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MENOPAUSE, ENDOMETRIAL CANCER AND METABOLIC SYNDROME: LINKS AND TREATMENTS

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30 Ottobre 2017

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STATEMENT OF ORIGINALITY

The work described in this thesis was carried out at the University Campus Bio-Medico, Rome.

The author designed the studies that are reported in this thesis and/or analyzed and described the results.

I hereby state that this thesis entitled **"Menopause, endometrial cancer and metabolic syndrome: links and treatment"** has not submitted for a degree or any other qualification at any other university.

Michela Angelucci

ABSTRACTS

RESEARCH PROJECT N°1

Efficacy of Myoinositol and Flavonoids in post-menopausal women affected by metabolic syndrome. A randomized crossover study

The aim of this study is to evaluate the efficacy of myo-inositol and soy isoflavones in reducing insulin resistance in postmenopausal patients with metabolic syndrome. Forty-two such patients were enrolled in the study and were randomised into two groups, G1 and G2. During the first year (time T0-T2), group G1 (21 patients) was administered myoinositol 2 g + soya isoflavones (genistein 200 mg) once daily while group G2 was treated with diet and exercise only. After one year, the treatments were crossed over: during the second year (time T2-T4), group G2 (21 patients) was administered myoinositol 2 g + soya isoflavones (genistein 200 mg) once daily, while group G1 stopped the pharmaceutical treatment and was treated with diet and exercise only. Patients were evaluated at baseline (T0) and every 6 months (T1-T4) for body mass index (BMI), abdominal circumference (CA), basal glucose (BG), triglycerides (TG), low density lipoprotein (LDL), and high-density lipoprotein (HDL). Myo-inositol in association with soy isoflavones produced a highly significant improvement in serum levels of BG and TG compared with the groups treated with diet and exercise only. There was no significant change in BMI in either group from T0 to T4. Supplementation with myo-inositol and soy isoflavones may be considered a reliable option in the treatment of metabolic syndrome in postmenopausal women.

RESEARCH PROJECT N°2

The role of novel biomarker HE4 in endometrial cancer: A case control prospective study

The aim of the study was to explore the clinical of serum human epididymis secretory protein E4 (HE4) and CA125 in endometrial carcinoma. From January 2010 to April 2012, serum specimens were collected from consecutive cases of endometrial carcinoma and from cases of uterus benign disease (control group). The CA125 normal value is considered less than 35 U/mL. Two HE4 cut-off are considered: less than 70 pmol/L and less than 150 pmol/L. The specificity analysis was performed using the Mann–Whitney test for the CA125 and HE4 series. The level of statistical significance is set at $p<0.05$. The sensitivity of CA125 in detecting endometrial cancer is 19.8%, whereas the sensitivity of HE4 is 59.4 and 35.6% for 70 and 150 pmol/L cutoff, respectively. Thus the specificity of HE4 is 100% (positive predictive value=100%, negative predictive value=71.52 and 61.31% considering the two HE4 cut-offs, respectively), whereas the CA125 specificity is 62.14% (positive predictive value033.9 %, negative predictive value044.14%) in detection of endometrial cancer. Combining CA125 and HE4, the sensitivity to detect endometrial cancer is 60.4 and 34.6 %, at HE4 cut off of 70 and 150 pmol/L, respectively, with a specificity of 100%. HE4 may be a new tool for preoperative evaluation and postoperative surveillance of endometrial cancer patients, with a positive predictive value=100 %. HE4 at cut-off of 70 pmol/L yields the best sensitivity and specificity.

STATEMENT OF ATTRIBUTION

Authors of the following trial:

The role of novel biomarker HE4 in endometrial cancer: a case control prospective study

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PART I

1. MENOPAUSE AND ENDOMETRIAL CANCER

1.1. ENDOMETRIAL CANCER

1.1.1 ENDOMETRIAL CANCER: THE SIZE OF THE PROBLEM

Endometrial cancer (EC) is the most common gynecological malignancy in Europe and North America. Worldwide, it is the fifth most commonly diagnosed cancer in women after breast, lung, and colorectal cancers. In the United States, approximately 49.560 cases of EC were diagnosed in 2013, making it the fourth most common cancer in women. The death rate has more than doubled during the past 20 years and has risen by 8% since 2008 [1-2]. EC has increased by over 40% in the United Kingdom since 1993, to 7536 cases in 2007 and 1741 deaths in 2008 [3]. It mostly occurs in postmenopausal women in their sixth and seventh decades of life [4]. The median age at diagnosis is 62 years (Fig. 1).

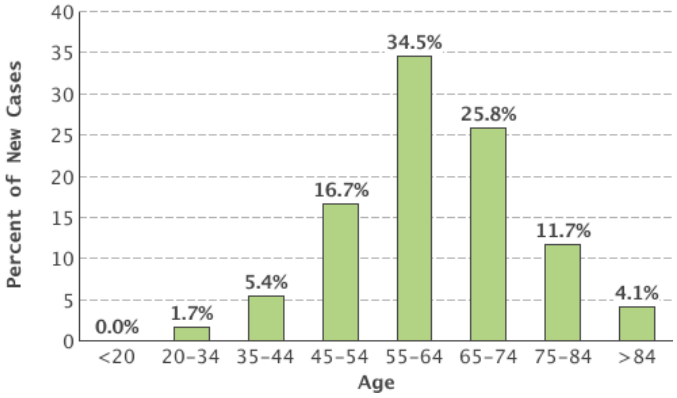

Fig.1 - Percent of New Cases by Age Group (SEER.CANCER.GOV)

Worldwide, higher EC incidence rates are observed in industrialized and Northern European populations and lower rates in developing countries [5]. White women had the highest rate (26.3%), followed by Black (24.8%), Hispanic (21.9%), Asian/Pacific Islander (20.5%), and American Indian/Alaska Native (19.9%) women.

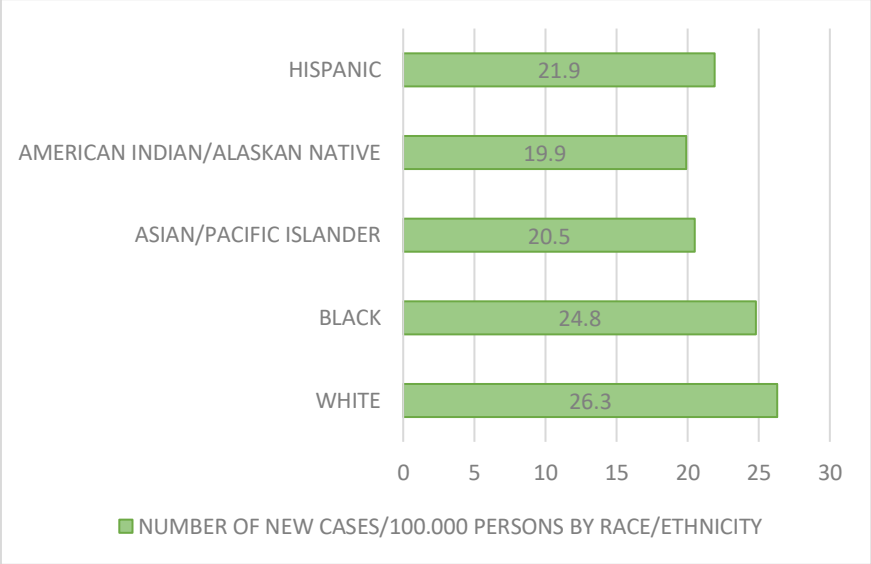

Fig. 2 - Number of New Cases per 100,000 Persons by Race/Ethnicity: Endometrial Cancer (SEER.CANCER.GOV)

Different incidence rates by race could be due to differences in lifestyle, socioeconomic status, and genetic predisposition [6]. Although the incidence of EC is 30% lower in African Americans than white Americans, black African women usually present with advanced stage disease and have a 4-times higher mortality rate than white women. The high risk of death in black women is attributed to poor access to primary health care, advanced stage at diagnosis, tumor characteristics and delay in treatment [7]. Early stage at diagnosis may be a reflection of high economic status and educational level, which usually lead to increased awareness and ultimately create the racial gap in life expectancy and survival rate [8].

The incidence of EC is about 10 times higher in developed countries than developing countries [9]. This could be due to various factors such as increased life expectancy, increased calorie intake and hence obesity, adjuvant Tamoxifen use for breast cancer, and a reduction in fertility rates. The risk of the disease has been shown to rise in Asian and African emigrants to developed countries, possibly due to changes in environmental risk factors [9,10]. It has been estimated that approximately one-third of the cancer deaths that occur in the US each year are due to modifiable lifestyle factors, including poor nutrition, physical inactivity, and excess weight [11]. Although the incidence of EC is high in developed countries, the death rate is low [12], probably because most patients present at an early stage. Ninety percent of women present with abnormal uterine bleeding and about 75% present with early stage disease. It is unclear whether the association between geography and the incidence of EC is a true geographic factor or a surrogate for other factors such as socioeconomic status, the medical care system, and lifestyle factors including diet.

1.1.2. ENDOMETRIAL CANCER: HISTOTYPES

EC develops along two distinct pathways with defined molecular alterations and distinct histologic and clinical features. Type I disease accounts for the majority of ECs (80-90%). Endometrioid tumor is the most common histological finding. These tumors may show microsatellite instability and mutations in PTEN, PIK3CA, K-ras, and CTNNBI. It has not been definitively established whether the etiology of this tumor type is behavioral, social, genetic, or all of these. Type I is associated with

unopposed estrogen exposure and is often preceded by premalignant disease (abnormal uterine bleeding).

In contrast, type II ECs are non-endometrioid with two main histologic subtypes: papillary serous adenocarcinoma, and clear cell adenocarcinoma. They are often only detected at an advanced stage, and tend to be more aggressive than endometrioid adenocarcinomas. No hormonal risk factors have been identified for this type, and there is no readily observed premalignant phase. Women with type II tend to be older than those with type I and the disease is detected at a more advanced stage. Type I is estrogen dependent and develops through the hyperplasia-carcinoma sequence, whereas type II cancers are not estrogen dependent and develop independently of the endometrial hyperplasia pathway [13].

Although type II accounts for only 10% of all ECs, it is responsible for 50% of relapses and associated deaths with a 5-year, all stage, overall survival rate of just 35% [14]. Type II cancers typically arise in an atrophic endometrial background, and often have deep myometrial penetration and demonstrate lymph node spread. They usually occur 5–10 years later than type I tumors [13].

1.1.3. ENDOMETRIAL CANCER: RISK FACTORS

Factors that increase the risk of EC include early age at menarche [14], late age at first delivery, small number of children, and short period or lack of breastfeeding [15]. Earlier menarche increases the risk of developing EC up to 9 fold compared with late menopause (after 55 years). Long term use of combination oral contraceptives reduces the risk [16]. Short or irregular cycles and a late age at

menopause are associated with an increased risk [15]. Longer ovulatory cycles seem to be directly correlated with the risk of EC [17]. Another risk factor is hormone replacement therapy (HRT), but this is largely confined to estrogen-only therapy [18]. The risk of EC is directly correlated with increasing age [16-18]. Over 90% of cases are diagnosed after the age of 50 years, making EC more common in post- than in premenopausal women [19]. Advanced age is also considered as a predictor of poor outcome [20]. Setiawan and colleagues described a 67% higher risk of EC in women whose menopause occurred between 50 and 54 years compared with those reaching menopause before 45 years. The risk increased by 79% with menopause after 55 years [21]. A family history of EC is associated with a two to three fold increased risk in premenopausal women [22]. In women less than 50 years old, about 9% of EC is due to mutations in mismatch repair genes (MSH1, MSH2, MSH6), which result in Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch II syndrome [23]. A family history of other cancers such as uterine and intestinal cancer is directly associated with an increased risk of EC, suggesting a genetic link [22,23]. Women who have had breast or ovarian cancer also have an increased risk of developing EC [24]. The risk increases almost 3 fold among *BRCA1* carriers (which is less than the effect on the risk of breast cancer), while no increase in risk is found in *BRCA2* mutation carriers [25,26]. Elevated serum estrogen levels are associated with chronic anovulation, increasing the risk of developing EC [27]. Women with polycystic ovary syndrome (PCOS) and women with estrogen-secreting ovarian tumors thus have an increased risk of developing EC, especially in reproductive age [28]. Anovulation or oligo-ovulation associated with PCOS chronically exposes the endometrium tissue to estradiol, leading to abnormal cell proliferations and

consequently to neoplastic changes, even at a young age. Most young patients (<40 years) with EC suffer from chronic anovulation. Almost a third of women with EC also have PCOS [29]. Insulin resistance and PCOS, both components of MetS, may play a central role in the pathogenesis of EC [30]. Medication such as clomiphene citrate, used to induce ovulation in the treatment of infertile women, increases estradiol levels and is considered a risk factor for endometrial cancer [31]. Lower parity and/or nulliparity [32] were found to increase the risk of developing EC up to four fold, while multiparity decreases the risk by up to 70% [32,33]. Furthermore, any additional birth [after the birth of the second child] decreased the risk by 10% for every new child. This is because parity alters the hormonal balance towards higher progesterone and lower estrogen, which suppresses endometrial mitotic activity. Shedding of the endometrial tissue during delivery could lead to elimination of initiated or precancerous cells [34]. HRT can be used to treat the symptoms of menopause. The hormones most commonly used are estrogen and progestins. These hormones are often used together, but some women are given estrogen alone, estrogen therapy (ET). In the past ET was used to treat symptoms such as hot flushes [35-37]. ET increases the risk of EC 5 fold. Since the 1980s, this finding has resulted in a huge reduction in the use of ET by postmenopausal women who have not undergone hysterectomy [38]. Combination estrogen and progestin therapy is recommended for women with an intact uterus [39]. The risk of EC is positively correlated with high-fat diet or high energy intake from animal sources [40,41]. Vegetarian or vegan diet and high intake of fruits and nutrients such as fibers and vitamins are associated with a reduced risk [42,43], while high calorie intake increases the risk up to 3 fold [44]. This can be explained in two ways. First, a high fat diet can lead to the development

of obesity, itself considered a risk factor for EC. Second, a high-fat diet boosts estrogen metabolism, which also increases the risk of EC [45]. Dietary differences may therefore be another reason behind the differing incidence of EC from country to country. African Americans, who tend to eat a high-fat diet with less fruits and vegetables, are at a high risk compared with white Americans [46,47]. A variety of epidemiologic studies have examined the association between obesity and EC. Obesity is clearly identified as a fundamental risk factor in both pre- and postmenopausal women [48]. Obese women have a 2-22 fold increased risk of developing EC compared to women with a normal BMI [49-55]. The risk of EC rises when obesity is associated with infertility or amenorrhea, as is the case in PCOS [53]. This is because obesity increases insulin resistance and estrogen exposure, which is already high in infertile women and women with anovulation or amenorrhea [53-55]. The risk of EC increases 1.2 fold for each 5 kilograms of weight gain [56]. The association of body fat distribution with EC has been well characterized: upper body fat is more strongly associated than lower body fat [56,57]. Obesity is associated with a poorer prognosis and increased mortality for both pre- and postmenopausal women. Since obesity is also associated with insulin resistance and hyperinsulinemia the positive relationship with the risk of EC becomes even stronger [58]. Obesity can improve the effect of diabetes, as most patients with Type II diabetes are obese. Several studies [58-60] showed a positive relationship between the risk of EC and diabetes. In comparison with non-diabetic woman, a diabetic woman has a 2 to 3 fold increased risk of developing EC [60,61]. Friberg and colleagues found a more than 6 fold increase in the risk of EC when diabetes is associated with obesity. The risk grows to 10 times when obese diabetic women do not exercise [62].

Tamoxifen is a selective estrogen receptor modulator often used to treat women with an estrogen receptor positive breast cancer [63]. Tamoxifen stimulates endometrial proliferation and the thickness of the endometrium increases in line with the duration of the treatment [64]. The relative risk is 2.0 for 2–5 years of tamoxifen therapy and 6.9 for >5 years when compared to non-users [65]. In addition, the elevated risk of EC with long term Tamoxifen use is usually associated with a poor prognosis and poor survival rate [65,66].

1.1. ADVANCED AGE AND ENDOMETRIAL CANCER

The risk of EC is positively correlated with increasing age [4-6]. The mean age for diagnosis of endometrial adenocarcinoma is 61 years, although most cases diagnosed between the ages of 50 and 60 years. Ninety percent of cases occur in women older than 50 years. Only 20% are pre-menopausal, with approximately 5% developing the disease below the age of 40 [13]. Investigation of age standardized incidence rate in different age groups revealed an increase across most age groups from 0-79 years, most markedly in age groups 60-69 and 70-79 years [1]. Many studies have also suggested that older age at diagnosis of EC predicts poorer outcomes [67]. Some authors believe that EC among the elderly is intrinsically more aggressive than in younger patients [68,69]. Older women typically present with higher tumor grades, more advanced stage disease, deeper myometrial invasion (MI), and less favorable histology. Possible factors also include, the likelihood of surgical under staging and receiving less than optimal adjuvant radiotherapy or chemotherapy [70-84]. Outcome differences may simply represent differences in treatment between young and older

patients. Elderly patients are less likely to undergo lymph node sampling [85] and receive adjuvant RT than younger patients, even in the presence of adverse prognostic factors [86,87]. Literature evidence suggests that elderly women with gynecologic malignancies tolerate chemotherapy rather well [88]. Because of the relatively high rate of recurrence in elderly EC patients, they should be encouraged to participate in clinical trials investigating the benefits of adjuvant chemotherapy in patients with high-risk EC. It is important to encourage not only older patients but also, at times, the treating physician to try to complete the prescribed treatment to obtain the highest rate of control, given that radiation toxicity is not correlated with age. This encouragement is important because elderly patients in general are underrepresented in cancer clinical trials [89]. It remains unclear, however, whether age is a true prognostic factor in EC. In a review of 819 patients with Stage I-II EC from the Gynecologic Oncology Group database, Zaino et al. demonstrated that the RR increased from 1 for patients aged ≤45 years at the time of diagnosis to 2 for patients aged 55 years, 3.4 for patients aged 65 years, and to 4.7 for patients aged ≥75 years [90]. In the prospective randomized trial of Postoperative Radiation Therapy in Endometrial Carcinoma (PORTEC) for Stage I disease, Creutzberg et al. [84] reported that age ≥60 years was an independent predictor of death from EC (hazard ratio of 3.1 and 95% CI, 1.2-8; P=0.02). The data in the literature also suggest that there is an incremental increase in the risk of dying from EC with increasing age. Abeler et al. reported on 181 patients with clear cell endometrial carcinoma and found older age to be a significant predictor of poor disease-free survival on multivariate analysis (P 0.03). Stewart et al. reported on 119 patients with high-risk features (i.e. deep myometrial invasion or aggressive histology) who were treated with whole

abdominal radiation therapy [91]. Here to, older age was a significant predictor of poor disease-free survival on multivariate analysis. [92]. Another study by Alektiar et al. found age ≥70 years to be an independent predictor of poor locoregional control. The negative impact of advanced age remained manifest even after using disease free survival rather than overall survival as an endpoint (RR: 3 and 95% CI, 1-9; P 0.03) [20]. All these studies showed that the influence of advanced age is independent of other poor prognostic factors such as deep myometrial invasion or aggressive histologic types. In contrast, Mundt et al. found that age is not an independent prognostic factor for recurrence in these patients. The authors suggested that the higher rates of recurrence and poorer survival rates reported in the elderly are most likely the result of imbalances in pathologic factors and less aggressive therapy [69]. In relation to histotype, in a study by Evans et al., the increase in EC incidence was confined to type 1, while type 2 was static over the 12-year study period [93]. The most significant increases are seen in the 60-79 age groups, suggesting that the estrogenic impact on the development of type 1 persists throughout the perimenopausal years. Whereas there is a need for new and more effective target therapy for type 2 EC, type 1 needs research in prevention strategies that can target this persistent risk, such as long-term prophylaxis with oral or intrauterine device-delivered progestin.

1.2. HORMONAL ACTIVITY IN MENOPAUSE

1.2.1. EXTRAGONADAL PRODUCTION OF ESTROGEN

Before the menopause, the ovaries are the major source of estrogen and progesterone production (Fig. 3). After ovulation, membranous granulosa cells remaining in the follicle begin to take up lipids and characteristic yellow lutein pigment. Progesterone is produced from these active secretory cells; the progesterone level therefore drops after the menopause [94,95]. Estrogen can still be produced but progesterone cannot, and women may be exposed to estrogenic effects with low progesterone activation after the menopause.

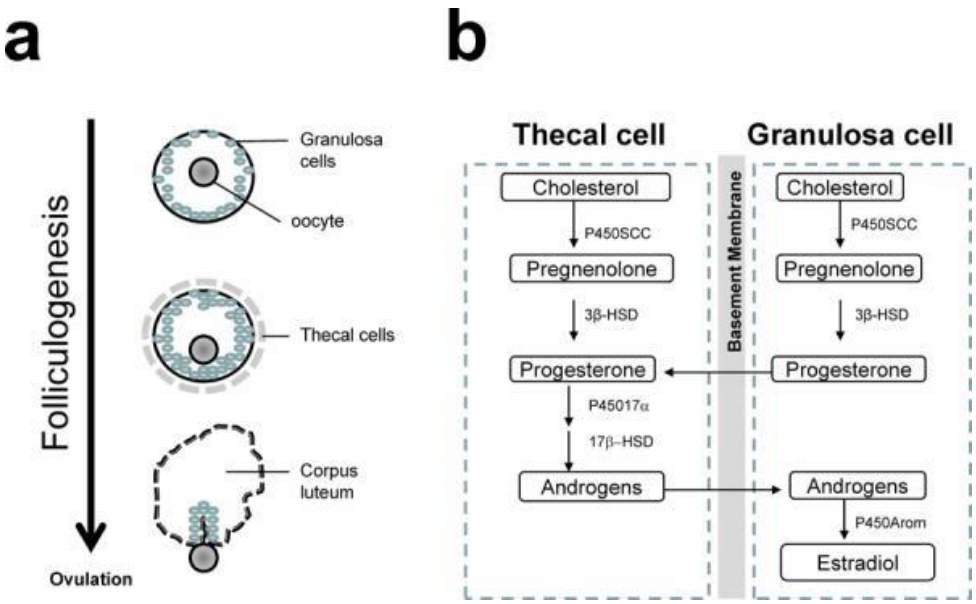

Fig. 3 - Estrogen synthesis in the ovary: (a) Folliculogenesis. A primordial follicle consists of an oocyte and a layer of granulosa cells at the beginning of folliculogenesis. Thecal cells form a layer surrounding the granulosa cells when the follicle is activated. At end of folliculogenesis, thecal cells luteinize to form the corpus luteum after ovulation. (b) Cell-specific estrogen synthesis in the ovary. Production of estrogens starts with the synthesis of pregnenolone from cholesterol, catalyzed by the cytochrome P450 side chain cleavage enzyme (P450scc). Pregnenolone is then converted to progesterone by 3-beta-hydroxysteroid dehydrogenase (3β-HSD) in both thecal and granulosa cells. Progesterone is converted to androgens via cytochrome P450 17α-hydroxylase (P45017α) and 17-beta-hydroxysteroid dehydrogenase (17β-HSD) in thecal cells during the follicular phase. The conversion of 17β-estradiol is catalyzed by aromatase (P450Arom) in granulosa cells.

After the menopause, the ovaries stop synthesizing estrogen and progesterone. A shift in their balance toward more estrogen increases the risk of developing EC. In postmenopausal women estrogen is produced in many extragonadal sites and acts locally at these sites as a paracrine or even intracrine factor. These sites include the mesenchymal cells of adipose tissue, including the breast, bone osteoblasts and chondrocytes, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain [95]. Estrogens are a class of steroid hormones that regulate the development and function of female reproductive organs. In the ovary, estrogen synthesis begins in theca cells with androgen synthesis and ends with conversion of androgens to estrogens in granulosa cells by the enzyme aromatase. Like other steroid hormones, estrogens enter cells passively and bind to estrogen receptors, which then regulate the transcription of downstream estrogen-responsive genes. 17β-estradiol is the most common and potent form of estrogen in mammals. It is also produced in many extragonadal organs, including the adrenal glands, brain, adipose tissue, skin, pancreas [95-96], and other sites yet to be identified. The discovery of these sites greatly expands our knowledge of the novel roles of estrogens beyond the reproductive system [96]. The first discovery of extragonadal estrogen synthesis was made in 1974 by Hemsell and his colleagues, when they made the unexpected observation that androgens were converted to estrogens in adipose tissue [97]. Since then, many other extragonadal sites of estrogen synthesis have been discovered. Adipose tissues are the main source of circulating estrogen after the ovaries in women, and their contribution to total circulating estrogens increases with age [98]. The chemical structure and biological activity of the estrogens synthesized in the extragonadal sites are no different from those produced by the ovaries.

However, there are unique features differentiating extragonadal from gonadal synthesis. A major difference is in the biochemical pathway. The tissues and cells of the extragonadal sites of estrogen synthesis are unable to synthesize C19 steroids, the precursors of estrogen synthesis, but can convert them to estrogens, a critical and rate-limiting step mediated by Cyp19 aromatase. Hence, extragonadal estrogen synthesis is dependent on an external source of C19 precursors [99] and aromatase expression. Because C19 steroids can be supplied to a local tissue via the circulation and are converted to estrogens in any tissue where aromatase is expressed, the presence of aromatase expression in a local tissue confirms extragonadal estrogen synthesis. Table 1 lists the peripheral tissues that express aromatase and are therefore able to convert C19 precursors to estrogens. Estrogen is involved in the pathogenesis of a number of hormone responsive diseases, including breast, endometrial, and ovarian cancers, which are more prevalent in postmenopausal women. These malignant tumors express aberrantly higher levels of aromatase than their nonmalignant counterparts [99,100]. Considering the association of aromatase with estrogen-dependent tumors/cancers, inhibitors of this enzyme have been targeted for the treatment and development of hormone responsive diseases. Aberrantly higher expression of aromatase and, thus, estrogen synthesis, has been demonstrated in malignant endometrial tissues [100].

1.2.2. ESTROGEN TYPES

There are three major forms of physiological estrogens in women: estrone, 17β-estradiol, and estriol [101] (Fig 4).

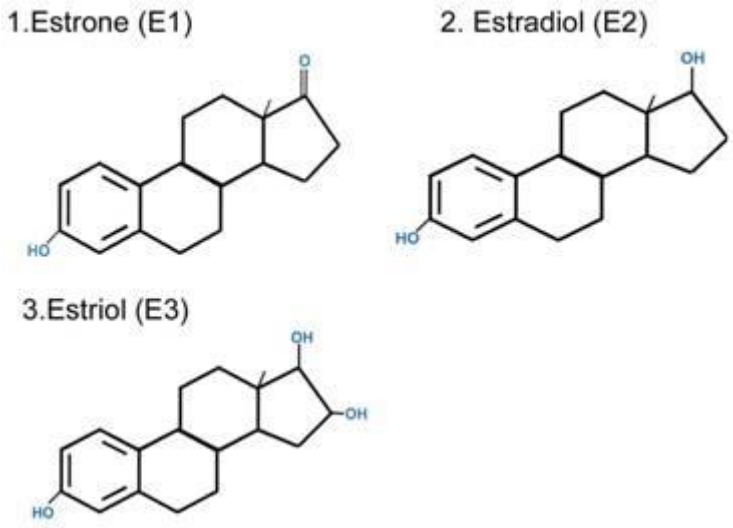

Fig. 4 - Molecular structures of the estrogens. The basic 18-carbon estrane nucleus, shared by estrone, 17β-estradiol, and estriol, is modified in each by differences in the number and arrangement of the hydroxyl groups.

Estrone is synthesized in skin and adipose tissues from circulating androstenedione of adrenal origin and is the main form of estrogen produced in postmenopausal women [102]. 17β-estradiol is the most potent estrogen and is the major estrogen product synthesized in premenopausal ovaries. It is the biologically active estrogen in postmenopausal as well as premenopausal women, even if circulating levels are low. It is synthesized either by reduction of estrone in extragonadal sites including skin and adipose tissue or by direct aromatization of circulating testosterone. Estriol is the least potent estrogen and is synthesized in large quantities in the placenta. Ashihara showed that women with Type 1 EC had a higher serum 17β-estradiol level in the ovarian vein than those with type 2 tumors [102]. One of the substrates for 17β-

estradiol synthesis in extraovarian tissues is testosterone. Elevated levels of plasma androstenedione and testosterone are associated with an increased risk of EC in both pre- and postmenopausal women [40]. The conversion of androstenedione and testosterone to estrone and 17β-estradiol respectively is catalyzed by aromatase (Fig 5).

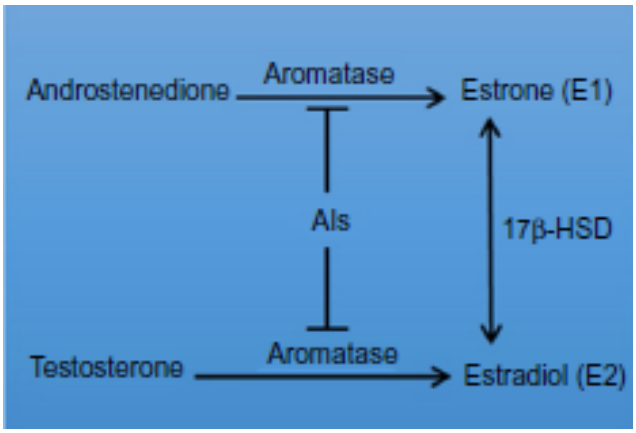

Fig. 5 - Schematic representation of extragonadal estrogen synthesis. The estrogen precursors androstenedione and testosterone, which primarily originate in the adrenal gland in postmenopausal women, are converted to estrogen through the action of aromatase in peripheral tissues such as adipose tissue and skin, and locally in malignant breast, endometrial and ovarian tissues.

Consequently, it has been reported that intratumoral expression of aromatase is considerably higher in malignant EC than in disease-free tissues [103]. This suggests that production of estrogens in situ, due to aberrantly high aromatase expression, is a key factor in the development and progression of EC [104]. Aromatase is overexpressed in the majority of malignant endometrial tumors and can be effectively targeted for the prevention and treatment of various types of EC. The functional roles of estrogens are mediated mostly by estrogen receptors, that are nuclear receptor transcription factors. Therefore, a tissue that expresses one or more estrogen receptors is considered to be a target of estrogenic regulation. Type I EC is associated with a high expression of estrogen receptors [105].

2. MENOPAUSE AND METABOLIC SYNDROME

2.1. METABOLIC SYNDROME: DEFINITION

Biologically, menopause is defined as the permanent cessation of ovulation, characterized by the end of menstruation for at least 12 months [106,107]. Natural menopause in the worldwide population occurs between 45 and 55 years of age. In Italy, it occurs on average at approximately 51 years [108]. During this physiological period, several metabolic and hormonal changes occur. Depletion of sex steroid hormones is an important consequence of normal ageing and gonadal failure, potentially increasing vulnerability to disease in hormone-responsive tissues, including the brain, bone and cardiovascular system, and affecting adversely quality of life [109]. The most typical symptoms of hormonal menopause (follicle-stimulating hormone (FSH) >30 IU/l, 17β-estradiol <30 pg/ml, FSH/luteinizing hormone (LH) >1) include vasomotor symptoms such as hot flashes and night sweats, urogenital atrophy, osteopenia and osteoporosis, psychiatric disorders, sexual dysfunction, skin lesions, cardiovascular disease, MetS, cancer, and finally, metabolic disorders and obesity. Metabolic syndrome (Mets) has been studied since the early 80s; its alternative name, Syndrome X, was originally coined by Gerald Reaven [109] in 1988. It involves obesity, raised blood pressure, hyperglycemia or insulin resistance and dyslipidemia. These are all important cardiovascular risk factors, and obesity is considered the biggest contributor to cardiac dysfunction in postmenopausal women. Cardiovascular disease patterns are different in men and women. Women usually develop cardiac dysfunction ten years later than men [110], due to the cardioprotective effects of estrogen, which protects against atherosclerosis

and also exerts a direct protective effect against ischaemia-reperfusion injury in the myocardium. [111, 112]. Despite this, the risk of coronary artery disease seems to be particularly high in women, due to MetS. The overall incidence of MetS is 20-30% in the general middle-aged population and the incidence varies from 8 to 24% in men and from 7 to 46% in women [113]. In 1999 the WHO suggested a working definition of MetS, progressively refined [114], as glucose intolerance, impaired glucose tolerance (IGT) or diabetes mellitus (DM), and/or insulin resistance, together with two or more of the components listed below:

1. Raised arterial pressure, i.e. ≥140/90 mmHg

2. Raised plasma triglyceride (≥150 mg/dl) and/or low HDL-C (<35 mg/dl in men and <39 mg/dl in women)

3. Central obesity, i.e. waist/hip ratio (WHR) >0.9 in men and >0.85 in women and/or body mass index (BMI) >30 kg/m^2

4. Microalbuminuria, i.e. urinary albumin excretion rate ≥20 µg/minute or albumin/creatine ratio ≥30 µg/mg

The European Group for the Study of Insulin Resistance (EGIR) proposed a modification of the WHO definition, using the term insulin resistance syndrome rather than MetS [115]. Their diagnostic criteria included elevated plasma insulin (>75th percentile) plus two other factors from the following:

1. Abdominal obesity: waist circumference (WC) ≥94 cm in men and ≥80 cm in women
2. Hypertension: ≥140/90 mmHg or antihypertensive treatment

3. Elevated triglycerides (≥150 mg/dl) and/or reduced HDL-C (<39 mg/dl for both men and women)
4. Elevated plasma glucose: impaired fasting glucose (IFG) or IGT, but no diabetes

Notably, the EGIR focused more on abdominal obesity than did the WHO, but unlike the WHO, they excluded patients with type 2 DM because insulin resistance was viewed primarily as a risk factor for diabetes. This definition was followed by a simpler definition, proposed by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) [116]. According to this definition, a subject has MetS if he or she has three or more of the following criteria:

1. Abdominal obesity: WC ≥102 cm in men and ≥88 cm in women
2. Hypertriglyceridemia: ≥150 mg/dl (1.695 mmol/l)
3. Low HDL-C: <40 mg/dl in men and <50 mg/dl in women
4. High blood pressure (BP): >130/85 mmHg
5. High fasting glucose: >110 mg/dl

This differs from the WHO definition on several fronts. The NCEP ATP III did not believe that insulin resistance is mandatory for the development of MetS and hence suggested the term metabolic syndrome instead of the previously used term insulin resistance syndrome. This definition recognizes central obesity as the culprit, and hence BMI, a parameter for general obesity, is not included. Central obesity is quantified using WC instead of WHR, as used by the WHO. This definition considers low HDL and high triglycerides as separate components (both of them being individually atherogenic) rather than viewing dyslipidemia as a single component. The cutoff points used for BP and HDL are stringent in comparison with those

suggested in the WHO definition, but by avoiding the need for clamp techniques and measurement of microalbuminuria, the NCEP ATP III definition is much more practically applicable. The latter also considers proinflammatory state and prothrombotic state as components of MetS, though they are not included in the criteria defining MetS. The American Association of Clinical Endocrinologists (AACE) also preferred the term insulin resistance syndrome over MetS [117]. The major criteria they considered were IGT, elevated triglycerides, reduced HDL-C, elevated BP, and obesity. They did not specify a given number of criteria for diagnosis, but left it to clinical judgment. They suggested that factors like family history of atherosclerotic cardiovascular disease or type 2 DM, polycystic ovary syndrome, and hyperuricemia be considered while exercising clinical judgement. Patients with type 2 DM were excluded from the definition of insulin resistance syndrome. The various components suggested by the AACE are as follows:

1. Some degree of glucose intolerance
    - IFG/IGT
2. Abnormal uric acid metabolism
    - Plasma uric acid concentration
    - Renal uric acid clearance
3. Dyslipidemia
    - Triglycerides
    - HDL-C
    - LDL particle diameter [small, dense LDL-particles]
    - Postprandial accumulation of TG-rich lipoproteins

4. Hemodynamic changes
    - Sympathetic nervous system activity
    - Renal sodium retention
    - Blood pressure (~50% of patients with hypertension are insulin resistant)
5. Prothrombotic factors
    - Plasminogen activator inhibitor-1
    - Fibrinogen
    - Markers of inflammation
    - C-reactive protein, white blood cell count, etc.
6. Endothelial dysfunction
    - Mononuclear cell adhesion
    - Plasma concentration of cellular adhesion molecules
    - Plasma concentration of asymmetric dimethylarginine
    - Endothelial-dependent vasodilatation

The American Diabetes Association lowered the fasting plasma glucose threshold used to identify individuals with IFG from 110 mg/dl to 100 mg/dl. Subsequently, the NCEP ATP III has also suggested lowering the fasting plasma glucose threshold to 100 mg/dl [13]. Researchers worldwide preferred using the NCEP ATP III definition because it was relatively simple and clinically applicable. However, various researchers noted that the WC cutoffs it suggests were not applicable in other countries. Though the WC cutoffs suggested by various groups differ, the generally accepted cutoffs for Asians are 90 cm for men and 80 cm for women [117-119].

According to the new International Diabetes Federation (IDF) definition, MetS is the combination of the following variables [20]:

• Central obesity (defined as waist circumference ≥94 cm for Caucasian men and ≥80 cm for Caucasian women, with ethnicity specific variants for other groups) plus any two of the following four factors:

• Raised triglyceride (TG) level: ≥150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality

• Reduced HDL cholesterol: <40 mg/dL (1.03 mmol/L) in males and <50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality

• Raised blood pressure: systolic BP ≥130 or diastolic BP ≥85 mmHg, or treatment of previously diagnosed hypertension

• Raised fasting plasma glucose (FPG) ≥100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes. If above 5.6 mmol/L or 100 mg/dL, an oral glucose tolerance test is strongly recommended but is not necessary to diagnose the syndrome.

The main metabolic changes in the menopause are the increase in central (intra-abdominal) body fat, the increase in blood glucose and insulin levels, and the shift toward a more atherogenic lipid profile (increased LDL and TG levels, and lipoprotein (a) levels and reduced HDL). Postmenopausal women therefore have higher total cholesterol, LDL cholesterol, TG, and lipoprotein (a) levels and lower HDL cholesterol levels than premenopausal women. Chedraui et al. demonstrated that MetS women displayed significantly higher levels of adipsin, leptin, resistin, insulin, and homeostasis model assessment estimated insulin resistance (HOMA-IR)

values and lower adiponectin levels. These differences were mainly observed in women with abdominal obesity, regardless of whether they met the criteria for MetS or not. In this same sense, lower adiponectin levels were significantly associated with low HDL-C and high triglyceride levels; while higher insulin and HOMA-IR values were associated with high triglyceride and glucose levels respectively [21]. In another paper, Chedraui et al. reported that postmenopausal women with MetS showed higher interleukin 6 (IL-6) (inflammation) and lower urokinase-type plasminogen activator (uPA) levels (endothelial dysfunction). Moreover, IL-6 levels were higher in women with abdominal obesity, low HDL-C and high triglyceride levels. Women with low HDL-C and high triglyceride levels presented significantly lower uPA levels. These were mainly related to metabolic and lipid abnormalities [120]. Recent studies suggest that the changing hormone pattern could be the reason for metabolic changes.

2.2. THE ROLE OF ESTROGENS AND THEIR RECEPTORS IN FAT METABOLISM

In mouse models, sex steroids are required to regulate adipocyte metabolism and also influence the sex-specific remodeling of particular adipose sediments [121,122]. Factors that control fat distribution in humans are partially determined by sex hormone concentrations [123]. Men, on average, have less total body fat but more central/intra-abdominal adipose tissue, whereas women tend to have more total fat that favors gluteal/femoral and subcutaneous sediments [124]. Weight and abdominal fat distribution differ between women of reproductive age and menopausal women [125]. The decrease in estrogen levels in menopausal women is associated with the

loss of subcutaneous fat and an increase in abdominal fat [126]. The relevance of estrogens in subcutaneous fat accumulation is evident; in fact, estrogen hormone therapy in men also increases the amount of subcutaneous fat [127]. As already noted, 17β-estradiol is the most potent estrogen in humans, followed by estrone and estriol [128]. Estrogen function is mediated by estrogen receptors (ERs) in the nuclear receptor superfamily representing a large group of transcriptional regulators. Two types of ER have been identified, alpha (ERα) and beta (ERβ) receptors [129,130]. In the signaling pathway, ligand activated ER dissociates from its chaperone heat-shock protein and binds as a dimer directly to an estrogen response element (ERE) in the promoter of target genes [131,132], although the action of 17β-estradiol was previously thought to be subject to an action in gene expression regulation. There is now increasing evidence of non-nuclear cytosolic or plasma membrane-associate receptors that mediate non-genomic and rapid effects of several steroid hormones [133-135]. In this manner, the traditional estrogen nuclear receptors have been found to function outside the nucleus through direct nongenomic effects. Several membrane-signaling activation mechanisms can explain the rapid responses to 17β-estradiol [136]. These include activation of kinase, phosphatase, and phospholipase that can mediate both calcium-dependent signaling and downstream nongenomic physiological responses, such as effects on cell cycle, cell survival, and energy metabolism [135,136]. Human subcutaneous and visceral adipose tissues express both ERα and ERβ [137], whereas only ERα mRNA has been identified in Brown adipose tissue. ERα plays a major role in the activity of adipocytes and sexual dimorphism of fat distribution. Female and male mice that lack ERα have central obesity and severe insulin resistance, and are diabetic. ERα polymorphism in humans

seems to be associated with higher risk factors for cardiovascular diseases [138]. Furthermore, estrogen seems to promote and maintain the typically female fat distribution characterized by accumulation of adipose tissue, especially in the subcutaneous fat sediments, with only modest accumulation of intra-abdominal adipose tissue [139]. In fact, estradiol directly increases the number of antilipolytic α2A-adrenergic receptors in subcutaneous adipocytes [140]. Lipolysis in humans is controlled primarily by the action of β-adrenergic (lipolytic) and α2A-adrenergic (antilipolytic) receptors. Visceral adipocytes exhibit a high α2A/β ratio and are stimulated by epinephrine; in contrast, no effect of estrogen on α2A-adrenergic receptor mRNA expression in adipocytes from the intra-abdominal fat sediment was observed [141]. However, it is important to highlight that the effects of estrogens differ according to the route of administration and their lipolytic influence or how fat accumulation affects specific regions of the body [142].

2.3. THE ROLE OF TESTOSTERONE IN METABOLIC SYNDROME

The menopause-related predominance of testosterone appears to be a key hormonal change associated with the incidence of MetS, independent of aging and other standard cardiovascular disease (CVD) risk factors. Estrogen exerted a direct positive effect on CVD risk in women, a benefit that was lost as women transitioned from a premenopausal to a postmenopausal state and experienced a loss of estrogen [114]. However, these data show that the change in estrogen level is a non-significant predictor of MetS risk. It is more likely that the progressive androgenicity of the hormone pattern exerts a direct negative effect on CVD risk. Recent clinical trials show that estrogen replacement does not protect against CVD [143].

Testosterone is associated with insulin resistance, hyperinsulinemia, low HDL levels, high blood levels of glucose and triglycerides, and diabetes mellitus [144], and epidemiologic data show that androgens are associated with hemostatic and inflammatory markers [145].

2.4. LIPID METABOLISM DISORDERS IN MENOPAUSE

The dramatic decrease in estradiol during the menopausal transition leaves the vasculature vulnerable to lipids and to cardiovascular disease (CVD) [146]. Dyslipidemia in menopause is characterized by an increase in LDL and a drop in HDL levels. In the Healthy Women Study, total and LDL cholesterol rose and HDL and HDL2 cholesterol declined in perimenopausal women who had ceased menstruating for at least 1 year compared to age-matched premenopausal women who were still menstruating [147]. In addition, both the Los Angeles Atherosclerosis Study and the SWAN Heart Women demonstrated that the antiatherogenic effect of HDL diminishes in women around the age of menopause [148,149] and it was suggested that this may be related to changes in the lipoprotein subclass profile observed during the menopausal transition, accelerating the development of atherosclerosis. Another study demonstrated, in 31 women 6 months after hysterectomy and bilateral salpingo-oophorectomy, a significant increase in triglycerides (TG), total cholesterol, and LDL and a slight, but non-significant, increase in HDL [150]. Kabir et al. compared 30 women with normal and surgical menopause, showing that TG was higher and LDL cholesterol was lower in the surgical group [151]. A similar study, in two groups each of 50 subjects, demonstrated

lower HDL and higher very low-density lipoprotein (VLDL) cholesterol in the surgical group [152].

2.5. INSULIN RESISTANCE IN MENOPAUSE

One of the most important pathophysiological components of MetS is insulin resistance. The mechanism is complex: insulin resistance with inadequate compensatory hyperinsulinemia reduces the normal suppression of free fatty acid (FFA) from adipose tissue exerted by insulin. The increased levels of FFA may impair peripheral glucose uptake, increase hepatic gluconeogenesis, and reduce hepatic clearance of insulin [153]. Menopause is associated with increased insulin resistance but the pathophysiology is unclear. Several papers highlighted increased fasting insulin and fasting glucose levels in postmenopausal compared with premenopausal women [154-157]. Over the last twenty years, many studies have shown that HRT in postmenopausal women significantly influences glucose metabolism [158-163], but only a handful have clearly demonstrated a significant reduction in the incidence of diabetes among postmenopausal women taking HRT [162,163]. It is possible that the estrogens reduce the incidence of diabetes by improving endothelial function. Impaired endothelial function lowers its permeability, and diminished peripheral blood flow may limit insulin delivery and promote insulin resistance [164,165]. In fact, the effectiveness of insulin is limited by its interstitial concentration. The estrogen deficiency that characterizes the postmenopausal period gives rise to endothelial dysfunction [166], so by restoring estrogen levels, endothelial function improves [167]. It should also be added that some studies have observed that transdermal 17β-estradiol seems to possess an insulin-like action on the endothelial

cells. After 2 hours of hyperglycemia induction, the plasma concentrations of soluble adhesion molecules are elevated in both patients with type 2 diabetes and healthy individuals [168]. In contrast, insulin reduces intercellular adhesion molecule-1 levels in endothelial cells [169]. Clinically, insulin treatment is accompanied by a reduced level of circulating soluble adhesion molecules [170]. Seljeflot et al. [171] found substantial reductions in E-Selectine and vascular cell adhesion molecule-1 after treatment with transdermal estradiol. These results might be explained by the effects of estrogen on glucose and insulin metabolism, although there is contrasting literature evidence in relation to this. Not all studies found estrogen to act incisively on glucose metabolism [172-175], but there is some evidence that estrogen therapy improves insulin sensitivity [176,177] and has a beneficial effect on hepatic gluconeogenesis, reducing the hepatic production of glucose [178]. In any case, ageing itself is associated with an increased risk of MetS and diabetes [179]. Other risk factors for diabetes in menopause include obesity, low physical activity, poor diet, smoking, excessive alcohol consumption and certain medications. Furthermore, impaired vitamin D3 metabolism and calcium deficiency (typical in postmenopausal women) translate into an increased risk of both types of DM Type 1 and Type 2 [159].

2.6. PREVALENCE OF MetS AMONG POSTMENOPAUSAL WOMEN

The prevalence of MetS varies considerably worldwide and among studies. This could be due to the size of the caseload, the use of different definitions of MetS, gender, ethnicity, urbanization level, socioeconomic and environmental differences, genetic factors, and lifestyle. The figure 6 shows the prevalence of MetS among postmenopausal women worldwide.

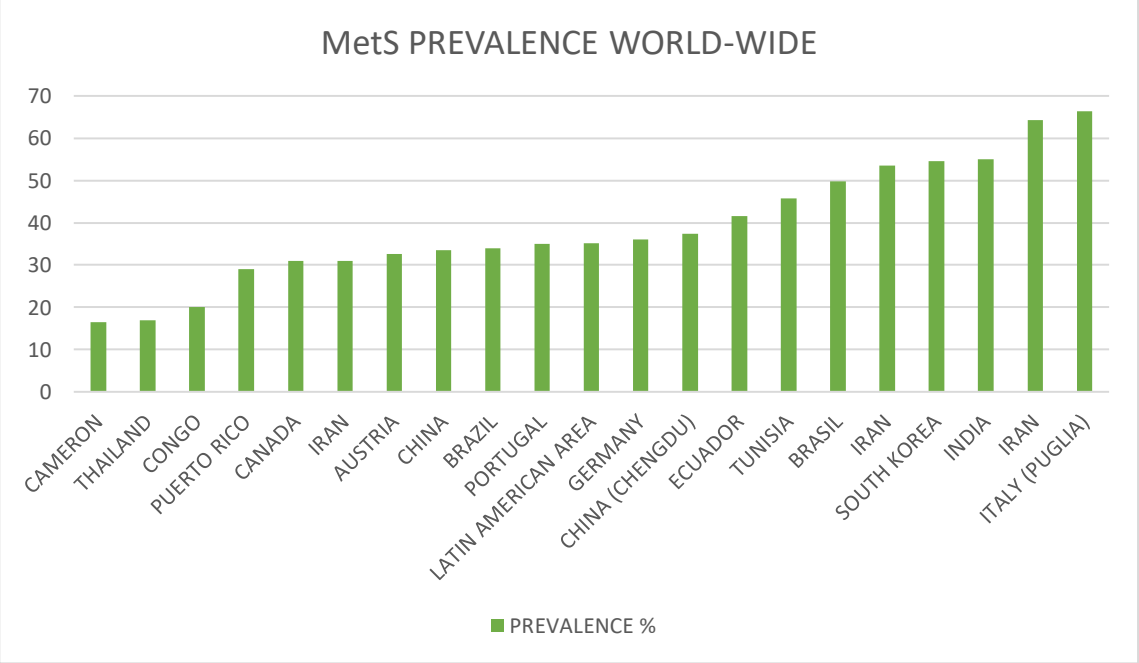

Fig. 6 - MetS prevalence (percent) in postmenopausal women worldwide

The lowest prevalence was found in Thailand, at 16.9% [180], Cameroon (16.5%) [181] and Congo (20%) [182], while almost one-third of the Latin American postmenopausal population (35%) was affected [183]. These data are similar to those found in other studies that included middle aged women: Germany (36.1%) [184], Portugal (35%) [185], Austria (32.6%) [186], China (33.5%) [187], Canada [31%] [188], Puerto Rico (29%) [189], and Ecuador (41.5%) [190]. The highest values were

found in Tunisia (45.7%) [191], Brazil (49.8%) [192], South Korea (54.6%) [193], Iran (64.3%) [194], India (55%) [195], and Italy (66.4%) [196].

Unexpectedly, Italy showed the highest prevalence: this could be due to the small population studied. Puglia is just a small region of Italy. The lifestyle and genetic characteristics of Puglian women differ significantly from those of other Italians - even more so than Thai or African women - which might explain this high prevalence. In addition, the diet in Puglia is typically high in carbohydrates, and its inhabitants have a progressively sedentary lifestyle. A similar effect has been produced by the rapid globalization of diet and increasing switch to nontraditional fast foods in urban areas of South Korea, India, Iran and Tunisia. The prevalence of MetS in pre- and postmenopausal women ranges from 6% to 65% [184,185,190-192,194,196]. An increased postmenopausal incidence has been shown in many studies throughout the world. Various features associated with MetS emerge during the menopausal transition, including obesity, atherogenic lipid profiles, diabetes, hyperinsulinism and hypertension. Physiological changes in this period, especially changes in reproductive endocrine function, might contribute to the risk of MetS. This may in part be due to increasing estrogen deficiency [197]. The figure 7 shows the prevalence of MetS in pre- and postmenopausal women worldwide.

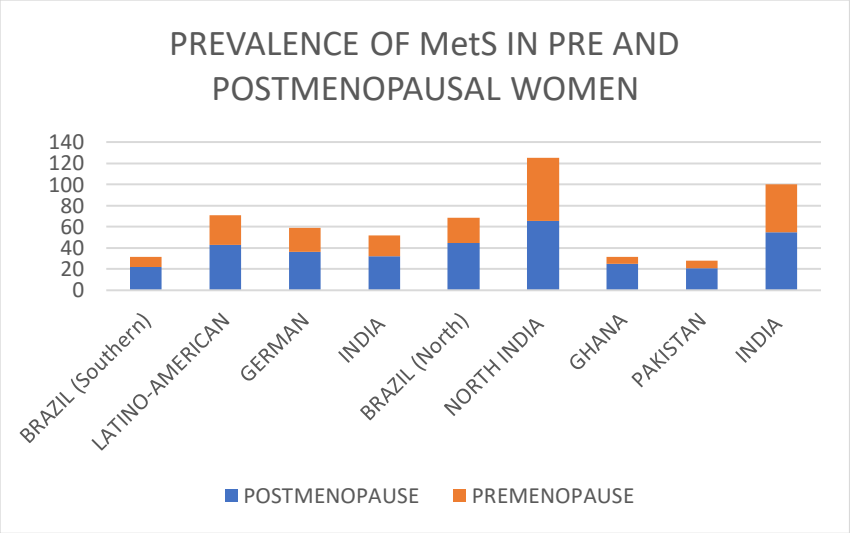

Fig. 7 - Prevalence of Mets in pre- and postmenopausal women

In a recent Brazilian study, the prevalence of MetS was higher in the postmenopausal group (22%) than in the premenopausal population (9%) [192]. Deibert et al. [184] found MetS in 36.1% of postmenopausal German women against 22.7% of premenopausal women. In an Indian study [193], the prevalence of MetS was found to be 45% in premenopausal and 55% in postmenopausal women. According to a study of 323 women between 40 and 65 years by Neto *et al.* [192] in Brazil, the prevalence was 44.4% in postmenopausal women and 24% in premenopausal women using NCEP criteria and 61.5% and 37% respectively using IDF criteria.

As already noted, the prevalence of MetS increases through the transition period from pre- to postmenopause, from 6.7% in the third decade to 43.5% in the seventh decade. Some authors have identified age itself as the main risk factor behind this [180-197]. Many of the features of MetS (central obesity and dyslipidemia with elevated TG, reduced HDL, and increase in LDL) emerge alongside estrogen deficiency in postmenopausal women. MetS therefore seems to play a key role in cardiovascular

disease (CVD) in postmenopausal women. As the average life expectancy of women after the menopause is now 20-30 years, the medical impact of MetS is significant [198]. The emergence of the features of MetS may be a direct result of ovarian failure or, alternatively, an indirect result of the metabolic consequences of central fat redistribution with estrogen deficiency. It is not clear whether the transition to menopause increases cardiovascular risk in all women or only those who develop the features of MetS. It should also be borne in mind that physical activity and lean muscle in women naturally diminish with age [199]. Body composition shifts to more fat and less muscle, slowing down the rate at which the body metabolizes biomolecules and resulting in weight gain, and above all central fatness, culminating in metabolic abnormalities and a higher prevalence of MetS. Current evidence suggests that multiple risk factors for CVD emerge in the postmenopausal period, but the features of MetS may be present even before the menopause. More research is clearly needed to further characterize the mechanisms by which women develop these metabolic changes with menopause [200]. In a recent Ghanaian study, the prevalence of MetS for the total population was 14.4%, 25.6%, 29.2%, and 30.4% using WHO, NCEP ATP III, IDF and Harmonization (H_MS) criteria respectively [201]. These data confirm that the prevalence of MetS can differ depending on the criteria used. In all studies, independently of the definition of MetS used, there was a statistically significant relationship between MetS, postmenopausal stage and increased age. For example, in the abovementioned Ghanaian study the prevalence of MetS was higher in the postmenopausal group (25.2%, 41.1%, 43.0% and 43.9% for WHO, NCEP ATP III, IDF and H_MS respectively) than the premenopausal population (6.3%, 14.7%, 18.9% and 23.1% respectively).

Neto et al [192] also analyzed the data separately according to NCEP and IDF criteria. Using the NCEP criteria, the overall prevalence of MetS was 34.7% (112 cases); 44.4% of postmenopausal women had MetS compared to 24% of premenopausal women-OR=2.52 (CI=1.56 to 4.079, $p<0.001$; and there was no statistically significant relationship ($p=0.228$) between race and MetS, despite its greater frequency among black women (42.9%) in comparison with white women (37.9%) and mixed-race women (31%). In contrast, according to IDF criteria, the overall prevalence was 49.85% (161 cases); MetS was present in 61.5% of postmenopausal women and in 37% of premenopausal women-OR=2.72 (CI=1.74 to 4.27), $p<0.001$; and here too, there was no statistically significant relationship between race and MetS ($p=0.323$), even though MetS was again more prevalent in black women (59.2%), followed by mixed race women (49.2%) and white women (46%). In conclusion, assessing MetS prevalence in postmenopausal women worldwide has many difficulties. Differences in genetic profile, diet, physical activity, age, and lifestyle influence the prevalence of MetS and its components, as do sociocultural influences, a sedentary lifestyle, and different levels of urbanization among the populations. Only the standardization of diagnostic criteria makes it possible to compare the prevalence in different populations.

3. METABOLIC SYNDROME AND ENDOMETRIAL CANCER

3.1. METABOLIC SYNDROME AND MALIGNANT TRANSFORMATION

MetS has emerged as a clinical condition that might predispose to malignant disease, alongside weight and other metabolic risk factors such as insulin resistance, diabetes, hypertension, and dysglycemia.

3.1.1. INSULIN RESISTANCE/HYPERINSULINEMIA

Insulin resistance is defined as a condition in which the normal cellular response to insulin is reduced. The pancreatic β cells respond by secreting more insulin, leading to increased circulating insulin concentrations (hyperinsulinemia) to maintain normal plasma glucose concentrations [202]. An Italian study by Giovannucci et al. confirmed that insulin promotes colon cancer in vivo using a rat tumorigenesis model. Insulin injections five times per week induced colon carcinogenesis [203]. This could be attributable to farnesylation of oncogene RAS promoted by insulin, which allows RAS translocation to the plasma membrane for cell signaling; the majority of colon tumors present RAS mutations that activate this oncogene. Furthermore, the insulin receptor is upregulated in colon and breast tumors [203-204].

Insulin is a major anabolic hormone that can stimulate cell proliferation and, in vivo, indirectly stimulates insulin-like growth factor-1(IGF-1). Like insulin, IGF-1 plays an important role in cell proliferation. Insulin can stimulate IGF-1 production by upregulating growth hormone receptors in the liver [1]. Hyperinsulinemia can also increase IGF-1 bioavailability by decreasing hepatic secretion of IGF-binding protein (IGFBP)-1 and -2, so that more IGF-1 is free to bind to its receptor on normal and cancerous cells. The IGF-1 receptor is overexpressed in breast and colon cancers

[205], and its activation stimulates the p21 ras/MAPK cell proliferation pathway and the PI3K/AKT cell survival pathway [206]. IGF-1 also stimulates angiogenesis, by increasing vascular endothelial growth factor (VEGF) production in colon, endometrial, breast, and prostate cancer cells. [207-209]. Finally, it has been suggested that insulin and free IGF-1 regulate the bioavailability of sex steroids that affect the development of hormone-dependent tumors such as endometrial, breast and prostate cancer. Normally, SHBGs produced by the liver circulate while bound to estrogens and androgens to inhibit their receptor binding and cell growth effects. Hyperinsulinemia and IGF-1 inhibit the synthesis of the SHBGs, promoting sex hormone-dependent cancers by increasing the bioavailability of estrogen and testosterone [210].

3.1.2 OBESITY

3.1.2.1. AROMATASE

A central feature of MetS is obesity, which is expressed by an increased waist-hip ratio (WHR) or body mass index (BMI), reflecting an increase in adipose tissue. Circulating concentrations of estradiol in postmenopausal women are directly related to BMI [211]. Estradiol can be formed from the conversion of androgens via the cytochrome P450 enzyme complex known as aromatase, which is present in adipocytes and adipocyte stromal tissue. Many breast and endometrial cancers are dependent on estradiol for tumor growth. Consequently obesity (BMI >30 kg/m2) predisposes to increased estrogen production and is associated with a two to five fold increase in the risk of EC [212] and a two fold increase in the risk of breast cancer in postmenopausal women [213].

Circulating estrogens are an important pathological mechanism linking obesity with breast and EC development in postmenopausal women. Increased adiposity raises the production of interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) cytokine, potent inducers of aromatase activity, and thus the production of estradiol, a potent growth factor for estrogen receptor-positive breast and endometrial cancers [214]. Accordingly, aromatase inhibitors (anastrozole, exemestane and letrozole) are recommended for use in postmenopausal women with hormone-dependent breast cancer [215] and those at risk of EC [212]. Anastrozole was recently used to successfully reverse endometrial hyperplasia in obese postmenopausal women [216]. Clinical trials of letrozole as a primary or adjuvant therapy for patients with EC are now underway; so far the results look promising [217,218].

3.1.2.2. ADIPOKINES

Obesity is characterized by the excessive accumulation and storage of fat in the body. Adipocytes, or fat cells, are not just simple storage sites for triglycerides but a complex endocrine organ able to secrete hormones, cytokines, and other proteins with signaling properties [collectively termed "adipokines"]. This diverse group has a role in processes such as appetite and energy balance, inflammation, insulin resistance/sensitivity, angiogenesis, lipid metabolism, cell proliferation, and atherosclerosis [219]. Many of these functions are related to either MetS or cancer, and they may serve as a link between these two conditions.

There are more than 50 adipokines with diverse functions affecting glucose homeostasis, insulin sensitivity, angiogenesis, adipogenesis, inflammation, and cell

proliferation, apoptosis, and differentiation [219]. Cytokines secreted by adipocytes are known to promote insulin resistance and increase circulating triglycerides, features of MetS [220]. Inflammation has also been linked to many types of cancer, including gastric, pancreatic, esophageal, liver, bladder, and colorectal cancers because it influences the growth, apoptosis, and proliferation of tumor and stromal cells. TNF-α activates nuclear factor-KB, which increases the production of NO, a substrate for reactive oxygen species (ROS) formation, and stimulates other inflammatory cytokines. ROS and inflammatory cytokines lead to insulin resistance and glucose intolerance [220,221]. Thus ROS, inflammatory cytokines, and insulin resistance can instill a vicious circle, as free fatty acids, glucose, and insulin further stimulate nuclear factor-KB activation. Invading white blood cells within the tumor stroma are also an important source of TNFα, IL-6, IL-10, and other cytokines within tumors. Thereby, increased circulating cytokine levels from adipocytes promote cancer progression by contributing to inflammation. Leptin is an adipocyte-specific hormone that serves as a metabolic signal to the brain, causing inhibition of appetite and increased basal metabolism to promote use of the stored energy (fat). Circulating leptin levels are thus directly related to adiposity [222]. However, obese patients develop resistance to leptin, becoming hyperleptinemic and more susceptible to the components of MetS. In addition to its association with obesity and insulin resistance, increased plasma leptin is associated with prostate, colon, breast, and endometrial cancer [223]. Leptin stimulates proliferation via MAPK signaling in prostate cancer cells and MCF-7 breast cancer cells; however, in transformed breast epithelial cells it has also been shown to activate STAT3, ERK, and AP-1 pathways, leading to cell proliferation. It also contributes to the metastasis of cancer cells by stimulating

angiogenesis in vitro and in vivo [223]. It therefore directly stimulates cancer cells and may serve as an important link between obesity and cancer. Adiponectin is the most abundant adipocyte-derived factor, accounting for 0.05% of serum proteins. Its anti-angiogenic, anti-inflammatory and anti-apoptotic properties may mediate its anti-tumor effects. Its levels are significantly reduced in obese patients (BMI >30 kg/m2) [224]. The primary mechanism by which it increases insulin sensitivity is the activation of 5-AMP-activated protein kinase (AMPK) in the muscle [by globular adiponectin] or in the liver [by full-length adiponectin] [225,226]. Adiponectin also reduces plasma free fatty acid concentrations and has anti-inflammatory and anti-atherosclerotic properties. It is inversely correlated with the risk of breast, endometrial, and gastric cancer [226]. Angiogenesis is the process of new blood vessel formation from pre-existing vasculature and is a critical process for tumor formation and metastasis. One of the most important proangiogenic factors secreted by adipocytes is vascular endothelial growth factor (VEGF). VEGF and its receptors (VEGFR-1 and VEGFR-2) modulate endothelial cell proliferation and migration as well as survival, vascular permeability, and tubulogenesis. VEGF is secreted by human fat cells, especially omental fat [227]. Serum VEGF was positively associated with visceral fat accumulation but not subcutaneous fat, as assessed by computerized tomography scans at the umbilical level [228]. The adipokine visfatin, also known as NAMPT/pre-B-cell enhancing factor, has been linked to several inflammatory disease states and cancer. It also regulates growth, apoptosis, and angiogenesis in mammalian cells [229], while Fukuhara et al. reported it to be an insulin-mimetic adipokine [230]. These diverse roles suggest that it may be an important component in physiological and disease states, especially in MetS-related cancers. Recent studies

demonstrated that high serum levels and tissue expression of visfatin were correlated with various cancers including breast cancer [231], colorectal cancer [232], prostate cancer [233], and gastric cancer [234]. Visfatin may therefore be an obesity-induced adipokine involved in the development of MetS-related cancers. Adipokines influence insulin resistance by increasing or decreasing insulin sensitivity. Because insulin resistance is directly related to MetS and cancer, adipokines may play a crucial role in linking these two diseases. The evidence that adipokines and proinflammatory cytokines derived from adipose tissue promote carcinogenesis [either by promoting insulin resistance or by directly influencing cancer cells] is considered intermediate.

3.1.2.3. IMPAIRED GLUCOSE REGULATION/HYPERGLYCEMIA

MetS is characterized by increased circulating glucose levels. Cancer cells have an accelerated metabolic rate and a corresponding high demand for glucose. To accommodate this, they have an enhanced ability to take up and use glucose. Glucose transporter proteins and especially GLUT1, GLUT 3 and GLUT12 are increased in many tumors [235]. GLUT3 has been detected in lung, ovarian, and gastric cancers but not in the corresponding non-cancerous tissues. GLUT12 has been found in prostate and breast cancer, but not in benign prostatic hyperplasia, and was reduced or absent in non-cancerous breast tissue. Several studies of patients with different tumor types (including breast cancer) have confirmed that increased glucose uptake/accumulation by tumors correlates with a higher grade of tumor, increased metastatic potential, reduced response to therapy, and poorer survival. If it is true that excess glucose levels favors cancer development, a combination of calorie restriction, exercise and a high-antioxidant diet should inhibit its development.

Numerous dietary components, such as olive oil, tea polyphenols, soy, resveratrol, fresh fruit and vegetables, omega-3 fatty acids, selenium, and others with antioxidant activity have demonstrated cancer-protective effects in breast and colon cancer in vitro and in vivo [236]. ROS have been considered as a potential intermediate in hyperglycemia-related cancer development. But it is also important to consider mechanistic evidence for hypertriglyceridemia-generated ROS. Mitochondria are the cell powerhouses, producing energy in the form of ATP as well as ROS [237]. Increased oxidative stress in fat has been demonstrated as an important pathogenic mechanism in MetS. Adipocyte NADPH oxidase production of ROS is specifically increased in fat in an obese mouse model, and NADPH oxidase inhibition improved blood glucose, insulin, and triglyceride concentrations in this model. These data suggest that ROS in fat may be a cause of MetS and a possible therapeutic target for obesity-associated MetS [238]. In addition, it has been proposed that excessive cytosolic triglyceride accumulation in non-adipose tissue such as liver and muscle enhances the ROS produced by respiring mitochondria by inhibiting adenosine nucleotide translocator and thereby decreasing ADP [239]. Thus, increased triglyceride/fatty acids and obesity contribute to the development of oxidative stress and ROS accompanying MetS.

3.2. METABOLIC SYNDROME AND RISK OF ENDOMETRIAL CANCER

Previous epidemiological data showed a strong association between the risk of EC and obesity (as measured by both BMI and waist circumference [239]), MetS and hyperinsulinemia. Premenopausal women with MetS, have an almost two fold increased risk of EC, largely due to increasing waist circumference. After menopause, this jumps to a 60-230% higher risk [240]. A 2015 study using the SEER-Medicare database showed that EC was significantly associated with MetS (OR 1.39) and its separate components, including overweight/obesity (OR 1.95), impaired fasting glucose (OR 1.36), hypertension (OR 1.31), and high triglycerides (OR 1.13). The association with the other component factors remained even after adjusting for overweight/obesity [241]. Another study by Shou et al. [242] suggested that patients affected by MetS with abnormalities in systolic and diastolic blood pressure have an increased occurrence of EC. These results are supported by a larger Italian study carried out in 2014 in which the risk estimates for EC were independently calculated for the components of MetS, including BMI and/or waist circumference (2.21), hyperglycemia (1.81), hypertension (1.81), and hypertriglyceridemia (1.17) [243]. Shan et al. [244] found that insulin resistance and hyperinsulinemia are key events in early endometrial hyperplasia and might even be factors initiating the development of EC. One large case–control study showed that patients with uncontrolled diabetes had a relative risk of developing EC of 5.563, whereas patients with controlled diabetes had a lower increased risk (RR 1.331) [245]. One study also showed that hyperinsulinemia and insulin resistance are both prevalent among overweight but non-obese women with EC, thus it is imperative that even overweight patients be screened for insulin resistance and encouraged to maintain a normal weight [246].

Excess weight is an established risk factor for EC. However, while studies have suggested a relation between the individual components of MetS and EC, very little research has been conducted to determine whether MetS better predicts the risk of EC than do weight [247], diabetes [248,249], hypertension [250,251], dyslipidemia, or dysglycemia alone [252-255,245]. In a recent study, Fredenreich [256] examined four different definitions of MetS that were all associated with an elevated risk of EC. The largest increased risk was for the modified IDF definition that used a more appropriate cut-point for central adiposity (a waist circumference of 88 rather than 80 cm) and hence recognized the importance of elevated waist circumference as a key EC risk factor. A large study by Bjorge et al. [257] included 917 EC cases from a total of 287.320 participants pooled from seven European cohorts, with a follow-up of 10 years. They found a statistically significant, 37% increased risk per unit increase in MetS score, constructed by adding individual z-scores computed for BMI, blood pressure, glucose, cholesterol, and triglycerides, standardized separately for sub-cohorts and for fasting time [257]. A case–control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort [58] concluded that MetS was directly associated with EC, with an approximate doubling in risk observed depending on which definition of MetS was used (relative risk (RR) 1.68; 95% CI: 1.23–2.31 for the IDF definition and OR 2.12; 95% CI: 1.51–2.97 for the NCEP definition). The EPIC study investigators also observed that the risk of EC increased with the number of MetS factors present (3 factors present OR 2.57; 95% CI: 1.52-4.32, 4 or 5 factors present OR 2.95; 95% CI: 1.60-5.45). The abovementioned Italian hospital-based case–control study found that the MetS definition most strongly associated with EC included BMI >30 kg/m2 and at least 2

of hypertension, diabetes, and hyperlipidemia (OR 8.40; 95% CI: 3.95-17.87) [255]. This risk was 2.37 (95% CI: 1.59-3.53) for the other 3 MetS components when obesity was excluded from the MetS definition. This study also found a significant increase in risk with increasing number of components for all MetS definitions examined. One small study from China found that central obesity, higher triglycerides, lower HDL-C, and abnormal plasma glucose were independent risk factors for EC, in addition to MetS [242]. Finally, a Norwegian study by Furberg at al. found a nearly 2 fold, but not statistically significant, elevated risk with a cluster of metabolic abnormalities, although they excluded obesity from their MetS definition [253]. The link between higher EC risk and central adiposity, history of hypertension, and history of diabetes as single risk factors has been well established [251]. A limited number of studies have also examined the association between risk of EC and levels of blood glucose or blood lipids individually, including several cohort [252,253,257,258] and case-control studies [245,254,255]. Most of these were limited in that the blood samples collected were non-fasting (no food intake for at least 8 hours). The most consistent finding in these studies was an elevated risk of EC with higher levels of triglycerides [217,218,228] and blood glucose [216-218]. The increased risk with elevated triglycerides were in the order of 25% to 230% [245,252,257]. When adjusted for BMI, the association with triglycerides is often attenuated [252,257], suggesting that a large part of this association is mediated by obesity. In conclusion, glucose and triglycerides are the primary contributors to increased risk for EC in patients with MetS.

3.3. METABOLIC SYNDROME AS CAUSE OF DEATH IN ENDOMETRIAL CANCER

Most patients affected by EC have early-stage disease (stages I-II) at diagnosis [261]. This determines a good outcome after treatment, with a 5-year survival rate as high as 96%. Despite their good prognosis, however, they frequently have associated several comorbidities, that may influence decision making for follow-up treatment and thus may affect consequent prognosis. Such comorbidities include diabetes mellitus, hypertension, dyslipidemia, obesity, or a combination of these [259-262] Medical morbidities including the components of MetS, especially obesity or diabetes mellitus, are clinically associated with EC as risk factors for cancer as well as prognostic factors for treatment outcomes. A Gynecologic Oncology Group (GOG) trial [263] showed that obesity does not increase disease recurrence rates. However, it does increase mortality rates in women with a diagnosis of early stage EC. Similar studies show that obese EC survivors with diabetes have a reduced life expectancy compared with their counterparts without diabetes or obesity [259]. Although the mortality rate in EC patients is low, the relative risk of dying for obese EC patients is significantly higher than for those with normal BMI (RR 2.53 for BMI 30-34, RR 6.25 for BMI >40) [230].

Given the high survival rates for low-grade localized cancers, these patients were most likely to survive their cancer diagnosis and go on to develop and die from cardiovascular conditions [264]. In the NIH-AARP Diet and Health Study, an increased BMI was correlated with an increase in 5-year all-cause mortality and EC

specific mortality, but not in cardiovascular mortality. However, the correlation between 10-year cardiovascular mortality and BMI became statistically significant (HR 4.08) at the extremes of BMI [265,266]. These studies suggest that survival after EC is rising, but that it increases the risk of dying from cardiovascular disease. In a majority of cases, early-stage EC alone does not lead to death, but these women will have an increased all-cause and obesity-related mortality later in life. In conclusion, based on these findings, EC therapy should include treatment of obesity and cardiovascular disease.

3.4. THERAPEUTIC STRATEGIES: HOW TO REDUCE OBESITY-RELATED MORTALITY IN ENDOMETRIAL CANCER PATIENTS

3.4.1. DIET AND EXERCISE

Both dietary modification and physical activity are associated with a decreased risk of EC. A weight loss of 5-10% has been shown to reverse insulin resistance, reduce IGF-1 levels, and prevent the development of T2DM [267]. A 2008 study of about 43000 postmenopausal women enrolled in the American Cancer Society Cancer Prevention Study II Nutrition Cohort [1992-2003] showed that moderate physical activity was associated with a 33% decrease in the risk of EC. This benefit was maximized in overweight or obese participants [268]. In terms of diet, a 2009 Italian case-control study showed that a diet low in vegetables may exert an unfavorable effect on endometrial disease. The beneficial role of vegetables may be due to their content of vitamins, other micronutrients, or components such as flavonoids and phytoestrogens, known to have antioxidant and anticarcinogenic properties. They may also influence female hormone levels and availability, given that EC is strongly

correlated with unopposed estrogens [269]. Another study focusing on exercise, BMI, and quality of life in survivors showed that lack of exercise and high BMI were associated with reduced quality of life. Furthermore, 70% of study participants were obese and were not meeting health exercise guidelines [270]. Unfortunately, despite the evidence suggesting that improving diet and increasing exercise can improve survival and quality of life, studies show that EC patients are not able to follow these lifestyle modifications [246]. In one study about changes in diet, exercise, and use of complementary medicine, 86% of participants were obese. Between the preoperative and 6-month postoperative periods, weight, exercise, and fruit and/ or vegetable intake did not change, but complementary medicine use significantly increased. This study shows that women who are obese or have a sedentary lifestyle before treatment of EC are resistant to changes in their lifestyle after treatment. They also turned to complementary medicine rather than lifestyle modification, suggesting that they may have received inadequate counseling from their gynecologist or found it difficult to successfully implement healthy lifestyle changes [271]. In a literature review carried out by Zhang et al., only 14.2% of EC patients proved able to exercise safely without supervision based on their health at the time of diagnosis. Factors likely to lead to exclusion from unsupervised community and/or home-based exercise programs included older age at diagnosis and higher BMI [272]. When placed in structured lifestyle intervention care groups, EC survivors are able to lose weight and incorporate healthy lifestyle changes. The SUCCEED trial showed that significant 6-month and 12-month changes were achievable, but recommended a longer follow-up. The rate of adherence in this study was 84%, and follow-up data were available from 92% of participants at 6 months and 79% at 12 months, showing that survivors are

more likely to follow diet and exercise regimens when enrolled in a supervised program [273]. As a follow-up to their study on complementary medicine use, von Gruenigen et al. randomized 45 overweight and obese EC survivors into a control group and an intervention group that received counseling on weight loss, healthy diet, and physical activity. After 1 year, the intervention group lost significantly more weight than the control group. Furthermore, their physical activity increased by 16.4 metabolic equivalents compared with a drop in 1.3 metabolic equivalents in the control group. Most importantly, the women in the intervention group also had increased quality of life scores compared with the control group [271].

3.4.2. MEDICATIONS

The primary medical management for women with endometrial hyperplasia without atypia is currently high-dose progestin therapy [274]. Medications targeted for hypercholesterolemia and type II diabetes can also be used to inhibit endometrial proliferation [275]. In patients with endometrial hyperplasia, prescribing these medications can not only stabilize their obesity-related comorbidities, but also prevent further malignant transformation. Prescription of these medications is also a viable option in patients who have failed high-dose progestin therapy and wish to avoid a hysterectomy. A 2015 retrospective cohort study of 985 EC cases showed improved disease-specific survival (81%) in women taking statins at the time of diagnosis and staging compared with nonusers (74%). Women taking aspirin for cardiovascular health in addition to a statin had particularly low disease-specific mortality (HR 0.25) in comparison with patients taking neither aspirin nor a statin [276].

Metformin not only inhibits gluconeogenesis but also improves insulin sensitivity and peripheral glucose uptake [275]. Additional research suggests that it also activates growth inhibitory pathways, improves signal transduction pathways, and lowers sex steroid hormone levels in postmenopausal women [277,278]. Two separate case reports have shown that insulin-sensitizing agents such as metformin can cause regression in patients with atypical endometrial hyperplasia resistant to progestin therapy. In one of these reports, the patients were obese, insulin-resistant, and nulliparous. In both reports, endometrial biopsy after initiation of the insulin-sensitizing agents showed proliferative endometrium [279,280]. In a recent interventional study, women with atypical hyperplasia or endometrioid adenocarcinoma received metformin twice daily for 4 weeks before surgery. Pre-surgical metformin was associated with reduced proliferation compared with a control group. Notably, formerly undiagnosed insulin resistance and/or diabetes were very common in the enrolled patients [281]. In conclusion, the relationship between EC and insulin resistance and the benefits of metformin as a chemotherapeutic drug are clear. Given all the evidence, metformin and/or statins should be considered for obese gynecologic patients with abnormal endometrial lining, whether or not in association with progestins.

3.4.3. BARIATRIC SURGERY

For severely obese patients, bariatric surgery is associated with a significant reduction in hormone receptors and can be considered a viable option [282]. A systematic review and meta-analysis carried out in 2015 showed a significant risk reduction for EC associated with bariatric surgery (pooled RR 0.40) [283]. Furthermore, a study of 6596 USA patients (including both men and women) who underwent gastric bypass surgery showed an overall reduction in cancer risk (HR 0.76). Interestingly, this study also found that the risk reduction was most pronounced in women (HR 0.73). The hazard ratio for EC specifically dropped significantly to 0.22 after bariatric surgery.

Ward et al. carried out a retrospective cohort study of women admitted with and without a diagnosis of obesity and/or a history of bariatric surgery. In this study a history of bariatric surgery was associated with a 71% risk reduction for uterine malignancy, and an 81% risk reduction if the weight loss was maintained after surgery [284]. Current data clearly demonstrate that bariatric surgery can result in clinically significant weight loss that not only reduces the risk of developing EC but also mediates cardiovascular comorbidities. Furthermore, cost-effective analysis of bariatric surgery in obese EC patients shows it to be a cost-effective option in comparison with routine care [285]. Despite the well-defined relationship between EC and obesity, hyperinsulinemia, and MetS, medical practitioners are not counseling their obese EC patients on the benefits of weight loss. Furthermore, EC patients are most likely to suffer reduced quality of life and to eventually die of their medical comorbidities rather than their cancer. It is therefore imperative that gynecologic oncologists routinely incorporate weight-loss counseling during the diagnosis,

treatment, and follow-up care for EC. Potential options include self-directed or supervised diet and exercise programs, medical management with insulin-sensitizing agents and/or statins, and if necessary, for severely obese patients only, bariatric surgery. As medical and surgical care providers, gynecologic oncologists have a unique opportunity to provide weight loss counseling and continuity of care for their cancer patients. Given the growing number of women who are obese worldwide, this is an opportunity we cannot afford to miss.

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PART II

4. EFFICACY OF MYOINOSITOL AND FLAVONOIDS IN POSTMENOPAUSAL WOMEN AFFECTED BY METABOLIC SYNDROME: A RANDOMIZED CROSSOVER STUDY.

4.1 INTRODUCTION

4.1.1 INOSITOL

Inositol (also called 1,2,3,4,5,6-cyclohexanehexol or cyclohexanehexol) is a molecule commonly referred to as a vitamin-B. However, this term is not completely appropriate, as inositol is more correctly a pseudo-vitamin because of its prevalence in average diets and its importance in the human body. Inositol is known as a cyclic polyol that is a precursor for the phosphorylated compounds phosphoinositides. Phosphoinositides are involved in signal transduction, and interact with other secondary messengers, including diacylglycerol (DAG) and inositol triphosphate (IP3) [1]. Inositol is a polyol and a member of the cyclohexane group, all of which have a hexagon shape with six hydroxyl groups surrounding the structure. There are nine different inositol stereoisomers: Myoinositol (MI), Epi-inositol, Cis-inositol, Allo-inositol, Muco-inositol, Scyllo-inositol, Neo-inositol, L-Chiro-inositol, and D-Chiro-inositol (DCI) (Fig.8). MI and DCI are the most predominant isomers and have important functions in human physiology. MI is uniquely defined by having a lone axial hydroxyl group on C2, where the other eight isomers are equatorial [2].

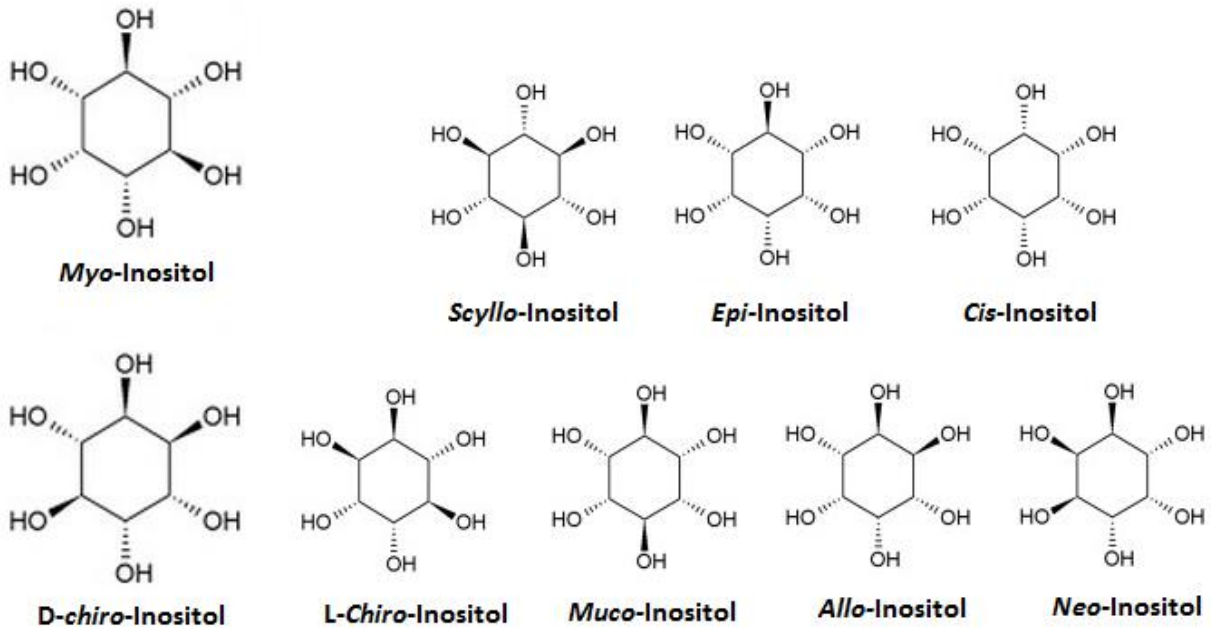


Fig. 8 - The nine different inositol molecules or stereoisomers.

Inositol occurs naturally in a variety of vegetable and animal food products, as well as in the human body. Inositol is a component of an average human diet in low concentrations. The typical dietary intake of MI ranges from 225 to 1,500 mg/day per 1,800 kcal body weight. MI can be typically found in milk products, fruits (e.g. mandarin oranges), vegetables, grains, meat, and fish. Inositol is not considered an essential nutrient for human nutrition, since MI and DCI can both be synthesized in the human body from glucose [3]. MI is the precursor of various phosphorylated derivatives, including IP3 [4-5]. There are 63 possible phosphorylated variants, which are divided into groups based on how many phosphate groups they possess (six variants of IP1, fifteen of IP2, twenty of IP3, fifteen of IP4, six of IP5, and a single IP6 molecule known as inositol hexaphosphate or phytic acid) [6]. Based on the structures of IP5 and IP6, enzymes may create pyrophosphorylated derivatives by

adding pyrophosphate groups to the D1, D3, or D5 carbons. These derivatives are referred to as IP7-IP9 [6-8] (Fig. 9).

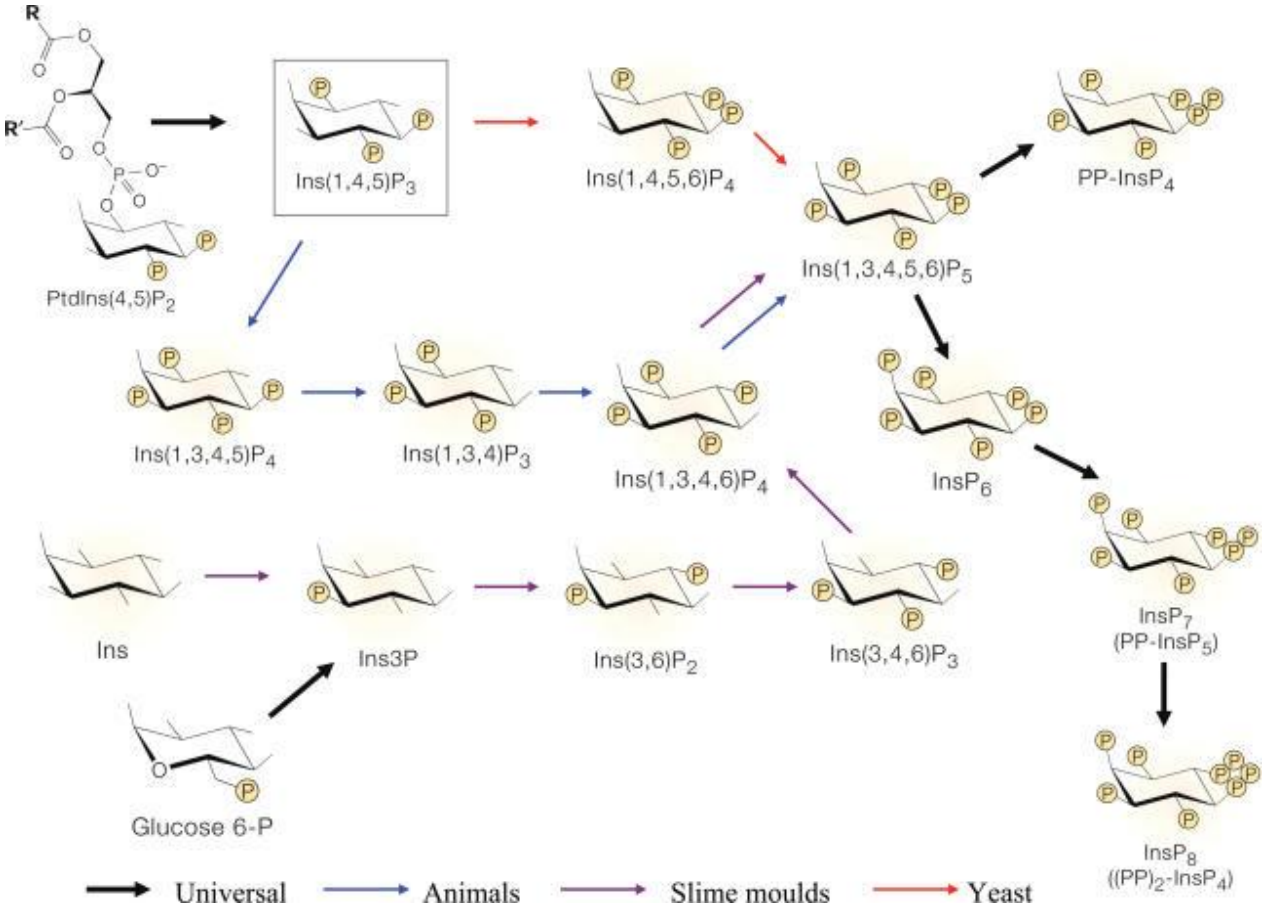


Fig. 9 - The eukaryotic inositol phosphate metabolism pathway.

One way to visualize and understand the phosphorylate inositol derivatives is to use a "turtle diagram" (Fig. 10), where the axial group of the carbon is the turtle's head (carbon 2) and the first carbon is the right frontal flipper, which is usually anchored to the cell membrane. Visualizing inositol in this manner can clarifying its numerous enantiomers [9].

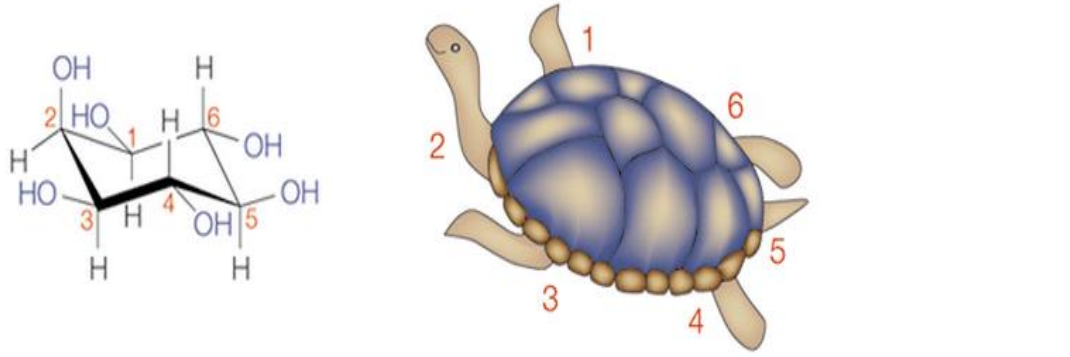


Fig. 10 - A diagram of the "turtle" image representing the inositol phosphates.

MI initially does not have any phosphate groups. The addition of phosphate groups to different positions can result in over 70 different signalling molecules within cells. These are categorized into groups based on how many phosphates they possess and are referred to as IP1-IP9. Another group of MI derivatives includes the phosphatidylinositol polyphosphates (PIPS), which are lipid based signalling molecules [10].

4.1.1 INOSITOL: PHARMACOLOGY

Inositol is taken up into tissue via a sodium-dependent inositol co-transporter. This co-transporter also mediates glucose uptake and can competitively inhibit inositol uptake [11], similar to DCI. However, MI has a ten-fold greater affinity for this transporter than does DCI [12]. Inositol is commercially available in powder or soft gel form. The application of a soft gel inositol has been shown to reduce the requirement of 2-4 g MI powder to 600-1,200 mg of the MI soft gel [13]. Supplementation of MI (5 g for one week and double the next week) in persons with metabolic syndrome was able to decrease apolipoprotein B and Low Density Lipoprotein-Cholesterol (LDL-C), while increasing choline plasmalogen, while the ratio of the two remained unaffected. These changes did not occur in persons without metabolic syndrome [14].

4.1.1.2 INOSITOL: MECHANISM OF ACTION

MI and DCI are intracellular signalling mediators of the insulin signalling pathway (Fig. 11).

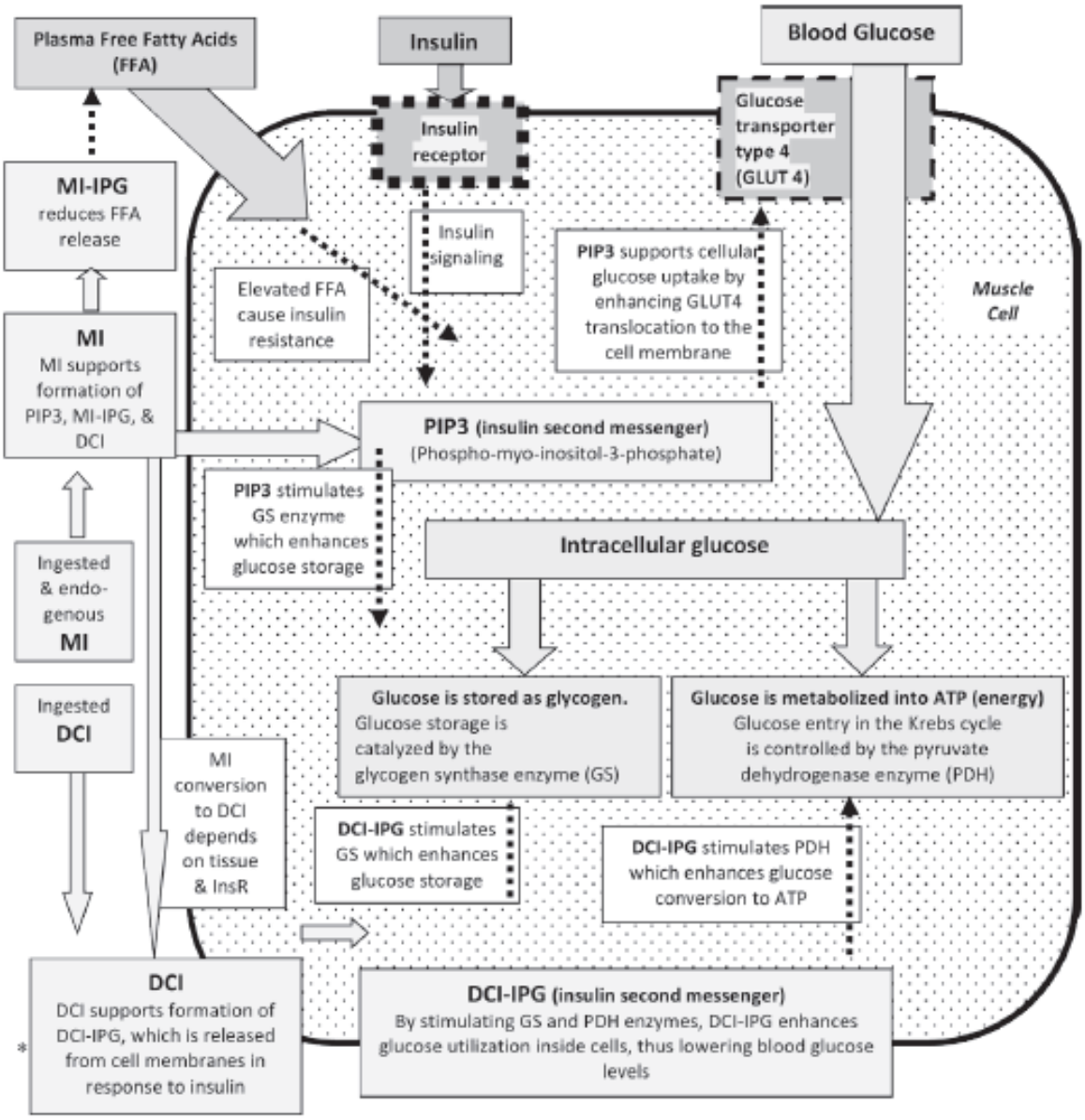


Fig.11 - Roles of MI and DCI in supporting insulin stimulated glucose entry and its utilization inside cells.

When DCI levels are low, glucose metabolism is impaired, which explains, in part, the state of insulin resistance. Inositols and their derivatives support improved glucose metabolism, as follows:

1. MI-derived phosphoinositol-3-phosphate (PIP3) upregulates glucose transport inside the cells by stimulating GLUT4 translocation to the cell membrane [10].

2. DCI-derived DCI-IPG supports enhancement of glucose conversion to ATP by increasing its transport in the Krebs cycle. This is achieved by stimulation of the pyruvate dehydrogenase (PDH) enzyme [10,15].

3. MI and DCI derivatives PIP3 and DCI-IPG, respectively, increase glucose storage as glycogen inside cells. This is achieved by the stimulation of the glycogen synthase enzyme (GS) [10,15,16].

4. MI-derived MI-IPG supports downregulation of free fatty acid (FFA) release from adipose tissues by inhibiting adenylate cyclases [15].

This effect is beneficial for the human body because FFAs have been shown to impair glucose disposal, thus causing insulin resistance and increased triglyceride synthesis [17].

Inositol (specifically MI) is a secondary messenger of insulin signalling via inositol phosphoglycans [18]. MI converts to DCI at rates that are specific to each type of tissue [15]. However, MI to DCI conversion has been found to be much lower in patients with type 2 diabetes, Polycystic Ovarian Syndrome (PCOS), gestational diabetes, or any insulin-resistant state when compared to normal controls through measurements from blood, tissue, and urine samples [19-22]. The increase in urine

concentrations is directly correlated to reduced insulin receptor activity in skeletal muscle [23]; therefore, urine levels of MI and DCI are biomarkers for insulin resistance [21]. MI is converted to DCI by the epimerase enzyme. This enzyme's activity is inversely correlated with the degree of insulin resistance [15,16]. Researchers have categorized epimerase downregulation as an "enzyme defect" associated with syndromes that display insulin resistance. However, there are reasons to believe that this so-called defect may not simply represent a random genetic mutation, but may be the result of evolutionary pressure for adaptation to variable food intake and survival, making selected genetic types more susceptible to developing insulin resistance [24-26]. Thus, the downregulation of epimerase may be viewed instead as a genetically programmed metabolic switch meant to downregulate glucose utilization, thus favouring metabolism of fat for fuel. Specifically, epimerase inhibition results in the reduction of DCI produced from MI in various tissues, while intracellular glucose disposal is influenced by the DCI-derived cellular mediator DCI-IPG. Researchers speculate that this adaptation may have occurred during an "evolutionary type" of insulin resistance triggered by famine, when body fat sediments release more FFAs. In contrast, a "modern type" of insulin resistance often occurs in the setting of excess caloric intake, especially from fat, and overall high body fat. However, these two distinct metabolic types are similar in the sense that they both display elevated plasma FFAs. Excess FFAs have been shown to impair glucose disposal through well-known metabolic switches, which can cause or aggravate insulin resistance [27].

4.1.1.3 INOSITOL: OPTIMAL DOSING

Oral ingestion of MI induces rapid GLUT4 translocation. Oral ingestion of 1,000 mg/kg of MI in mice is able to exert an acute hypoglycaemic effect when paired with 2 g/kg of glucose, without also reducing blood glucose and with slightly less efficacy than the same oral dose of D-pinitol. D-pinitol is thought to be related to an increase in GLUT4 translocation [28]. In animal studies, large oral doses of MI (compared to the human dose of 80 mg/kg) may aid in glucose deposition when taken with carbohydrates, with no inherent hypoglycaemic effect. Supplementation of 2,000 mg MI twice daily for six months in postmenopausal women with metabolic syndrome is associated with improvements in all biomarkers of glucose metabolism (insulin and glucose levels, as well as sensitivity to insulin) [29]. The benefits appear to be slightly greater when the trial was extended to a full year [30]. In women with insulin resistance, supplemental MI appears effective at improving insulin sensitivity. Increased urinary inositol metabolites are seen in patients with insulin resistance (e.g. patients with type 2 diabetes and PCOS), and in pregnant women with gestational diabetes [31]. Supplementation of 4,000 mg inositol daily during pregnancy is associated with improved biomarkers of gestational diabetes; specifically, the decline in insulin sensitivity and rise in glucose were both significantly attenuated [32]. In a study in women with PCOS who achieved pregnancy, administering the same dose of inositol daily for the duration of pregnancy was associated with a significantly reduced risk of developing gestational diabetes (from 52% to 17.2%) [33]. There was no overall change in average weight gain during pregnancy associated with supplementation of inositol [34]. Overall, supplementation with inositol throughout pregnancy halves the risk of developing gestational diabetes, and improves insulin

sensitivity. Supplementation with MI at low doses (200 mg) in women with PCOS is less effective than at 1,200-2,000 mg. Although there has been some success in using low-dose inositol in obese women, there was only limited success when used to treat merely overweight women [35-36]. Generally, the weight-loss effects of inositol treatment in women with PCOS are only seen in those with a Body Mass Index (BMI) exceeding 37 [37]. Interestingly, women whose insulin resistance is not due to PCOS fail to find a therapeutic benefit with even 4,000 mg daily for a year [38]. For women with PCOS who are known to have difficulty losing weight, supplementation with inositol is able to help alleviate this difficulty, though this benefit may be dependent on a diagnosis of PCOS [39]. Based on the pharmacokinetics of inositol supplementation, it is possible to split the daily dose to half the dose every 12 hours in order to maintain continuous therapeutic levels of inositol. It is best to take inositol on an empty stomach, especially not with meals high in carbohydrates, since inositol competes with glucose for absorption in the gut and uptake from the bloodstream into cells [40]. Patients should ensure that they maintain an adequate intake of zinc, manganese, and magnesium, as these minerals have important roles in inositol transport and metabolism. Other supplements, such as lipoic acid and N-Acetylcysteine (NAC), may have additive synergistic effects in improving glucose metabolism. In summary, MI and DCI can be considered conditionally essential nutrients for conditions such as METs and PCOS, where dysglycemia and insulin resistance play critical roles. The clinical studies discussed herein demonstrate that average dietary inositol intake and endogenous inositol production need to be supplemented with MI and DCI in order to improve glucose metabolism homeostasis. This paradigm is also supported by the decreased MI urine levels observed in these

conditions. Thus, MI and DCI should be consider essential supplements for treatment of PCOS, METs, gestational diabetes, and possibly type 2 diabetes.

4.1.2 D-CHIRO INOSITOL VERSUS MYOINOSITOL: BIOLOGIC DIFFERENCES

Inositol is present in cells in both a free form and as a component of membrane phosphoinositides. Inositol is involved in a variety of cellular functions, including growth, survival, development, function of peripheral nerves, osteogenesis, and reproduction. In their conjugated form, inositols are components of cellular membranes and have crucial functions in membrane integrity and intracellular signalling. Phosphatidylinositol is the precursor of phosphatidylinositol phosphate and phosphatidylinositol diphosphate (PIP2), which upon hydrolysis by phospholipase C (PLC) results in inositol 1,4,5 trisphosphate. Inositol 1,4,5 trisphosphate functions as second messenger for membrane receptors coupled to PLC, and is involved in the signalling mechanisms of many autacoids, hormones, and neurotransmitters [41]. Both MI and DCI can influence the intracellular metabolic processes by activating key enzymes involved in oxidative and non-oxidative glucose metabolism [42,43]. MI is involved in the metabolism, transport, and breakdown of glucose and its conversion to glycogen. DCI is involved in the insulin signalling pathway and in the stimulation of serial enzymes that are involved in the regulation of glucose metabolism, including pyruvate dehydrogenase phosphatase (PDHP), protein phosphatase 2C (PP2C), and inositol-phosphate glycan [42,44]. MI and DCI work in synergy in the glucose metabolism pathway. MI induces the translocation of glucose transporters to the cell membrane in order to enhance glucose cellular uptake

[5,6], and DCI stimulates pyruvate dehydrogenase and supports ATP production through the Krebs' cycle [42]. Recent data indicate that DCI glycans specifically stimulate insulin secretion in pancreatic B-cells [45]. These mechanisms are summarized in Fig. 12.

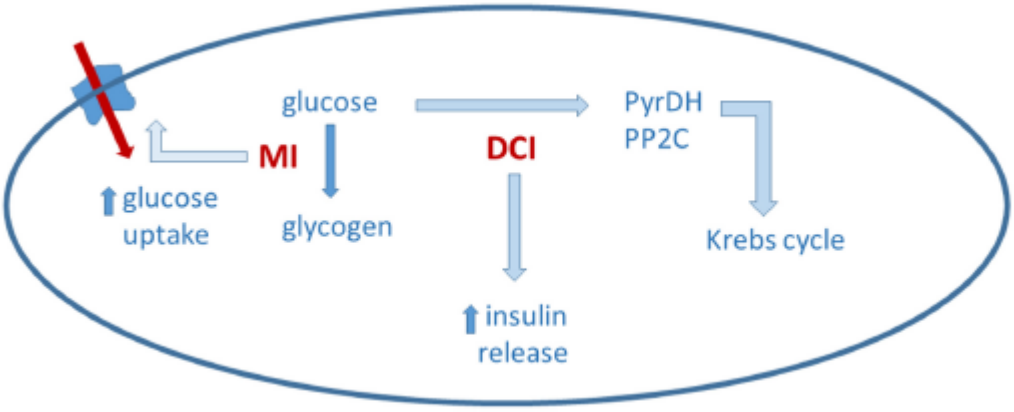


Fig.12 - MI and DCI may act in a complementary way on glucose metabolism..

MI and DCI mediate different mechanisms of action for insulin. Specifically, MI is converted to inositolphosphoglycan insulin second messenger (MI-IPG) and is involved in cellular glucose uptake. DCI is converted to IPG insulin second messenger (DCI-IPG) and is involved in glycogen synthesis [46-48]. Significant variability has been noticed in the ratio of MI and DCI in fat, muscle, and liver. These differences reflect the distinct functions that the two isomers are likely to play in those tissues. In the cell, the relative proportion of MI to DCI is actively maintained as MI is enzymatically transformed into DCI through NAD–NADH-dependent epimerases, depending on specific tissue requirements [42-46]. The epimerase conversion of MI to DCI is under insulin control; however, in patients with type 2 diabetes, reduced tissue insulin sensitivity leads to decreased epimerase activity and hence

downregulation of DCI synthesis [46]. In ovarian tissue, MI and DCI play different roles, especially in the physiology and thus potential treatment of PCOS [9]. In the ovary, DCI is involved in insulin-induced androgen synthesis [10]., whereas MI mediates glucose uptake and follicle stimulating hormone (FSH) signalling [49-52]. In the past two decades, several studies have reported the effectiveness of MI and DCI in improving pathologic conditions associated with insulin resistance, including PCOS, metabolic syndrome, and infertility [53]. While DCI effects are restricted to insulin signalling transduction, MI has been demonstrated to exert other noticeable activities in ensuring oocyte quality and maturation. In human ovaries, 99% of the intracellular pool of inositol is composed of MI, and the remaining 1% is DCI [54]. In patients affected by PCOS, epimerase activity is increased in theca cells, causing a deficit of MI [55]. Reduced intraovarian MI leads to disruptions in ovarian MI and DCI concentrations. This may adversely affect glucose uptake and metabolism of both oocytes and follicular cells, which impairs FSH signalling. Since oocytes are characterized by high glucose consumption, this defect compromises oocyte quality [33,34,37].

4.1.2.1 D-CHIRO INOSITOL

DCI is a key component of the insulin transduction pathway in cells. However, while DCI treatment improves the systemic consequences of insulin resistance by modulating insulin activity in non-ovarian tissues, DCI exerts controversial effects on oocyte function. In 1999 Nestler et al. used DCI to treat PCOS. In their study, 19 out of 22 obese hyperinsulinemic PCOS patients treated with 1.2 g/d DCI showed restored ovulation compared with the placebo group (86% ovulated versus only 27% in the placebo group) [56]. In this study, DCI treatment for PCOS women decreased the insulin response to orally administered glucose. This decrease was most likely due to improvement in peripheral insulin sensitivity. Serum androgen concentrations also decreased in the women who received DCI treatment. Most likely, administration of exogenous DCI restored diminished intracellular DCI and the DCI phosphoglycan content in skeletal muscle and other insulin targeted tissues to normal levels. These encouraging results obtained during this first pilot DCI-based trial [56] also suggest that DCI could be used to treat women with PCOS. These results also indicate that DCI may prove useful in the treatment of other disorders with insulin resistance. After publication of these innovative results, Insmed Pharmaceuticals obtained a U.S. patent in 1999 claiming the effectiveness of DCI in the treatment of PCOS. A follow-up study was performed in 2002 by the same group, this time in lean women with PCOS [57]. In accordance with the earlier study [18], supplementing with DCI was associated with improved insulin sensitivity, reduction in circulating free testosterone, and increased frequency of ovulation [57]. Insmed Pharmaceuticals subsequently began a large multi-centre placebo-controlled trial of DCI supplementation in women with PCOS, this time doubling the dose of DCI to 2,400

mg daily [48]. The results were surprising: higher dose DCI treatment failed to replicate the findings of the two previous studies [56,57] in terms of improving ovulatory frequency. On the contrary, higher DCI dosing paradoxically decreased oocyte quality. The lack of efficacy in the latter trial may have been due to the high dose of DCI. These results actually align with a recent trial by Rosalbino et al. [58] in which increasing DCI dosage progressively negatively influenced oocyte and embryo quality. Similar results have been obtained when treating PCOS women scheduled for IVF with metformin at 500 mg three times per day [59]. Metformin decreased the number of dominant follicles, retrieved oocytes, and metaphase II stage oocytes, despite the increased gonadotropin administered. Baillargoen et al. showed that metformin increased the insulin stimulated release of DCI phosphoglycans. Therefore, we can speculate that metformin promotes glycogen synthesis and further reduces ovary energy status through DCI phosphoglycans [60]. This evidence may explain the opposite results seen in the two Nestler trials [48,49]. In the first study, DCI supplementation restored ovulation, probably due to the peripheral action of DCI which allowed epimerase to physiologically restore the ovary. In the second trial, the excess of DCI reduced the previously observed positive impact. These results support the "DCI paradox hypothesis." The DCI paradox hypothesis in the ovary was published in 2011 by Unfer et al. [61]. They suggested that in women with PCOS, hyperinsulinemia likely stimulates epimerase activity in the ovary, resulting in overproduction of DCI and concomitant depletion of MI. The resulting deficiency of MI could be responsible for the poor oocyte quality and impairment of FSH signalling. This hypothesis has resulted in a clinical focus on the use of MI and DCI supplementation to restore normal ovary function. In fact, a correlation between MI

concentration in follicular fluid and high oocyte quality has been found in a number of studies that reported MI supplementation is able to improve oocyte quality [62,63]. Finally, although DCI is useful in the treatment of PCOS patients to reduce insulin resistance, it has no effect on ovulation frequency. Interestingly, a recent Italian study by Maurizi et al [64] showed for the first time that DCI and folic acid oral supplementation may be an adjuvant treatment in overweight or obese insulin-treated type 1 diabetes patients.

4. 1. 2. 2 MYOINOSITOL

Several studies have confirmed the role of MI in improving oocyte quality [12,24,25,27]. In particular, the levels of MI in follicular fluid is positively correlated with embryo quality [11]. Furthermore, in 2002 Chiu et al showed a direct correlation of MI concentration in follicular fluid with high oocyte quality [62]. An Italian trial evaluated MI administration in 25 PCOS patients [63], and suggested the effectiveness of MI in the treatment of infertility in women with PCOS. The authors reported an increase in the frequency of spontaneous menstrual cycles and pregnancies in women with PCOS who received MI in combination with folic acid. Several other studies support these findings [55,61,64,65]. These findings emphasize that PCOS patients with hyperinsulinemia typically have enhanced MI to DCI epimerization in the ovary. This results in an increased DCI:MI ratio (i.e. overproduction of DCI), which in turn leads to MI deficiency in the ovary. This MI depletion could ultimately be responsible for the poor oocyte quality observed in these patients [66]. Furthermore, based on the observation that MI supplementation reduces the need for recombinant FSH administrated during *in vitro* fertilization

cycles [63], it is likely that MI deficiency in the ovary also impairs FSH signalling, resulting in an increased risk of ovarian hyperstimulation syndrome in PCOS patients. Unfer et al. conducted a comparative study on the effects of MI versus DCI supplementation on oocyte quality in PCOS patients. They reported that the number of mature oocytes was significantly higher, with a parallel diminution in the number of immature oocytes, in the MI group compared to the DCI group, even though the total number of oocytes retrieved did not differ between the two treatment groups [67]. A potential explanation for this phenomenon is the tissue-specific nature of insulin resistance in women with PCOS. Indeed, although muscle and liver are insulin-resistant in women with PCOS, the ovaries retain normal insulin sensitivity.

4.1.3 ISOFLAVONES

Isoflavones, also called phytoestrogens, are plant-derived compounds with oestrogen-like biologic activity and a chemical structure similar to that of oestradiol [68]. Isoflavones are a class of phytochemicals, and represent a broad group of nonsteroidal compounds with diverse structures originally derived from plants that bind oestrogen receptors (ERs) in animals and humans. Isoflavones have greater affinity for ER-β than for ER-α, and possess both oestrogen agonist and antagonist properties. The isoflavone family includes the biochemicals genistein, daidzein, glycitein, biochanin A, and formononetin [69]. Genistein and daidzein are found in high concentrations in soybeans and soy products, as well as in Trifolium Pratense (red clover), Japanese Arrowroot (kudzu), and in the Apios Americana (American Groundnut) [70]. Soy is the most widely used isoflavone-containing food. Soy proteins can be obtained by extracting protein from the whole bean, and is often a

rich source of isoflavones [71]. The primary isoflavones found in soybeans are genistein, daidzein, and glycitein (Fig. 13).

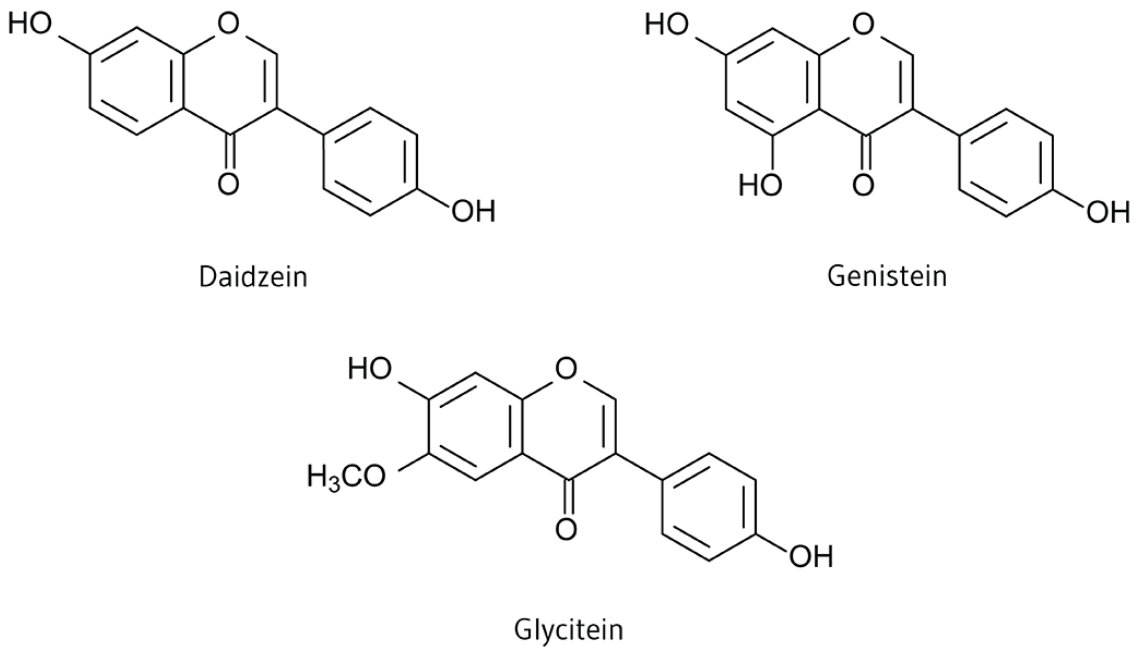


Fig.11 - The chemical structures of the major Soy isoflavone aglycones.

The relative amounts of these isoflavones vary depending on the localization within the soybean. The whole soybean contains equal amounts of genistein and daidzein, with smaller amounts of glycitein. The germ of the soybean, however, which has an overall higher concentration of isoflavones, has approximately four times more daidzein than genistein, and relatively high concentrations of glycerine. Some soy supplements are made from soy germ and thus have this isoflavin profile [72]. However, only approximately 30% of North American women have the ability to metabolize daidzein to equol (Fig. 14), making products made from soy germ potentially more difficult to metabolize.

Equol is a nonsteroidal oestrogen-like compound that binds to both ERs, but with a high affinity for ERα. Thus, equol is often referred to as an ERα agonist. Equol is metabolized from daidzein by intestinal bacteria. Equol is thought to be a stable compound, which remains in the system for several days after a soy challenge [73].

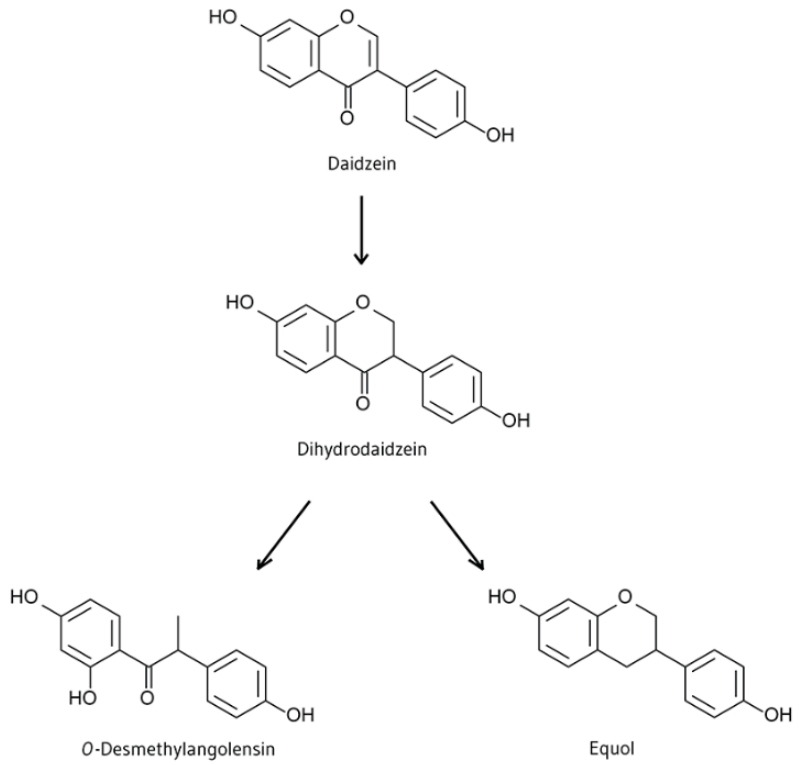


Fig.12 - The chemical structures of daidzein metabolites.

Equol (7,4′-dihydroxy-isoflavan) is a nonsteroidal oestrogen of the isoflavone family. It was originally isolated from equine urine in 1932 [68]. Four decades later, in 1982, it was identified in human urine [74]. In 1968, equol was determined to be a bacterial metabolite of daidzein [75], one of the two predominant phytoestrogens found in soy and soy-derived products. Equol is unique in having a chiral centre at C-3 due to the lack of a double bond in the heterocyclic C-ring. As a result of a chiral carbon at position C-3 of the molecule, equol exists in two enantiomeric forms, R-(+) equol

and S-(-) equol. The latter is the natural diastereoisomer produced by intestinal bacteria in the intestine of humans and rats [76]. Only S(Y)-equol is detected in the plasma of equol-producing women and is the only form thought to have biological activity. Equol can be produced by intestinal bacteria in some, but not all, adults.

Biotransformations that take place after oral administration of soy isoflavones are summarized in Fig. 15.

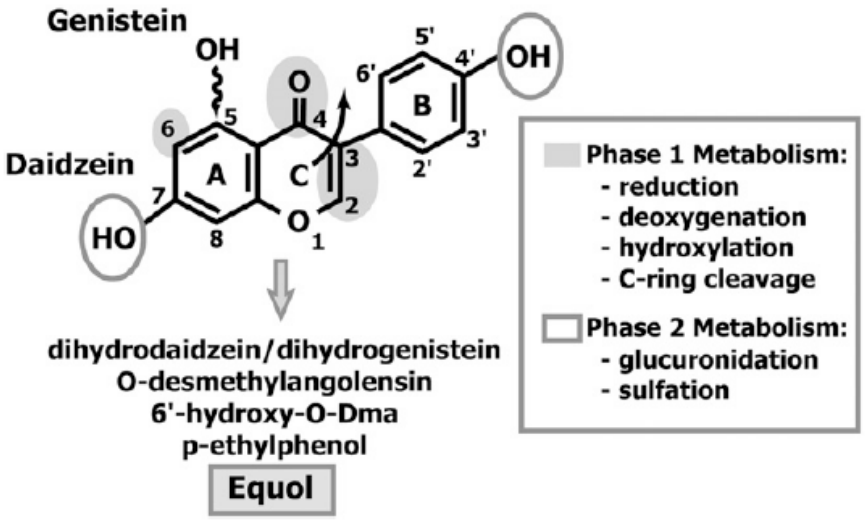


Fig. 15 - The production of S-(-)equol from daidzin.

Isoflavones were first considered to be phytoestrogens because they bind to both ERs and because of their effects on infertility. They can function as antioestrogens by binding ER in place of potent physiologic oestrogens, thus blocking biologic oestrogen from exerting its normal biologic effects [77,78]. During the 1980s, Akiyama et al. demonstrated the role of genistein in inhibiting protein tyrosine kinases, which correlated with upregulation of the epidermal growth factor receptor

(EGFR) and overexpression of the platelet-derived growth factor receptor (PDGFR), both of which are involved in breast cancer [79]. Therefore, isoflavones can reduce the risk for oestrogen-dependent cancers, including ER-positive breast cancer. Genistein arrests cell cycle progression at the G2–M transition, prevents apoptosis, has antioxidant properties, modifies eicosanoid metabolism, and inhibits angiogenesis [78,79].Other roles within the cell have also been proposed, including functioning as antioxidants [70,71], inhibitors of DNA topoisomerases, and in steroid synthesis and metabolism [80,81]. Recent studies on S(Y)- and R(+)-equol showed that S(Y)-equol is a better agonist for ER-β, with agonist properties comparable to that of genistein. This role for S(Y)-equol challenges earlier studies about this metabolite [82]. Isoflavones can naturally occur in soy food, [82] or can be isolated in extracts, supplements [76,83,84], as pure compounds [76,85], or as stable-isotope labelled analogues [86,87]. Regardless of the form, the bioavailability of these isoflavones remains similar. However, the rate of absorption of the isoflavones daidzein and genistein as glycosides are distinctly different from those of daidzein and genistein in their aglycone form. This discrepancy has recently been suggested to be an important difference that could ultimate influence the efficacy of isoflavone treatment [88].

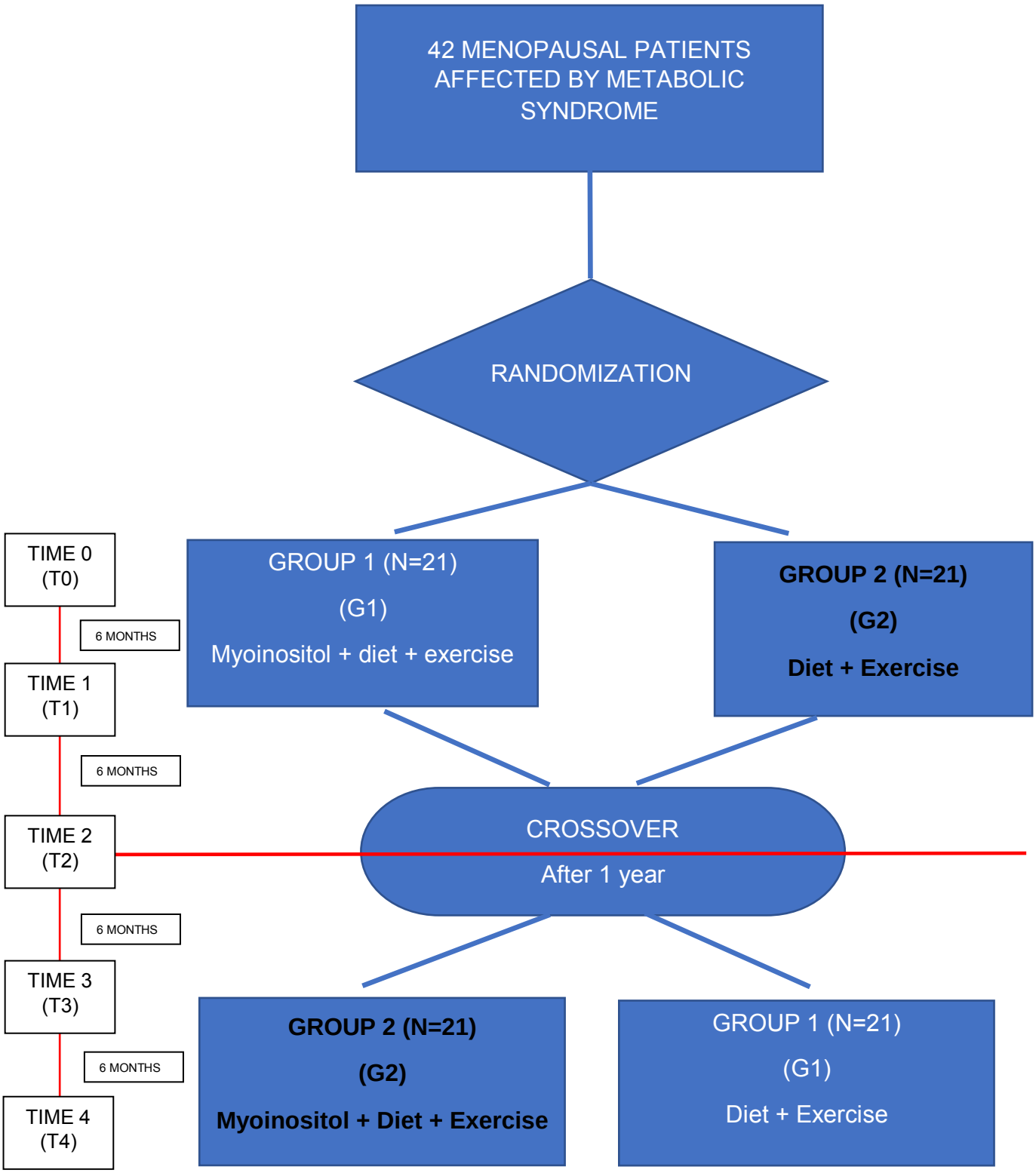
4.1.3.1 ANTI-DIABETIC EFFECTS OF ISOFLAVONES

Eating foods with a low glycaemic index (GI) improves blood sugar control and blood lipid levels in patients with non-insulin-dependent diabetes. Legumes, especially soybeans, have a very low GI and are an ideal food choice for diabetic diets [89,90,91]. Studies have also found that isoflavones in soybeans significantly reduce the risk of type 2 diabetes by decreasing patients' overall GI [92,93]. This observation may be explained by the fibre, tannin, and phytic acid in soybeans [94,95]. Thus, soybeans may play an important role in the dietary program for women with diabetes and for those with an elevated risk of developing diabetes.

Blood sugar control may also be improved by eating carbohydrates that are high in soluble fibre [96]. Soy fibre is extremely fermentable in humans, and therefore may have more physiologic benefits than other types of fibre [97]. Furthermore, it has been demonstrated that 25 g of supplemental soy fibre per day significantly reduced insulin response and total plasma cholesterol in patients [98]. Finally, soy isoflavones are able to effectively increase insulin secretion without any change in glucose disposal or decrease in plasma adiponectin concentrations [94]. This data supports the use of soy food and soy extracts to reduce the risk of type 2 diabetes and its related complications. Decreased circulating oestrogen during menopause is often responsible for glucose metabolism dysregulation, and it is one of the most important features of MetS. Hormone replacement therapy (HRT) shows some benefit in controlling cardiometabolic disease, improving glucose tolerance, and lowering the risk diabetes. Unfortunately, many studies suggest that HRT-treated women show an increased incidence of breast cancer, adverse cardiovascular consequences (i.e.

venous thrombosis), and potential interactions with serum lipoproteins [99,100]. Consequently, the development of compounds targeting ER-mediated pathways appears to be a promising and low risk method to reduce potential complication in postmenopausal women with MetS [101]. A recent Italian trial [102] showed that genistein treatment for up to one year significantly decreased insulin resistance, fasting glucose, and insulin levels, thus improving overall glucose homeostasis. A recent meta-analysis indicated that soy isoflavones significantly reduced total cholesterol and LDL-C, but did not change HDL-C [103]. The positive association between isolated soy isoflavones and HDL-C has been documented in postmenopausal women [104]. Homeostatic control of both glucose and lipid metabolism could be related to adipokine production. Adiponectin has been linked to insulin sensitivity and exerts insulin-sensitizing effects [105]; its levels decreased in obese patients and in patients with type 2 diabetes [106]. Adiponectin has a strong anti-inflammatory and anti-atherogenic potential [107]. Indeed, any treatment that increases adiponectin levels may improve insulin resistance [108]. Nonetheless, low concentrations of adiponectin are associated with increased prevalence of MetS, especially in postmenopausal women [109]. In an Italian study [102], genistein significantly increased adiponectin and visfatin serum levels compared with placebo. In conclusion, it is possible that the use of phytoestrogens could improve MetS parameters without adverse events or extreme side effects. We need further randomized clinically trials comparing isoflavones, HRT, and metformin to establish a usable guideline for treatment aimed at preventing type 2 diabetes and metabolic syndrome.

4.2 STUDY DESIGN



4.3 PATIENTS AND METHODS

Forty-two outpatient postmenopausal women diagnosed with metabolic syndrome who were referred to the Policlinic Campus Bio Medico in Rome, Italy were enrolled in a two-year study. In order to define metabolic syndrome, we used the Adult Treatment Panel III of the National Cholesterol Education Program criteria, and all 42 women met at least three of the five reported necessary criteria. At the time of recruitment, all women were treated with low-calorie diets and aerobic exercise (walking for 40 minutes five times a week). Patients were divided into two group, G1 and G2, randomly. During the first year (time T0-T2), the G1 group (21 patients) received 2 g MI + soy isoflavones (genistein 200 mg) once a day. The G2 group (21 patients) was treated with only diet and exercise. After one year, the therapies were switched. Thus, during the second year (time T2-T4), the G2 group (21 patients) received 2g MI + soy isoflavones (200 mg) once a day. The G1group stopped MI treatment and was then treated with only diet and exercise. The characteristics of all 42 patients at baseline are reported in Table 1. They were evaluated at baseline (T0) and every six months for four follow-up times (T1-T4). Patients were evaluated for: BMI, Abdominal Circumference (CA), Serum Glucose (BG), Triglycerides (TG), Low Density Lipoprotein (LDL), and High Density Lipoprotein (HDL). Patient enrolment started in January 2012 and finished in December 2013. Participants gave written informed consent before beginning the study.

	PATIENT	AGE	BMI	AC (cm)	BG	TRIG	HDL	LDL
GROUP 1	B. A.	51	28	97	103	220	37	189
	A. L.	46	26	95	107	215	52	160
	V. P.	59	32	128	140	270	30	286
	G. L.	52	27	97	107	226	50	161
	M. M. A.	55	27	100	108	223	42	160
	O. F.	63	28	110	100	290	35	227
	P. T.	50	29	108	110	257	39	199
	M. E.	58	28	106	110	221	30	204
	P.M. A.	54	30	130	121	356	37	229
	N. M. P.	51	28	108	108	280	36	196
	C.A.	47	27	105	97	240	40	172
	V. G.	49	29	110	103	220	37	209
	M. G.	58	28	107	98	225	38	184
	V. A.	50	30	115	120	307	30	248
	R.A.	57	30	117	118	298	31	224
	A. R. G.	59	28	109	96	220	40	168
	E.M. G.	55	27	104	95	235	52	168
	D.V.V.	57	30	115	120	270	60	156
	P. E.	58	26	97	112	220	45	167
	O. N.	66	27	106	115	295	42	194
	D. L.G.	65	26	93	109	220	50	153
GROUP 2	F. M. A.	50	29	110	115	245	47	209
	V. B.	50	26	95	109	225	42	190
	C. M. P.	48	30	128	126	322	32	234
	O. L.	57	27	98	108	215	40	207
	P. L.	53	28	110	112	230	42	257
	F.A.	47	30	128	125	330	35	259
	C. I.	53	27	100	110	225	40	193
	T. M.	52	27	98	107	256	40	214
	A. M.	59	26	96	106	235	35	196
	R. L.	53	29	112	115	280	57	152
	T. S.	53	27	103	112	295	50	157
	R. L.	46	30	120	126	370	55	246
	L.I.	55	28	108	118	288	39	178
	C.S.	57	29	113	128	260	41	207
	B. C.	55	27	106	116	290	46	221
	S. I.	52	26	98	108	237	38	172
	U. M.	54	28	110	110	278	40	224
	V.M. A.	56	28	107	108	245	38	181
	D. M.	52	29	115	120	295	45	217
	N. R.	54	26	95	107	240	42	192
	N. C.	56	27	100	112	289	38	162

Table 1 - Characteristics of patients at T0 (basal time)

4.4 STATISTICAL ANALYSIS

Mean values and standard deviations of the dependent measurements across the four time intervals in which the data collection took place are reported in Table 2.

Descriptive Statistics

	GROUP	Mean	Std. Deviation	N
BMI_Delta	1	-,4762	,67964	21
	2	-2,0952	1,22085	21
	Total	-1,2857	1,27424	42
Ca_Delta	1	-7,6667	3,85141	21
	2	-11,7619	4,89801	21
	Total	-9,7143	4,82011	42
Glic_Delta	1	-3,9048	5,43971	21
	2	-15,7619	5,47636	21
	Total	-9,8333	8,06654	42
HDL_Delta	1	2,9048	7,16174	21
	2	3,4762	7,08956	21
	Total	3,1905	7,04424	42
Trig_Delta	1	-28,1905	19,20838	21
	2	-63,2857	24,03153	21
	Total	-45,7381	27,87696	42
LDL_Delta	1	-3,8095	10,27433	21
	2	-38,1905	15,40655	21
	Total	-21,0000	21,67948	42

Table 2 - Analysis of Variance (ANOVA)

First, in order to rule out the possibility that the two groups had different baseline values for the target dependent variables, six univariate Analysis of Variance (ANOVA) were performed to compare the measured values (BMI, CA, BG, TG, HDL, and LDL) between the two groups. No significant differences were found between groups for any of the target dependent variables. Next, multivariate analysis of variance (MANOVA) was performed on the standard deviation measured at T1 in order to analyse whether significant differences emerged between those individuals assigned to G1 (i.e. those who initially received the treatment) and G2 (i.e. those who had not received the treatment yet). Results from the MANOVA analysis showed a significant multivariate effect on the DVs between the groups (Wilks $\lambda = 0.590$; $F = 4.047$; df = 6, 35; $p = 0.003$; $\eta^2 = 0.41$). The multivariate effect was also qualified by significant univariate differences for the levels of GB ($M_{Group1} = 104.095$; $M_{Group2} = 111.524$; $F = 7.703$; df = 1, 40; $p = 0.008$; $\eta^2 = 0.16$), TG ($M_{Group1} = 224.286$; $M_{Group2} = 257.238$; $F = 11.350$; df = 1, 40; $p = 0.002$; $\eta^2 = 0.22$), and LDL ($M_{Group1} = 177.238$; $M_{Group2} = 199.048$; $F = 4.742$; df = 1, 40; $p = 0.035$; $\eta^2 = 0.11$), while the other DVs did not display significant differences between G1 and G2. At T2 (12 months) after G1 received treatment, the differences between G1 and G2 increased, with the exception of BMI and CA, whose differences were still not significantly different. The MANOVA analysis yielded a significant main effect of Group on the dependent measures at T2 (Wilks $\lambda = 0.342$; $F = 11.232$; df = 6, 35; $p < 0.001$; $\eta^2 = 0.66$). The ANOVA for follow-up after significant MANOVA showed that at T2, individuals belonging to G1 (i.e. treated six months before) displayed lower levels of GB ($M_{Group1} = 97.429$; $M_{Group2} = 112.190$; $F = 42.961$; df = 1, 40; $p < 0.001$; $\eta^2 = 0.52$), TG ($M_{Group1} = 210.524$; $M_{Group2} = 252.143$; $F = 18.093$; df = 1, 40; $p < 0.001$; $\eta^2 = 0.31$), HDL

(M_{Group1} = 38.095; M_{Group2} = 41.143; F = 7.891; df = 1, 40; p = 0.008; η^2 = 0.17), and LDL (M_{Group1} = 174.000; M_{Group2} = 194.952; F = 4.589; df = 1, 40; p = 0.038; η^2 = 0.10) than those assigned to G2 (i.e. not already treated). At T3, the treatment procedure was reversed: individuals assigned to G2 were treated, while individuals in G1 were not treated anymore. MANOVA analysis was conducted to test if the differences between the same set of dependent variables at T3 between the two groups was significantly different (Wilks λ = 0.420; F = 8.049; df = 6, 35; $p < 0.001$; η^2 = 0.58). Notably, however, at T3 there was only one dependent variable exhibiting significant differences between groups. At T3, individuals in G2 displayed significantly lower BMIs than those in G1 (M_{Group1} = 27.67; M_{Group2} = 26.19; F = 18.340; df = 1. 40; $p < 0.000$, η^2 =0.31). The significant differences between the two groups emerged at T2 were switched off by the treatment administered to individuals assigned to G2. Finally, at T4 (i.e. when individuals in G2 were treated 6 months before, and individuals in G1 18 months before), multivariate differences between G1 and G2 emerged (Wilks λ = 0.452; F = 7.061; df =6, 35; $p < 0.001$, η^2 = 0.55), qualified by significant univariate differences among almost all the dependent variables. For instance, individuals in G2 at T4 exhibited lower scores for BMI (M_{Group1} = 27.67; M_{Group2} = 25.71; F = 29.133; df = 1, 40; $p < 0.000$; η^2 = 0.42), CA (M_{Group1} = 99.81; M_{Group2} = 95.38; F = 3.782; df = 1, 40; p = 0.05; η^2 = 0.09), GB (M_{Group1} = 105.48; M_{Group2} = 98.43; F = 9.550; df = 1, 40; p = 0.004; η^2 = 0.19), TG (M_{Group1} = 224.57; M_{Group2} = 205.76; F = 5.837; df = 1, 40; p = 0.02; η^2 = 0.13), and LDL (M_{Group1} = 189.24; M_{Group2} = 165.05; F = 5.307; df = 1, 40; p = 0.03; η^2 = 0.12), with no significant differences emerging for HDL (M_{Group1} = 43.52; M_{Group2} = 45.48; F = 2.614; df = 1, 40; p = 0.114; η^2 = 0.06).

At this stage, it was not apparent whether such differences could be ascribed to a decrease in the levels of the target dependent variables after G2 was treated, or to an increase in the levels of the same variables in G1 due to the time elapsed since treatment, or possibly to both. Accordingly, paired sample t-tests were utilized to compare the different values of each DV in G1 and G2 across time intervals. Results from the paired sample t-tests are discussed in the results.

4.5 RESULTS

We used ANOVA analysis to compare the two groups. At time T0, there were no significant differences between the G1 and G2. We thus concluded that the two groups were as not significantly different at the beginning of the experiment and represented a valid comparison. For all the considered variables, the differences between the variance within groups and the variance between groups, were considered to be significant if they had a P-value > 0.005 (Table 3).

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
BMI_t0 * Gruppo	Between Groups	(Combined)	1,167	1	1,167	,531	,470
	Within Groups		87,810	40	2,195		
	Total		88,976	41			
C.A (cm) * Gruppo	Between Groups	(Combined)	1,167	1	1,167	,012	,913
	Within Groups		3.891,810	40	97,295		
	Total		3.892,976	41			
GLIC BAS * Gruppo	Between Groups	(Combined)	242,881	1	242,881	2,974	,092
	Within Groups		3.266,190	40	81,655		
	Total		3.509,071	41			
TRIG * Gruppo	Between Groups	(Combined)	2.784,857	1	2.784,857	1,767	,191
	Within Groups		63.042,762	40	1.576,069		
	Total		65.827,619	41			
HDL * Gruppo	Between Groups	(Combined)	20,024	1	20,024	,373	,545
	Within Groups		2.146,952	40	53,674		
	Total		2.166,976	41			
LDL * Gruppo	Between Groups	(Combined)	1.090,381	1	1.090,381	1,013	,320
	Within Groups		43.062,762	40	1.076,569		
	Total		44.153,143	41			

Table 3 - Results for ANOVA analyses at T0.

At T1 after six months of treatment for G1, significant differences between the groups were observed, including differences in the levels of: BG (p = 0.008), TG (p = 0.002), and LDL (p = 0.035). For these variables, G1 showed significant variation (p >

0.005). However, no significant differences were observed for BMI or AC between the two groups (Table 4).

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
BMI_t1 * Gruppo	Between Groups	(Combined)	,095	1	,095	,020	,889
	Within Groups		194,190	40	4,855		
	Total		194,286	41			
Ca_t1 * Gruppo	Between Groups	(Combined)	16,095	1	16,095	,199	,658
	Within Groups		3.232,476	40	80,812		
	Total		3.248,571	41			
GB_t1 * Gruppo	Between Groups	(Combined)	579,429	1	579,429	7,733	,008
	Within Groups		2.997,048	40	74,926		
	Total		3.576,476	41			
TG_t1 * Gruppo	Between Groups	(Combined)	11.401,524	1	11.401,524	11,350	,002
	Within Groups		40.182,095	40	1.004,552		
	Total		51.583,619	41			
HDL_t1 * Gruppo	Between Groups	(Combined)	30,857	1	30,857	1,669	,204
	Within Groups		739,714	40	18,493		
	Total		770,571	41			
LDL_t1 * Gruppo	Between Groups	(Combined)	4.994,381	1	4.994,381	4,742	,035
	Within Groups		42.130,762	40	1.053,269		
	Total		47.125,143	41			

Table 4 - Results for ANOVA analyses at T1

At T2, the G1 group stopped MI treatment. At this time point, as shown in Table 3, BG, TG, LDL, and HDL variables all had significant differences between G1 and G2. Furthermore, at time T2, HDL was higher in G2 than in G1 (Table 5).

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
BMI_t2 * Gruppo	Between Groups	(Combined)	,595	1	,595	,375	,544
	Within Groups		63,524	40	1,588		
	Total		64,119	41			
Ca_t2 * Gruppo	Between Groups	(Combined)	82,881	1	82,881	1,182	,284
	Within Groups		2.805,905	40	70,148		
	Total		2.888,786	41			
GB_t2 * Gruppo	Between Groups	(Combined)	2.288,095	1	2.288,095	42,961	,000
	Within Groups		2.130,381	40	53,260		
	Total		4.418,476	41			
TG_t2 * Gruppo	Between Groups	(Combined)	18.187,524	1	18.187,524	18,093	,000
	Within Groups		40.209,810	40	1.005,245		
	Total		58.397,333	41			
HDL_t2 * Gruppo	Between Groups	(Combined)	97,524	1	97,524	7,891	,008
	Within Groups		494,381	40	12,360		
	Total		591,905	41			
LDL_t2 * Gruppo	Between Groups	(Combined)	4.609,524	1	4.609,524	4,589	,038
	Within Groups		40.174,952	40	1.004,374		
	Total		44.784,476	41			

Table 5: Results for ANOVA analyses at T3

At T2, G2 started MI treatment. At T3, no significant differences between the two groups were observed, except in BMI (Table 6).

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
BMI_t3 * Gruppo	Between Groups	(Combined)	22,881	1	22,881	18,340	,000
	Within Groups		49,905	40	1,248		
	Total		72,786	41			
Ca_t3 * Gruppo	Between Groups	(Combined)	13,714	1	13,714	,232	,633
	Within Groups		2.366,762	40	59,169		
	Total		2.380,476	41			
GB_t3 * Gruppo	Between Groups	(Combined)	4,667	1	4,667	,016	,901
	Within Groups		11.984,667	40	299,617		
	Total		11.989,333	41			
TG_t3 * Gruppo	Between Groups	(Combined)	1.314,881	1	1.314,881	1,839	,183
	Within Groups		28.601,619	40	715,040		
	Total		29.916,500	41			
HDL_t3 * Gruppo	Between Groups	(Combined)	6,881	1	6,881	,564	,457
	Within Groups		488,095	40	12,202		
	Total		494,976	41			
LDL_t3 * Gruppo	Between Groups	(Combined)	1,929	1	1,929	,002	,967
	Within Groups		45.362,857	40	1.134,071		
	Total		45.364,786	41			

Table 1 - Results for ANOVA analyses at T3

At T4, the two groups showed significant differences for all variables except for HDL. G2 had an average lower BMI than G1 (TABLE 7).

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
BMI_t4 * Gruppo	Between Groups	(Combined)	40,024	1	40,024	29,133	,000
	Within Groups		54,952	40	1,374		
	Total		94,976	41			
Ca_t4 * Gruppo	Between Groups	(Combined)	205,929	1	205,929	3,782	,059
	Within Groups		2.178,190	40	54,455		
	Total		2.384,119	41			
GB_t4 * Gruppo	Between Groups	(Combined)	521,524	1	521,524	9,550	,004
	Within Groups		2.184,381	40	54,610		
	Total		2.705,905	41			
TG_t4 * Gruppo	Between Groups	(Combined)	3.714,881	1	3.714,881	5,837	,020
	Within Groups		25.458,952	40	636,474		
	Total		29.173,833	41			
HDL_t4 * Gruppo	Between Groups	(Combined)	40,024	1	40,024	2,614	,114
	Within Groups		612,476	40	15,312		
	Total		652,500	41			
LDL_t4 * Gruppo	Between Groups	(Combined)	6.144,381	1	6.144,381	5,307	,027
	Within Groups		46.312,762	40	1.157,819		
	Total		52.457,143	41			

Table 7 - Results for ANOVA analyses at T4

Age seems not to have a significant effect on any variables (TABLE 8).

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
Intercept	Pillai's Trace	,271	2,101[b]	6,000	34,000	,079	,271
	Wilks' Lambda	,729	2,101[b]	6,000	34,000	,079	,271
	Hotelling's Trace	,371	2,101[b]	6,000	34,000	,079	,271
	Roy's Largest Root	,371	2,101[b]	6,000	34,000	,079	,271
ETA	Pillai's Trace	,136	,893[b]	6,000	34,000	,511	,136
	Wilks' Lambda	,864	,893[b]	6,000	34,000	,511	,136
	Hotelling's Trace	,158	,893[b]	6,000	34,000	,511	,136
	Roy's Largest Root	,158	,893[b]	6,000	34,000	,511	,136
Gruppo	Pillai's Trace	,830	27,625[b]	6,000	34,000	,000	,830
	Wilks' Lambda	,170	27,625[b]	6,000	34,000	,000	,830
	Hotelling's Trace	4,875	27,625[b]	6,000	34,000	,000	,830
	Roy's Largest Root	4,875	27,625[b]	6,000	34,000	,000	,830

a. Design: Intercept + ETA + Gruppo

b. Exact statistic

Table 8 - Results of multivariate tests for age

Treatment with MI significantly affected the results for all considered variables (Table 9).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	BMI_Delta	29,626[a]	2	14,813	15,636	,000	,445
	Ca_Delta	211,507[b]	2	105,754	5,565	,007	,222
	Glic_Delta	1588,271[c]	2	794,136	28,689	,000	,595
	HDL_Delta	13,804[d]	2	6,902	,133	,876	,007
	Trig_Delta	12962,937[e]	2	6481,469	13,375	,000	,407
	LDL_Delta	12568,855[f]	2	6284,428	36,575	,000	,652
Intercept	BMI_Delta	4,561	1	4,561	4,814	,034	,110
	Ca_Delta	124,326	1	124,326	6,543	,015	,144
	Glic_Delta	250,686	1	250,686	9,056	,005	,188
	HDL_Delta	2,237	1	2,237	,043	,836	,001
	Trig_Delta	903,975	1	903,975	1,865	,180	,046
	LDL_Delta	565,682	1	565,682	3,292	,077	,078
ETA	BMI_Delta	2,102	1	2,102	2,219	,144	,054
	Ca_Delta	35,412	1	35,412	1,864	,180	,046
	Glic_Delta	112,057	1	112,057	4,048	,051	,094
	HDL_Delta	10,375	1	10,375	,200	,657	,005
	Trig_Delta	30,342	1	30,342	,063	,804	,002
	LDL_Delta	157,331	1	157,331	,916	,345	,023
Gruppo	BMI_Delta	22,355	1	22,355	23,598	,000	,377
	Ca_Delta	129,898	1	129,898	6,836	,013	,149
	Glic_Delta	1199,538	1	1199,538	43,334	,000	,526
	HDL_Delta	6,695	1	6,695	,129	,721	,003
	Trig_Delta	11852,190	1	11852,190	24,458	,000	,385
	LDL_Delta	11002,544	1	11002,544	64,034	,000	,621
Error	BMI_Delta	36,946	39	,947			
	Ca_Delta	741,064	39	19,002			
	Glic_Delta	1079,562	39	27,681			
	HDL_Delta	2020,672	39	51,812			
	Trig_Delta	18899,182	39	484,594			
	LDL_Delta	6701,145	39	171,824			
Total	BMI_Delta	136,000	42				
	Ca_Delta	4916,000	42				
	Glic_Delta	6729,000	42				
	HDL_Delta	2462,000	42				
	Trig_Delta	119725,000	42				
	LDL_Delta	37792,000	42				
Corrected Total	BMI_Delta	66,571	41				
	Ca_Delta	952,571	41				
	Glic_Delta	2667,833	41				
	HDL_Delta	2034,476	41				
	Trig_Delta	31862,119	41				
	LDL_Delta	19270,000	41				

a. R Squared = ,445 (Adjusted R Squared = ,417)
b. R Squared = ,222 (Adjusted R Squared = ,182)
c. R Squared = ,595 (Adjusted R Squared = ,575)
d. R Squared = ,007 (Adjusted R Squared = -,044)
e. R Squared = ,407 (Adjusted R Squared = ,376)
f. R Squared = ,652 (Adjusted R Squared = ,634)

Table 2 – Test of between-subjects effects. Treatment with MI significantly affected the results for all considered variables.

We also utilized t-tests to compare all the variables among the two groups. In both groups, the overall BMI did not change significantly from T0 to T4. At the end of the treatment in G1 (i.e. T2), the BMI remained unchanged when compared to the values in G2. In particular, G1 was unresponsive to treatment with MI, and the mean BMI even increased at T3. At T3-T4, BMI remained constant. Conversely, in G2, the mean BMI continued to decrease at all time points, independent of inositol treatment (Fig.16).

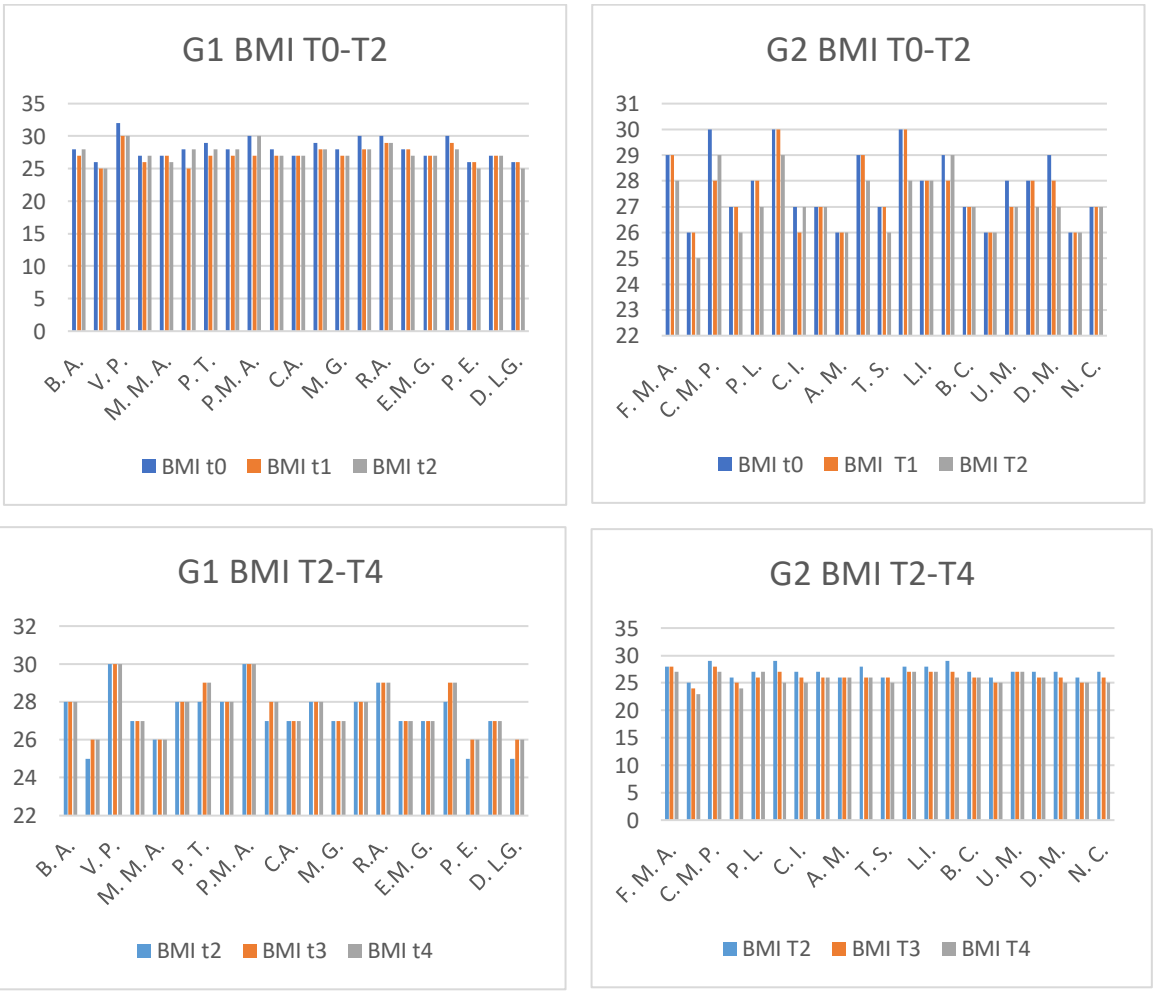


Fig. 16 – BMI differences between the two groups from T0 to T4

The mean AC levels had significant variation within the groups over time. In particular, in G1, AC levels decreased significantly during the MI treatment; a trend which stopped at T3. In G2, AC levels decreased continually from T1 to T4 (Fig. 17).

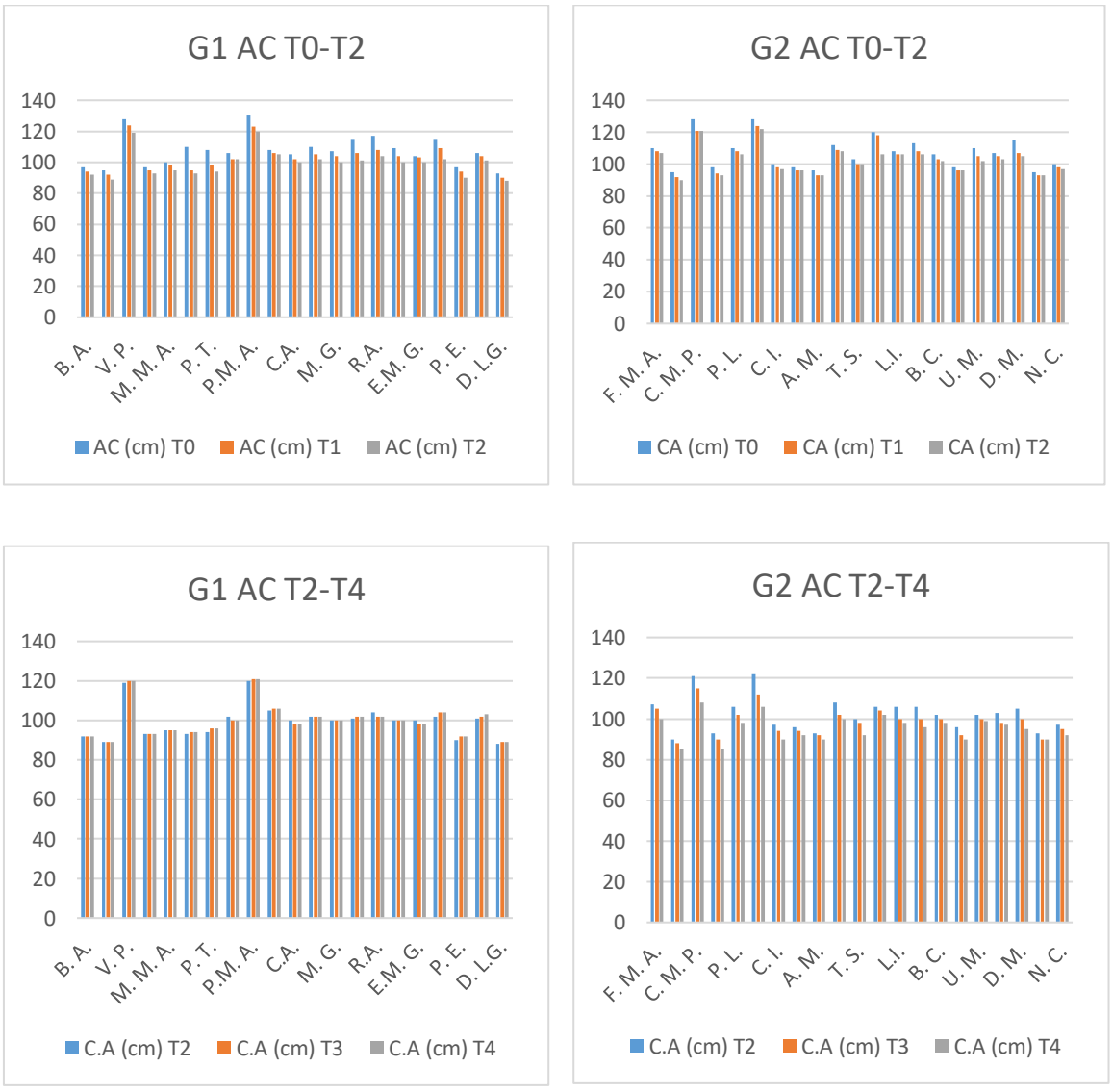


Fig. 17 – AC differences between the two groups from T0 to T4

In G1, mean BG levels decreases significantly at T1 and T2. In G2, BG levels started to decrease at T1, then significantly decreased at T3. At T4, BG levels remained constant for both groups (Fig.18).

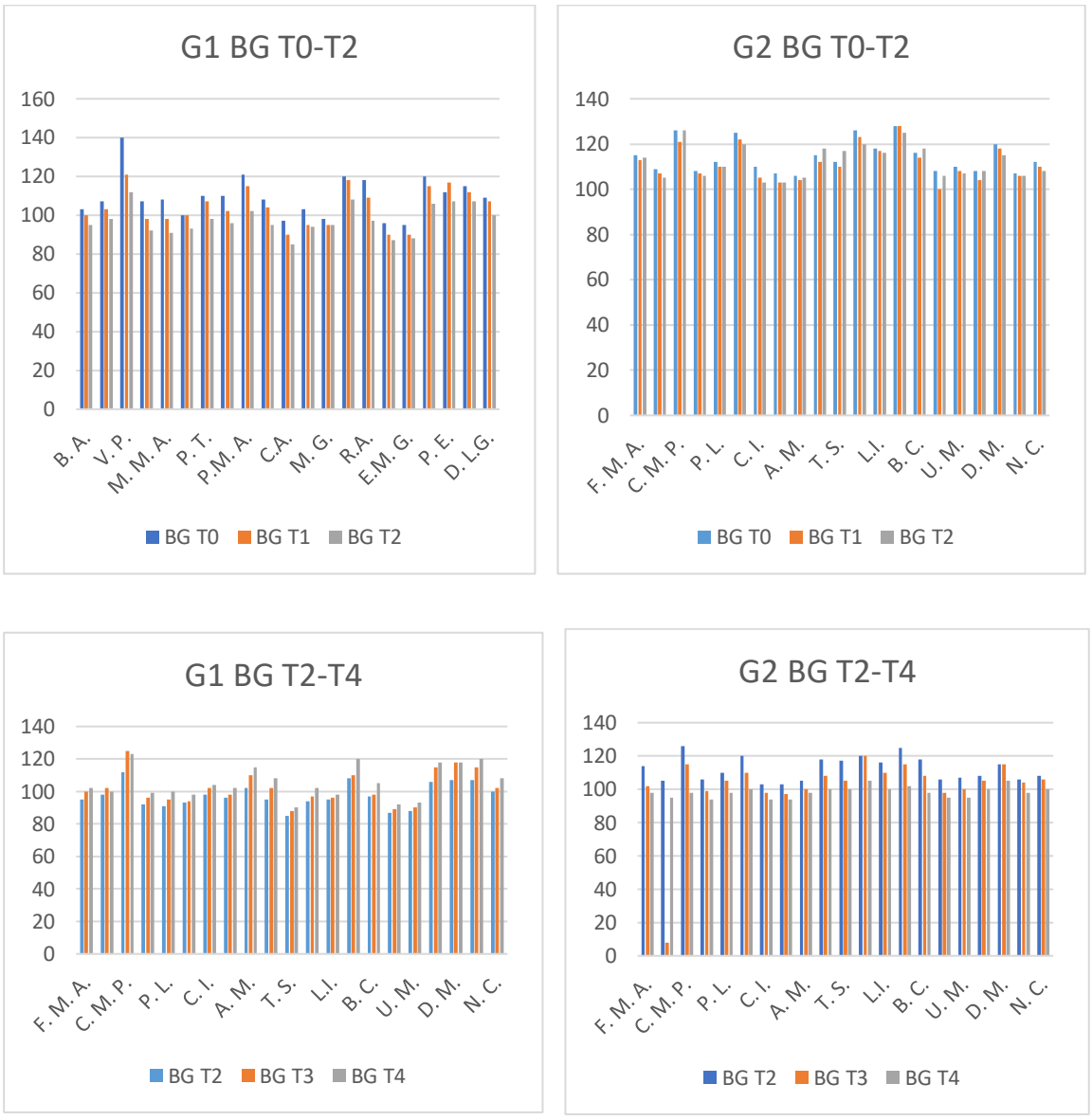


Fig. 18 – BG differences between the two groups from T0 to T4

In G1, TG levels decreased at T1-T,2 and increased again when the patients stopped MI treatment (T3-T4). In G2, TG levels started to decrease gradually from T1-T2, even though the MI treatment had not yet started. In T3-T4, during MI treatment, this value decreased considerably (Fig. 19).

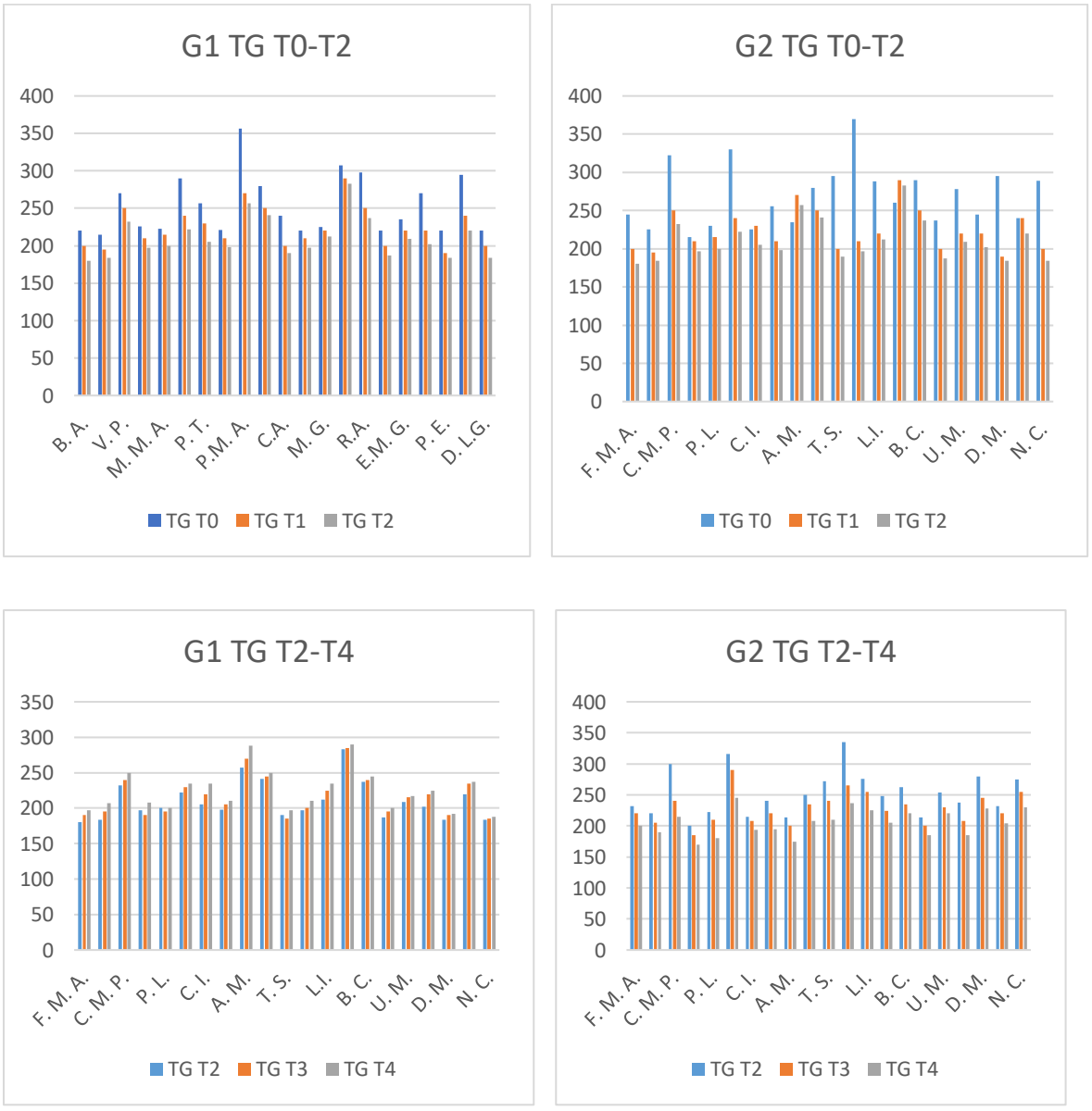


Fig. 19 – TG differences between the two groups from T0 to T4

When analysing HDL levels, MI administration did not cause appreciable variations before T1. Despite MI treatment, HDL levels continued to drop between T1 and T2. HDL levels remained constant between T2 and T4. In G2, HDL levels started to decrease at T1, even though this group had not been treated with MI in T1-T2. In G2, HDL levels showed significant variation at the end of the treatment (T4) (Fig. 20).

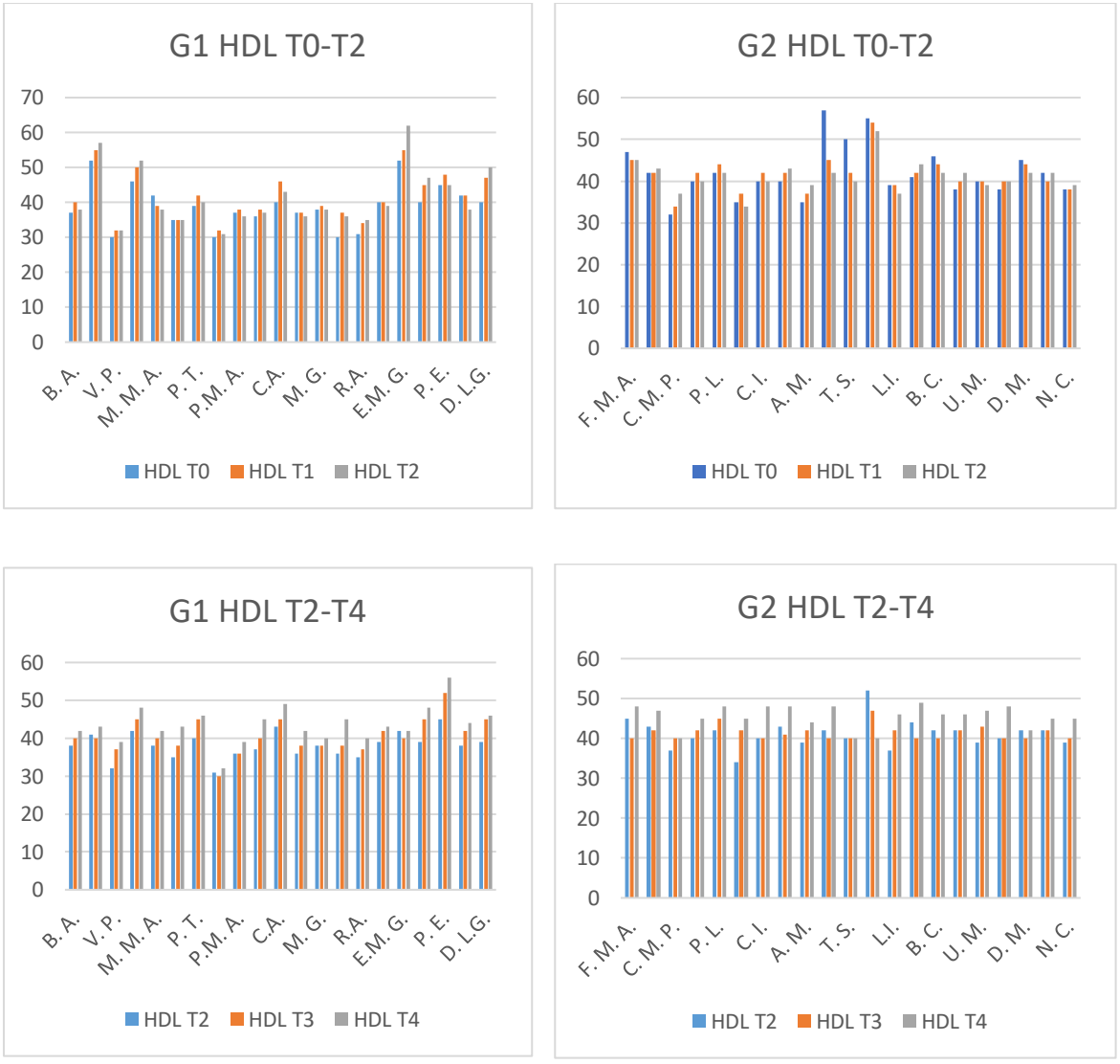


Fig. 20 – HDL differences between the two groups from T0 to T4

In G1, LDL levels decreases between T0 and T2. When the MI treatment stopped (T3-T4), LDL values increased. In G2, LDL levels decreased between T0 to T4, both before and during MI treatment.

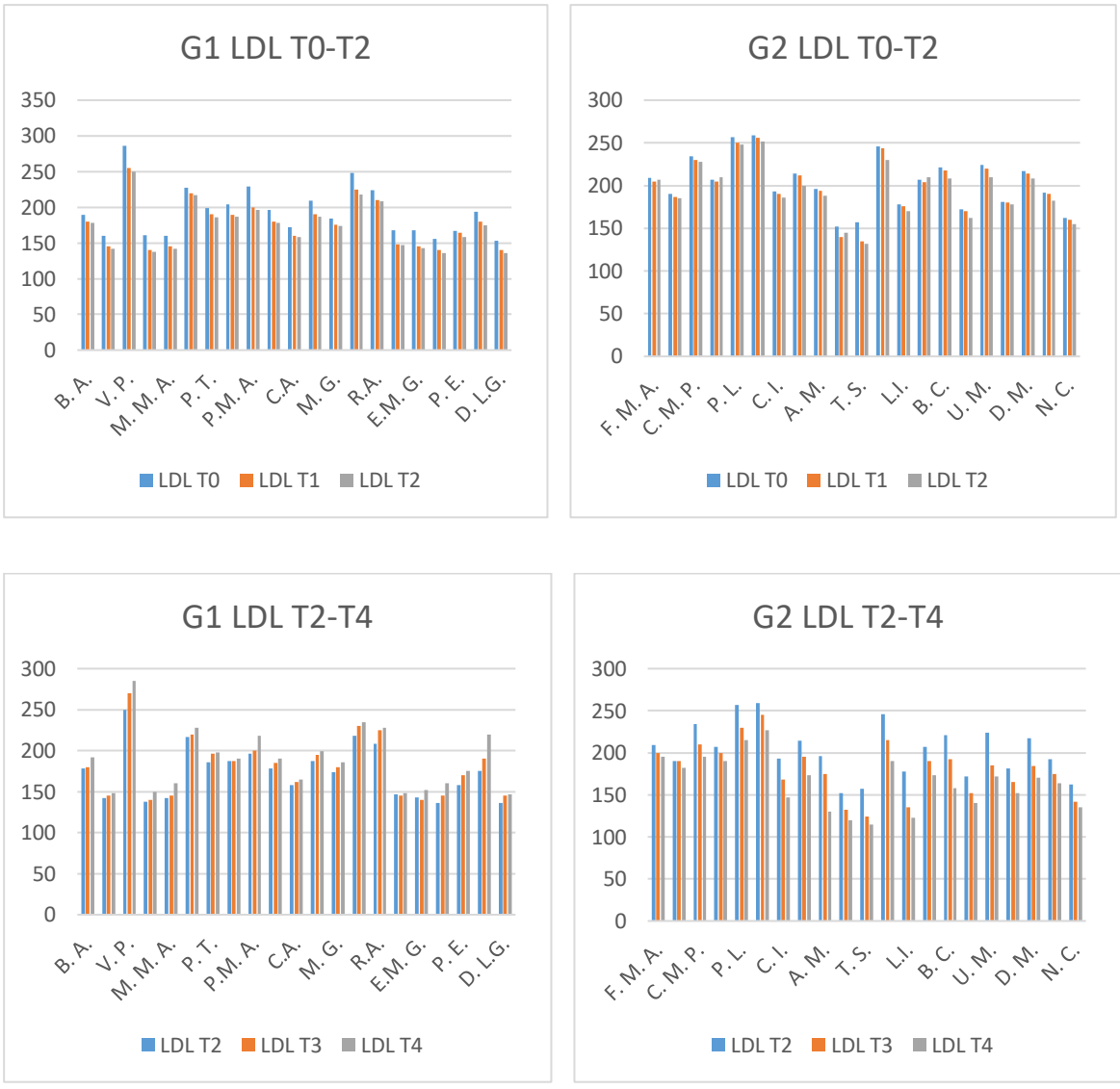


Fig. 20 – HDL differences between the two groups from T0 to T4

4.6 DISCUSSION

The outcomes from our study showed significant effects of MI treatment on BG, TG, and LDL levels in the first months of therapy. Combination of MI with isoflavones and had positive results in all parameters evaluated. Only BMI did not change significantly from T0 to T4 in both groups. We also noted a persistence trend in our results: during T3-T4, G1 treatment was stopped, but the evaluated parameters maintained a significant difference from basal levels with only diet and exercise. G2 during T0-T2 did not start MI treatment and did show significant variations in measured parameters with only diet and exercise. These results are in accordance with previous studies. In particular, the first study of inositol treatment alone (MI 2 g two times a day) in 80 menopausal women with metabolic syndrome reported highly significant differences after treatment for all parameters analysed, with the exception of BMI and waist circumference. The authors noted that this may be due to the short treatment period and/or the low overall adherence to diet. Additionally, in the placebo group, after six months a reduction in HOMA, total cholesterol, BMI, and waist circumference was noted in both groups, though with more favourable values observed in the MI treatment group (31). A follow-up study by the same group confirmed the findings of their previous trial. They followed the 80 women with metabolic syndrome for 12 months. In the group treated with diet plus MI, almost all of the measured metabolic markers improved, reducing the risk of cardiovascular disease for these women. Additionally, 20% of the women no longer had metabolic syndrome. A significant decrease in TG levels was also noted after the first six months (32). The most important result from their studies was the critical reduction in serum insulin levels and, consequently, insulin resistance. This reduction was two

times greater compared to previous studies using other insulin-sensitizing drugs such as pioglitazone, rosiglitazone, and metformin, which are considered the gold standard therapies for patients with insulin resistance [110-112]. In 2016, D'anna et al published a randomized trial testing inositol in 40 postmenopausal women. They analysed the effectiveness of MI plus melatonin (20 patients) compared to MI treatment alone (20 patients) [113]. When used in combination with melatonin, MI was effective at half the normal dose (2g) compared to the efficacy of MI alone (4g). The correlation between insulin levels and abdominal circumference or BMI was significant in both groups, but the MI plus melatonin group showed the greatest difference. In this study, the authors also evaluated endometrial thickness, and found that it decreased in both groups, as well as serum insulin levels. These findings suggest that, for some parameters, these changes may have been due to MI alone, while for other parameters, metformin can modulate the effects of MI. Another Italian trial [114] studied the effectiveness of inositol treatment with alpha lipoic acid as a dietary supplement in insulin-resistant patients to increase insulin sensitivity. Postmenopausal women affected by MetS show the highest incidence of breast cancer in the female population. The correlation between MetS and breast cancer incidence has been widely highlighted in the literature worldwide, and MetS is now considered a modifiable risk factor for breast cancer [115]. In a recent Italian study [114] after only six months of treatment with inositol and alpha lipoic acid, combined with a low-calorie diet, postmenopausal women at risk for breast cancer showed significant reduction in HOMA-IR score (>20%) and lipid profile control when compared to the placebo group. Serum insulin levels decreased in 89.3% of patients treated with the MI supplement. According to the lipid profile, reduction in TG levels (43.2%) was

evident in the treatment group ($p < 0.0001$). Significant increases in HDL-C levels (48.6%) were found in the group treated with inositol plus alpha lipoic acid when compared to the placebo group. Therefore, it is clear that MetS control can be considered a first step towards primary breast cancer prevention. A recent trial by D'anna et al [116] demonstrated a protective effect on cardiovascular markers with six months administration of a combination of MI, soy isoflavones, and cocoa polyphenols in postmenopausal women with metabolic syndrome. The authors also evaluated as a secondary outcome the improvement of adiponectin, resistin, Visfatin (a peptide secreted by adipose tissue), and bone-alkaline phosphatase (Bone-ALP, a marker of bone turnover) levels. Adiponectin is an adipocyte-specific secreted protein that sensitizes the liver and muscle to the action of insulin [117]. It is the only adipocyte-derived hormone to be downregulated in the insulin-resistant state, so the levels of adiponectin strongly correlate with basal insulin levels and insulin sensitivity. In this trial, adiponectin levels were significantly increased after 12 months in the treated group compared to the control. This result is in accordance with a recent study by same group where the isoflavone genistein aglycone was used [102]. These studies suggest that low concentrations of adiponectin are associated with increased prevalence of metabolic syndrome, especially in postmenopausal women [109]. The insulin-sensitizing effect of cocoa polyphenols, however, is not yet clear. In conclusion, inositol should be considered for treatment of metabolic syndrome, with the positive effects of reducing the risk cardiovascular disease and cancer. Our studies validated previous studies linking inositol therapy with alpha lipoic acid, melatonin, isoflavones, and cocoa polyphenols. Our results showed that this combination was very effective in treating metabolic syndrome. Our results support

confirming these data in larger international and multi-centric studies. Our study was limited by the few Italian studies available with limited data, and a short follow-up time. The effectiveness of inositol in treating PCOS patients affected by insulin resistance is already established. However, there is little data on how postmenopausal patients are affected by metabolic syndrome, and consequently by insulin resistance. The number of postmenopausal patients in our study is still too low, and the type of supplement used in previous studies is too variable to draw overall conclusions. It is important to note that the type of inositol we analysed in treating postmenopausal patients is MI. Considering the effectiveness of DCI on lowering the glycaemic and lipemic profiles in pre-diabetic and diabetic patients, it would also be useful to perform a randomized control study comparing MI and DCI treatment of postmenopausal patients with metabolic syndrome.

5. THE ROLE OF NOVEL BIOMARKER HE4 IN ENDOMETRIAL CANCER: A CASE CONTROL PROSPECTIVE

5.1 INTRODUCTION

In developed countries, endometrial cancer represents the most common gynecologic cancer [118]. In the USA, approximately 42.160 cases are diagnosed annually; 7.780 deaths occur and over 4,000 new cases are diagnosed in Italy yearly [119]. The diagnosis is usually performed in an early stage and this results in better prognosis, with a 5-year overall survival rate of 80–85 % and a cancer-specific survival rate of 90– 95 % [120,121]. Abnormal bleeding from the uterus after menopause is the most common and early developed symptom of endometrial cancer. However, almost 15 % of endometrial cancers occur in women without vaginal bleeding [122]. The role of tumor markers in endometrial cancer is still debated. However, some serum tumor markers have been studied during recent years. CA15-3 and CA125 have been found to be elevated in only 36% [123] and 24.6% [124] of endometrial cancer patients, respectively. Elevated CA125 levels have been demonstrated to correlate with advanced disease [125]. Thus, the challenge to find a preoperative tool for endometrial cancer diagnosis and staging is still open. Human epididymis protein 4 (HE4) is a novel tumor marker that circulates in the bloodstream and is overexpressed in patients with serous and endometrioid epithelial ovarian carcinomas and recurrent ovarian cancer [126,127]. However, only few studies on HE4 in endometrial cancer exist in literature up to now. Moore has been the first to investigate the role of HE4 in endometrial cancer, demonstrating an improvement in sensitivity compared with that of CA 125, even if just in endometrioid endometrial cancer [125,128].

The aims of this prospective comparative study are:

– To evaluate HE4 sensitivity and specificity in all histotypes of consecutive endometrial cancer patients;

– To correlate the preoperative HE4 levels with different prognostic factors: stage of disease, myometrial and cervical invasion, lymph node status, and histotypes.

5.2 PATIENTS AND METHODS

Starting January 2010 to April 2012, all patients with endometrial cancer at prior endometrial biopsy, referred to the Division of Gynaecologic Oncology of the University Campus Bio-Medico of Rome, were prospectively included in the study. The institutional internal review board approved the study. Inclusion criteria for enrollment were as follows: (1) aged between 18 and 80 years; (2) Eastern Cooperative Oncology Group performance status 0–2 according to World Health Organization criteria; (3) informed consent obtained from the patients. Exclusion criteria included: (1) abnormal cardiac, hematological, renal, respiratory, and/or hepatic functions; (2) presence of a secondary malignancy; (3) concomitant benign and/or malignant adnexal pathologies. All patients had radiologic imaging by pelvic ultrasound. Concerning operative features, all patients diagnosed with endometrial cancer at prior endometrial biopsy underwent surgery consisting in hysterectomy and bilateral salpingo-oophorectomy with complete surgical staging. Staging included also pelvic washing, bilateral pelvic, and/ or para-aortic lymph node dissection. Only patients with complete surgical staging and a pathologically confirmed endometrial cancer were considered in the study. The control group, consisted of age-matched patients with benign uterine disease, received vaginal, laparoscopic, or laparotomic

hysterectomy. The day before surgery, blood samples were obtained. All sera were acquired following a standard collection protocol. Briefly, samples were collected in a red top vacutainer, clotted 60-90 min and centrifuged for 10 min at 1,300×g. Serum fractions were aliquoted and stored at -80 °C until analysis. HE4 levels were determined using the HE4 EIA assay (Fujirebio Diagnostics). The HE4 EIA is a solid phase, noncompetitive immunoassay based upon the direct "sandwich" technique using two monoclonal antibodies, 2 H5 and 3D8, directed against two epitopes in the CWFDC domain of HE4. During the enzyme reaction, a blue color developed if the antigen was present. The intensity of the color was directly proportional to the amount of HE4 present in the samples. CA125 levels were evaluated by a one-step "sandwich" radioimmunoassay. Polystyrene beads coated with M11 capture antibody reacting with molecules containing OC 125-reactive determinants were incubated with control or patients' serum samples, standards, and tracer (125I-labeled mouse monoclonal OC 125 antibody) aliquots. The bound radioactivity observed was proportional to the concentration of the OC 125 reactive determinant (antigen). Normal levels of CA125 were considered to be less than 35 U/mL. Several studies are trying to determine for HE4 the cutoff point that provides the best accuracy, in terms of minimal false-negative and false-positive results. For this study, we consider two cutoff: normal values less than 150 pmol/L, according to the manufacturer's indications, and also less than 70 pmol/L, as suggested by Moore et al. [125]. Using the statistical software MedCalc Software Version No. 11.6.1.0, we firstly analyzed the chance to describe the collected values as normal distributions. The series of CA125 and HE4 values detected in the carcinoma patients did not satisfied the Kolmogorov–Smirnov test for normal distribution. So, we performed the analysis

using the nonparametric test (Mann–Whitney for independent samples) for comparing the CA125 and HE4 series. The level of statistical significance was set at $p<0.05$. In terms of diagnostic accuracy of the assays, the performance was assessed on the estimation of receiver operating characteristic (ROC) curve for endometrial cancer cases versus uterine benign cases. The area under the ROC curve (AUC) was calculated by MedCalc Software Version No. 11.6.1.0.

5.3 RESULTS

Starting January 2010 to April 2012, serum samples were obtained from 101 patients with surgically staged endometrial cancer (study group) and from 103 patients with benign uterine disease (control group). Patients of both groups matched for age (64.9 versus 63 years, not statistically significance), performance status (1 versus 1, not statistically significance), and BMI (23.7 versus 24.2, not statistically significance). Concerning preoperative blood samples, mean preoperative CA125 plasma concentration for cancer patients is 39.26±57 U/mL (range 1.8–300.5) and mean preoperative HE4 plasma concentration for cancer patients is 128.07± 120 pmol/L (range 12.52–734.12). In this group, CA125 levels above the cutoff are detected in 20/101 (19.8 %) patients, HE4 levels above the cutoff of 70 pmol/L are detected in 60/101 (59.4 %) patients, while HE4 levels above the cutoff of 150 pmol/L are detected in 36/101 (35.6 %) cancer patients. Mean preoperative CA125 plasma concentration in control group is 36.4±46.04 U/mL (range 3.2–381.7) and mean preoperative HE4 plasma concentration for control group is 40.16±13.99 pmol/L (range 11–59.28). In control group, CA125 levels above the cutoff are detected in

37/103 (35 %) patients; HE4 levels above the cutoff of 70 and of 150 pmol/L are never detected. The sensitivity of CA125 in detecting cancer patients is 19.8 % whereas the sensitivity of HE4 is 59.4 and 35.6 % for 70 and 150 pmol/L cutoff, respectively. In both cases the specificity of HE4 is absolute (100 %), whereas the CA125 has a lower specificity of 62.14 % with a positive predictive value of 33.9 %. These data are summarized in Table 10.

	Number (n101)	**Mean**	**Sensitivity %**	**Specificity %**	**PPV * (%)**	**NPV ** (%)**
Ca125>35 U/mL	20	39.27	19.8	62.1	33.9	44.1
HE4>70 pmol/L	60	128.07	59.4	100	100	71.5
HE4>150 pmol/L	36		35.6	100	100	61.3

Table 3 Test accuracy in detecting malignant endometrial disease

** Positive predictive value*

*** Negative predictive value*

Comparing CA125 and HE4 levels in the two groups, we did not find a statistically significant difference ($p=0.3879$) of CA125 levels, but we found a statistically significant difference of HE4 levels ($p<0.0001$). Distributions of the CA125 and HE4 values in cancer and control groups have been drawn in two graphs (Fig. 21). In Fig.1a, the 35-U/mL threshold has been drawn to highlight that the majority of carcinoma patients lie below this level. In Fig. 1b, the HE4 cutoff of 70 and 150 pmol/L have been traced to underline that all control patients remain below the lowest cutoff chosen in this work. HE4 statistical specificity and sensitivity analysis studied in the two selected groups of patients showed that the area under the ROC curve was

0.864 (95 % CI 0.809–0.908) (Fig. 22a). CA125 statistical specificity and sensitivity analysis studied in the two selected groups of patients showed that the area under the ROC curve was 0.546 (95 % CI 0.475–0.616) (Fig. 22b). Statistically significant difference has been found (p< 0,01) comparing CA125 and HE4 ROC-AUC (Fig. 23). Combining CA125 and HE4, the sensitivity to detect endometrial cancer is 60.4 and 34.6 %, at HE4 cutoff of 70 and 1502 pmol/L, respectively, with a specificity of 100 %. Within the 101 patients with surgically staged endometrial cancer, 50 (49 %) were diagnosed with stage I disease, 12 (12 %) with stage II disease, 36 (36 %) with stage III disease, and 3 (3 %) with stage IV disease. Operative features of the two study groups are exposed in Table 11. Concerning analysis of HE4 values according to prognostic factors, we found a mean serum HE4 value of 85.8 pmol/L for Stage I, 147.8 pmol/L for Stage II, 140.4 pmol/L for Stage III, and 588.3 pmol/L for Stage IV. Analyzing stage I, patients with stage IA disease (<50 % myometrial invasion) have a significantly lower median serum HE4 value than patients with stage IB disease (>50 % myometrial invasion; 63.4 vs 108.7 pmol/L; p0 0.012). Moreover, we found a statistically significant difference comparing median HE4 value in patients with Stage I versus Stage II (p00.0011) and in patients with Stage III versus patients with Stage IV (p<0.001) (Table 12). Concerning the histology, endometrioid cancer patients have a median HE4 value of 130.7 pmol/L comparing with non-endometrioid cancer patients with a median HE4 value of 56.8 pmol/L (p<0.001).

	Endometrial Cancer Group (n=101)	Control Group (n=103)
MEDIAN AGE (RANGE)	64.9 (34–85)	63.8 (31–74)
FIGO stage		
I	50 (49 %)	-
II	12 (12 %)	-
III	36 (36 %)	-
IV	3 (3 %)	-
HISTOLOGY		
Endometrioid adenocarcinoma G1	3 (3 %)	-
Endometrioid adenocarcinoma G2	50 (49 %)	-
Endometrioid adenocarcinoma G3	42 (42 %)	-
Non-endometrioid carcinoma	6 (6 %)	-
Benign uterine disease	-	103(100 %)
TYPE OF SURGERY		
Pelvic lymphadenectomy	101 (100 %)	-
Para-aortic lymphadenectomy	56 (56 %)	-
Vaginal hysterectomy		48 (47 %)
Laparotomic hysterectomy	70 (70 %)	32 (31 %)
Laparoscopic hysterectomy	31 (30 %)	23 (22 %)
MYOMETRIAL INVASION		
≤1\2	49(48 %)	-
≥1\2	54(52 %)	-
CERVICAL INVOLVEMENT		
Negative	65 (64 %)	-
Positive	36 (36 %)	-
ADNEXAL METASTASIS		
Negative	95 (94 %)	-
Positive	6 (6 %)	-

Table 4 The clinical characteristics of the study group and control group patients

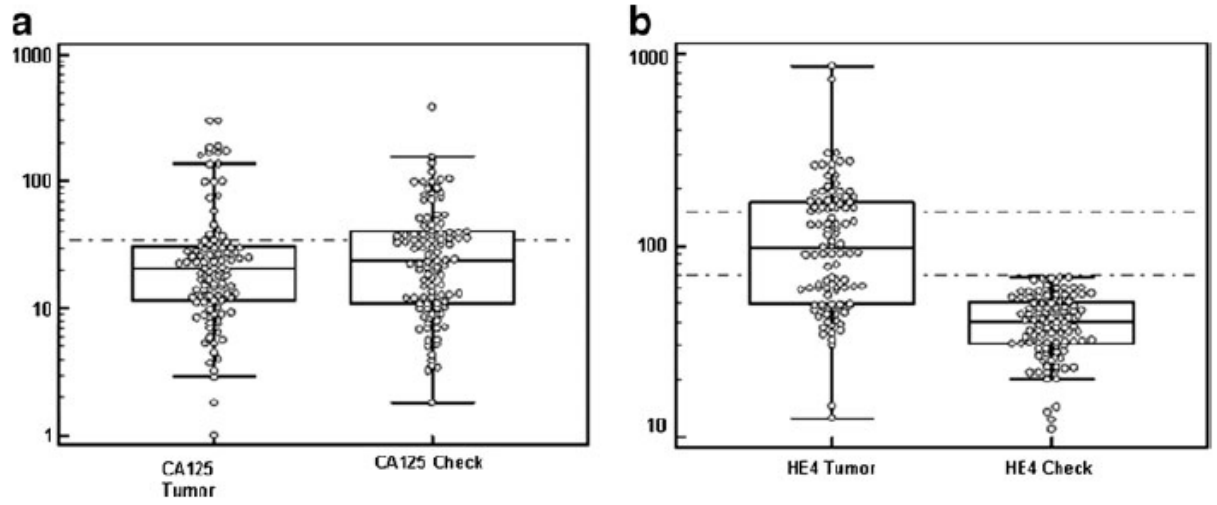


Fig. 21

a Comparison among control and carcinoma patients CA125 serum levels.

b Comparison among control and carcinoma patients HE4 serum levels

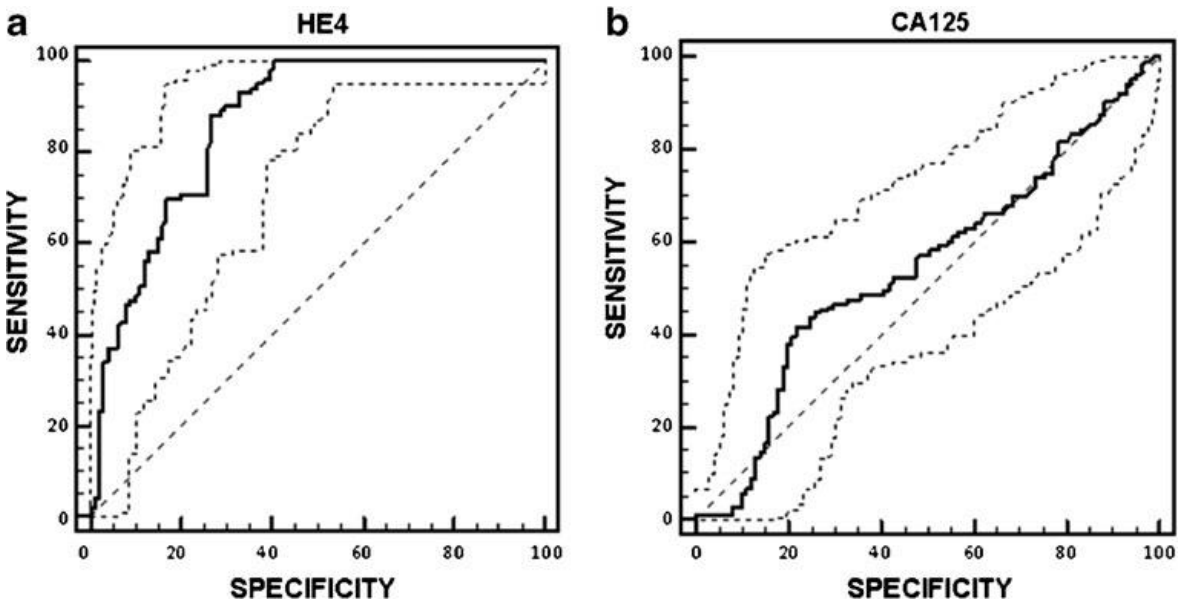


Fig. 22

a HE4 ROC curve was 0.864 (95 % CI 0.809–0.908).

b CA125 ROC curve was 0.546 (95 % CI 0.475–0.616)

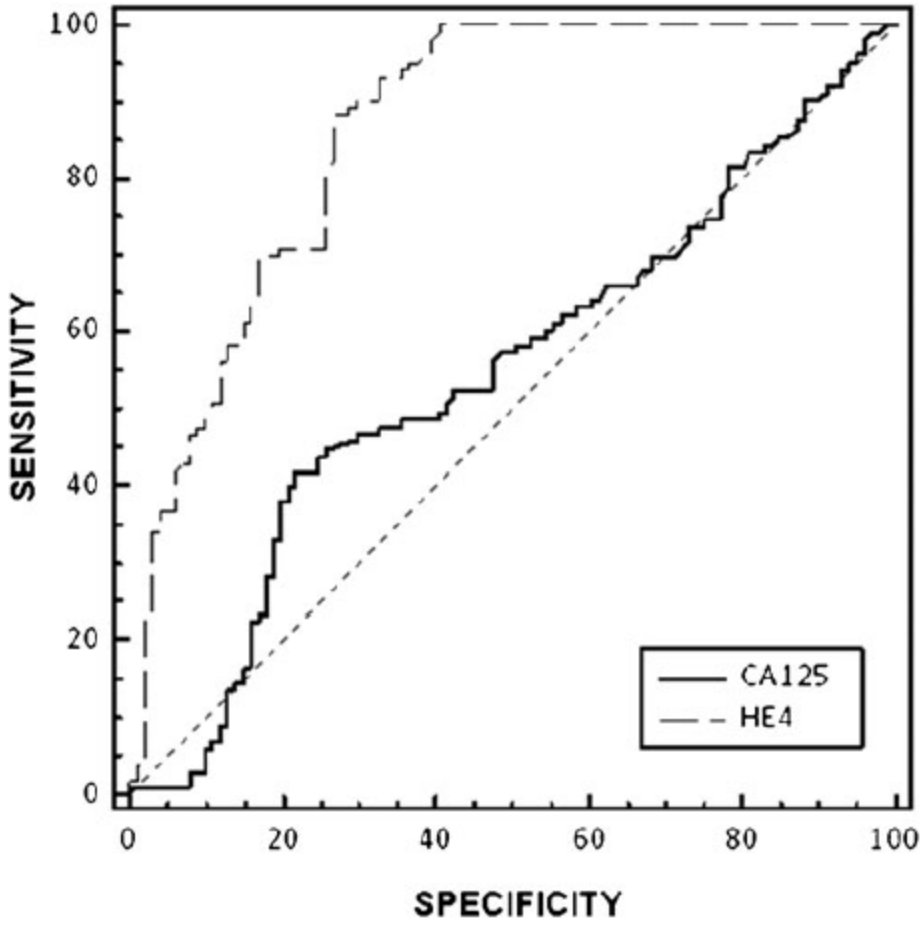


Fig. 23 Comparison of CA125 and HE4 ROC curves

5.4 DISCUSSION

Endometrial carcinoma is generally considered a malignancy with favorable prognosis because the majority of patients show their disease early by postmenopausal bleeding and therefore can be diagnosed at the first stage. However non-endometrioid cancer patients with a median HE4 value of 56.8 pmol/L ($p<0.001$). almost 15 % of endometrial cancers occur in women without vaginal bleeding [123]. The role of tumor markers in endometrial cancer is still debated, and the challenge to find a preoperative tool for endometrial cancer diagnosis and staging is still open. In literature, several studies have investigated the role of different serum markers in endometrial cancer such as CEA, CA72.4, CA19.9, CA15.3, and M-CSF, resulting elevated in only 20% to 30% of patients [125,130-135]. The most commonly used tumor marker in endometrial cancer is CA125. Presurgical CA 125 levels were shown to be related to the stage of the disease, myometrial invasion depth, peritoneal cytology, and lymph node metastasis [133,134,136-140]. However, another study reports that only 10% of patients with stage I and II disease have elevated CA125 levels [141]. In addition, Beck et al. demonstrated that only 15 % of stage I uterine cancer patients, 33% of stage II, and 62% of stage III patients have elevated CA125 levels [142]. Furthermore, serum CA 125 levels are often elevated in disease-free endometrial cancer patients who have undergone abdominal radiation [143]. In our study, CA125 sensitivity and specificity in detecting endometrial cancer is 19.8 and 62.1% respectively, considering all stages. Other serum markers for endometrial cancer, such as HE4, are now under investigation. HE4 is a novel tumor marker that circulates in the bloodstream and it is overexpressed in patients with serous and endometrioid epithelial ovarian carcinomas [127]. In literature, only two

studies investigated the HE4 role in endometrial cancer diagnosis. Moore found that HE4 provided 46% sensitivity for endometrioid adenocarcinoma of the endometrium in all stages at 95% specificity [124]. Bignotti et al. found that HE4 had a sensitivity of 67% at a specificity of 95% compared with CA125 alone, considering all endometrial cancer stages [125]. Moore and Bignotti considered only endometrioid endometrial cancer and used as control group healthy postmenopausal women. Our study is the first in literature that includes all endometrial cancer histotypes and a surgical control group. In the present study, HE4 sensitivity in detecting endometrial cancer is 59.4% (using 70 pmol/L as cutoff), with a specificity of 100 %. Concerning the HE4 role in endometrial cancer staging, there are only two studies up to now in literature. Moore et al. found that HE4 differentiated women with less than 50% myometrial invasion (stage I A) from those with more than 50 % myometrial invasion (stage I B) with 94% sensitivity and a negative predictive value (NPV) of 97%. These findings suggest that serum HE4 concentrations greater that 70 pmol/l can be helpful in identifying almost 95% of patients with stage I disease who ultimately need surgical staging while excluding more than 95% of patients who do not [144]. Kalogera et al. found that HE4 is elevated in a high proportion of EC patients and it is correlated with myometrial invasion (>5%, $p<0.001$) [145]. Our data confirm the preliminary results of Moore and Kalogera. In fact, in our cancer group, median serum HE4 value is 85.8 pmol/L for Stage I, 147.8 pmol/L for Stage II, 140.4 pmol/L for Stage III, and 588.3 pmol/L for Stage IV. In addition, patients with stage IA disease (<50% myometrial invasion) have a significantly lower median serum HE4 value than patients with stage IB disease (>50% myometrial invasion; 63.4 vs 108.7 pmol/L; $p<0.012$). Moreover, we found a statistically significant difference

comparing median HE4 value in patients with Stage I versus Stage II ($p<0.001$) and in patients with Stage III versus patients with Stage IV ($p<0.001$). We also found that the lymph node status correlates with the HE4 values. In fact, there is a statistical significant difference comparing stage I versus stage III ($p<0.001$). These findings suggest that HE4 could be useful as a preoperative indicator to identify patients suitable for pelvic and para-aortic lymphadenectomy. This is further corroborated by a recent study by Kamei et al. who showed that HE4 expression was closely associated with lymph node involvement in breast cancer patients [146]. Moreover, concerning the histology, endometrioid cancer patients have a median HE4 value of 130.7 pmol/L comparing with non-endometrioid cancer patients with a median HE4 value of 86.8 pmol/L ($p<0.001$). These results are in accordance with the literature evidence of HE4 overexpression in patients with endometrioid histotype in epithelial ovarian carcinomas [137]. In conclusion, our results confirm that HE4 is an accurate and sensitive serum marker for detection of endometrial cancer patients, exhibiting a better diagnostic performance compared to CA125. In particular, HE4 cutoff of 70 pmol/L yields the best sensitivity and specificity, with a positive predictive value of 100% and NPV equal to 71.52% for the 70 pmol/L cutoff. We also found that HE4 marker was never increased in patients with benign disease, differently from the CA125 results. Combining CA125 and HE4, the sensitivity to detect endometrial cancer is 60.4% at HE4 cutoff of 70 pmol/L with a specificity of 100%. Our data suggest that serum HE4 may offer preliminary risk stratification prior to definitive surgery. Large prospective clinical studies are certainly necessary to support these findings and to assess the potential of HE4 as a new tool for preoperative evaluation and postoperative surveillance of endometrial cancer patients.

	STAGE I	STAGE II	STAGE III	STAGE IV	p
MEAN HE4 (pmol/L)	85.8 IA IB 63.4 108.7	147.8	140.3	588.3	Stage IA vs IB p<0.05 Stage I vs II p<0.05 Stage II vs III p00.07 Stage III vs IV p<0.05

Table 5 Mean HE4 levels and stage correlation

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- ✓ "Use of MYOCET as a valid drug in patients affected by ovarian cancer, suffering from chemotherapy side effects" 17th INTERNATIONAL MEETING OF THE EUROPEAN SOCIETY OF GYNAECOLOGICAL ONCOLOGY Milan (from 11th to 14th of September 2011) (POSTER)

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