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**EFFECTS OF HORMONE REPLACEMENT THERAPY ON  
POSTMENOPAUSAL WOMEN WITH TYPE 2 DIABETES MELLITUS.**

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## LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
Apo	Apolipoprotein
ALT	Alanine tranferase
AST	Aspartate transferase
BAP	Bone alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
CABG	Coronary artery bypass grafting
cAMP	Cyclic adenosine monophosphate
CEE	Conjugated equine oestrogens
cGMP	Cyclic guanine monophosphate
CHD	Coronary Heart Disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DM	Diabetes Mellitus
DVT	Deep venous thrombosis
FFA	Free fatty acid
FSH	Follicle stimulating hormone
GGT	Gamma-Glutamyl transpeptidase
HDL	High-density lipoprotein
HepG2 cells	Human heptoma line cells
HRT	Hormone Replacement Therapy
IGT	Impaired glucose tolerance
IR	Insulin resistance

IL-6	Interleukin 6
LDL	Low Density Lipoprotein
LH	Luteinising hormone
LPL	Lipoprotein lipase
MI	Myocardial infarction
MPA	Medroxyprogesterone acetate
MMP	Matrix metalloproteinases
NASH	Non-alcoholic steatohepatitis
PAI	Plasminogen activator inhibitor
PTCA	Percutaneous Transthoracic Coronary Angiogram/plasty
SHBG	Sex hormone binding globulin
SMC	Smooth muscle cell
TG	Triglycerides
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
tPA	Tissue plasminogen activator
VLDL	Very-low-density cholesterol
W/H Ratio	Waist to hip ratio

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## DECLARATION

The initiation and design of the study described in this thesis was my own work, as was the analysis of the results. The work was carried out in the Biochemistry laboratory of Glasgow Royal Infirmary NHS University Trust between the years 1998 and 2004.

I personally carried out the venesection and patient assessment and was responsible for care and administration of the study medication.

Some of the results described have been published in peer review journals or presented at professional meetings. These include:

McKENZIE J, SATTAR N, MacCUIISH AC.

Hormone Replacement Therapy in type 2 diabetes- A Pilot Study.

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PATERSON K, SATTAR N.

A randomised double-blind placebo-controlled trial of a low dose continuous combined hormone replacement therapy in women with type 2 diabetes.

Oral presentation at European Association for the Study of Diabetes Meeting. Glasgow 2001.

MCKENZIE J, KELLY A, CRAWFORD L, PATERSON KP, SATTAR N.

A Randomised Double-Blind Placebo-Controlled Trial of the Effects on Bone Metabolism of a Low Dose Continuous Combined Hormone Replacement Therapy in Women with Type 2 Diabetes.

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Metabolic, Inflammatory, and Haemostatic Effects of a Low-Dose Continuous Combined HRT in Women With Type 2 Diabetes: Potentially Safer With Respect to Vascular Risk. *Obstetrical & Gynecological Survey*. 59(7):524-525, July 2004.

McKENZIE J, JAAP AJ, GALLACHER S, KELLY A, CRAWFORD L, GREER I, RUMLEY A, PETRIE JR, LOWE GD, PATERSON K, SATTAR N.

Metabolic, inflammatory and haemostatic effects of a low-dose continuous combined HRT in women with type 2 diabetes: potentially safer with respect to vascular risk? *Clinical Endocrinology*. 59(6):682-689, December 2003.

MCKENZIE J, FISHER BM, JAAP AJ, STANLEY A, PATERSON K, SATTAR N.

Effects of HRT on liver enzyme levels in women with type 2 diabetes: a randomised placebo-controlled trial. *Clinical Endocrinology*. In press.

**CHAPTER 1**  
**INTRODUCTION**

Hormone replacement therapy (HRT) is widely accepted as an effective treatment for symptoms associated with the menopause. Many of the preparations are licensed and recommended as prophylaxis or treatment for postmenopausal osteoporosis [1]. HRT may also have a role in the prophylaxis of cardiovascular disease this remains controversial. Epidemiological studies suggest a reduction in coronary heart disease of around 50% in postmenopausal women taking oestrogen [2]. There is confusion amongst the medical profession over the prescribing of HRT since many cautions are included in the product literature. These include the use in women with diabetes. Women with diabetes may have more to gain from their use of HRT than women without.

## **1.1 Type 2 Diabetes Mellitus**

### **1.1.1 Insulin Resistance**

Insulin resistance is the underlying mechanism behind type 2 diabetes mellitus (type 2 DM). This may be defined as a reduced biological response to a physiological amount of insulin. There is a broad range of insulin sensitivity in an apparently healthy population. Up to 25% of the population may have levels of insulin resistance equal to that seen in patients with type 2 diabetes [3]. When a non-diabetic person consumes excessive calories and gains weight the body becomes markedly resistant to the actions of insulin. Insulin resistance initially results in lower levels of uptake of glucose from the blood into the target organs, predominantly skeletal muscle and to a lesser extent adipose tissue [4]. This leads to hyperglycaemia. The response to this is for more insulin to be secreted from the pancreatic  $\beta$  cells resulting in relative hyperinsulinaemia. In the majority of people with diabetes in the early or pre diagnosis state this elevation in secretion of insulin will ensure that euglycaemia is maintained as tissue insensitivity is overcome.

Eventually pancreatic  $\beta$  cell failure begins and the hyperinsulinaemic state becomes inadequate to maintain euglycaemia and hyperglycaemia ensues. At this point patients are classified as suffering from type 2 diabetes.

The association between hypertension, hypertriglyceridaemia, low HDL-cholesterol and high LDL-cholesterol is well-established [5]. The "insulin resistance syndrome", "multiple metabolic syndrome", "Reaven's syndrome" or "Syndrome X" includes these factors and insulin resistance or type 2 diabetes and clotting abnormalities i.e. increased fibrinogen and plasminogen activator inhibitor-1. Visceral obesity, hyperuricaemia, impaired fibrinolysis and microalbuminuria have also been added to the original description of the syndrome.

The most important peripheral tissue in glucose homeostasis, in quantitative terms, is skeletal muscle. Anything which affects the insulin sensitivity of skeletal muscle will have a profound effect on glucose homeostasis of the whole body [6]. Adipose tissue also plays a very important role in glucose homeostasis as it has a pivotal role in the integration of carbohydrate and lipid metabolism and hence energy supplies.

The prevalence of insulin resistance can be seen to vary between populations, gender, and different age groups and also depends upon the metabolic variables used to define the syndrome. Impaired actions of insulin i.e. insulin resistance may result from 2 mechanisms. Firstly the defect may be at the receptor level. A reduced number of insulin receptors are present or a reduction in the receptor's affinity for insulin. This may occur as a consequence of chronic hyperinsulinaemia i.e. down regulation. Secondly there may be post-receptor defects. These are defects in intracellular events distal to the binding site. This accounts for the majority of insulin resistance seen in type 2 diabetes. These defects appear to be species, tissue and or disease dependent [7].

Insulin resistance is influenced by both genetic and environmental factors. Although it is largely associated with obesity especially abdominal obesity [8] it can develop in lean individuals [9]. With the euglycaemic insulin clamp technique [10] it has been shown that tissue sensitivity to insulin declines by approximately 30-40% when an individual becomes greater than 35-40% over ideal body weight [11] [12] [13] [14].

It is clear that a substantial proportion of the population exist with a state of insulin resistance, which is compatible with developing diabetes but also increases the risk of developing coronary heart disease (CHD). The current view is that there is an inherited (or acquired) predisposition present in certain individuals from which various components of the metabolic syndrome can emerge. The existence of this predilection allows for the clustering of the elements of the syndrome in some individuals but also for the dissociation of the elements seen in others. In young individuals obesity, type 2 DM, hypertension and atherosclerotic cardiovascular disease are uncommon. By the age of 70 over 50% of individuals have evidence of CVD and 45-50% are obese and hypertensive. The incidence of type 2 DM is somewhat lower (10-12%) although in certain populations it is much higher [15].

Most obese individuals have normal glucose tolerance as their insulin resistance is matched by increased insulin secretion. The mechanism for this enhanced insulin secretion, especially in cases where plasma glucose concentrations were not elevated, was initially thought to be a consequence of exposure of the liver to elevated free fatty acid (FFA) levels generated by enlarged intra-abdominal fat stores [16]. The levels of FFA's are increased in most obese individuals primarily because of an increase in lipolysis in the expanded fat cell mass [17]. Elevated FFA concentrations produce peripheral and hepatic insulin resistance, which in normal individuals leads to, FFA

potentiated, glucose stimulated, insulin release. In DM it is thought that FFA's fail to stimulate insulin secretion. This leads to unchecked hepatic and peripheral insulin resistance which in turn lead to increased hepatic gluconeogenesis and decreased utilisation of glucose in the peripheral tissues [18]. Studies have also shown that there is reduced post-prandial FFA metabolism in abdominal obese, insulin resistant patients as compared to peripherally fat individuals so perpetuating the problem [19].

In obesity FFA's may cause both peripheral insulin resistance and compensatory increase in insulin secretion [20]. The consequence of long term high FFA exposure appears to be more complex. Compared to glucose FFA's are weak insulin secretagogues.

The cytokine tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) may also have a role in peripheral insulin resistance in obesity. TNF- $\alpha$  levels are elevated in the adipose tissue of obese insulin resistant individuals [21]. The mechanism by which TNF- $\alpha$  may influence insulin resistance may be by the release of TNF- $\alpha$  from adipose tissue. Levels of TNF- $\alpha$  in the circulation are low and neutralisation of TNF- $\alpha$  by antibody for 4 weeks has little effect on insulin resistance in obese patients with type 2 DM [22]. TNF- $\alpha$  may act locally on muscle [23] or may act indirectly via another factor. This factor may be FFA's. This latter hypothesis is supported by observations that neutralisation of TNF- $\alpha$  in Zucker rats is associated not only with an increase in insulin sensitivity but also a decrease in FFA levels [21] and that infusion of TNF- $\alpha$  in humans leads to an increase in plasma FFA levels [24].

There is a 4-hour lag period between the rise of plasma FFA's and the onset of inhibition of insulin stimulated glucose uptake. This may prevent insulin resistance in non-obese individuals after a fat rich meal as FFA levels seldom remain elevated that long. In obese individuals FFA levels are persistently elevated and peripheral insulin resistance

mediated by FFA's may occur. The elevated FFA levels will stimulate the release of insulin to compensate. The increase in FFA's may in addition increase hepatic gluconeogenesis [25]. In obese patients genetically prone to developing DM the ability to release compensatory insulin may be lost eventually. Thus FFA increased insulin resistance and increase gluconeogenesis would go unchecked leading to hyperglycaemia. This in turn would produce progressively more islet  $\beta$ -cell desensitisation and more peripheral resistance [18].

At the skeletal muscle level, high FFA's compete with glucose for insulin stimulated glucose metabolism-the glucose-fatty acid cycle [26] leading to the inhibition of both glucose oxidation and storage. Strong negative associations have been found between waist; hip ratio and insulin stimulated glucose oxidation and also insulin stimulation of glycogen synthase activity. It has also been found that FFA's inhibit the uptake of glucose in a dose dependant manner throughout the physiological range of plasma FFA concentration (~50 to ~800 $\mu$ mol/l) [27]. The contribution of FFA's to insulin resistance is at maximum only 50%. At levels of comparable euglycaemia and low plasma FFA concentration (<100  $\mu$ mol/l) levels of insulin stimulated glucose uptake in-patients with diabetes are half of that seen in normal individuals [28]. It should also be noted that the increased insulin resistance induced by FFA's has a physiological role to preserve glucose for metabolism in the CNS at times when glucose is scarce i.e. times of starvation, or prolonged exercise and also in late pregnancy. It is only in the obese state that they become pathological.

### 1.1.2 Dyslipidaemia

The original concept written of the insulin resistance syndrome by Reaven in his seminal paper in 1988 was of an association of insulin resistance with hypertriglyceridaemia and with low HDL-cholesterol levels. The potential link with LDL-cholesterol concentration and composition was not considered [9]. It was not until 1991 that it was realised that an insulin resistant state does not lead to large changes in LDL-cholesterol levels but rather to increases in levels of LDL apolipoprotein (apo) B levels [29]. There is 1 apoB molecule for each LDL-cholesterol molecule so the presence of increasing apoB concentrations without concomitant rise in LDL-cholesterol levels suggests an increase in the number of cholesteryl ester depleted LDL molecules which are small and dense [30]. The production of apoB appears to be substrate-regulated [31]. Conversely it has been shown that high cholesterol levels, associated with large buoyant LDL-cholesterol particles are not indicative of a hyperinsulinaemic state [32].

Adipose tissue is the body's largest energy store and as such has a central role to play in co-ordinating the use of energy. The storage and release of energy is largely controlled by insulin via inhibition of lipolysis and enhancement of lipogenesis. Lipoprotein lipase (LPL) in adipose tissue hydrolyses triacylglycerol (triglyceride) in circulating triacylglycerol containing lipoproteins making FFA's available for uptake into tissues and subsequent storage as triacylglycerol. This process is enhanced by insulin in the healthy state by enhancing transcription of LPL [33]. Insulin has the opposite effect in skeletal muscle promoting energy utilisation rather than storage [33].

The most common dyslipidaemia associated with DM is raised triglycerides due to an excess of very low-density lipoprotein (VLDL). This is caused by reduced clearance of VLDL by insulin sensitive enzyme LPL and in type 2 DM by increased VLDL

production [34]. The presence of small dense LDL phenotype is also associated with hypertriglyceridaemia [35]. It is thought that this is due to an over production of apoB due in turn to an increased availability of FFA's.

The smaller dense LDL-cholesterol molecules are less able to react with the LDL receptors, which increases the proportion of LDL-cholesterol, which can react with receptors for modified LDL on macrophages thereby increasing the formation of foam cells. In addition the metabolism of smaller dense LDL-cholesterol is slower resulting in longer residence of these particles in the circulation. Once again increasing its atherogenic potential [36] [37].

### 1.1.3 Prothrombotic Changes

Many abnormalities consistent with a procoagulant state are also associated with type 2 DM [38]. In type 2DM an increase in platelet aggregation, via decreased cAMP and cGMP production, is seen. The synthesis and activity of thromboxane, procoagulants, fibrinogen and von Willebrand's factor are known to be increased. The concentration and activity of antithrombin factors including antithrombin III is in contrast decreased. All of these lead to an increased predisposition to thrombosis [38]. This tendency to coagulation is further enhanced by a decrease in fibrinolysis due to a decrease in tPA activity and an increase in PAI-1 synthesis and activity (directly increased by insulin and IGF-1) [39]. This tendency is also increased by a decrease in the concentration of  $\alpha$ 1 antiplasmin [39].

From the Framingham data fibrinogen levels were seen to rise across the range of blood sugar levels. A relationship between fibrinogen levels and cardiovascular events was

established in the Gothenburg study, the Northwick Park study as well as the Framingham study [40] [41] [42]. In the Framingham heart study in particular the risk of CHD was seen to be strongly associated with antecedent fibrinogen levels even after adjustment for diabetes and the standard risk factors. Antecedent fibrinogen levels were also related to risk of MI in men with a borderline significance in women. When multivariate analysis was carried out and corrections applied for fibrinogen levels there remained a residual effect for glucose intolerance. Thus fibrinogen does not account for the residual effect of diabetes on incidence of CVD [43].

#### 1.1.4 Endothelial Dysfunction

The endothelium provides not only a barrier and selective transport functions in vessel walls but also a surface facilitating assembly of prothrombinase derived through both tissue factors and the intrinsic pathway of the coagulation system. Endothelial cells can elaborate t-PA, PAI-1, prostacyclin, procoagulant factors e.g. von Willebrand factor, cytokines, adhesion molecules including selectins and growth factors which can influence vascular smooth muscle migration and proliferation. Disturbance of endothelial dependent vasomotor function has been demonstrated in the coronary and forearm vessels of non-diabetic subjects both with established CHD [44] and those with risk factors for CHD [45] and may be important in the pathogenesis of atherothrombotic diseases.

There is considerable evidence in favour of endothelial dysfunction in type 2 DM, in particular in-patients with microalbuminuria [46]. Microalbuminuria may be thought of

as the end result of increased vascular permeability secondary to endothelial dysfunction [47] [48]. It is also well established that CHD mortality increases with urinary albumin excretion rates in type 2 DM [49]. Microalbuminuria in type 2 DM is diminished by angiotensin converting enzyme (ACE) inhibitors known to potentiate bradykinin induced elaboration of t-PA by endothelium and to diminish elaboration of PAI-1 mediated metabolism of angiotensin [50] [51]. Serum ACE levels are associated with CHD risk and are found to be elevated in female patients with type 2 DM. This elevation has been traced to an insertion/deletion polymorphism in the ACE gene [52] which in turn has been linked to CHD mortality especially in patients with type 2 DM and few other risk factors [53].

Research has shown that the fibrinolytic parameters PAI-1 and t-PA antigen, are strongly related to insulin resistance, HDL-cholesterol, triglyceride, body mass index, waist to hip ratio and blood pressure [54]. The concentration of PAI-1 is noted to be increased in type 2 DM and in cardiovascular disease. Sobel et al found an increase in both total PAI-1 (free plus plasminogen activator-complexed PAI-1) and a decrease in total urokinase plasminogen activator (free and receptor bound) in specimens of atheroma taken from patients with diabetes when compared to those without [39]. This suggests that in patients with a hyperinsulinaemic state as in type 2 DM there is an elevation of intramural PAI-1, which may contribute to an increase in thrombosis and accelerated vasculopathy. It has also been suggested that insulin may act either directly or indirectly via lipoprotein changes in the cells that synthesis PAI-1. Insulin is known to increase expression of PAI-1 in HepG2 cells (a human hepatoma line) and in concentrations consistent with type 2 DM insulin and proinsulin, its precursor, greatly increased elaboration of PAI 1 from these cells [55].

Juhan-Vague et al found that patients with angina pectoris exhibit elevated concentrations of PAI-1 in plasma [56]. Gray et al also demonstrated that patients with diabetes presenting [57] with acute MI had higher levels of PAI-1 and these could be correlated with level of glycosylation of haemoglobin and admission glucose. These findings are in accordance with those of Hamsten et al [58] [59] and Meade et al [41] who demonstrated high levels of t-PA inhibition in survivors of MI compared to control subjects. Impaired endogenous fibrinolytic activity may accelerate atherosclerosis by exposing vascular luminal wall surfaces to persistent and recurrent thrombi and clot-associated mitogens [39] [60].

Even modest caloric restriction can modify concentrations of insulin in elderly obese subjects, diminish elevated PAI-1 levels in blood and normalise several biochemical markers indicative of accelerated thrombosis [61]. PAI-1 expression in Hep G2 cells is also decreased by exposure to gemfibrozil [62]. This may explain the favourable impact of fibrates on mortality in the Helsinki Heart study affecting the fibrinolytic system rather than by an effect on lipids alone [63]. No association has been found between level of glycosylated haemoglobin and level of abnormal fibrinolysis. There is evidence to suggest that poor metabolic control may prevent clot lysis in that glycosylated fibrin is more resistant to plasmin digestion [64] and accumulation of fibrin is reported to occur in those tissues most affected by diabetic complications [65].

In addition other components of the insulin resistance syndrome may have a further detrimental effect on the endothelium. Increased plasma levels and oxidation of small, dense LDL-cholesterol leads to impaired endothelium-dependent vasodilatation by reducing prostacyclin synthesis, increasing endothelin production and release and inactivation nitric oxide, [66] [67] while low HDL levels are associated with abnormal coronary artery vasoconstriction [68]. Oxidised LDL-cholesterol has also been shown to

promote rapid adhesion of neutrophils to endothelium by up regulation of neutrophil adhesion receptors [69] and induction of cytokine release from human blood mononuclear cells [70].

## **1.2 Diabetes and Coronary Heart Disease.**

### **1.2.1 Epidemiology**

For patients with diabetes (type 1 or 2) the risk of cardiovascular heart disease is greatly increased both for type 1 and type 2 [71]. In a survey in USA cardiovascular mortality was six fold greater in men and four fold greater in women than in subjects without DM, independent of other risk factors e.g. hypercholesterolaemia or hypertension [38]. The cardiovascular mortality for women with type 2 DM is four times that found in the general population [43]. The presence of type 2 DM also exacerbates the effect of other known cardiovascular risk factors. It leads to adverse changes in lipoprotein metabolism, body fat composition, blood pressure, fibrinolysis and the vasculature. The central mechanism behind these changes is insulin resistance.

There is a clearly recognised and researched sequence of events from impaired glucose tolerance to type 2 diabetes and then on to microvascular complications i.e. nephropathy, retinopathy and neuropathy. The same relationship does not appear to hold for macrovascular complications i.e. CHD, CVD, and PVD. In general, over 70 % of patients with DM die of macrovascular disease, mostly CHD. The increase in CHD is not related exclusively to glycaemic control [72]. Some studies suggest that patients with both established type 2 DM and those with impaired glucose tolerance (IGT) show the same relative risk of CHD. One possible reason for this is that macrovascular risk is

associated with glycaemic levels lower than those employed currently to diagnose DM. This level is based on the risk of microvascular disease [73] [71].

The MRFIT study [74] examined the predictors of CVD mortality among men with and without diabetes and also the independent effect of diabetes (type 1 and 2) on cardiovascular mortality and morbidity. Approximately 350,000 men, aged 35-57, were followed up for an average of 12 years. The absolute risk of CVD death was much higher for diabetic than nondiabetic men of average age group, ethnic background and risk factor level. Overall the relative risk was 3.0 times higher when adjustments were made for age, race, income, serum cholesterol level, systolic blood pressure and reported smoking history ( $p < 0.0001$ ).

Diabetes is the only common condition, which increases the risk of heart disease in women to a level approaching that seen in men [75]. In the Rancho Bernardo Study and its subsequent follow up study, diabetes was found to be an independent predictor for heart disease in both men and women but most strongly in the women. The relative hazard of ischaemic heart disease deaths in diabetics being 1.8 in men and 3.3 in women after adjusting for age and 1.9 and 3.3 respectively after adjusting for age, blood pressure, cholesterol levels, body mass index and smoking using a Cox regression model [76]. Conventional risk factors do not account for this excess, and attention has focused on the possible contribution of abnormalities of fibrinolysis and coagulation in type 2DM.

### 1.2.2 Lipids

Early primary and secondary cardiovascular prevention trials of lipid lowering therapy have included diabetic patients, although in very small numbers. (Table 1.1, 1.2-statin

trials) Subgroup analysis of the WOSCOPS trial [77], a large primary cardiovascular prevention trial of 6595 patients, 66 of whom had type 2 DM, using pravastatin, revealed that the reduction in cholesterol and in morbidity and mortality from CHD from the use of pravastatin was the same in those with or without DM. Pravastatin reduces the incidence of fatal and nonfatal coronary events in middle-aged men with moderately elevated cholesterol (LDL-cholesterol >4mmol/l) and no history of myocardial infarction by 31%. (95% CI 17-43 p<0.001) The need for coronary angiography and revascularisation procedures was also lowered by approximately 31(95% CI 10-47 p=0.007) and 37 % (95% CI 11-56 P=0.009) respectively. The number of patients with diabetes was too small for subgroup analysis to be meaningful (approximately 1% of patients). The same is true of the AFCAPS/TEXCAPS study using lovastatin for primary prevention.

In the Helsinki Heart trial [78] [79] a primary prevention trial of 4081 patients using the fibrate gemfibrozil, 135 participants had DM. In this group there were 8 events due to CHD in the placebo group (n=76) and only 2 in the treatment group (n=59). The reduction in risk in these 2 groups was not significant (Table 1.3-fibrate trials). Subgroup analysis of the VA-HIT trial [80], which also utilised gemfibrozil in 2531 patients, 633 of whom had DM, but for secondary prevention of CHD revealed a significant reduction in the primary combined endpoint of death and nonfatal MI and stroke of the order of 24% which was consistent with that seen in patients without type 2 DM. Analysis of trial evidence shows that in patients with DM and dyslipidaemia the development of proteinuria is a particularly strong predictor of CHD risk and as such these patients should then be treated as in secondary CHD prevention [81].

Subgroup analyses from the three large secondary prevention statin studies i.e. 4S [86] [83], CARE [84] and LIPID [85] studies have been published (Table 1.1, 1.2-statin trials). In the 4S study 202 patients with DM were recruited (~4% of study population).

There was a 43% decrease in all cause mortality (p nonsignificant) and a 55% decrease in CHD incidence (p=0.002) in those treated with simvastatin. This result is equivalent to that seen in patients without diabetes. The patients in the 4S study were unrepresentative of the diabetic population as a whole as individuals with triglyceride levels above 2.5mmol/l were excluded. Thus many patients with typical DM dysliproteinaemia were excluded.

In the CARE study 586 patients with diabetes (~15% of the study population) were recruited. Treatment with pravastatin in this group lowered the CHD incidence by 25% (95% CI 0-43, p=0.05) a level comparable with that seen in patients without diabetes. The exclusion criteria for this study were plasma cholesterol >6.2mmol/l, LDL-Chol > 4.5mmol/l and triglyceride > 4.0mmol/l. The subgroup included patients with previously diagnosed DM, those with DM diagnosed during the study by a glucose tolerance test and also those with impaired glucose tolerance. It is not clear whether patients were stratified in the study during randomisation as to the presence or absence of DM.

In the LIPID study only 164 patients with diabetes were recruited (~2% of the study group). A fall of 19% in deaths due to CHD was found in this group with pravastatin treatment but this was not significantly different from patients with DM in the placebo group

**Table 1.1****Statin Trials.**

Trial	WOSCOPS [77]	AFCAPS/TEXCAPS [82]	4S [83]	CARE [84]	LIPID [85]
Drug	pravastatin	lovastatin	simvastatin	pravastatin	pravastatin
Total recruited	6595	6605	4444	4159	9014
No. DM	66 (1%)	60 (0.9%)	202 (4.5%)	586 (14.1%)	164 (1.8%)
No. Women	0	600 (9.1%)	800 (18%)	582 (13.9%)	1532 (17%)

**Table 1.2**

**Statin Trials.** (Figures are significant unless otherwise stated.)

End Point (% redn)	Total Popn	DM	Women
<u>WOSCOPS</u>			
Total Mortality	22	22	0
CHD Incidence	31	31	0
<u>AFCAPS/TEXCAPS</u>			
Total Mortality	ns	ns	ns
CHD Incidence	37	37	46
<u>4S</u>			
Total Mortality	30	ns	ns
CHD Incidence	33	55	ns
CHD Mortality	42	ns	ns
CABG/Angioplasty	37	32	49
<u>CARE</u>			
Total Mortality	ns	ns	ns
CHD Incidence	23	25	51
CHD Mortality	24	3	ns
CABG/Angioplasty	27	32	45
<u>LIPID</u>			
Total Mortality	22	ns	ns
CHD Incidence	24	ns	ns
CHD Mortality & nonfatal MI	25	19	ns

**Table 1.3****Fibrate Trials** (Figures are significant unless otherwise stated.)

End point (%Reduction)	Total Popn	DM
<u>Helsinki Heart Trial</u> (gemfibrozil)[79] [78]		
Total Mortality	ns	ns
CHD Incidence	34	ns
CHD Deaths	26	ns
<u>VA-HIT</u> (gemfibrozil) [80]		
Total Mortality	ns	ns
CHD Deaths	22	ns
CHD Incidence	23	ns
Combined end point(death, nonfatal MI & CVA)	24	24

It has become clinical practice to give lipid lowering statin therapy to patients with diabetes whose cholesterol is raised over 5mmol/l when triglycerides are less than 5mmol/l despite diet therapy. Hypertriglyceridaemia is more common in patients with diabetes and the most appropriate lipid lowering drug is less certain when hypertriglyceridaemia is pronounced or when triglycerides are raised but cholesterol is relatively low [87]. Whether such patients would benefit more from fibrates alone or a combination of fibrate and statin is at present the subject of clinical trials.

### 1.2.3 Hypertension

Implicit within the insulin resistance syndrome it is seen that hypertension is very common in patients with type 2DM. 40% of patients at diagnosis of DM have hypertension [88]. Insulin resistance is linked to hypertension [9]. The exact mechanism of association remains to be resolved. Possibilities include insulin-mediated renal sodium reabsorption in the proximal tubule, increased sympathetic activity and catecholamine secretion and increased activity of the renin-angiotensin system [9].

The Hypertension in Diabetes (HDS) study revealed hypertension is strongly related to obesity and is highly predictive of cardiovascular complications [88]. In type 2 DM, hypertension does not usually indicate the presence or development of nephropathy. If present, hypertension will accelerate the decline of renal function in patients with established nephropathy [89]. The United Kingdom Prospective Diabetes Study (UKPDS), which is the follow on clinical study of the HDS have provided important new evidence on treating hypertension in patients with type2 DM (Table 1.4, 1.5 - hypertension trials)

In the UKPDS study 1148 hypertensive patients (mean age 56, mean BP at entry 160/94mmHg) were recruited [90] [91]. Patients were then randomly allocated to receive tight control of blood pressure (n=758) or less tight control (n=390). Follow up was for a median time of 8.4 years. Mean blood pressure during follow up was significantly reduced in the group assigned tight control (144/82mmHg) compared to the group assigned to less tight control (154/87mmHg) ( $p<0.0001$ ). Reductions in risk in the group assigned to tight control compared to less tight control were 24% in diabetes related end points (95% CI 8-38%,  $p=0.0046$ ), 32% in deaths related to diabetes (95% CI 6-51%,  $p=0.019$ ), 44% in strokes (95% CI 11-65%,  $p=0.013$ ) 21% in MI ( $p=0.13$ ) and 37% in microvascular end points (95%CI 11-56%,  $p=0.0092$ ). There was non-significant reduction in all cause mortality. ( $p=0.17$ ). After 9 years of follow up, 29% of patients in the group assigned to tight control required 3 or more treatments to lower BP to the desired level. Captopril and atenolol were equally effective in reducing blood pressure to a mean of 144/83mmHg and 143/81mmHg respectively and reducing the risk of macrovascular end points. A similar proportion of patients required 3 or more antihypertensive agents at the end of follow up in the 2 groups. Analysis in this trial was done on intention to treat basis.

All patients with type 2 DM should be treated to attain a blood pressure of <80mmHg diastolic whether it is with an atenolol or ACE inhibitor based regimen. Subgroup analysis of other outcome trials also supports the use of other antihypertensive medication. Thiazide diuretics, which were previously contraindicated in DM, substantially improve the prognosis of patients with hypertension and type 2 DM [94]. In the Syst-Eur trial treatment based on nitrendipine, a dihydropyridine calcium antagonist, also had clear benefit in patients with isolated hypertension and DM [97] (table 1.4, 1.5-hypertension trials).

**Table 1.4****Hypertension Trials**

Trial	UKPDS [90] [91]	HOT [92] [93]	SHEP [94]	SYST-EURO [95] [96]
Drug	Captopril, atenolol	felodipine	chlorthalidone	nitrendipine
Total recruited	1148	18790	4763	4695
No. DM	1148	1501 (8%)	583 (12%)	492 (10%)
No. Women	920 (80%)	8831 (47%)	2715 (57%)	3138 (67%)

**Table 1.5****Hypertension Trials** (Figures are significant unless otherwise stated.)

End Point (% redn)	Total Popn	DM
<u>UKPDS</u>		
Total Mortality	ns	ns
CHD Incidence	21	21
CVA Incidence	44	44
Microvascular Incidence	37	37
<u>HOT</u>		
Total Mortality	9	9
CHD Deaths	15	29
CHD Incidence	53	51
CVA Incidence	45	48
<u>SHEP</u>		
Total Mortality	15	26
CHD Incidence	29	56
CVA Incidence	38	22
<u>SYST-EURO</u>		
Total Mortality	ns	55
CHD Deaths	ns	76
CHD Incidence	26	69
CVA Incidence	38	73

## **1.3 Women and Cardiovascular Disease.**

### 1.3 Epidemiology

Cardiovascular disease (CVD) particularly coronary heart disease is the leading cause of death among women aged 60 years and older [98] [99] [100]. Cardiovascular disease deaths, primarily from CHD outnumber the next 16 causes of death in women combined, including all cancers [101]. Women are 4 to 8 times more likely to die of CVD than of any other disease. Since 1980 death from CVD has declined dramatically in men, whereas it has increased in women. Since 1984 annual CVD mortality in women in USA has exceeded that of men by about 50,000 a year.

It is a well-recognised fact that women live longer than men. This difference is apparent from birth and continues to rise with age especially as fewer women now die in childbirth. In most industrialised countries the largest reason for this sex differential is coronary heart disease. The sex ratio remains almost constant across countries with varying diets, cultural habits and heart disease rates at 2.5-4.5 [102]. This holds even though women in general are more obese have higher blood pressure, higher plasma cholesterol, higher fibrinogen levels and more diabetes than men. There is a gender-mediated difference that could be explained most simply by the hypothesis that oestrogen is protective.

Despite evidence to the contrary a common perception exists that CVD affects mainly men and is not a serious problem for women. This perception has arisen partly from the sex difference at age of first presentation of symptoms. In general, in subjects without DM, CHD begins a decade later in women than men. Women develop angina 10 years later and have their first myocardial infarction (MI) 20 years later than men [98] [103].

An additional reason for the misconception is that chest pain is less likely to be associated with substantial coronary artery disease in women. This has resulted in sex differences in outcomes after diagnosis of angina. In the Framingham study although more women (47%) than men (29%) had angina as a presenting symptom it led to more serious disease in only 19% of women compared to 44% of men [104]. Similarly men were found to have 43% of MI's compared to 29% in women. In the Myocardial Infarction and Intervention Registry (MITI) study [105] and a further multicentre study which examined the numbers of patients presenting with acute chest pain that went onto develop MI, [106] more men had MI's than women.

A possible explanation for this is that chest pain syndromes in women are more likely to be accompanied by normal coronary arteries at angiography. In the Coronary Artery Surgery Study (CASS) 50% of women and 17% of men with chest pain had minimal or no coronary arterial narrowing [107]. Women with chest pain may either not have CHD or if present the prognosis for their CHD is benign. A consequence of this has been that the participation of women in both primary and secondary prevention trials has been minimal until recently.

In addition, exercise treadmill testing is less specific in women than in men thus it has not been utilised as a diagnostic tool as widely in women as in men [108] [109] [110]. The specificity and sensitivity can be greatly increased by the addition of thallium to the treadmill test [111]. Women are also less likely to undergo coronary angiography or percutaneous transluminal coronary angioplasty (PTCA). Among patients with ejection fraction of less than 0.40 after MI enrolled in the Survival and Ventricular Enlargement (SAVE) study, men were twice as likely to have undergone a cardiac procedure prior to the index infarction even though women reported greater functional disability from

angina before the MI [112]. Once a diagnosis of CHD is made in women referral rates for PTCA and coronary artery bypass grafting (CABG) are equivalent [113].

Despite these difficulties, early diagnosis of CHD in women is important as two thirds of sudden deaths occur in women with no CHD history [98]. Women also have a poorer prognosis and more severe outcome after MI, PTCA and CABG compared to men [98]. Women are more likely to die from a first MI than men and for those who survive there is a higher risk of re-infarction and death [98]. In the Framingham Heart Study, 44% of women who had an MI died within 1 year, compared to 27% of men [99]. Some of the sex differences in prognosis from CHD between the sexes can be attributed to the greater age of women at which they develop the disease, as outlined before. Age is a non-modifiable risk factor for CHD hence the importance of early diagnosis is once again reinforced.

### 1.3.2 Lipids

Natural menopause confers a 3-fold increase in CHD risk [99]. In the Nurses' Health Study bilateral oophorectomy lead to an 8 fold increase in CHD risk [114]. After the age of 50 cholesterol levels remain static in men. Levels of low density lipoprotein (LDL) cholesterol increase on average by 0.05mmol/l per year in women from age 40 to 60 in women [115]. At least part of this increase is due to declining oestrogen levels leading to down-regulation of LDL-receptors in the liver [116] [117]. A high LDL-cholesterol level is a strong predictor of CHD risk in women under 65 but its predictive power lessens after this age [118]. At menopause levels of total cholesterol, very low density (VLDL) cholesterol and triglycerides (TG) have also been observed to increase as do small dense LDL cholesterol particles [116] [117]. The increase in the latter, being associated with a 3-fold increase in MI risk [119].

In the National Health and Nutrition Examination Surveys (NHANES), a cross sectional study, high-density lipoprotein (HDL) cholesterol levels were observed to be lower in men than in women and did not appear to change with age [115]. In 2 further longitudinal studies levels of HDL-cholesterol decreased in postmenopausal women [117] [120]. It is now appreciated that low HDL-cholesterol is a stronger predictor of CHD mortality in women than in men especially in women 65 and over [121] [118] [122]. The HDL2 cholesterol subfraction is especially prone to fall after the menopause. This subfraction is thought to be more cardioprotective than either the HDL-1 or HDL-3 sub-fractions [117]. In a study of women over 70 years of age those with a HDL cholesterol of less than 0.9mmol/l had a relative risk of CHD mortality twice that of women with HDL cholesterol levels of 1.6mmol/l and above [122] [123].

The ratio of total cholesterol to HDL cholesterol is also important in CHD risk assessment. In the Framingham Heart Study, the 8 year risk of heart disease was 7% for women with a ratio less than 5, 12% for those with a ratio of 5 to 7 and 20% for those with a ratio over 7 [99].

Increased TG levels may also be important in CHD risk and mortality in women. An analysis of population based prospective studies found a statistically significant increase in the risk of incident CVD of 14% in men and 37% in women after adjustment for HDL cholesterol level and other risk factors [124]. A prospective study of 1405 postmenopausal women carried out for an average of 14 years reported a strong correlation between TG levels and death due to CVD [125]. TG levels over 4.50mmol/l were associated with a greater than 3 fold increase in risk of CVD mortality. Increases in TG levels are important in postmenopausal women especially when HDL cholesterol

falls below 1.03mmol/l [126] [127]. Average or high HDL cholesterol levels appear to attenuate the increase in CHD risk due to elevation of TG levels.

As mentioned earlier, female participation in both primary and secondary cardiovascular prevention trials has been limited. (See tables). Most studies have included men only or a small number of women. Only 2 primary prevention lipid lowering trials have included a large enough number of women without CHD to make analysis meaningful. The first study randomised patients including 1184 women to receive colestipol or placebo. Follow up of these patients was for on average 2 years and a fall of 10% was observed in total cholesterol over this time. Unfortunately no effect was found on CHD mortality. (RR 0.93, 95% CI 0.38-2.26) It can be seen from the wide confidence intervals that this study may not have been powered to show any effect on CHD mortality [128].

The main primary prevention trial including women was the Air Force/ Texas Coronary Atherosclerosis Study (AFCAPS/TexCAPS) (table 1.1-statin trials). Approximately 600 women with average total and LDL cholesterol levels and below average HDL cholesterol levels (mean 1.03mmol/l) were recruited. At the end of the first year of treatment with lovastatin LDL cholesterol had been reduced by 25%, total cholesterol by 18% and triglycerides by 15%. Over the same time period HDL cholesterol levels rose by 6%. All compared to placebo. After an average follow up time period of 5.2 years the risk of acute major coronary events (fatal and nonfatal MI, unstable angina and sudden cardiac death) was reduced by 37% in the treatment group compared to the placebo group ( $p < 0.001$ ). The reduction in risk was greater in the women recruited compared to the men (46% cf. 37%). This difference was not significant due to the small number of events in the female subgroup (7 in the lovastatin group vs. 13 in the placebo group). The trial unfortunately had insufficient power to examine CHD mortality or total mortality [82].

Women were slightly better represented in the major secondary prevention trials. (Table 1.1) In the 4S study 800 women were included in the recruitment to be randomised to receive simvastatin [129]. At the mid point of the trial active treatment had reduced LDL cholesterol levels by 37.4% in women and significantly reduced the risk of the combined end point (all cause mortality, CHD death, non fatal MI or resuscitated cardiac arrest) by 34% in both men and women. The need for PTCA or CABG was reduced by 49% in women (95%CI 0.30-0.86). Total mortality was not reduced in women (RR 1.16, 95% CI 0.68-1.99). There was also no reduction in CHD mortality in the females. These findings reflect a lower total mortality and CHD mortality in the women in the placebo group than the men (6% and 4% cf. 13% and 8% respectively). The authors of the study thought the lower mortality figures in the females could have been due to women being recruited more on the basis of a history of angina alone than their male counterparts and since in women this is not always an indicator of abnormal coronary arteries as previously discussed, this may have affected the outcome data. The importance of considering the sex differences in CHD in men and women when examining trial results which recruit on the basis of angina alone rather than CHD confirmed by angiography or on basis of a confirmed MI is important.

In the Cholesterol and Recurrent Events (CARE) trial [130] 582 women with a confirmed history of MI and mean LDL cholesterol of 3.59mmol/l were randomized to receive pravastatin or placebo. After 5 years of follow up the women in the active treatment group had a 43% reduction in CHD death or nonfatal MI. This is compared to the men in the same group who showed a reduction of only 21%. Although CHD mortality was significantly reduced in men but not in women, women had a greater reduction in nonfatal MI (51%) compared with men (15%). The need for PTCA or CABG was

decreased by 48% and 39%, respectively, in women compared with 17% and 24% in men. Women experienced a 56% reduction in stroke ( $p=0.07$ ).

In the LIPID trial [85] 1532 of the 9014 recruits were women with confirmed angina or MI. After 6.2 years of follow up a non-significant 11% reduction in CHD death or nonfatal MI was reported in the women (cf. a significant 26% reduction in men). Significant outcome data was reported for the whole group but not for the female cohort alone.

A sub-study of the Canadian Coronary Atherosclerosis Intervention Trial (CCAIT) examined the effect of lovastatin over 2 years of treatment on coronary angiograms in women with diffuse coronary atherosclerosis, various coronary risk factors and total cholesterol between 5.69 and 7.76mmol/l. Lovastatin lowered LDL cholesterol by 32% and total cholesterol by 24% compared to the levels in the placebo group. It also decreased the progression of atherosclerosis seen on repeated angiograms [131].

It can therefore be seen that primary and secondary trials of statins have shown substantial benefits in women. Up to 46% reduction in major coronary events has been demonstrated as well as significant benefits in lipoprotein levels.

### 1.3.3 Hypertension

Elevated systolic and diastolic blood pressure confers an increased risk of CHD in both men and women. Women with hypertension have a 4-fold risk of heart disease compared to normotensive women. This compares to a 3-fold increase in men with hypertension [121] [132]. Isolated systolic hypertension in older women, is of particular concern in terms of CHD risk [121]. The prevalence of hypertension increases with age and because

of their survival advantage women with hypertension outnumber men with hypertension in the older age groups. There is an estimated prevalence of hypertension of 60% in women over 45 years of age, (identified as a blood pressure >140/90mmHg or use of antihypertensive medication) [121].

Women have been well represented in the hypertension trials. (Table 1.3) No subgroup analysis has been published in these.

## **1.4 Current treatment of postmenopausal women with type 2 DM**

### **1.4.1 Primary Prevention**

#### **1.4.1.1 Estimating Risk**

In the past investigators with the Framingham Heart Study developed CHD risk equations for use by clinicians in predicting the development of coronary disease in individuals free from disease [133]. These equations reflected the study investigators experience of follow up from 1950 to the mid 1960's [134]. A handbook of these equations was published in 1973 [135]. Further modifications have been made since then taking account of HDL cholesterol levels measured in the Framingham cohort from 1968 and also older age group experience. Data has also now been incorporated from the Framingham Offspring Cohort study. Estimates of CHD risk have now been produced that reflect the approximate combination impacts of total and HDL cholesterol, systolic or diastolic blood pressure, cigarette smoking, diabetes and left ventricular hypertrophy as measured by echocardiography. Risk prediction charts have been drawn up jointly by the British Cardiac Society, the British Hypertension Society, the British

Hyperlipidaemia Association and the British Diabetic Association and management can be adjusted accordingly. From these charts risk is estimated as less than 15%, 15-30% or greater than 30% over the next 10 years.

Blood pressure and serum cholesterol levels exhibit a log linear relation with risk of CHD in prospective epidemiological studies. There seems to be little evidence of a lower threshold. For a given proportional reduction in risk factor a proportional reduction in CHD risk will be seen. The absolute risk reduction i.e. the benefit to the patient will depend on the patient's baseline absolute risk. Evidence from clinical trials has unequivocally shown that individuals with an absolute CHD risk as low as 15% (equivalent to a cardiovascular risk of 20%) over 10 years do benefit from blood pressure and lipid lowering interventions that lower coronary and cardiovascular morbidity and mortality. The cost and logistical implications for such an intervention are prohibitive. Professional bodies have recommended that, at a minimum, individuals with an absolute CHD risk of 30% over 10 years be targeted for comprehensive risk factor management. This should include as appropriate blood pressure and lipid lowering therapy. There is evidence from both local and national audits e.g. the ASPIRE study that the risk factors in this group of patients has not as yet been fully addressed [136]. The exceptions to treatment in the context of absolute CHD risk are patients with malignant hypertension, familial hypercholesterolaemia or other inherited dyslipidaemia or patients with diabetes with associated target organ damage.

#### 1.4.1.2 Treatment of Diabetes

Treatment includes aggressive management of glycaemia. Each 1% reduction in HbA1c is associated with a 21% reduction in diabetes related death and in particular a 14% reduction in MI over 10 years. No lower threshold has been found for this effect [137].

Intensive treatment with insulin in patients with type 1 DM can lead to a 40% reduction in cardiovascular events [138]. In the UKPDS a significant decrease in risk of microvascular but not macrovascular disease in patients with type 2 DM was seen when glycaemia was treated regardless of treatment regimen i.e. insulin or sulphonylurea [139]. The reduction in incidence of MI did reach borderline significance ( $p=0.052$ ) which the researchers felt indicated that the beneficial effect of intensive glucose control outweighed the theoretical risk of the antidiabetic agent. This would suggest that metabolic control of diabetes is important in prevention of CVD. Intensified insulin regimens are known to be associated with a less atherogenic profile of lipoproteins than intensive therapy with oral hypoglycaemic agents [140] [141].

#### 1.4.1.3 Treatment of Hypertension

Collins and MacMahon examined in a meta-analysis 17 trials involving almost 50,000 patients with a mean follow up of 4.9 years. A fall in diastolic blood pressure on treatment of 5-6mmHg was achieved. This was associated with a significant reduction in fatal and nonfatal stroke (38%) and fatal and nonfatal MI (16%). No significant differences were found between the trials examined [142]. All cause mortality was also seen to fall by 12% due to nonvascular deaths being evenly distributed between the treatment groups. A 20-25% reduction in CHD events was expected rather than 16% observed. It may be that most of the randomised, controlled trials of blood pressure treatment on CHD risk provide an underestimate of the reduction due to several factors. These included the fact that the trials were usually short term, relatively low risk patients were recruited to the trials preferentially, those with concomitant disease or end organ damage were excluded generally and up to 25% of patients assigned to the placebo arm

of trials (those with the highest blood pressure) were switched to active therapy. These factors converge to reduce the absolute risk reduction detected [143].

Hypertension can be addressed by lifestyle modification and drug therapy to achieve a diastolic BP <80mmHg and systolic BP <140mmHg [92]. Cardiovascular risk increases across the whole range of blood pressure. Intervention is recommended from a level at which trial evidence has shown intervention reduces cardiovascular risk. Evidence from both observational and randomised trials show that cardiovascular risk is equally strongly linked to either systolic or diastolic blood pressure [5].

#### 1.4.1.4 Lipid Lowering Therapy

Meta-analyses of primary prevention trials have in general shown that reduction of cholesterol leads to a lowering of CHD risk [144] [145] [146] [147] [148] [149]. This is true whether the method of reduction is diet or diet and drugs. It is estimated that for a 10% fall in cholesterol there is a 25% fall in CHD risk [144]. This is equivalent to an average 0.6mmol/l fall in most trials. This fall in CHD risk is achieved after only 2 years on treatment.

It is recommended in the SIGN 40 guidelines that lipid lowering therapy is commenced if 10year CVD risk is >30% when serum total cholesterol is >5mmol/l [150]. Women should be considered for lipid lowering at the same threshold as men.

Triglycerides are an independent risk factor of cardiovascular disease in type 2 DM [151]. Trials are currently underway to assess the reduction in CVD risk by lowering TG levels.

#### 1.4.1.5 Antiplatelet Therapy

The use of aspirin and other antiplatelet drugs had not been recommended for primary prevention of CHD until the results of the Hypertension Optimal Treatment (HOT) trial were published. In this trial low dose (75mg) aspirin was used in conjunction with medication to control blood pressure in patients with atherosclerotic complications or who had target organ damage due to hypertension e.g. left ventricular hypertrophy, proteinuria or renal impairment. The group who received aspirin was seen to benefit more than those who did not [92]. Additional evidence as to the validity of prescribing aspirin for primary prevention has been provided by the MRC thrombosis prevention trial of aspirin and warfarin in which men at high risk of CHD, regardless of presence of hypertension, benefited from aspirin therapy [152]. Antiplatelet therapy, in the form of aspirin in the first instance, is commenced if CVD risk is >20% over 10years [153].

#### 1.4.2 Secondary Prevention

Most of the evidence for prevention in this area comes from trials undertaken after MI. Patients with angina and those following cardiovascular intervention especially percutaneous transluminal coronary angioplasty have not been extensively studied, but where evidence does exist e.g. aspirin in angina or lipid lowering following coronary artery bypass grafting the results are generally consistent with those found post-MI [154] [85].

For married couples there is a concordance for lifestyle and risk factors such as obesity, hypertension lipids and glucose [155]. There is no trial evidence in favour of stopping

smoking following the development of CHD, but observational data shows that the risk of recurrent disease is reduced by ~50% within 1 year of stopping and a favourable effect on mortality is sustained for more than 10 years [156]. In the action on secondary prevention through intervention to reduce events (ASPIRE) study 1 in 5 patients had resumed smoking at follow up [136]. Uncertainty exists about the use of nicotine replacement therapy in patients with CHD, or other atherosclerotic disease as some of the cardiotoxic effects of smoking are attributable to nicotine. A short-term trial of transdermal nicotine in patients with CHD has shown no significant rise in cardiovascular risk [157].

#### 1.4.2.1 Treatment of Hypertension

Raised blood pressure continues to be a risk factor for subsequent cardiovascular events after an MI [158]. Twenty five percent of patients with hypertension also have a history of angina, MI or both [159]. Despite this no clinical trials of antihypertensive treatment in patients with established CHD have been carried out. Results from primary prevention trials are therefore extrapolated to secondary prevention in treatment of hypertension.

The use of  $\beta$  blockers during and after MI leads to a 23% reduction in mortality in survivors [160]. The use of rate limiting calcium antagonists (verapamil and diltiazem) in MI survivors leads to a reduction in all cause mortality as well as deaths from recurrent MI. The benefits are restricted to patients without left ventricular impairment [161].  $\beta$  blockers are the preferred treatment option post-MI and verapamil or diltiazem are recommended if  $\beta$  blockers are contraindicated or not tolerated.

Angiotensin converting enzyme (ACE) inhibitors are indicated for patients following MI, primarily for those with evidence of heart failure in the acute phase and for those in whom there is left ventricular dysfunction i.e. ejection fraction less than 40% [162] [163].

The relationship between death and blood pressure is J-shaped and concern has been raised that lowering of the diastolic blood pressure may lead to increasing rates of death due to CHD especially in those hypertensive patients with co-existing CHD or left ventricular hypertrophy [164] [165]. These concerns were based on the knowledge that maximum cardiac blood flow occurs during diastole. The theory has not been supported by trials of treatment of hypertension in the elderly [94], a proportion of whom were likely to have at least preclinical CHD or in trials of the treatment of heart failure in which low levels of blood pressure were attained [162].

#### 1.4.2.2 Lipid Lowering Therapy

Serum cholesterol and HDL-cholesterol continue to be risk factors for recurrent CHD events after MI [166]. A meta-analysis was conducted, before the major statin trials reported. This included 21 trials employing diet, drugs (clofibrate, gemfibrozil, cholestyramine, colestipol, niacin) or partial ileal bypass surgery. The mean total cholesterol was 6mmol/l and the average reduction achieved was 10%. With this reduction a 10% reduction in mortality was achieved (95% Confidence Interval 3-16%  $p=0.008$ ). There was no effect on non-cardiac mortality [145]. Prior to the statin trials angiographic evidence was also sought for confirmation of effects of lipid lowering. These revealed a significant lowering of the rate of angiographic progression and higher rates of angiographic regression of atheroma regardless of treatment moiety employed [167].

The strongest evidence that lowering of cholesterol is beneficial in patients with existing CHD comes from the 3 main statin trials discussed earlier i.e. the 4S trial, the CARE trial and the LIPID trial. There is strong evidence that following an MI or identification of unstable angina statin therapy should be prescribed for patients with a total cholesterol of 5mmol/l or greater. The benefits for secondary prevention of CHD in patients with cholesterol levels under 5mmol/l have not been established as yet. Similarly the benefit to patients with cerebrovascular disease or peripheral vascular disease is the subject of ongoing trials. In the ASPIRE study 78% of men and 86% of women had a cholesterol concentration of greater than 5mmol/l. Only a minority of patients were on statin therapy and of these 50% had not had sufficient dose to reduce their cholesterol level below 5mmol/l. Another approach based on trial evidence is to reduce LDL cholesterol by 33% in secondary prevention. This will usually be achieved if the statin doses used in the trials are prescribed [168].

#### 1.4.2.3 Antiplatelet therapy

There is no single trial of aspirin used in patients with CHD which give definitive results. A meta-analysis of antiplatelet drug trials shows a 31% reduction in nonfatal re-infarctions, a 42% reduction in nonfatal strokes and a 13% reduction in cardiovascular mortality. Aspirin in the dose range 75-325mg alone is as effective as aspirin in combination with either dipyridamole or sulphinyprazole or at higher dose. It is recommended all patients with established CHD should be prescribed aspirin [154].

Oral anticoagulation with the coumarins e.g. warfarin, in MI survivors lowers the risk of reinfarction, coronary death, and stroke. Due to the considerable side effects these drugs

are usually reserved for those patients with large anterior MI, left ventricular aneurysm, paroxysmal tachycardias, chronic heart failure and systemic embolic disease [169].

### 1.5 Summary

Type 2 DM is caused by an increase in insulin resistance (IR). The cause of this increase is complex and includes presence of increasing FFA's. Type 2 DM is associated with dyslipidaemia (hypertriglyceridaemia and low HDL-c) endothelial dysfunction, a prothrombotic state and hypertension in Reaven's Syndrome. It is also a recognized independent risk factor for cardiovascular disease.

Cardiovascular disease is the leading cause of death in women over 60. Despite this it often remains undiagnosed or misdiagnosed due to misconceptions and difficulties with testing. Women also have a much poorer prognosis from CVD than men. Menopause leads to a 3-fold increase in cardiovascular risk. The fall in oestrogen levels lead to deleterious effects on lipids and endothelium. Women have largely been excluded from the major primary and secondary cardiovascular prevention trials but do seem to benefit from lowering of LDL-c, blood pressure as well as antiplatelet therapy and better diabetic control.

## CHAPTER 2

### HORMONE REPLACEMENT THERAPY (HRT)

In 1936, Mocquot and Moricard described the first clinical use of androgens for the treatment of menopausal symptoms in women. In 1943, Salmon and Geist reported the effects of use of oestrogen therapy combined with either testosterone or methyltestosterone in postmenopausal women. Since then many postmenopausal women have been treated with ovarian hormones in an attempt to alleviate the symptoms of menopause and more recently in the hope of preventing osteoporosis and reducing the risk of ischaemic heart disease. The postmenopausal period has been simplistically considered an endocrine deficiency state and replacement therapy has been seen as restoring the premenopausal endocrine state. None of the available HRT regimens entirely mimic the pattern of hormone secretion prior to menopause. The most frequently used and most studied replacement regimen has been oestrogen alone. The relatively recent addition of progestogen does make the preparations more like those seen physiologically but they may negate some of the clinical benefits of oestrogen alone.

## **2.1 Types of HRT.**

### **2.1.1 Oral Oestrogen**

This is the mainstay of HRT therapy. Oral administration of oestrogen leads to hormone concentrations in hepatic sinusoidal blood that are 4 to 5 times higher than those in peripheral blood [170]. This first pass effect promotes the hepatic synthesis and secretion of several coagulation factors and lipid apoproteins that may or may not be beneficial.

Conjugated equine oestrogen (CEE) is by far the most widely used oestrogen in the USA but not widely used in Europe. Most of the long-term epidemiological data is with this formulation. It is a complex mixture containing mostly oestrone (50%) and equilin

(25%) with small amounts of 17 hydroxyequilin, equilin, 17 $\beta$ -oestradiol and 17 $\beta$ -dihydroequilenin, all in the sulphate ester form. Equilenin has pronounced hepatic effects and a prolonged half-life.

Greater use is made of oestradiol valerate and mixtures of oestradiol, oestrone and oestriol in Europe. Orally ingested oestradiol is metabolized to oestrone within the intestinal mucosa and liver, increasing serum oestrone concentrations. Although oestrone itself is a weak oestrogen it is in reversible equilibrium with oestradiol and thus acts as a source of oestradiol. There is a virtual absence of ovarian oestradiol secretion in postmenopausal women. Oestriol is not converted into oestradiol and has considerably less biological activity [170].

### 2.1.2 Parenteral Oestrogen

Oestrogen can be administered parenterally thus avoiding the first pass effect in the liver. Oestradiol applied to the skin in patches containing the hormone in alcoholic solution is absorbed into the circulation at a steady state for 3 to 4 days [171]. Oestradiol pellets can be implanted subcutaneously. The pellets last for several months but the rate of decline in serum oestradiol concentrations varies widely and can be associated with marked side effects [172]. Direct delivery of oestradiol into the systemic circulation can also be achieved by means of vaginal pessaries or rings or by topical applications of gel.

### 2.1.3 Combined Oestrogen and Progestogen Preparations

Due to the increased risk of endometrial hyperplasia and carcinoma with oestrogen alone most women who have not undergone hysterectomy are treated with a progestogen in addition to oestrogen. Synthetic progestogens are used as progesterone is very poorly

absorbed even in the micronised form. It also causes somnolence in the natural form. Progestogens have some androgenic activity especially 19-nortestosterone derivatives e.g. norgestrel and norethisterone. C-21 pregnane derivatives e.g. medoxyprogesterone acetate, hydrogesterone, medrogestrone and megestrol acetate are very weak androgens [170].

To avoid the continued menstruation that usually accompanies the addition of cyclic progestogen therapy and oestrogen, continuous combined oestrogen and progestogen therapy has been introduced. It is associated with a 40% reduction in incidence of breakthrough bleeding especially in the first 6 months. The only organ that actually needs progesterone is the uterus and so providing it locally by means of a progesterone-impregnated intrauterine device may prove effective and avoid any systemic effects of progesterone.

## **2.2 Characteristics of Women who use HRT.**

The RCGP oral contraception study cohort [173] was an observational study of 23,000 women attending 1400 general practitioners who were using oral contraception and a similar number of age matched control subjects who were followed up from 1968 until 1990. Women were followed regularly and all information on pregnancies, illnesses and death as well as any hormones prescribed for how long and whether the dosage was in accordance with the manufacturer's recommendations. By the end of 1990 approximately 20% of the population had used HRT, 9% were current users. This agreed with a national survey of prescribing habits of general practitioners carried out in 1989, which estimated that approximately 9% of female patients aged between 40 and 64 were using HRT [174]. This is much lower than the figure found in 1976 among the recruits for the American Nurses Health Study where 53% of postmenopausal women had used HRT. This was a

selected population [175]. Two more recent studies carried out in UK in 1995 have shown an increased prevalence of use of HRT with 15-20% of women aged 45-64 now being current users [176] [177].

From these studies it appears that women who use HRT tend to be of higher socio-economic or educational status. They tend to have had a hysterectomy, used the oral contraceptive pill in the past or to have a history of headache, migraine or nonpsychotic psychiatric illness. A history of ischaemic heart disease, hypertension or diabetes made women less likely to receive HRT therapy. The frequency of prescribing in a 1996 review was 24.5% in all women, 21.8% in hypertensive women and only 11.1% in women with type 2 DM [178]. Recent prescribing figures from Ireland have shown a peak of prescribing of continuous combined HRT in April 2002 with 106 age adjusted rate per 1000 of General Medical Services population to a low of 79.6 one year later. [179]

## **2.3 Known Benefits of HRT**

Possible benefits of HRT include decrease menopausal symptoms, prevention of osteoporosis and prevention of MI and CVA. Possible side effects include increased incidence of endometrial carcinoma, increased incidence of breast cancer and increased incidence of deep venous thrombosis and pulmonary embolism.

### **2.3.1 Menopausal Symptoms**

HRT is widely accepted as an effective treatment for symptoms associated with the menopause. Features of oestrogen deficiency e.g. hot flushes, vaginal dryness and atrophy of the breasts respond well to replacement. The vaguer symptoms e.g. loss of libido, loss of self-esteem, depression, weight gain and loss of concentration generally although not always seem to respond less well [180].

### 2.3.2 Osteoporosis.

Osteoporosis is defined as a reduction in bone mass per unit volume. Bone density declines with age as bone resorption exceeds new bone formation and after the menopause this process accelerates. Loss of ovarian function is the single most important factor in the aetiology of osteoporosis, but many others including white race, smoking, low level of physical activity, low dietary intake of calcium and vitamin D and possibly high dietary sodium and protein intake are also important. By the age of 70, 50% of women will have sustained at least one osteoporotic fracture [181]. Of those suffering a hip fracture up to 20% will die as a direct result and many suffer prolonged pain and immobility [182]. It has been calculated that HRT started early in menopause and continued for at least 5 years could reduce by 50% the overall incidence of osteoporotic fracture [183].

Oestrogen therapy protects against postmenopausal osteoporosis, no matter which preparation of oestrogen is used. The first evidence of the beneficial effect of oestrogen on the bony skeleton came from women who had undergone oophorectomy [184]. Oestrogen inhibits bone resorption and therefore prevents bone loss and may increase bone in postmenopausal women. Most studies have used conjugated equine oestrogen (CEE). One long term randomized study used CEE 2.5mg and 10mg of medoxyprogesterone acetate (MPA) for 7 days of each month lead to prevention of

cortical, in this case metacarpal bone, loss if treatment was started 3 years after menopause. In the same study cortical bone was shown to increase by 8% in 10 years if treatment started before 3 years had elapsed [185]. The results of this study suggest that adding a progestogen to oestrogen therapy was not detrimental to the effect. One further study of progestogen alone did show some beneficial effect [186].

The daily dose of CEE required to prevent bone loss has been calculated to be 0.625mg [187], but half of this dose may suffice when it is combined with supplemental calcium [188]. Oestradiol valerate studies have shown a clear dose related bone protection effect [189] and, in one trial, an increase in bone density [190]. Discontinuation of therapy is followed by immediate resumption of bone loss at a rate similar to that in women naive of the preparation [191]. Treatments with percutaneous oestradiol gel, transdermal oestradiol and oestradiol implants (with or without testosterone) have all been shown to cause gains in bone density. Once again a dose response effect is seen [192] [193] [194].

Treatment with oestrogen in postmenopausal women not only prevents bone loss but also prevents vertebral and femoral fractures [195] [196] [197]. From the Framingham Study [198] a long lasting protective effect was achieved against hip fractures when oestrogen was given in the first 4 years following menopause. Treatment should be continued for at least 5 years to obtain significant benefits in terms of reducing fracture risk. This result has been confirmed in computer modeling studies that predicted a 28% decrease in femoral neck fractures if oestrogen is given for 10 years, a 40% decrease with 15 years and a 55% decrease with 20 years of therapy [199]. The reduction in fracture risk exceeds that expected from bone density measurement [200]. It has been estimated that the risk of fractures increases 4 fold for each decrease of 1 standard deviation (SD) in bone mineral density at the hip and that 66% of femoral neck fractures occur in women whose BMD at the femoral neck is below the lowest quartile [201].

The findings of research into the effects of type 2 DM on bone mass are extensive but findings are inconsistent. Researchers have reported lower, equal or greater bone mass in people with type 2 DM relative to control subjects free from DM [202] [203] [204] [205] [206] [207]. The largest study has shown that bone mineral density is decreased in type 2 DM and the level of reduction correlates with both duration of DM and the level of deficit in insulin secretion [208]. Bone turnover in type 2 DM with good metabolic control is believed to be equal to or lower than bone turnover in people without DM [204].

The form of treatment of DM may also be important to bone mass. Women on diet therapy alone have similar bone density to those without DM. In contrast women on oral hypoglycaemics are at higher risk (RR 1.80, 95% CI 1.03-3.16) and women on insulin therapy are at even higher risk of hip fracture (RR 2.66, 95% CI 1.52-4.64) [208]. In addition diabetic complications of retinopathy, neuropathy and angiopathy may influence the fracture event independently of bone mass [210].

Part of the inconsistency in results from trial could be caused by the heterogeneous groups studied (pre-menopausal vs. postmenopausal or different diabetes treatment groups) or by potential confounding by obesity. In animal studies longstanding insulin dependent DM results in osteoporosis probably related to a decrease in osteoblast function [34].

Women with type 2 DM may be theoretically protected from osteoporosis due to their tendency to obesity. This is due to the increased amount of adipose tissue producing metabolically active steroids and insulin related growth factors, which may stimulate bone formation.

## **2.4 Risks of HRT**

### **2.4.1 Endometrial Carcinoma**

Endometrial carcinoma is a common gynaecological malignancy. Studies have investigated various endogenous and exogenous risk factors for endometrial cancer. To date more than 30 observational trials have confirmed that unopposed oestrogen increases the risk of endometrial cancer [211]. The excess risk increases with dose and duration of oestrogen. Ten years of unopposed oestrogen increases the risk 8.2-fold [212]. The increase in risk becomes apparent within 2 years of starting therapy and persists for many years after the preparation is stopped. The tumour induced is usually but not always better differentiated and less invasive and have a much better prognosis than those which occur spontaneously [213] [214].

The excess of endometrial carcinoma is prevented in a dose and duration dependent fashion by the addition of cyclic progestogen therapy [215] [216]. The duration of progestogen administered per cycle is of paramount importance. The administration for 12 days each cycle of minimal quantities of progestogens prevents the development of endometrial abnormalities [217]. The rare endometrial cancer observed in women taking combined therapy (oestrogen plus a cyclic progestogen) may reflect poor compliance with progestogen moiety of regimen [218].

Oestrogen alone also increases the incidence of endometrial hyperplasia which is a premalignant condition. In a trial of 3 years of HRT 33% of women in the unopposed

oestrogen group developed adenomatous or atypical endometrial hyperplasia. Less than 1% of women in the oestrogen plus either MPA or micronised progestogen developed similar hyperplasia [138].

The association of diabetes and increased incidence of endometrial cancer has been reported in many studies. Increased levels of estrogen found in the serum of patients with DM do not give proper explanation of the phenomenon. One study [219] included 148 patients who, due to endometrial cancer underwent surgery. The control group consisted of 212 patients undergoing surgery due to cervical cancer. In the case group there were 18.9% of those with diabetes in comparison with the control group with the percentage of only 6.1. Ki-square test showed a statistically significant difference in the incidence of DM between the case (with endometrial cancer) and control (with cervical cancer) groups ( $p < 0.005$ ). Relative risk (RR) of endometrial cancer was 3.57 for patients with DM in comparison with those without it. From this study it appears that women with DM develop endometrial cancer nearly 3.6 times as often as women without it. Further data from the literature shows RR of endometrial cancer in patients with DM to be between 1.3 and 2.7 [34] [220] [221].

The link between cancer and type 2 DM has been most widely studied in colon cancer. A population based study by Yancik et al [222] found a significant increases in colon cancer risk and overall mortality among patients with type 2 DM (RR 1.37, 95% CI 1.05-1.79) after adjustment for age, sex and tumour stage. The authors concluded that this was mediated through hyperinsulinaemia. Insulin has been shown to be a tumour promoter in animal models [223] and elevated circulating insulin and C-peptide levels have been related prospectively to colon cancer risk [224] [225]. In addition circulating levels of insulin-like growth factor I (IGF-I) which promotes cell proliferation and inhibits apoptosis has been positively associated with colon cancer in several studies. The

relationship between cancer and type 2 DM is likely to be complex as while a hyperinsulinaemic state occurs in the early development of type 2 DM in the later stages a cell depletion leads to a hypoinsulinaemic state [226] [227].

#### 2.4.2 Breast Cancer

One of the main worries regarding HRT therapy has been its association with breast cancer. The relationship between HRT and breast cancer is far from clear with studies reporting decreased, unchanged and increased risk. Results from an Oxford study [228] and the Nurses' Health Study [229] do seem to have some accord. The most recent follow up from the Nurses' Health Study representing 12 years follow up of 480,112 person years of follow up has demonstrated a significantly higher risk of breast cancer among women currently using oestrogen than those who have never used oestrogen. No increase was found between those currently using HRT and those who had taken oestrogen but later discontinued treatment. The increased surveillance of women taking HRT cannot account for this finding. Combined oestrogen and progestogen is also associated with increased risk. Further the effect of oestrogen does not appear to depend on dose used or duration of treatment.

The chance of any women experiencing breast cancer during her lifetime is 1 in 9 and therefore even a slight increase in risk will yield a substantial increase in number of cancers. There is a suggestion that there is a lower mortality due to breast cancer associated with HRT use than that occurring spontaneously [230].

Two meta-analyses of the studies of oestrogen therapy and breast cancer have been carried out. The authors of the first analysis concluded that treatment of postmenopausal women with 0.625mg or less of CBE per day did not increase the risk of breast cancer.

They also concluded that the wide variation in risk in women treated with higher doses implied the presence of other risk factors [231]. In the second analysis the authors concluded that the risk of breast cancer was increased after 15 years of therapy but not before. This study did include pre-menopausal women and several preparations including oestradiol and HRT with and without progestogen. The importance of family history was commented upon [232]. Both analyses included case control studies only and cohort studies were excluded. An exponential curve fitting process was applied to the results which each analysis gained but this may have been flawed as short term data was plentiful and robust compared to any longer term data which was consequently weaker. A third smaller and earlier meta-analysis did conclude that there was no excess risk of breast cancer with oestrogen replacement therapy [233].

WHI[234]

Breast density on radiographs increases in the first year of hormone therapy in approximately one third of women. This makes interpretation of mammograms more difficult [235]. Density of mammography has been found to be a marker for increased risk of breast cancer. Increased breast density is associated with a two-fold increase in risk of breast cancer [236].

Women who have had breast cancer are usually excluded from receiving oestrogen therapy. Small-scale studies suggest that breast carcinoma, contrary to earlier belief, may not be reactivated by oestrogen therapy [237] [238]. Most postmenopausal women with breast cancer are treated with the anti-oestrogen, tamoxifen. Tamoxifen does have some oestrogenic activity especially in bone and has some effects on lipoprotein metabolism.

Overall there is appears to be no apparent increased risk of breast cancer in women who have type 2DM. The outcome in patients diagnosed with breast cancer that have coexisting DM is however poorer than those without. The relative risk of death being

1.76 (95% CI 1.23-2.52), when all other variables are removed [239]. Goodwin et al [240] reported on insulin levels in 512 non-diabetic women with early stage breast cancer. They found that the highest levels of fasting insulin were significantly associated with distant recurrence and death even after adjustment for BMI, age, hormonal receptor status and other prognostic factors for breast cancer.

#### 2.4.3 Prothrombotic Changes.

Short-term oral oestrogen alone or in combination with progestogen results in a procoagulant state in women without DM. The balance between pro-coagulant - anticoagulant shifts to increase the risk of deep venous thrombosis three fold in the first year of exposure [241]. A variety of thrombotic factors are noted to change. Clotting factors such as Factor VII rise as do markers of thrombin formation i.e. prothrombin fragments F1+2 and fibrinopeptide A [242]. While fibrinogen, antithrombin and protein S fall [243] [244]. In addition, oestrogens may also improve platelet function [245].

Four observational studies have provided evidence for an association between postmenopausal HRT and venous thromboembolic events (VTE). In those studies the risk of VTE amongst current users of HRT was 2 to 3.6 times higher than the risk in nonusers [246]. The absolute risk is low being 3 cases per 10,000 treated women per year

## 2.5 HRT and Cardiovascular Risk

### 2.5.1 Hypertension.

Concern has been expressed regarding the use of HRT in women who have co-existing hypertension. Much of this concern is again based on the known association between high dose oestrogen containing oral contraceptives and hypertension. It is well recognized that there is a slight increase in blood pressure in most women taking the oral contraceptive pill, which adds to their relative risk for increased cardiovascular disease [247]. This small rise is enough to raise the blood pressure to 140/90 mmHg or more in approximately 5% of women during a 5-year period. In more than half, the blood pressure returns to normal when oral contraceptive use is stopped [248]. In a few women, severe hypertension occurs, leading to malignant phase hypertension and renal damage [249]. Although an association between malignant hypertension and the oral contraceptive pill is well recognized, the number of cases reported is small, and the clinical course and outcome of the condition in OC pill users are uncertain.

It is uncertain whether oral contraceptives directly cause hypertension *de novo*, or whether they simply exaggerate an existing propensity to develop hypertension. The exact mechanism of oral contraceptive-induced hypertension is uncertain, but changes in circulatory haemodynamics, haemorheological abnormalities, the renin-angiotensin-aldosterone system, insulin sensitivity and erythrocyte cation transport have been identified [250] [251] [252].

The situation with HRT is even less clear as menopause itself is associated with a rise in blood pressure which is independent of the effects of age [253]. There are only three

published studies to date examining the effect of HRT in hypertensive women. Overall in two of the studies, albeit in small numbers [254] [255], systolic blood pressure fell. In the third study Lip et al did not find any significant change in blood pressure over a median follow-up of 18 months in hypertensive women receiving HRT whether it was an oral or transdermal preparation [256]. It was concluded that HRT did not adversely affect blood pressure in this group of hypertensive women receiving HRT whether it was an oral or transdermal preparation.

### 2.5.2 Endothelial Dysfunction.

Oral oestradiol has been shown in women without DM to reduce PAI-1 activity by around 50%. This is explained in part by a reduction in PAI-1 antigen levels [257] [243] and also by a small increase in tissue plasminogen activator (tPA) activity. Research in women with DM suggests that oral oestradiol is associated with a similar fall in PAI-1 activity [258].

Improvement in endothelial dependent vasodilatation has been observed during infusion of  $\alpha$ -oestradiol into coronary arteries of postmenopausal women without DM undergoing angiography [259]. Oestrogen also selectively potentiates endothelial-dependent vasodilatation in forearm resistance vessels [260] and potentiates endothelial-independent vasodilatation in patients with cardiovascular risk factors [261]. This may be related to decreased levels of LDL-cholesterol oxidation leading to increased nitric oxide levels and prostacyclin production and decreased endothelin levels [67] [262]. Oestrogens also have a calcium channel blocking effect which may help to decrease the speed of production of atherosclerotic plaques in coronary arteries [263]. The addition of progesterone does not

appear to attenuate the beneficial effects of oral oestrogen on the endothelium and it has been thought it may even add to its effect [264] [265].

#### 2.5.4 Insulin Resistance

In recent years evidence has emerged to suggest that oral HRT preparations, unlike oral contraceptives, do not adversely affect insulin sensitivity. Two randomised placebo controlled trials [266] [258] have provided promising results. Andersson et al [266] treated 25 women with type 2 DM with 2mg of  $17\beta$ -oestradiol for 3 months in a double blind crossover fashion. As well as conventional exclusion criteria (e.g. thromboembolic disease), patients on insulin therapy were omitted from the study. They observed significant reductions of around 20% in fasting glucose and a 14% reduction in HbA1c in the oestradiol treated group. C-peptide concentrations also fell by around 16% and there was a trend for an increase in whole body glucose disposal. Hepatic glucose production was not assessed in this study. Brussard et al [258] also examined the metabolic effects of 2mg  $17\beta$ -oestradiol in a similar group of patients for a shorter time period (6 weeks), but employed a more straightforward double-blind design, and in addition excluded patients on metformin therapy. Nevertheless the results were consistent demonstrating a smaller (3.5%) but significant reduction in HbA1c. There was no effect of oestrogen replacement on whole body glucose disposal but suppression of hepatic glucose production by insulin was significantly enhanced, particularly in those patients with triglyceride levels less than 2.0mmol/l at baseline. In both studies weight increased slightly but significantly with oestradiol treatment suggesting the improvements in glucose metabolism was unrelated to changes in BMI.

The two studies have also strongly suggested that unopposed oral oestradiol might improve glycaemic control in patients with diabetes. The predominant mechanism for this improvement appears to be enhancing the effect of oestradiol on insulin sensitivity in the liver rather than the periphery. Oral HRT preparations are likely to offer more pronounced effects with respect to glycaemic control than transdermal preparations. Consistent with this possibility, Mosnier-Pudar et al [267] observed no change in plasma HbA1c or fructosamine after 6 months of transdermal oestradiol therapy in women with DM.

In contrast to 'natural' oestrogen, information on the metabolic effects of conjugated oestrogens in subjects with DM is currently lacking. Subjects without DM some deterioration of glucose tolerance and increased plasma insulin concentrations have been seen in those receiving oral HRT containing conjugated equine oestrogen with or without progestogen [268].

#### 2.5.5 Lipids

Data relating to the cardiovascular benefits of HRT had previously been derived largely from observational studies. These studies may have been prone to various levels of bias including selective use of HRT by younger healthy women with healthier lifestyles and a more favourable CHD risk profile [2] [76]. The studies also mainly used unopposed oestrogen alone. The biases in the observational studies would increase the apparent oestrogen benefit. A meta-analysis of 22 randomised trials of short-term oestrogen therapy in which cardiovascular events were given as reason to leave trial or as adverse events while in trial revealed a risk ratio of 1.39 in users compared to non users [269].

This would seem to be unlikely if oestrogen reduces the risk of CVD by 30% as was thought from observational trials [76].

In the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial [270], 875 postmenopausal women were recruited to receive either oral conjugated equine oestrogen (CEE) (0.625mg/day) alone or with medoxyprogesterone acetate (MPA) or micronised progesterone or placebo for 3 years. The group who received CEE alone showed a 10% reduction in LDL cholesterol levels when compared to the control group. The addition of either MPA or progesterone did not alter the effect on LDL-cholesterol levels.

In a study of 31 women by Walsh et al [271] the effect of differing doses of CEE on plasma lipids was examined. A dose of 0.625mg/day lead to a 15% reduction in LDL-cholesterol and 1.25mg/day lead to a 19% reduction in LDL-cholesterol ( $p < 0.05$ ).

Differences between different oestrogen preparations have also been reported. McManus et al [272] demonstrated a 13.8% ( $p < 0.01$ ) reduction in LDL-cholesterol with CEE (0.625mg) ( $p < 0.05$ ) which compared to a 7.8% reduction with oestradiol valerate (1mg) and 12.7% ( $p < 0.05$ ) with oestradiol (2mg) and MPA (1mg). No significant effect on LDL-cholesterol was seen with either oestradiol (2mg) and norethisterone (1mg) or transdermal oestradiol (50 $\mu$ g). All preparations were given for 4 weeks in total. In a longer study of 3 months of treatment LDL cholesterol levels decreased by 15% ( $p < 0.001$ ) with CEE (0.625mg) compared to 14% ( $p < 0.005$ ) due to oestradiol (2mg). Again no effect was seen with transdermal oestradiol (0.1mg twice per week) [269]. Variations in percentages of LDL reduction between studies of HRT have also been attributed to differences in baseline levels in LDL cholesterol in the women recruited [273].

The beneficial reductions in LDL cholesterol seen in trials are often accompanied by a beneficial increase in HDL cholesterol. In the PEPI trial [270] the CEE (0.625mg) group showed a 10% increase in HDL cholesterol during the first year of the study. The effect was slightly diminished over the following 2 years but remained 7% above baseline at completion of the trial. The addition of MPA further decreased the increase in HDL cholesterol to 4%. The addition of micronised progesterin did not alter the effect of CEE alone.

Studies to examine other oestrogen preparations rather than CEE have found a similar effect on HDL cholesterol. The 3 month study by Walsh et al, [271] previously discussed, found that CEE, 0.625mg or 1.25mg, increased HDL cholesterol by 16 and 18% respectively, while micronised oestradiol, 2mg, increased the HDL cholesterol by 15%. Transdermal oestradiol, 0.1mg per week had no effect. The McManus group also examined the effect of differing oestrogen preparations on HDL cholesterol levels and found oral oestradiol valerate (1mg), CEE (0.625mg), or transdermal oestradiol (50µg/day) for 4 weeks increased the HDL cholesterol levels by 7.1% ( $p < 0.01$ ), 6.3% ( $p < 0.05$ ) and not significantly respectively. A substudy of this group among 18 of the trials participants who were non-hysterectomised demonstrated a significant fall in HDL cholesterol levels when norethisterone (1mg) was added to the oral oestradiol (2mg). In contrast, the addition of MPA (5mg) to the oestradiol lead to a non significant fall in HDL cholesterol levels ( $p > 0.05$ ).

A further benefit of HRT on lipid levels is the reduction seen in lipoprotein (a) (Lp(a)) levels. A randomized double blind crossover study [274] of 100 women who had undergone hysterectomy showed a 10% reduction in Lp(a) levels after 6 months of treatment with oral oestradiol (2mg). After 12 months no further reduction in Lp(a) levels were seen. Similarly CEE (0.625mg) for 2 months lead to a 24% decrease in

Lp(a) levels and after 6 months led to a 32% reduction in Lp(a) levels in 2 separate trials [273] [274]. Transdermal oestradiol (50µg/day, twice per week) led to a 10% reduction in Lp(a) levels [274]. Data from the PEPI trial showed that progestins did not effect the Lp(a) lowering effect of CEE [270] [277]. A combination HRT has been shown to have a significant ( $p=0.02$ ) 16% reduction in Lp(a) levels, but only a small percentage of the women studies experienced a drop in their Lp(a) levels to less than 30mg/dl which is thought to be the critical level in CHD risk [278].

The reduction in LDL-C seen with HRT is due to up-regulation of LDL receptor numbers whereas the increase in HDL-C is due to increased apolipoprotein-A1 production, but is also due to the down regulation of lipoprotein lipase in the liver. This explains why topical oestrogen preparations have no effect on lipoprotein levels as hepatic first pass does not occur.

Several studies have shown that HRT results in an increase in triglyceride (TG) levels. A 24% and 38% increase in total TG levels was seen in women receiving 0.625mg and 1.25mg of CEE for 3 months. Oral oestradiol has also been shown to increase total TG levels by 24%. Transdermal oestradiol has not been shown to have any effect on TG levels [270]. Similar results were found in the PEPI trial [270] with CEE alone. The addition of progestins appears to have little effect on TG levels [270] [279]. In a retrospective subset analysis by the Menopause Study Group postmenopausal women with a baseline TG level of greater than 1.808mmol/l upon receiving continuous or cyclical CEE plus MPA experienced a 13.7% reduction in TG levels [280]. This could be an important finding in diabetic women who often have elevated TG levels.

With respect to lipids and lipoprotein levels in type 2 DM, results from the ARIC study, a large randomised controlled trial including women with type 2 DM, suggest that

women with type2 DM have a blunted response to the HDL-raising effects of oestrogen (6% rise vs. 16% in control subjects) and an exaggerated hypertriglyceridaemic response (25% vs. 16%) [281]. Differences in LDL-cholesterol, apo A1 and apo B between hormone users and nonusers in diabetic and non-diabetic women were similar. These results were based upon cross-sectional analysis and unknown selection factors may have influenced the use of HRT in some women. There was no information reported on the formulations (conjugated oestrogens vs. natural oestradiol) and the doses used.

The lipid results from randomised trials of unopposed oestradiol in women with DM however have been more encouraging. These suggest that HDL-cholesterol increases by around 20% in women with diabetes treated with oral oestradiol [266] [282], which is a figure comparable to data from studies in women without DM. Furthermore the rise in HDL was predominantly in the cardio-protective HDL2 subtraction and, as LDL-cholesterol was reduced by 15-24%, the LDL: HDL ratio declined by around one third. Importantly triglyceride concentration increased only marginally and non-significantly in both studies (3-12%), alleviating concerns arising from the ARIC study of an exaggerated rise in this parameter in women with DM. Indeed if anything the changes in triglyceride concentrations were slightly less than those seen previously in studies in women without DM [271] and may reflect an oestradiol-mediated enhanced suppression of hepatic triglyceride synthesis by insulin. That is improved insulin sensitivity similar to the effects on glucose metabolism. In line with the lack of change in triglyceride concentrations, Brussard et al observed no significant change in LDL particle size, in contrast to expectations. Oestradiol replacement also had no appreciable effect on the ability of LDL-cholesterol to be oxidised [282] [283]. There is currently no information on the effects of HRT in women with DM on circulating lipid peroxide and antioxidant concentrations.

A previous study of a combination of transdermal oestradiol and natural progesterone in women with type 2 DM observed no change in plasma levels of cholesterol, HDL-cholesterol, triglycerides and apolipoproteins A1 and B [267].

In the Nurses Health Study [284] the beneficial effects of HRT with respect to cardiovascular disease in women without DM were greatest among those who used combined HRT when compared to women who took oestrogen alone or did not use HRT. Thus the addition of a progestogen which was predicted to negate some of the cardiovascular protection of oestrogen did not appear to do so.

A favourable change in lipoprotein profile was initially thought to be the major determining factor in HRT and cardiovascular risk; however it has become clear that any alteration in lipoprotein profile could explain only 30-50% of the potential vascular benefits seen with oestrogen [2].

#### 2.5.5. HRT and Cardiovascular Trials.

In summary therefore as our study was commenced in early 1998 current understanding at that time was that HRT should be licensed and has proven benefit in the treatment of the menopause and in the prophylaxis and treatment of osteoporosis. HRT could also have a role to play in the prophylaxis of cardiovascular disease although this remained controversial. Observational studies pointed to a reduction of approximately 50% in cardiovascular disease in women taking HRT. Laboratory studies also identified many mechanisms by which HRT could have beneficial effects e.g. improving lipid profiles. However, there remained potential concerns regarding the potential for unmeasured confounding. First, women who took HRT were often healthier, better educated and also more likely to seek medical care. Second, epidemiological study design and

methodology could have increased the likelihood of a favourable outcome being found. Further, the results of these studies were typically reported in terms of relative risk rather than absolute risk i.e. the likelihood of a cardiovascular event in a user was compared to non users rather than the likelihood of a cardiovascular event at all. This is illustrated by examining the results of the Nurses Health Study. This epidemiological study has often been cited as being a well designed study that strongly supports a CHD prevention role for hormones as a large number of subjects were included and the results were comprehensively recorded over a long time period. [284] In the 10 year follow up the relative risk of heart problems in users versus non users was 0.56 (after adjustment for age and cardiovascular risk factors) i.e. a reduction in risk of approximately 50%. Absolute risk was not reported but examination of the data reveals that 405 heart problems were reported in the 48,470 women over the 10 year period. This equates to a rate of heart problems of 0.12% per woman per year or approximately 1% per women over the 10 years in the study. From this it can be seen that 99% of the women studied did not have a heart problem over the 10 years whether they used HRT or not.

In addition prior to 1998 most of the epidemiological studies compared cardiovascular outcomes in users of HRT to non users of HRT. The effects of HRT were not compared to other intervention. Again this can be illustrated by the results of the Nurses Health Study where lifestyle interventions were statistically controlled for to avoid confounding rather than analysed as variables [284]. When Hu et al looked at this in the context of a 31% reduction in CHD among participants in the Nurses Health Study between 1982 and 1992, HRT could account for 9% of this reduction but lifestyle changes accounted for a larger percentage, smoking reduction 13% and improved diet 16% and harmful lifestyle changes had the opposite effect, obesity raising it by 7% [285].

The first large randomized placebo controlled trial of HRT with clinical cardiovascular endpoints was the Heart and Estrogen/progestin Replacement Study (HERS) was published in 1998 [241]. This was a secondary prevention trial. HERS randomised 2763 menopausal women with an intact uterus, up to 80 years of age, with established coronary heart disease manifest as MI, CABG, angioplasty or angina with at least 50% angiographic narrowing of a major coronary artery to 0.625mg of CEE plus 2.5mg of MPA daily or placebo. The primary outcome measure was the combination of nonfatal MI and coronary death. At the end of 4.1 years of follow up, despite the predicted favourable fall in LDL-c concentration and rise in HDL-c concentration there was no difference in the primary outcome in the active group when compared to the placebo group (172 events in treatment group, 176 in placebo group).

The HERS investigators concluded that this HRT regimen did not reduce overall coronary risk in women with established CHD and noted a trend toward an early increase in risk (RR 1.52) and a possible later decrease (RR 0.75) although the CI over this time period was wide and ranged over 0 (0.50-1.13). To ascertain if the possible coronary risk reduction seen in years 4-5 of the HERS trial would persist and possibly lead to an overall decrease in the risk of CHD in the treatment group an open labeled event surveillance study was carried out on 93% of the HERS participants who completed the trial and wished to continue on with HRT (HERS II). This extended the total mean observation time to an average of 6.8 years. At the end of this study again no significant difference in CHD events was observed in the HRT group compared to the placebo group. Adjustments for potential confounders including statin use and selective analysis of women adherent to randomized treatment assignment did not produce significant differences in outcome [286].

Concern was expressed by the HERS investigators over a 2.08-fold increase in venous thromboembolism and a 40% excess of gall bladder disease requiring surgical intervention. Given the lack of CHD benefit this level of harm becomes even more important. Paradoxically the HERS investigators found a decreased incidence of diabetes in the HRT arm of the study (6.2% HRT group vs. 9.5% in the placebo group). The relative hazard of diabetes in the HRT group was calculated as 0.65 (95% CI 0.48-0.89). This remained unchanged after adjusting for baseline demographic characteristics and laboratory and medication variables. The number of patients needed to be treated with CEE+MPA to prevent one case of incident DM was 30(95%CI 18-103) [287]. Any benefit from this would need to be weighed against the adverse events seen by the investigators.

Confirmation of the HERS results from a number of other secondary CHD prevention studies then began to appear. These included:

1. The Papworth HRT Atherosclerosis Study (PHASE) [288] which randomized 225 menopausal women with established CHD to transdermal oestrogen plus progestin versus placebo. No evidence for cardiac prevention was seen. A non significant increase in coronary events was seen in the HRT group with event rates highest in the 2<sup>nd</sup> and 4<sup>th</sup> years of the study. Each year a nonsignificant increase in DVT was also seen for women in the HRT group.
2. The Estrogen Replacement and Atherosclerosis (ERA) angiographic trial [289] randomized 309 women mean age 66 years to CEE or placebo if they had previous hysterectomy and CEE plus MPA or placebo if not. Coronary angiograms were carried out at baseline and after 3.2 years of treatment. No difference in angiographic regression or progression was seen in the HRT versus placebo groups.

3. Women's Angiographic Vitamin and Estrogen (WAVE) trial [290] randomized 423 women with at least 15-75% coronary stenosis at baseline angiogram to CEE, CEE plus MPA or placebo dependent on hysterectomy status and Vitamin C plus E versus placebo in a 2-by-2 factorial design. Follow up was for 2.8 years and at this time repeat angiograms showed nonsignificant worsening of the coronary arteries. Additionally in the HRT group there was a significant increased risk of death and nonfatal MI and a trend to an increase in risk in the Vitamin treated group.
4. The Estrogen in the Prevention of ReInfarction Trial (ESPRIT) [291] randomized 1017 women aged 50-69 who were post MI to oestradiol or placebo and followed them for 2 years. No difference in reinfarction, cardiac death and all cause mortality was seen but 50% of HRT group failed to comply with study medication and 37% of the control group started on HRT.
5. The Women's Estrogen-progestin Lipid Lowering Hormone Atherosclerosis Regression Trial (WELL-HART) [292] randomized 226 women with documented CHD to 17  $\beta$  oestradiol plus sequential MPA or placebo. Mean age of participants was 63. The women had their LDL-cholesterol level reduced to below 130mg/dl with a combination of diet plus statin therapy and a baseline angiogram was carried out. The women were then followed up for an average of 3.3 years and repeat angiograms performed. No significant effect on progression or regression of coronary atherosclerosis was seen in the treatment group when compared to the placebo group.

In 2002 as our study was concluding another pivotal clinical trial on the role of HRT in cardiovascular disease prevention was reported [234]. This was a primary prevention trial which was planned to run until 2005. It was stopped early after 5.2 years of follow up due to health safety concerns, i.e. an increased risk of invasive breast cancer that

exceeded the preset trial stopping boundaries, in addition to a lack of global risk benefit, again based on a pre-established global risk score. This was one arm of the Women's Health Initiative Trial (WHI). This arm of WHI randomized 16,608 women aged 50-79 with an intact uterus to 0.625mg of CEE plus 2.5mg of MPA or placebo. The trial was stopped due to a 26% (95% CI of HR 1.00-1.59) increased risk of breast cancer. Other results included a doubled risk of PTE (95% CI of IIR 1.39-3.25), a 29% increased risk of CHD (95% CI of HR 1.02-1.63 and a 41% increased risk of CVA (95% CI of HR 1.07-1.85). Benefits seen included a 37% decreased risk of colon cancer a 33% decreased risk of hip fracture and a 24% decreased risk of total fracture. No effect was seen on all cause mortality. In clinical terms this would equate to for every 10,000 treated for 1 year with HRT an anticipated 7 further CHD events, 8 further strokes, 8 PTE, 8 invasive breast cancers with 6 fewer colon cancers and 5 fewer hip fractures. From this it can be seen importantly that breast cancer, CHD, CVA and PTE provide almost equal contributions to harm from HRT. The investigators based on these results concluded that HRT should not be recommended for the primary prevention of CHD.

Subsequent analyses of the results have shown that most of the excess CHD risk was of MI rather than angina, revascularisation or heart failure. The excess risk was most apparent (as in the HERS study) in the first year of the trial. The group most at excess risk of MI during this time was the youngest cohort of women aged 50-59 years [293].

Subsequently the oestrogen only arm of WHI was also prematurely halted due to health safety concerns and a lack of benefit in 2004 (planned to run to 2005). Follow up of 10,739 women with hysterectomy for 6.8 years with 0.625mg of CEE or placebo had shown no effect on risk of CHD, with an increase in CVA risk similar to that seen in the CEE/MPA arm of the trial i.e. 12 more CVA for every 10,000 women treated for 1 year.

There was a decreased risk of hip fracture (RR 0.61), a non significant decrease in breast cancer risk (RR 0.77) and a nonsignificant decrease in risk of colon cancer (RR 1.08)

Thus, over the time of our investigations of HRT in diabetes, the evidence for a role for HRT in the prevention of CHD had altered dramatically. At the inception of our trial, HRT seemed, based on observational data and animal studies, to have much to offer women in both primary and secondary prevention of CHD especially in women with increased risk of CHD e.g. women with T2DM. However, around the time of its completion, randomized controlled trials had indicated that HRT should not be commenced or continued for either primary or secondary prevention of CHD. The latter advice is now widely accepted by all national bodies. Women at increased risk for CHD should be given conventional CHD risk reducing agents, and there is not role whatsoever presently for hormonal preparations in CVD risk reduction.

Table 2.1

## HRT and Cardiovascular Events Trials.

Trial	Size (n)	Active agent used	1 <sup>o</sup> or 2 <sup>o</sup> prev <sup>n</sup> .	Age baseline (yrs)	Follow Up (yrs)	No of events	Odds ratio (95% CI) for CHD/CVA	Other comments
WHI [234]	16608	CEE+MPA	2 <sup>o</sup>	63	5.2	CHD 286 CVA 212	CHD 1.29, CVA-1.41 95% confidence intervals needed	Early rise in CHD risk in year 1 (add the OR and 95%CI), later decline
HERS [241]	2762	CEE+MPA	1 <sup>o</sup>	67	4.1	CHD deaths - 242 (C 122, active 132)	CHD 0.99 (0.81-1.22)	93% continue into HERS II trial no sign decrease in CHD events.
ESPRIT [291]	1017	E2	2 <sup>o</sup>	62.6	2.0	Re-infarction / cardiac death 123 (61 C, 62 E2)	Re-infarction/ cardiac death 0.99 (0.70-1.41)	Unopposed E2 employed to avoid influence of progestogens. High drop out rate.
WELLSHART [292]	226	E2 alone & E2+MPA arms	2 <sup>o</sup>	63.5	3.3	Cardiac * 50 (16 C, 16 E2, 18 E2+MPA) Cardiac + revasc n=67 (23 C, 19 E2, 25 E2+MPA)	Cardiac* 1.02 (0.76-1.34)	No effect on progression of coronary atherosclerosis
PHASE [288]	225	transdermal E2 + prog	2 <sup>o</sup>	64	2.5	Angina, MI, cardiac death - 80 (27 C, 53 active)	Angina, MI, Cardiac death 1.49 (0.84-1.95)	Testing Transdermal preparation.
WAVE [290]	423	CEE+MPA VITD+C	2 <sup>o</sup>	65	2.8	Cardiac deaths 22 (14 active, 8 C)	Cardiac death 1.8 (0.75-4.3). Death, nonfatal MI, CVA 1.9 (0.97-3.6)	Potential for harm suggested by both interventions.
ERA [289]	309	E2 alone & E2+MPA arms	2 <sup>o</sup>	66	3.2	Cardiac death 9 (3 C, 4 E2, 2 E2+MPA) MI 19 (7C, 6 E2, 6 E2+MPA) Any CHD event 91 (34 C, 29 E2, 28 E2+MPA)	Cardiac Death 0.99 (0.78-1.32) MI 0.87 (0.64-1.23) Any CHD event-0.86 (0.74-1.24)	No effect on progression of coronary atherosclerosis. No late benefit.

\* - cardiac death, nonfatal MI, Unstable angina, CVA, TIA, DVT, PTE

## **CHAPTER 3**

### **PILOT STUDY**

### **3.1 Introduction**

An open label pilot study of Kliofem, a continuous combined HRT of oestradiol 2mg and norethisterone 1mg was carried out on 10 postmenopausal women recruited from a general diabetic clinic. This was carried out to allow power calculations to be carried out for the main study and also to assess the side effect profile in our chosen group of patients.

## 3.2 Methods

### 3.2.1 Patients Recruited

Ten postmenopausal women with type 2 DM were recruited from the general diabetic outpatient clinics at Glasgow Royal Infirmary University NIIS Trust to the pilot study. The inclusion and exclusion criteria were as for the main study.

Patients were included regardless of their treatment for diabetes whether it was diet, oral hypoglycaemic agents or insulin.

The ethics committee of the Trust approved the study and all women gave informed consent prior to entry into the study.

This was a non-blinded non-randomised study of the oral continuous combined HRT preparation Kliofem (oestradiol 2mg, norethisterone 1mg) carried out over a 6-week period.

Anthropometric measurements and fasting blood samples for lipoprotein and metabolic parameters were obtained at baseline and after 6 weeks of therapy.

### 3.2.2 Laboratory Analysis

The reproductive hormones: oestradiol, luteinising (LH), follicle stimulating hormone (FSH), testosterone and sex hormone binding globulin (SHBG) were measured using

semi-automated 'Immulite' technology (DPC, Los Angeles, USA). Plasma total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol were determined by a modification of the standard Lipid Research Clinics Protocol. The intra-assay and inter-assay coefficients of variation (CVs) for lipid measures were both less than 3%. Fibrinogen, factor VII and factor IX, activated protein C (APC) ratio and tissue plasminogen activator (t-PA) antigen were measured in citrated plasma (0.11 M trisodium citrate; 9:1:vol:vol) as previously described [294][295] [296]. The APC ratio measurement was an APTT-based test rather than a factor-V prediluted test. The intra-assay CVs for these haemostatic mediators were all less than 5%. C-peptide was measured using the DPC Immulite 2000 analyser with a CV of <7%. Plasma glucose was measured using the glucose oxidase method (Glucose Reagent Kit - Olympus AU5200, Olympus Optical Co Ltd).

C-reactive protein concentration was measured using an in-house sensitive double antibody sandwich ELISA as described previously [297]. The assay was linear up to 5mg/l and logarithmic thereafter, and had a lower detection limit of 0.10 mg/l. The inter- and intra-assay coefficients of variation were less than 10% across the range of measured results. Sensitive IL-6 was measured by double antibody sandwich ELISA (R & D systems) with an intra-assay CV of 8%.

### 3.2.3 Statistical Analysis

Both parametric and nonparametric tests were used to assess differences from the baseline. Nonparametric testing was applied to triglyceride, VLDL-Cholesterol and leptin results, as these were not normally distributed unlike the other results.

### 3.3 Results

The baseline values and the effects of treatment on insulin resistance, lipids, lipoprotein profile, fibrinolysis, leptins and androgens are shown in tables 3.1-3.4 respectively.

Mean age of patients at inclusion was 59. Two patients were on metformin, 3 on gliclazide/ glibenclamide and 2 were on diet therapy. The average mean BP was 120mmHg. Mean duration of diabetes was 3 years and of menopause was 9 years. Vaginal bleeding led to the withdrawal of 3 patients prior to completion of 6 weeks of treatment and they were excluded from any further analysis. Compliance was assessed by measurement of plasma oestradiol levels, which were on average 349pmol/l (range 140 to 700pmol/l).

No significant differences were found in the body mass index or blood pressure either systolic or diastolic during the time on treatment or until washout measurements were made. The body mass index decreased by 0.5% during treatment and continued to fall during the washout period. Neither decrease reached a significant level. The waist to hip ratio and blood pressure levels fell, but again the change did not reach significance.

The levels of HbA1c and C peptide decreased by a significant amount during the time on treatment ( $p=0.02$ ,  $p=0.05$  respectively). This was not sustained off treatment.

**Table 3.1****Baseline Characteristics/Insulin Resistance in Pilot Trial**

	Baseline	6weeks	washout	p treatment	p washout
BMI(Kg/m <sup>2</sup> )	30.04(4.98)	29.98(4.52)	29.77(4.9)	0.9	0.4
Waist:hip ratio	0.79(0.11)	0.82(0.09)	0.85(0.09)	0.2	0.2
Systolic BP(mmHg)	120(14)	117(9)	116(14)	0.49	0.31
<b>Insulin/Glycaemia</b>					
HbA1c	8.73(1.57)	7.82(1.93)	8.21(1.81)	0.02	0.2
C peptide	1.22(0.35)	0.99(0.99)	0.98(0.41)	0.05	0.16

**Table 3.2****Lipids**

	Baseline	6weeks	washout	p treatment	p washout
Cholesterol (mmol/l)	5.4(1.82)	4.79(1.34)	5.21(1.68)	0.05	0.4
LDL-C (mmol/l)	3.61(1.4)	3.34(1.13)	3.35(1.39)	0.4	0.35
IDL-C (mmol/l)	0.95(0.25)	0.89(0.15)	1.06(0.22)	0.25	0.05
LDL/HDL	3.91(1.66)	3.72(1.09)	3.57(1.67)	0.67	0.22
Triglyceride(mmol/l)	2.34(1.17)	1.97(0.83)	2.07(0.82)	0.09	0.4

**Table 3.3****Haemostatic Indices**

	Baseline	6weeks	p treatment
Factor VII (IU/dl)	175(45.3)	135.7(30.9)	0.001
Fibrinogen (g/l)	3.51(0.51)	3.72(0.59)	0.1
vWF	153.9(50.6)	142(49.3)	0.22
Leptin	24.55(14)	22.55(10.5)	0.93

**Table 3.4****Androgens**

	Baseline	6weeks	washout	P treatment	p washout
Testosterone	1.71(0.41)	1.41(0.55)	1.49(0.52)	0.07	0.33
FAI	7(4.3)	4.4(3.4)	6.6(4.4)	0.03	0.57

Total cholesterol levels fell to a significant degree while on treatment ( $p=0.05$ ). This is seen to be a result of the cumulative fall in LDL-cholesterol, HDL-cholesterol and VLDL-cholesterol all of which were seen to fall but levels did not reach conventional research significance of 0.05. VLDL-cholesterol did fall to a significance of  $p=0.1$ . The same level of significance was seen in the level of fall of triglyceride levels. These changes were not sustained off treatment. In contrast HDL-cholesterol levels rose significantly after treatment was discontinued ( $p=0.05$ ). Ratio of LDL/HDL-cholesterol did not change significantly over either period in the study.

Factor VII levels fell by 22.5% during treatment ( $p=0.001$ ). No correlation between the fall in factor VII levels and triglyceride levels was found. Fibrinogen levels rose to some degree of significance ( $p=0.1$ ). Leptin and von Willebrand factor levels did not change to a significant degree during treatment.

Free androgen index decreased significantly while on treatment ( $p=0.03$ ) compared to off treatment when no significant reduction was found. Testosterone also decreased while on treatment but not to such a degree of significance.

### 3.4 Discussion

This study demonstrated that in the short term, in a small number of patients, an oral combined HRT preparation resulted in an improvement in control of type 2 diabetes as indicated by the decrease in HbA1c and C peptide. The fall in HbA1c and C peptide of 10% and 19% respectively are in line with studies carried out previously in postmenopausal women with type 2 diabetes using oestradiol alone [282] [266].

The waist to hip ratio was seen to rise in this study while on HRT therapy. The BMI in contrast fell during the study. However neither result reached significance.

The lowering of the total cholesterol reflects the lowering of LDL-cholesterol. This is thought to be due to oestrogen induced up regulation of LDL receptors in the liver. The drop in LDL-cholesterol in this sample did not reach significance. The fall in HDL-cholesterol while on treatment was balanced by the fall in LDL-cholesterol as indicated by the non-significant fall in the LDL/HDL-cholesterol ratio. The rise anticipated in HDL-cholesterol from studies in non-diabetic women was not seen. The results we observed were a balance of the oestrogenic increase in HDL-cholesterol and the progestogen induced reduction in HDL-cholesterol. The more androgenic norethisterone is seen to predominate. The observed change in HDL-cholesterol may also reflect observations in the ARIC study that in diabetic women the oestrogenic response is blunted [281].

Oestrogens are known to increase triglyceride, and in turn, VLDL-cholesterol synthesis. However androgenic progestogens antagonise this effect and reduce triglyceride synthesis in the liver. This response is seen to predominate in our study. The results observed did

however allay our concerns regarding the hypertriglyceride response experienced in previous studies [281].

The changes in factor VII levels seen in our study are not as anticipated. Factor VII is widely associated with CHD and in the HERS study the observed early increased risk of CHD events was thought to be due to an immediate prothrombotic effect [241]. This effect was likely to be due to a rise in Factor VII and fibrinogen. While a rise in fibrinogen of some significance was seen, the factor VII levels were seen to fall to a greater extent. No correlation was found between the fall in triglyceride and factor VII levels

This study revealed that HRT was safe for short-term use in postmenopausal women with type 2 DM. The high dropout rate allowed us to make some changes to our study design in particular a lower dose preparation was chosen to lessen the side effects experienced by participants.

### 3.5 Power Calculations

From the pilot study it was known that the minimum clinically significant difference for changes in LDL-cholesterol was 0.38mmol/l. Large studies into oestradiol replacement therapy have shown that the standard deviation of changes in LDL-cholesterol are approximately 0.48mmol/l. For the main study an 80% probability of detecting the above difference at a 5% significance level was required. Calculations using these figures revealed a standard difference of 1.26. Tables were then used to calculate that 36 patients would be needed for the study i.e. 18 patients in each group. A drop out rate of 20% was allowed for (as this had been the figure seen in the pilot study) and so it was anticipated that 44 patients in total would need to be recruited to allow the main study to have the strength required to show a significant difference in LDL-cholesterol the main outcome measure.

## CHAPTER 4

### METABOLIC, INFLAMMATORY AND HAEMOSTATIC EFFECTS

#### 4.1 Introduction

Until the publication of the Heart and Oestrogen/Progestogen Replacement Study (HERS) [241] and more recently of the Women's Health Initiative (WHI) study [234], many women and their physicians were convinced of the cardio-protective effects of Hormone Replacement Therapy (HRT). In HERS, women with established CHD were randomised to 0.625 mg/d conjugated equine oestrogen (CEE) plus 2.5 mg/d medroxy-progesterone acetate (MPA)) or matching placebo. The HRT group experienced an elevation in coronary heart disease (CHD) risk in the first year of use and no overall difference in events over four years [241]. The WHI used the same preparation in a primary prevention setting and also reported an increased risk of CHD and of stroke in the active arm compared to placebo [234], and more recently a potential deleterious effect on cognitive function [287]. Therefore, despite a beneficial effect of this preparation on LDL and HDL cholesterol concentrations, other effects may be unfavourable; the search for these is receiving intense scrutiny. Most current attention has focused on possible triglyceride-raising, pro-coagulant and pro-inflammatory effects of CEE and 2mg oestradiol containing HRT's [299] [243] [300] [244] [301] [302] but data on preparations containing low dose oestradiol combined to norethisterone, particularly from randomised placebo-controlled trials, are sparse.

Women with type 2 diabetes have a markedly elevated baseline risk for CHD. A recent report from a prospective observational study [303] suggested that HRT use leads to a significantly increased risk of death from all causes and ischaemic heart disease among women with diabetes. Current users of HRT with diabetes had a near ten-fold increased risk of myocardial infarction (9.2, 95% CI, 2.0 to 41.4) compared with never users with

diabetes. By contrast, Ferrera et al [304] noted that among women with diabetes who did not have a recent myocardial infarction, current HRT use was associated with a significant 16% lower risk of acute myocardial infarction. Thus HRT effects on CHD risk in diabetes are controversial and randomised trials are required.

HRT preparations are not homogeneous with respect to metabolic effects: metabolic actions are profoundly altered according to route of delivery, dose and chemical nature of the combined oestrogenic and progestogenic preparations [305]. Data from trials with differing designs suggest that lower doses of oestradiol (1mg) or transdermal preparations may have fewer deleterious and perhaps even beneficial effects on inflammatory and haemostatic pathways [302] [306][307] [301][308]. In addition, there is an increasing awareness that androgenic progestogens such as norethisterone may offer several advantages over MPA particularly with respect to coagulation and inflammatory parameters [307] [308].

The aim of the present randomised double-blind placebo-controlled study therefore was to examine the metabolic effects of a novel continuous combined preparation containing 1mg oestradiol and 0.5 mg norethisterone in women with type 2 diabetes. We comprehensively assessed key pathways, including lipids and glycaemic parameters, and haemostatic and inflammatory pathways, known to be influenced by hormonal regulation and relevant to CHD risk. The hypothesis was that this low dose oestradiol preparation combined with norethisterone would continue to reduce LDL-cholesterol, limit any triglyceride rise and would have fewer potentially adverse effects on key coagulation and inflammatory parameters than observed with conventional CEE/MPA-based preparations.

## 4.2 Methods

### 4.2.1 Subjects Recruited

From December 1998 to September 2000, 50 women with type 2 diabetes aged under 70 years of age were recruited from general diabetic clinics in Glasgow Hospitals. Women randomised were clinically and biochemically postmenopausal i.e. at least one year since last menses and a follicle stimulating hormone (FSH) concentration of greater than 20 IU/l. Menopause could be either natural or surgically-induced. A normal pelvic examination and mammogram within the year prior to inclusion in the trial was also required.

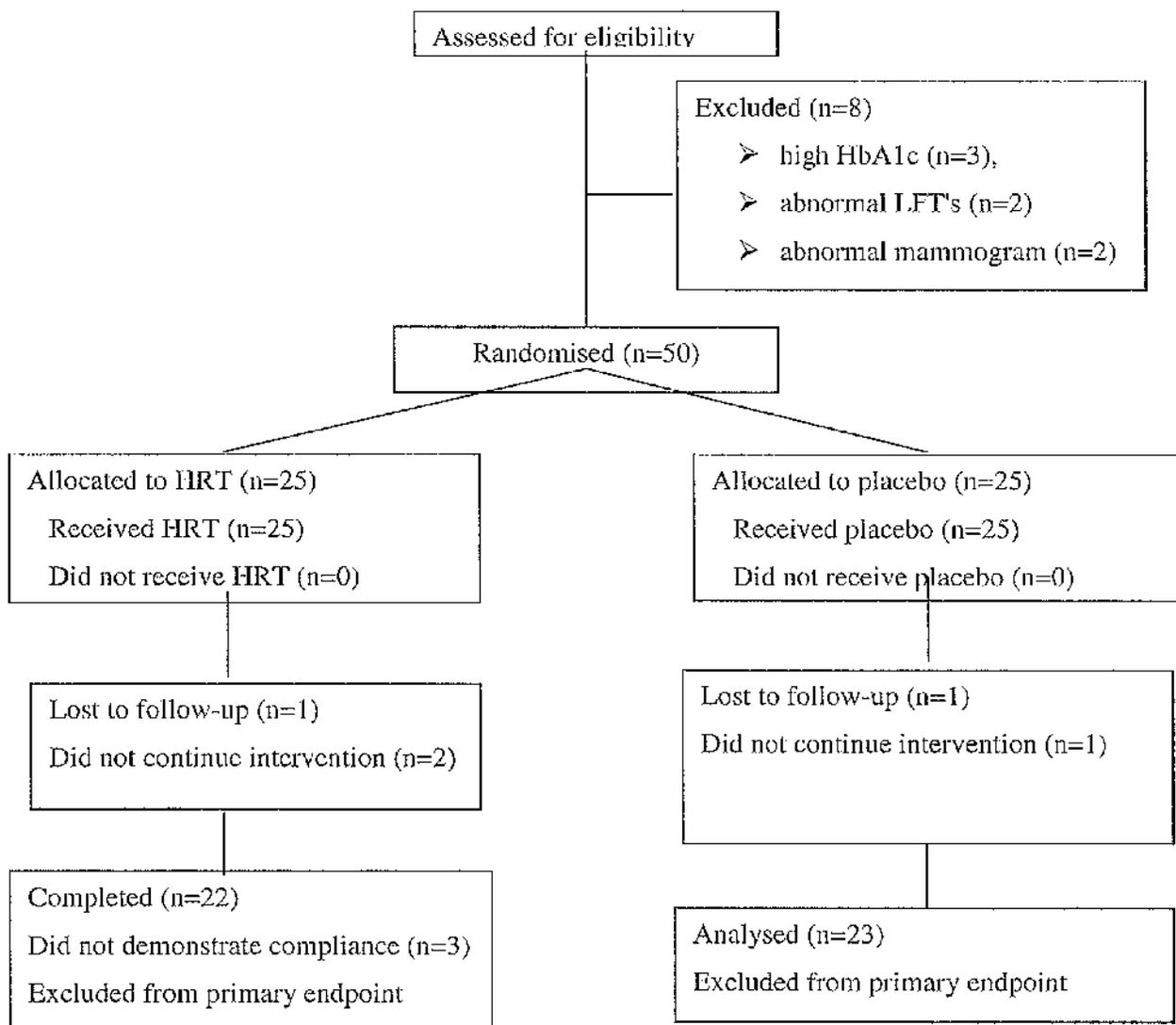
Exclusion criteria comprised: poor glycaemic control; severe hypertriglyceridaemia ( $>10$  mmol/l); moderate to severe hypertension (systolic  $>160$  mmHg, diastolic  $>110$  mmHg); renal impairment (serum creatinine greater than twice the upper limit of normal range); liver disease (serum transaminases and bilirubin greater than twice the upper limit of normal range); or established cardiovascular, cerebrovascular, or peripheral vascular disease. Subjects with either a personal history of - or first-degree relative with - breast cancer were excluded.

Women eligible at screening were randomised to prescription of either active medication (1mg oestradiol plus 0.5mg norethisterone) or identical placebo daily for six months. Randomisation was effected in blocks of four using computer-generated numbers. Compliance was assessed by returned medication packs at the final visit and by oestradiol and gonadotrophin concentration measures at the final visit. A requirement of oestradiol to rise by more than 10pmol/l and FSH to decline by more than 5 U/L was pre-defined.

The participating hospitals' local research ethical committees granted approval. All subjects gave written informed consent to a single investigator (JM). As far as possible, existing medications for glycaemic control, blood pressure or lipid lowering (detailed in Table 4.1) were not altered throughout the course of the study.

We determined that our sample size had 80% power to detect a 5% reduction in LDL-cholesterol and 90% power to detect a 5% reduction in factor VII with  $\alpha=0.05$ .

**Figure 1: Trial Outcome Flow Chart**



**Table 4.1.**

Baseline characteristics of study groups. Mean (SD) reported.

	Active	Placebo
Age (years)	60.7 (5.5)	61.3 (4.8)
BMI (Kg/m <sup>2</sup> )	30.5 (6.5)	29.8 (5.61)
Waist circumference (cm)	93.9 (11.3)	93.7 (13.6)
Years post menopausal	14.6 (8.5)	14.2 (6.3)
Smokers (Y/N)	6/19	5/22
Systolic blood pressure (mmHg)	152 (17)	151 (21)
Diastolic blood pressure (mmHg)	87 (8)	83 (9)
Medications		
Diet alone (n)	5	3
Oral hypoglycaemics (n)	10	11
Insulin (n)	4	9
Anti-hypertensives (n)	10	10
Lipid-lowering agents (n)	5	5

#### 4.2.2 Study visits

Women attended the Diabetes Centre, Glasgow Royal Infirmary, Glasgow at baseline and 6 months, having fasted for 10 hours and avoided heavy exercise, alcohol and caffeine in the preceding 24 hours. Subjects rested prone for 15 minutes prior to blood pressure recordings being taken in triplicate (mean recorded). The women also had anthropometric measurements to include height (cm), weight (kg), and waist and hip circumferences (cm). From these body mass index (BMI) was calculated as  $\text{weight}/(\text{height})^2$  in  $\text{kg}/\text{m}^2$ , as well as waist to hip ratio (WHR).

#### 4.2.3 Laboratory methods

The reproductive hormones: oestradiol, luteinising (LH), follicle stimulating hormone (FSH), testosterone and sex hormone binding globulin (SHBG) were measured using semi-automated 'Immulite' technology (DPC, Los Angeles, USA). Plasma total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol were determined by a modification of the standard Lipid Research Clinics Protocol. The intra-assay and inter-assay coefficients of variation (CVs) for lipid measures were both less than 3%. Fibrinogen, factor VII and factor IX, activated protein C (APC) ratio and tissue plasminogen activator (t-PA) antigen were measured in citrated plasma (0.11 M trisodium citrate; 9:1:vol:vol) as previously described. The APC ratio measurement was an APTT-based test rather than a factor-V prediluted test. The intra-assay CVs for these haemostatic mediators were all less than 5%. C-peptide was measured using the DPC Immulite 2000 analyser with a CV of <7%. Plasma glucose was measured using the glucose oxidase method (Glucose Reagent Kit - Olympus AU5200, Olympus Optical Co Ltd).

C-reactive protein concentration was measured using an in-house sensitive double antibody sandwich ELISA as described previously [308]. The assay was linear up to 5mg/l and logarithmic thereafter, and had a lower detection limit of 0.10 mg/l. The inter- and intra-assay coefficients of variation were less than 10% across the range of measured results. Sensitive IL-6 was measured by double antibody sandwich ELISA (R & D systems) with an intra-assay CV of 8%.

#### 4.2.4 Statistical analysis

Mean differences in changes from baseline between the two treatment groups were compared using the unpaired t-test: the 95% confidence interval for change in active group data relative to change in control group data are presented. Adjustment for baseline concentrations was made by linear regression. Baseline data are presented as mean and SD or median and interquartile range (IQR) for parameters exhibiting skewed distribution.

## 4.3 Results

### 4.3.1 Subjects recruited

58 women were screened as potential recruits into the study. Of these, 50 were randomised (Figure 1). The eight women not entered had unacceptably high HbA1c (n=3), abnormal liver function tests (n=2), abnormal mammogram (n=2) or abnormal pelvic examination (n=1). Five women did not complete the study due to either relocating or personal reasons. Thus, 45 women completed the study. Of the 22 in the active group, 19 demonstrated adherence to study medications by predetermined criteria. Data from women falling outside these criteria were omitted from subsequent analyses. There were no serious adverse events. Breast tenderness and breakthrough bleeding were reported by three women on active treatment and by none on placebo.

Table 4.1 demonstrates the baseline characteristics of women completing the study. The two groups allocated to treatment were similar in age, BMI, blood pressure and years since menopause. All categories of diabetes therapy were represented and similar percentages were taking anti-hypertensive or lipid-lowering agents. The treatment groups also showed similar baseline hormonal and lipid concentrations (Tables 4.2 and 4.3)

**Table 4.2.**

Sex hormone changes in active and placebo groups. Baseline data are given as mean (SD) or median (interquartile range).

	Active Baseline	Mean Change	Placebo Baseline	Mean Change	*Difference (95% CI)	p
LH (IU/l)	35 (26-39)	-23.7	37 (24-44)	-0.4	-23 (-32 to -15)	<0.001
FSH (IU/l)	53 (49-83)	-41.8	60 (48-81)	-2.5	-39 (-50 to -28)	<0.001
Oestradiol (pmol/l)	63.3 (15.9)	+165	65.4 (14.6)	2.0	163 (111 to 215)	<0.001
Testosterone (nmol/l)	1.23 (0.63)	-0.12	1.19 (0.33)	0.0	-0.12(-0.42 to 0.17)	0.41
SHBG	33 (21-52)	+15.7	37 (27-49)	+1.4	14.3 (0.52 to 28.1)	0.042

LH = luteinising hormone, FSH = follicle stimulating hormone, SHBG= sex hormone binding globulin

\* difference is change active relative to change in placebo

**Table 4.3**

Lipids and insulin/glycaemia changes in active and placebo groups. Baseline data are given as mean (SD) or median (interquartile range).

	Active baseline	Mean Change	Placebo baseline	Mean change	Difference	p
Cholesterol (mmol/l)	6.02 (1.07)	-0.62	5.68 (0.97)	-0.13	-0.49 (-0.05 to -0.90)	0.020
LDL-C (mmol/l)	4.14 (0.93)	-0.55	3.80 (1.00)	-0.10	-0.44 (-0.79 to -0.08)	0.018
HDL-C (mmol/l)	1.30 (0.32)	-0.07	1.36 (0.29)	-0.06	0.01 (-0.10 to 0.10)	0.83
Chol:IDL-C ratio	4.88 (1.58)	-0.28	4.46 (1.53)	+0.20	-0.48 (-0.99 to 0.00)	0.05
Triglyceride (mmol/l)	1.75 (1.15- 2.35)	-0.034	1.68 (1.25- 2.21)	+0.16	-0.19 (-0.58 to 0.19)	0.31
C-peptide (nmol/l)	0.97 (0.65- 1.29)	-0.18	0.79 (0.49- 1.51)	0.09	-0.27 (-0.44 to -0.09)	0.003
Glucose (mmol/l)	12.4 (4.2)	-1.74	11.3 (3.2)	0.42	-2.16 (-4.06 to -0.28)	0.026
HBA1c (%)	10.2 (1.8)	-0.37	10.2 (1.3)	0.22	-0.59 (-1.45 to 0.27)	0.17

#### 4.3.2 Sex Hormone Changes.

A significant reduction in gonadotrophin concentrations and an elevation in oestradiol (all  $P < 0.001$ ) and sex hormone binding globulin (SHBG) levels ( $P = 0.042$ ) was observed in those women randomised to active therapy as a group (Table 4.2). Total testosterone was not altered significantly but free androgen index fell significantly ( $p < 0.001$ , data not shown).

#### 4.3.3 Lipid and Insulin/Glycaemic Changes

Table 4.3 demonstrates Lipid and glycaemic changes in the groups. Both total (10%) and LDL cholesterol (13%) concentrations were reduced significantly with active treatment ( $p < 0.05$ ) but HDL cholesterol and triglyceride were not altered. Similarly, fasting C-peptide was reduced by 19% ( $p < 0.01$  vs change in placebo). Fasting glucose was also reduced ( $p < 0.05$ ) in the active arm as was HbA1c but the latter change did not reach significance ( $p > 0.10$ ).

#### 4.3.4 Haemostatic and Inflammatory Changes

Haemostatic and inflammatory variables are presented in Table 4.4. Significant reductions in Factor VII levels ( $p < 0.001$ ), and t-PA antigen and IL-6 concentrations ( $p < 0.02$ ) were observed without significant alteration in factor IX, APC resistance (APC ratio), fibrinogen or CRP concentrations.

**Table 4.4**

Haemostatic and inflammatory changes in active and placebo groups. Baseline data are given as mean (SD) or median (interquartile range).

	Active baseline	Mean Change	Placebo Baseline	Mean Change	Difference	p
Factor VII (IU/dl)	160 (36)	-26.7	152 (31)	5.50	-32 (-43 to -21)	<0.001
Factor IX (IU/dl)	163 (42)	+7.00	155 (40)	+3.60	3.7 (-9.9 to 16.3)	0.49
APC Ratio	2.74 (0.6)	+0.25	2.74 (0.5)	+0.23	0.0 (-0.25 to 0.25)	0.99
tPA-antigen (ng/ml)	14.9 (5.6)	-2.01	12.7 (3.8)	+0.97	-2.98 (-5.00 to -0.95)	0.005
Fibrinogen (g/l)	3.91 (0.68)	+0.02	3.89 (0.92)	-0.12	0.14 (-0.19 to 0.47)	0.39
CRP (mg/l)	5.05 (4.46-8.53)	+1.15	3.37 (1.76-8.10)	+0.72	0.73 (-2.27 to 3.72)	0.62
IL-6 (pg/ml)	3.46 (2.48-4.89)	-0.32	3.55 (2.11-4.47)	+1.10	-1.42 (-2.55 to -0.29)	0.015

Significance values were also checked with adjustment for baseline concentrations of all parameters measured. The results were in keeping with unadjusted values: specifically, reductions in cholesterol (adjusted  $p=0.032$ ), factor VII (adjusted  $p<0.001$ ), tPA-antigen (adjusted  $p=0.01$ ) and IL-6 (adjusted  $p=0.045$ ) were similar and CRP remained similarly unchanged (adjusted  $p=0.96$ ).

Finally, we determined the number of patients randomised to HRT or placebo that achieved 10% decline in LDL cholesterol and factor VII concentration using an intention to treat analysis. A 10% decline in either parameter is clinically relevant. Ten of the 25 in the HRT group had >10% decline in LDL cholesterol compared to only 4 in the placebo group ( $p=0.059$ , Chi-square test). For factor VII, 16 of the 25 in the HRT group had >10% decline in factor VII whereas only 1 of the 25 in the placebo group did so ( $P<0.0001$ ).

#### 4.4 Discussion

Our study is one of the very few randomised double-blind placebo-controlled trials of HRT in diabetic women, albeit in an older group than those who would normally receive HRT. More importantly, it is the largest study to date to examine metabolic actions of a novel continuous combined preparation containing 1mg oral oestradiol and 0.5mg norethisterone in a high CHD-risk population. The key results were lowered plasma LDL cholesterol, Factor VII, t-PA antigen and IL-6 concentrations, and statistically similar triglyceride, Factor IX, APC resistance and CRP levels. This pattern of effects differs markedly from the profile produced by normal dose HRT containing CEE and MPA used in HERS and WHI which increase triglyceride, factor VII and promote a doubling in CRP concentrations [241] [299]. As a result, an HRT containing low dose oestradiol and norethisterone may be more suitable for women who have an elevated risk of CHD (e.g. those with type 2 diabetes), who require HRT for menopausal symptom relief or bone protection. Formal clinical trials are required to test this suggestion.

The potentially better portfolio of metabolic effects of the HRT in this study may result either from the use of a low oestradiol dose or from the use of a more androgenic progestogen, norethisterone. More likely is that the balance of effects deriving from this HRT lean more towards androgenic rather than oestrogenic actions. This is an important point since conventional wisdom has dictated the use of non-androgenic progestogens to minimise any HDL-cholesterol reducing effect [299]. Clearly, this course of action needs re-evaluation as HDL-cholesterol was unchanged in the present study.

With respect to the inflammation cascade, HRT's containing 2mg oestradiol or CEE elevate CRP concentrations almost two-fold [300] [294]. In the observational arm of the

(large) WHI, current HRT use was associated with higher CRP but, interestingly, similar IL-6 levels in those women not taking HRT [309]. Moreover, transdermal delivery of oestradiol appears not to be associated with elevation of CRP [294] [306] [296] which therefore may represent a “first pass” effect on hepatic CRP synthesis [302]. Since elevated CRP levels in men and women are independently linked to risk for CHD and stroke [310], the HRT-induced CRP rise has been put forward as a potential pathway explaining the results of HERS and WHI [234] [241]. It should be acknowledged, however, that even though vascular literature suggests several mechanisms whereby CRP may be directly atherogenic [310], it is not yet known whether the HRT-induced CRP rise leads directly or indirectly to a biologically adverse outcome.

Our data demonstrate a lack of significant rise in CRP in those assigned active therapy compared to the placebo group ( $p=0.62$  crude,  $p=0.96$  adjusted difference). Although power may be an issue here, studies in similar size to the present one, but employing CEE- or 2mg oestradiol-based preparations, have demonstrated significant, near two-fold elevations in CRP [301] [311]. A lack of CRP rise in the present study concurs with results from two other studies in non-diabetic women that used lower oestradiol doses (1mg) [301] [312]. Moreover, HRT combining oral norethisterone with transdermal oestradiol may lower CRP [308]. Any tendency to an oestradiol-induced CRP elevation in the present study may have been attenuated by the oral norethisterone. The reduction in IL-6 concentration is of interest as androgens exhibit anti-inflammatory effects in several tissues [313]. Because of the variability of circulating inflammatory markers and the wide confidence interval in the result reported in this and studies of similar size [314] [315], larger studies using low dose HRT preparations are now urgently required to confirm our findings. In this respect, a potentially lower CHD risk with lower doses of

HRT has recently been suggested by Ferrera et al [304] in their analysis of data from the Northern California Kaiser Permanent Diabetes Registry.

That triglyceride did not rise in this study is also relevant to CHD risk. Oral oestrogens, particularly CEE-based preparations, significantly increase circulating triglyceride concentrations by increasing hepatic synthesis of triglyceride-rich particles [305]. Increases in triglyceride concentration may enhance plaque instability by affecting platelet and endothelial function, as well as altering coagulation and vascular inflammation [316] [317]. Triglyceride concentration is independently linked to CHD risk, particularly in women [318]. Indeed, the HERS investigators speculated that the 10% rise in triglyceride concentration in their study may have contributed to the early increase in CHD events despite the positive changes in other lipid parameters [241]. In general, increase in triglyceride concentration is less pronounced with oral oestradiol-containing HRT's compared to CEE-based HRT's and absent with transdermal delivery [305]. Moreover, androgens decrease triglyceride concentration [305] [307], thereby opposing any tendency for an oestradiol-mediated increase.

Consistent with the reduction in factor VII coagulation activity (by 17%) with active treatment in this study, similar reductions have been reported with HRT's combining transdermal oestradiol with either oral MPA (10 mg) [319] or oral 1mg norethisterone [306]. By contrast, oral 2mg oestradiol or 0.625mg CEE alone increase factor VII activity, whereas transdermal oestradiol has a negligible effect [243] [294] [306]. These data strongly suggest that oral progestogens reduce factor VII coagulation activity. The reduction in factor VII herein therefore indicates an overall hormonal balance favouring a dominant norethisterone action. Although elevated factor VII coagulation activity has been associated with an increased risk of coronary thrombosis in one study of men [41].

Similar data in women are lacking. The relevance of HRT-mediated changes in factor VII levels therefore requires clarification.

A further potential benefit noted in our study was a reduction in fasting glucose concentration. Whether this reflects an improvement in insulin action must be viewed with a degree of caution since we did not directly measure insulin action using clamp techniques, and many women were taking insulin therapy. Future studies should measure insulin action directly. Nevertheless, although HbA<sub>1c</sub> was not significantly altered, an improvement in insulin sensitivity or reduced hepatic glucose production accords with findings of two previous randomised placebo controlled trials in women with diabetes with unopposed oral 2mg 17 $\beta$ -oestradiol alone [282] [266]. The recent report of a significant reduction in the incidence of diabetes (by 35%) in women with coronary disease assigned to active treatment in HERS study as compared to those given placebo [320] suggests that conventional HRT may lessen risk of diabetes. Whether low dose HRT containing oestradiol and norethisterone has the same (or even larger) effect deserves further study.

The observed reduction in tPA-antigen (which largely measures circulating t-PA-PAI complexes) is in keeping with a reduction in PAI-1, which was not directly measured. PAI-1 has also been shown to fall in oral HRT studies [294] [321] [322]. Alternatively, the fall in t-PA may reflect reduced endothelial disturbance (i.e. t-PA release). Interestingly, elevated tPA-Ag (but not PAI-1) levels independently predicted CHD event rate in a recent meta-analysis of prospective studies in general populations [294].

There are several notable strengths of this study. Firstly, it is one of the very few randomised double-blind placebo-controlled trials of HRT in diabetic women, a group at

elevated baseline risk of CHD and at potentially elevated CHD risk with conventional HRT therapy [303]. Secondly, the novel low dose preparation was very well tolerated and the side-effect profile was excellent, helping maintain investigator blinding. Thirdly, we tracked adherence to study medication by measuring oestradiol and gonadotrophin concentrations. Finally, we assessed a number of key pathways for CHD risk simultaneously, an approach that facilitated a more comprehensive assessment of the overall balance of metabolic, haemostatic and inflammatory effects of the low dose HRT used. A limitation of our study is the modest number of patients recruited. Nevertheless, statistically significant changes detected in several key parameters indicate sufficient power to detect meaningful changes in pathways of interest; moreover, the results have biological plausibility. Our data therefore provide a strong basis for future studies examining the clinical safety of low dose HRT containing oestradiol and norethisterone in preference to conventional HRT containing CEE and MPA, supporting an emerging consensus that lower doses may be the safer option for many women [304] [318].

In conclusion, our study shows that low dose HRT containing 1mg oestradiol and 0.5mg norethisterone generates a vastly different portfolio of metabolic and haemostatic action compared to that observed with use of conventional higher dose HRT. Specifically, triglyceride, factor IX, APC resistance and CRP levels were statistically similar, whereas IL-6 and factor VII levels were significantly reduced and LDL-cholesterol lowering was retained. On the basis of our data, we suggest that a preparation containing low dose oestradiol combined with norethisterone may be more suitable for women who require HRT for menopausal symptom relief or bone protection but who are at higher risk of CHD, such as those with type 2 diabetes. However, before definitive recommendations are made, such novel formulations should be assessed in a large randomised controlled trial powered for cardiovascular endpoints.

## **CHAPTER 5**

### **EFFECTS ON BONE MINERAL DENSITY**

## 5.1 Introduction

Hormone replacement therapy (HRT) is routinely used to treat menopausal symptoms and to prevent osteoporosis. Until recently, it was also commonly perceived that HRT might protect against coronary heart disease (CHD). However, recent data from two large prospective studies using 0.625 mg conjugated equine oestrogens (CEE) combined with medroxyprogesterone acetate (MPA) have demonstrated increased CHD risk with active therapy in largely healthy postmenopausal women [241] or those with prevalent CHD [234]. These findings have resulted in the abandonment of the recommendation of HRT for cardioprotection. Women with type 2 diabetes have a markedly elevated baseline risk for CHD. A recent report from a prospective observational study [303] suggested that HRT use leads to a significantly increased risk of death from all causes and ischaemic heart disease among women with diabetes. Current users of HRT with diabetes had a near ten-fold increased risk of myocardial infarction (9.2, 95% CI, 2.0 to 41.4) compared with never users with diabetes.

There is an emerging consensus that lower doses may be the safer option for many women [304] [323]. Moreover, experts in the field are beginning to recommend use of lower dose oral preparations, perhaps containing oestradiol rather than CEE and progestogens other than MPA [324].

Postmenopausal women with diabetes are at markedly elevated CHD risk and recent data suggests this group may also be at significantly elevated risk of hip fractures [209]. Strategies to prevent osteoporosis may be especially warranted in women with diabetes.

Such women may therefore receive particular benefit from HRT but little data exists on the effects of low dose preparations on bone mineral density (BMD). Here, we examined if six months treatment with a low dose continuous combined HRT containing 1mg oestradiol and 0.5 mg norethisterone significantly increases BMD relative to placebo and reduces serum bone alkaline phosphatase (BAP) in post menopausal women with type 2 diabetes.

## 5.2 Methods

### 5.2.1 Subjects recruited

50 women aged under 70 years of age were recruited from general diabetes clinics in Glasgow Hospitals. Women randomised were clinically and biochemically postmenopausal i.e. with at least one year since last menses and a follicle stimulating hormone (FSH) concentration of greater than 20 IU/l. A normal pelvic examination and mammogram within the year prior to inclusion in the trial was also required. Women were included with a widespread of glycaemic control but all had an HbA1c of < 10 and no evidence of significant hypertriglyceridaemia, hypertension, and renal or liver disease. In addition, none had cardiovascular, cerebrovascular, or peripheral vascular disease. Subjects with either a personal history of - or first-degree relative with - breast cancer were excluded.

### 5.2.2 Study Visits.

Women eligible at screening were randomised to prescription of either active medication or identical placebo daily for six months. Compliance was assessed by oestradiol and gonadotrophin concentration measures at the final visit. A requirement of oestradiol to rise by more than 10 pmol/l and FSH to decline by more than 5 U/L was pre-defined. The participating hospitals' local research ethical committees granted ethical approval. Existing medications for glycaemic control, blood pressure or lipid lowering (detailed in Table 4.1) were not altered throughout the course of the study.

The BMD in the lumbar spine (L2-L4) and hip were measured using dual-energy x-ray absorptiometry (Lunar DPX+). The densitometer was calibrated daily using the local Lunar spine phantom. Daily spine phantom quality control was within 1% of the reference value. The coefficients of variation for the anatomic phantoms during the baseline scans were 0.46% for the spine and 0.58% for the hip. The software program for the DXA scan had a comparison feature that permitted identical regions of interest to be used on the repeat scans. For the hip these regions of interest were total hip, femoral neck, Ward's triangle, femoral shaft and trochanter. During the course of the trial the in vivo coefficients of variation were 1.2% or less for measures of spine and hip.

The same patients also underwent a series of metabolic tests, the results of which have already been presented elsewhere [325].

### 5.2.3 Statistical analysis

Baseline data are presented as mean and SD. Mean differences in changes from baseline between the active and placebo groups were compared using the unpaired t-test: the 95% confidence interval for change in active group data relative to change in control group data are presented. Adjustment for baseline concentrations was made by linear regression.

## 5.3 Results

### 5.3.1 Subjects recruited

Of the 50 women randomised, five did not complete the study due to either relocating or personal reasons. Of the women completing the study, 22 were in the active group and of these 19 adhered to study medications as evidenced by an increase in oestradiol ( $>10$  pmol/l) and fall in FSH ( $>15$  U/L). There were no serious adverse events. Three women (16%) reported breast tenderness and breakthrough bleeding on active treatment but none on placebo.

Table 4.1 demonstrates the baseline characteristics of women completing the study. The groups were similar in age, BMI, blood pressure and years since menopause. All categories of diabetes therapy were represented and similar percentages were taking anti-hypertensive or lipid-lowering agents. A significant reduction in gonadotrophin concentrations and an elevation in oestradiol (both  $p<0.001$ ) was observed in women randomised to active therapy (data not shown).

### 5.3.2 Bone Mineral Density (BMD) and Bone Alkaline Phosphatase (BAP) changes.

Table 5.1 shows the BMD changes in both groups. Significant changes were seen at all sites - total hip, Ward's triangle, femoral shaft and trochanter - except, as anticipated, the neck of femur. In addition, serum bone alkaline phosphatase decreased in the active group by 23% but marginally increased in the placebo group such that relative change

was highly significant ( $p < 0.001$ ). The changes in BMD remained significant after adjustment for baseline BMD, BMI, years post-menopausal and serum BAP.

**Table 5.1**

Baseline and changes over six months in bone mineral density (BMD) and serum bone alkaline phosphatase (BAP) in women randomised to HRT and placebo.

	Active Baseline	Mean Change (%)	Placebo Baseline	Mean Change (%)	Difference	p	*p
BMD							
L2-L4	1.213	+0.023	1.083	-0.021	0.073	0.004	0.006
Neck	0.905	+0.001	0.825	-0.001	0.003 [-	0.83	0.63
Wards	0.763	+0.003	0.710	-0.028	0.031	0.063	0.028
Trochanter	0.825	+0.015	0.798	-0.038	0.053	0.020	0.010
Shaft	1.168	+0.021	1.114	-0.014	0.035	0.028	0.045
Total	1.003 (0.041)	+0.012 (1.19)	0.945 (0.032)	-0.018	0.030 [0.004 to	0.027	0.040
Serum BAP	20.54 (1.40)	-4.79 (23.3)	21.59 (1.55)	1.31	-6.10 [-2.94 to -9.26]	<0.001	<0.001

\*p = p-value adjusted for baseline BMD (or BAP), years post-menopausal, and baseline BMI.

## 5.4 Discussion

We demonstrate for the first time that low dose continuous combined HRT benefits BMD in a group of postmenopausal women with diabetes, a group with markedly elevated CHD risk. Interestingly, recent data indicate that doses of CEEs-MPA lower than 0.625 mg/d effectively increase BMD in early postmenopausal women [326]. The relevance of our data on an HRT containing low dose oestradiol (as opposed to conjugated oestrogens) and norethisterone (as opposed to MPA) is that such an HRT is more likely to avoid many of the adverse metabolic effects and associated elevated CHD risk seen with conventional higher dose HRT and thus the excess risk for CHD [241] [234]. Indeed, we have already reported statistically similar triglyceride, factor IX, APC resistance and CRP levels with this preparation in these patients, whereas IL-6 and factor VII levels were significantly reduced and LDL-cholesterol lowering was retained [325]. This profile of effects differs markedly from that obtained by CEE-MPA based HRT which increases triglyceride, CRP and factor VII. Therefore, we have suggested that the preparation used herein is likely to avoid the elevated CHD risk seen with more conventional preparations as used in HERS and WHI.

It is important to note that the extent of improvement in BMD with 6 months treatment of the novel low dose combined oestradiol/norethisterone preparation was in the range of 1-2%. This improvement is at least equivalent, perhaps even superior, to effects seen with lower dose CEE, as reported in a recent multicentre US trial conducted in early postmenopausal women [326].

There are several notable strengths of this study. Firstly, it is one of the very few randomised double-blind placebo-controlled trials of HRT in diabetic women, a group at elevated baseline risk of CHD and at potentially elevated CHD risk with conventional HRT therapy [234]. Secondly, the novel low dose preparation was very well tolerated and the side-effect profile was excellent, helping maintain investigator blinding. In terms of clinical benefit, fewer side effects will only serve to enhance compliance such that long-term therapy, critical for osteoporosis prevention, is more sustainable. Finally, we tracked adherence to study medication by measuring oestradiol and gonadotrophin concentrations.

In conclusion, we have shown for the first time that a low dose HRT containing 1 mg oestradiol and 0.5 mg norethisterone improves BMD in women with type 2 diabetes and does so by approximately the same magnitude of low dose CEE-based preparations. Since women with type 2 diabetes are at elevated risk for CHD and conventional CEE/MPA-based HRT's increase such risk, there is an increasing need to consider lower dose preparations containing differing oestrogens and progestogens. Accordingly, our data provide important reassurance that lower dose HRT can be confidently prescribed for protection against bone loss in women with type 2 diabetes.

## **CHAPTER 6**

### **LIVER EFFECTS**

## 6.1 Introduction

Insulin resistance plays a central role in type 2 DM and glucose intolerance. There is also a strong association between type 2 DM and liver disease. Approximately 75% of patients with diabetes may have non-alcoholic fatty liver (NAFL) [327]. Additionally, the role of the liver in the pathogenesis of type 2 diabetes is increasingly considered. Both directly determined liver fat content [328] and circulating levels of alanine aminotransferase (ALT) [329] [330] [331] and gamma-glutamyltransferase (GGT), [332] [333] [334] which reflect liver fat content, have been shown by ourselves and others to be associated with diabetes risk, independently of alcohol consumption, in prospective studies.

The Women's Health Initiative (WHI) [234] randomized 15,000 women to receive either 0.625mg of conjugated equine oestrogens and 2.5mg of medroxyprogesterone acetate or placebo each day. The study was halted after an average of 5.6 years because of adverse cardiovascular events in the intervention arm. Evaluation of the data after the end of the study did, however, reveal that women randomised to active therapy had a lower incidence of self-reported diabetes relative to placebo recipients, with a hazard ratio of 0.79 (nominal 95% CI 0.67–0.93). This hazard ratio was unaffected by adjustment for changes in body mass index or waist circumference.

In view of the above observations, data indicating HRT users have a better profile of liver function tests are of interest. For example, in the National Health and Nutrition Evaluation Survey (NHANES) III data [115] on liver enzymes in ~12,000 subjects representative of the U.S. population, lower levels of NAFLD were seen in postmenopausal women taking hormone replacement therapy (HRT). Whilst the latter data are observational and maybe confounded by unmeasured factors, a beneficial effect

of HRT to lessen liver fat accumulation may be speculated. Randomized controlled data to directly test this speculation are absent. We had an excellent opportunity to examine changes in liver function tests in a randomized double-blind placebo-controlled trial of a low dose continuous combined HRT (1mg 17 $\beta$ -oestradiol and 0.5mg norethisterone acetate) in postmenopausal women with type 2 diabetes. We have already shown this preparation to offer a beneficial metabolic profile inclusive of lower lipids, glucose and cytokine concentrations [325]. Thus, we tested the hypothesis that liver function test concentrations would be improved by HRT relative to placebo effects in women with type 2 diabetes.

## 6.2 Methods

### 6.2.1 Subjects Recruited

The subject recruitment has been described in detail previously. [325] Briefly, 50 women with type 2 diabetes aged under 70 years of age who were clinically and biochemically postmenopausal were recruited. A normal pelvic examination and mammogram within the year prior to inclusion in the trial was also required. Exclusion criteria included poor glycaemic control, severe hypertriglyceridaemia ( $>10$  mmol/l), moderate to severe hypertension (systolic  $>160$  mmHg, diastolic  $>110$  mmHg), renal impairment (serum creatinine greater than twice the upper limit of normal range), liver disease (serum transaminases and bilirubin greater than twice the upper limit of normal range).

Established cardiovascular, cerebrovascular, or peripheral vascular disease also excluded selection. Subjects with either a personal history of, or first-degree relative with, breast cancer were excluded.

Women eligible at screening were randomised to prescription of either active medication (1mg oestradiol plus 0.5mg norethisterone) or identical placebo daily for six months. Randomisation was effected in blocks of four using computer-generated numbers. Compliance was assessed by returned medication packs at the final visit and by oestradiol and gonadotrophin concentration measures at the final visit. A requirement of oestradiol to rise by more than 10pmol/l and FSH to decline by more than 5 U/L was pre-defined.

The participating hospitals' local research ethical committees granted approval. All subjects gave written informed consent to a single investigator (JM). As far as possible,

existing medications for glycaemic control, blood pressure or lipid lowering (Table 4.1) were not altered throughout the course of the study.

### 6.2.2 Study visits

Women attended the Diabetes Centre, Glasgow Royal Infirmary, Glasgow at baseline and 6 months, having fasted for 10 hours and avoided heavy exercise, alcohol and caffeine in the preceding 24 hours. Subjects rested prone for 15 minutes prior to blood pressure recordings being taken in triplicate (mean recorded). The women also had anthropometric measurements to include height (cm), weight (kg), and waist and hip circumferences (cm). From these body mass index (BMI) was calculated as  $\text{weight}/(\text{height})^2$  in  $\text{kg}/\text{m}^2$ , as well as waist to hip ratio (WHR).

### 6.2.3 Laboratory methods

The reproductive hormones: oestradiol, luteinising (LH), follicle stimulating hormone (FSH), testosterone and sex hormone binding globulin (SHBG) were measured using semi-automated 'Immujite' technology (DPC, Los Angeles, USA). ALT and AST were determined on fresh samples using standard reagents by reaction rate assay based on the conversion of NADH to NAD. All AST, ALT, GGT and alkaline phosphatase (ALP) analyses were conducted in the same laboratory with adherence to external quality control. The between batch CV for their determination was <5%.

#### 6.2.4 Statistical analysis

Baseline ALT, AST and GGT were skewed and geometric means and SD are presented. Changes in ATL, AST and ALP were normally distributed and thus difference in change from baseline between the two treatment groups was compared using the unpaired t-test; the 95% confidence intervals for change in active group data relative to change in control group data are presented. Adjustment for baseline concentrations was made by linear regression. Changes in unadjusted GGT in active and placebo groups were skewed so we compared changes using logged values.

## 6.3 Results

### 6.3.1 Subjects Recruited

58 women were recruited from a general diabetic clinic and screened as potential recruits into the study. Of these, 50 were randomised. The eight women not entered had unacceptably high HbA1c (n=3), abnormal LFT's (n=2), abnormal mammogram (n=2) or abnormal pelvic examination (n=1), the latter two categories necessitating further investigations. Five women did not complete the study due to either moving home or other personal reasons. Forty-five women therefore completed the study and of the 22 in the active group, 19 demonstrated compliance as evidence by an increase in oestradiol and fall in FSH. None of the women in either group suffered any serious adverse effects. Breast tenderness and breakthrough bleeding were reported in three women in the active group but none in the placebo arm.

Table 4.1 demonstrates the baseline characteristics of women completing the study. The groups were similar in age, BMI, blood pressure and years since menopause. All categories of diabetes therapy were represented and similar percentages were taking anti-hypertensive or lipid-lowering agents. A significant reduction in gonadotrophin concentrations and an elevation in oestradiol (both  $p < 0.001$ ) was observed in women randomised to active therapy (data not shown).

### 6.3.2 Changes in Liver Function Tests

Table 6.1 demonstrates baseline and 6 month alterations in serum ALT, AST and ALP concentrations in placebo and active groups. It was of note that all three analytes

declined in concentration in the active group relative to placebo. The mean changes for all three analytes were pronounced. Figure 2 demonstrates significant reduction in serum GGT levels in the active relative to placebo recipients. Baseline levels were not significantly different in women randomized to active or placebo groups. In order to test for any potential confounding by baseline levels, we further compared changes in parameters with adjustment for baseline levels and noted that significant active-placebo differences were retained (Table 4.1).

When we examined relationship between reductions in relation to baseline concentrations (Table 6.2, Figure 3), we noted very strong correlations in the active group for all four analytes whereas only AST was borderline significant in the placebo group. Similarly, the magnitude of reduction in AST, ALT and GGT were highly correlated in the active group (majority  $p < 0.01$ ) whereas no such significant associations were noted in the placebo group.

**Table 6.1**

Baseline and changes over six months in liver function tests in women randomised to HRT and placebo.

	Active Baseline	Mean change	Placebo Baseline	Mean change	Delta for active vs. placebo	p value delta	*p value delta
ALT	28.7 (1.9)	-13.1(16.8)	20.7 (1.6)	1.27 (5.6)	-14 (-23 to -6)	0.002	0.004
AST	22.1 (1.5)	-5.8 (7.3)	18.7 (1.5)	3.4 (7.0)	-9.2 (-14 to -5)	<0.001	<0.001
ALK Phos	217.6 (40)	-54.21 (31)	210.9 (51)	6.6 (29)	-61 (-80 to -42)	<0.001	<0.001

\*p value=p value delta adjusted for baseline

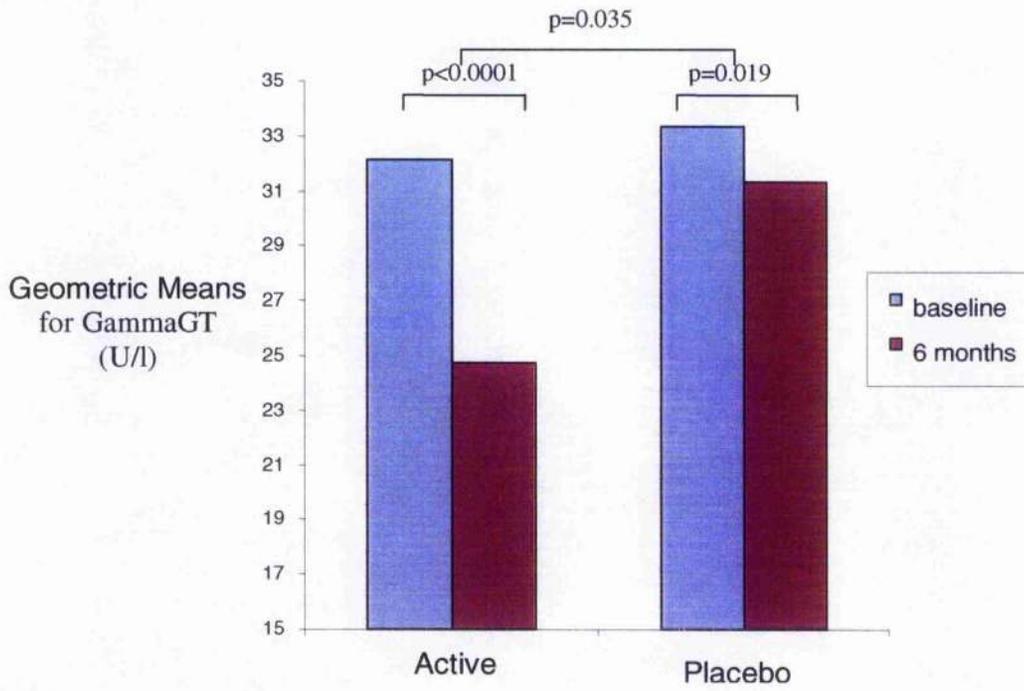
**Table 6.2.**

Correlation of change with baseline liver function tests concentrations in active and placebo group.

	Active	Placebo
ALT change vs baseline ALT	R=0.93, p<0.001	R=0.17, p=0.44
AST change vs baseline AST	R=0.90, p<0.001	R=0.44, p=0.04
ALP change vs baseline ALP	R=0.68, p=0.001	R=0.11, p=0.63
GGT change vs baseline GGT	R=0.87, p<0.001	R=-0.30, p=0.17

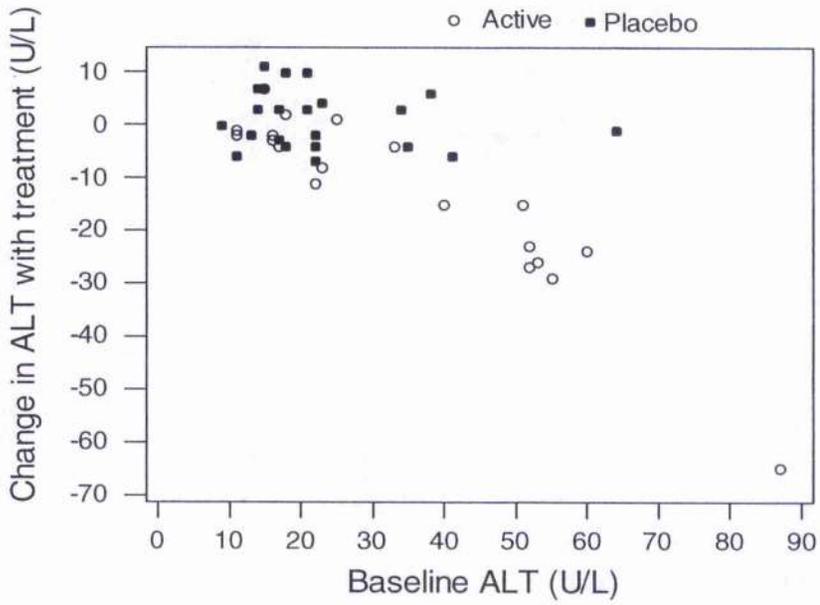
**Figure 2**

Reduction in serum GGT levels in the active relative to placebo participants.



**Figure 3.**

Association of change with baseline ALT levels in women randomized to placebo or active therapy.



## 6.4 Discussion

In a randomized placebo controlled study we have demonstrated that an HRT containing 1mg 17 $\beta$ -oestradiol and 0.5mg norethisterone can significantly lessen circulating concentrations of routinely measured liver function tests (ALT, AST, GGT and ALP) in women with type 2 diabetes. Such observations are relevant for a number of reasons. Most importantly, excess liver fat is a common occurrence in obesity and in patients with type 2 diabetes. Indeed, the average levels of ALT and AST in the women in this study were much higher than recently suggested normal levels for women from a recent large population survey. A subset of patients with NAFL, previously thought to be a benign non-progressive condition, can develop advanced fibrosis, cirrhosis and hepatocellular carcinoma [335]. The presence of obesity and/or type 2 DM are the strongest predictors of fibrosis [336]. Secondly, excess liver fat, even within the subclinical range as demonstrated by ALT or GGT levels, signal elevated risk for type 2 diabetes and perhaps indicate a role for liver fat accumulation in the pathogenesis of type 2 diabetes. Finally, a recent large multicentre study suggested HRT may reduce risk for type 2 diabetes but mechanisms were not examined [234]. Our results suggest an HRT mediated reduction in liver fat could account for some of the above observations.

A possible mechanism by which HRT may lessen liver function tests is by a reduction in liver fat. Elevations in GGT, ALP, AST and in particular ALT have been shown to correlate with excess liver fat and insulin resistance and ALT and GGT are now well described as predictors of type 2 diabetes [329] [330] [331] [332] [333] [334]. Elevated ALT is now used, together with other metabolic markers, as a risk factor signaling excess liver fat and the need to further investigate NAFL. Thus the parallel reductions in ALT, AST and GGT in the active group would be consistent with a reduction in liver fat.[335][336] The significant reduction in fasting glucose reported previously in this

study [325] would be in keeping with lower fat accumulation since hepatic fat accumulation is linked to increased hepatic gluconeogenesis [337]. Although part of the reduction in ALP may come from effects on bone metabolism, the relative extent of change in the active group in this parameter (~25%) would suggest an effect on liver generated ALP is also likely. Previous studies have suggested that elevations in ALP may also be linked to NAFL [338].

We cannot determine directly by our study design whether the oestradiol or norethisterone component of the HRT was responsible for the beneficial effect on liver tests. Androgenic progestogens, such as norethisterone lessen circulation concentrations of a range of factors produced by the liver (e.g. triglyceride, Factor VII etc), whereas oestrogens do the reverse [244]. One could speculate that androgenic progestogens up-regulate hepatic beta-oxidation, leading in turn to lower hepatic fat accumulation. Triglyceride (albeit not significantly) and factor VII concentrations were lowered in this study with active therapy [325] suggesting a dominance of the androgenic effect with the HRT used. Whilst direct evidence for our supposition is lacking, other data indicate oral oestrogens may increase triglyceride concentrations by 'decreasing' hepatic beta-oxidation [339]. It is also relevant that previous studies have shown a beneficial effect of unopposed oestradiol on liver insulin sensitivity [266] [282], but effects on liver function tests or liver fat were not measured. Finally, it is notable that PPAR gamma agonists may exert their insulin sensitizing effects in part by reducing liver fat content [340].

## CHAPTER 7

### DISCUSSION

## 7.1 Summary of study.

The purpose of this study was to examine the effects of a low dose continuous combined HRT preparation on insulin sensitivity, lipid profile, clotting factors and markers of endothelial function and inflammation in postmenopausal women with type 2 DM. This study showed a significant fall in fasting insulin ( $p=0.043$ ) and C-peptide ( $p=0.003$ ). Fasting glucose ( $p=0.026$ ) also declined significantly, but there were no significant effects on HBA1c or BMI which did not fall. The study would suggest that in terms of glucose metabolism and control of diabetes a low dose combined continuous HRT would not lead to any deterioration in control, but may be as our trial was not powered to detect such a change.

The changes in lipid profiles in our patients were as expected from previous work with total and LDL-cholesterol both showing significant falls ( $p= 0.020$  and  $0.018$  respectively). The HDL-cholesterol did not unfortunately rise, but due to the fall in LDL-cholesterol the ratio of total: HDL-cholesterol did fall by a significant amount ( $p=0.05$ ). Triglyceride concentrations were also not significantly altered. Haemostatic and inflammatory variables showed a significant fall in Interleukin-6 concentration, Factor VII and tissue plasminogen activator antigen concentrations, ( $p=0.015, <0.001$  and  $0.005$  respectively) but CRP, factor IX and APC resistance did not alter significantly.

It would seem that IIRT containing 1mg oestradiol and 0.5mg norethisterone may avoid the adverse metabolic and haemostatic effects potentially implicated in the elevated CHD and stroke risk. Specifically, triglyceride, factor IX, APC resistance and CRP levels were statistically similar, whereas IL-6 and factor VII levels were significantly reduced and LDL-cholesterol lowering was retained. The above can only be surmised as our trial did

not include enough women or for a long enough time period for us to make any comments on CHD and stroke risk and only examined surrogate markers.

## 7.2 Studies of HRT in Women with DM

A recently reported study of the effect of a continuous combined HRT, Kliofem (2mg 17 $\beta$ -oestradiol, 1mg norethisterone acetate) as used in our pilot study, recruited 150 postmenopausal women with either type 2(94) or type 1 DM(56) into a randomised controlled study over a 1 year treatment period [341]. Seventy patients failed to complete the study and may affect any results that were found. This HRT was found to have neutral effects total and LDL cholesterol and triglycerides in either type 1 or type 2 DM. A difference was found in the effect of HRT on HDL-cholesterol in the 2 treatment groups. There was a significant decrease ( $p<0.001$ ) in patients with type 1 diabetes but no decrease in those with type 2 diabetes. The mean difference of the change in HDL cholesterol between the 2 types of diabetes being 0.12 ( $p=0.008$ ). The researchers offered no explanation for this difference but thought it warranted further study [341]. This result must be assessed on the background of the differences in lipid abnormalities in patients with type 1 and type 2 DM i.e. patients with type 2 DM typically have low HDL cholesterol levels but patients with type 1 DM have normal or elevated HDL cholesterol. This elevated HDL cholesterol does not confer a cardiovascular benefit possibly due to the HDL cholesterol subfractions which are elevated [341].

One observational study of Danish Nurses which recently reported could not find any significant association between current or ever use of HRT and ischaemic heart disease, MI or death from all causes [303]. They found that the effect was modified by the presence of DM. Women with DM who were current users of HRT had significantly

increased risk of ischaemic heart disease (HR 4.2, 95% CI 1.4-12.5), MI (9.2, 2.0-41.5) and death from all causes (3.2, 1.4-7.5) compared with women with diabetes who had never used HRT [303]. The HRT used could be, oestrogen alone or oestrogen plus progestogen, but in all cases the doses used were higher than used in our study [303].

### **7.3 Randomised Controlled Studies of HRT**

The first primary and secondary cardiovascular prevention trials in women without type 2 DM reported during this study and as discussed earlier no benefits from HRT have been seen. The first to report was the Heart and Estrogen/Progestin Replacement Study (HERS) [241] which was designed to assess the efficacy of HRT for secondary prevention of cardiovascular endpoints. This trial reported that a fixed combination of 0.625mg per day of CEE and 2.5mg of MPA had no effect on fatal and nonfatal cardiac events compared to placebo. This study of 2763 American women with a mean age of 67 years followed up for an average of 4.1 years. After 4 years of follow-up there were 179 CHD events in the HRT group and 182 in the placebo group with 57 events in the HRT group and 38 in the placebo group in year 1 (relative hazard (RH) 1.5). In the first 4 months of the trial there was a RH of 2.3. In years 4 and 5 in contrast there were 40 events in the HRT group and 53 in the placebo group (RH 0.75).

The average duration of follow up was 7 months less than originally planned as enrollment of a large percentage of participants took place within the final 6 months of the 18 month recruitment period. A higher than expected crossover rate between the groups was also encountered in the HERS trial. There was a 1.7% per year cross over rate from placebo to HRT (1% per year was expected) and an 18% crossover from HRT to placebo in the first year (5% was expected). Also the event rate in both groups was much lower than expected, being 3.3% per year rather than 5% which was expected. It

was predicted that the event rate in the placebo group would be 24% when the actual rate was 13%. The explanation given for this lower event rate was that more women than ever are treated with statins, aspirin,  $\beta$ -blockers and angiotensin-converting enzyme inhibitors all of which decrease events in patients with CHD [342].

The HERS investigators reported on the observation that changes in lipid levels with HRT are not predictive of cardiovascular outcomes in women with heart disease [343]. It would seem that baseline levels of HDL-c and on treatment levels of LDL-c are significantly associated with cardiovascular events, but reduction in LDL-c had only a modest association with events and increases in HDL-c and triglyceride levels had no association with cardiovascular risk. The reasons for this observation may be that any beneficial effects in LDL-c and HDL-c are opposed by a rise in TG levels.

Soon after the HERS trial had reported the large primary prevention trial which was designed to define the risks and benefits of strategies that could potentially reduce the incidence of heart disease, breast and colorectal cancer and fractures in postmenopausal women was prematurely stopped. This was due to the preset threshold for increased numbers of breast cancer being breached. This trial was one component of the Women's Health Initiative (WHI) [234] the first randomized primary prevention trial of postmenopausal hormones for CHD. Between 1993 and 1998 the WHI enrolled 161,809 postmenopausal women in the age range 50-79 into a set of clinical trials at 40 clinical centres in USA. The primary outcome for the HRT trials component was CHD. Hip fractures were a secondary outcome. Invasive breast cancer was designated a primary adverse outcome. Additional clinical outcomes chosen as secondary outcomes that could be affected by HRT included other cardiovascular disease, endometrial, colorectal and other cancers and other fractures. 16,608 women with intact uterus were recruited to the oestrogen and progestin component of the trial. They were randomized to receive either

placebo or a continuous combined combination of 0.625mg CEE and 2.5mg MPA (as in the HERS study). The trial was stopped 3 years early based on health risks that exceeded health benefits after an average follow-up of 5.2 years. A parallel trial of oestrogen alone in women who have had a hysterectomy is continuing to its planned date of completion in 2005 when average follow-up will be 8.5 years. The trial was stopped because women in the active treatment group had an increased risk of breast cancer (hazard ratio (HR) 1.26, 95% CI 1.00-1.59) which was above a predetermined level. Several other results were also causing concern including increased CHD (HR 1.29, 95% CI 1.02-1.63), stroke (HR 1.41, 95% CI 1.07-1.85) and pulmonary embolism (HR 2.13, 95% CI 1.39-3.25). Beneficial results included decreases in colorectal cancer (HR 0.63, 95% CI 0.43-0.92) and hip fracture (HR 0.66, 95% CI 0.45-0.98). Numbers of deaths in the active and placebo group were similar. Only 3.5% were lost to follow up and during the study compliance reached 48% in the active group and 62% in the placebo group. This exceeded that expected and may influence the intention to treat figures which have yet to be reported.

Most adverse events began appearing within the first 2 years of the trial but increased cancer risk did not occur until after year 3. Results in subgroup analyses are consistent suggesting there is not any particular group of patients who would benefit more from the HRT studied. The authors concluded that results from WHI indicated that the combined HRT preparation of 0.625mg CEE and 2.5mg MPA should not be initiated or continued for the primary prevention of CHD.

The findings of WHI as regards preventing CHD concurs with the HERS and HERS II findings in women with clinically apparent CHD. The findings in WHI for stroke are also consistent with those in HERS although somewhat more extreme. The findings as regards venous thromboembolism are consistent in both WHI and HERS trials. Although

it was the prevalence of breast cancer that crossed a designated safety boundary and led to the early termination of WHI the breast cancer results are not unexpected from earlier observational data [228] [229]. The 26% excess risk is also consistent with a non-significant 27% increase found in the HERS II trial. The reduction in colorectal cancer in the hormone group is consistent with observational studies which have suggested fairly consistently that users of HRT may be at lower risk of colorectal cancer [344].

The results of the HERS and WHI trials lead to much discussion. The validity of the results was questioned. In particular the age of the women in the trial was seen as a possible reason for the lack of benefit. Animal studies had previously shown that when CEE was initiated at time of oophorectomy there was a 50-70% decrease in coronary plaque, but if CEE was started years later no benefit to plaque formation or stabilization was seen. [345] This may suggest that HRT has less effect on limiting the atherosclerotic process if plaques are already developed as may have occurred in WHI and in HERS trials. The average age of the women in the WHI (CEE+MPA) trial was 63, the sample including only 10% in the lowest cohort of 50-54 years and 20% between 54-59 years. No information in either trial was given as to age at menopause of the subjects which may be important in defining cardiovascular status, which changes with age and more rapidly after menopause. [2] Similarly in the women randomized to HRT in WHI, 36% had hypertension, 49% were current or past smokers. It is possible therefore that although subjects were designated as healthy the process of atherosclerosis was likely to be active in these women. Supporters of HRT suggested that if loss of hormones permits rapid progression of atherosclerosis then early intervention with HRT perhaps in the perimenopausal period would be more effective. Prescribing of HRT to older women for cardioprotection until the time of the trial had become common clinical practice that the trials were designed to evaluate and as mentioned earlier, in the WHI (CEE+MPA) trial the women at greatest risk were found to be in the youngest cohort.

The generalisability of the results from HERS and WHI were also questioned on the basis of the HRT preparation used. CEE is a mixture of steroids extracted from equine urine and is uncertain composition, but its main active ingredient is sodium oestrone sulphate. After menopause women lose oestradiol (major ovarian hormone) whereas the levels of oestrone (largely produced in peripheral tissues) remains unchanged. In this way it can be seen that CEE does not replace oestradiol. CEE and oestradiol by definition are both oestrogen as they can bind to and activate oestrogen receptors but the pharmacological properties are known to vary considerably and may have influenced the final outcomes of the trials. A Swedish study found a reduced risk of MI for oestradiol compared to oral oestriol or vaginal oestriol/dienoestrol. [346]

In vitro studies using human aortic smooth muscle cells (SMC) demonstrated that oestrogens present in CEE were significantly less potent compared with oestradiol in inhibiting mitogen induced SMC growth and mitogen activated protein kinase activity. Abnormal growth of SMC plays a role in CHD and the lack of antiproliferative action with CEE could be partly responsible for the negative outcomes of HERS and WHI. [347] In the Estrogens in the Prevention of Atherosclerosis (EPAT) study which was a primary prevention study where women were randomized to oestradiol or placebo after hysterectomy progression of intimal thickening was seen in repeat angiography after 3.2 years of follow up. [348] These findings may provide evidence that use of differing oestrogens may have a differential effect on CHD effects of HRT. Much of the observational data that demonstrated a positive effect of HRT however was seen with CEE.

The use of MPA in both the HERS and WHI has also been blamed for the lack of CHD effect. In support of this idea in the PEPI trial CEE caused beneficial effects on LDL-C

and HDL-C levels that were attenuated by MPA. [270]. The interpretation of this is that MPA may decrease the protective effects of oestrogen on the cardiovascular system. This observation is not supported by the observation that CEE and CEE+MPA were equipotent in inhibiting atherosclerosis in nonhuman primates and also in the WHI CEE alone study no protective effect on CHD was seen even though lipids were favourably changed. In the Nurses Health Study a similar risk reduction for CHD was seen among women taking CEE alone or CEE+ MPA. Also in the ARIC study reductions in intimal medial thickness were similar in women receiving oestrogen alone or oestrogen plus MPA. [281]

Examination of the results of HERS and WHI trials seemed to suggest that the effects of HRT changed over time in the study. Some reports were that HRT was only harmful at the initiation of treatment and could be beneficial by the end. The relative risk was seen to change over time in the HERS trial with RR in the first year of trial being 1.52 and the RR in years 4 to 5 being 0.75 however in no year did HRT users have a lower risk than non users and in the final year of the trial the RR had a very wide confidence interval crossing 0 of 0.50-1.13. These findings did lead to a unifying hypothesis of the effects of HRT on the cardiovascular system of "early harm and late benefit." [349] This was based on 3 possible processes induced by HRT. These were

1. Early plaque erosion/rupture made worse by CEE+ MPA
2. Long term reduction in plaque formation by the beneficial effects of HRT on lipids
3. Relative antagonism of vasculoprotective effects of oestrogen by MPA.

If HRT is begun under conditions where vulnerable plaques have accumulated i.e. women years after the menopause as in the HERS and WHI trials the effects of plaque erosion and rupture will dominate initially –early harm- and it may take some years for

the decreased plaque formation caused by the positive lipid effects to be seen-late benefit. There is a possible biological mechanism for this hypothesis in that oestrogens promote the production of matrix metalloproteinases (MMPs) which contribute to erosion and rupture of vulnerable preformed plaque (early harm) but which facilitate remodeling of stable plaque (late benefit).[349]

The aim of this hypothesis is to explain both the observational trial data and the controlled trial data. It would assert that if HRT is started at the time of menopause, as in the observational trials, when vulnerable coronary plaque is limited HRT should produce a decrease in CHD. At this time more of a benefit should be expected either from oral oestrogens (more of a rise in HDL-c than transdermal oestrogens)[350] or transdermal oestrogens (less of a rise in inflammatory markers e.g. CRP) than oral oestrogen). [290] If instead HRT is commenced years after menopause as in WHI and HERS trials when vulnerable coronary plaque load is extensive there will be a sharp increase in CHD events within the year or two after therapy is begun largely reflecting inflammation induced and/or MMP induced plaque erosion and rupture. If HRT is continued there will be fewer events as time progresses with a net benefit after 5-6 years as seen in WHI and HERS. As the initial increase in CHD events is due to the release of prothrombotic and proinflammatory factors from the liver stimulated by oral oestrogens these problems could be lessened by the use of transdermal oestrogens at this stage. If some of the positive effects of oestrogen on the cardiovascular system are negated by progestogens especially MPA there should be greater benefits seen in women taking oestrogen alone or with more androgenic progestogens e.g. norethisterone which is consistent with some observational studies. [75] Also if the increased risk of breast cancer seen in HERS and WHI is due to constant exposure of the breast to circulating progestogen as has been suggested than this risk could be lessened if progestogens used were less likely to

increase breast density i.e. norethisterone rather than MPA[349] or if the progestogen could be given locally to the uterus e.g. by a progestogen impregnated uterine coil.

The most consistently adverse effect in both the HERS and the WHI studies, as well as others has been the increase in triglyceride levels. In the WHI there was a 6.9% rise in the treatment group compared to the placebo group levels [234]. This is consistent with the rise seen in the HERS study treatment group compared to the placebo group of 10% in the first year [241]. The rise in triglycerides may also result in an overestimate of the decrease in LDL-cholesterol based on the Friedwald equation for estimation of LDL-cholesterol levels as was carried out in both of the large trials. As we have discussed earlier elevated blood triglycerides are an important risk factor for both CHD and stroke especially among women [35]. The elevated triglycerides being associated with higher levels of small dense LDL-cholesterol which are particularly atherogenic. [37] They are also associated with lower HDL-cholesterol levels. The elevation in the triglycerides seen with CEE containing HRT is due to greater production of VLDL-cholesterol in the liver. This is not seen with oestradiol containing HRT preparations as in our own study. After correction for lipid levels in the HERS trial it can be seen that neither baseline nor on treatment triglycerides are related to CHD incidence. Clearly other factors apart from lipoprotein levels are important. Likely to be chief among these are the effects of HRT on fibrinolysis and endothelial function.

Studies had demonstrated that CRP and IL-6 are increased by HRT [244] [320]. Clinical epidemiological studies have also consistently shown that elevated CRP is a risk factor for CHD in women. Elevated CRP has been linked to changes in complement and induces tissue factors and adhesion molecules. The WHI demonstrated that baseline levels of CRP and IL-6 were associated with a 2-fold increase in risk of developing CHD. In the WHI HRT was associated with increasing CRP levels but no such change was seen

in the IL-6 levels, suggesting that the preparation used was not stimulating a generalized inflammatory response. In our own study the CRP levels were not altered significantly and the IL-6 levels were seen to fall significantly. Care must be taken when interpreting this result.

The increase in risk of stroke and CHD in the WHI trial was surprising given that at the end of the first year of treatment the LDL-cholesterol had decreased by 12.7% in the active treatment group compared to the placebo group. The level of HDL-cholesterol had also increased by 7.3% over the same time period in the treatment group compared to the placebo group. Similarly in the HERS trial an 11% reduction in LDL-c and an 11% increase in HDL-c was seen. Although the lipid changes are smaller than those achieved by statins if one assumes an almost 2% reduction in CHD for every 1% decline in LDL-cholesterol and an almost 2 to 3% decrease for every 1% rise in HDL-cholesterol then there would have been an anticipated fall in primary CHD incidence of between 30 and 35% and a 24% reduction in CHD incidence based on secondary prevention induced by simvastatin [351][352] (see table 7.1). The lack of effect seen may be as neither of the trials was powered to fully detect a significant benefit associated with either LDL-c reduction or HDL-c elevation.

Another secondary prevention of CHD trial has had similar results. The preparation under investigation in this incidence was bezafibrate, a fibric acid derivative [353]. In this study a fall in triglycerides by 21% and a rise in HDL-c of 18% did not significantly reduce the overall risk of recurrent cardiovascular events. Although LDL-c reduction and HDL-c elevation are established strategies for secondary prevention of CHD the clinical effect of lipid lowering agents on secondary CHD prevention cannot be predicted solely by their effects on lipid levels.

The effect of bone density was also examined in our study and in the HERS II and WHI trials. Significant changes were seen at all sites examined including total hip, Ward's triangle, femoral shaft and trochanter after adjustment for baseline BMD, BMI and years since menopause. No change was seen at the neck of femur. Serum bone alkaline phosphatase also decreased in the group on active treatment by 23%. The fall seen in the control group of this substance over the treatment time period made this result even more significant ( $p < 0.001$ ). The HERS II trial reported no decrease in the risk of fracture in their treatment group indicated by a lack of increase in BMD [354].

**Table 7.1**

Comparing effects in our trial, IIRT trials and statin trials.

Trial	Ours	HERS[241]	WHI[234]	4S[83]
No. DM (%)	100	2.7	4.4	4.5
Reduction in LDL-C	13.2	10.9	12.7	35.0
Change in HDL-C	-5.4	11.0	7.3	8.0
Change in TG's	-1.9	9.0	6.9	-10.0
Duration of Treatment (yrs).	0.5	4.1	5.2	4
CHD Incidence (RR)	na	0.99	1.29	0.67

The investigators did however indicate that this may have been due to the absence of routine spinal radiographs in the trial which would have meant that some vertebral fractures would have been missed and also that fewer of the women recruited had osteoporosis than would have been expected for a group of women of this age. They pointed out that clinical trials of bisphosphonates have found an effect on the risk of fracture in women with osteoporosis but not in women with normal bone density [355] The WHI is the first trial with definitive data supporting the ability of postmenopausal HRT to prevent fractures at the hip, vertebrae and other sites.

The investigation of effects on liver function tests was not a pre-specified end-point of our original study but rather a post hoc analysis stimulated by the results of the WHI study suggesting a protective effect of HRT on risk for type 2 DM. The results of this study suggest HRT containing oestradiol and norethisterone can lessen circulating concentrations of liver function tests in women with diabetes possibly by reducing liver fat content. Whilst these data do not advocate a role for HRT for this reason, improved understanding of the mechanisms for such an effect may lead to new therapies to treat individuals with NAFL.

#### 7.4 Future Prospects

The results of our trial would suggest that this low dose preparation could be of benefit in the prevention of CHD in postmenopausal studies but the overriding evidence from the large randomized controlled trials of HRT with clinical rather than surrogate end points is that HRT has no place in the prevention, either primary or secondary, of CHD or indeed of stroke in postmenopausal women.

Research into the area of inflammatory indices and their effect on CHD risk is an area of expansion at present and any role that HRT may have in their modulation would be of interest to our future understanding of the vascular effects of HRT.

Future studies with HRT are unlikely to be conducted but studies of selective estrogen receptor modulators (SERMS) e.g. Raloxifene, are ongoing. Selective estrogen receptor modulators improve cardiovascular risk factors, reduce the risk of vertebral fracture, and are associated with a reduced incidence of invasive breast cancer in postmenopausal women with osteoporosis. In the Raloxifene use for the Heart (RUTH) trial 10,101 postmenopausal women were randomized to receive either 60mg of raloxifene or placebo per day. Approximately half of the women had documented coronary heart disease (CHD) (n = 5,031); the remainder had multiple CHD risk factors that increased their risk for a CHD event (n = 5,070). The RUTH cohort is the largest group of postmenopausal women at increased risk of CHD events ever assembled in a clinical trial, and is the first trial designed to determine the effect of a selective estrogen receptor modulator on the risk of CHD events. The results of this trial are eagerly awaited. [356]

### **7.5 Recommendations for the use of HRT.**

For menopausal vasomotor symptoms –lowest dose for shortest time possible, consider topical preparations

For postmenopausal osteoporosis-use of non oestrogen treatments is recommended e.g. bisphosphonates, calcitonin. HRT only recommended when osteoporosis risk outweighs the risk of HRT preparation used.

For CHD prevention- HRT has no place here and other methods of reducing CHD should be employed e.g. diet and exercise, smoking cessation, control of hypertension, control of DM, statin therapy and antiplatelet therapy.

The US Food and Drug Administration (FDA) has recommended that all HRT preparations should have a box on the packaging warning of possible harm from HRT to include risk of heart disease, heart attack, stroke and breast cancer. The label also has to indicate that HRT preparations are only recommended for vasomotor symptoms of menopause, for vulvovaginal atrophy and for prevention of postmenopausal osteoporosis.

## REFERENCES

1. Royal College of Physicians. Osteoporosis. Clinical Guidance for Prevention and Treatment. London: Royal College of Physicians, 1999.
2. Barrett-Connor E, Bush T. Oestrogen and Coronary Heart Disease in Women. *JAMA* 1991;265:1861-1867.
3. Despres J, Murette A. Relation of components of insulin resistance syndrome to coronary disease risk. *Current Opinion in Lipidology* 1994;5:274-289.
4. Vranic M. Glucose turnover. A key to understanding the pathogenesis of diabetes. *Diabetes* 1992;41:1188-1206.
5. Kannel W, Dawber T, McGee D. Perspectives on systolic hypertension the Framingham Study. *Circulation* 1986;61:1179-1182.
6. Moller D, Flier J. Insulin resistance- mechanisms; syndromes and implications. *N Eng J Med* 1991;325:938-948.
7. Livingstone C, Dominiczak A, Campbell I, Gould G. Insulin resistance; hypertension and GLUT 4. *Clin Sci* 1995;89:109-116.
8. Despres J. Abdominal Obesity as important component of insulin resistance syndrome. *Nutrition* 1993;9:452-459.
9. Reaven G. Role of Insulin Resistance in Human Disease. *Diabetes* 1988;37:1595-1607.
10. DeFronzo R, Tobin J, Andres R. The glucose clamp technique a method for quantifying insulin secretion and resistance. *Am J Physiol* 1979;6E:214-223.

11. DeFronzo R, Sherwin R, Hendler R, Felig P. Insulin binding to monocytes and insulin action in human obesity; starvation and refeeding. *J Clin Invest* 1978;62:204-213.
12. Bonnadona R, Groop L, Kraemer N, DeFronzo R. Obesity and insulin resistance in man a dose response study. *Metabolism* 1990;39:452-459.
13. Kolterman O, Insel J, Saekow M, Olefsky J. Mechanism of insulin resistance in human obesity evidence for receptor and postreceptor defects. *J Clin Invest* 1980;65:1273-1284.
14. Bogardus C, Lillioja S, Mott D, Reaven G, Kashiwagi A, Foley J. Relationship between obesity and maximal insulin-stimulated glucose uptake in vivo and in vitro in Pima Indians. *J Clin Invest* 1984;73:800-805.
15. DeFronzo R, Ferrannini E. Insulin Resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidaemia and atherosclerotic cardiovascular disease. *Diabetes Care* 1991;14:173-194.
16. Peiris A, Mueller R, Smith G, Struve M, Kissebah A. Splanchnic insulin metabolism in obesity Influence of body fat distribution. *J Clin Invest* 1986;78:1648-1657.
17. Jensen M, Haymond M, Rizza R, Cryer P, Miles J. Influence of body fat distribution on free fatty acid metabolism in obesity. *J Clin Invest* 1989;83:1168-1173.
18. Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 1996;45:3-10.

19. Roust L, Jensen M. Postprandial free fatty acid kinetics are abnormal in upper body obesity. *Diabetes* 1993;42:1567-1573.
20. Unger R. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM; genetic and clinical implications. *Diabetes* 1995;44:863-870.
21. Hotamisligil G, Spiegelman B. Tumour necrosis factor alpha a key component of the obesity-diabetes link. *Diabetes* 1994;43:1271-1278.
22. Hurel S, Ofei F, Wells A, et al. Tumour necrosis factor alpha and insulin sensitivity in humans effects in vivo of antibody blockade in obese NIDDM patients and in vitro upon human cultured myotubes. *Exp Clin Endocrinol Diabetes* 1996;104 (suppl 2):59-60.
23. Maggs D, Jacob R, Rife F, et al. Interstitial fluid composition of glycerol, glucose and amino acids in human quadriceps muscle and adipose tissue evidence for significant lipolysis in skeletal muscle. *J Clin Invest* 1995;96:370-377.
24. Van Der Poll T, Romijn J, Endert E, Borm J, Buller H, Sauerwein H. Tumour necrosis factor mimics the metabolic response to acute infection in healthy humans. *Am J Physiol* 1991;24:E457-E465.
25. Fanelli C, Calderone S, Epifano I., et al. Demonstration of a critical role for free fatty acids in mediating counterregulatory stimulation of gluconeogenesis and suppression of glucose utilisation in humans. *J Clin Invest* 1993;92:1617-1622.
26. Randle P, Garland P, Hales C, Newsholme E. The glucose-fatty acid cycle- Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963:785-789.

27. Boden G, Chen X, Ruiz J, White J, Rosetti L. Mechanisms of fatty acid induced inhibition of glucose uptake. *J Clin Invest* 1994;93:2438-2446.
28. Boden G, Chen X. Effects of fat on glucose uptake and utilisation in patients with non-insulin-dependent diabetes. *J Clin Invest* 1995;96:1261-1268.
29. Despres J. Obesity and lipid metabolism relevance of body fat distribution. *Current Opinion in Lipidology* 1991:25-15.
30. Reaven G, Chen Y, Jeppesen J, Maheux P, Krauss R. Insulin resistance and hyperinsulinaemia in individuals with small dense low-density lipoprotein particles. *J Clin Invest* 1993;92:141-146.
31. Sniderman A, Cianflone K. Substrate delivery as a determinant of hepatic apoB secretion. *Arterioscler Thromb* 1993;13:629-636.
32. Selby J, Austin M, Newman B, et al. LDL subclass phenotypes and the insulin resistance syndrome in women. *Circulation* 1993;88:381-387.
33. Braun J, Severson D. Regulation of the synthesis, processing and translocation of lipoprotein lipase. *J Biochem* 1992;287:337-347.
34. Dunne F, Keane H, Jenkins D, Wright A. Hormone replacement therapy and diabetes mellitus. *Clin Endocrinol* 1996;44:615-620.
35. Sniderman A, Wolfson C, Teng B, Frankun F, Bachorik P, Kwiterovich P. Association of hyperapobetalipoproteinaemia with endogenous hypertriglyceridaemia and atherosclerosis. *Ann Intern Med* 1982;97:833-839.
36. Velazquez E, Winocour P, Kesteven P, Alberti K, Laker M. Relation of lipid peroxides to macrovascular disease in type 2 diabetes. *Diabetic Med* 1991;8:752-758.

37. Austin M, Mykkanen L, Kuusisto J, Edwards K, Nelson C, Haffner S.  
Prospective study of small LDL's as a risk factor for non insulin dependent diabetes mellitus in elderly men and women. *Circulation* 1995;92:1770-1778.
38. Sobel B. Altered fibrinolysis and platelet function in the development of vascular complications of diabetes. *Current Opinion in Endocrinology and Diabetes* 1996;3:355-360.
39. Sobel B, Woodcock-Mitchell J, Schneider D, Holt R, Marutsuka K, Gold H.  
Increased plasminogen activator inhibitor type 1 in coronary artery atherectomy specimens from type 2 diabetic compared with non-diabetic patients a potential factor predisposing to thrombosis and its persistence. *Circulation* 1998;97:2213-2221.
40. Wilhelmsen L, Svardsudd K, Korsan-Bengtson K for the Gothenberg Heart Study.  
Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med* 1984;311:501-505.
41. Meade T, Chakrabart IR, Haines A for the Northwick Heart Study. Haemostatic function and cardiovascular death early results of a prospective study. *Lancet* 1980;1:1050-1054.
42. Kannel W, Wolf P, Castelli W, D'Agostino R. Fibrinogen and risk of cardiovascular disease. *JAMA* 1987;258:1183-1186.
43. Kannel W, D' Agostino R, Wilson P, Belanger A, Gagnon D. Diabetes, fibrinogen and risk of cardiovascular disease. The Framingham experience. *Am Heart J* 1990;120:672-676.

44. Forstermann U, Mugge A, Alheid U, Haverich A, Frolich J. Selective attenuation of endothelium-mediated vasodilatation in atherosclerotic human coronary arteries. *Circ Res* 1988;62:185-190.
45. Vita J, Treasure C, Nabel E, et al. Coronary vasomotor response to acetylcholine relates to risk factors for coronary heart disease. *Circulation* 1990;81:491-497.
46. Chen J, Gall M, Deckert M, Jensen J, Parving H. Increased serum concentration of von Willebrand factor in non-insulin dependent diabetic patients with and without diabetic nephropathy. *BMJ* 1995;311:1406-1407.
47. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen K, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage: the Steno hypothesis. *Diabetologia* 1989;32:219-226.
48. Stehower C, Nauta J, Zeldenrust G, Hackeng W, Donker A, den Ottolander G. Urinary albumin excretion, cardiovascular disease and endothelial dysfunction in non-insulin dependent diabetes mellitus. *Lancet* 1992;340:319-323.
49. Mattock M, Morrish N, Viberti G, Keen H, Fitzgerald A, Jackson G. Prospective study of microalbuminuria as predictor of mortality in NIDDM. *Diabetes* 1992;41:736-741.
50. Vaughan D, Lazos S, Tong K. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin angiotensin system and thrombosis. *J Clin Invest* 1995;95:995-1001.
51. Ridker P, Gaboury C, Conlin P, Seely E, Williams G, Vaughan D. Stimulation of plasminogen activator inhibitor in vivo by infusion of angiotensin II. Evidence of

- a potential interaction between the renin angiotensin system and fibrinolytic function. *Circulation* 1993;87:1969-1973.
52. Rigart B, Hubert C, Alhene-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin 1 converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990;86:1343-1346.
53. Keavney B, Dudley C, Stratton I, et al. UK Prospective Diabetes Study (UKPDS) 14: Association of angiotensin-converting enzyme insertion/deletion polymorphism with myocardial infarction in NIDDM. *Diabetologia* 1995;38:948-952.
54. Potter van Loon B, Kluit C, Radder J, Blankenstein M, Meinders A. The cardiovascular risk factor plasminogen activator inhibitor type 1 is related to insulin resistance. *Metabolism* 1993;42:945-949.
55. Sneider D, Sobel B. Augmentation of synthesis of plasminogen activator inhibitor type 1 by insulin and insulin-like growth factor type 1 implications for vascular disease in hyperinsulinaemic states. *Proc Natl Acad Sci USA* 1991;88:9959-9963.
56. Juhan-Vague I, Thompson S, Jespersen J. Involvement of the haemostatic system in the insulin resistance syndrome. *Arterioscler Thromb* 1993;13:1865-1873.
57. Gray R, Yudkin J, Patterson D. Impaired fibrinolytic activity in patients with acute myocardial infarction. *Clin Sci* 1991;80(suppl24):23.

58. Hamsten A, Wiman B, de Faire U, et al. Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. *N Engl J Med* 1985;313:1557-1563.
59. Hamsten A, de Faire U, Walldius G. Plasminogen activator inhibitor in plasma risk factor for recurrent myocardial infarction. *Lancet* 1987;23-9.
60. McGill J, Schneider D, Arfken C, Lucore C, Sobel B. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. *Diabetes* 1994;43:104-109.
61. Calles-Escandon J, Ballor D, Harvey-Berino J, Andes P, Tracey R, Sobel B. Amelioration of the inhibition of fibrinolysis in obese elderly subjects by moderate caloric restriction. *Am J Clin Nutr* 1996.
62. Nordt T, Kornas K, Fujii S, Sobel B. Gemfibrozil inhibits the induction of plasminogen activator inhibitor type 1 by insulin and its precursors. *Circulation* 1995;91:555-558.
63. Manninen V, Tenkanen L, Koskinen P, et al. Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study; Implications for treatment. *Circulation* 1992;85:37-45.
64. Brownlee M, Vlassara H, Cerami A. Non-enzymatic glycosylation reduced the susceptibility of fibrin to degradation by plasmin. *Diabetes* 1983;32:680-684.
65. Gough S, Grant P. The fibrinolytic system in diabetes mellitus. *Diabetic Medicine* 1991;8:898-905.

66. Stewart D, Monge J. Hyperlipidaemia and endothelial dysfunction. *Curr Opin Lipidol* 1993;4:319-324.
67. Tikkanen M. Mechanism of cardiovascular protection by postmenopausal hormone replacement therapy. *Cardiovascular Risk Factors*. 1993;3:138-143.
68. Kuhn F, Mohler E, Satler L, Reagan K, Lu D, Rackley C. Effects of high-density lipoprotein on acetylcholine induced coronary vasoreactivity. *Am J Cardiol* 1991;68:1425-1430.
69. Lehr H, Krombach F, Munzing S, et al. In vitro effects of oxidised low density lipoproteins on CD11b/CD18 and L-selectin presentation on neutrophils and monocytes with relevance for the in vivo situation. *Am J Path* 1995;146:218-227.
70. Thomas C, Jackson R, Ohlweiler D, Ku G. Multiple oxidation products in LDL induce IL-1beta release from human blood mononuclear cells. *J Lipid Res* 1994;35:417-427.
71. Haffner S, Stern M, Hazuda P, et al. Cardiovascular risk factors in confirmed pre-diabetic individuals does the clock for coronary heart disease start ticking before the onset of clinical diabetes? *JAMA* 1990;263:2893-2898.
72. Krolewski A, Warran J, Vassania P, et al. Evolving natural history of coronary heart disease in diabetes mellitus. *Am J Med* 1990;90 (suppl 2A):565-615.
73. Fuller J, Shipley M, Rose G, et al. Coronary heart disease risk and impaired glucose tolerance. *Lancet* 1980;i:1373-1376.
74. Stamler J, Vaccaro O, Neanton J, for the MR FIT Writing Group. Diabetes, other risk factors and 12-year cardiovascular mortality for men screened in the multiple risk factor intervention trial. (MR FIT). *Diabetes Care* 1993;16:434-444.

75. Barrett-Connor E. Fortnightly Review. Hormone replacement therapy. *BMJ* 1998;317:457-461.
76. Barrett-Connor E. Postmenopausal oestrogen and prevention bias. *Ann Intern Med* 1991;115:455-456.
77. Shepherd J, Cobbe S, Ford I, et al. Prevention of Coronary Heart Disease with pravastatin in men with hypercholesterolaemia. *N Engl J Med* 1995;333:1301-1307.
78. Koskinen P, Manttari M, Manninen V, Huttunen J, Heinonen O, Frick M. Coronary heart disease incidence in NIDDM patients in the Helsinki Heart Study. *Diabetes Care* 1992;15:820-825.
79. Frick M, Elo O, Haapa K, et al. Helsinki Heart Study: Primary prevention trial with gemfibrozil in middle aged men with dyslipidaemia. *N Eng J Med* 1987;317:1237-1245.
80. Rubins H, Robins S, Collins D, et al. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high density lipoprotein cholesterol. *N Eng J Med* 1999;341:410-418.
81. Durrington P. Serum high density lipoprotein cholesterol in diabetes mellitus an analysis of factors which influence its concentration. *Clin Chem Acta* 1980;104:11-23.
82. Downs G, Clearfield M, Weiss S, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels results of AFCAPS/TEXCAPS; Air Force/Texas coronary atherosclerosis study. *JAMA* 1998;279:1615-1622.

83. Pyorala K, Pedersen TR, Kjekshus J, Faergeman O, Olsson AG, Thorgeirsson G. Cholesterol lowering with simvastatin improves prognosis of diabetic patients with coronary heart disease: A subgroup analysis of the Scandinavian Simvastatin Survival Study (4S) *Diabetes Care* 1997;20:614-620.
84. Goldberg RB, Mellies MJ, Sacks FM, et al. Cardiovascular events and their reduction with pravastatin in diabetic and glucose-intolerant myocardial infarction survivors with average cholesterol levels: subgroup analyses in the Cholesterol and Recurrent Events (CARE). *Circulation* 1998;98:2513-2519.
85. The Long Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N Engl J Med* 1998;339:1349-1357.
86. Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease. The Scandinavian Simvastatin Survival Study;(4S). *Lancet* 1994;334:1383-1389.
87. Durrington P. Statins and fibrates in the management of diabetic dyslipidaemia. *Diabetic Medicine* 1997;14:513-516.
88. The Hypertension in Diabetes Study Group. Hypertension in diabetes study (HDS) I; Prevalence of hypertension in newly presenting type 2 diabetic patients and the association of risk factors for cardiovascular and diabetic complications. *J Hypertens* 1993;11:309-317.

89. Gall M, Hougaard P, Borch-Johnsen K, et al. Risk factors for development of incipient and overt diabetic nephropathy in patients with non-insulin dependent diabetes mellitus. *BMJ* 1997;314:783-788.
90. UK Perspective Diabetes Study Group. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes. (UKPDS 38). *BMJ* 1998;317:703-713.
91. UK Perspective Diabetes Study Group Group UPDS. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes. (UKPDS 39). *BMJ* 1998;317:713-720.
92. Hansson L, Zanchetti A, Carruthers S, et al. Effects of intensive blood pressure lowering and low dose aspirin in patients with hypertension principal results of the hypertension optimal treatment (HOT) randomised trial. *Lancet* 1998;351:1755-1762.
93. The HOT Study Group. The Hypertension Optimal Treatment Study (the HOT study). *Blood Pressure* 1993;2:62-68.
94. Systolic Hypertension in Elderly Patients (SHEP) Co-Operative Research Group. Prevention of stroke by antihypertensive treatment in older persons with isolated systolic hypertension. *JAMA* 1991;265:3255-3264.
95. Tuomilehto J, Rastenyte D, Burkenhager W, Thijs L, Antikainen R, Bulpitt C, et al. Effects of calcium channel blockade in older patients with diabetes mellitus and systolic hypertension. *N Eng J Med* 1999;340:677-684.

96. Staessen J, Fagard R, Thijs L, et al. Randomised double blind comparison of placebo and active treatment for older patients with isolated systolic hypertension. *Lancet* 1997;350:757-764.
97. Langford H. All cause mortality in the hypertension detection and follow-up program findings for the whole cohort and for persons with less severe hypertension with and without other traits related to mortality. *Prog Cardiovasc Disease* 1986;29(suppl pt 3).
98. Eaker E, Chesebro J, Sacks F, Wenger N, Whisnant J, Winston M. Cardiovascular disease in women. *Circulation* 1993;88:1999-2009.
99. Kannel W, Wilson P. Risk factors that attenuate the female coronary advantage. *Arch Intern Med* 1995;155:57-61.
100. Rich-Edwards J, Manson J, Hennekens C, Buring J. The primary prevention of coronary heart disease in women. *N Eng J Med* 1995;332:1758-1766.
101. American Heart Association. 1999 Heart and Stroke statistical update. 1998:1-29.
102. Kalin M, Zumoff B. Sex Hormones and coronary disease: a review of the clinical studies. *Steroids* 1990;55:330-352.
103. Murabito J. Women and cardiovascular disease: contributions from the Framingham Heart Study. *J Am Med Womens Assoc* 1995;50:35-55.
104. Lerner D, Kannel W. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26year follow-up of the Framingham population. *Am Heart J* 1986;111:383-390.

105. Maynard C, Litwin P, Martin J, Weaver W. Gender differences in the treatment and outcome of acute myocardial infarction: results from the Myocardial Infarction Triage and Intervention Registry. *Arch Intern Med* 1992;152:972-976.
106. Cunningham M, Lee T, Cook E, et al. The effect of gender on the probability of myocardial infarction among emergency department patients with acute chest pain: a report from the multicentre chest pain study group. *J Gen Intern Med* 1989;4:392-398.
107. Chaitman B, Bourassa M, Davis K, et al. Angiographic prevalence of high risk coronary artery disease in patient subsets (CASS study). *Circulation* 1981;64:360-367.
108. Weiner D, Ryan T, McCabe C, et al. Exercise stress testing: correlations among history of angina, ST-segment response and prevalence of coronary artery disease in the Coronary Artery Surgery Study (CASS study). *N Eng J Med* 1979;301:230-235.
109. Barolsky S, Gilbert C, Faruqui A, et al. Differences in electrocardiographic response to exercise of women and men: a non-Bayesian factor. *Circulation* 1979;60:1465-1474.
110. Detry J, Kapita M, Cosyns J, et al. Diagnostic value of history and maximal exercise electrocardiography in men and women suspected of coronary heart disease. *Circulation* 1977;56:756-761.
111. Hung J, Chaitman B, Lam J, et al. Noninvasive diagnostic test choices for the evaluation of coronary artery disease in women: a multivariate comparison of

- cardiac fluoroscopy, exercise electrocardiography and exercise thallium perfusion scintigraphy. *J Am Coll Cardiol* 1984;4:8-17.
112. Steingart R, Packer M, Hamm P, et al for the Survival and Ventricular Enlargement Investigators. Sex differences in the management of coronary artery disease. *N Engl J Med* 1991;325:226-230.
113. Welty F. Cardiovascular disease and dyslipidaemia in women. *Arch Intern Med* 2001;161:514-522.
114. Colditz G, Willett W, Stampfer M, Rosner B, Speizer F, Hennekens C. Menopause and the risks of coronary heart disease in women. *N Eng J Med* 1987;316:1105-1110.
115. Johnson C, Rifkind B, Sempos C, et al. Declining serum total cholesterol levels among US adults: the National Health and Nutrition Examination Surveys.(NHANES) III. *JAMA* 1987;269:3002-3008.
116. Campos H, McNamara J, Wilson P, Ordovas J, Schaefer E. Differences in low density lipoprotein subfractions and apolipoproteins in pre-menopausal and postmenopausal women. *J Clin Endocrinol Metab* 1988;67:30-35.
117. Stevenson J, Crook D, Godsland I. Influence of age and menopause on serum lipids and lipoproteins in healthy women. *Atherosclerosis*. 1993;98:83-90.
118. Manolio T, Pearson T, Wenger N, Barrett-Connor E, Payne G, Harlan W. Cholesterol and heart disease in older persons and women: a review of an NHLBI workshop. *Ann Epidemiol* 1992;2:161-176.

119. Austin M, Breslow C, Hennekens C, Buring J, Willett W, Krauss R. Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA* 1988;260:1917-1921.
120. Matthews K, Meilaha E, Kuller L, Kelsey S, Caggiula A, Wing R. Menopause and risk factors for coronary heart disease. *N Eng J Med* 1989;321:641-646.
121. Mosca L, Manson J, Sutherland S, Langer T, Barrett-Connor E. Cardiovascular disease in women a statement for healthcare professionals from the American Heart Association. *Circulation* 1997;96:2468-2482.
122. Walsh J, Grady D. Treatment of hyperlipidaemia in women. *JAMA* 1995;274:1152-1158.
123. Corti M, Guralnik J, Salive M, et al. HDL cholesterol predicts coronary heart disease mortality in older persons. *JAMA* 1995;274:539-544.
124. Austin M, Hokanson J, Edwards K. Hypertriglyceridaemia as a cardiovascular risk factor. *Am J Cardiol* 1998;81:7B-12B.
125. Bass K, Newschaffer C, Klag M, Bush T. Plasma lipoprotein levels as predictors of cardiovascular death in women. *Arch Intern Med* 1993;153:2209-2216.
126. LaRosa J. Triglycerides and coronary risk in women and the elderly. *Arch Intern Med* 1997;157:961-968.
127. Castelli W. The triglyceride issue: a view from Framingham. *Am Heart J* 1986;112:432-437.

128. Dorr A, Gunderson K, Schneider J, et al. Colestipol hydrochloride in hypercholesterolaemic patients: effect on serum cholesterol and mortality. *J Chronic Disease*. 1978;31:5-14.
129. Meittinen T, Pyorala K, Olsson A, et al for the Scandinavian Simvastatin Survival Study group. Cholesterol lowering therapy in women and elderly patients with myocardial infarction or angina pectoris. *Circulation* 1997;96:4211-4218.
130. Lewis S, Sacks F, Mitchell J, et al for the CARE investigators. Effect of pravastatin on cardiovascular events in women after myocardial infarction: the Cholesterol and Recurrent Events (CARE) trial. *J Am Coll Cardiol* 1998;32:140-146.
131. Waters D, Higginson L, Gladstone P, et al for the CCAIT Study group. Effects of cholesterol lowering on the progression of coronary atherosclerosis in women: a Canadian Coronary Atherosclerosis Intervention Trial (CCAIT) sub-study. *Circulation* 1995;92:2404-2410.
132. Kitler M. Differences in men and women in coronary artery disease, systemic hypertension and their treatment. *Am J Cardiol* 1992;70:1077-1080.
133. Kannel W, McGee D, Gordon T. A general cardiovascular risk profile. The Framingham Study. *Am J Cardiol* 1976;38:46-51.
134. Truett J, Cornfield J, Kannel W. A multivariate analysis of the risk of coronary heart disease in Framingham. *J Chronic Dis* 1967;20:511-524.
135. Coronary Risk Handbook. Estimating the risk of coronary heart disease in daily practice. 1973.

136. ASPIRE Steering Group. A British Cardiac Society survey of the potential for secondary prevention of coronary heart disease ASPIRE (action on secondary prevention through intervention to reduce events). Preliminary results. *Heart* 1996;75:334-342.
137. Stratton I, Adler A, Neil H, et al. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes. (UKPDS35). *BMJ* 2000;321:405-412.
138. The Diabetes Control and Complication (DCCT) Trial Research Group. Effect of intensive diabetes management on macrovascular events and risk factors in the Diabetes Control and Complications Trial. *Am J Cardiol* 1995;75:894-903.
139. UK Perspective Diabetes Study Group. Intensive blood glucose control with sulphonylurea or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998;352:837-852.
140. Taskinen M, Packard C, Shepherd J. Effect of insulin therapy on metabolic fate of apolipoproteins in NIDDM. *Diabetes* 1990;39:1017-1027.
141. Romano G, Patti L, Inzelli F, et al. Insulin and sulphonylurea therapy in NIDDM patients are the effects on lipoprotein metabolism different even with similar blood glucose control? *Diabetes* 1997;46:1601-1606.
142. Collins R, MacMahon S. Blood pressure, antihypertensive drug treatment and the risks of stroke and of coronary heart disease. *Br Med Bull* 1994;50:272-298.

143. Linjer E, Hansson L. Underestimation of the true benefits of antihypertensive treatment an assessment of some important sources of error. *J Hypertens* 1997;15:221-225.
144. Law M, Wald N, Thompson S. By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *BMJ* 1994;308:367-373.
145. Law M, Thompson S, Wald N. Assessing possible hazards of reducing serum cholesterol. *BMJ* 1994;308:373-379.
146. Gould A, Rossouw J, Santanello N, et al. Cholesterol reduction yields clinical benefit. A new look at old data. *Circulation* 1995;91:2274-2282.
147. Davey-Smith G, Song F, Sheldon T. Cholesterol lowering and mortality, the importance of considering initial level of risk. *BMJ* 1993;306:1367-1373.
148. Holme I. An analysis of randomised controlled trials evaluating the effect of cholesterol reduction on total mortality and coronary heart disease incidence. *Circulation* 1990;82:1916-1924.
149. Smith G, Pekkanen J. Should there be a moratorium on the use of cholesterol lowering drugs? *BMJ* 1992;304:431-434.
150. Scottish Intercollegiate Guidelines Network. Lipid Lowering for the prevention of coronary heart disease: a national clinical guideline. *SIGN 40* 1999.
151. Fontbonne A, Eschwege E, Cambien F, et al. Hypertriglyceridaemia as a risk factor of coronary artery disease mortality in subjects with impaired glucose tolerance or diabetes. Results from the 11 year follow-up of the Paris Perspective Study. *Diabetologia*. 1989;32:300-304.

152. The Medical Research Council General Practice Research Framework. Thrombosis prevention trial randomised trial of low-intensity oral anticoagulation with warfarin and low dose aspirin in the primary prevention of ischaemic heart disease in men at high risk. *Lancet* 1998;351:233-241.
153. Scottish Intercollegiate Guidelines Network. Antithrombotic therapy: a national clinical guideline. (SIGN 36). *Edinburgh* 1999;SIGN.
154. Antiplatelet Trialists Collaboration. Collaborative overview of randomised trial of antiplatelet therapy ; prevention of death, myocardial infarction and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994;308:81-106.
155. Sacket D, Anderson G, Milner R. Concordance for coronary risk factors among spouses. *Circulation* 1975;52:589-595.
156. Daly L, Wood D, Kinmonth A, et al. Long term effect on mortality of stopping smoking after unstable angina and myocardial infarction. *BMJ* 1983;287:324-326.
157. Joseph A, Norman S, Ferry L, et al. The safety of transdermal nicotine as an aid to smoking cessation in patients with cardiac disease. *N Engl J Med* 1996;335:1792-1798.
158. Browner W, Hulley S. Effect of risk factor status on treatment criteria. Implications of hypertension trials. *Hypertension* 1989;13:51-56.
159. British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Association. Joint British recommendations on prevention of coronary heart disease in clinical practice. *Heart* 1998;80(2S):1S-29S.

160. Yusuf S, Peto R, Lewis J, et al. Beta blockade during and after myocardial infarction an overview of the randomised trials. *Pro Cardiff Dis* 1983;371:335-371.
161. The Danish Study Group of Verapamil in Myocardial Infarction. Effect of verapamil on mortality and major events after myocardial infarction (the Danish verapamil infarction trial II- DAVIT II). *Am J Cardiol* 1990;66:779-784.
162. Pfeffer M, Braunwald E, Moye L, on behalf of the SAVE Investigators. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement (SAVE) trial. *N Engl J Med* 1992;327:669-677.
163. The Acute Infarction Ramipril Efficacy Study Investigators. Effect of ramipril on the mortality and morbidity of survivors of acute myocardial infarction with evidence of heart failure (AIRE). *Lancet* 1993;342:812-828.
164. Cruikshank J, Thorp J, Zacharias F. Benefits and potential harm of lowering high blood pressure. *Lancet* 1987;581-584.
165. D'Agostini R, Belanger A, Kannel W, et al. Relationship of low diastolic blood pressure to coronary heart disease death in the presence of myocardial infarction. The Framingham study. *BMJ* 1991;303:385-389;.
166. Pekkanen J, Linn S, Heiss G. Ten year mortality from cardiovascular disease in relation to cholesterol level among men with and without pre-existing cardiovascular disease. *N Engl J Med* 1990;322:1700-1707.

167. Ericsson G, Hamsten A, Nilsson J, et al. Angiographic assessment of effects of bezafibrate on progression of coronary artery disease in young male post-infarction patients. *Lancet* 1996;347:849-853.
168. Thompson G. What targets should lipid-modulating therapy achieve to optimise the prevention of coronary heart disease? *Atherosclerosis* 1997;131:1-5.
169. Smith P, Arneson H, Holme I. The effect of warfarin on mortality and reinfarction after myocardial infarction. *N Engl J Med* 1990;323:147-152.
170. Kuhl H. Pharmacokinetics of oestrogens and progestogens. *Maturitas* 1990;12:171-197.
171. Powers M, Schenkel L, Darley P, Good W, Balestra J, Place V. Pharmacokinetics and pharmacodynamics of transdermal dosage forms of 17 $\beta$ -oestradiol: comparison with conventional oral oestrogens used for hormone replacement. *Am J Obstet Gynecol* 1985;152:99-106.
172. Gangar K, Cust M, Whitehead M. Symptoms of oestrogen deficiency associated with supraphysiological plasma oestradiol concentrations in women with oestradiol implants. *BMJ* 1989;299:601-602.
173. Moorhead T, Hannaford P, Warskyj M. Prevalence and characteristics associated with use of hormone replacement therapy in Britain. *British Journal of Obstetrics and Gynaecology* 1997;104:290-297.
174. Wilkes H, Meade T. Hormone replacement therapy in general practice a survey of doctors in the Medical Research Council General Practice Research Framework. *BMJ* 1991;302:1317-1320.

175. Hemminki E, Malin M, Topo P. Selection of postmenopausal therapy by women's characteristics. *J Clin Epidemiol* 1993;46:211-219.
176. Lancaster T, Surman G, Lawrence M, et al. Hormone replacement therapy characteristics of users and nonusers in a British general practice cohort identified through computerised prescribing records. *J Epidemiol Commun Health* 1995;49:389-394.
177. Griffiths F, Jones K. The use of hormone replacement therapy results of a community survey. *Fam Pract* 1995;12:163-165.
178. Feher M, Isaacs A. Is hormone replacement therapy prescribed for postmenopausal diabetic women? *Br J Clin Pract* 1996; 50:431-432.
179. Usher C, Teeling M, Bennett K and Feely J. Effect of Women's Health Initiative trial on hormone replacement therapy prescribing. *British Journal of Clinical Pharmacology*. 2004;57:667.
180. Kumar P, Clark M. *Clinical Medicine*. third ed: Balliere Tindall, 1995.
181. Jensen G, Christiansen C, Boesen J, Hegedus V, Transbol I. Epidemiology of postmenopausal spinal and long fractures a unifying approach to postmenopausal osteoporosis. *J Orthopaedic Research* 1982;166:75-81.
182. Stevenson J, Whitehead M. Postmenopausal osteoporosis. *BMJ* 1982;285:585-588.
183. Stevenson J, Lees B, Devenport M, Cust M, Ganger K. Determinants of bone density in normal women risk factors for future osteoporosis. *BMJ* 1989;38:30-35.
184. Aitken J, Hart D, Lindsay R. Oestrogen replacement therapy for the prevention of osteoporosis after oophorectomy. *BMJ* 1973;3:515-518.

185. Nachtigall L, Natchtigall R, Natchigall R, Beckman E. Oestrogen replacement therapy: a 10 year prospective study in the relationship to osteoporosis. *Obstet Gynaecol* 1979;53:277-281.
186. Lindsay R, Hart D, Purdie D, Ferguson M, Clark A, Kraszewski A. Comparative effects of oestrogen and progestogen on bone loss in postmenopausal women. *Clin Sci Mol Med* 1978;53:193-195.
187. Lindsay R, Hart D, Clark D. The minimum effective dose of oestrogen for prevention of postmenopausal bone loss. *Obstet Gynecol* 1984;63:759-763.
188. Ettinger B, Genant H, Cann C. Postmenopausal bone loss is prevented by treatment with low dosage oestrogen with calcium. *Ann Intern Med* 1987;106:40-45.
189. Christiansen C, Christensen M, Larsen N, Transbol I. Pathophysiological mechanisms of oestrogen effect on bone metabolism: dose response relationships in early postmenopausal women. *J Clin Endocrinol Metab* 1982;55:1124-1130.
190. Munk-Jensen N, Pors Nielsen S, Obel E, Bonne Ericksen P. Reversal of postmenopausal vertebral bone loss by oestrogen and progestogen: a double blind placebo controlled study. *BMJ* 1988;296:1150-1152.
191. Christiansen C, Christensen M, Transbol I. Bone mass in postmenopausal women after withdrawal of oestrogen/gestagen replacement therapy. *Lancet* 1981;1:459-461.
192. Riis B, Thomsen K, Strom V, Christiansen C. The effect of percutaneous oestradiol and natural progesterone on postmenopausal bone loss. *Am J Obstet Gynecol* 1987;156:61-65.

193. Adami S, Suppi R, Bertoldo F, et al. Transdermal oestradiol in the treatment of postmenopausal bone loss. *Bone Miner* 1989;7:79-86.
194. Savvas M, Studd J, Fogelman I, Doolcy M, Montgomery J, Murby B. Skeletal effects of oral oestrogen compared with subcutaneous oestrogen and testosterone in postmenopausal women. *BMJ* 1988;297:331-333.
195. Hutchinson T, Polansky S, Feinstein A. Postmenopausal oestrogen protects against fractures of the hip and distal radius: a case control study. *Lancet* 1979;2:705-709.
196. Weiss N, Ure C, Ballard J, Williams A, Daling J. Decreased risk of fractures of hip and lower forearm with postmenopausal use of oestrogen. *N Eng J Med* 1980;303:1195-1198.
197. Ettinger B, Genant H, Cann C. Long-term oestrogen replacement therapy prevents bone loss and fractures. *Ann Intern Med* 1985;102:319-324.
198. Kiel D, Felson D, Anderson JJ, Wilson P, Moskowitz M. Hip fracture and the use of oestrogens in postmenopausal women: the Framingham Study. *N Engl J Med* 1987;317:1169-1174.
199. Bechetz P. Drug therapy: hormonal treatment of postmenopausal women. *N Eng J Med* 1994;330:1062-1071.
200. Kanis J. Oestrogens, the menopause and osteoporosis. *Bone* 1996;19(suppl 5):18-90.
201. Johnston CJ, Slemenda C, Melton L. Clinical use of bone densitometry. *N Eng J Med* 1991;324:1105-1109.

202. van Daele P, Stlok R, Burger H, et al. Bone density in non-insulin dependent diabetes mellitus: The Rotterdam Study. *Ann Intern Med* 1995;122:409-414.
203. Sosa M, Dominguez M, Navarro M, et al. Bone mineral metabolism is normal in non-insulin dependant diabetes mellitus. *J Diabetes Complications* 1996;10:201-205.
204. Piepkorn B, Kann P, Forst T, Andreas J, Pfutzner A, Beyer J. Bone mineral density and bone metabolism in diabetes mellitus. *Horm Metab Res* 1997;29:584-591.
205. Meema H, Meema S. The relationship of diabetes mellitus and body weight to osteoporosis in elderly females. *Can Med Assoc J* 1967;96:132-139.
206. Johnston C, Hui S, Longcope C. Bone mass and sex steroid concentrations in postmenopausal caucasian diabetics. *Metabolism* 1985;34:544-550.
207. Haffner S, Bauer R. The association of obesity and glucose and insulin concentration with bone density in pre-menopausal and postmenopausal women. *Metabolism* 1993;6:735-738.
208. Wagasugi M, Wakao R, Tawata M, Gan N, Koizumi K, Onaya T. Bone mineral density as measured by dual energy X-ray absorptiometry in patients with non insulin dependent diabetes mellitus. *Bone* 1993;14:29-33.
209. Nicodemus K, Folsom A. Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. *Diabetes Care* 2001;24:1192-1197.
210. Ziegler R. Diabetes mellitus and bone metabolism. *Hormone metabolism and research* 1992;26:90-94.

211. Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Pettit D. Hormone replacement therapy and endometrial cancer risk a meta-analysis. *Obstet Gynaecol* 1995;85:304-313.
212. Barrett-Connor E. Hormone replacement and cancer. *Br Med Bulltin* 1992;48:345-355.
213. Robboy S, Bradley R. Changing trends and prognostic features in endometrial cancer associated with exogeneous oestrogen therapy. *Obstet Gynecol* 1979;54:269-277.
214. Chu J, Schweid A, Weiss N. Survival among women with endometrial cancer: a comparison of oestrogen users and nonusers. *Am J Obstet Gynecol* 1982;143:569-573.
215. Gambrell Rj. Prevention of endometrial cancer with progestogens. *Maturitas* 1986;8:159-168.
216. Persson I, Adami H, Bergkvist I., et al. Risk of endometrial cancer after treatment with oestrogens alone or in conjunction with progestogens: results of a prospective study. *BMJ* 1989;298:147-151.
217. Whitehead M, Townsend P, Pryse-Davies J, Ryder T, King R. Effects of oestrogens and progestogens on the biochemistry and morphology of the postmenopausal endometrium. *N Eng J Med* 1981;305:1599-1605.
218. Beresford S, Weiss N, Voigt L, McKnight B. Risk of endometrial cancer in relation to use of oestrogen combined with cyclic progesterone therapy in postmenopausal women. *Lancet* 1997;349:458-461.

219. Stephanovic A, Trezic M, Kontic O, et al. Incidence of diabetes mellitus as a risk factor of endometrial cancer. *Acta Obstetrica et Gynecologica Scandinavica* 1997;76:86.
220. La Vecchia C, Negri E, Franceschi S, D'Avanzo B, Boyle P. A case control study of diabetes mellitus and cancer risk. *Br J Cancer* 1994;70:950-953.
221. Maatela J, Aromaa A, Salmi T, Pohja M, Vuento M, Gronroos M. The risk of endometrial cancer in diabetic and hypertensive patients a nation-wide record-linkage study in Finland. *Annals Chirurgiae et Gynaecologiae* 1994;208:20-24.
222. Yancik R, Wesley M, Ries L, et al. Co-morbidity and age as predictors of risk for early mortality of male and female colon carcinoma patients: a population based study. *Cancer* 1998;82:2123-2134.
223. Tran T, Medline A, Bruce W. Insulin promotion of colon tumours in rats. *Cancer Epidemiol Biomarkers Prev* 1996;5:1013-1015.
224. Kaaks R, Toniolo P, Akhmedkhanov A, et al. Serum C-peptide, insulin like growth factor (IGF)-I, IGF-binding proteins and colorectal cancer risk in women. *J Natl Cancer Inst* 2000;92:1592-1600.
225. Schoen R, Tangen C, Kuller L, et al. Increased blood glucose and insulin, body size and incident colorectal cancer. *J Natl Cancer Inst* 1999;91:1157-1154.
226. Iju F, Manson J, Liu S, et al. Prospective study of adult onset diabetes mellitus and risk of colorectal cancer in women. *J Natl Cancer Inst* 1999;91:542-547.

227. La Vecchia C, Negri E, Decarli A, et al. Diabetes mellitus and colorectal cancer risk. *Cancer Epidemiol Biomarker Prev* 1997;6:1007-1010.
228. Colditz G, Stampfer M, Willett W, Hennekens C, Rosner B, Speizer F. Prospective study of oestrogen replacement therapy and risk of breast cancer in postmenopausal women. *JAMA* 1990;264:2648-2653.
229. Colditz G, Stampfer M, Willett W, et al. Postmenopausal hormone use and risk of breast cancer: a 12 year follow-up of the Nurses' Health Study. In Mann RD ed. *Hormone replacement therapy and breast cancer risk*. Carnforth, England: Parthenon. 1992:63-77.
230. Bergkvist L, Adams H, Persson I, Bergstrom R, Krusemo U. Prognosis after breast cancer diagnosis in women exposed to oestrogen and oestrogen-progestogen replacement therapy. *Am J Epidemiol* 1989;130:221-228.
231. Dupont W, Page D. Menopausal oestrogen replacement therapy and breast cancer. *Arch Intern Med* 1991;151:67-72.
232. Steinberg K, Thacker S, Smith S, et al. A meta-analysis of the effect of oestrogen replacement therapy on the risk of breast cancer. *JAMA* 1991;265:1985-1990.
233. Armstrong B. Oestrogen therapy after the menopause - boon or bane? *Med J Aust* 1988;148:213-214.
234. Writing group for the Womens' Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. Principal results from the Women's Health Initiative Randomized Controlled Trial. *JAMA* 2002;288:321-333.

235. Laya M, Larson E, Taplin S, White E. Effect of oestrogen replacement therapy on the specificity and sensitivity of screening mammography. *J Natl Cancer Inst* 1996;88:643-669.
236. Boyd N, Byng J, Jong R, et al. Quantitative classification of mammographic densities and breast cancer risk results from the Canadian National Breast Screening Study. *J Natl Cancer Inst* 1995;87:670-675.
237. Stoll B, Parbhoo S. Treatment of menopausal symptoms in breast cancer patients. *Lancet* 1988;1:1278-1279.
238. Creasman W. Oestrogen replacement therapy: is previously treated cancer a contraindication? *Obstet Gynecol* 1991;77:308-312.
239. Yancik R, Wesley M, Ries L, Havlik R, Edwards B, Yates J. Effect of age and co-morbidity in postmenopausal breast cancer patients aged 55 years and older. *JAMA* 2001;285:885-892.
240. Goodwin P, Emis M, Pritchard K, et al. Fasting insulin and outcome in early stage breast cancers: results of a prospective cohort study. *J Clin Oncol* 2002;20:42-51.
241. Hulley S, Grady D, Bush T, et al. Randomised trial of oestrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women (HERS) trial. *JAMA* 1998;280:605-613.
242. Caine Y, Bauer K, et al. Coagulation activation following oestrogen administration to postmenopausal women. *Throm Haemost* 1992;68:392-395.

243. Kroon U, Silfverstolpe G, Tengborn L. The effects of transdermal oestradiol and oral conjugated oestrogens on haemostasis. *Thromb Haemost* 1994;71:420-423.
244. Cushman M, Legault C, Barrett-Connor E, et al. Effect of postmenopausal hormones on inflammation-sensitive proteins: the Postmenopausal Estrogen/Progestin Interventions (PEPI) Study. *Circulation* 1999;100:717-722.
245. Bar J, Tepper R, Fuchs J, Pardo Y, Goldberg S, Oloradia J. The effect of oestrogen replacement therapy on platelet aggregation and adenosine triphosphate release in post-menopausal women. *Obstet Gynaecol* 1993;81:261-264.
246. Grady D, Hulley S, Furberg C. Venous thromboembolic events associated with hormone replacement therapy. *JAMA* 1997;278:4-77.
247. Lip G, Beevers M, Beevers D. Malignant hypertension in young women is related to previous hypertension in pregnancy, not oral contraceptive. *QJM* 1997;90:571-575.
248. Weir J. Oral contraceptives and hypertension. *Hypertension* 1988;11(suppl2):11-15.
249. Lim K, Isles C, Hodsmann G, Lever A, Robertson J. Malignant hypertension in women of childbearing age and relation to the oral contraceptive pill. *BMJ* 1987;294:1057-1059.
250. Walters W, Lim Y. Haemodynamic changes in women taking oral contraceptives. *J Obstet Gynecol Br Commonwealth* 1970;77:1007-1012.
251. Crane M, Harris J, Winsor W. Hypertension, oral contraceptive agents and conjugated oestrogens. *Ann Intern Med* 1971;74:13-21.

252. Godsland I, Walton C, Felton C, Proudler A, Patel A, Wynn V. Insulin resistance, secretion and metabolism in users of oral contraceptives. *J Clin Endocrinol Metab* 1992;74:64-70.
253. Staessen J, Bulpitt C, Fagard R, Lijnen P, Amery A. The influence of menopause on blood pressure. *Hypertension* 1989;3:427-433.
254. Pfeffer R, Kurosaki T, Charlton S. Oestrogen use and blood pressure in later life. *Am J Epidemiology* 1979;110:469-47.
255. Jespersen C, Arnung K, Hagen C, et al. Effects of natural oestrogen therapy on blood pressure and renin-angiotensin system in normotensive and hypertensive menopausal women. *J of Hypertension* 1983;161-364;.
256. Lip G, Beevers M, Churchill D, Beevers G. Hormone replacement therapy and blood pressure in hypertensive women. *J Hum Hypertens* 1994;8:491-494.
257. van Wersch, JW, Ubachs, JM, van-de-Ende A, van Enk, A. The effect of two regimens of hormone replacement therapy on the homeostatic profile in postmenopausal women. *Eur J Clin Chem Clin Biochem* 1994;32:449-453.
258. Brussard H, Gevers Leuven J, Frolich M, Kluft C, Krans H. Short-term oestradiol replacement therapy improves insulin resistance, lipids and fibrinolysis in postmenopausal women with NIDDM. *Diabetologia* 1997;40:843-849.
259. Gilligan D, Quyyumi A, Cannon R. Effects of physiological levels of oestrogen on coronary vasomotor function in post-menopausal women. *Circulation* 1994;90:786-791.

260. Leiberman E, Gerhard M, Uehata A, et al. Oestrogen improves endothelium dependent flow mediated vasodilatation in postmenopausal women. *Ann Intern Med* 1994;121:936-941.
261. Gilligan D, Badar D, Panza J, Quyyumi A, Cannon R. Acute vascular effects of oestrogen in postmenopausal women. *Circulation* 1994;90:786-791.
262. Sacks F, Gerhard M, Walsh B. Sex hormones, lipoproteins and vascular reactivity. *Curr Opin Lipidol* 1995;6:161-166.
263. Collins P, Rosano G, Jiang C, Lindsay D, Sarrel P, Poole-Wilson P. Cardiovascular protection by oestrogen- a calcium antagonist effect? *Lancet* 1993;341:1264-1265.
264. Spagnoli L, Palmeiri G, Mauriello A, Orlandi A, Bancheri C, Pasetto N. High-dose synthetic progestogens inhibit foam and smooth muscle cell proliferation and atherosclerotic plaque formation in aortas of rabbits fed a hypercholesterolaemic diet. *Atherosclerosis*. 1990;82:27-36.
265. Clarkson R, Anthony M, Klein K. Effects of oestrogen treatment on arterial wall structure and function. *Drugs* 1994;47(suppl 2):42-51.
266. Anderson B, Mattsson L, Hahn L, et al. Oestrogen replacement therapy decreases hyperandrogenicity and improves plasma lipids in postmenopausal women with Non-Insulin Dependent Diabetes Mellitus. *J Clin Endocrinol Metab* 1997;82:638-643.
267. Mosnier-Pudar H, Faguer B, Guyenne T, Tchobroutsky G. Effects of deprivation and replacement by percutaneous 17 $\beta$  oestradiol and oral progesterone on blood pressure and metabolic parameters in menopausal patients with non-

- insulin dependent diabetes. *Archives des Maladies du Coeur et des Vaisseaux* 1991; 84:1111-1115.
268. Stevenson J, Crook D, Godsland I, Collins P, Whitehead M. Hormone replacement therapy and the cardiovascular system: non-lipid effects. *Drugs* 1994;47(suppl 2):35-41.
269. Hemminki F, McPherson K. Impact of postmenopausal hormone replacement therapy on cardiovascular events and cancer pooled data from clinical trials. *BMJ* 1997;315:149-153.
270. The Writing Group for the PEPI Trial. Effects of oestrogen and oestrogen/progestin regimens on heart disease risk factors in postmenopausal women: The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. *JAMA* 1995;273:199-208.
271. Walsh B, Schiff I, Rosner B, Greenberg L, Ravnihar V. Effects of postmenopausal oestrogen replacement on the concentrations and metabolism of plasma lipoproteins. *N Eng J Med* 1991;325:1196-1204.
272. McManus J, McEneny J, Thompson W, Young I. The effect of hormone replacement therapy on the oxidation of low density lipoprotein in postmenopausal women. *Atherosclerosis* 1997;135:73-81.
273. Mosca L. The role of hormone replacement therapy in the prevention of postmenopausal heart disease. *Archives of Internal Medicine* 2000;160:2263-2272.

274. Haines C, Chung T, Chang A, Masarei J, Tomlinson B, Wong E. Effects of oral oestradiol on Lp(a) and other lipoproteins in postmenopausal women. *Arch Intern Med* 1996;156:866-872.
275. Shewmon D, Stock J, Rosen C, et al. Tamoxifen and oestrogen lower circulating lipoprotein (a) concentrations in healthy postmenopausal women. *Arterioscler Thromb* 1994;14:1586-1593.
276. Lobo R, Notelovitz M, Bernstein L, Khan F, Ross R, Paul W. Lp(a) lipoprotein: relationship to cardiovascular disease risk factors, exercise and oestrogen. *Am J Obstet Gynecol* 1992;166:1182-1190.
277. Espeland M, Marcovina S, Miller V, et al. Effect of postmenopausal hormone replacement on lipoprotein (a) concentration. *Circulation* 1998;97:979-986.
278. Mosca L, Jahnige K, Giacherio D, Christman G, Johnson T. Beneficial effect of hormone replacement therapy on lipoprotein (a) levels in postmenopausal women. *Prev Cardiol* 1999;2:51-58.
279. Lobo R, Pickar J, Wild R, Walsh B, Hirvonen E. Metabolic impact of adding medoxyprogesterone acetate to conjugated oestrogen therapy in postmenopausal women. *Obstet Gynaecol* 1994;84:987-995.
280. Pickar J, Wild R, Walsh B, Hirvonen E, Lobo R, for the Menopause Study Group. Effects of the different hormone replacement therapy regimens on postmenopausal women with abnormal lipid levels. *Climacteric* 1998;1:26-32.
281. Robinson J, Folsom A, Nabulsi A, Watson R, Brancati F, for the Atherosclerosis Risk in Community Study (ARIC) Investigators. Can

- postmenopausal hormone replacement improve plasma lipids in women with diabetes? *Diabetes Care* 1996;19:480-485.
282. Brussard H, Gevers Leuven J, Kluft C, et al. Effect of 17 $\beta$ -oestradiol on plasma lipids and LDL oxidation in postmenopausal women with type II diabetes mellitus. *Arterioscler Thromb Vasc Biol* 1997;17:324-330.
283. Ravi-Subbiah M, Kessler B, Agrawal M, Rajan R, Abplan W, Rymaszewski Z. Antioxidant potential of specific oestradiols on lipid peroxidation. *J Clin Endocrinol Metabol* 1993;77:1095-1100.
284. Grodstein F, Stampfer M, Manson J. Postmenopausal oestrogen and progestin use and the risk of cardiovascular disease. *N Eng J Med* 1996;15:453-461.
285. Hu F, Stampfer M, Manson J, Grodstein F, Colditz G, Speizer F, et al. Trends in the incidence of coronary heart disease and changes in diet and lifestyle in women. *New England Journal of Medicine*. 2000;343:530-537
286. Grady D, Herrington D, Bittner V, et al. for the HERS Research Group. Cardiovascular disease outcomes during 6.8 years of hormone therapy; Heart and Estrogen/progestin Replacement Study follow-up (HERS II). *JAMA*. 2002;288:49-57.
287. Kanaya AM, Herrington D, Vittinghoff E, Lin F, Grady D, et al. Glycaemic effects of postmenopausal hormone therapy: the Heart and Estrogen/progestin Replacement Study: a randomised, double blind, placebo controlled trial. *Annals of Internal Medicine*. 2003;138:1-9.
288. Clark SC, Kelleher J, Lloyd-Jones H, et al. A study of hormone replacement therapy in postmenopausal women with ischaemic heart disease: The

- Papworth HRT Atherosclerosis Study. *British Journal of Obstetrics and Gynaecology*. 202;109:1056-1062.
289. Herrington DM, Reboussin DM, Brosnihan KB, et al. Effects of estrogen replacement on the progression of coronary artery atherosclerosis. *N Engl J Med*. 2000;343:522-529.
290. Waters DD, Alderman EL, Hsia J, et al. Effects of hormone replacement therapy and antioxidant vitamin supplements on coronary atherosclerosis in postmenopausal women. A randomized controlled trial. *JAMA*. 2002;288:2432-2440
291. The ESPRIT Team. Oestrogen therapy for prevention of re-infarction in postmenopausal women: A randomized placebo controlled trial. *Lancet*. 2002;360:2001-2008.
292. Hodis HN, Mack WJ, Azen SP, et al for the Women's Estrogen progestin Lipid Lowering Hormone Atherosclerosis Regression Trial Research Group. Hormone therapy and the progression of coronary artery atherosclerosis in postmenopausal women. *N Engl J Med*. 2003;349:535-545
293. Manson JE, Hsai J, Johnson KC, et al. for the Women's Health Initiative Investigators. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med*. 2003;349:523-534.
294. Lowe G, Danesh J, Rumley A. Tissue plasminogen activator antigen and coronary heart disease: a new prospective study and meta-analysis. *Blood Coag Fibrinolys* 2001;12:A1-A2.
295. Woodward M, Lowe GD, Rumley A, et al. Epidemiology of coagulation factors, inhibitors and activations markers: The Third Glasgow MONICA survey. Illustrative reference ranges by age, sex and hormone use. *Br J Haematol* 1997;97:775-784.

296. Lowe G, Yranell J, Sweetnam P, et al. Fibrin D-dimer, tissue plasminogen activator, plasminogen activator inhibitor and the risk of major ischaemic heart disease in the Caerphilly Study. *Thromb Haemost* 1998;79:129-133.
297. Sattar N, Perera M, Small M, Lumsden M. Hormone replacement therapy and sensitive C-reactive protein concentrations in women with type 2 diabetes. *Lancet* 1999;354:487-488.
298. Rapp SR, Espeland MA, Shumaker SA, et al. Effect of Estrogen plus Progestin on Global Cognitive Function in Postmenopausal Women: The Women's Health Initiative Memory Study: A Randomized Controlled Trial. *JAMA* 2003;289: 2663-2672.
299. Pettit D. Hormone replacement therapy and heart disease prevention. *JAMA* 1998;280:650-652.
300. Ridker P, Hennekens C, Rifai N, Buring J, Manson J. Hormone replacement therapy and increased plasma concentration of C-reactive protein. *Circulation* 1999;100:713-716.
301. van Baal W, Kenemans P, Emeiss J, et al. Long term effects of combined hormone replacement therapy on markers of endothelial function and inflammatory activity in healthy postmenopausal women. *Fertil Steril* 1999;71:663-670.
302. Lowe G, Upton M, Rumley A, McConnachie A, O'Reilly D, Watt G. Different effects of oral and transdermal hormone replacement therapies on factor IX, APC resistance, tPA, PAI and C-reactive protein- a cross-sectional population survey. *Thromb Haemost* 2001;86:550-556.

303. Lokkegaard E, Pedersen A, Heitmaa B, et al. Relation between hormone replacement therapy and ischaemic heart disease in women: prospective observational study. *B M J* 2003;326:426-428.
304. Ferrara A, Quesenberry CP, Karter AJ, Njoroge CW, Jacobson AS, Selby JV. Current use of unopposed oestrogen and oestrogen plus progestin and the risk of acute myocardial infarction among women with diabetes: the Northern California Kaiser Permanente Diabetes Registry 1995-1998. *Circulation* 2003;107:43-48.
305. Knopp R, Zhu X. Multiple beneficial effects of oestrogen on lipoprotein metabolism. *J Clin Endocrinol Metab* 1997;82:3952-3954.
306. Vehkavaara S, Silveira A, Hakala-Ala-Pietila T, et al. Effects of oral and transdermal oestrogen replacement therapy on markers of coagulation, fibrinolysis, inflammation and serum lipids and lipoproteins in postmenopausal women. *Thromb Haemost* 2001;85:619-625.
307. Perera M, Sattar N, Petrie J, et al. The effects of transdermal oestradiol in combination with oral norethisterone on lipoproteins, coagulation and endothelial markers in postmenopausal women with type 2 diabetes. A randomised, placebo-controlled study. *J Clin Endocrinol Metab* 2001;86:1140-1143.
308. Sattar N, Perera M, Small M, Lumsden M. Hormone replacement therapy and sensitive C-reactive protein concentrations in women with type 2 diabetes. *Lancet* 1999;354:487-488.

309. Pradhan AD, Manson JE, Rossouw JE, et al. Inflammatory biomarkers, hormone replacement therapy, and incident coronary heart disease: prospective analysis from the Women's Health Initiative observational study. *JAMA* 2002;288:980-987.
310. Ridker P. Role of inflammatory biomarkers in prediction of coronary heart disease. *Lancet* 2001;358:946-948.
311. Manning P, Sutherland WH, Allum AR, de Jong SA, Jones SD. Effect of hormone replacement therapy on inflammation-sensitive proteins in postmenopausal women with Type 2 diabetes. *Diabetic Med* 2002;19:847-852.
312. Stork S, von Schacky C, Angerer P. The effect of 17 $\beta$ -oestradiol on endothelial and inflammatory markers in postmenopausal women: a randomized, controlled trial. *Atherosclerosis* 2002;165:301-307.
313. Gornstein RA, Lapp CA, Bustos-Valdes SM, Zamorano P. Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *J Periodontol* 1990;70:604-609.
314. van Baal W, Kenemans P, van der Mooren M, Kessel H, Emeis J, Sthouwer C. Increased C-reactive protein levels during short term hormone replacement therapy in healthy postmenopausal women. *Thromb Haemost* 1999;81:925-928.
315. Zanger D, Yang B, Ardans K, et al. Divergent effects of hormone therapy on serum markers of inflammation in postmenopausal women with coronary artery disease on appropriate medical management. *J Am Coll Cardiol* 2000;36:1797-1802.

316. Sattar N, Petrie J, Jaap AJ. The atherogenic lipoprotein phenotype and vascular endothelial dysfunction. *Atherosclerosis* 1998;138:229-235.
317. Dichtl W, Nilsson L, Goncalves I, et al. Very low density lipoprotein activates nuclear factor-kappa in endothelial cells. *Circ Res* 1999;84:1085-1094.
318. Hokanson J, Austin M. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high density lipoprotein cholesterol level: a meta-analysis of population based prospective studies. *J Cardiovas Risk* 1996;3:213-219.
319. Kroon U, Tenghorn L, Rita H, et al. The effects of transdermal oestradiol and oral progestogens on homeostasis variables. *Br J Obstet Gynaecol* 1997;104:32-37.
320. Kanaya AM, Herrington D, Vittinghoff E, et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Oestrogen/progestin Replacement Study. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 2003;138:1-9.
321. Koh K, Mincemoyer R, Bui M, et al. Effects of hormone replacement therapy on fibrinolysis in postmenopausal women. *NEJM* 1997;336:683-690.
322. Andersen L, Mincemoyer R, Bui M, et al. Effects of hormone replacement therapy on fibrinolysis in postmenopausal women. *Am J Obstet Gynecol* 1999;180:283-289.
323. Herrington DM. Hormone replacement therapy and heart disease: replacing dogma with data. *Circulation* 2003;107:2-4.

324. Stevenson J, Whitehead M. Hormone replacement therapy. *BMJ* 2002;325:113-114.
325. McKenzie J, Jaap AJ, Gallacher S, et al. Metabolic, inflammatory and haemostatic effects of a low-dose continuous combined HRT in women with type 2 diabetes: potentially safer with respect to vascular risk? *Clinical Endocrinology*. 2003;59:682-689.
326. Lindsay R, Gallagher J, Kleerekoper M, Pickar J. Effect of lower doses of conjugated equine oestrogens with and without medoxyprogesterone acetate on bone in early postmenopausal women. *JAMA* 2002;287:2668-2676.
327. Malnick S, Beergable M, Knobler H. Non-alcoholic fatty liver: a common manifestation of a metabolic disorder. *Quarterly Journal of Medicine* 2003;96:699-709.
328. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab* 2002;87:3023-3028.
329. Ohlson LO, Larsson B, Bjorntorp P, et al. Risk factors for type 2 (non-insulin-dependent) diabetes mellitus. Thirteen and one-half years of follow-up of the participants in a study of Swedish men born in 1913. *Diabetologia* 1988;31:798-805.
330. Vozarova B, Stefan N, Lindsay RS, et al. High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. *Diabetes* 2002; 51:1889-1895.

331. Sattar N, Scherbakova O, Ford I, et al. Elevated alanine aminotransferase predicts new-onset type 2 diabetes independently of classical risk factors, metabolic syndrome, and C-reactive protein in the West of Scotland Coronary Prevention Study. *Diabetes* 2004;53:2855-2860.
332. Perry I, Wannamethee S, Shaper A. Prospective study of serum gamma-glutamyl transferase and risk of NIDDM. *Diabetes Care* 1998;21:732-737.
333. Lee D, Jacobs D, Gross M, et al. Gamma-glutamyl transferase is a predictor of incident diabetes and hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. *Clin Chem* 2003;49:1358-1366.
334. Nakanishi N, Suzuki K, Tatara K. Serum gamma-glutamyl transferase and risk of metabolic syndrome and type 2 diabetes in middle-aged Japanese men. *Diabetes Care* 2004; 27:1427-1432.
335. Matteoni C, Younossi Z, Gramlich T, Boparai N, Liu Y, McCullough A. Nonalcoholic fatty liver disease; A spectrum of clinical and pathological severity. *Gastroenterology* 1999;116:1413-1419.
336. Angulo P, Keach J, Batts K, Lindor K. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999;40:1356-1362.
337. Shah P, Basu A, Rizza R. Fat-induced liver insulin resistance. *Curr Diab Rep* 2003;3:214-218.
338. Angulo P. Mechanisms of disease. Non-alcoholic fatty liver disease. *N Engl J Med* 2002;346:1221-1231.

339. O' Sullivan A, Hoffman D, Ho K. Estrogen, lipid oxidation and body fat. *N Engl J Med.* 1995;333:669-670.
340. Tiikkainen M, Hakkinen A, Korsheninnikova E, Nyman T, Makimattila S, Yki-Jarvinen H. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. *Diabetes* 2004;53:2169-2176.
341. Scott A, Dhindsa P, Forsyth J, Mansell P. Effect of hormone replacement therapy on cardiovascular risk factors in postmenopausal women with diabetes. *Diabetes, Obesity and Metabolism.* 2004;6:16-22.
342. Blumenthal R, Zacur EI, Reis S, Post W. Beyond the null hypothesis-do the HERS results disprove the estrogen/coronary heart disease hypothesis? *Am J Cardiol* 2000;85:1015-1017.
343. Shiplack M, Chaput L, Vittinghoff E, et al. Lipid changes on hormone therapy and coronary heart disease events in the Heart and Estrogen/progestin Replacement Study (HERS). *American Journal of Obstetrics and Gynaecology.* 2003;146:870-875.
344. Grodstein F, Newcomb P, Stampfer M. Postmenopausal hormone therapy and risk of colorectal cancer: a review and meta-analysis. *Am J Med* 1999;106:574-582.
345. Mikkola TS, Clarkson TB. Estrogen replacement therapy, atherosclerosis and vascular function. *Cardiovascular Research.* 2002;53:605-619.

346. Grodstein F, Stampfer MJ, Falkeborn M, Naessen T, Persson I.  
Postmenopausal hormone therapy and risk of cardiovascular disease and hip fracture in a cohort of Swedish women. *Epidemiology*. 1999;10:476-480.
347. Dubey RK, Gillespie DG, Zacaria LC, Imthurn B, Jackson EK, Keller PJ.  
Clinically used oestrogens differentially inhibit human aortic smooth muscle cell growth and MAP kinase activity. *Arterioscler Thromb Vasc Biol*. 2000;20:964-972.
348. Hodis HN, Mack WJ, Lobo RA, Shoupe D, Sevanian A, Mahrer PR, Selzer RH, Liu CH, Azen SP. Estrogen in the prevention of atherosclerosis. A randomized double blind, placebo controlled trial. *Ann Intern Med*. 2001;135:939-953.
349. Phillips LS, Langer RD. Postmenopausal hormone therapy: critical appraisal and a unified hypothesis. *Fertility and Sterility*. 2005;83:558-566.
350. Adami S, Rossini M, Zamberlan N, Bertoldo F, Dorizzi R, Lo Cascio V.  
Long term effects of transdermal and oral oestrogens on serum lipids and lipoproteins in postmenopausal women. *Maturitas*. 1993; 17:191-196.
351. Skouby S. Consequences for HRT following the HERS II and WHI reports: the primum non nocere is important, but translation into quo vadis is even more essential. *Acta Obstetricia et Gynecologica Scandinavica* 2002;81:793-798.
352. Pedersen T, Olsson A, Faergeman O, et al. Lipoprotein changes and reduction in the incidence of major coronary heart disease events in the Scandinavian Survival Study (4S). *Circulation* 1998;97:1453-1460.
353. The writing group of the Bezafibrate Infarction Prevention Study.  
Secondary prevention by raising HDL cholesterol and reducing triglycerides in

patients with coronary artery disease: the Bezafibrate Infarction Prevention (BIP) Study. *Circulation* 2000;102:21-27.

354. Cauley J, Black D, Barrett-Connor E, et al. Effects of hormone replacement therapy on clinical fractures and height loss: the Heart and Oestrogen/Progestin Replacement Study.(HERS). *Am J Med* 2001;110:442-450.
355. Cummings S, Black D, Thompson D, et al. Effect of alendronate on risk of fracture in women with low bone mineral density but without vertebral fractures. *JAMA* 1998;98:114-118.
356. Wenger NK, Barrett-Connor E, Collins P, Grady D, Kornitzer M, Mosca L, et al. Baseline characteristics of participants in the Raloxifene Use for The Heart (RUTH) trial. *American Journal of Cardiology*. 2002;90:1204-1210.

