saeid et al., 2021). The relation of FTO gene SNPs with breast cancer is not consistent in the literature. In a study to determine the association of FTO gene mutations in Chinese population, FTO rs16953002 AA genotype significant increased breast cancer risk compared to GG genotype, while FTO rs1477196 AA genotype showed significant decreased breast cancer risk compared to GG genotype (the latter association was only found in women with BMI < 24 kg/m2) (Zeng et al., 2015). Results of a meta-analysis to determine the association of FTO polymorphisms and breast cancer risk showed no association between FTO gene rs9939609 polymorphisms and breast cancer risk (Jafari Nedooshan et al., 2017).

Kaklamani et al. were the first to investigate the relationship of genetic variants of the obesity-associated gene ADIPOQ with breast cancer risk, a study that was performed in New York City. Two functional SNPs +276 G→T (rs1501299) and +45 T→G (rs2241766) were found to be associated with increased risk of breast cancer (Kaklamani et al., 2008). However, these findings were not reproduced in a study of Turkish breast cancer patients (Erbay et al., 2016).

In a study by Jung et al., the GWAS meta-analysis-identified SNPs for IR phenotypes in white postmenopausal women were correlated with breast cancer risk. Out of the 58 loci that were found to reach genome-wide significance, 29 loci were associated with breast cancer risk. SNPs associated with increased risk of breast cancer included 3 SNPs in G6PCs (one of them being rs13431652 index SNP), SNP in NR5A2 (rs10919774 index SNP), 24 SNPs in MTRR/LOC729506 (one of them being rs1885458) and SNP in DOCK1 (Jung et al., 2018).

IL-6 gene polymorphisms were also found to be significantly associated with breast cancer. However, no association was found between polymorphisms within IL1 gene cluster and breast cancer risk (Hefler et al., 2005). IL-18 promoter polymorphism was also reported to increase breast cancer susceptibility (Back et al., 2014).

Chemokines induce inflammation, changing the tumour micro-environment and play a role in tumour growth and metastasis. Genotype GG of CXCL12rs1801157 polymorphisms was found to be more prevalent in breast cancer patients in the Pakistani population, thus allele G is possibly associated with increased breast cancer risk (Khalid & Hanif, 2017).

2.7 Role of genetic factors in endometrial carcinogenesis

Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer – the most common cause of hereditary colorectal cancer), an autosomal dominant inherited cancer susceptibility syndrome is associated with 2.3% of endometrial cancers (Hampel et al., 2007). It is caused by germline mutation in DNA mismatch repair genes: MLH1, MSH2, MSH6 or PMS2. Mutations in MLH1 and MSH2 have high penetrance, i.e. are associated with high lifetime risk of Lynch Syndrome. MLH2 gene mutation accounts for 50-60% of Lynch syndrome associated endometrial cancer (Bonadona et al., 2011).

Multiple genetic markers for obesity, obesity-related SNPs, were correlated with endometrial cancer risk. 7 loci were significantly associated with endometrial cancer risk, even after adjustment for BMI. These included loci in the FTO gene, MC4R and TMEM18 (Olson, 2002).

FTO gene was found to be overexpressed in endometrial carcinoma tissue. The increased oestrogen production such as that associated with obesity caused increased FTO nuclear accumulation and enhanced endometrial cancer cells proliferation (Zhu et al., 2016). In a meta-analysis involving databases from January 1984 to April 2015, it was suggested that FTO gene polymorphism (rs9939609) may be associated with increased endometrial cancer risk (Huang et al., 2017). FTO rs8050136 polymorphism or FTO rs12927155 were however not found to be associated with endometrial cancer carcinogenesis (Zhao et al., 2016; Gaudet et al.,

2010). In a meta-analysis looking at nine case-control studies involving 3601 non-Hispanic white women with endometrial carcinoma and 5275 controls, FTO rs9939609 AA genotype was also positively associated with endometrial cancer risk (OR 1.17, CI 1.03-1.32). However, after adjusting for BMI, this association was no longer apparent – suggesting that FTO rs9939609 is a potential susceptibility marker in women at higher risk of endometrial cancer (Lurie et al., 2011).

The Shanghai Endometrial Cancer genetics study concluded that loci within MC4R gene and TMEM18 are associated with increased endometrial cancer risk (Lurie et al., 2011). Both MC4R gene and TMEM are responsible for the regulation of appetite and food consumption (Huang, T. et al., 2017; Larder et al., 2017). MC4R rs17782313 was not however associated with higher endometrial cancer risk (Lurie et al., 2011).

Genes related to inflammatory markers, e.g., interleukins, were also investigated regarding endometrial cancer risk. IL-6 gene polymorphisms, -174 CC genotype and IL-6 -174 C allele were found to have a positive association with endometrial cancer (Wang et al., 2016). IL-32, which is responsible for the activation of other inflammatory mediators, is also associated with increased endometrial cancer risk, through its gene polymorphisms: TT genotype of rs28372698 and CC genotype of 12934561 (Yu et al., 2015).

2.8 Developing a personalized approach for early detection and prevention of breast and endometrial cancer

The aim of prevention strategies is to decrease the chance of developing breast and/or endometrial cancer or their complications. Thus, focus is on promotion of good health and prevention of these diseases and their associated morbidity and mortality. There are three levels of prevention strategies that can be implemented to decrease these hormone-dependent malignancies.

2.8.1 Tertiary prevention

Tertiary prevention is implemented in patients who already have developed symptoms. It aims to reduce disease severity and its associated morbidity, to slow/stop disease progression by modalities such as chemotherapy or rehabilitation and to prevent disease complications by screening for complications (Kisling and Das, 2022).

2.8.2 Secondary prevention - Screening and early detection

Secondary prevention occurs in the form of screening. It targets healthy appearing individuals before the onset of signs and symptoms and aims to diagnose disease in its early subclinical state (Kisling and Das, 2022). Prevention of breast and endometrial cancer remains challenging with early diagnosis being one of the best approaches to improve prognosis and survival rate.

Mammography has been used for screening of breast cancer since 1980s with the aim of early detection and better treatment outcome (Winters et al., 2017). The European Commission Initiative on Breast Cancer (ECIBC) recommends mammography screening (for women who are asymptomatic with an average breast cancer risk) every 2-3 years for women aged 45-49 years, every 2 years for women aged 50-69 years and every 3 years for women aged 70-74 years. Digital breast tomosynthesis can be used as screening tool instead of digital mammography for women aged 50-69years (European Commission Initiative on Breast Cancer, 2022). The American Cancer Society (ACS) on the other hand, recommends annual screening with mammography from 45-54 years, while women aged 40-44 years who wishes to screen should also have the opportunity for annual screening. Women aged 55 years or more are recommended to undergo biennial screening or choose to continue annual screening (Smith et al., 2017). Women at high risk of breast cancer (including women with a known BRCA gene mutation, women treated with chest radiation) are advised to carry out annual mammography and MRI (Smith et al., 2017)

In a study to determine the effectiveness of mammography to reduce the stage specific incidence of breast cancer in Dutch women who had been invited to biennial screening (between 1989-2012) concluded that this screening programme had minimal impact on the incidence of advanced breast cancer (Autier et al., 2017). Autier et al debated the use of mammography as the screening tool for breast cancer mainly due to overdiagnosis (of around 20% or more of breast cancer cases diagnosed with screening) and the improved treatment strategies even for patients with advanced disease (Autier & Boniol, 2018).

There is no recommended screening test for endometrial cancer for asymptomatic women. Transvaginal ultrasound assessment of the endometrial thickness in postmenopausal asymptomatic women did not show a high predictive value for endometrial cancer. Consideration of the patient's risk factors for endometrial malignancy should also be carried out and referral for hysteroscopy and biopsy if malignancy suspected (Tsikouras et al., 2016).

In view that endometrial cancer typically carries an early presentation and high detection rates (85%), it makes it unlikely for a screening test to detect it an earlier stage, reducing mortality rate (*National Cancer Institute.* 2021). The ACS advices that postmenopausal women should be aware of the risks and the presenting symptoms of endometrial cancer and to seek advice if symptoms arise. Screening with endometrial biopsy (starting testing at 35years) is only advocated for asymptomatic women who have or are at very high risk of endometrial cancer including known/suspected Lynch syndrome mutation (Smith et al., 2017; Morrison et al., 2021).

2.8.3 Primary Prevention – identifying population at risk and development of personalised risk-stratified breast/endometrial cancer prevention strategies

The word 'primary' means earliest – it refers to measures that are implemented before the disease occur; reducing/eliminating risk factors and promoting measures that can be protective for the disease. The multinational European study - Health Lifestyle in Europe by Nutrition in Adolescence (HELENA) study, for

example is looking at aspects including: the dietary intake, physical activity, body composition, nutritional status and genotype in adolescences to aid development of strategies for promotion of healthy lifestyles and disease prevention (HELENA Study).

Epidemiological studies helped to provide a better insight about the risk factors of breast/endometrial cancer, and thus a better understanding of the pathogenesis of these hormone dependent malignancies. Such risk factors include increasing age, history of early menarche and late menopause, family history of breast/uterine cancer, obesity and being physically inactive (*Gynecologic Cancers|CDC* 2021). Three components can be proposed to be play a role in the pathogenesis of postmenopausal breast cancer and endometrial cancer. These include metabolic syndrome/insulin, steroids/hormonal, and inflammation. Risk factors related to these components of carcinogenesis can be used to develop risk assessment tools to identify patients' risk of developing the respective cancer.

Figure 2.14 is showing a schematic showing a personalised approach for early detection and prevention of breast cancer. It involves:

1) Risk assessment: Women are initially assessed using a validated risk assessment tool to determine their estimated risk.
2) Risk stratification: Women are then stratified into risk groups – low, intermediate, high and very high risk.
3) Risk tailored intervention: In the case of breast cancer, this may include early mammography screening, supplemental screening with other modalities or

different screening intervals. Women at high risk will be offered prophylactic/primary treatment (medical/surgical) (Figure 2.14)

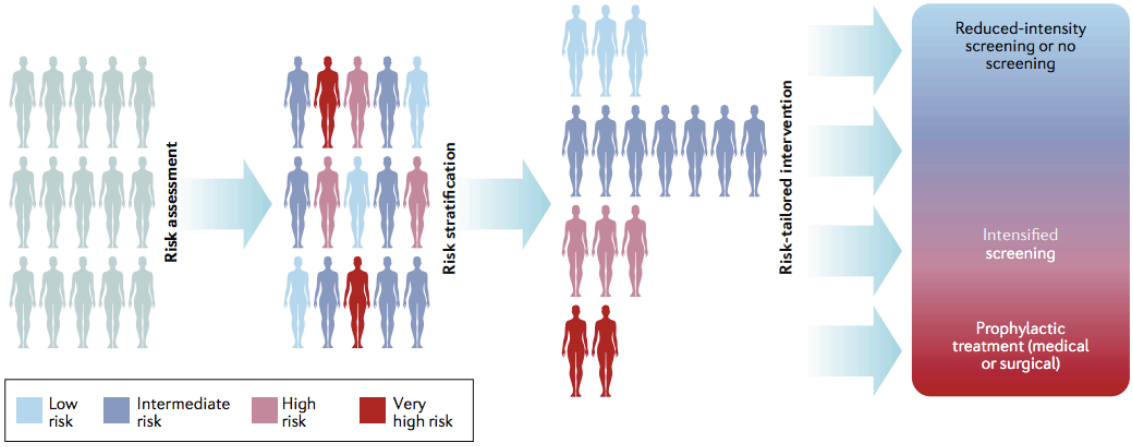


Figure 2.14 A schematic showing a personalised approach for early detection and prevention of breast cancer. Women are initially assessed using a validated risk assessment tool to determine their estimated risk. Women are then stratified into risk groups – low, intermediate, high and very high and will receive risk-tailored interventions.

Several risk-prediction models for use in women in the general population to determine breast cancer risk have been developed. The Gail model (National Cancer Institute's Breast Cancer Risk Assessment Tool) is a population-validated breast cancer risk assessment tool which has proved to give good results in predicting the 5-year risk of developing invasive breast cancer in women with no previous history of any breast cancer or carcinoma in situ and in women who have no genetic mutation that is associated with increased breast cancer risk (including BRCA1 and BRCA2). The expected/observed invasive breast cancer ratio was calculated to be 1.09 (CI 1.00-1.18). The following parameters are used in this assessment tool: age, ethnicity, previous breast biopsies (number of breast biopsies and whether there was any atypical hyperplasia), age of menarche, age of first live birth and number of first-degree relatives who had breast cancer. An elevated breast cancer risk is

defined as a calculated Gail Model 5-year risk >1.7%. These patients are candidates for consideration of possible interventions for risk reduction (Nickson et al., 2018). The Breast Cancer Surveillance Consortium (BCSC) (Tice et al., 2008) included 1,095,484 women undergoing mammography and incorporated also the BI-RADS breast density (radiological assessment of breast tissue density on mammogram) apart from age, race/ethnicity, family history (first relative) of breast cancer and history of breast biopsy in the prediction model. After 5.3years of follow-up, 14,766 women were diagnosed with invasive breast cancer, with an expected/observed ratio of 1.03 (CI 0.99-1.06). This model is however not applicable to patients who had previous breast cancer/DCIS/breast augmentation/mastectomy or are younger than35/older than 74years.

As discussed above, breast cancer and endometrial cancer have multiple established risk factors – lifestyle and anthropometric factors, hormonal and biochemical factors, family history and genetic variants. Polygenic risk scores (PRS) summarise the combined effect of the total number of SNPs included in the score and are currently being used to predict breast cancer risk.

In a study using data from the Breast Cancer Association Consortium (BCAC) including 94,075 breast cancer subjects and 75,017 controls of European ancestry, PGSs were evaluated to determine which PGS can be used in the prediction of breast cancer. The best performing PGS (313 SNPs) has an odds ratio for overall disease of 1.61 (95% CI: 1.57-1.65) and the lifetime risk of overall breast cancer is 32.6% (in the top centile of PRSs) (Mavaddat et al., 2019).

Risk models including clinical data, epidemiological pathological and genetic for breast cancer are developing to help predict more accurately cancer risk. B-CAST and BRIDGES (Horizone et al., 2020) are datasets that provide a platform for estimating breast cancer risk and have been used to develop breast cancer risk prediction models (Individualized Coherent Absolute Risk Estimator, iCARE and BOADICEA) (Choudhury et al., 2020; Lee et al., 2019).

Work is also being done to develop efficient models for early diagnosis and prediction of endometrial cancer. An epidemiological risk model conducted on 201,811 Western European women (from eight countries in the European Prospective Investigation into Cancer and Nutrition cohort), including BMI, menopausal status, age of menarche, age at menopause, OCP use showed a predicted efficiency over 5years of up to 77% (Husing et al., 2016). A retrospective case-control risk model carried out on women in central China had a higher prediction accuracy rate – 91.17% for the internal validation set efficacy (Wang et al., 2022)

Such work is lacking on the Maltese population. Logistic regression models for risk factors for breast and endometrial cancer which are obtained from this study can be used to formulate risk assessment models for breast and endometrial cancer specific for the Maltese population. By calculating the odds ratio of the individual risk factors, the population at increased risk of breast/endometrial cancer can be identified.

2.9 Aims and Objectives

The hormone-dependent female malignancies have been shown to be linked to the metabolic status and to the genetic profile of the individual. These malignancies are common accounting for a significant degree of worldwide morbidity and mortality in the female population.

2.9.1 Research Question

> Can a composite screening tool using biological risk factors, metabolic, hormonal or genetic markers related to the metabolic syndrome be developed to identify women at increased risk of developing these hormone-dependant malignancies in the Maltese population?

2.9.2 Aims

- In the light of the literature review, this study aims to investigate which markers: - biological risk factors, biochemical markers of the metabolic syndrome, hormonal or genetic markers – are associated with breast and/or endometrial cancer.
- Also, it is aimed to evaluate the association between established polygenic risk scores related to type 2 diabetes and insulin resistance and the risk of postmenopausal hormone driven malignancies

- To compare the performance of a polygenic risk score relative to clinical/anthropometric predictors in classifying cancer from control patients

2.4.3 Objectives

- To recruit a case population comprising patients with postmenopausal breast and endometrial cancer, and matched controls using defined inclusion and exclusion criteria
- To describe clinical and biochemical characteristics of the case and control population, and compare the prevalence of the markers of the metabolic syndrome: biological risk factors, metabolic and genetic factors in patients with breast and endometrial cancer as compared to the general population.
- To extract genomic DNA and undertake low-pass whole genome sequencing, on the case and control population to derive genome-wide SNP genotype data in the study population.
- Following appropriate quality control procedures, to extract SNP genotype data from the sequenced data. The choice of SNPs was based on two defined polygenic risk scores related to insulin resistance and type 2 diabetes derived from the literature.
- To explore the association between aggregate polygenic risk scores and the risk of endometrial and breast cancer using statistical modelling.

Chapter 3

Methods

3.1 Research plan

The study was a retrospective, non-interventional, case control study. It did not in any way interfere with the routine management of either breast or endometrial cancer offered to the women.

3.1.1 Definition of study cohort

Subjects who satisfied the inclusion/exclusion criteria described below were recruited. These subjects were traced from the histopathological records of Mater Dei Hospital, the only University teaching hospital in Malta. Women who were non-Maltese (of non-Maltese genetic origin) were excluded. For the cancer cohorts, premenopausal patients with confirmed breast/endometrial malignancy were excluded. The normal cohort was recruited through the registry of hysterectomy specimens with normal findings – premenopausal patients and those with history of breast malignancy were excluded from this cohort. The author personally called all these subjects starting from the ones with the most recent diagnoses, and these were invited to enrol in the study. There was no particular selection. Details regarding the study was given as well as further details on the tests being carried out. These subjects attended Mater Dei Hospital and the author of the study carried out the interview, measured anthropometric data and carried out venepuncture for blood sample collection.

3.1.2 Inclusion Criteria

A sample of three study populations was recruited:

- Study Group 1: Patients with a history of endometrial carcinoma.

- Study Group 2: Patients with a history of breast carcinoma.
- Study Group 3: A control group including women with histologically confirmed absence of endometrial carcinoma (patients undergoing hysterectomy and result was benign) and have no history of breast carcinoma.

All the patients recruited were postmenopausal (i.e. had cessation of menstruation for 12months or more) patients of Maltese ethnicity (of Maltese genetic origin, identified by their national identity card number which incorporates place of birth code and their ability to communicate in the Maltese language).

3.1.3 Exclusion Criteria

- Premenopausal patients
- Non-Maltese patients

3.1.4 Ethics

Ethical approval was obtained from the University of Malta Ethics Committee Board (UREC number 44/2016). A copy of the ethics approval is included in appendices (appendix 1). All subjects signed an informed consent for participation in this study, including for bloodletting (for biochemical/hormonal/genetic testing). The sample bottles were pseudonymised, with the primary identifier being securely filed by the principal investigator.

3.1.5 Consent

Subjects that accepted to participate in the study received an information sheet about the study, including the aim and importance of the research. By participating

the patients were tested for medical conditions, and in the event of newly diagnosed condition is found – referral to relevant physician is done. It was explained that the blood tests taken for genetic analysis will help give more insight on the genetic risk for breast/endometrial cancer. The procedure involved, and the risks/inconvenience and benefits incurred by the study were also discussed in the information sheet. They were informed that participation was on voluntary basis and that all information collected was confidential. The contact number of the investigator was included in the information sheet should they have any queries. They were then asked to sign a written consent form in which subjects gave permission to use the information and their blood sample for the study (appendix 2 and 3).

3.1.6 Measures to minimise bias

The three study cohorts were identified from the histological registers of the department of pathology at MDH and were invited to participate by the researcher. All patients who met the inclusion criteria and exclusion criteria and agreed to participate were enrolled. The direct contact of the researcher with the patient at invitation may have brought in an element of participation bias through indirect persuasion. However, such bias should not have in any way influenced the observations. While the control group was confirmed by histological study of hysterectomy specimens, such was not the case in regards to breast cancer and assumption of absence of disease was only based on a negative past history.

3.1.7 The study population

From around 600 patients contacted, 300 patients accepted to be recruited in this study - 132 patients were diagnosed with breast cancer; 90 patients were diagnosed with endometrial cancer (four patients had both endometrial and breast cancer- these cases developed endometrial malignancy after they were recruited in the study while on treatment for their primary malignancy) and 82 patients had normal histological findings. A smaller proportion of women with normal histological findings accepted the recruitment invitation compared to the cancer population. Recruitment was carried out over four years; it was affected by the COVID pandemic and the imposed measures implemented by the public health department to decrease hospital attendance to the bare essentials. According to sample power calculations, the ideal study number was 383 (calculated as per formula below). Bigger numbers especially in the control group would potentially have resulted in more statistically significant results.

$$\text{Sample size} = \frac{\frac{z^2 \times p\,(1-p)}{e^2}}{1 + \left(\frac{z^2 \times p\,(1-p)}{e^2 N}\right)}$$

N = population size; e = Margin of error (percentage in decimal form); z = z-score. The z-score is the number of standard deviations a given proportion is away from the mean (Sample size calculator)

3.2 Biological profile of the populations

3.2.1 Clinical data collection

Each subject was interviewed and information regarding relevant previously described risk factors for endometrial and breast malignancy was obtained, including: history of hypertension, diabetes mellitus type 2, parity, history of

hormonal treatment or tamoxifen use, history of polycystic ovary syndrome, family history (first degree relatives) of hypertension/diabetes type 2/respective cancer and reproductive factors such as: parity, age of menarche/menopause, breastfeeding. When analysing menopause and reproductive age, patients who had undergone surgical menopause (after hysterectomy) as well as women who had cessation of menses following chemotherapy were excluded. (The interview template/information sheet is included in the appendices, (appendix 4).

3.2.2 Anthropometric data collection

The following anthropometric data was measured according to established protocols (John A., 1995).

- *Standing height (cm)*: the subject stood erect on a calibrated clinical stadiometer, with both heels together and weight evenly distributed on both feet and the head in the Frankfort horizontal plane (a line passing horizontally from the ear canal to the lowest point of the eye orbit parallel to the floor) while the horizontal bar is lowered to the highest point of the subject's head
- *Weight (kg)*: the subject in lightweight clothes and with shoes removed, weight was recorded using an electronic scale. The weight and height were measured on the same scale.
- *Waist circumference (cm)*: measured in standing position with back straight. A measuring tape is aligned with the top of the hip bone and wrapped around the waist, midway between lower margin of last palpable rib and superior border of the iliac crest. It was ensured that the tape is parallel to the floor

and not twisted. The measurement was taken on exhalation and to the nearest 0.5cm.

- *Hip circumference (cm):* measured in the standing position using a measuring tape, at the level of maximum extension of the buttocks, also taken to the nearest 0.5cm.
- *The Body mass index and waist-to height ratio* were calculated.

3.3 Blood sample collection

All patients underwent phlebotomy for blood investigations on one occasion in the morning after an overnight fast of around 12-14 hours (water only was allowed) by the same observer (the author of the study). Following forearm venepuncture, samples were taken from each patient for the following:

- K_2-EDTA vacutainer bottles for complete blood count, Haemoglobin A1c (HbA1c) and for DNA determination.
- Serum separation tube for measurement of lipid profile (including HDL, LDL, total triglycerides, and cholesterol), hormone profile (including testosterone, progesterone, LH, FSH, prolactin, oestradiol and SHBG) and for fasting serum insulin. The latter is taken so as to calculate Homeostatic model of insulin resistance (HOMA-IR) assay, to quantify insulin resistance.
- Fluoride oxalate tube for fasting glucose analysis.

The EDTA bottle for DNA determination was stored at -20degrees centigrade till DNA extraction was carried out. The serum bottle (to be used for insulin assay) was inverted 5 times, allowed to clot for a minimum of 30minutes and transferred to the

laboratory in a portable cooler. It was then immediately centrifuged at 2000g (relative centrifuge force, rcf) for 10minutes. The serum was then separated from the clot using a sterile Pasteur pipette, aliquoted in labelled 100µL containers and frozen to -80degrees centigrade till subsequent analysis. All samples were processed according to good laboratory practice guidelines.

3.4 Biochemical profile of the populations

Information regarding the analytic procedures of the respective tests was collected through the manuals of the individual analysers.

3.4.1 Complete blood count analysis

The complete blood count analysis was carried out using a multiparameter automated haematology analyser from Sysmex – XN-Series. A specific reagent in Lysercell WNR causes modification of phospholipids on the cell membrane and haemolysis of reticulocytes and mature red blood cells (that do not have a nucleus). It also differentially disrupts the cytoplasmic membrane of nucleated red blood cells and white blood cells. Then a labelling fluorescent agent in Fluorocell WNR was used to stain organelles and nucleic acids. The stained cell intensities are expressed as 2D scattergram, known as WNR scattergram, with the vertical axis corresponding to the forward-scattered light, FSC (which represent cell size) and horizontal axis corresponding to the side fluorescent light, SFL (which represent cell size). Different cell types have different FSC and SFL intensities and thus will locate on different areas of the scattergram (Matthews et al., 1985).

3.4.2 Glucose assay

Glucose estimation was analysed using Roche/Hitachi cobas c analyser utilizing a hexo-kinase assay and UV test. Hexokinase is used to catalyse the phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate is then oxidized by NADP glucose-6-phosphate dehydrogenase to gluconate-6-phosphate. The NADPH produced by this reaction is measured photometrically; this is directly proportional to the glucose concentration.

3.4.3 Cholesterol assay

Cholesterol estimation was done on the cobas c analyser which utilizes an enzymatic colorimetric assay. Oxidizisation of cholesterol produces cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxide, in the presence of peroxidase, is then involved in the oxidative coupling of phenol and 4-aminophenazone to form a red quinone-imine dye. Cholesterol concentration is directly proportional to the colour intensity of the dye, which is determined by measuring the increase in absorbance at 500-550nm (Allain et al., 1974) .

3.4.4 HDL-Cholesterol assay

Roche/Hithachi cobas c system was used for HDL-cholesterol assay, utilizing a homogenous enzymatic colorimetric test. LDL, VLDL and chylomicrons selectively binds with dextran sulphate and magnesium ions to form water-soluble complexes which are resistant to polyethylene glycol (PEG)-modified enzymes. In the presence of water, PEG-cholesterol esterase acts on HDL-cholesterol esters to break it down quantitatively into free cholesterol and fatty acids. PEG-cholesterol oxidase acts on

HDL-cholesterol in the presence of oxygen, oxidizing cholesterol to Δ^4-cholesterone and hydrogen peroxide. The hydrogen peroxide generated reacts with 4-amino-antipyrine and High Sensitivity Direct Agglutination (HSDA) in the presence of peroxidase to form a purple-blue dye. The cholesterol concentration is directly proportional to the colour intensity and is measured photometrically (Mori et al., 1992).

3.4.5 LDL-Cholesterol assay

Quantitative determination of LDL-Cholesterol was performed using Roche/Hitachi cobas c system, using an enzymatic colorimetric assay. LDL cholesterol esters are first broken down into free cholesterol and fatty acids by the enzyme cholesterol esterase. Free cholesterol is then oxidized by cholesterol oxidase to form Δ^4-cholesterone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide reacts with 4-aminoantipyrine and HSDA to form a blue-purple dye. The colour intensity of the purple-blue dye is measured photometrically and is directly proportional to the cholesterol concentration.

3.4.6 Triglycerides assay

Triglycerides assay was performed on cobas analyser using an enzymatic colorimetric assay. Triglycerides are hydrolysed to glycerol and free fatty acids (by the action of lipase). Glycerol kinase then phosphorylates glycerol to glycerol-3-phosphate. Oxidation of glycerol-3-phosphate produce dihydroxyacetone phosphate and hydrogen peroxide, in a reaction catalysed by glycerol phosphate. The reaction of hydrogen peroxide with 4-aminoantipyrene (4-AAP) and N-Ethyl-N-

[3-sulphopropyl]-m-anisidine (ESPA) using a peroxidase, results in a purple colour, which can be measure at absorbance of 540nm (Nägele et al., 1984).

3.4.7 Insulin assay

The quantitative determination of insulin was done on a cobas e analyser, using an immunoassay. The first incubation involves insulin, a biotinylated monoclonal insulin-specific antibody, and a monoclonal insulin-specific antibody (labelled with a ruthenium complex). For the second incubation, streptavidin-coated microparticles are added. The complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell. This is followed by magnetic transfer of microparticles onto the surface of the electrode. ProCell/ProCell M then removes unbound substances. A voltage is applied to the electrode, inducing chemiluminescent emission that is measured by a photomultiplier.

3.4.8 HbA1c assay

HbA1c assay was performed on Roche/Hithachi cobas c system, to determine the quantitative amount of mmol/mol haemoglobin and % HbA1c in the whole blood. A haemolysing reagent containing a detergent – tetradecyltrimethylammonium bromide (TTAB) is used to eliminate leucocytes; leucocytes are not lysed by TTAB. HbA1c and haemoglobin levels are determined from the obtained hemolysate by two independent reactions:

HbA1c: HbA1c reacts with anti-HbA1c specific antibody (R1) to form a soluble antigen-antibody complex. Since the HbA1c molecule has only one epitope per b-

globin for specific antibody testing, insoluble immune complex formation does not take place. Then R3, buffer/polyhapten, is added. The polyhapten has numerous epitopes per molecule and reacts with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complexes which can be measured turbidimetrically at 340nm.

Haemoglobin: the released haemoglobin in the haemolyzed sample is converted to a derivative that can be measured spectrophotometrically.

The final result is expressed as mmol/mol HbA1c or % HbA1c and is calculated from HbA1c/Hb ratio as follows:

mmol/mol HbA1c according to IFCC: HbA1c (mmol/mol) = (HbA1c/Hb) x1000

% HbA1c according to DCCT/NGSP: HbA1c (%) = (HbA1c/Hb) x 91.5 + 2.15

3.4.9 Homeostasis model of assessment insulin resistance - HOMA-IR

HOMA-IR gives an indication of the presence and the extent of insulin resistance, the higher HOMA-IR the more the insulin resistance. It can be calculated by multiplying fasting blood glucose (mmol/ml) and fasting insulin level (μU/ml) and the result divided by 22.5.

3.5 Definition of metabolic syndrome

The cut-off levels for the criteria of the metabolic syndrome were taken according to International Diabetes Federation (IDF) metabolic syndrome definition. Patients' characteristics were correlated with these criteria cut-offs (Table 3.1). According to IDF, patients will be diagnosed with metabolic syndrome if they have central obesity

and two of the other factors. If BMI is equal or more than 30 kg/m^2, it is classified as central obesity and waist circumference is not relevant.

Table 3.1 International Diabetes Federation metabolic syndrome (Alberti et al., 2006)

Central obesity	Waist circumference, females (Europoids) ≥ 80 cm as a measure of central obesity
Raised triglycerides	≥ 1.7 mmol/l (150 mg/dl) or specific treatment for this lipid abnormality
Reduced HDL-cholesterol	< 1.29 mmol/l (50 mg/dl) in females or specific treatment for this lipid abnormality
Hypertension	Systolic: ≥130 mmHg or Diastolic: ≥ 85 mmHg or treatment of previously diagnosed hypertension
Raised fasting plasma glucose	Fasting plasma glucose ≥ 5.6 mmol/l (100 mg/dl) or previously diagnosed Type 2 diabetes If > 5.6 mmol/l or 100 mg/dl, oral glucose tolerance test is strongly recommended but is not necessary to define presence of the syndrome

3.6 Hormonal analysis

One patient with normal histological findings was on hormone replacement therapy at the time of patient recruitment, and therefore she was excluded from this analysis. Information regarding hormonal analysis was obtained through Standard

Operation Procedures issued from Clinical Chemistry Section Mater Dei Hospital as well as through manuals of the individual analysers.

3.6.1 Oestradiol assay

Oestradiol concentration was determined using ADVIA Centaur XP Enhanced Oestradiol assay which uses a competitive assay format. A releasing agent is used to release endogenous oestradiol from its binding proteins. An anti-oestradiol monoclonal antibody labelled with acridinium ester is added to bind to the available oestradiol. An oestradiol derivative capture solid phase is then added – this will compete with oestradiol for the binding of the acridinium-labelled antibody. After washing, acid and base are dispensed, to cause a chemiluminescent reaction. The amount of oestradiol present is inversely proportional to the amount of relative light units detected.

3.6.2 FSH, LH, testosterone, SHBG and progesterone assays

FSH, LH, testosterone, SHBG and progesterone assays were carried out on Siemens Immulite 2000 XPi, using a solid-phase, chemiluminescent immunometric assay. Assay-specific antibody or antigen-coated polystyrene beads are used as solid phase. A bead is dispensed into a specially designed Reaction Tube. This Reaction Tube serves as a vessel for incubation, wash and signal development. The sample is first incubated with an alkaline phosphatase-labelled reagent and the reaction mixture is then separated from the bead by spinning the Reaction Tube. The fluid is transferred to a Coaxial Sump Chamber and four discrete washes occur. The bead remains in the Reaction Tube with no residual unbound label. The bound label is

quantified using dioxetane substrate. When this chemiluminescent substrate reacts with the alkaline phosphatase label bound to the bead, light is emitted. The amount of light emitted is detected by the Photomultiplier Tube and is proportional to the amount of the analyte.

3.6.3 Prolactin assay

Prolactin assay was carried out using ADVIA Centaur Prolactin assay. It is a two-site sandwich immunoassay using constant amounts of two antibodies and direct chemiluminometric technology. The first antibody used, in the Lite reagent, is a polyclonal goat anti-prolactin antibody labelled with acridium ester in buffer with sodium azide and preservatives. The second antibody is found in the Solid Phase and is a monoclonal mouse anti-prolactin antibody covalently coupled to paramagnetic particles in buffer. The blood sample is first mixed with Lite Reagent and incubated, followed by the addition of Solid Phase, and left for incubation. The system then automatically separates, aspirates, and washes the cuvettes with reagent water. Acid Reagent and Base Reagent is then added to initiate a chemiluminescent reaction. The amount of the relative light units detected by the system shows a direct relationship to the amount of prolactin in the patient's sample.

3.7 Genetic profile of the populations

3.7.1 DNA extraction from whole blood

DNA extraction and purification from whole blood was carried out using QIAamp Blood Midi (Spin Protocol). The procedure followed was that provided in the QIAamp® DNA Midi/Maxi Handbook (Qiagen 2015).

Firstly, the reagents were prepared. These included:

Table 3.2 Reagents used during DNA extraction using QIAamp Blood Midi

QIAGEN Protease stock solution	stored at 2-8^{o}C
QIAamp DNA Blood MIDI Kits	5.5 ml distilled water was pipetted into the vial of lyophilized QIAGEN Protease. Once dissolved, QIAGEN Protease remained stable for 2months stored at 2-80^{0}C
Buffer AL	Buffer AL was stored at room temperature (15-25^{0}C). Before use, it was mixed thoroughly by shaking.
Buffer AW1	Buffer AW1 was stored at room temperature (15-25^{0}C). Before first use, ethanol (96-100%) was added.
Buffer AW2	Buffer AW2 was stored at room temperature (15-25^{0}C). Before first use, ethanol (96-100%) was added.

Prior to the extraction process, the frozen blood samples were thawed on a rotator and mixed manually, ensuring that the sample was well-mixed and fully thawed.

Below are the procedure steps:

1. 100 μl QIAGEN Protease was pipetted into the bottom of a 15 ml labelled centrifuge tube.

2. 1 ml of blood was added and mixed briefly.

3. 1.2 ml of Buffer AL was added and mixed thoroughly by inverting the tube 15times followed by vigorously shaking for at around 1 minute. A homogenous solution forms when there is adequate lysis.

4. The sample was then left to incubate at 70^0C for 10minutes so that the DNA yield will reach a maximum.

5. 1 ml of ethanol (96-100%) was added to the sample and mixed by inverting the tube 10 times. This was then followed by vigorous shaking to ensure efficient binding and the yielding of a homogenous solution.

6. The sample was carefully transferred onto the QIAamp MIDI column placed in a 15 ml centrifuge tube. The cap was closed, and sample was centrifuged at 1850 x g (3000rpm) for 3 minutes. Care was taken to always hold the closed QIAamp Midi columns in an upright position to prevent liquid passing through the ventilation slots on the rims of the columns.

7. The QIAamp Midi column was then removed. The filtrate was discarded and the QIAamp Midi column placed back into the 15 ml centrifuge tube.

8. 2 ml of Buffer AW1 was carefully added to the QIAamp Midi column. Care was taken not to moisten the rim. The cap was closed, and the sample centrifuged at 4500 x g (5000rpm) for 1minute.

9. 2 ml Buffer AW2 was then carefully added (without moistening the rim) to the QIAamp Midi column. The cap was closed and centrifuged at 4500 x g (5000rpm)

for 15 minutes. This removes all traces of Buffer AW2 from the QIAamp Midi column before elution.

10. The QIAamp Midi column was placed in a clean 15 ml centrifuge tube, and the collection tube containing the filtrate was discarded. A wet paper tissue was used to wipe any spillage off QIAamp Midi column before it was inserted into the 15 ml centrifuge tube.

11. DNA is eluted by pipetting 200 µl of Buffer AE directly onto the membrane of the QIAamp Midi column and the cap closed. It was then left to incubate at room temperature for 5 minutes and centrifuged at 4500 x g (5000rpm) for 2 minutes.

3.7.2 Assessing DNA purity and quantification

UV spectrophotometry using NanoDrop 2000c spectrophotometer was used to enable assessment of DNA purification and concentration.

The pedestal surface of the spectrophotometer was initially wiped to remove any residue. Blanking was then done – by pipetting a total volume of 1.2 µL of the blank (Buffer AE) on the pedestral surface. A value of 0 ng/µL meant that the spectrophotometer was blanked and that there were no functional errors. After blanking, it was ensured that each DNA sample was well-mixed and the pedestal surface is cleaned again. 1.2 µL of the extracted DNA sample was then pipetted onto the spectrophotometer and the arm of the spectrophotometer put down. For each DNA sample, the concentration and absorbance ratio (A260/A280) were noted. The pedestal surface was wiped after every DNA sample and blanking was performed after 30 readings measured.

DNA samples with concentrations of 50 ng/μl or more and A260/A280 ratio between 1.8-2.0 were considered of good quality for further downstream analysis and genotyping.

In order to determine concentration of DNA, the spectrophotometer measures the absorbance at a wavelength of 260 nm (A260). The purity of extracted DNA can be affected by the presence of other protein or organic solvents from the DNA extraction process. Thus, the spectrophotometer also measures the DNA sample absorbance at a wavelength of 280 nm (A280) in order to retrieve the A260/A280 ratio. The smaller the ratio, the higher the amount of contamination that is present in the sample and the less pure the sample is.

An example of a spectrophotometric curve is given below. It is showing the absorbance curves of DNA samples 170 and 171 and of the elutes of 170 and 171. Purification concentration is adequate for all samples as evidenced by the nucleic acid concentration and the A260/A280 ratio. Extracted samples that did not meet the required parameters were re-extracted and re-analysed.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
2	170	Owner	18.05.2018 11:48:50	142,2	ng/µl	2,845	1,476	1,93	2,49	DNA	50,00
3	171	Owner	18.05.2018 11:51:33	112,6	ng/µl	2,252	1,187	1,90	2,54	DNA	50,00
4	170elute2	Owner	18.05.2018 12:06:14	87,3	ng/µl	1,746	0,943	1,85	2,06	DNA	50,00
5	171elute2	Owner	18.05.2018 12:07:43	69,3	ng/µl	1,387	0,753	1,84	1,96	DNA	50,00

Method Information

Parameter	Value
Workbook File	C:\Documents and Settings\Owner\My Documents\1852018 run1.twbk
Application	Nucleic Acid
Data range	220nm 350nm
Analysis wavelength	260 nm

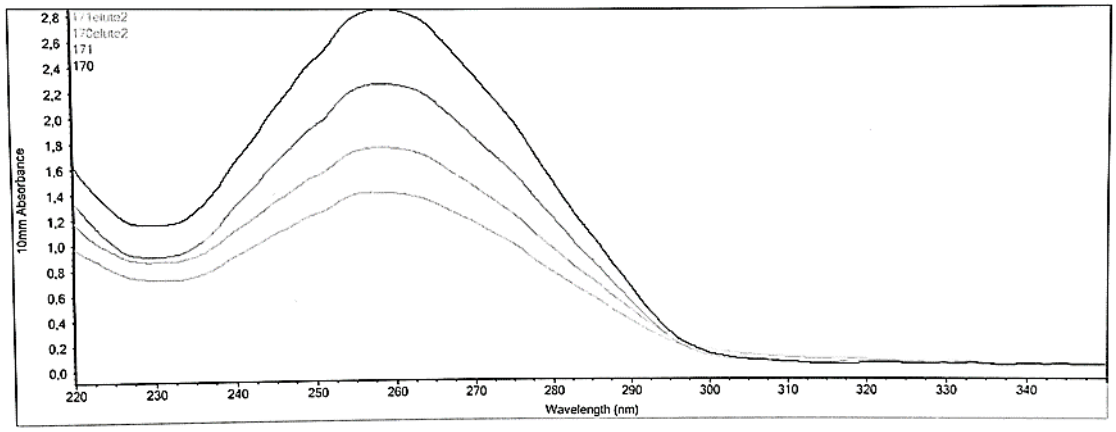


Figure 3.1 Sample of spectrophotometry result

3.7.3 DNA storage

The eluted DNA was stored in coded 0.5 mL screw-capped tubes in 96-well storage format (Micronic®) at -20°C for later use.

3.7.4 Whole Genome Sequencing

Figure 3.2 illustrates the workflow related to the genetic analysis. Steps 1-4 are already detailed above. After making sure that the guidelines for sample submission are met (Table 3.3), the extracted DNA was transferred and shipped using Therma freeze for Low-Pass Whole Genome Sequencing Analysis (LP-WGS) at GENEWIZ Azenta Life Sciences, Germany. LP-WGS was used as an alternative to single nucleotide polymorphisms (SNPs) array. This technique is ideal for rapid SNP

genotyping across the whole genome of the patients recruited. It is an innovative technology that has never been fully explored in local studies.

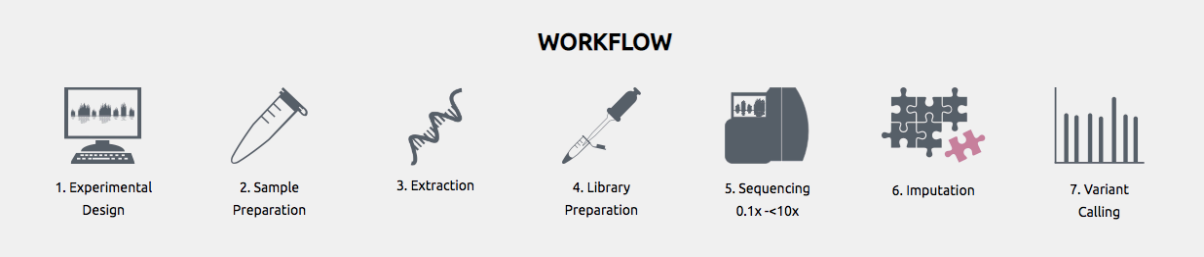


Figure 3.2 The workflow related to DNA analysis by low pass whole genomic sequencing analysis (LP-GWS) (GENEWIZ, 2022)

Table 3.3 Criteria met before transfer to LP-GWS (GENEWIZ, 2022)

Sample Type	Genomic DNA
Sample Purity (OD260/280)	1.8-2.0
Minimum Amount:	500 ng
Concentration	Normalized to 20 ng/µL
Resuspension Buffer	Water, elution buffer, or low TE (DNA Elution buffer)

At Genewiz, sequencing was carried out using Illumina platforms - a short sequencing read on the platform is aligned against genomic strips from human genome 38 (HG38). Sequencing is followed by genotype imputation, referenced against the 1000Genome Phase 3 imputation reference panel. It is 99% accurate in variant call detection when compared to the conventional genotyping arrays. Variant calling is the last step in LP-GWS where reads that are different from the reference genome, variants, are identified and saved as vcf file. (GENEWIZ, 2022; Pan et al., 2019; Koboldt et al., 2020)

Standard quality control was carried out – SNPs with minor allele frequency (MAF) less than 0.05 or alleles that were out of Hardy-Weinberg equilibrium ($p<0.0001$) were excluded. It was also ensured that individuals had a genotyping rate of >5%.

Principal components analysis (PCA) of the low-pass whole genome sequencing data, which was analysed in this study, was performed. The Maltese genome data was mapped to the reference PCA coordinates from samples of the Human Genome Diversity Project (HGDP) and to the imputed Population Reference Sample (POPRES) dataset as the reference panel for analysis of individuals of European ancestry.

In summary, the HGDP dataset includes genotypes across 632,958 autosomal SNPs for 938 unrelated individuals from 53 worldwide populations, and the POPRES dataset includes 318,682 autosomal SNPs for 1,385 unrelated individuals from 37 European populations (Novembre et.al, 2008; Li et.al, 2008; Wang et.al, 2015).
Estimation of individual ancestry is possible using LASER (Locating Ancestry from SEquence Reads). This program functions by directly analysing shotgun sequence reads without calling genotypes. Analysis is possible by constructing a sample-specific PCA map for sequence-read analysis of each individual. This overlaps with the reference PCA space (which is constructed using genotypes of a set of reference individuals). Overlapping of the sample specific PCA map with the reference PCA map allows the identification of the coordinates' ancestral background; making it ideal to correct for population stratification in GWASs (Wang et.al, 2015).

Published polygenic risk scores (of European ancestry) related to diabetes mellitus and insulin resistance were used to determine the association of these SNPs with increased risk of breast and/or endometrial cancer. Below is a summary of the references of the genetic risk scores used (Table 3.3). The choice of the GRSs was determined keeping in mind (A) the aims of this study - to investigate markers

associated with increased risk of breast and endometrial cancer, and to evaluate the association between established polygenic risk scores related to type 2 diabetes and insulin resistance and the risk of postmenopausal hormone driven malignancies (B) the finding from the logistic regression model that BMI is a strong predictor of both breast and endometrial cancer; both DM and insulin resistance are associated with high BMI.

Table 3.4 References of the genetic risk scores used in the study

	Authors	Description of literature
GRS 1	Polfus et al., 2021	Genome-wide association meta-analysis of type 2 DM - a unweighted GRS of 582 variants in risk loci for type 2 diabetes were used.
GRS 2	Aly et al., 2021	Genome-wide association and GRS analysis of diabetes and insulin resistance - a unweighted GRS consisting of 223 polymorphisms related to insulin resistance were used.

Each GRS, (defined by the aggregate sum of risk alleles at each locus) was z-transformed. Z-score transformation enables standardisation of the two GRS that have differing numbers of risk alleles and thus enables their direct comparison. Z score transformation was achieved as follows:

$$Z\ score = (\chi - \mu)/\ \sigma$$

where χ = score, μ = mean, σ = standard deviation

Z scores are divided into quintiles – Quintile 1 is regarded as the lowest (first) quintile, having the lowest number of risk alleles while quintile 5 has highest

number of risk alleles. Logistic Regression models were then used to estimate the odds ratios and 95% confidence intervals (CI) for the association between GRS quintiles and breast and/or endometrial cancer risk.

3.8 Statistical analysis

The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test for normality of a number of clinical variables (Table 4.1).

Graphic analysis using the normal Q-Q plot was used to assess for a normal distribution of quantitative variables. This showed that almost all continuous variables had a skewed non-normal distribution. Therefore, non-parametric tests were used and data was presented using medians and interquartile range (IQR) for continuous variables. Categorical variables were expressed as proportions (%). Statistical analysis was performed using IBM SPSS Statistics v24.

Mann-Whitney U-test and Kruskal-Wallis ANOVA tests were used to compare the distribution of continuous variables across two or three categories respectively. The chi-square test was used to compare categorical variables. Statistical significance was determined at cut-off p-values of $P<0.05$, as per convention.

The null hypothesis proposes that no statistical difference exists between samples and the difference observed is accidental; it is accepted if the p value exceeds the 0.05 level of significance. On the other hand, the alternative hypothesis proposes

that the difference observed is true and is accepted if the p value is less than the 0.05 criterion.

Logistic regression analysis, univariate and multivariate unconditional logistic regression models were carried out. Logistic models allow examination of the relationship between different variables of interest, where the dependent/outcome variable was group – breast cancer group, endometrial cancer group or normal group. Such analysis identifies which factors have the greatest impact on the development of breast or endometrial cancer. It calculates the crude odds ratios (ORs) and their 95% confidence intervals (C.I.) to evaluate the effects of different factors on cancer risk. ORs measure the association between an exposure and an outcome as well as the magnitude of the effect of the respective risk factors on disease outcome. An OR >1 indicates positive association, i.e., exposure is associated with higher odds of the disease outcome, whilst OR < 1 indicates a negative association, i.e. exposure is associated with lower odds of the disease outcome. The magnitude of the OR is the "strength of the association"; the further the OR from 1.0, the more likely the more likely that the relationship between exposure and disease is casual. The precision of ORs is determined through the use of the 95% CI – a narrow CI indicates high precision while a wide CI suggests a low level of OR precision.

Receiver operating characteristics curve (ROC) analysis was used to compare how selected variables, including z-transformed polygenic risk scores, discriminate each cancer cohort from the controls. ROC analysis computes the area under the ROC curve, with values of 0.5 indicative of no discrimination. The higher AUC value indicates a better performance in disease classification.

Chapter 4

Results

4.1 Introduction

300 patients were recruited - 132 patients were diagnosed with breast cancer, 90 patients with endometrial cancer (four patients had both endometrial and breast cancer) and 82 patients had histological confirmed absence of endometrial cancer after hysterectomy and no history of breast cancer. The age at diagnosis of breast cancer ranged from 47 to 79 years (median age 60.0 years) while the age at diagnosis of endometrial cancer ranged from 52 to 88 years (median age 66.0 years). The age of women with normal histological findings who served as controls ranged from 45 to 82 years (median age 63.0 years).

4.2 Histology

4.2.1 Breast carcinoma cohort

The most prevalent histological diagnosis was invasive ductal carcinoma grade 2, accounting for 40.91% of cases (Figure 4.1).

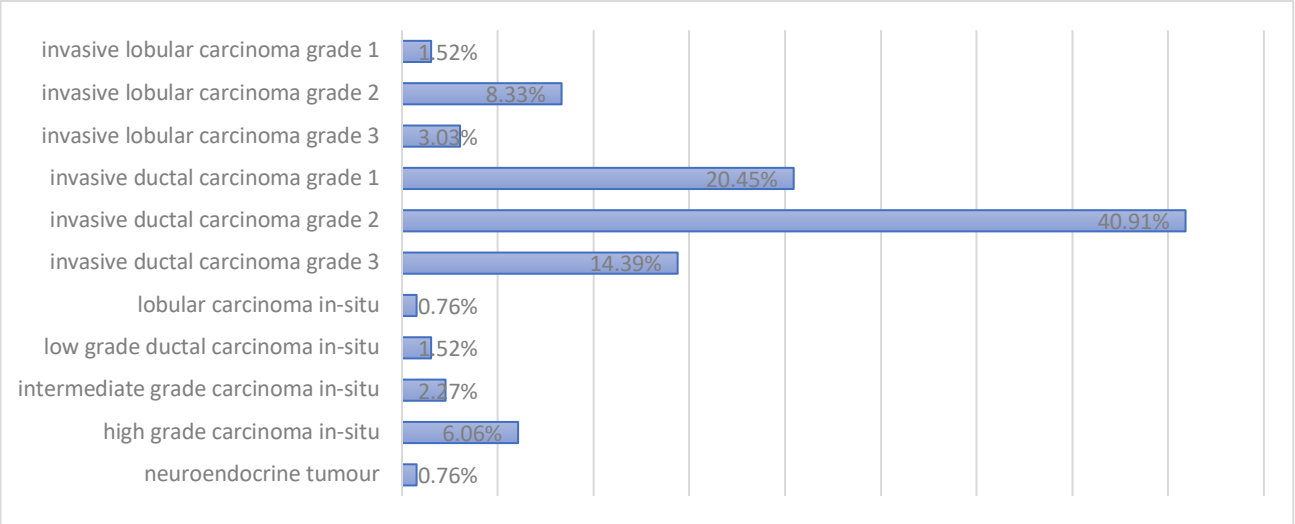


Figure 4.1 The percentage distribution of the respective histological diagnoses of breast carcinoma

4.2.2 Endometrial carcinoma cohort

91.1% of patients with endometrial cancer had endometrioid adenocarcinoma. Two patients had associated mixed villoglandular morphology. The remaining 8.9% of the sample population with endometrial cancer exhibited serous papillary histology (6.7%) and mucinous endometrial adenocarcinoma (2.2%).

The majority of the endometrioid endometrial adenocarcinoma cases were staged at FIGO stage 1 (86.9%); 5.95% were staged as FIGO stage 2 and 7.1% as FIGO stage 3 (Figure 4.2). The cases diagnosed with serous papillary adenocarcinoma were also mostly Stage 1 (3 cases FIGO 1a, 2 cases FIGO 1b and a case FIGO 2), while the cases of mucinous adenocarcinoma were both Stage 1a.

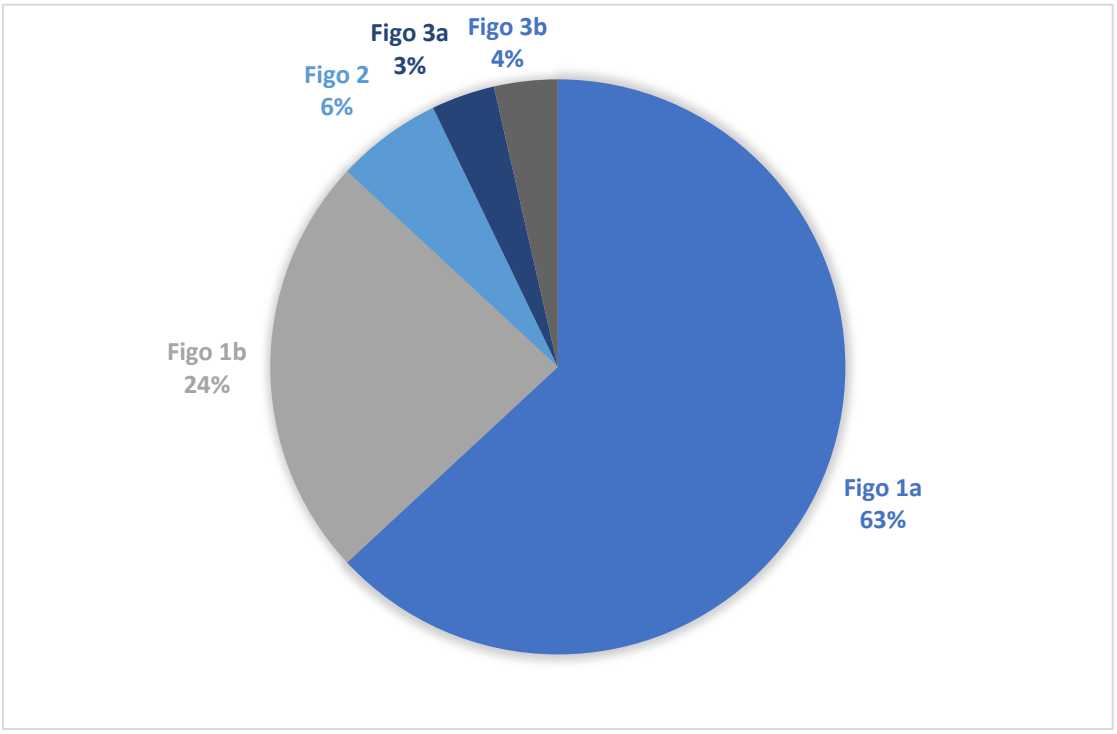


Figure 4.2 The percentage prevalence of the respective Figo stages of endometrioid endometrial adenocarcinoma

4.3 Clinical and biochemical/hormonal characteristics

The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test for normality of a number of clinical variables. Our paraments, almost all, had a significance level <0.05, thus showing that these variables do not follow a normal distribution (Table 4.1).

Table 4.1 Tests of Normality of a number of clinical variables

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
BMI	0.526	136	**<0.01**	0.060	136	**<0.01**
waist circumference (cm)	0.061	136	0.20	0.963	136	**0.001**
hip circumference (cm)	0.107	136	**<0.01**	0.909	136	**<0.01**
cholesterol	0.046	136	0.20	0.989	136	**0**.361
triglycerides	0.164	136	**<0.01**	0.761	136	**<0.01**
HDL	0.082	136	**0.03**	0.946	136	**<0.01**
LDL	0.045	136	0.20	0.993	136	0.734
HbA1C	0.201	136	**<0.01**	0.836	136	**<0.01**
fasting glucose	0.158	136	**<0.01**	0.809	136	**<0.01**
Follicle stimulating hormone	0.065	136	0.20	0.960	136	**<0.01**
Luteinizing hormone	0.115	136	**<0.01**	0.900	136	**<0.01**
oestradiol	0.213	136	**<0.01**	0.731	136	**<0.01**
progesterone	0.380	136	**<0.01**	0.548	136	**<0.01**
prolactin	0.346	136	**<0.01**	0.197	136	**<0.01**
testosterone	0.414	136	**<0.01**	0.445	136	**<0.01**
SHBG	0.143	136	**<0.01**	0.908	136	**<0.01**
Insulin (uU/ml)	0.160	136	**<0.01**	0.855	136	**<0.01**
reproductive years	0.190	136	**<0.01**	0.725	136	**<0.01**
breastfeeding, no of months	0.314	136	**<0.01**	0.542	136	**<0.01**

4.3.1 Breast Carcinoma Cohort

When clinical and biochemical characteristics of the breast cancer population were compared with the control group (Tables 4.2, p value a), key differences were as follows:

- Menarche age. The median age of menarche in patients with history of breast cancer (12.0 years) was found to be lower than the median age of menarche of patients with normal histological findings (13.0 years), p=0.02.
- Parity. More women with breast cancer were nulliparous (16.7%) when compared to controls (7.3%); the difference just reaching statistical significance, with p-value of 0.049.
- Breastfeeding. The proportion of women who breastfed was found to be statistically lower than that for the controls (40.9% vs 59.1%: p=0.007); difference in the median duration of breastfeeding also showed statistical significance difference ($p< 0.01$).
- Family history. Higher number of patients with breast cancer had family history of breast cancer when compared to the controls: 43.2% vs 25.6%, p= 0.009.
- BMI. The cohort of women with a history of breast cancer had a statistically significant higher BMI than the control group: 29.4 vs 28.5 kg/m^2, p=0.04.
- Hormonal levels. Serum levels of FSH and LH were statistically lower in the breast cancer cohort when compared to the controls; while SHBG was found to be statistically higher; $p<0.01$, $p<0.01$, $p=0.02$ respectively.

There was no significant difference between breast cancer population and the population with normal histology when comparing:

- Median menopausal age. In the population with normal histological findings, the median menopausal age was 50.0 years which is the same as that of breast cancer patients.
- Reproductive years. Both populations had 38 years of reproductive years.
- Miscarriage rate. Miscarriage rate between these two groups did not reach statistical significance (20.5% vs 24.4%).
- History of PCOS. Patients with a history of breast cancer had a lower rate of PCOS (9.1%) when compared with the group of women with normal histological findings (11.0%). However, this difference was not found to be statistically significant.
- History of hormonal use. Both OCP and HRT were seen more common in the normal cohort when compared to patients with breast cancer; however, this difference was not statistically significant.
- Medical history. Patients with a history of breast cancer were found to have a non-significant higher percentage of hypertension, hypercholesterolaemia and diabetes mellitus when compared with patients with normal histological findings (48.5% vs 39.0%, 41.7% vs 34.1%, 12.9% vs 11.1% respectively).
- Smoking. Patients with history of breast cancer were found to have the higher percentage of smoking history (22.0%) as compared to women with normal histological findings (12.2%) – this difference however was not found to be significant.
- Family history. Family history of diabetes mellitus and hypertension were found to be at a higher percentage in patients who were diagnosed with

breast cancer as compared to patients with normal histological findings: 64.4% vs 54.9% and 62.9% vs 57.3% respectively, however did not reach statistical significance.

- Median waist-hip ratios. No difference in the median waist-hip ratios was found, both 86cm.
- Biochemical levels. The median levels of triglycerides and HbA1c are higher in patients with a history of breast cancer as compared to those with normal histology. However, differences didn't reach statistical significance. Patients with history of breast cancer had a lower HOMA-IR result when compared with patients with normal but the difference was not statistically different.
- Metabolic syndrome. Patients with breast cancer history had a higher percentage of metabolic syndrome when compared with normal histology group – 38% vs 32.4% but this difference was also not statistically significant.
- Hormonal levels. The median levels of testosterone were found to be same in patients with breast cancer history when compared with patients with normal histological findings. The median levels of oestradiol, progesterone and prolactin were higher (but do not reach statistical significance) in patients with a history of breast cancer as compared to those with normal histology. The respective values for normal vs breast cancer cohort include oestradiol level: 65.5 vs 61; progesterone levels: 0.64, IQR 0.21 vs 0.64 IQR 0.11; prolactin levels: 137 vs 121.

Table 4.2a Characteristics of participants

	Overall		Normal		BC		EC		BC ad EC	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Age at diagnosis	63.00	13.00	63.00	13.00	60.00	10.00	66.00	14.00	63.00	13.00
Menarche age	12.00	3.00	13.00	2.00	12.00	2.00	12.00	2.00	12.00	2.00
Menopause age	50.00	5.00	50.00	5.00	50.00	5.00	51.00	5.00	50.50	4.00
Reproductive years	38.00	6.00	38.00	6.00	38.00	5.00	39.00	7.00	38.00	6.00
Breastfeeding (months)	0.00	6.00	1.00	8.00	0.00	6.00	0.00	0.00	0.00	3.00
BMI	29.97	8.45	28.48	6.58	29.43	7.51	33.95	9.20	31.11	9.00
Waist:hip ratio	0.86	0.09	0.86	0.09	0.86	0.09	0.87	0.10	0.86	0.09
FSH	55.45	43.55	63.00	36.50	46.70	40.80	57.80	46.80	51.15	45.15
LH	22.35	18.55	24.50	23.00	19.80	14.80	25.80	21.00	21.25	17.00
Oestradiol	61.00	40.00	65.50	35.00	61.00	45.00	58.50	38.00	60.00	39.00
Progesterone	0.64	0.11	0.64	0.21	0.64	0.11	0.64	0.01	0.64	0.06
Prolactin	129.50	75.50	137.00	85.50	121.00	65.00	151.00	94.00	128.00	69.00
Testosterone	0.69	0.00	0.69	0.00	0.69	0.00	0.69	0.00	0.69	0.00
SHBG	58.10	42.70	55.85	34.05	67.50	48.10	45.20	34.40	58.65	48.20
Cholesterol	5.39	1.28	5.37	1.22	5.38	1.28	5.43	1.35	5.40	1.29
Triglycerides	1.26	0.80	1.05	0.56	1.27	0.80	1.35	0.73	1.31	0.79
HDL	1.60	0.59	1.63	0.65	1.64	0.60	1.52	0.54	1.59	0.59
LDL	3.16	1.26	3.19	0.97	3.15	1.31	3.11	1.46	3.14	1.34
HbA1C	5.70	0.70	5.60	0.70	5.70	0.70	5.80	1.00	5.80	0.60
HOMA-IR	2.47	2.47	2.23	1.38	2.45	2.73	2.72	3.24	2.58	2.72

Characteristics of participants were classified as overall (total participants), Normal/controls, Breast carcinoma (BC), endometrial carcinoma (EC), BC and EC.

Table 4.2b The p values of the characteristics of participants

	p value a	p value b	p value c	p values d	p values e
Age at diagnosis	NS	**0.02**	**<0.01**	NS	NS
Menarche age	**0.02**	**0.01**	0.45	**0.01**	**0.01**
Menopause age			NS		
Reproductive years			NS		
Breastfeeding (months)	**<0.01**	**0.12**	**<0.01**	**<0.01**	**0.01**
BMI	**0.04**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
Waist:hip ratio		NS		0.94	0.97
FSH	**<0.01**	0.89	**<0.01**	**0.03**	**0.03**
LH	**<0.01**	0.93	**<0.01**	**0.03**	**0.02**
Oestradiol		NS		0.20	0.18
Progesterone	0.06	**0.01**	0.30	**0.01**	**0.02**
Prolactin	0.05	0.08	**<0.01**	0.66	0.64
Testosterone		NS		0.98	0.97
SHBG	**0.02**	**0.01**	**<0.01**	0.80	0.74
Cholesterol			NS		
Triglycerides	0.05	**<0.01**	0.19	**0.01**	**0.01**
HDL			NS		
LDL			NS		
HbA1C		**<0.01**	0.12	**0.01**	**0.02**
HOMA-IR	NS	**0.01**	**<0.01**	**0.03**	**0.04**

p value a: BC vs Normal; p value b: EC vs Normal; p value c: BC vs EC; p value d: BC and EC vs Normal; p value e: Endometrioid EC and BC vs Normal; NS:not significant

The tables below (Table 4.3-4.5) show association of breast cancer with parity, history of breastfeeding and family history of breastfeeding; these three factors exhibited significant association with breast cancer.

Table 4.3 Association of breast cancer with parity

			Group		
			Breast cancer	Normal	Total
Parity	Nulliparous	Count	22	6	28
		Percentage	16.7%	7.3%	131.1%
	Multiparous	Count	110	76	186
		Percentage	83.3%	92.7%	86.9%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

More patients with breast cancer were nulliparous compared to controls, p=0.049

Table 4.4 Association of breast cancer with history of breastfeeding

			Group		
			Breast cancer	Normal	Total
History of breastfeeding	Yes	Count	54	49	103
		Percentage	40.9%	59.8%	48.1%
	No	Count	78	33	111
		Percentage	59.1%	40.2%	51.9%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

Less patients with breast cancer breastfed compared to normal controls, p=0.007

Table 4.5 Association of breast cancer with family history of breast cancer

			Group		
			Breast cancer	Normal	Total
Family history of breast cancer	Yes	Count	57	1921	78
		Percentage	43.2%	25.6%	36.4%
	No	Count	75	61	136
		Percentage	56.8%	74.4%	63.6%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

More patients with breast cancer had family history of breast cancer, p=0.009

The tables showing other associations which did not reach significance are included in Appendix 6.

The following figure illustrate history of hypertension, diabetes mellitus type 2 and hypercholesterolaemia in breast cancer group compared to controls (Figure 4.2).

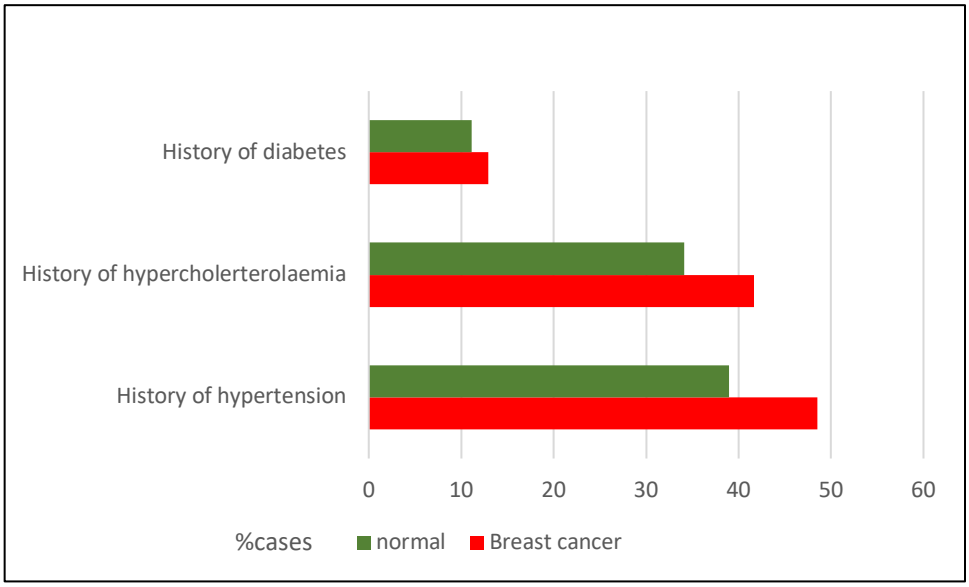


Figure 4.2 Clustered Bar graph of breast cancer and normal groups by history of diabetes mellitus, hypercholesterolaemia and hypertension: more cases of patients with breast cancer had history of diabetes, hypercholesterolaemia and hypertension

4.3.2 Endometrial cancer cohort

When comparing clinical and biochemical factors between women with endometrial cancer and the control group of women with normal histological findings after hysterectomy (Tables 4.2, p value b), the following were the key differences:

- Age of menarche. Women with endometrial cancer had earlier age of menarche when compared with women with normal histological findings, 12.0 years vs 13.0 years, p=0.01.
- Parity. More women with a diagnosis of endometrial carcinoma were nulliparous when compared to the control group (20% vs 7.3%), p=0.017.
- History of breastfeeding. Women with endometrial cancer breastfed less when compared to the normal controls (25.8% vs 58.8%, p<0.01)
- Medical history. Patients with a history of endometrial cancer were found to suffer more from hypertension and diabetes mellitus when compared with patients with normal histological findings – 72.2% vs 39.0% and 26.7% vs 11.0%, p=<0.01 and p=<0.01 respectively (Figure 4.4).
- Family history. Patients with endometrial cancer were found to have higher percentage of family history of hypertension, 76.7% vs 57.3% (p=0.007).
- BMI. The BMI of patients with history of endometrial cancer was found to be significantly higher than that of patients with normal histology– 33.9 vs 28.4, p<0.01.
- Biochemical levels. Serum triglycerides level, HbA1c and HOMA-IR were found to be higher in patients with a history of endometrial cancer when compared to women with normal histology (p<0.01, p<0.01, p=0.01 respectively).

- Metabolic syndrome was significantly higher in the endometrial cancer cohort compared to controls (82.9% vs 45.1%, p<0.01).
- Hormonal levels. Progesterone and SHBG were found to be significantly lower in endometrial cancer cohort compared with normal cohort (p=0.01 and p=0.01 respectively).

The following associations did not reach statistical significance:

- Menopause age. The age of menopause was found to be higher in the endometrial cancer group when compared to the normal controls, 51.0 vs 50.0years, the difference however was not significant.
- Reproductive age. When comparing the reproductive years in both populations, it was found to be longer in patients with endometrial cancer when compared to the normal controls (39.0 vs 38.0), the difference also however was not significant.
- Miscarriage rate. Women with endometrial carcinoma were found to have marginally lower miscarriages than the controls (22.2% vs 24.4%), difference however did not reach statistical significance.
- Duration of breastfeeding. The median number of months of breastfeeding carried out by patients with a history of endometrial cancer was not significantly different.
- History of PCOS. A marginally higher percentage of history of PCOS was found in the endometrial cancer group compared with the normal group, 11.1% vs 11.0%; the difference was also not significant.

- Medical history. Patients with a history of endometrial cancer were found to have higher percentages of history of hypercholesterolemia, difference however was not significant (44.4% vs 35.4%).
- Family history. Patients with endometrial cancer were found to have higher percentages of family history of diabetes mellitus and endometrial cancer as compared with patients with normal histological findings – 60.0% vs 54.9% and 20.0% vs 9.8%, not reaching significance.
- Hormonal use. Both OCP use and HRT use were found to be marginally lower in women with endometrial cancer compared with normal controls (9.2% vs 10.0% and 10.3% vs 11.3% respectively); both differences being not significant.
- Smoking. History of smoking was found at a higher percentage in patients with endometrial cancer as compared with women with normal histological findings – 16.7% vs 12.2%. However, this difference was also not significant.
- WHR. The median WHR of patients with history of endometrial cancer was found to be similar to that of patients with normal histology (0.87 vs 0.86 respectively).
- Hormonal levels. Patients with history of endometrial cancer exhibited lower levels of FSH, LH, oestradiol, prolactin and testosterone when compared with women with normal histology - these differences were not significant.

The following tables (Table 4.6-4.11) depict the association of endometrial cancer with factors that show statistically significant association with endometrial cancer. These factors include: parity, history of breastfeeding, history of hypertension,

history of diabetes mellitus, presence of the metabolic syndrome and family history of hypertension.

Table 4.6 Association of endometrial cancer with parity

			Group		
			Endometrial cancer	Normal	Total
Parity	Nulliparous	Count	18	6	24
		Percentage	20.0%	7.3%	14.0%
	Multiparous	Count	72	76	148
		Percentage	80.0%	92.7%	86.0%
Total		Count	41	72	172
		Percentage	100.0%	100.0%	100.0%

More patients with endometrial cancer were nulliparous compared to normal controls, p=0.017

Table 4.7 Association of endometrial cancer with history of breastfeeding

			Group		
			Endometrial cancer	Normal	Total
History of breastfeeding	Yes	Count	23	47	70
		Percentage	25.8%	58.8%	41.4%
	No	Count	66	33	99
		Percentage	74.2%	41.3%	58.6%
Total		Count	89	80	169
		Percentage	100.0%	100.0%	100.0%

Less patients with endometrial cancer breastfed compared with normal controls, p<0.01

Table 4.8 Association of endometrial cancer with history of hypertension

			Group		
			Endometrial cancer	Normal	Total
History of hypertension	Yes	Count	65	32	97
		Percentage	72.2%	39.0%	56.4%
	No	Count	25	50	75
		Percentage	27.8%	61%	43.6%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

More patients with endometrial cancer suffered from hypertension, p<0.01

Table 4.9 Association of endometrial cancer with history of diabetes mellitus

			Group		
			Endometrial cancer	Normal	Total
History of diabetes mellitus	Yes	Count	24	9	33
		Percentage	26.7%	11.0%	19.2%
	No	Count	66	73	139
		Percentage	73.3%	89.0%	80.8%
Total		Count	90	83	172
		Percentage	100.0%	100.0%	100.0%

More patients with endometrial cancer suffered from diabetes, p=0.009

Table 4.10 Association of endometrial cancer with presence of metabolic syndrome

			Group		
			Endometrial cancer	Normal	Total
Presence of metabolic syndrome	Yes	Count	55	25	80
		Percentage	61.1%	30.5%	46.5%
	No	Count	35	57	92
		Percentage	38.9%	69.5%	53.5%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

More patients with endometrial cancer suffered with metabolic syndrome compared to normal controls, p<0.001

Table 4.11 Association of endometrial cancer with family history of hypertension

			Group		
			Endometrial cancer	Normal	Total
Family history of hypertension	Yes	Count	69	47	116
		Percentage	76.7%	57.3%	67.4%
	No	Count	21	35	56
		Percentage	23.3%	42.7%	32.6%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

More patients with endometrial cancer had history of hypertension compared to normal controls, p=0.007

The tables showing other associations with endometrial cancer which did not reach significance are included in Appendix 6.

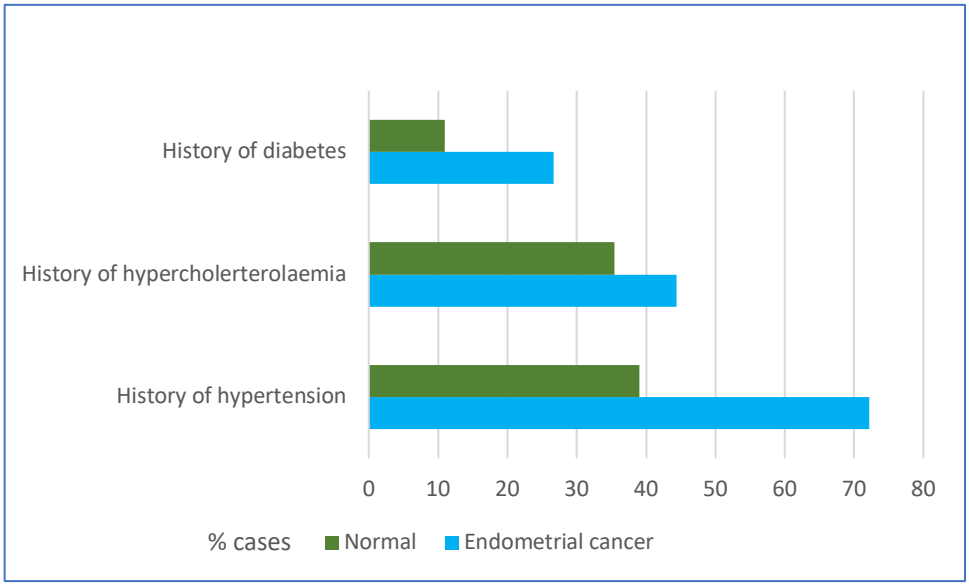


Figure 4.3 Clustered Bar graph of endometrial cancer and normal groups by history of diabetes, hypercholesterolaemia and hypertension: more patients with endometrial cancer had history of diabetes, hypercholesterolaemia and hypertension compared to normal controls

Figure 4.3 is illustrating the relative % cases in endometrial cancer cohort compared to controls that suffered from diabetes, hypertension or hypercholesterolaemia.

4.3.3 Comparing breast and endometrial cohorts

The following figure (Figure 4.4) summarizes the risk factors associated with breast and/or endometrial cancer.

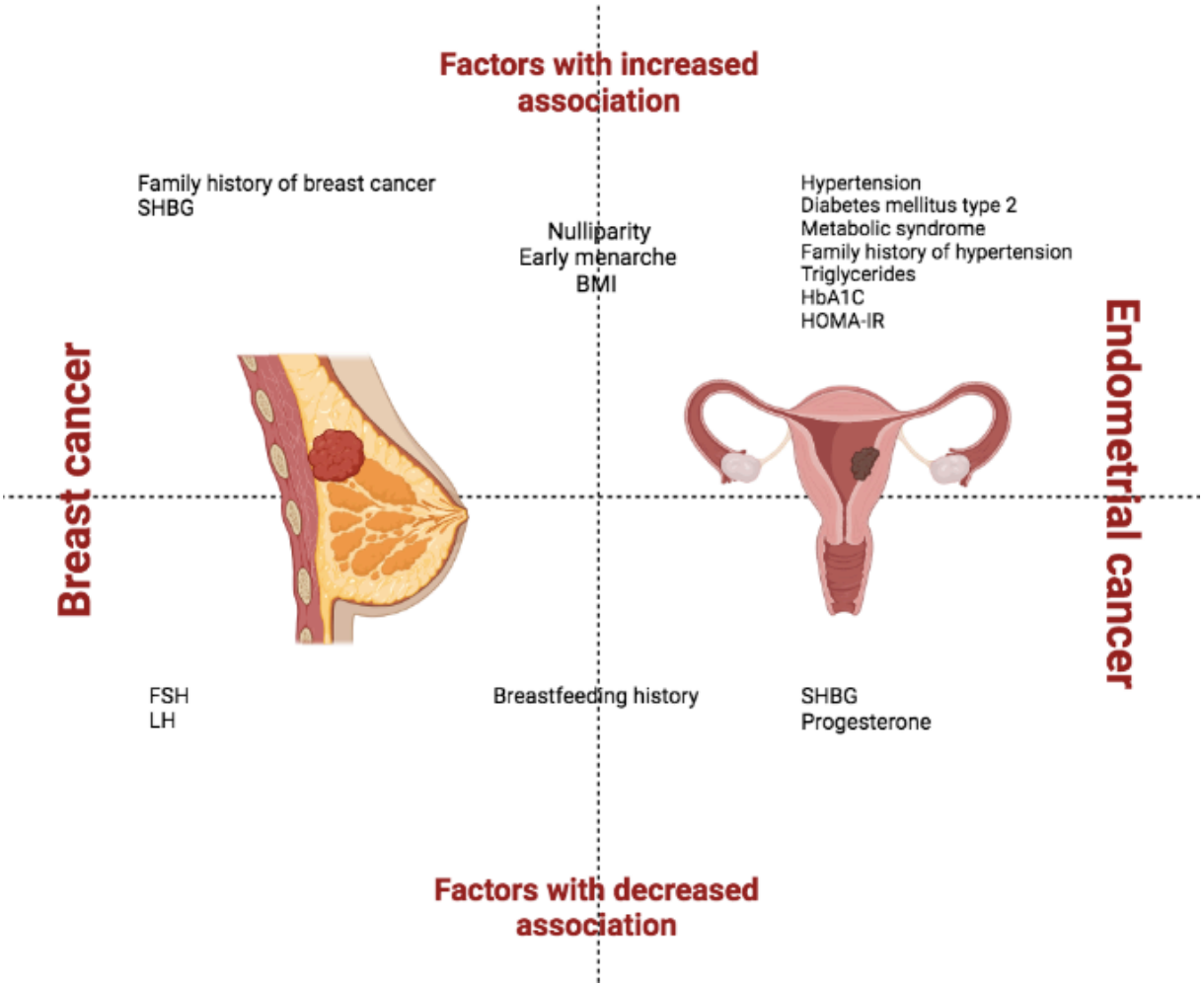


Figure 4.4. Summary of clinical/biochemical factors that showed significant association with breast and endometrial cancer

It is important to note that:

- Nulliparity, early menarche and high BMI showed increased association with both breast and endometrial cancer
- Breastfeeding history showed decreased association with both malignancies.

When comparing the risk factors of the breast cancer cohort with endometrial cancer cohort, factors that had p value higher than 0.05 signify non-statistically significant difference and thus similar occurrence between the groups. Such factors include:

- menarche age
- menopause age
- reproductive years
- WHR
- serum oestradiol
- serum progesterone
- serum testosterone
- cholesterol
- triglycerides
- HDL
- LDL
- HbA1c (Table 4.1, p value c).

4.3.4 Comparing cancer cohorts with controls

When comparing the risk factors in breast and endometrial cancer cohorts taken together, as aggregate group, against the normal cohort, the following factors reached statistical significance (p values d in Table 4.2):

- age of menarche
- number of months of breastfeeding
- BMI
- serum level of FSH, LH and progesterone
- triglycerides, HbA1c and HOMA-IR

When minor subtypes of endometrial carcinoma were excluded from the analysis (serous papillary carcinoma and mucinous adenocarcinoma, 8 patients), retaining only endometrioid type endometrial adenocarcinoma and breast carcinoma, consistent findings were observed (p values e in Table 4.2).

4.3.5 Comparing the three cohorts

The following figures (Figures 4.5-4.8) show the factors with significant association with breast and endometrial cancer, i.e., parity, breastfeeding history, menarche age and BMI, illustrating the respective percentages in the three study cohorts.

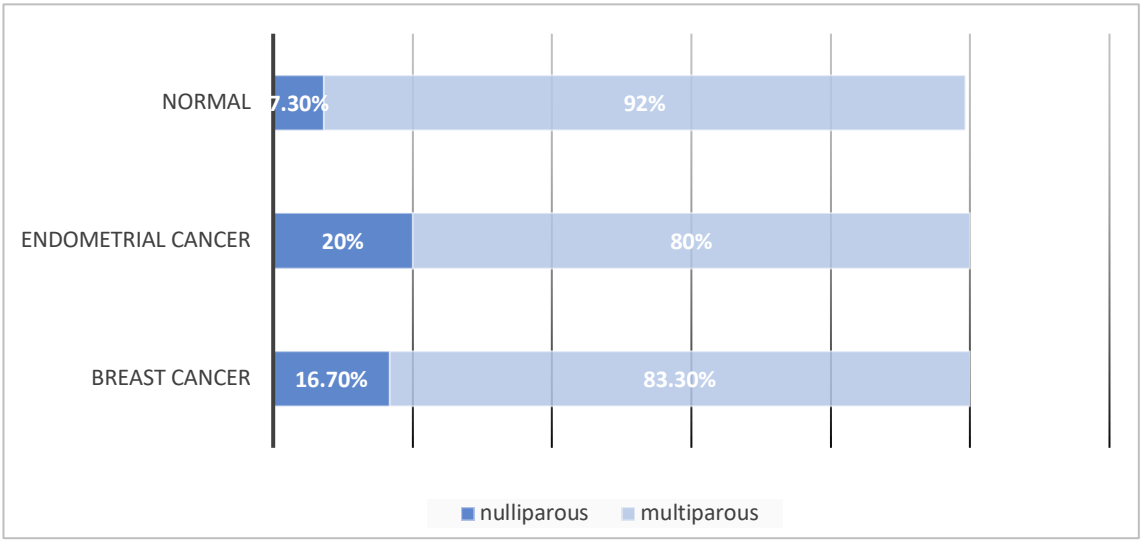


Figure 4.5 A stacked bar graph showing the percentages of nulliparous women and multiparous women in the three cohorts. Significant difference was found between the breast cancer cohort with controls and endometrial cancer cohort with controls.

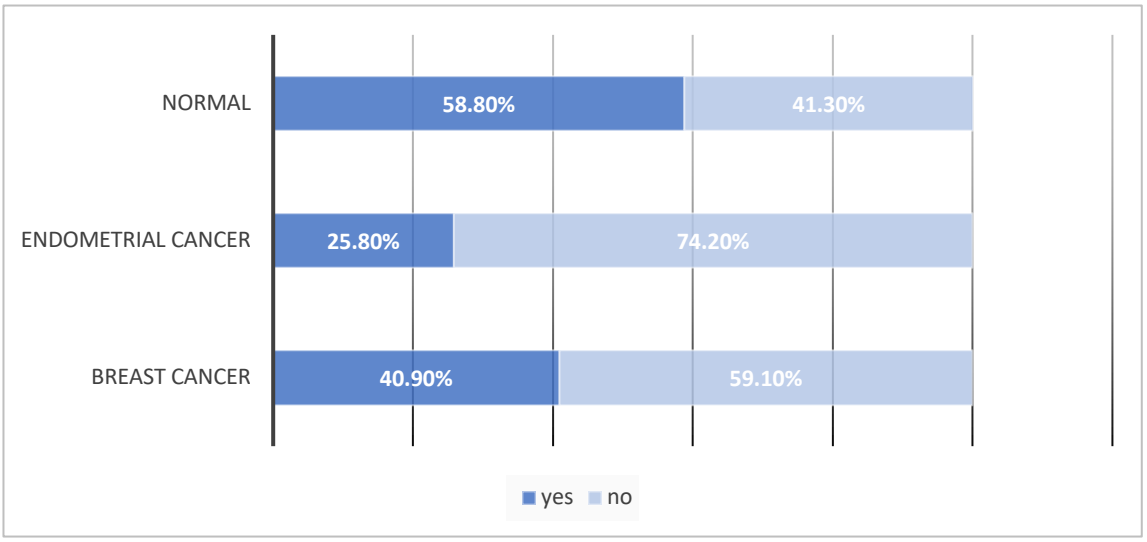


Figure 4.6 A stacked bar graph showing the percentages of women who breastfed compared with those who never breastfed in the three cohorts. Significant difference was found between the breast cancer cohort with controls and endometrial cancer cohort with controls.

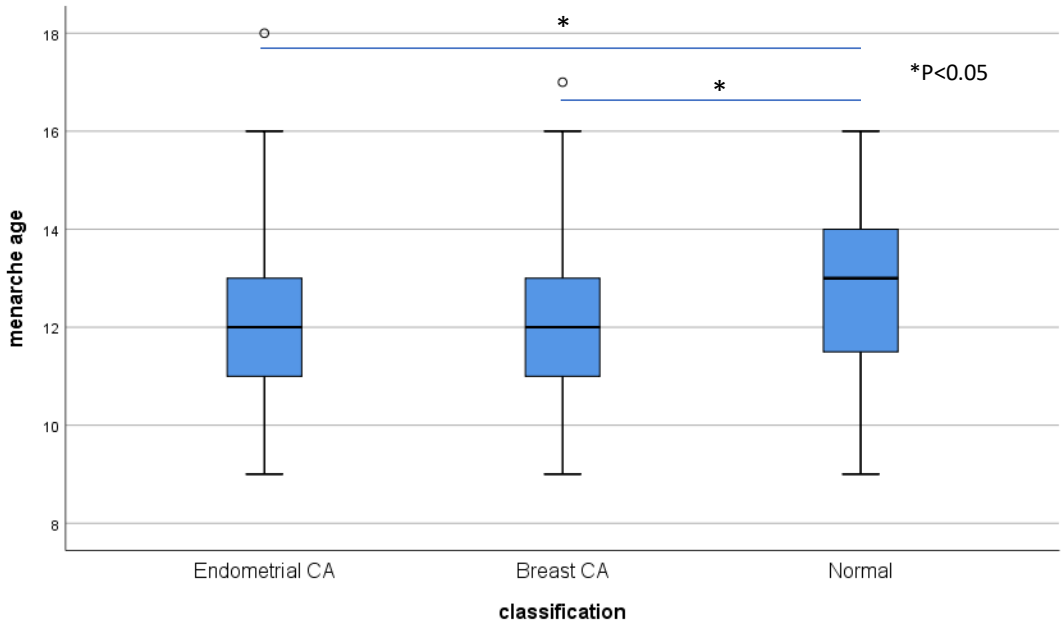


Figure 4.7 A box chart showing the difference in menarche age in the three cohorts: statistical difference is obtained between endometrial and breast cancer respectively when compared with normal controls

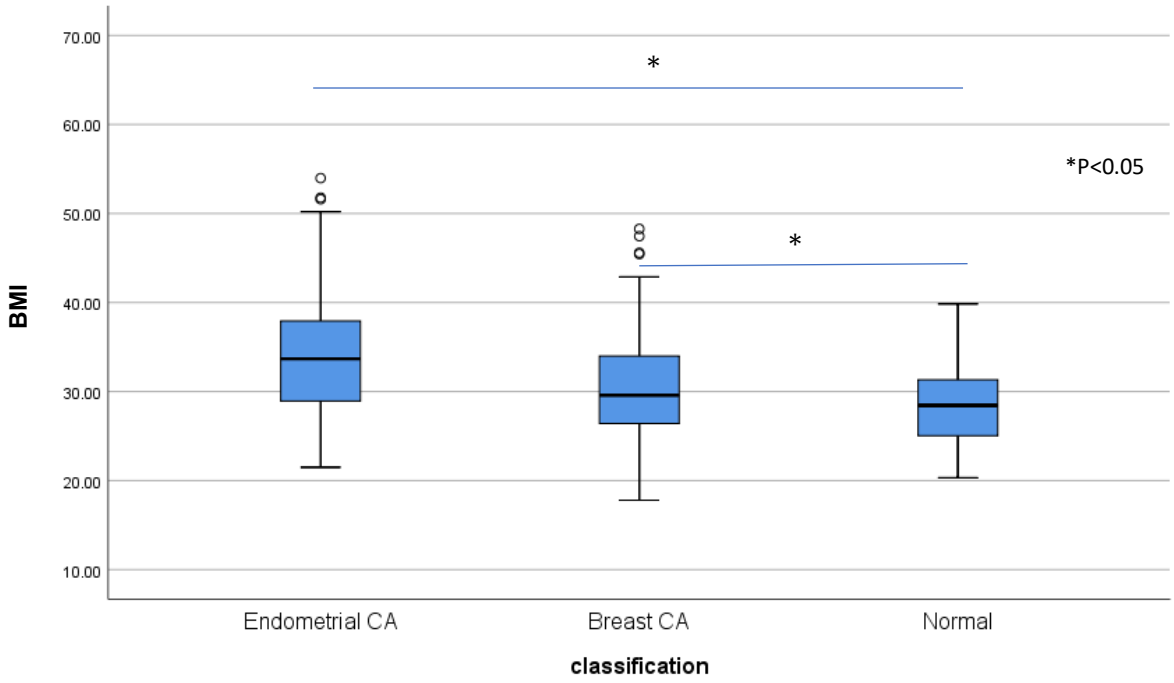


Figure 4.8 A box chart showing graphical representation of BMI in the three cohorts: statistical difference is obtained between endometrial and breast cancer respectively when compared with normal controls

It is evident that the normal control population has the lowest BMI, followed by the breast cancer cohort and the endometrial cancer cohort (Figure 4.8).

The three box plots that follow aid visualization of the relation of the three populations and their respective BMIs with (A) menarche age, (B) menopause age, (C) HOMA-IR (Figure 4.9). The normal control cohort has older age of menarche when compared with the breast cancer and endometrial cancer cohorts: median age of menarche were 13years, 12years and 12years respectively. Patients in the endometrial cancer cohort who had early age of menarche appear to have higher BMI (lighter shade of blue scale). The median menopause age for the normal and breast cancer cohort was 50years, while that of endometrial cancer cohort was 51years. No obvious difference in BMI can be noted across the three cohorts with varied menopausal age. HOMA-IR was found to be lowest in the normal cohort (median 2.23) when compared to the breast cancer and endometrial cancer cohorts, 2.45 and 2.72 respectively. It's interesting to observe that patients with lower HOMA-IR had lower BMI – darker blue colour scale.

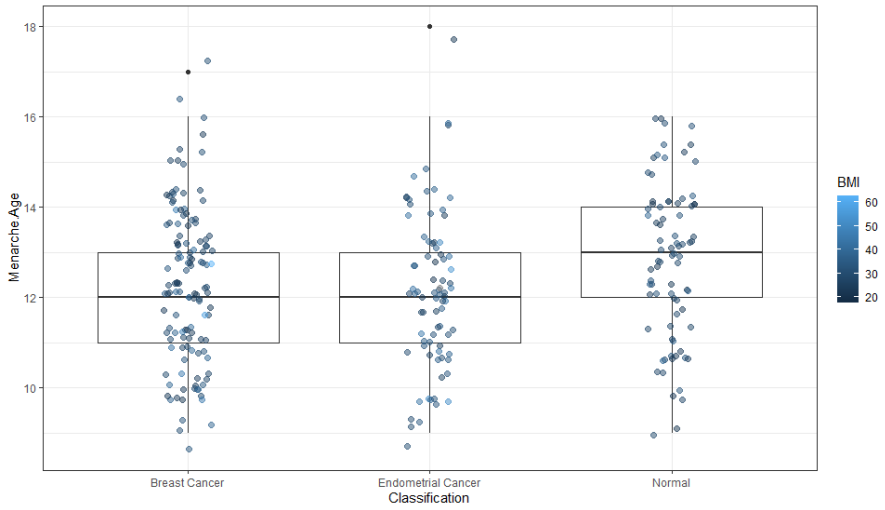


Figure 4.9A

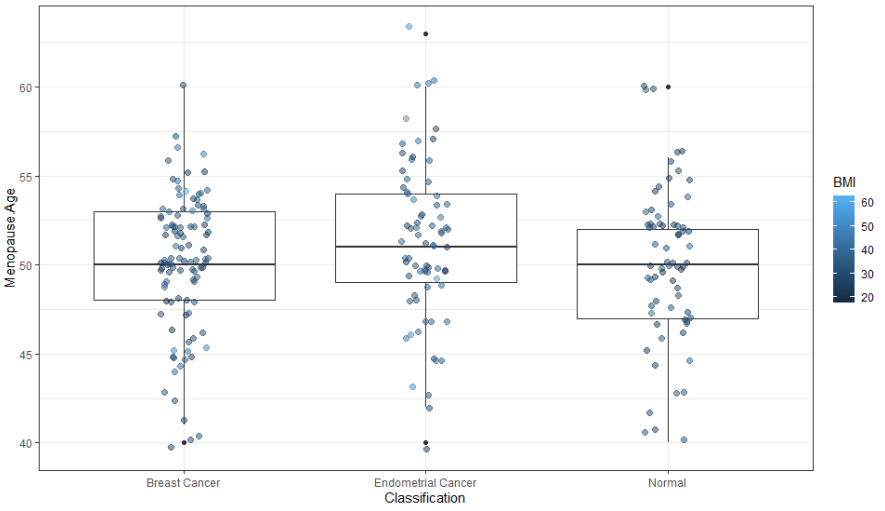


Figure 4.9B

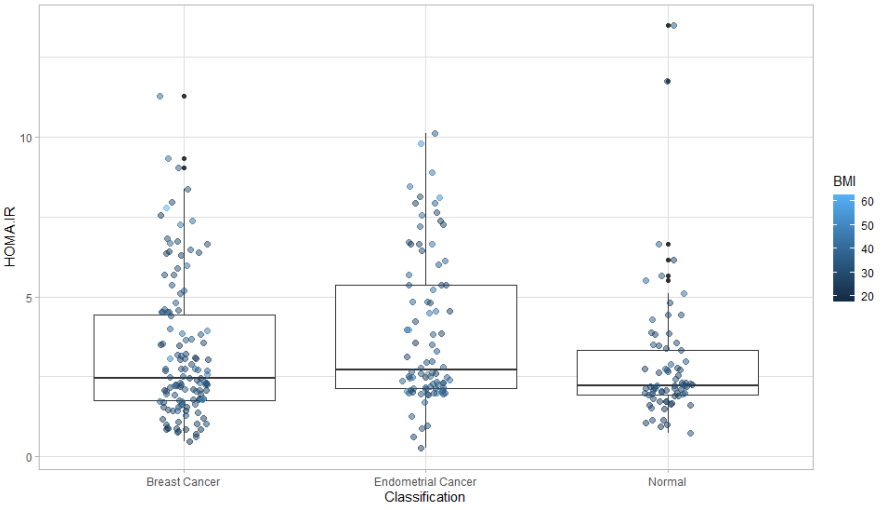


Figure 4.9C

Figure 4.9 Box plots showing the relation of the three populations with (A) menarche age, (B) menopause age, (C) HOMA-IR; the blue colour scale showing their BMIs

4.3.6 Correlation between the different risk factors

Figure 4.10 is a correlation heatmap showing a graphical representation of the correlations between the different risk factors. Positive correlation is strongest (p=0.5 or above) between progesterone and prolactin, FSH and LH, HbA1c and fasting plasma glucose (FPG), cholesterol and LDL, menopausal age and reproductive years. Negative correlation is strongest between triglyceride level and HDL (p=-0.5). For example, a value of 0.5 between HbA1c and FPG means that increasing HbA1c is associated with increasing FPG while a value of -0.5 between triglycerides and HDL means that high triglycerides is associated with low HDL.

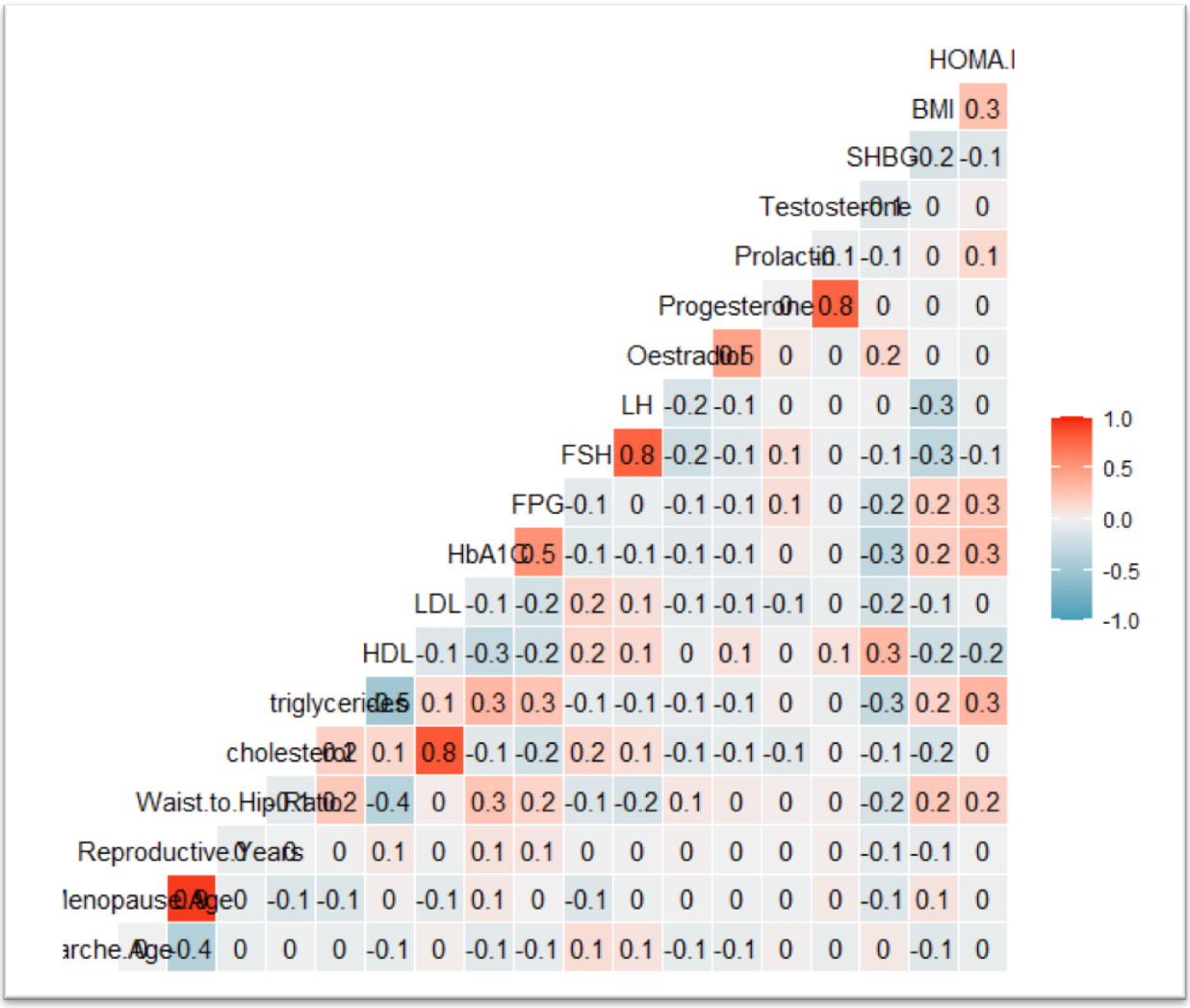


Figure 4.10 Correlation matrix heatmap showing Pearson Correlation p-values (positive correlations are in red, negative correlations are in blue)

4.3.7 Logistic Regression Analysis

In this section we explored the clinical risk factors with breast and endometrial cancer respectively.

4.3.7.1 Breast cancer

The logistic regression model (Table 4.12) identifies BMI and history of breast cancer as risk factors for breast cancer, with BMI having the lowest P-value and family history as the parameter with the strongest effect size. Conversely, a history of breastfeeding is protective.

A significant positive correlation was found between breast cancer and:

- Family history of breast cancer. The odds ratio of family history of breast cancer is 1.48 (95% CI 1.01-2.18), which indicates that having a first relative with breast cancer increases the odds of having breast cancer (rather than being normal) by 48.1%.
- BMI. The odds ratio of BMI is 1.04 (95% CI 1.01-1.07), which means that for every 1kg/m^2 increase in BMI, the odds of having breast cancer increases by 3.9%.

Breastfeeding history was found to negatively correlate with breast cancer risk. The odds ratio of breastfeeding is 0.665 (95% CI 0.45-0.98) which indicates that breastfeeding decreases the odds of having breast cancer by 33.5%.

Table 4.12 Logistic regression model for breast cancer risk factors.

	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Parity	.80	0.477	.43	1.48
Breastfeeding	.67	**0.039**	.45	.98
FH breast cancer	1.48	**0.046**	1.01	2.18
Menarche age	1.24	0.284	.84	1.85
BMI	1.04	**0.024**	1.01	1.07

BMI and family history (FH) of breast carcinoma were identified as disease risk factors, and history of breast feeding had a protective effect.

4.3.7.2 Endometrial Cancer

The logistic regression model for risk factors for endometrial cancer is shown in Table 4.13. It identifies BMI as a significant predictor of endometrial cancer risk. The odds ratio of BMI is 1.08 which indicates that for every increase in 1kg/m^2 the odds of having endometrial cancer increases by 8.4%.

A negative correlation was found between endometrial cancer risk and breastfeeding. History of breastfeeding is associated with a decreased risk of endometrial cancer, odds ratio of 0.54, which indicates a 46.2% decrease in endometrial cancer risk with breastfeeding.

Table 4.13 Logistic regression model for endometrial cancer risk factors

	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Breastfeeding	.54	**0.007**	.34	.84
Menarche age	1.23	0.406	.75	2.01
BMI	1.08	**<0.01**	1.04	1.13
History of HT	1.51	0.082	.95	2.41
History of DM	1.39	0.299	.75	2.61
FH of HT	1.29	0.300	.80	2.10

BMI exhibited a significant positive correlation with endometrial cancer while history of breast feeding was protective

Increasing BMI was identified as the best predictor for both breast and endometrial cancer (OR 1.04 95% CI 1.01-1.07 and OR 1.08 95%CI 1.04-1.13 respectively).

4.4 Genetic Results

The workflow of the project including DNA extraction from whole blood, assessment of DNA purity, storage and genetic profiling, are detailed in the methodology. After stringent quality control measures, LP-WGS was carried out on 293 DNA samples.

We performed PCA of the LP-WGS data analysed in this study. Mapping of the Maltese genome data to the reference PCA coordinates from samples of the HGDP shows that the Maltese cohort mapped close to both the European and Middle Eastern data points, suggesting a shared genetic ancestry between the populations. To further refine this analysis, we also mapped the WGS data to the imputed POPRES dataset as the reference panel for analysis of individuals of European ancestry (Figure 4.11). This analysis shows significant partial overlap between the Maltese and Southern / Southwestern European datasets, and a relatively distinct cluster from Western and Central European data points.

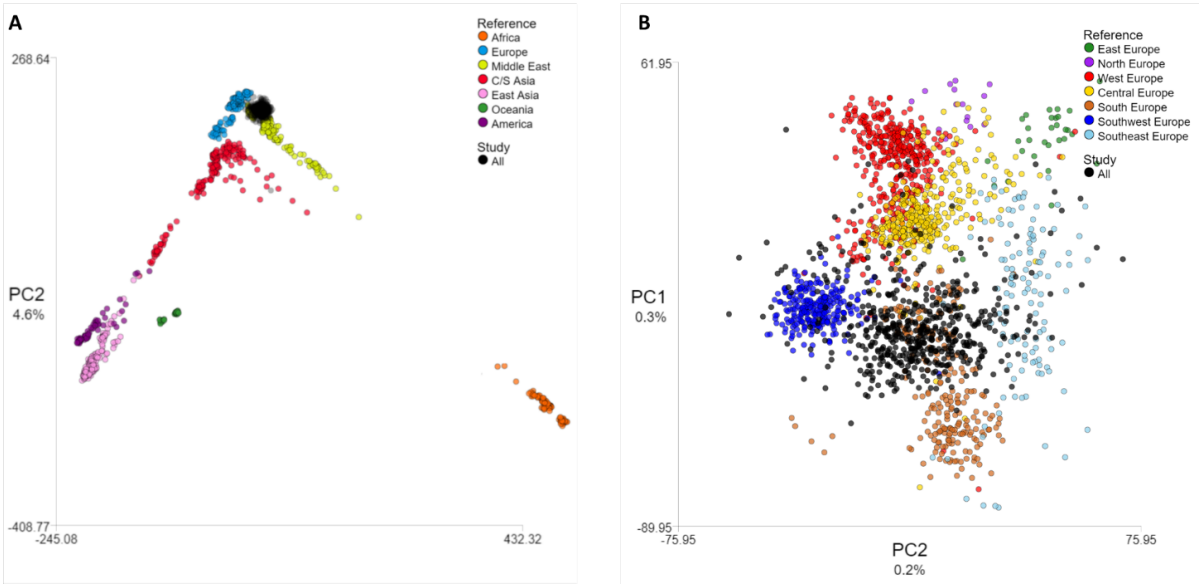


Figure 4.11 (A) The Maltese WGS dataset (in black) is mapped to the reference PCA coordinates for samples from the HGDP reference panel. (B) The Maltese WGS dataset is mapped to reference coordinates from the POPRES dataset.

The genetic link in Maltese postmenopausal women between diabetes mellitus type II and insulin resistance with breast/endometrial cancer was established. This was performed by determining whether the following GRSs (of European ancestry) were associated with increased breast/endometrial cancer risk:

- GRS 1, Polfus et al., 582 SNPs known to be associated with diabetes mellitus type II
- GRS 2, Aly et al., 223 SNPs known to be associated with insulin resistance

4.4.1 Genetic association analysis with GRS 1 (Polfus et. al, 2021)

Correlation between this GRS (582 risk loci for type 2 diabetes) and quantitative metabolic/anthropometric parameters identified significant correlation between this GRS and HbA1C, fasting plasma glucose (FPG), waist circumference and waist-hip ratio. The Spearmann correlation coefficient and p value is shown in table 4.14.

Table 4.14 Correlation between Zscore Polfus et al, 2021 and fasting plasma glucose (FPG), HbA1C and waist circumference (WC)

		FPG	HbA1C	WC
Spearman's Correlation Z Score Polfus et al 2021	Spearmann Correlation Coefficient	.120*	.165**	.181**
	Sig. (2-tailed)	**0.040**	**0.005**	**0.002**
	N	291	286	276

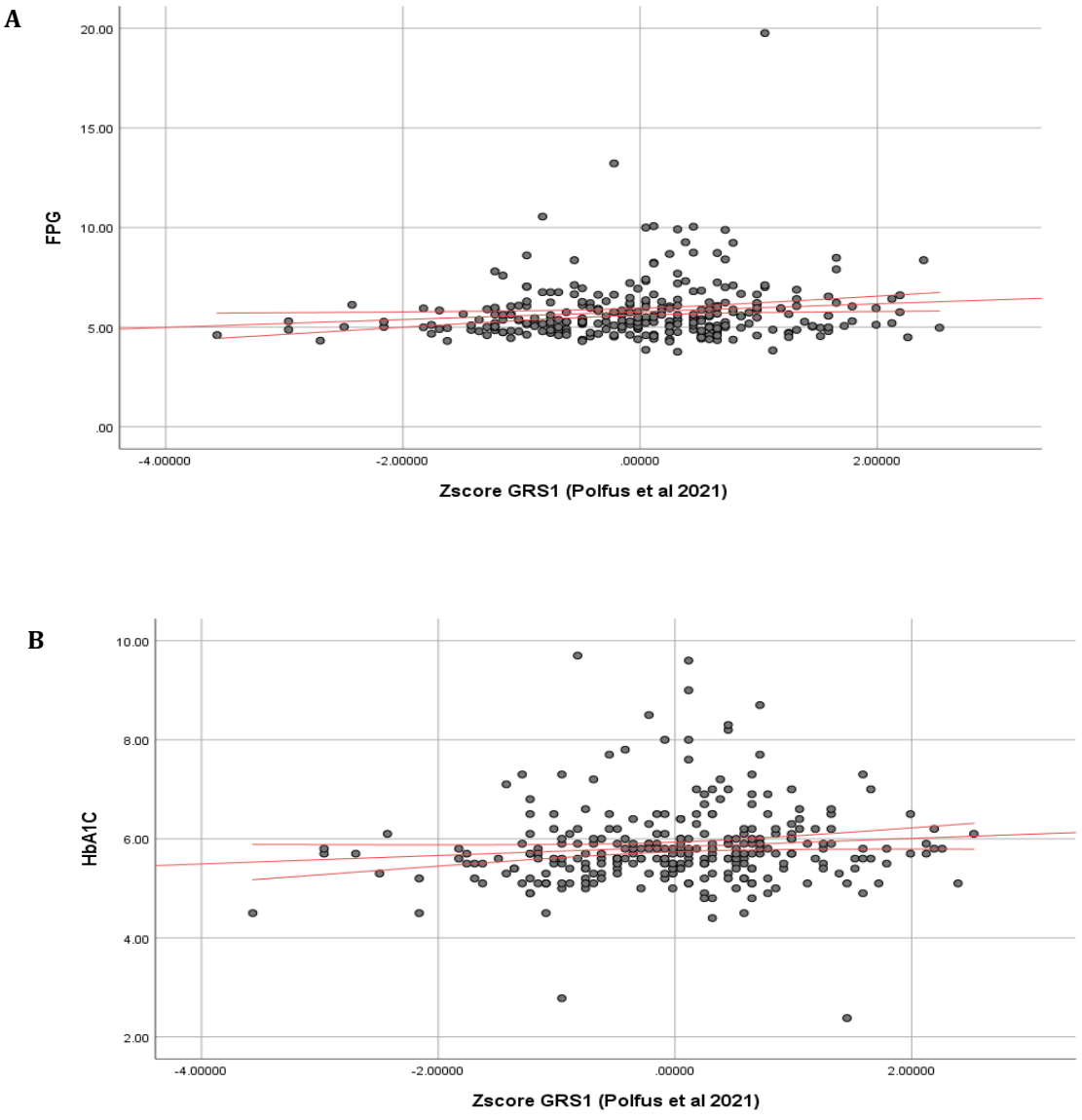
*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

All three parameters: FPG, HbA1C and WC showed significant correlation with this score, p=0.04, 0.005, 0.002 respectively

Each GRS, (defined by the aggregate sum of risk alleles at each locus) was z-transformed. Z-score transformation enables standardisation of the two GRS that have differing numbers of risk alleles and thus enables their direct comparison.

Figure 4.12 is showing the linear relationship between FPG, HbA1C and WC and the number of variants involved. The middle line is the regression fit line (line of best fit); with the line above it signifying the upper limit of the 95% confidence interval and line below it representing the lower limit of the 95% confidence interval.



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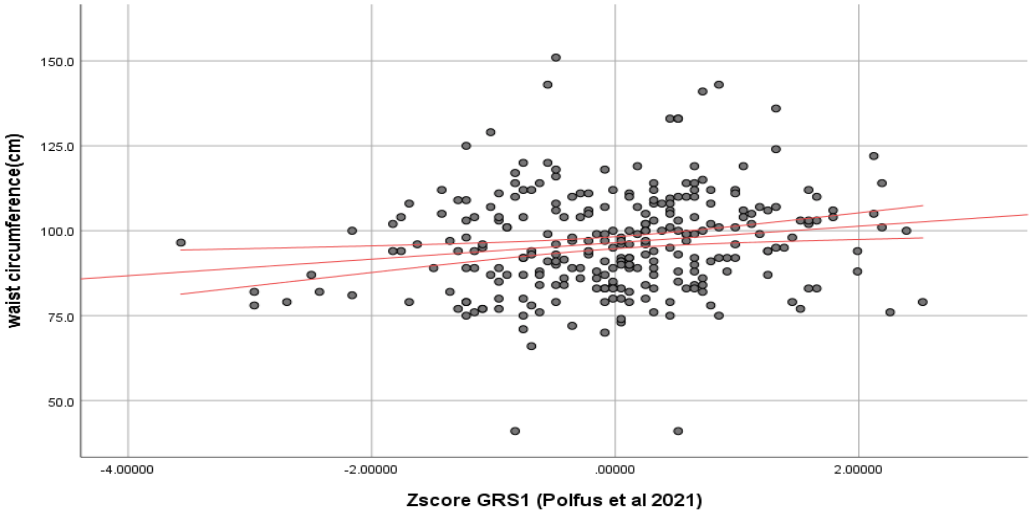

Figure 4.12 Scatter plot graphs showing association of fasting glucose, FPG (A), HbA1c (B), waist circumference, WC (C) with GRS 1 (Polfus et al., 2021) – Relationship is linear: the middle line is the regression fit line (line of best fit); with the line above it signifying the upper limit of the 95% confidence interval and line below it representing the lower limit of the 95% confidence interval.

The mean Z-score (together with the 25th and 75th percentiles) for breast and endometrial cancer as aggregate group compared with the normal controls is shown in Table 4.15.

Table 4.15 Mean Z scores (together with the 25th and 75th percentiles) for breast and endometrial cancer as aggregate group compared with the normal controls.

Z-score Polfus et al 2021		
	Breast and endometrial cancer	**Normal**
Median	0.22	-0.35
75th Percentile	0.75	0.32
25th Percentile	-0.49	-1.02

The values are higher in the cancer group (aggregate group of breast and endometrial cohorts) compared with the controls.

Higher median Z-scores (as well as higher 25th and 75th percentiles), ie greater number of alleles of GRS1 (Polfus et al) – established SNPs related to diabetes mellitus type II (T2DM), were found in the cancer cohort when compared to the normal control cohort. Comparison of the breast and endometrial cancer cohorts (as aggregate) with controls thus identified a higher burden of T2DM risk alleles in breast and endometrial cancer.

Figure 4.13 shows the GRS Z-score of the breast and endometrial cancer patients together compared to controls, with a clear shift to the right indicating enrichment for the T2DM risk polymorphisms in the case population.

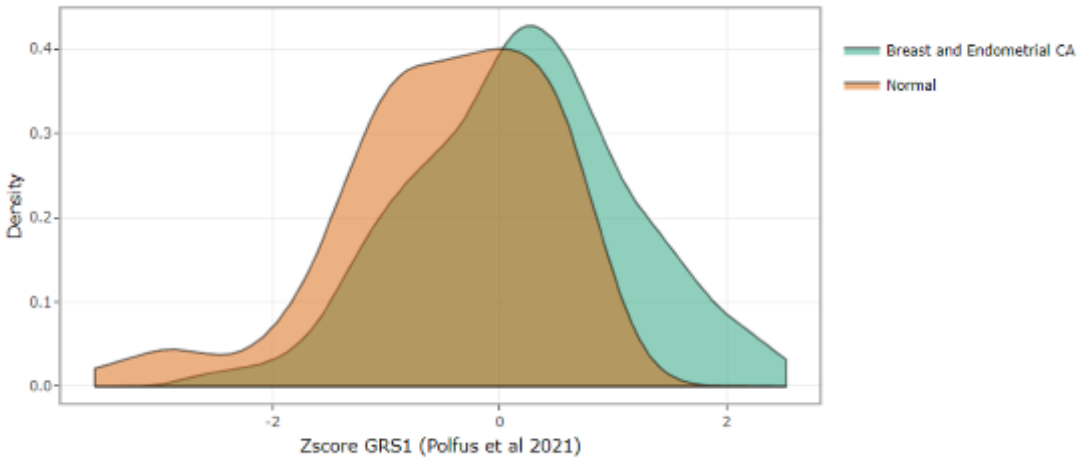

Figure 4.13 Distribution of the alleles (GRS 1) for the breast/endometrial cancer populations, as aggregate group compared with controls; a clear shift to the right indicates enrichment for the T2DM risk polymorphisms (GRS1, Polfus et al, 2021) in the breast/endometrial population

Figure 4.14 and Figure 4.15 show the respective distribution of GRS Z-score of the breast cancer population and endometrial cancer population separately compared to controls, the first as a density distribution plot while the latter as a violin plot

(wider sections depict more observations, thinner sections correspond to less observations).

The median GRS for the patient cohorts is shown in Table 4.15. Consistent with the findings obtained when comparing the two malignancies together as aggregate, comparing breast and endometrial cancer separately with controls also showed higher burden of the T2DM alleles (higher medians and higher 25th and 75th percentiles) as well as a clear right shift of the density distribution graph of both cancer groups compared to controls. (Figure 4.14). Of note, the endometrial cancer cohort exhibited the highest Z-score median and the greatest right shift. Increasing the number of controls will probably show less overlap and better distinction between the cancer and normal cohorts.

Table 4.16 Median Z scores (together with the 25th and 75th percentiles) for breast and endometrial cancer, shown separately, compared with the normal controls.

Z score Polfus et al 2021			
	Breast carcinoma	**Endometrial carcinoma**	**Normal**
Median	0.11	0.32	-0.35
75th Percentile	0.65	0.82	0.32
25th Percentile	-0.62	-0.39	-1.02

The values are higher in the cancer group compared with the controls.

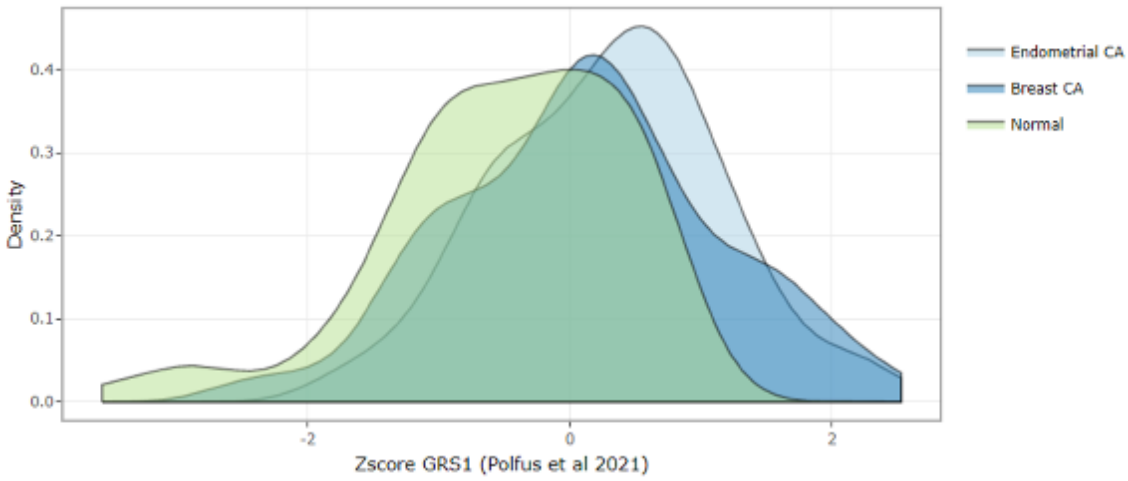

Figure 4.14 A distribution plot comparing the allele distribution (GRS 1) for breast and endometrial cancer group, shown separately, compared with controls; a clear shift to the right indicates enrichment for the T2DM risk polymorphisms (GRS1, Polfus et al, 2021) in the case populations.

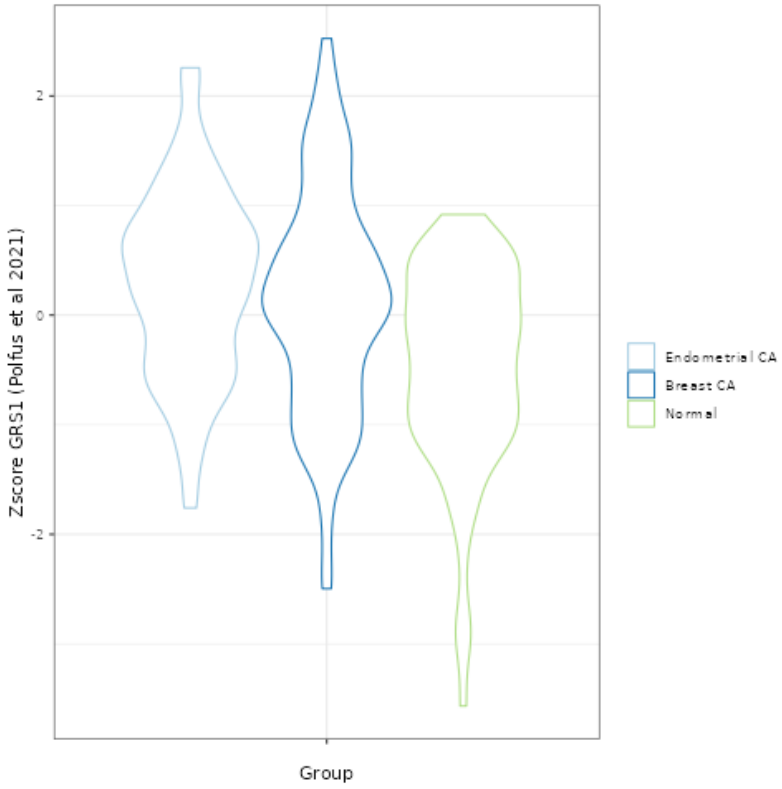

Figure 4.15 A violin plot showing risk score distribution of GRS1 (Polfus et al, 202) Zscores –depicting the distribution of the Z-scores in the three populations – wider sections depict more observations, thinner sections correspond to less observations.

As outlined in the methods, Z-scores were categorised into quintiles - Quintile 1 is regarded as the lowest (first) quintile, having the lowest number of alleles while quintile 5 has the highest number of alleles. Dividing GRS into quantiles helps to evaluate the extent of genetic burden - patients in the lowest level (Quintile 1) having the lowest genetic predisposition while patients in Quintile 5, the highest genetic predisposition.

Logistic Regression modelling was then used to derive the odds of breast and/or endometrial cancer per GRS Z-score quintile, relative to the first quintile (which has the lowest burden of risk alleles). In this analysis, breast and endometrial cancer were considered as the dependent response variable - initially as an aggregate cancer cohort and subsequently as separate groups. The Z-transformed GRS was used as the independent predictor.

In Model 1, no adjustment for confounding factors was implemented, and crude ORs are presented. Adjustment for confounding factors was then performed as follows: in model 2, adjustment for age at diagnosis, fasting insulin, fasting plasma glucose and waist:hip ratio was implemented; in model 3, triglyceride levels were also incorporated into the regression model in view of the established association between fasting triglyceride levels and insulin resistance (Klop et al, 2013).

Below is a table (Table 4.17) showing logistic regression models of GRS 1 (Polfus et al., 2021) with the populations of breast and endometrial cancer together (as aggregate cohort).

Table 4.17 Logistic regression models of GRS1 (Polfus et al., 2021) with aggregate breast/endometrial cancer, unadjusted and adjusted for variable parameters.

GRS 1 (Polfus et al., 2021)	Odds ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Model 1 Unadjusted				
Quintile 5	**10.01**	**<0.01**	3.53	28.40
Quintile 4	**2.49**	**0.018**	1.17	5.32
Quintile 3	2.18	0.055	.98	4.84
Quintile 2	1.74	0.149	.82	3.71
Quintile 1	1	-	.	.
Model 2 Adjusted for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio				
Quintile 5	**20.60**	**<0.01**	5.30	80.11
Quintile 4	**2.98**	**0.014**	1.25	7.13
Quintile 3	2.27	0.069	.94	5.52
Quintile 2	2.40	0.047	1.01	5.69
Quintile 1	1	.	.	.
Model 3 Adjusted for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio, triglycerides				
Quintile 5	**21.74**	**<0.01**	5.44	86.80
Quintile 4	**3.19**	**0.010**	1.32	7.73
Quintile 3	2.37	0.058	.97	5.81
Quintile 2	2.36	0.052	.99	5.644
Quintile 1	1	.	.	.

In crude (unadjusted) models, cancer risk was found to be statistically significantly higher in quintile 5 – OR 10.01, p<0.01 and quintile 4 – OR 2.49, p=0.018. This indicates that the odds of having cancer in Quintile 5 (the quintile having the highest number of alleles) of GRS1 was 10.01 higher than Quintile 1 (the quintile with the least number of risk alleles) – signifying a strong association with T2DM risk alleles. Quintile 4 had an odd of 2.49 greater than the odds of exposure among controls. These relations were preserved even after adjusting for different parameters (Table 4.17). This increased odds of exposure among cancer cases signify a positive association or a risk factor for the disease. Thus, a higher number of alleles of GRS 1 (Polfus et al, 2021) was found to be significantly associated with breast and endometrial cancer.

The following two tables, Table 4.18 and Table 4.19 show the regression models for the associations between GRS1 (Polfus et al., 2021) with breast and endometrial cancer separately (after adjustment for age at diagnosis, fasting insulin, fasting plasma glucose, waist: hip ratio, triglycerides).

Table 4.18 Logistic regression models of GRS1 (Polfus et al., 2021) with breast cancer (adjusted for variable parameters)

GRS 1 (Polfus et al., 2021)	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Adjusted for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio, triglycerides				
Quintile 5	**11.07**	**0.001**	2.79	43.98
Quintile 4	2.02	0.151	.78	5.24
Quintile 3	1.99	0.154	.77	5.17
Quintile 2	1.39	0.49	.55	3.53
Quintile 1	1	.	.	.

Table 4.19 Logistic regression models of GRS1 (Polfus et al., 2021) with endometrial cancer (adjusted for variable parameters)

GRS 1 (Polfus et al., 2021)	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Adjusted for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio, triglycerides				
Quintile 5	**191.03**	**<0.01**	24.64	1480.74
Quintile 4	**16.80**	**0.001**	3.32	84.99
Quintile 3	**6.75**	**0.025**	1.27	35.86
Quintile 2	**13.10**	**0.001**	2.81	61.10
Quintile 1	1	.	.	.

After adjusting for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio, triglycerides, breast cancer risk was found to be statistically significantly higher in

quintile 5 only - OR 11.07, p=0.001, while endometrial cancer risk was found to be statistically significantly higher in quintile 5 OR 191.03, p<0.01, quintile 4 – OR 16.80, p=0.001, quantile 3 – OR 6.75, p=0.025 and quantile 2 – OR 13.09, p=0.001. Thus, alleles of GRS 1 (Polfus et al., 2021) were found to be significantly positively associated with endometrial cancer risk even at the lower quantile level.

Of note was the very high OR of endometrial cancer. This may be due to the very broad confidence interval, likely secondary to the small number of cases compared to controls in the top quintile. Larger numbers are required to evaluate the relevance of this findings.

4.4.2 Genetic association analysis with GRS 2 (Aly et al., 2021)

A greater number of alleles of GRS 2 (Aly et al., 2021) -including 223 polymorphisms related to insulin resistance, was found in breast and endometrial cancer cohort when compared to controls. Table 4.20 shows the difference in the mean and median Z-score of the breast/endometrial cancer population (as aggregate) compared to controls; while Table 4.21 shows the differences in mean Z-scores for breast and endometrial cancer cohorts separately compared to normal cohort.

Table 4.20 Mean and Median Z score GRS 2 (Aly et al., 2021) of breast/endometrial cancer vs controls

	Breast and Endometrial CA		Normal	
	Mean	Median	Mean	Median
Zscore GRS2 (Aly et al, 2021)	.28	.37	-.74	-.83

A higher mean / median Z-score GRS2 (Aly et al., 2021) was found in breast and endometrial cancer cohort when compared to controls.

Table 4.21 Mean Z scores (together with the 25th and 75th percentiles) for breast and endometrial cancer compared with the normal controls.

Z score Aly et al 2021			
	Breast carcinoma	**Endometrial carcinoma**	**Normal**
Median	0.37	0.37	-0.83
75th Percentile	0.92	0.87	-0.13
25th Percentile	-0.33	-0.33	-1.24

The values are higher in the cancer groups compared with the controls.

The following figures show the distribution of alleles in this GRS for endometrial/breast cancer populations together (Figure 4.16) and separate (Figure 4.17) when compared to controls. Of note is the clear shift to the right of the breast/endometrial aggregate cohort, indicating enrichment for the insulin resistance risk polymorphisms (GRS2, Aly et al, 2021) in the breast/endometrial population. The distribution of alleles GRS 2 of the breast cancer and endometrial cancer cohort were observed to be very similar (Figure 4.17 and Figure 4.18).

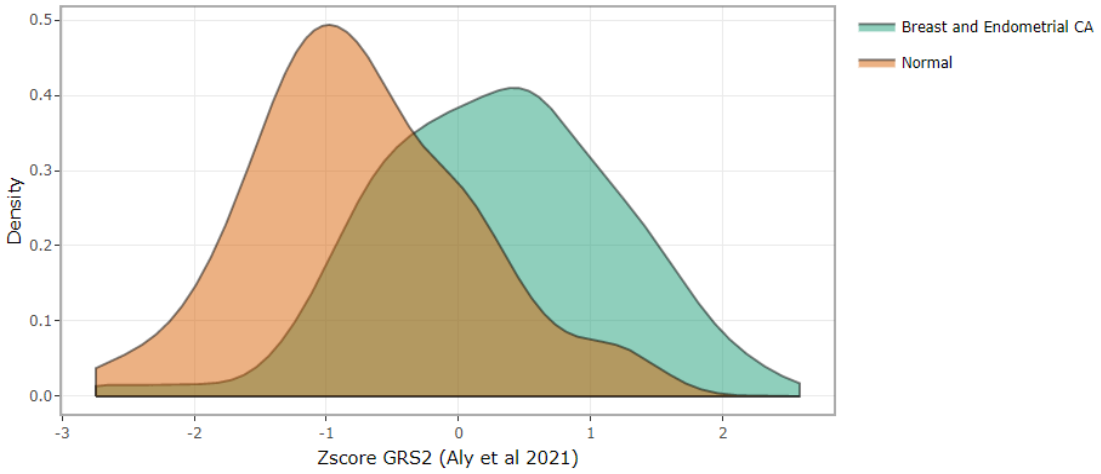

Figure 4.16 Distribution of the alleles (GRS 2) for the breast/endometrial cancer populations (as aggregate) and controls; a clear shift to the right indicates enrichment for the insulin resistance risk polymorphisms (GRS2, Aly et al, 2021) in the breast/endometrial population

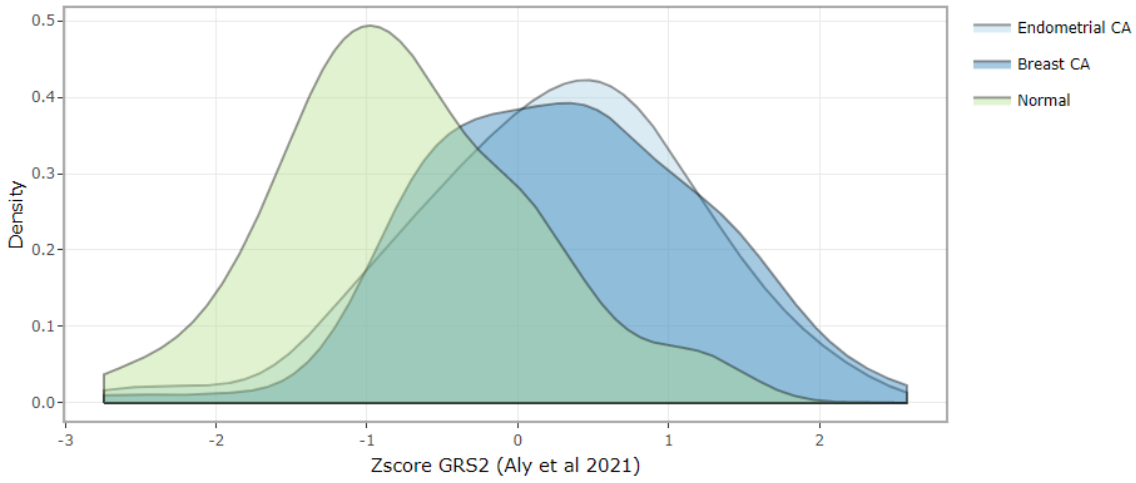

Figure 4.17 A distribution plot of the alleles (GRS 2) for the breast and endometrial cancer populations respectively and controls - showing a clear shift to the right indicates enrichment for insulin resistance (GRS2, Aly et al, 2021) in the case populations.

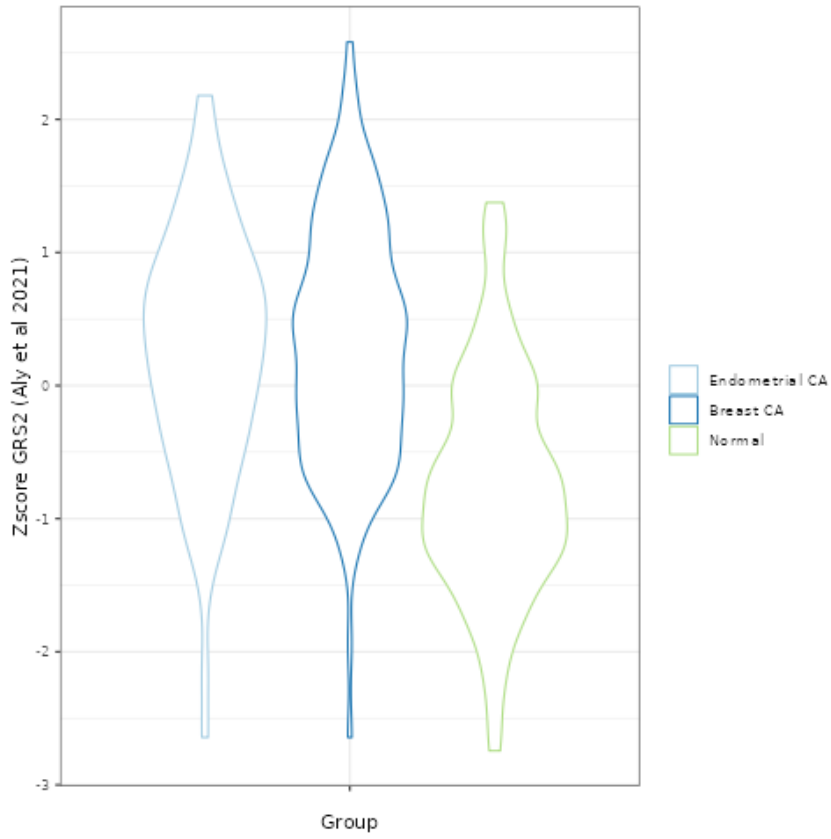

Figure 4.18 A violin plot showing risk score distribution of GRS2 (Aly et al, 2021) Z-scores–depicting the distribution of the Z-scores in the three populations – wider sections depict more observations, thinner sections correspond to less observations.

Table 4.22 is showing logistic regression models of GRS 2 with the populations of breast and endometrial cancer together. In Model 1, no adjustment for confounding factors was implemented, and crude ORs are presented. Adjustment for confounding factors was then performed as follows: in model 2, adjustment for age at diagnosis, fasting insulin, fasting plasma glucose, waist:hip ratio and triglycerides was implemented; in model 3, serum oestradiol level, family history (FH) of endometrial cancer, FH of Breast cancer and menopause age were also incorporated into the regression model (Table 4.22).

Without controlling for anything, GRS 2 was found to be statistically significant higher in all quintiles. This increase in cancer risk remained statistically significantly higher even after adjusting for various parameters including age at diagnosis, fasting Insulin, fasting glucose, waist: hip ratio, triglycerides, oestradiol, FH of endo cancer, FH of Breast cancer, menopause age (Table 4.22, Model 3).

Table 4.22 Logistic regression models of GRS2 (Aly et al., 2021) with breast and endometrial cancer unadjusted and adjusted for variable parameters.

GRS 2 (Aly et al, 2021	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Model 1 Unadjusted				
Quintile 5	**24.39**	**<.01**	7.83	75.95
Quintile 4	**21.81**	**<.01**	7.68	61.95

Quintile 3	**6.62**	**<0.01**	2.98	14.69
Quintile 2	**4.59**	**<0.01**	2.09	10.07
Quintile 1	1	.	.	.
Model 2 Adjusted for age at diagnosis, fasting Insulin, fasting glucose, waist: hip ratio, triglycerides				
Quintile 5	**48.60**	**<0.01**	10.33	228.62
Quintile 4	**23.82**	**<0.01**	7.83	72.41
Quintile 3	**8.46**	**<0.01**	3.40	21.04
Quintile 2	**4.41**	**0.001**	1.84	10.52
Quintile 1	1	.	.	.
Model 3 Adjusted for age at diagnosis, fasting Insulin, fasting glucose, waist: hip ratio, triglycerides, oestradiol, FH of endo Ca, FH of Breast Ca, menopause age				
Quintile 5	**43.41**	**<0.01**	7.99	235.73
Quintile 4	**37.70**	**<0.01**	9.61	147.90
Quintile 3	**9.21**	**<0.01**	2.99	28.38
Quintile 2	**4.47**	**0.008**	1.47	13.57
Quintile 1	1	.	.	.

When separating the cancer population into breast and endometrial cancer respectively, association of GRS 2 with both breast cancer and endometrial cancer risk was still preserved (Table 4.23 and Table 4.24).

Table 4.23 Logistic regression models of GRS2 (Aly et al, 2021) with breast cancer adjusted for variable parameters.

GRS 2 (Aly et al, 2021)	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Adjusted for age at diagnosis, fasting Insulin, fasting glucose, waist:hip ratio, triglycerides				
Quintile 5	49.27	**<0.01**	9.71	249.76
Quintile 4	27.2	**<0.01**	7.87	94.04
Quintile 3	9.04	**<0.01**	3.27	24.96
Quintile 2	5.01	**0.002**	1.82	13.71
Quintile 1	1	.	.	.

Table 4.24 Logistic regression models of GRS2 (Aly et al., 2021) with endometrial cancer adjusted for variable parameters.

GRS 2 Aly et al, 2021)	Odds Ratio (OR)	Significance	95% Confidence Interval for OR	
			Lower	Upper
Adjusted for age at diagnosis, fasting Insulin, fasting glucose, waist:hip ratio, triglycerides				
Quintile 5	**48.60**	**<0.01**	10.33	228.62
Quintile 4	**23.82**	**<0.01**	7.83	72.41
Quintile 3	**8.46**	**<0.01**	3.40	21.04
Quintile 2	**4.41**	**0.001**	1.84	10.52
Quintile 1	1	.	.	.

4.4.3 Summary of % Z-score GRS in the different quintiles

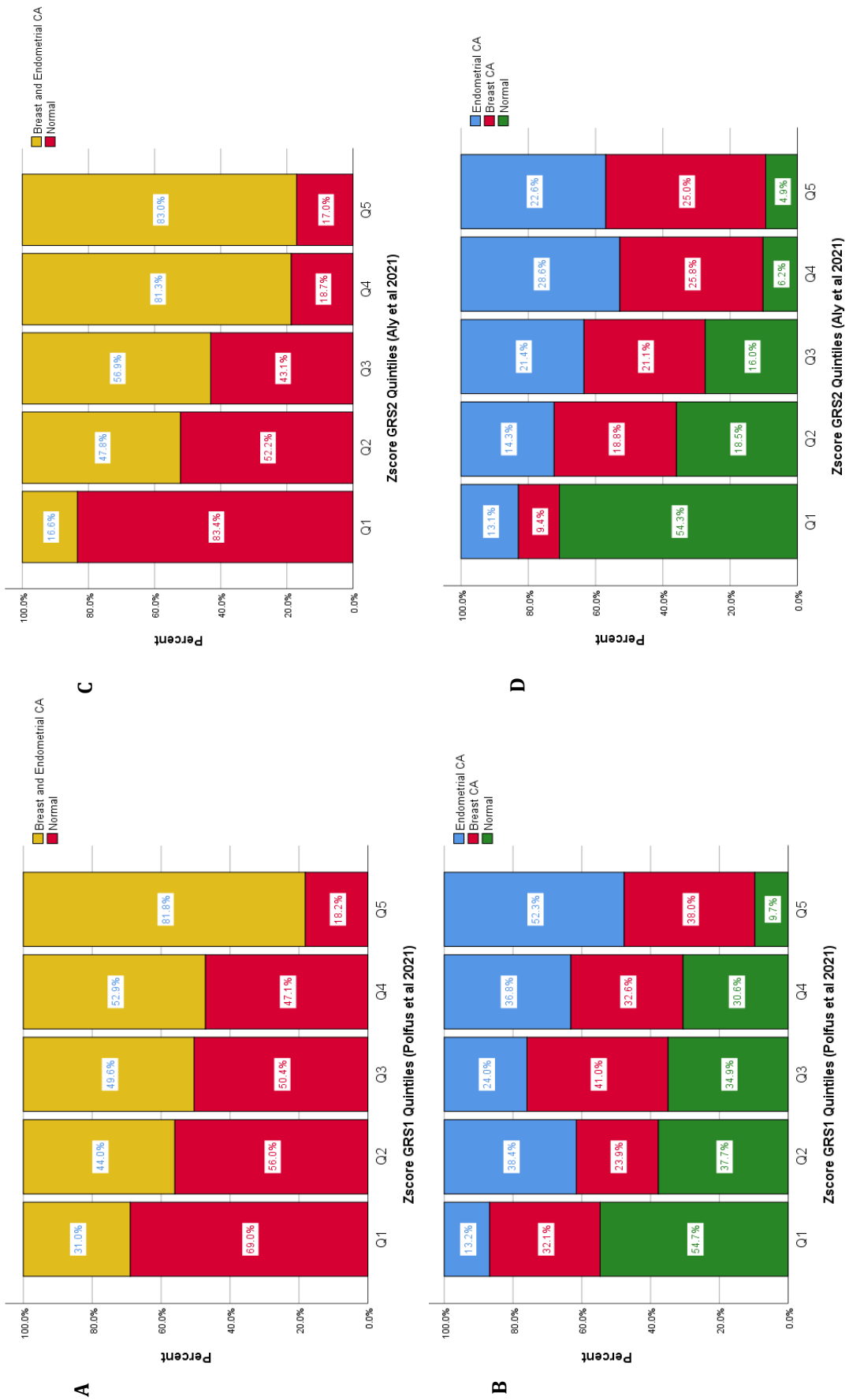

Figure 4.19 Graphs showing the % Z-score GRS in respective Quintiles. Figure A describes the % distribution of Zscore GRS 1 in the normal cohort compared with breast/endometrial cancer cohort as aggregate while in Figure B, endometrial and breast cancer cohort are separate. Figure C describes the % distribution of Zscore GRS 2 in breast/endometrial cancer cohort as aggregate compared with controls while in Figure D, endometrial and breast cancer cohort are separate. It is evident that higher % of the normal cohort occupies Quintiles 1 (with the least risk alleles). The malignancy cohort, both as aggregate or separate cohorts, occupies mostly the higher Quintiles – higher burden risk polymorphisms in the malignancy cohorts.

4.5 Risk prediction model performance

The risk predictive performance was evaluated using the area under the receiver operating characteristics curve (ROC) (Figures 4.20 and 4.21). A higher score signifies a better discriminatory parameter. An area under the ROC curve of 0.5 suggests no discrimination. The higher the value the better the test is as a predictive test for screening, with more than 0.8 considered an excellent test to predict the disease (Mandrekar, 2010). Reference line is included as a baseline – if a parameter falls below the reference line, it is not sensitive or specific to be used as a predictor.

BMI was used together with GRS1 and GRS 2 in the risk predictor model. BMI was included in this ROC curve because from the logistic regression models, BMI was identified as the best predictor for both breast and endometrial cancer (OR 1.039; 95% CI 1.01-1.07 and OR 1.084; 95%CI 1.04-1.13 respectively).

4.5.1 Breast cancer risk prediction performance

Table 4.25 and Figure 4.20 show that BMI, Zscore GRS 1(Polfus et al., 2021) and Zscore GRS 2 (Aly et al., 2021) reach an area under the ROC curve more than 0.5, the findings being statistically significant ($p=0.031$, $p<0.01$, $p<0.01$ respectively). Thus, all three parameters – BMI, GRS1 and GRS 2 can be used as breast cancer risk predictors. GRS 2 is however best predictor for breast cancer, having the highest sensitivity and specificity, with an area under the ROC curve of 0.81.

Table 4.25 Area under the receiver operating characteristic for breast cancer prediction including significance, standard error and 95% confidence intervals are shown for BMI, Z-score genetic risk score (GRS) 1 and Z score of GRS 2.

Area Under the ROC Curve					
Test Result Variable(s)	**Area**	**Standard Error**	**Significance**	**95% Confidence Interval**	
				Lower	**Upper**
BMI	0.59	0.04	**0.031**	0.51	0.66
Zscore GRS Polfus et al, 2021	0.66	0.04	**<0.01**	0.58	0.72
Zscore GRS Aly et al, 2021	0.81	0.03	**<0.01**	0.74	0.87

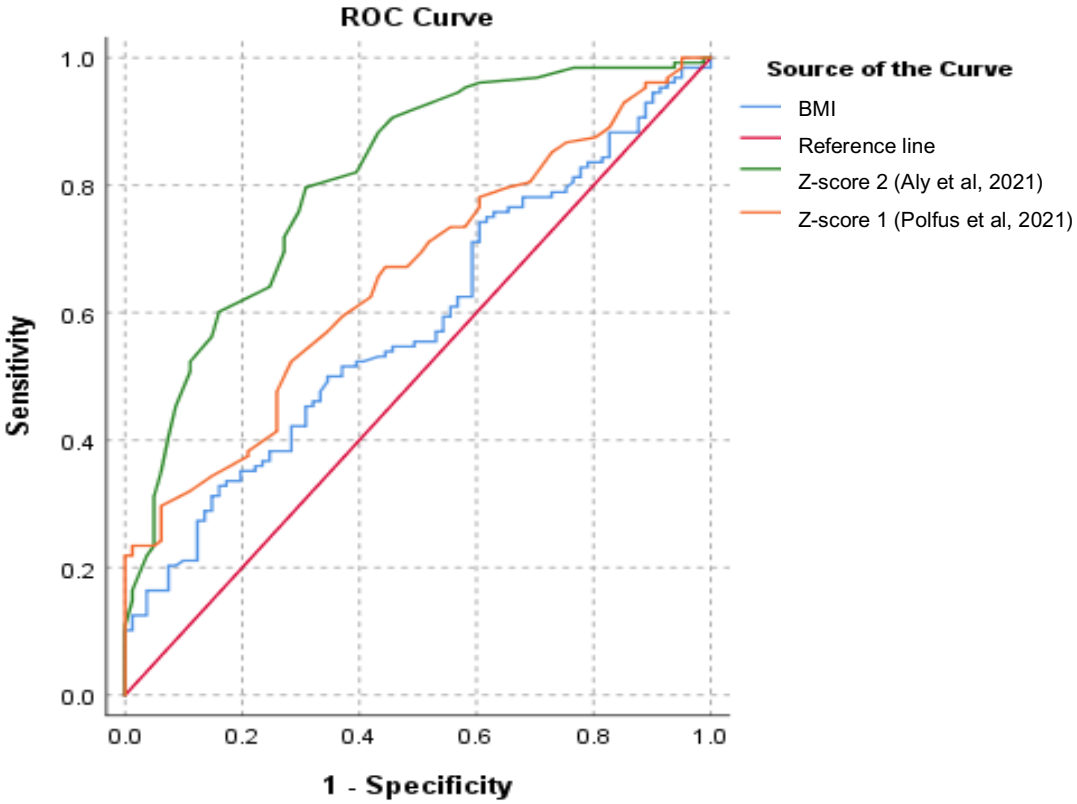

Figure 4.20 Receiver operating characteristic curves for breast cancer prediction are shown for BMI, Z-score genetic risk score (GRS) 1 (Polfus et al, 2021) and Z score of GRS 2 (Aly et al, 2021). The red line is the refence line; GRS 2 is the best predictor.

4.5.2 Endometrial cancer risk prediction performance

Table 4.26 and Figure 4.21 show the area under the ROC curve for BMI, GRS1 and GRS 2 as predictors for endometrial cancer. Similar findings to the breast cancer risk predictor model were found. The three parameters: can be used as risk predictors but GRS 2 is the best predictor for endometrial cancer, having the highest sensitivity and specificity, with an are under the ROC curve of 0.80.

Table 4.26 Area under the receiver operating characteristic for endometrial cancer prediction, including significance, standard error and 95% confidence intervals are shown for BMI, Z-score genetic risk score (GRS) 1 (Polfus et al, 2021) and Z score of GRS 2 (Aly et al, 2021).

Area Under the ROC Curve					
Test Result Variable(s)	**Area**	**Standard Error**	**Significance**	**95% Confidence Interval**	
				Lower	**Upper**
BMI	0.74	0.04	**<0.01**	0.67	0.82
Zscore Polfus et al, 2021	0.73	0.04	**<0.01**	0.65	0.80
Zscore Aly et al, 2021	0.80	0.04	**<0.01**	0.73	0.87

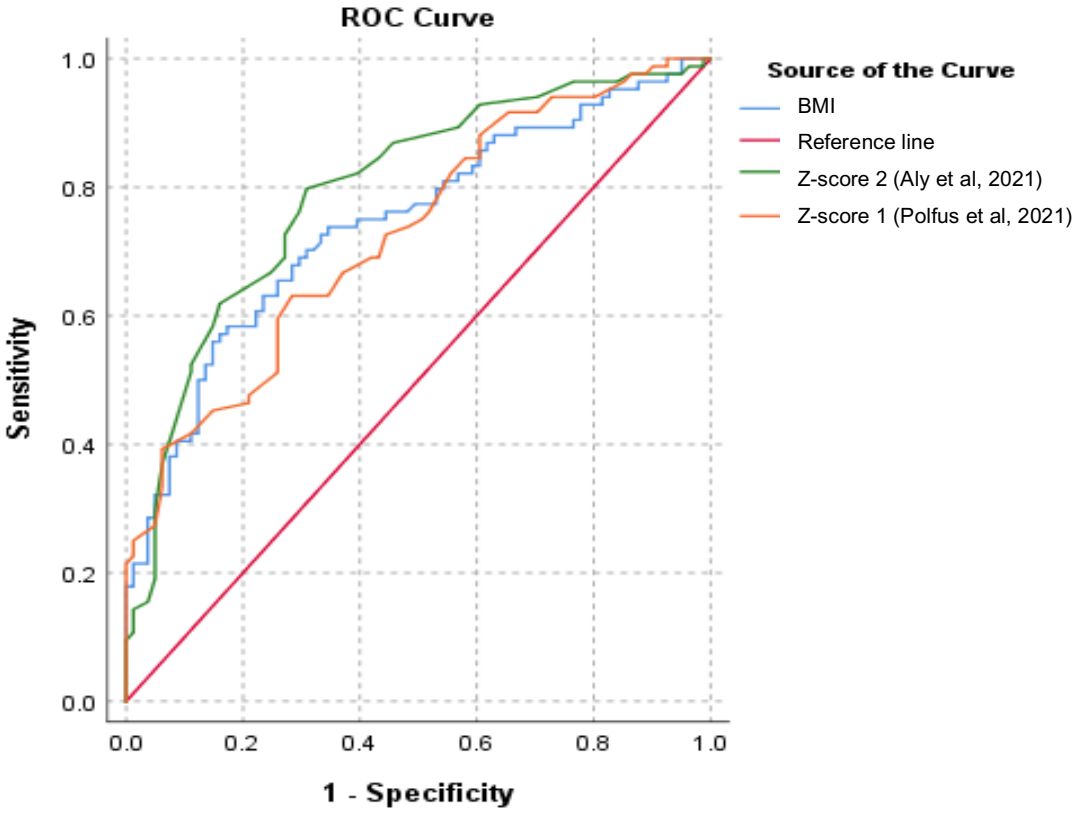

Figure 4.21 Receiver operating characteristic curves for endometrial cancer are shown for BMI, Z-score genetic risk score (GRS) 1 (Polfus et al, 2021) and Z score of GRS 2 (Aly et al, 2021). The red line is the refence line and corresponds to an area under the curve of 0.5.

Chapter 5

Discussion

5.1 Summary of findings

The present study is an original work whereby the principal investigator recruited, interviewed, measured anthropometric paraments and took bloods from the participants. The biochemical/hormonal tests were carried out at MDH according to hospital protocols while DNA extraction and checking DNA purification were carried out by the investigator at the genetics laboratory UOM. DNA analysis was done using LP-GWS at GENEWIZ, Germany.

When evaluating the association of risk factors: clinical, biochemical, metabolic, hormonal and genetics with the hormone dependent malignancies – postmenopausal breast cancer and endometrial cancer the following were observed:

- Nulliparity, early menarche and high BMI showed positive association with both breast and endometrial cancer ($p=0.49$ vs $P<0.01$, $p=0.02$ vs $p=0.01$, $p=0.04$ vs $p<0.01$ respectively).
- Breastfeeding showed a negative association with both malignancies. Breastfeeding was significantly less ($p=0.007$) and duration of breastfeeding shorter ($p<0.01$) in the breast cancer cohort compared to controls. History of breastfeeding was also less in the endometrial cancer cohort compared to controls, $p<0.01$.
- Other factors exhibiting positive association with breast carcinoma compared controls were family history of breast cancer ($p=0.009$), high serum SHBG level ($p<0.01$). The following factors showed negative association with breast cancer: serum FSH level ($p<0.01$) and LH level ($p=0.02$).

- Apart from nulliparity, early menarche and high BMI, the following factors also showed positive association with endometrial cancer: history of hypertension ($p<0.01$), history of diabetes mellitus type 2 ($p<0.01$), history of the metabolic syndrome ($p<0.01$), family history of hypertension ($p=0.007$) and serum triglycerides ($p<0.01$), HbA1C ($P<0.01$) and HOMA-IR ($p=0.01$) levels. Serum levels of SHBG and progesterone showed a negative association with endometrial cancer, with $p=0.01$ and $p=0.01$ respectively.

- Logistic regression analysis model for breast cancer identifies BMI and history of breast cancer as risk factors for breast cancer; BMI has OR 1.04 95% CI 1.01-1.07 ($p=0.24$) while family history of breast cancer has OR 1.48 95% CI 1.01-2.18 ($p=0.046$). Conversely, a history of breastfeeding is protective, OR 0.67 95% CI 0.45-0.98 ($p=0.039$).
- Logistic regression analysis for endometrial cancer identified BMI as risk factor for endometrial cancer, with OR1.08 95% CI 1.04-1.13 ($p<0.01$) and breastfeeding history as protective factor, OR 0.54 95% CI 0.34-0.84 ($p=0.007$).
- Increasing BMI was identified as the best predictor for both breast and endometrial cancer.

- Genetic association analysis with GRS1 (Polfus et al., 2021) showed higher median Z scores in the breast and endometrial cancer cohorts (when analysed as aggregate and also when analysed separately) compared to controls. This implies higher burden of T2DM risk alleles.

- Logistic Regression models of GRS1 (Polfus et al., 20121), crude unadjusted and adjusted models, showed that OR of cancer risk for breast/endometrial cancer (as aggregate group) was higher in quintile 5 compared with lower quintiles. When analysed as aggregate, the cancer cohort had a significantly higher OR in Quintiles 4 and 5, confirming higher burden of diabetes mellitus type II risk alleles in these quantiles
- Logistic regression models (adjusted for age at diagnosis, fasting insulin, fasting glucose, waist: hip ratio, triglycerides) done for breast and endometrial cancer as separate cohorts also showed highest values of OR in quintile 5 compared with lower quantiles. In the breast cancer cohort significance is reached in Quintile 5 while in the endometrial cancer cohort significant difference in OR was observed from quintiles 2-5 when these were compared to quintile 1. Thus, GRS1 (Polfus et al., 2021) can be considered as a significant risk factor for both breast and/or endometrial cancer, stronger for the latter.
- Similar to the genetic association analysis with GRS1 (Polfus et al., 2021), genetic association analysis with GRS2 (Aly et al., 2021), which included insulin resistance risk alleles showed higher median Z scores in the breast and endometrial cancer cohorts (when analysed as aggregate and also when analysed separately) compared to controls. The cancer cohorts had a significantly higher OR in Quintiles 2-5, confirming high burden of insulin resistance risk alleles. GRS2 can be considered as a significant risk factor for breast and/or endometrial cancer, correlation also preserved in the lower Quintiles.

- Risk prediction model performance, as evaluated by ROC, showed that BMI, GRS1 (Polfus et al., 2021) and GRS2 (Aly et al., 2021) can be used as risk predictors for both breast and endometrial cancer. GRS 2 (Aly et al., 2021) was the best predictor for both malignancies, having the highest sensitivity and specificity.

5.2 Breast and endometrial cancer incidence – upward trends

The western lifestyle characterized by increase weight gain and physical inactivity is associated with changes in the metabolic and hormonal milieu including hyperoestrogenaemia, insulin resistance and inflammation. These mechanisms have been repeatedly reported to be key in the development of the hormone dependent malignancies – breast and endometrial cancer.

Worldwide the burden of obesity and the metabolic syndrome is steadily on the increase, as are the incidences of breast and endometrial cancer. Overweight/obesity rate more than doubled between 1980 to 2013 (Ng et al., 2014) accounting for around 30% of world's population (WHO, 2013). The Maltese population is no exception, but rather it is on the forefront. Malta ranks the second country with the highest overweight/obese individuals according to the WHO European Health Report 2018 (WHO, Europe, 2019). The high prevalence of obesity, together with the high incidence of diabetes mellitus and insulin resistance in Malta are clearly linked with the high rates of breast and endometrial cancer reported in this population – the ASR (world) of breast cancer in Malta was 89.5 per 100 000 vs 47.8 per 100 000 worldwide (IARC, 2020) while the ASR (world) of endometrial

cancer in Malta is 17.8 per 100 000 vs 8.4 per 100 000 worldwide (WHO, 2018; Bray et al., 2018).

Moreover, changes in sexual and reproductive practices worldwide are also associated with an increased risk of breast/endometrial cancer. Such risk factors include postponing (or avoidance) of pregnancy and reducing duration (or avoidance) of breastfeeding. Birth rates in the US and Europe and other industrialized countries with high economic activity are on the decline (Skakkebaek et al., 2019). Worldwide rates of breastfeeding are also low – only 1 in 5 babies are breastfed for 12months in high-income countries while only 1 in 3 babies are breastfed for the first 6 months in low and middle-income countries. The worldwide breastfeeding rate at 12months is lowest in the UK (<1%) and Ireland (2%) (Victora et al., 2016) . The latest report of Malta's National Obstetric Information System showed that infant feeding at discharge from hospital decreased from 59.36% in 2015 to 56.54% in 2016 (Gatt & Borg, 2017).

5.3 Clinical and biochemical/hormonal characteristics

In this section we discuss in further detail the results of the clinical/biochemical/hormonal risk factors of these hormone dependent malignancies – postmenopausal breast cancer and endometrial cancer. Table 5.1 summarises our findings as well as compares them with results from the literature.

Risk factor	Breast carcinoma		Endometrial carcinoma	
	Evidence from the literature	**Results from current study**	**Evidence from the literature**	**Results from current study**
Peak age (Western Europe)	65-69 years	60years	65-69 years	66years
Medical conditions DM Hypertension Metabolic syndrome	inconsistent ↥ ↥	↑ ↑ ↑	inconsistent ↥ ↥	↥ ↥ ↥
Total cholesterol HDL Triglycerides	↥ ↓ inconsistent	↑ ↑ ↑	↥ ↓ ↥	↑ ↓ ↥
HbA1C HOMA-IR	↑ ↥	↑ ↓	↥	↥ ↥
Family history of respective cancer	↥	↥	↥	↑
Family history of DM Family history of HT	↑ -	↑ ↑	↥ -	↑ ↥
Tobacco consumption	↥	↑	↓	↑
BMI	↥	↥	↥	↥
WHR	inconsistent	-	↥	↑
Parity - nulliparity	↥	↥	↥	↥
History of breastfeeding Mean duration of breastfeeding	↓ ↓	↓ ↓	↓ ↓	↓ -
Early menarche Late menopause	↥ ↥	↥ -	↥ ↥	↥ ↑
PCOS	inconsistent	↓	↥	↑

Combined HRT COCP	↥ ↥	↓ ↓	↧ ↧	↓ ↓
Oestradiol Testosterone SHBG	↥ inconsistent inconsistent	↑ - ↥	↥ inconsistent ↧	↓ ↓ ↧
FSH LH	↥ ↥	↧ ↧	inconsistent inconsistent	↓ ↓

Table 5.1 A table comparing the findings from the current study with results from the literature. (Underlined are those that reached statistical significance; ↑ and ↓ arrows signify increased or decreased association; - means no difference between cancer and control)

The median age of the cohort population diagnosed with breast cancer is 60.0 years, which is lower than the peak age in Western Europe (65-69years) (Ferlay et al., 2012) but more similar to that of the US (62years) (SEER, 2016). The median age of patients diagnosed with endometrial cancer was 66.0years. This is in parallel to the peak age group in Western Europe, which is between 65-69years (Ferlay et al., 2012) while it is higher than US average age at diagnosis of endometrial cancer which is 60years (American cancer society, 2022).

More women with breast or endometrial carcinoma in the present study were nulliparous when compared to the normal control group respectively, both being statistically significant ($p=0.049$ and $p<0.01$ respectively). Parity has long been studied in association with breast and endometrial cancer risk and has been proposed as protective factor for these malignancies (Savona-Ventura & Grech, 1986; Akbari et al., 2011; Wu et al., 2015).

History of breastfeeding did reach statistically significant difference between both the breast cancer cohort and endometrial cancer cohort when compared to the normal control cohort ($p=0.007$ and $p<0.01$ respectively). Lower percentages of

women with breast/endometrial cancers reported ever breastfeeding. The number of months of breastfeeding were also lower in patients with a history of breast/endometrial cancer compared with the normal cohort, however the difference here only reached significance for the breast cancer cohort ($p<0.01$). Breastfeeding has also long been studied in association with these hormone dependent malignancies and has also been proposed as a protective factor in multiple studies (Akbari et al., 2011; Ma et al., 2018; Yang & Jacobsen, 2008; Collaborative Group on Hormonal Factors in Breast Cancer, 2002; Zhan et al., 2015).

Patients with history of breast/endometrial cancer had a menarche age which was statistically lower than the normal cohort ($p=0.02$ and $p=0.01$). This reflects data from the Collaborative Group on Hormonal Factors in Breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2012) which concluded that the younger the menarche age the higher the risk for breast cancer and data from the Black women's Health Study which concluded that menarche age at less than 11years was associated with higher risk of endometrial cancer when compared with menarche age of 12-13years (Sponholtz et al., 2017).

Our data showed no statistically significant difference between age of menopause in breast/endometrial cancer cohorts when compared to the normal cohort. However, the Collaborative Group on Hormonal Factors in Breast cancer found an increased breast cancer risk with increased menopausal age while epidemiological evidence does show consistently that age of menopause is positively correlated with endometrial cancer (Dunneram et al., 2019; Wernli et al., 2006; Jung et al., 2016).

The duration of the reproductive years, i.e. the duration of ovarian hormone exposure has also been studied. It has been proposed that the longer the exposure, the increased risk for breast and endometrial cancer (Jung et al., 2016). The present study did show a longer exposure to ovarian hormones in the breast/endometrial cancer cohort compared with the normal controls, but such differences were not statistically significant.

PCOS was found in a lower percentage in the breast cancer cohort as compared to controls, while it was found marginally higher percentage in the endometrial cancer group compared with the normal group - both differences however were not statistically significant. Although there is no consensus in the literature regarding the association of PCOS with breast cancer (Agnoli et al., 2010), studies did show association of PCOS with endometrial cancer (Ding et al., 2018).

Patients with breast/endometrial cancer were found to have higher percentage of HT, hypercholesterolaemia, DM and metabolic syndrome when compared with normal controls. However statistical significance was only reached when comparing history of HT, DM and presence of metabolic syndrome in the endometrial cancer cohort with the normal control cohort ($p<0.01$, $p<0.01$, $p<0.01$). Epidemiological studies do show a positive association between hypercholesterolaemia, DM and metabolic syndrome with breast cancer (De Bruijn, et al., 2013; Agnoli et al., 2010; Kitahara et al., 2011) while multiple studies also show an association between HT, DM and metabolic syndrome with an increased endometrial cancer risk (Friberg et al., 2007; Alberti, 2006).

Positive associations were found for HOMA-IR, HbA1C and triglycerides and endometrial cancer risk, but not with breast cancer risk. Studies do show higher levels of insulin and HbA1c in patients with endometrial cancer (Naed et al, 2015; Karaman et al, 2015). Dossus et al, also observed higher serum triglycerides in patients with endometrial cancer risk, however Kho et al noted that the role of triglycerides in endometrial carcinogenesis is inconclusive (Kho et al, 2021).

Excess body weight has been repeatedly reported to be strongly associated with increased breast and endometrial cancer risk (Renehan et al., 2008; Wang et al., 2016; Bhaskaran et al., 2014; Shaw et al., 2016; Yang et al., 2012; Sponholtz et al., 2016). Excess body weight has been associated with around 9% of female breast cancer while obesity has been attributed to at least 40% of endometrial cancer in the UK (Renehan et al., 2008). Interestingly, the Nurses' Health Study II showed that although body fatness in childhood/adolescence is associated with increased endometrial risk, it was inversely associated with pre-and postmenopausal breast cancer risk (Baer et al, 2020; Dougan et al, 2015). In the present study, BMI and WHR were the parameters taken to assess body fatness. The mean BMI of patients in the breast cancer cohort and of patients in the endometrial cancer cohort both were found to be statistically significantly higher than the BMI of the normal control cohort ($p=0.04$ and $p<0.01$ respectively). No statistically significant association was however found between WHR and breast or endometrial cancer risk respectively.

Smoking was found at a higher percentage in the breast cancer cohort when compared to controls, but association was found to be not significant (22% vs 12.2%, $p=0.072$), concluding that smoking in our study was not associated with

increased breast cancer risk. Epidemiological evidence regarding smoking and breast cancer is inconsistent. However, findings from the Generations Study and the Danish nurse cohort study do show an increased risk of breast cancer with smoking (Andersen et al., 2017; Jones et al., 2017). In fact, tobacco smoking is included as one of the probable causes of breast cancer by IARC. On the other hand, multiple studies including the National Institutes of Health-AARP Diet and Health Study showed that smoking decrease the risk of endometrial cancer (Felix et al., 2014). The findings of the present study however do not conform – the endometrial cancer cohort has a higher percentage of smokers compared to the controls, but this difference in history of smoking was not found to be statistically significantly associated with endometrial cancer.

The use of hormonal replacement therapy or hormonal use as oral contraception did not show any significant association with breast cancer risk. As opposed to the literature there were more users of both the COCP and HRT in the normal cohort group as compared to the breast/endometrial cancer cohort, although difference was not statistically significant. In the literature, there is robust data including results from the Million Women study and WHI, showing a positive association between HRT with breast cancer (Reeves et al., 2006; Manson et al., 2013; Pragout et al., 2018). Current/recent use of oestrogen-progesterone combined contraceptives and current use of combined oestrogen-progesterone HRT are currently classified by the IARC as being a cause for breast cancer (Cogliano et al., 2011). An opposite effect is seen with the use of COCP or HRT (oestrogen and progestin) on endometrial cancer risk – both are associated with decreased endometrial cancer risk (Mueck et al., 2010; Manson et al., 2013). Only the use of

cyclic/sequential combined HRT with less than 10days of progesterone appears to be associated with increased risk of endometrial cancer (Sjögren et al., 2016; Manson et al., 2013).

Multiple studies had shown that family history of breast cancer is a strong risk factor for breast cancer (Brewer et al., 2017; Kotsopoulos et al., 2020) while family history of endometrial cancer showed positive association with endometrial cancer (Johnatty et al., 2017). Our study confirms the association of family history of breast cancer with increased risk of breast cancer ($p=0.009$). However, family history of endometrial cancer, although was higher in endometrial cancer cohort, was not found significantly higher.

Analysis of the endogenous sex hormones in patients diagnosed with breast cancer showed statistically significant lower levels of FSH and LH ($p<0.01$ respectively). FSH and LH are the key glycoproteins that control the regulation of oestrogen and progesterone production. They are also suggested to be involved in postmenopausal breast cancer progression which typically have higher levels of FSH and LH (Sanchez et al., 2018; Zhou, J. et al., 2013). This is inconsistent with the present finding of low level of FSH and LH in the breast cancer cohort.

When comparing the breast cancer cohort with the normal controls, higher level of serum SHBG ($p=0.02$) was observed in the breast cancer cohort. The association of SHBG with breast cancer risk in the literature is inconsistent. Higher levels of SHBG were found in the postmenopausal women with ER-positive breast cancer in UKTOCS study (Fourkala et al., 2016). On the other hand, EHBCG reported that low

levels of SHBG are associated with increased breast cancer risk (Key et al., 2002). A recent study confirmed an inverse correlation between SHBG and ER+ breast cancer but also found a positive correlation between SHBG and ER- breast cancer (Dimou et al., 2019) .

In our study, serum SHBG and progesterone levels were found to be significantly lower ($p=0.01$ and $p=0.01$ respectively) in the endometrial cancer cohort compared with the normal cohort. The low levels of progesterone and SHBG found in the endometrial cancer cohort are consistent with evidence from the literature. Mullee et al, in an observational study of 159,702 women found an inverse association of SHBG with endometrial cancer risk (Mullee et al, 2021). Natural progesterone has long been described as being protective of endometrial cancer (Lieberman & Curtis, 2017). It is used even as treatment for early endometrial cancer (Stage 1A) when surgery is contraindicated.

Although the relation between oestradiol level and breast cancer risk was not statistically significant, the breast cancer cohort did show a higher median oestradiol level. It was however surprising that lower levels of oestradiol were obtained in the endometrial cancer cohort compared to the controls. This finding contrasts with the literature which strongly suggest that endometrial cancer is positively associated with bioavailable oestradiol level. It is possible that this difference can be explained due to the fact that the normal cohort population is obese (mean BMI 28.5).

In conclusion, our findings of associations of risk factors with breast/endometrial cancer are overall in conformity with evidence from the literature.

5.3.1 Comparing breast and endometrial cancer cohorts

Postmenopausal breast cancer and endometrial cancer are both considered as hormone dependent malignancies. Although their carcinogenesis pathways may not necessary be exactly the same, they do share common aetiologies. Our study did confirm that the following:

- Risk factors observed to have positive association with both malignancies: nulliparity, early menarche age, BMI
- Risk factors observed to have negative association with both malignancies: breastfeeding history
- Risk factors that showed similar occurrences in both malignancy cohorts: menarche age, menopause age, reproductive age, WHR, serum oestradiol, serum progesterone, serum testosterone, cholesterol, triglycerides, HDL, LDL, HbA1c

5.3.2 Logistic Regression Models

The logistic regression models for breast and endometrial cancer risk factors calculated the odds ratios of the respective risk factor included in the model. Of note BMI and breastfeeding history were included in both the logistic regression models for breast cancer risk factors and endometrial cancer risk factors.

Increasing BMI was identified as the best predictor for both breast and endometrial cancer. It could be noted that for every 1kg/m^2 increase in BMI, the odds of having breast cancer increased by 3.9% (OR=1.04; 95% CI 1.01-1.07; p=0.024) while the odds of having endometrial cancer increased by 8.4% (OR=1.08; 95% CI 1.04-1.13; p<0.01). As already discussed, obesity is a recognised risk factor for both breast and endometrial cancer. In fact, body fatness is classified as a cause of postmenopausal breast cancer by the International Agency for Research on Cancer (IARC) and the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR). Results from the Million Women Study (Yang et al., 2021) and Balck Women's Health Study (Sponholtz et al, 2016) confirms that BMI is strongly associated with endometrial cancer risk. Compared to normal-weight women (BMI <25kg/m^2), Shaw et al. observed that women who are obese (BMI >30, <35 kg/m2), were at 2.6-fold increased risk for endometrial cancer while women who are severely obese (BMI >35kg/m2) were at 4.7-fold increased risk for endometrial cancer (Shaw et al., 2016).

A significant positive correlation was also found between breast cancer and family history of breast cancer; having a first relative with breast cancer increases the odds of having breast cancer (rather than being normal) by 48.1%. (OR 1.48; 95% CI 1.01-2.18; p=0.046). Multiple studies have confirmed family history of breast cancer as a strong breast cancer risk factor (Brewer et al., 2017 & Kotsopoulos et al., 2020). Brewer et al, quoted an increased relative risk of 1.58 (95% CI 1.40-1.79) for postmenopausal women who had a family history of breast cancer. Family history of breast cancer is also used as one of the risk factors included in established risk-prediction models for breast cancer including Gail model and BCSC model.

Breastfeeding was noted to be protective for both breast and endometrial cancer. With breastfeeding, the odds of having breast cancer decreased by 33.5% (OD 0.665; 95% CI 0.45-0.98; p=0.039) and the odds of having endometrial cancer decreased by 46.2% (OR 0.54; 95% CI 0.34-0.84; p=0.007). Analysis of 47epidemiological studies by the Collaborative Group on Hormonal Factors in Breast Cancer, breastfeeding is observed to be a protective factor for breast cancer. Breast cancer risk was decreased by 4.3% for every 12months of breastfeeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002). The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) has also classified breastfeeding as protective factor for breast cancer. In a meta-analysis by Zhan et al, endometrial cancer risk was found to decrease by 1.2% for every month increase in breastfeeding (Zhan et al., 2015) and data from the meta-analysis from the Epidemiology of Endometrial cancer Consortium noted that the longer the duration of breastfeeding, the lower the endometrial cancer risk, levelling of this association was observed beyond 6-9months (Jordan et al., 2017).

5.4 Genetic Results

Genetic associations have been reported both with breast and endometrial malignancies. The most common cause of hereditary breast cancer is due to inherited mutation in BRCA1 or BRCA 2 gene while Lynch syndrome (autosomal dominant germline mutation in DNA mismatch repair genes – MLH1, MSH2, MSH6/PMS2) is linked to endometrial cancer. To our knowledge there have been no studies reported in the literature attempting to correlate these malignancies to GRSs related to the clinical components of the metabolic syndrome. Genome-wide

association studies show significant difference in performance of GRS across different ethnic groups (Polfus et al, 2021). This necessitates mapping of the Maltese genome data to the reference PCA coordinates from samples of the HGDP; noting that the Maltese cohort mapped close to the European data points and also to Middle Eastern data points. The genetic risk scores chosen were however both of European ancestry to avoid ethnic bias.

The choice of the GRSs was determined keeping in mind (A) the aim of this study - to investigate markers associated with increased risk of breast and endometrial cancer, and (B) the finding from the logistic regression model that BMI is a strong predictor of both breast and endometrial cancer.

GRSs with polymorphisms known to be associated with insulin resistance (GRS 2, Aly et al., 2021) and type 2 diabetes mellitus (GRS 1, Polfus et al., 2021) alleles were chosen. Insulin resistance is closely linked to obesity/high BMI - with increasing energy intake, hyperinsulinaemia results, which can develop into insulin resistance with chronic energy surplus. Also, both obesity and diabetes mellitus type II form part of the criteria of the metabolic syndrome. Multiple studies had confirmed positive association between history of metabolic syndrome and diabetes mellitus type II with breast/endometrial cancer (Bernard et al., 2016; Agnoli et al., 2010; Luo et al., 2014; Arthur RS et al., 2020).

Genetic association analysis with both GRS1 (Polfus et al, 2021) and GRS2 (Aly et al, 2021) showed higher median Z scores in the breast and endometrial cancer cohorts (when analysed as aggregate and also when analysed separately) compared to

controls. This implies higher burden of T2DM risk alleles and insulin resistance alleles.

Logistic Regression models of GRS1 (Polfus et al, 2021) and GRS2 (Aly et al, 2021) crude unadjusted and adjusted models, showed that OR of cancer risk for breast/endometrial cancer (as aggregate group and as separate cohorts) was higher in quintile 5 compared with lower quintiles. This confirms higher burden of diabetes mellitus type II and insulin resistance risk alleles in the higher quintiles.

5.5 Risk prediction model performance

What do we mean? How can we fit a model to the data to predict the future?

"Model development studies aim to derive a prediction model by selecting the relevant predictors and combining them statistically to a multivariable model." Collins et al, TRIPOD statement 2015

"summarize the effects of predictors to provide individualised predictions of the absolute risk of a diagnostic or prognostic outcome." Steyeberg, 2019

In our current study, the risk prediction models for breast and endometrial cancer as evaluated by using the area under the ROC, showed that BMI, GRS1 (Polfus et al., 2021) and GRS 2 (Aly et al., 2021) can be used as risk predictors for both malignancies in the postmenopausal Maltese population. GRS 2 (Aly et al., 2021) the GRS of SNPs known to be associated with insulin resistance phenotype, was found to be the best predictor for postmenopausal breast cancer and endometrial cancer, being the most sensitive and specific.

5.6 Implementation of prevention strategies

The identification of risk factors is key for the development of validated risk assessment tools and risk-prediction models which will stratify a population according to the individual risk.

Figure 5.1 includes a proposed clinical application of our findings to aid in stratification, starting with BMI, followed by the Aly et al, 2021 GRS thus identifying women at high risk. Our findings would supplement other identified risk prediction scores. If risk for breast/endometrial cancer is deemed to be high, primary prevention strategies can be considered to prevent disease. A personalised approach includes education of the population on their relative risk and incorporating risk-tailored interventions, which may be lifestyle changes (avoidance of potentially preventable risk factors), primary chemoprevention or surgery. For example, individuals identified as having high/moderate risk can be offered appropriate primary chemoprevention or surgery whereas those with low risk for disease would possibly be advised only lifestyle changes. Such plans would of course require large cohort longitudinal studies to ensure effectiveness.

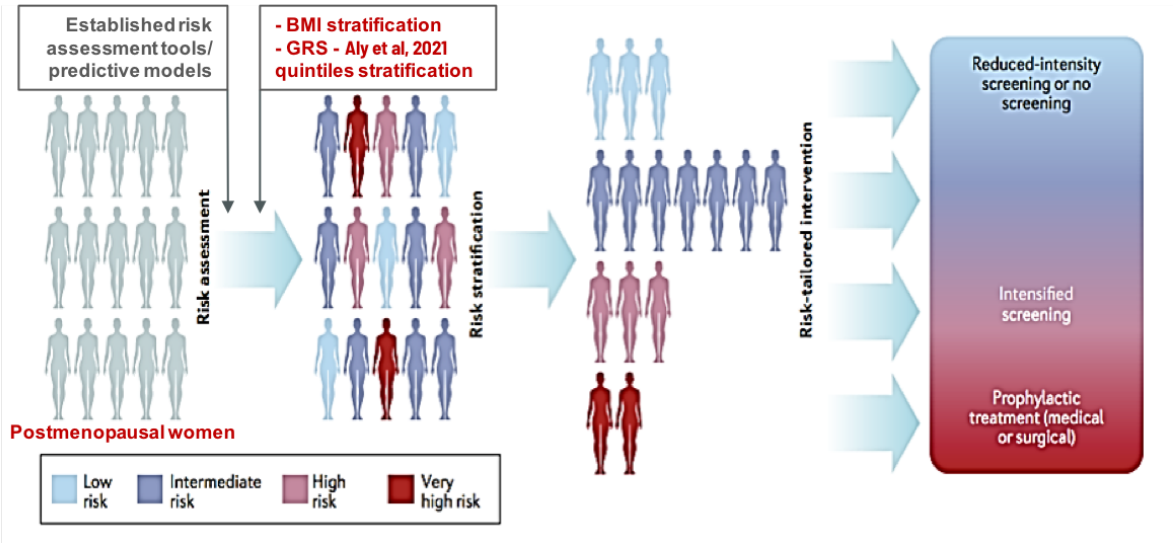

Figure 5.1 Clinical application of our study. BMI and GRS2 (Aly et al, 2021) can be used in the postmenopausal Maltese population to aid better risk stratification for breast/endometrial cancer, so patients at high risk can be identified and preventive strategies implemented.

5.6.1 Primary Prevention – avoidance of preventable risk factors

Effective primary prevention strategies are crucial to tackle the rise in breast/endometrial cancer globally. Education on cancer risk and potentially preventive factors for breast/endometrial cancer is of outmost important. These include:

- **Obesity:** Obesity is associated with increased risk of many cancers including cancer of the breast and endometrium. With the increased obesity pandemic, these cancers are on the increase. The Nurses' Health Study suggested that women who gained 25kg or more since the age of 18years or 10kg or more since menopause compared with those who maintained the same weight are at 1.45, 1.18 increased relative risk of invasive breast cancer respectively. On the other hand, epidemiological data shows that compared to normal-weight women (BMI <25kg/m^2), women who are obese (BMI >30, <35 kg/m2) were at 2.6-fold increased risk for endometrial cancer while women who are

severely obese (BMI >35kg/m2) were at 4.7-fold increased risk for endometrial cancer (Shaw et al., 2016). Dietary modification and exercise help prevent overweight and obesity together with the associated metabolic syndrome, the latter being itself also positively associated with these hormone dependent malignancies.

Women in Steady Exercise Research (WISER) Sister randomised controlled trial examined the effect of aerobic exercise on the clinical biomarkers – cholesterol, triglycerides, glucose, insulin, HOMA-IR and VO_2max (peak oxygen uptake – the maximum amount of oxygen the body can utilise while doing exercise) in women at high risk of developing breast cancer (e.g. BRCA1 or BRCA2 mutation and/or ≥ 18% lifetime risk according to prediction models). In women with BMI more than 25, exercise appeared to decrease insulin and triglycerides level and increasing VO_2max. Thus, the authors concluded that aerobic exercise may be considered in prescribed exercises doses to delay onset of disease in women at high genetic risk for breast cancer (Ehret CJ et al., 2021).

The effect of exercise on endometrial cancer risk was studied by Kitson et al. In their systematic review of the literature, a linear inverse relationship was observed between physical activity and endometrial cancer risk, effect being influenced by BMI (Kitson et al., 2022). Thus, exercise can be considered as an effective low cost primary prevention strategy for both breast and endometrial cancer.

- **Avoiding childbearing:** Before the first full term pregnancy, the breast is mainly composed of Type 1 and Type 2 lobules which are more susceptible to cancer mutations. During the first full term pregnancy, the breast lobules are fully matured containing the most cancer resistant type 4 lobules (Britt et al., 2007). Having a first birth before the age of 18 years is associated with about one-third the breast cancer risk when compared to having a first birth after the age of 35years (MacMahon et al., 1970). Also, according to the Collaborative Group on Hormonal Factors in Breast Cancer, there is an increased risk of 3% for each year older women had their first newborn while the relative risk of breast cancer was calculated to decrease by 7.0% for each birth. Parity is also protective for endometrial cancer; parous women were found to have an incident rate ratio of 0.77 (95% CI 0.57, 1.05) when compared to nulliparous in developing endometrial cancer.

- **Breastfeeding, the lack of:** The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) classified breastfeeding as protective factor for breast cancer. Breastfeeding is thought to decrease the risk for breast cancer by causing differentiation of breast tissue and by reducing the number of ovulatory menstrual cycles; decreasing breast cancer relative risk by 0.84 (Babalou, 2017). Ever breastfeeding was found to be protective for endometrial cancer - associated with an 11% decrease in endometrial cancer risk (Jordan et al., 2017). For every 6months increase in duration of breastfeeding, the endometrial cancer risk was decreased by 7% (relative risk 0.93; 95% CI 0.88, 0.97) (Ma et al., 2018). Efforts are made to support policies and programs to increase breastfeeding rates.

Breastfeeding, especially exclusive breastfeeding offers a magnitude of health benefits including decreasing childhood mortality and breast/endometrial cancer prevention. The WHO and UNICEF organized the Global Strategy for Infant and Young Child Feeding (IYCF) with the aim to achieve 90% universal coverage target (Jones et al., 2003).

- **Use of HRT:** Current use of combined oestrogen-progesterone HRT is classified as being a cause for breast cancer by the IARC (Cogliano et al., 2011). In the Million Women Study, current HRT use was found to be associated with a 66% increased risk of breast cancer than never users while results from the WHI concluded that there are 9 extra cases of breast cancer for every 10,000 postmenopausal women taking HRT. On the other hand, the rate of endometrial cancer was noted to be highest in women taking oestrogen only HRT and the risk increased with duration of use (Grosse et al., 2009) .

- **Use of COCP:** Current/recent use of oestrogen-progesterone contraceptives (combined) is classified as being a cause for breast cancer by the IARC (Cogliano et al., 2011). The earlier the age of starting OCP is suggested to be associated with an earlier age of breast cancer diagnosis (Imkampe & Bates, 2012). Although COCP use is a potential preventable risk factor for breast cancer, COCP use is suggested to be protective of endometrial cancer.

- **Alcohol consumption and smoking:** Alcoholic beverages are listed as a cause of breast cancer by the IARC and WCRF/AICR while tobacco smoking

is listed as a probable cause of breast cancer by the IARC (Cogliano et al., 2010). Women who drank 35-44g per day of alcohol had an increased relative risk of breast cancer of 1.32 (1.19-1.45, P<0.00001) while those who consumed ≥45g of alcohol per day were at an increased relative risk of 1.46 (1.33-1.61, P<0.00001) when compared to women who reported no alcohol intake. Smoking also increases breast cancer risk with an 18% increased risk of breast cancer in ever smokers and 27% increased risk in current smokers (when compared to never smokers) (Andersen et al., 2017). While both alcohol consumption and smoking are potential preventable risk factors for breast cancer, their role in endometrial carcinogenesis in different – smoking is suggested to exert a protective effect while the effect of alcohol is controversial.

5.6.2 Primary Chemoprevention

Chemoprevention refers to the use of chemical agents to prevent carcinogenesis in individuals who are asymptomatic. The side-effect profile of the agent must be weighed against the benefits from chemoprevention in particular the decreased risk of cancer development.

The ideal population targeted would be a population at high risk while the ideal chemoprevention agent would be one with low side-effect profile.

5.6.2.1 Breast cancer chemoprevention

- **Selective oestrogen receptor modular (SERM):** Tamoxifen and raloxifene have been shown to reduce the risk of invasive primary breast cancer in high risk women. The National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 Study found that tamoxifen reduced invasive breast cancer risk by 49% (two-sided P<0.00001) in high risk women. Women at high risk were 60years or older, 35-59years with a 5-year predicted breast cancer risk of ≥ 1.66% according to the Gail model or had a past history of lobular carcinoma in-situ. The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial showed that cases of invasive breast cases in the raloxifene group were similar to those of the Tamoxifen group (Vogel et al., 2006). FDA approved tamoxifen for primary breast cancer prevention in premenopausal and postmenopausal women at high risk in 1998 while raloxifene was approved for breast cancer prevention for postmenopausal women in 2007 (Waters, et al., 2012). Use of tamoxifen has however been shown repeatedly to be associated with endometrial hyperplasia, and increased risk of endometrial cancer, with a 1.5-6.9 increased risk in tamoxifen users (Hu et al, 2015).

- **Aromatase Inhibitors (AI):** The aromatase inhibitor exemestane was also shown to decrease risk of breast cancer in high risk postmenopausal women by 65% (P=0.002, 95% CI 0.18, 0.70). High risk women were defined as women ≥ 60years, or have a 5-year predicted breast cancer risk score (Gail model) of greater than 1.66%, or a previous diagnosis of atypical ductal/lobular hyperplasia/lobular CIS/ductal CIS with mastectomy. Use of

exemastane was associated with no serious side effects and minimal health-related quality-of-life changes (Goss et al., 2011). The American Society of Clinical Oncology developed a guideline on the use of chemoprevention for breast cancer in 2009 which was further updated in 2013 (Visvanathan et al., 2013). A potential algorithm for using breast cancer chemoprevention agents for women at high risk of developing breast cancer is shown in Figure 5.1.

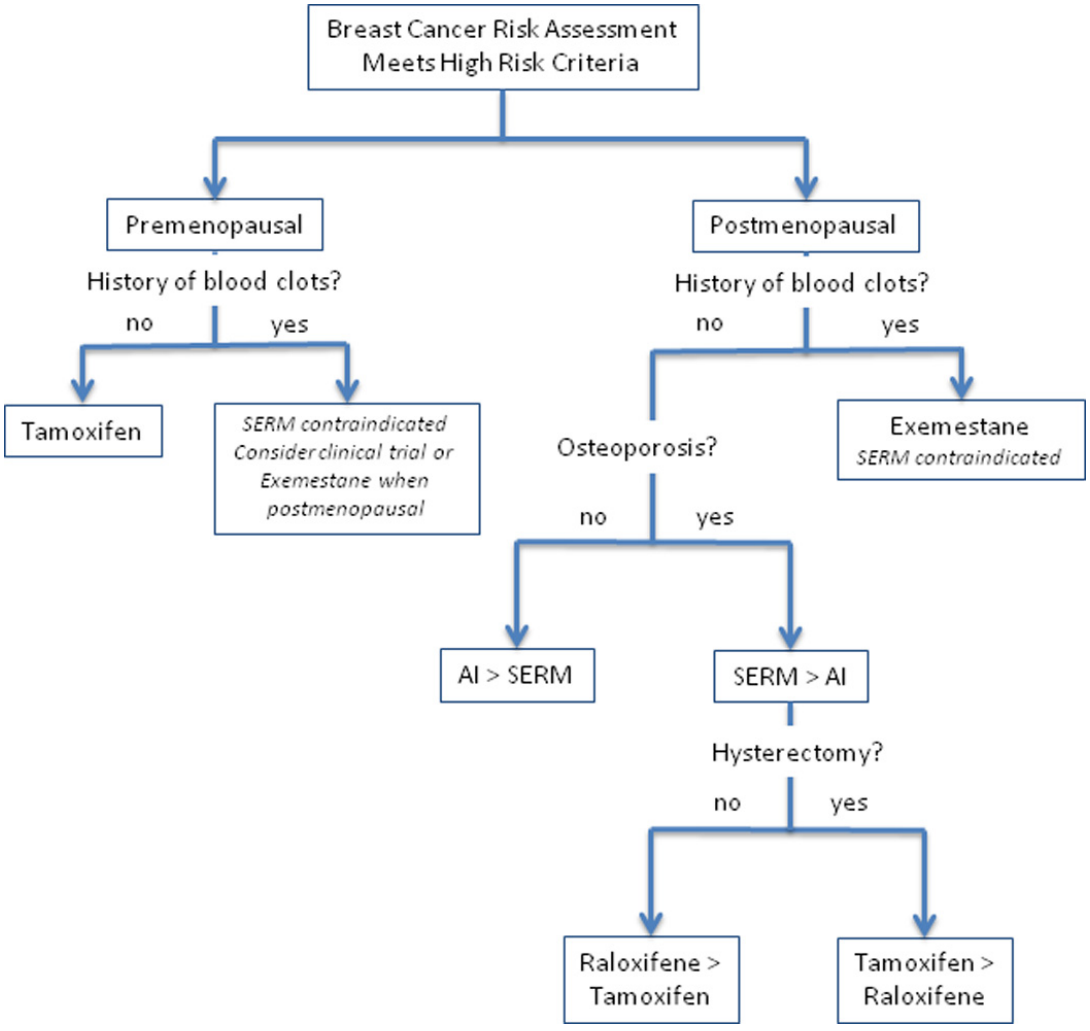

Figure 5.2 An algorithm to help determine which breast cancer chemoprevention agent to use for women at high risk of developing breast cancer (Reimers & Crew, 2012)

5.6.2.2 Endometrial cancer chemoprevention

- **Hormonal therapy:** Postmenopausal women with intact uteri and normal endometrial biopsy enrolled for WHI trial were randomized to conjugated equine oestrogen plus medroxyprogesterone acetate (n=8506) or placebo group (n=8506). Follow-up of these patients showed that women in the therapy group had decreased endometrial cancer risk: HR 0.65 (P=0.007; 95% CI 0.48, 0.89) (Chlebowski et al., 2016). Women with Lynch syndrome comprise a high-risk population for development of endometrial cancer – they carry a 40%-60% lifetime endometrial cancer risk. In a prospective multicentre randomized trial, the role of depo-medroxyprogesterone acetate and progestin-containing contraceptives on endometrial carcinogenesis in women with Lynch syndrome was studied. An endometrial biopsy was taken before and after completion of treatment. Results did show a decrease in endometrial proliferation with both OCP use and depo-medroxyprogesterone acetate and thus these agents are suggested to be potentially suitable chemo preventive agents for women at high risk of endometrial cancer (Lu et al., 2013).

- **Metformin:** Metformin, the first line treatment for type 2 diabetes and also used in conditions like PCOS, has been suggested as a potential agent for prevention of endometrial cancer. However, a meta-analysis looking at studies from 1980 to 2016 concluded that metformin was not found significantly associated with a decreased risk of endometrial cancer (P=0.7; OR=1.05, 95% CI:0.82, 1.35) (Chu et al., 2018). An Endometrial Cancer Chemoprevention Study of Metformin is a currently ongoing clinical trial to

assess the use of metformin as chemoprevention for endometrial cancer in women with BMI ≥30kg/m^2 and hyperinsulinaemia. The estimated study completion date is September 2022 (Lu et al., 2019).

- **Aspirin:** A meta-analysis to determine the association of aspirin use and cancer risk showed that aspirin is associated with a decreased endometrial cancer risk, having a relative risk of 0.92 (95% CI: 0.85, 0.99) (Qiao et al., 2018) .

5.6.3 Role of surgery

5.6.3.1 Role of surgery in breast cancer prevention

- The American Society of Breast Surgeons recommends a risk reducing bilateral mastectomy for patients without a history a breast cancer but with a known breast cancer related genetic mutation including BRCA1, BRCA2, TP53, or PTEN while it can be considered for patients with family history of breast cancer and CHEK2 CDH1, PALB2 or ATM genetic mutations (Chair & et al, 2019).

- Risk reducing salpingo-oophorectomy was also associated with lower breast cancer risk in BRCA1 mutation carriers (HR: 0.63; CI 0.41, 0.61) and BRCA2 mutation carriers (HR: 0.36; CI 0.16, 0.82) (Domchek et al., 2010).

5.6.3.2 Role of surgery in endometrial cancer prevention

- Prophylactic hysterectomy and bilateral salpingo-oophorectomy are suggested as preventive strategy for patients with Lynch syndrome (which carries an increased lifetime risk of both endometrial and ovarian cancer) especially after completion of childbearing. In a retrospective study looking at registries between 1973 and 2004, 315 patients were identified as carrying the high-risk germ-line mutations: MLH1, MSH2 or MSH6. No endometrial cancer was diagnosed in the hysterectomy group with 33% diagnosed in the control group ($P<0.001$). The prevented fraction or the efficacy of prophylactic surgery was thus 100% (Schmeler et al., 2006).

- Bariatric surgery was noted to be associated with a 71% decreased risk for endometrial cancer, the risk is decreased further to 81% if women maintain normal weight after the weight loss surgery (Ward et al., 2014).

5.6.4 Compliance with risk reducing prevention interventions

"Key to success of any intervention is engagement from those likely to benefit." (Derbyshire et al., 2022).

Padamsee et al., reviewed the uptake of multiple prevention interventions for breast cancer (Padamsee et al., 2017). Among cancer free BRCA mutation carriers, the rate of prophylactic surgical removal of ovaries and fallopian tubes (BPSO) ranged from 55% to 90% and the rate for bilateral prophylactic mastectomy (BPM) ranged from

11% to 50% (Skytte et al., 2010). Geographic differences in uptake of preventive surgical intervention exists (Skytte et al., 2010).

Uptake of tamoxifen for primary chemoprevention of breast cancer was noted to be very low: 1-5% and around half of those women started tamoxifen did not adhere to compliance and stopped before 5 years (Mallick et al., 2016). The use of these drugs for breast cancer chemoprevention was found to be low (Waters et al., 2012).

Reasons for this could be unawareness of the risk status for breast cancer, unfamiliarity with breast chemoprevention therapies and side-effects concerns including increased endometrial cancer risk with tamoxifen (risk ratio 2.53) (Fisher, B. et al., 1998). Other potential mechanisms that can affect compliance with risk reduction prevention strategies, including healthcare access financial and geographic, provider education, cultural differences, differing policies regarding risk management options in healthcare systems. On the other hand, anxiety related to cancer risk strongly motivates women to adhere to preventive strategies (Litton et al., 2009 & Dillard et al., 2013)

Strategies to increase uptake of SERMS use as breast chemoprevention include better awareness of the individual's breast cancer risk, better communication of these preventive therapies to physicians and minimizing side-effects profile. Enhancing uptake of anti-oestrogens for breast cancer chemoprevention in high risk populations does hold promise for breast cancer incidence reduction (Crew et al., 2017).

In a study to determine the willingness of women at high risk of endometrial cancer to engage in risk-reducing interventions showed that 94% of women were ready to lose weight, 91% agreed to eat more healthy, 87% to exercise more, 74% to take a pill every day and 49% were prepared to do the intra-uterine device - as strategies for primary prevention of endometrial cancer. Discussed barriers to compliance included willpower, forgetting, cost, social anxiety, possible side effects, finding time, physical fitness and previous bad experiences (Derbyshire et al., 2022)

5.7 Challenges in implementation of personalized risk-stratified prevention programmes

5.7.1. Behaviour change interventions

Michie et al., identified three criteria that need to be met for successful behaviour change interventions, as illustrated in the behaviour change wheel, Figure 5.2 (Michie et al., 2011).

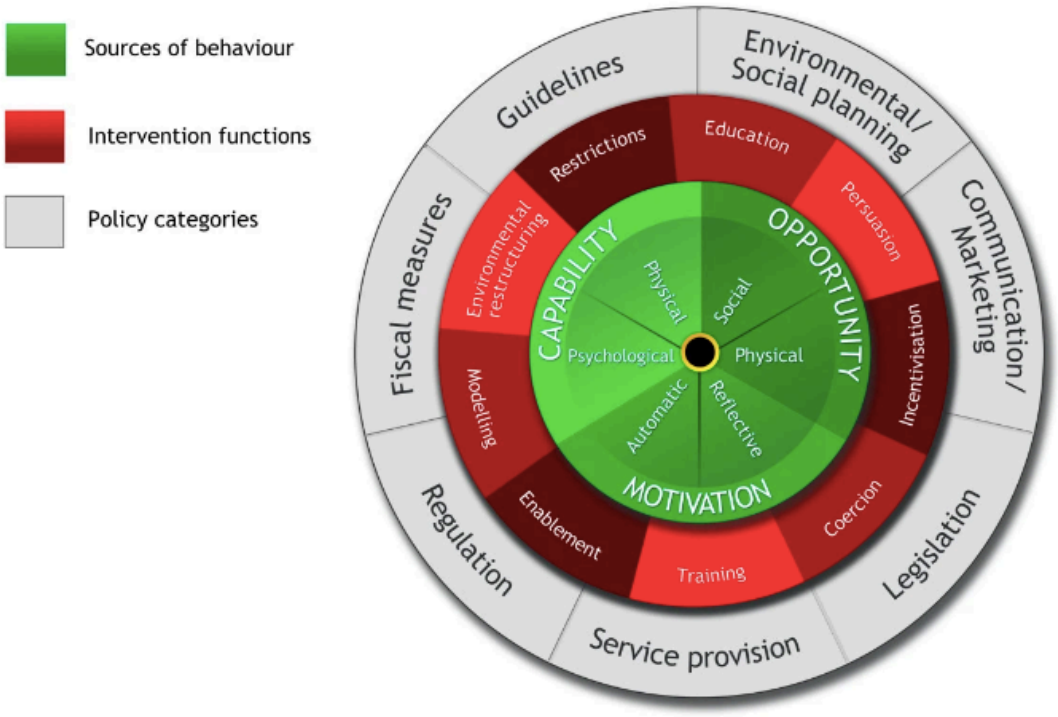

Figure 5.3 Behaviour change wheel (Michie et al., 2011)

At the centre of any framework proposing change in behaviour, three essential conditions (described as '*Sources of behaviour*') need to be in place:

1. Capability – capacity of the individual (physical/psychological) to engage in the desired intervention
2. Motivation – automatic or reflective brain processes that direct behaviour
3. Opportunity – social or physical factors that make the intervention possible.

Michie et al., described nine *intervention functions* (including education, persuasion, incentivisation, coercion, training, restriction, environmental restructuring, modelling and enablement) and seven *policy categories* (communication/marketing, guidelines, fiscal, regulation, legislation, environmental/social planning and service provision) when designing behaviour change interventions (Michie et al., 2011)

5.7.2 Development of framework and policies

Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) developed a statement to improve the quality of prediction models (Collins et al, 2015). Development and internal validation of a prediction model is followed by external validation and updating, software development and regulations and eventually clinical implementation.

As described by Pashayan et al, implementation of personalised risk-stratified programmes is very complex. It needs to consider the health-care system, the economic and political and social context. All stakeholders, including health care professionals, scientists, policymakers and the public need to work together using the latest evidence and tools to create a clear strategy for the implementation of individualised risk-prevention strategies – to reach the goal of improved population health, Figure 5.3 (Pashayan et al., 2020).

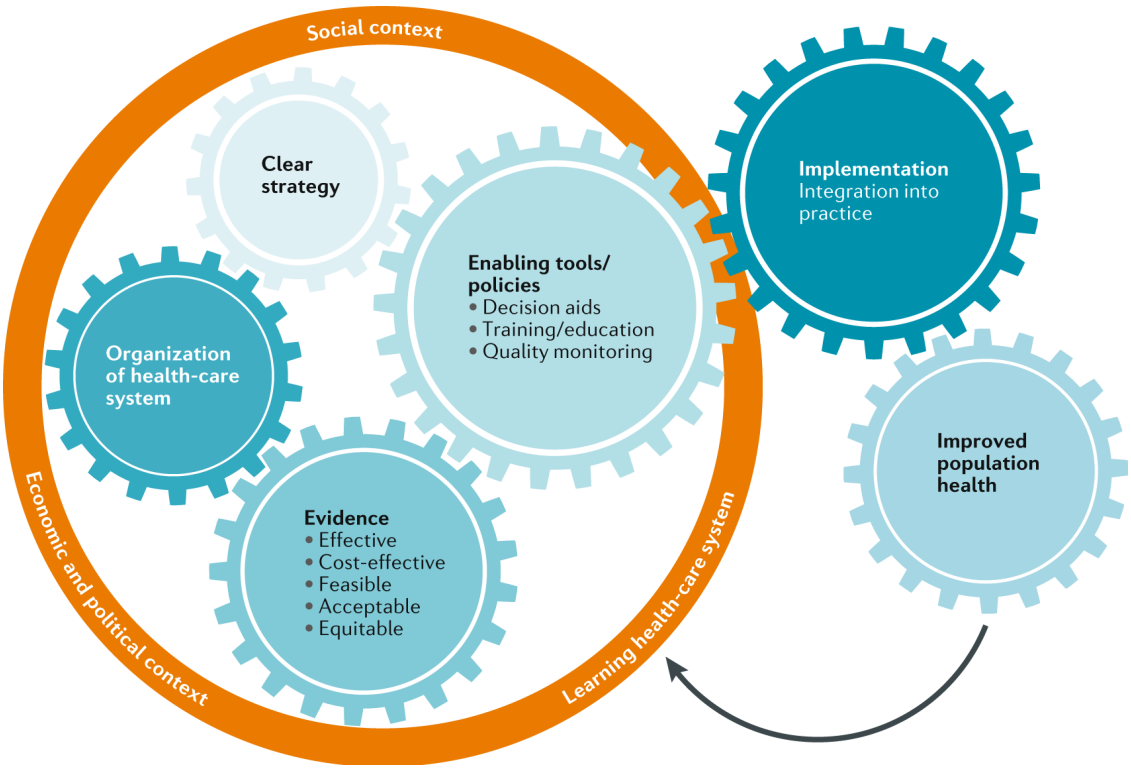

Figure 5.4 The different components involved in the implementation of risk-stratified strategies

Chapter 6

Conclusion

6.1 Strengths of the study

The strengths of this analysis are related to aspects of the methodology used. The data was collected by one investigator only, thus preventing observer error especially in the anthropometric measures. Also, biochemical/hormonal measures were carried at one laboratory, and there was only a small proportion of missing/haemolysed data results. Data collection was carried out from one population of Maltese ethnicity, therefore avoiding interracial variations. The genetic risk scores chosen were both of European ancestry to also avoid ethnic bias.

6.2 Limitations of the study

Since our study was confined to Maltese postmenopausal women, generalization of our results to other populations is limited. Another possible limitation is that the data set is relatively small and since features of the metabolic syndrome are very common in the Maltese population, a bigger sample might produce more statistically significant trends. (Recruitment of patients was affected by the COVID pandemic.)

In this study two GRSs were evaluated; further work using other GRSs can be done in the future.

6.3 Clinical application and novel findings of the study

Consistent with other studies on breast and endometrial carcinogenesis in postmenopausal women, the present study observed a positive association of early menarche, nulliparity and high BMI with both breast and endometrial cancer risk. Family history of breast cancer and high serum SHBG were also found to increase

breast cancer risk. The following factors showed a positive association with endometrial cancer: history of hypertension, history of diabetes, presence of the metabolic syndrome, family history of hypertension, high serum levels of triglycerides, HbA1c and HOMA-IR.

History of breastfeeding showed negative association with both breast and endometrial cancer risk. Our data also showed that serum FSH and LH levels are negatively associated with breast cancer risk while serum progesterone and SHBG show negative association with endometrial cancer risk.

Logistic regression models identified BMI as a risk factor and history of breastfeeding as protective factor for both breast and endometrial cancer. For breast cancer, family history of breast cancer also showed positive correlation.

Analysis of the GRS1 (Polfus et al., 2021) and GRS 2 (Aly et al., 2021) showed higher mean Z-scores in the cancer cohorts compared to controls, implying higher burden of T2DM and insulin resistance risk alleles in the cancer cohorts. Regression models, unadjusted and adjusted, identified significantly higher OR for both breast and endometrial cancer in the higher quintile. Risk prediction models (using are under the ROC curve) for breast and endometrial cancer showed that BMI, GRS1 (Polfus et al., 2021) and GRS 2 (Aly et al., 2021) can be used as risk predictors. GRS 2 (Aly et al., 2021), a GRS of SNPs known to be associated with insulin resistance, was found to be the best predictor for both malignancies.

In summary, this study determined the factors – biochemical, metabolic, hormonal which are associated with increased postmenopausal breast cancer and endometrial cancer. Logistic regression models proved that BMI was the best predictor for both malignancies. Polygenic risk scores with SNPs known to have association with diabetes mellitus type II and insulin resistance also showed good predictive value.

The results of the prediction models can help identify the high-risk populations, which after counselling can be offered breast/endometrial cancer prevention strategies. Such individualised interventions are urgently required to plateau the increasing trends of breast and endometrial malignancies.

To our knowledge, this study is the first GWAS analysis to investigate the Maltese postmenopausal population and the association between established GRS with risk of the hormone dependent malignancies – breast and endometrial cancer.

Further work can however be continued, including:

- Relate the risk factors: clinical, biochemical and genetic data to histological subtypes
- The proposed risk-stratification approach suggested by this study can be audited in a longitudinal study
- To use the genetic data to look at other potential contributing factors e.g. inflammatory markers in increasing risk of breast and endometrial cancer
- To assess performance of PRSs already published related to cancer risk vs PRSs related to obesity (data available from LP-WGS)

Chapter 7

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Chapter 8

Appendices

Appendix 1: Copy of University of Malta Research Ethics Committee Study Approval

L-UNIVERSITÀ TA' MALTA
Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA
Msida – Malta
Medical School
Mater Dei Hospital

Ref No: 44/2016

Tuesday 13th September 2016

Dr Alison Micallef Fava
Medina
Notary Zarb Street
Attard

Dear Dr Alison Micallef Fava,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

The role of biochemical markers and genetic susceptibility in the pathogenesis of hormone dependent malignancies

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Appendix 2: Consent Form (English)

CONSENT FORM Date _____

A. I give my permission to use my information and blood sample in the **present** study. ☐

B. I give my permission to use my information and blood sample in **future** studies under the following condition: (check one)

- I give my permission to be used in future research studies deemed important by the investigators. ☐
- I wish to be contacted if it is considered for further studies. After the study has been explained, I will decide if I want to be included or not. ☐
- Under no circumstances shall my information or sample be used for further studies. ☐

I have read the above information, or it has been read to me. I have understood it and had the opportunity to ask questions about it. I desire of my own free will and volition to participate in this study and accept the benefits and risks associated with this study.

_____ _____

Name and Surname Signature of research subject

_____ _____ _____

ID card number Telephone number Email address

_____

Address

_____ _____

Dr Alison Micallef Fava Prof Savona Ventura

Principal Investigator Supervisor

For further information: 79847820

Appendix 3: Consent Form (Maltese)

FORMOLA TAL-KUNSENS Data _____

A. Jiena nagħti il-permess tieghi biex jużaw l-informazzjoni u l-kampjun tad-demm tieghi f' dan l-istudju preżenti. ☐

B. Jiena nagħti il-permess tieghi biex jużaw l-informazzjoni u l-kampjun tad-demm tieghi fl-istudji futuri taħt il-kondizzjoni li ġejja (ghazel wahda)

- Nagħti il-permess tieghi biex jintużaw fl-istudji futuri ta' riċerka meqjusa importanti mill-investigaturi. ☐
- Nixtieq li nigi kkuntattjata jekk inkun ikkunsidrata ghal aktar studji. Wara li l-istudju jigi spjegat lili, jiena imbaghad niddeċiedi jekk irridx li nigi inkluża. ☐
- L-informazzjoni jew il-kampjun tieghi ma ghandhomx jintużaw ghal aktar studji. ☐

Qrajt l-informazzjoni t'hawn fuq, jew gie moqri lili. Fhimt u kelli l-opportunità li nistaqsi mistoqsijiet. Jien naccetta b'rieda hielsa u volontarjament biex nipparteċipa f'dan l-istudju u naċċetta l-benefiċċji u r-riskji assoċjati ma' dan l-istudju.

_____ _____

Isem u kunjom Firma tal-parteċipant fl-istudju

_____ _____ _____

ID card number Numru tat-telefon Email address

_____

Indirizz

_____ _____

Dr Alison Micallef Fava
Investigatur Principali
Ghal aktar informazzjoni: 79847820

Prof Savona Ventura
Supervisor

Appendix 4: Interview template/Information sheet

Data Sheet Number ____

Name and Surname	
ID number	
Contact number/s	

Date of birth	
Date of operation	
Histology (endometrial)	
Histology (breast)	
Age at diagnosis	

PERSONAL HISTORY

Parity	0	1	2	3
Miscarriages	0	1	2	3
Breastfeeding	yes		no	
History of PCOS	yes		no	
Age of menarche (years)				
Age of menopause (years)				
History of hormonal treatment	yes		no	

Hypertension	yes	no
Hypercholesteolaemia	yes	no
Diabetes mellitus	yes	no
History of tamoxifen use	yes	no
History of smoking *If yes: number of cigarettes*	Yes	no
Treatment at time of operation		

FAMILY HISTORY

Diabetes mellitus	yes	no
Hypertension	yes	no
Endometrial carcinoma	yes	no
Breast carcinoma	yes	no
Other		

Appendix 5: Transfer of Studies to PhD Degree letter

L-Università ta' Malta

Doctoral School
University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 3608/3254
doctoralschool@um.edu.mt

www.um.edu.mt

24 January 2020

Dr Alison Micallef Fava
Villa Medina
Notary Zarb Street
Attard ATD 9045

Student Code: 532983M

Dear Dr Micallef Fava

Transfer of Studies to Ph.D. Degree

I would like to inform you that Senate has agreed to the recommendation of the Board of the Faculty of Medicine and Surgery that you be allowed to transfer your studies to the Ph.D. degree, with effect from 1 February 2020.

A copy of the Ph.D. regulations is enclosed for your guidance. In particular your attention is being drawn to regulation 39. You are also advised to read the Principles of Procedure on the Supervision of Master's Dissertations and Doctoral Theses which may be found on the University website through the following link:
http://www.um.edu.mt/__data/assets/pdf_file/0018/104274/Procedures_for_Supervision_of_Masters_Dis.pdf

Your period of studies commenced on 1 October 2015 and, according to the regulations, you are expected to complete your studies by not later than 30 September 2021.

Yours sincerely

Colin Borg
Deputy Registrar

c.c. Dean, Faculty of Medicine and Surgery
Professor Charles Savona Ventura, Principal Supervisor
Director of Finance
Assistant Director, Scholarships Unit
Officer i/c Faculty of Medicine and Surgery
SIMS Office

Enc.

Appendix 6: Association tables

			Group		
			Breast cancer	Normal	Total
Miscarriages	Yes	Count	27	20	47
		Percentage	20.5%	24.4%	22.0%
	No	Count	105	62	167
		Percentage	79.5%	75.6%	78.0%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.457$, $p = 0.499$

Table 8.1 Association of breast cancer with miscarriages

			Group		
			Breast cancer	Normal	Total
History of PCOS	Yes	Count	12	9	21
		Percentage	9.1%	11.0%	9.8%
	No	Count	120	73	193
		Percentage	90.9%	89.0%	90.2%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.203$, $p = 0.652$

Table 8.2 Association of breast cancer with history of PCOS

			Breast cancer	Normal	Total
History of OCP use	Yes	Count	12	10	22
		Percentage	9.1%	12.2%	10.3%
	No	Count	120	72	192
		Percentage	90.9%	87.8%	89.7%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.528$, $p = 0.467$

Table 8.3 Association of breast cancer with history of OCP use

			Group		
			Breast cancer	Normal	Total
History of HRT use	Yes	Count	14	11	25
		Percentage	10.6%	13.4%	11.7%
	No	Count	118	71	189
		Percentage	89.4%	86.6%	88.3%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.387$, $p = 0.534$

Table 8.4 Association of breast cancer with history of HRT use

			Group		
			Breast cancer	Normal	Total
History of hypertension	Yes	Count	64	32	96
		Percentage	48.5%	39.0%	44.9%
	No	Count	68	50	118
		Percentage	51.5%	61.0%	55.1%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.1.83$, $p = 0.176$

Table 8.5 Association of breast cancer with history of hypertension

			Breast cancer	Normal	Total
History of hyper-cholesterolaemia	Yes	Count	55	28	83
		Percentage	41.7%	34.1%	38.8%
	No	Count	77	54	131
		Percentage	58.3%	65.9%	61.2%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 1.205$, $p = 0.272$

Table 8.6 Association of breast cancer with history of hypercholesterolaemia

			Group		
			Breast cancer	Normal	Total
History of diabetes mellitus Type 2	Yes	Count	17	9	26
		Percentage	12.9%	11.1%	12.2%
	No	Count	115	72	187
		Percentage	87.1%	88.9%	87.8%
Total		Count	132	81	213
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.146$, $p = 0.702$

Table 8.7 Association of breast cancer with history of diabetes mellitus type 2

			Group		
			Breast cancer	Normal	Total
History of smoking	Yes	Count	29	10	39
		Percentage	22.0%	12.2%	18.2%
	No	Count	103	72	175
		Percentage	78.0%	87.8%	81.8%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 3.243$, $p = 0.072$

Table 8.8 Association of breast cancer with history of smoking

			Breast cancer	Normal	Total
Family history of diabetes mellitus	Yes	Count	85	45	130
		Percentage	64.4%	54.9%	60.7%
	No	Count	47	37	84
		Percentage	35.6%	45.1%	39.3%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 1.921$, $p = 0.166$

Table 8.9 Association of breast cancer with family history of diabetes mellitus

			Group		
			Breast cancer	Normal	Total
Family history of hypertension	Yes	Count	83	47	130
		Percentage	62.9%	57.3%	60.7%
	No	Count	49	35	84
		Percentage	37.1%	42.7%	39.3%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.656$, $p = 0.418$

Table 8.10 Association of breast cancer with family history of hypertension

			Group		
			Endometrial cancer	Normal	Total
Miscarriages	Yes	Count	20	20	40
		Percentage	22.2%	24.4%	23.3%
	No	Count	70	62	132
		Percentage	77.8%	75.6%	76.7%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.113$, $p = 0.737$

Table 8.11 Association of endometrial cancer with miscarriages

			Group		
			Endometrial cancer	Normal	Total
History of PCOS	Yes	Count	10	9	19
		Percentage	11.1%	11.0%	11.0%
	No	Count	80	73	153
		Percentage	88.9%	89.0%	89.0%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.001$, $p = 0.977$

Table 8.12 Association of endometrial cancer with history of PCOS

			Group		
			Endometrial cancer	Normal	Total
History of OCP use	Yes	Count	8	8	16
		Percentage	9.2%	10.0%	9.6%
	No	Count	79	72	151
		Percentage	90.8%	90.0%	90.4%
Total		Count	87	80	167
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.031$, $p = 0.860$

Table 8.13 Association of endometrial cancer with history of OCP use

			Group		
			Endometrial cancer	Normal	Total
History of HRT use	Yes	Count	9	9	18
		Percentage	10.3%	11.3%	10.8%
	No	Count	78	71	149
		Percentage	89.7%	88.8%	89.2%
Total		Count	87	80	167
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.036$, $p = 0.851$

Table 8.14 Association of endometrial cancer with history of HRT use

			Endometrial cancer	Normal	Total
History of hyper-cholesterolaemia	Yes	Count	40	29	69
		Percentage	44.4%	35.4%	40.1%
	No	Count	50	53	103
		Percentage	55.6%	64.6%	59.9%
Total		Count	90	83	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 1.472$, $p = 0.225$

Table 8.15 Association of endometrial cancer with history of hypercholesterolaemia

			Group		
			Endometrial cancer	Normal	Total
History of smoking	Yes	Count	15	10	25
		Percentage	16.7%	12.2%	14.5%
	No	Count	75	72	147
		Percentage	83.3%	87.8%	85.5%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.691$, $p = 0.406$

Table 8.16 Association of endometrial cancer with history of smoking

			Group		
			Endometrial cancer	Normal	Total
Family history of diabetes mellitus	Yes	Count	54	45	99
		Percentage	60.0%	54.9%	57.6%
	No	Count	36	37	73
		Percentage	40.0%	45.1%	42.4%
Total		Count	91	82	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 0.461$, $p = 0.497$

Table 8.17 Association of endometrial cancer with family history of diabetes mellitus

			Group		
			Endometrial cancer	Normal	Total
Family history endometrial cancer	Yes	Count	18	8	26
		Percentage	20.0%	9.8%	15.1%
	No	Count	72	74	146
		Percentage	80.0%	90.2%	84.9%
Total		Count	90	82	172
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 3.509$, $p = 0.061$

Table 8.18 Association of endometrial cancer with family history of endometrial cancer

			Breast cancer	Normal	Total
Presence of metabolic syndrome	Yes	Count	63	30	93
		Percentage	47.7%	36.6%	43.5%
	No	Count	69	52	121
		Percentage	52.3%	63.4%	56.5%
Total		Count	132	82	214
		Percentage	100.0%	100.0%	100.0%

$X^2(1) = 2.555$, $p = 0.110$

Table 8.19 Association of breast cancer with presence of metabolic syndrome

Appendix 7 Poster Presentation

Micallef Fava A, Pace N, Savona-Ventura C. *The role of risk factors and biochemical markers in the susceptibility of hormone dependent malignancies.* 7th European Congress of Obstetrics and Gynaecology (EBCOG), 2-4sept 2021

THE ROLE OF RISK FACTORS AND BIOCHEMICAL MARKERS IN THE SUSCEPTIBILITY OF HORMONE DEPENDENT MALIGNANCIES

Introduction: Multiple studies have associated the global increase of postmenopausal breast and endometrial cancer with the worldwide increase in obesity and the metabolic syndrome.

Aim: To evaluate which markers can be utilized to develop a risk assessment screening diagnostic tool to identify individuals at increased risk of developing breast or endometrial cancer.

Method: Three populations were recruited: Patients with a history of endometrial carcinoma; patients with a history of breast carcinoma; and a control group. All patients recruited were postmenopausal of Maltese ethnicity. Each subject was interviewed, anthropometric data measured and a biochemical profile obtained.

Results: 195 patients were recruited: 80 patients were diagnosed with breast cancer, 44 patients were diagnosed with endometrial cancer (two patients had endometrial and breast cancer) and 73 patients had normal histological findings.

The study observed a positive correlation between early menarche and high BMI with both breast and endometrial cancer risk. Tobacco smoking and high level of SHBG were also found to increase breast cancer risk while a positive association between history of hypertension, presence of the metabolic syndrome and family history of endometrial cancer was found with endometrial cancer.

Menarche age less than 12years reached the highest specificity (74.6%) while BMI >25kg/m2 had the highest sensitivity (79.2%) for breast cancer. Family history of endometrial cancer reached the highest specificity (91.5%) while metabolic syndrome and BMI >25kg/m^2 had the highest sensitivity values (82.9% and 87.8% respectively) for endometrial cancer.

For every 1kg/m^2 increase in BMI, the odds of having breast cancer increased by 9% (OR=1.090) while the odds of having endometrial cancer increased by 19% (OR=1.190).

Conclusion: This study gave better understanding on the risk significance of various factors related to breast and endometrial carcinogenesis in the Maltese population.