RBC PARAMETERS, VITAMIN B12, FOLATE, HOMOCYSTEINE LEVELS AND MTHFR GENE POLYMORPHISM IN POSTMENOPAUSAL MALAY WOMEN AND THEIR RELATION TO HORMONE REPLACEMENT THERAPY

BY

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DEDICATION

I would like to dedicate this dissertation to my wife, whose support has been unwavering and to my beloved parents. Their moral and financial support has been beyond all limits.

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Mohamed Talal Al Jabr

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a	Alanine
Adenosyl-Cbl	Adenosylcobalamin
ATP	Adenosine Triphosfate
BHMT	Betaine Homocysteine Methyltransferase
BMD	Bone Mineral Density
bp	Base pair
CAD	Coronary Artery Disease
CbI	Cobalamins
CBS	Cystathionine B-Synthase
CHD	Coronary Heart Disease
CN-	Cyanide anion
CO_2	Carbon dioxide
CVD	Cardio Vascular Disease
df	degree of freedom
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
E3	Estriol
EDTA	Ethylenediamine Tetra Acetic Acid
EPT	Estrogen Progesterone Treatment
ET	Estrogen Therapy
FA	Folic Acid
FBC	Full Blood Count
FH_4	Tetrahydrofolate
FMP	Final Menstrual Period
FSH	Follicle Stimulating Hormone
Hb	Haemoglobin
Hct	haematocrit

Hcy	Homocysteine
HRT	Hormone Replacement Therapy
HUSM	Hospital Universiti Sains Malaysia
ICA	Internal Carotid Artery
IF	Intrinsic Factor
LH	Luteinizing Hormone
LW	Low molecular Weight
MAT	Methionine Adenosyltransferase
MCH	Mean Cell Hemoglobin
MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
Methyl-Cbl	Methylcobalamin
Methyl-FH_4	Methyl-Tetrahydrofolates
MI	Myocardial Infarction
MS	Methionine Synthase
MTHF	Methyltetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
N	Number
NC	Normal Control
NTC	No Template Control
O_2	Oxygen
PCR	Polymerase Chain Reaction
PCV	Paced Cell Volume
PGA	Pteroylglutamic Acid
PLT	Platelets
PML	Postmethionine Loading
POF	Premature Ovarian Failure
POS	Positive Control
R	Pearson correlation
RBC	Red Blood Cell

RDA	Recommended Daily Allowance
SAH	S-Adenosylhomocysteine
SAHH	S-Adenosyl-L-Homocysteine-Hydrolase
SAM	S-adenosylmethionine
SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
TCI	Transcobalamin I
TCII	Transcobalamin II
V	Valine
WHI	World Health Initiative
WHO	World Health Organization
X^2	Pearson Chi-square

PARAMETER-PARAMETER SEL DARAH MERAH, VITAMIN B12, FOLAT, ARAS HOMOSISTEINE DAN MUTAS GENE MTHFR DALAM KALANGAN WANITA MELAYU YANG TELAH MENOPAUS DAN HUBUNG KAIT DENGAN PENGGUNAAN TERAPI PENGGANTIAN HORMON

ABSTRAK

Latarbelakang: Hiperhomosisteinemia terjadi disebabkan oleh penurunan aras kofaktor-kofaktor (vitamin B12, B6 dan folat) dan / atau akibat kekurangan keberkesanan enzim methylene tetrahydrofolate reductase (MTHFR). Keadaan ini telah dikaitkan dengan risiko kejadian '*atherosclerosis'* yang merupakan salah satu faktor penyumbang kepada penyakit jantung koronari di kalangan wanita menopaus. Adalah tidak di ketahui samada perubahan pada parameter-parameter sel darah merah boleh terjadi tanpa melibatkan ketidak normalan pada aras vitamin B12 dan folat di kalangan wanita menopaus yang berkaitan dengan kehadiran hiperhomosisteinemia.

Objektif: utama kajian ini adalah untuk mengkaji hubungkait parameter-parameter sel darah merah, B12, folat, homosisteina dan polimorfisme gen MTHFR di kalangan wanita Melayu yang mengambil rawatan terapi penggantian hormon selepas menopaus dan yang tidak mengambil rawatan tersebut. Tujuan lain adalah untuk membandingkan paras homosisteina di antara wanita-wanita Melayu sebelum dan selepas menopaus.

Metodologi: Satu kajian keratan silang telah dijalankan di Hospital Universiti Sains Malaysia (HUSM). Seratus wanita Melayu menopaus secara semulajadi telah menjadi subjek kajian ini. Separuh dari mereka mengambil rawatan terapi penggantian hormon dan separuh lagi tidak mengambil rawatan tersebut. Sampel darah diambil dari subjek-subjek ini untuk ujian-ujian aras vitamin B12, serum dan sel darah merah folat dengan menggunakan kaedah ACCESS immunoasai. Aras homosisteina diukur dengan cara latex immunoasai secara '*automated'* sementara pengesanan polimorfisma gen MTHFR dilakukan ke atas subjek-subjek terpilih dengan menggunakan reagen attomol MTHFR 677C>T. Produk dari ujian '*polymerase chain reaction'* (PCR) kemudiannya di kesan dengan menjalankan ujian gel elektroforesis untuk menentukan jenis polimorfisma gen ini. Pengiraan darah lengkap dilakukan ke atas semua sampel dengan menggunakan mesin analisa hematologi dari Sysmex.

Keputusan: Umur subjek-subjek dalam kajian ini adalah di antara 50 – 60 tahun. Terdapat perbezaan yang ketara di antara aras homosisteina di kalangan wanita-wanita ini sebelum dan selepas menopaus. Walaubagaimanapun tiada perbezaan yang ketara secara statistik bagi paras homosisteina di kalangan wanita yang mengambil dan tidak mengambil rawatan terapi penggantian hormon. Purata aras B12 menunjukkan perbezaan yang ketara di kalangan wanita yang mengambil dan tidak mengambil hormon tersebut. Terdapat hubungkait di antara paras sel darah merah folat dan serum folat serta aras homosisteina. Tiada hubungkait di antara homosisteina dan parameter-parameter sel darah merah, B12 dan serum folat serta polimorfisme gen MTHFR di kalangan wanita-wanita ini.

Perbincangan/Kesimpulan: Purata aras homosisteina di kalangan wanita Melayu menopaus adalah lebih tinggi berbanding dengan sebelum menopaus. Kajian ini menunjukkan tiada kesan positif pada aras homosisteina di kalangan wanita yang mengambil rawatan terapi penggantian hormon. Hiperhomosisteinemia di kalangan wanita Melayu adalah disebabkan oleh pelbagai faktor dan polimorfisma gen MTHFR tidak menunjukkan hubungkait yang jelas dalam menyumbangkan keadaan ini dalam kajian ini. Kajian serupa yang melibatkan sampel yang lebih besar di masa hadapan dapat mengesahkan faktor-faktor penyumbang kepada hiperhomosisteinemia di kalangan wanita Melayu termasuk gen-gen lain yang ada kaitan dengan metabolisma folat dan B12.

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ABSTRACT

Background: Hyperhomocysteinemia occurs as a result of decreased levels of cofactors (vitamin B6, vitamin B12 and folate) and/or reduced efficiency of Methylenetetrahydrofolate reductase (MTHFR) enzyme. Hyperhomocysteinemia is an independent risk factor for atherosclerosis and may be one of the factors predisposing for coronary heart disease (CHD) in postmenopausal women. It is not well known whether changes in red blood cells parameters can occur independently of B12 and folate abnormalities in postmenopausal women in association with hyperhomocysteinemia.

Objectives: The main aim of this study was to investigate the association between red cell parameters, B12, folate, homocysteine level and MTHFR gene polymorphisms in postmenopausal Malay women on hormone replacement therapy (HRT) and those not receiving HRT. Another objective was to compare the homocysteine (Hcy) levels in post and premenopausal women.

Methodology: A comparative cross-sectional study was conducted at Hospital Universiti Sains Malaysia (HUSM). One hundred women were the subjects of this study, half of whom were HRT group and the other half without HRT. Blood samples were taken from all the subjects and tested for B12 and folate levels (serum and red

cell) using ACCESS immunoassay system. The concentration homocysteine was measured using an automated latex-enhanced immunoassay method. The MTHFR gene detection was performed for selected cases with high and normal Hcy levels using attomol® MTHFR 677C>T reagents. PCR products were detected by gel electrophoresis in 3.0% agarose gel. Full blood counts (FBC) were done for all samples using Sysmex haematology analyzer.

Results: The ages of the subjects were 50-60 years and all of them had natural menopause. There was a significant difference in the Hcy levels between premenopausal and postmenopausal women. However, Hcy level did not show any significant difference in postmenopausal women with and without HRT. B12 was significantly lower in subjects in the HRT group compared to non HRT group. There was a significant correlation found between serum folate and RBC folate and a significant correlation between the levels of Hcy and RBC folate. For the RBC parameters, B12 and folate levels and MTHFR gene polymorphisms, no relationship was established with Hcy level.

Conclusions: The Hcy level was higher in Malay postmenopausal women (both HRT group and non HRT group) compared with premenopausal Malay subjects. HRT did not show to have positive effects in on Hcy levels. Hyperhomocysteinaemia in postmenopausal Malay women may be multifactorial and the MTHFR gene polymorphisms were not found to be associated with this condition. In addition, red cell parameters were not shown to have any association with the other variables studied. It

was further concluded that bigger sample size with other genetic factors known to be involved in B12 and folate metabolism should be included in a similar study to confirm the factors contributing to the hyperhomocysteinaemia in postmenopausal women.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction and literature review

Menopause is a natural progression of women's physiology. However it can be a distressing stage for many women and is associated with increased risk of cardiovascular disease and osteoporosis. Hormone replacement therapy (HRT) has been the basis of the treatment of menopausal state. Despite the proven benefit of HRT, only 15% of post menopausal women currently use HRT (Amato *et al*., 2002) and of those who started HRT, nearly 30% of them subsequently stopped (Ryan *et al*., 1992). The main reasons for not taking HRT were concerns over its side effects, safety and efficacy of the treatment.

The Women's Health Initiative (WHI) Study stated an increase risk in cardiovascular disease, breast cancer, stroke and thromboembolic disease with conjugated equine estrogen plus medroxyprogesterone acetate compared with placebo (Prestwood *et al*., 2003). In view of this problem many women are increasingly turning to alternative medicine in an effort to manage their menopausal symptoms (Amato *et al*., 2002). However, there are still questions regarding the effectiveness of HRT and alternative medicines in managing menopausal symptoms and complications.

1.1 Menopause:

Menopause is defined as cessation of menses for at least 12 months. The diagnosis is therefore done retrospectively. Although there is a set point for menopause, it involves progressive changes in the hormonal and physiological status in women. Efforts have been made to categorize the stages of change in the menopausal continuum. This includes the menopause transitional period or premenopause which is divided into early and late phase. The early phase is categorized by irregularity in menstrual cycle of 7 days different from the normal cycle. In the late transitional period, there are 2 or more skipped cycles with an interval of amenorrhea of equal to or more than 60 days. The next stage is the actual postmenopausal stage which is divided into two phases, early and late menopause. The early menopause lasts for 5 years after the actual cessation of the menstrual flow and the late menopause begin after 5 years onwards (Soules *et al*., 2001).

The age of menopause has not changed much over the centuries; it has been fairly consistent between 50 to 52 years of age. There is also not much difference in the age of menopause between regions and across different ethnic groups. There are a variety of factors which have been linked to earlier age of menopause such as genetic, null parity, smoking, low body mass index, a diet high in vegetables fibers and cereals and autoimmune diseases (Lund, 2008).

1.2 Physiological changes:

In terms of physiological changes, menopause is generally marked by the decline in the oocytes and ovarian function. At birth the numbers of oocytes reach over a million, at around forty years of age this is reduced to a few thousands and by the time women reach menopause there are few or no oocytes left. It is the depletion of the oocytes that eventually leads to the final menstrual period (FMP) and the decline in the ovarian hormone production appears to reflect the decline in the ovarian follicle numbers.

The major gonadotrophins involved are the follicle stimulating hormone (FSH) and less importantly, the luteinizing hormone (LH). The menopause transition is characterized by a dynamic period whereby there is markedly changing hypothalamic-pituitary feedback between these hormones produced by the aging ovary. This physiological change occurs 10 years prior to the FMP. The hormonal changes in the premenopause transition lead to cycle irregularity and dramatic swings in estradiol from undetectable, to levels that are several times higher than those observed in those still in early reproductive age (Hall, 2004). With the decreasing number of follicles at menopause, estradiol level drops until it is no longer adequate to stimulate the endometrium leading to amenorrhea.

The level of inhibin A and inhibin B are also decreased. These hormones along with estradiol are secreted by the developing follicles and their main action is exerting a negative feedback to the hypothalamus, therefore reducing the secretion of FSH by the pituitary (Burger, 1996). At menopause with the reduced negative feedback by the inhibins, FSH tends to be higher to exceed 30 IU/l, a level considered as postmenopausal range.

Other hormones are also affected. Androgens secreted by both the adrenals and ovaries also decline as women age. However plasma testosterone levels fall only slightly after menopause. There is also a difference in the type of estrogen that predominates in the postmenopausal state. The main postmenopausal estrogen is estrone, which is produced by the conversion of adrenal androstenedione at the peripheral adipose tissue.

1.3 Effects of menopause:

1.3.1 Short term effects:

Vasomotor symptoms:

The most common symptoms experienced by menopausal women are vasomotor symptoms. Symptoms include hot flushes and night sweats and it can occur in as many as 88% of postmenopausal women (Feldman *et al*., 1985). However the prevalence of the symptoms is different across culture and regions. Women in Germany for example experience more hot flushes than those in Papua New Guinea (Kowalcek *et al*., 2005).

A study in Asia showed that only 30% of postmenopausal women in Malaysia complained of hot flushes compared to 70% in Thailand (Ko-En *et al*., 2010).

The mechanism of hot flushes has yet to be clearly defined. Although, many believe that the mechanism of hot flushes are related to the fall in estrogen levels, this alone does not account for the vasomotor symptoms since the levels of estrogen have not been found to correlate with hot flushes. It is hypothesized that the thermoregulatory zone is narrowed and more sensitive to subtle changes in core body temperature (Gracia and Freeman, 2004).This is thought to be more centrally mediated in the hypothalamus. It is still poorly understood how the changes in gonadal hormones level affect the thermoregulatory set point. Serotonin and norepinephrines are the other two hormones that are pivotal in the mechanism of hot flushes (Rapkin, 2007). Estrogen withdrawal may be correlated with a decline in both of these neuropeptides.

Sexual dysfunction:

Sexual dysfunction is highly prevalent during the menopausal transition. The Melbourne Women's Midlife Health Project which was a prospective study done in Australia reported the prevalence of sexual dysfunction to be as high as 88% in this group of women (Dennerstein *et al*., 2002). The reduction in sexual functioning adversely affects women's quality of life and also their sense of femininity (Ko-En *et al*., 2010).

Estrogen deficiency and androgen deficiency are associated with vaginal atrophy and dryness, and decrease in libido respectively (Van Voorhis, 2005), and these will affect sexual functioning. The presence of vasomotor symptoms such as night sweats, disturbed sleep and depression also lead to diminished libido in postmenopausal women which lead to sexual dysfunction (Reed *et al*., 2007). Hormonal factors are not the only factors contributing to sexual dysfunction. Other factors such as loss of sexual partner and the aging process itself are also implicated (Nappi and Lachowsky, 2009).

Urogenital atrophy:

The role of estrogen in maintaining normal urogenital structure and function is by stimulating the growth and development of vaginal epithelium, allowing it to remain thick, moist and supple. Vaginal atrophy occurs progressively with the dramatic fall in estrogen that occurs just before the final menstrual period. Clinically it is identified by the appearance of thin, pale and dry vaginal epithelium. It also lacks normal rogation and the introital size is often reduced.

There is also change in the vaginal pH with decrease in estrogen level. In women of reproductive age, vaginal pH is < 4.5. Vaginal pH taken from postmenopausal women was found to increase to between 6.0 and 7.5. This may lead to impairment in the local defense system and lead to vaginal and urinary tract infection (Van Voorhis, 2005).

Symptoms of vaginal atrophy include dryness, itching, vaginitis, and dyspareunia. It is a common symptoms reported by postmenopausal women. Vaginal dryness was a complaint in 51% of postmenopausal women in Hong Kong (Ko-En *et al*., 2010).

1.3.2 Long term effects:

The long term complication of menopause which have the greatest impact on health status both at the individual and public health levels are cardiovascular disease and osteoporosis.

Cardiovascular disease:

Cardiovascular disease is the major cause of death in women worldwide; the third Malaysian National Health Survey showed that the main cause of death of Malaysian women is due to cardiovascular disease (CVD) (Azmi *et al*., 2009). The figure is almost the same in other parts of developed countries like United State of America. There is a difference between the incidence of cardiovascular disease in men and women. The incidence of death due to cardiovascular disease is almost unheard of before women reach their 50s. However, by the age 70 years the incidence of CVD is equal in men and women, suggesting that menopause with it ensuing estrogen deficiency causes a rapid acceleration in CVD risk (Azmi *et al*., 2009).

The high incidence in cardiovascular disease is due to the increase in the risk factors associated with menopausal state. Menopausal state is associated with the emergence of features of metabolic syndrome which includes increase in central or intra abdominal body fat, a change toward more atherogenic lipid profile, with increased low density lipoprotein particles and increased insulin resistance (Knopp, 2002, Eaten and Anthony 2002). The mechanism for these changes may be a direct result of ovarian failure or, alternatively, an indirect result of the metabolic consequences of central fat redistribution with estrogen deficiency in postmenopausal women (Carr, 2003). There is however controversy regarding the relationship between weights gains and menopause. Previous data suggest that this is more related to aging rather than failure in ovarian function (Kolasa, 2002).

Another important risk factor for cardiovascular disease is sedentary life style. Menopausal women have been shown to lead more sedentary lifestyle compared to younger women (Kolasa, 2002). The role of other risk factors like C-reactive protein and homocysteinuria in linking menopause with cardiovascular disease are less well established (Kolasa, 2002). More studies need to be done to establish the associations between these factors.

When vitamin B12 level decreases too low, the methionine cycle breaks down which results in elevated levels of homocysteine. That cycle converts methionine into smaller molecules known as S-adenosylmethionine (SAMe). SAMe then breaks down into thousands of compounds and proteins that are vital for healthy cells, tissue and organs.

One of those breakdown products is homocysteine. Recycling of the homocysteine to getting quickly back into methionine needs the assistance of vitamin B12 and folic acid. If someone is deficient the normal function of the primary pathway of methionine cycle is disrupted and this will result in excess of homocysteine (Nygard et *al*., 1997).

Excess amount of homocysteine causes blood vessels to lose their elasticity, making it harder for them to dilate and damaging the inner lining. These changes allow cholesterol, collagen and calcium to attach to the inner walls of the blood vessels where they can form sticky deposits called as atherosclerotic plaque. These plaques narrow the arteries and increase the risk of artery disease, myocardial infarction, strokes and thrombosis clot (Nygard et *al*., 1997).

Coronary artery disease (CAD) or atherosclerotic heart disease is the end result of the accumulation of atheromatous plaques within the walls of the coronary arteries that supply the myocardium (the muscle of the heart) with oxygen and nutrients. It is sometimes also called coronary heart disease (CHD), but it is not the only cause, although CAD is the most common cause of CHD (Tortora & Grabowski 2003).

Osteoporosis:

Osteoporosis is a major health issue since it can lead to fracture with catastrophic consequences. The prevalence of osteoporosis is also rising worldwide. In Western countries for example United States of America, it is estimated that 54%

postmenopausal white women are osteopenic and 30% are osteoporotic as quoted by the International Osteoporosis Foundation in 2002. The National Osteoporosis Foundation in the USA reported that by the year 2010, about 12 million people over the age of 50 are expected to have osteoporosis (National Osteoporosis Foundation, 2002). There is an erroneous belief that Asian women suffer less osteoporosis and fall compared to their western counterpart. An audit released by the International Osteoporosis Foundation on the epidemiology of osteoporosis in Asia revealed that the incidence of hip fractures has risen by 2- to 3- fold over the past 30 years (International Osteoporosis Foundation, 2002).

The WHO defines osteoporosis based on bone mineral density, as a value of less than 2.5 standard deviation below the average in young women (T-score $\leq$ -2.5). This definition initially was more for epidemiological purpose, however in recent times this threshold value has been established as clinical diagnostic tool.

The postmenopausal state is associated with a decrease in the bone mineral density which leads to osteoporosis. There is a widely held belief that the decrease in the estradiol level directly contribute to this. Estradiol directly acts on osteoclast by reducing the rate of bone resorption (Steinweg, 2002). Bone loss in the postmenopausal women, therefore is the result of increase in the rate of bone remodeling and the imbalance in osteoclast.

The rate of bone loss differs in relation to period of menopause, with the most rapid loss occurring early in the premenopausal age. The rate of bone resorption then decrease in the late menopause (Sirola *et al*., 2003). There is also difference regarding the rate of bone loss at different site of the body. The lumbar spine has been shown to have more osteoporotic changes in early postmenopause compared with other sites such as the femur.

Menopausal and cancer risk:

Menopause in itself does not cause cancer, but the risk of developing cancer increases as a woman ages (Barrett-Connor *et al*., 2009). Women who have been through menopause are more likely to develop cancer because they are older. Among the cancers which are associated with late menopause are breast, ovarian, and endometrial cancers (Barrett-Connor *et al*., 2009). (Rossouw *et al*., 2002).

In terms of breast cancer, menopausal women was found in a study to have an additional increase in breast cancer risk if they had high breast density as measured by mammogram (Kerlikowske *et al*., 2009). This study also showed that advanced-stage breast cancer risk was increased 1.7-fold for postmenopausal HRT users who had very high density (BIRADS-4) compared to those with average density (BIRADS-2). The association between HRT use in postmenopausal women and the increased risk in breast cancer had been shown in various large studies most notably the Women's Health Initiative (WHI) trial (Rossouw *et al*., 2002).

Endometrial cancer is the commonest gynaecological cancer in the developed countries and it occurs mainly in the postmenopausal women. The main symptom of endometrial cancer is post menopausal bleeding. Postmenopausal women who presented with this symptom have to have their endometrial sampled to exclude the presence of any abnormality. In term of the effect of combined hormone replacement therapy on the cancer incidence, the WHI trial demonstrated the incidence of endometrial cancer during the 5-6 years of follow-up was 56 per 100000 person-years than observed in women taking the therapy or 13 fewer cases per 100000 person-years than observed in women taking placebo (Anderson *et al*., 2004). This indicated the protective effect of progestin against an increased risk of endometrial cancer associated with unopposed estrogen.

Ovarian cancer is also not associated with menopause but the incidence of the cancer increases as women go into postmenopause. The risk of postmenopausal women having the cancer is increased if they have additional risk factors such as low parity, infertility, early age of menarche, and late age of menopause (Berek and Hacker, 2005).The theory behind the increase risk of ovarian cancer with late age of menopause is thought to be related to the incessant ovulation theory. According to this theory with repeated damage and trauma to the ovarian epithelium during each ovulatory cycle, there is an increased potential for genetic mutation and ovarian neoplasm during the repair process. Incidence of ovarian cancer has also been shown to be associated with long term combined hormone therapy use (Anderson *et al*., 2004).

Cognitive function:

The effect of estrogen on cognitive function is an intriguing area of research. It is well known that normal aging causes a decline in certain cognitive function, and a decline in the estrogen associated with menopause may contribute to this process. In the past estrogen therapy has been associated with better performance on memory testing in postmenopausal women compared with postmenopausal controls who were not receiving estrogen therapy (Kawas *et al*., 1997). However data from the WHI do not show improvement in cognitive function in women taking either combined hormone replacement therapy or estrogen only therapy (Rossouw *et al*., 2002).

1.4 Therapeutic options:

There are three main therapies available in the management of menopausal complications which are hormonal replacement therapy (HRT), bisphosphonate and selective estrogen receptor modulator. The use of these agents is increasingly being challenged by the alternative medicines especially after the release of the result of Women Health Initiative Study. Among the popular alternative medicine used in menopausal symptoms are soy products, black cohosh, dong quai, red clover, ginseng and evening primrose oil (Black *et al*., 2006).

Hormone replacement therapy:

Hormone replacement therapy (HRT) has long been the mainstay of the treatment of menopausal symptoms and its complications especially osteoporosis. The essential component in HRT is estrogen, which is combined with progestogen to prevent endometrial hyperplasia in women with intact uterus (Barrett-Connor *et al*., 2006).

The oral estrogen commonly used is estradiol valerate 1mg or 2 mg, conjugated equine estrogen (CEE) 0.625 mg or 1.25 mg and estrone 1.25 mg. The minimum effective dose of estrogen has long been questioned. After the WHI, more interest has been shown to the use of low dose estrogen. The usually prescribed dosage of postmenopausal estrogen therapy has declined progressively and in the past 10 years, use of lower dose HRT has grown in popularity. Low-estrogen formulations that are starting to be used recently are 0.3mg CEE, 0.45 mg CEE and 0.5 mg or 1mg micronzed oral 17-estradiol and these are considered low dose HRT. This lower dose of oestrogen is also beneficial in terms of reduced breast tenderness, an effect that should also help to improve compliance and acceptability, particularly in older women (Barrett-Connor *et al*., 2006).

The progestogens used in HRT are nearly all synthetic. They are added to reduce the risk of endometrial hyperplasia and malignancy. The two classes of progestogens commonly used for in combination with estradiol is C-21 group (medroxyprogesterone acetate, dydrogesterone) derived from native progestogens and a C-19-*nor* group

(norethisterone, norgestrel) derived from testosterone. Dydrogesterone, a retro-progesterone derivative in particular is structurally closest to the body's own progesterone and fairly neutral effect on the protective properties of estrogen on bone metabolism (Schindler *et al*., 2003).

Currently progestogens are mainly given orally, however transdermal formulations of levonogestrel and norethisterone are becoming available. Progestogen is only required in women with intact uterus. It can be given either for 10-14 days every four weeks or for 14 days every 13 weeks or even continuously every day. The continuous formulations is suitable for women with early or late postmenopause while the monthly sequential regime is given for those in menopausal transition or premenopause stage (Cieraad *et al*., 2006).

The controversy with HRT started to appear after the release of the results from Women's Health Initiative (WHI) trial in 2002. The trial involved 27,347 predominantly healthy postmenopausal women aged 50 to 79 years old from 40 centers across America. The women were randomized to either receive 0.625 mg/d of CEE or 0.625mg/d CEE plus 2.5 mg/d of medroxyprogesterone acetate (MPA) based on their hysterectomy status and they were compared with placebo (Rossouw *et al*., 2002). The aim of the trial was to see the effect of HRT to a 'global health index' which includes coronary heart disease, breast cancer, fractures, stroke, pulmonary embolism, colorectal cancer and endometrial cancer (Rossouw *et al*., 2002).

For every 10,000 women per year who were taking combination HRT, there were eight more invasive breast cancers, seven more coronary heart disease events, eight more strokes and eight more pulmonary embolisms than in the placebo group. Physicians and patients were encouraged to use HRT for the shortest duration possible since the biggest increase in breast cancer was seen after 5 years. Similar result in term of breast cancer risk was seen in the Million Women Study conducted in England (Beral, 2003).The main limitation of WHI trial is that they include older women who were postmenopausal for many years and the result therefore cannot be extrapolated to the younger postmenopausal women.

A variety of drugs are used for prevention and treatment of CHD. Hormone replacement therapy (oestrogen) was reported to give benefit effects to reduce the risk of CHD in postmenopausal women (Smolders *et al*., 2005).

The main indication for HRT use currently is for the treatment of postmenopausal symptoms and it is no longer recommended for disease prevention (Dull, 2006). A Cochrane review concluded that oral estrogen or combined estrogen and progestogen hormone replacement therapy greatly reduces the frequency and severity of hot flushes and night sweats (MacLennan *et al*., 2004). The maximum benefit is usually seen within three months of treatment.

HRT has long been proven beneficial in the treatment of postmenopausal osteoporosis. Both observational studies and randomized clinical trial have demonstrated that estrogens or estrogens plus progestin increase bone density and reduce the risk of fractures by preventing bone loss mainly in the vertebral bone (Huot *et al*., 2008).

Recently, there has been increasing evidence that it is also beneficial in preventing fracture in the non-vertebral bone. A 5 year randomized controlled trial was done to compare the incidence of new non-vertebral bone fractures in non-osteoporotic postmenopausal women taking HRT or low dose vitamin D. This study showed the incidence of non-vertebral bone fractures is significantly reduced compared to the vitamin D group. The fall in fracture rate is also consistent with the increase in bone mass (Komulainen *et al*., 2008).

Femoston Conti is one of the examples of low dose hormone replacement therapy. It contains 17β-estradiol plus dydrogesterone with a multiple dosing range combination. The doses of estradiol include 1 and 2 mg, while the doses of dydrogesterone include 5, 10 and 20 mg. There are multiple studies which have shown the effectiveness of this treatment in managing menopausal symptoms and having bone protective effects. A study comparing the 17β-estradiol / dydrogesterone with conjugated equine estrogens(CEE)/norgestrel have shown that it was equally effective in managing climacteric symptoms while showing some advantages in terms of lipid profile and incidence of bleeding (Cieraad *et al*., 2006). A combination of 1 mg 17β-estradiol with a combination of 5, 10 and 20 mg of dydrogesterone had also been shown to have