THE UNIVERSITY OF HULL

INTERVENTIONS IN PATIENTS AT HIGH CARDIOVASCULAR RISK

being a Thesis submitted for the Degree of Doctor of Medicine

in the University of Hull

by

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To my husband John and my son Thomas: they are my inspiration.

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Publications

1. González S, Jayagopal V, Kilpatrick ES, Chapman T, Atkin SL. Effects of isoflavone dietary supplementation on cardiovascular risk factors in type 2 diabetes. **Diabetes Care 2007; 30(7):1871-1873.**

Abstracts/poster presentations

1. **S. González**, L.Cho, E.Kilpatrick, S.L. Atkin. Effects of metformin and hypocaloric diet on lipids and their biological variation in polycystic ovarian syndrome. **British Endocrine Society (BES). Endocrine Abstracts (2009) 19, P119.**

2. **González S,** Sugunendran S, Williams PE, Lawery A, Atkin SL. Growth hormone replacement effects in peripheral muscle and quality of life. **Endocrine Abstracts. The Endo Society, June 2007.** 89th Annual meeting. Toronto. **P3-474.**

3. **S. González**,V. Jayagopal, E.Kilpatrick, T.Chapman, S.L Atkin. Effects of isoflavones dietary supplementation on cardiovascular risk factors in type 2 diabetes. **DUK 2005**. Glasgow. UK. **P107.**

Abstract

Cardiovascular disease is a major cause of premature death in UK and an important contributor to escalating health costs. The risk of suffering a cardiovascular event is associated with multiple risk factors that can cluster in different pathological conditions such as type 2 diabetes, polycystic ovarian syndrome (PCOS) and hypopituitarism. Therefore, interventions in these patients at high risk of cardiovascular disease can be potentially beneficial to improve cardiovascular health and this is explored in the different chapters of this thesis.

Current knowledge of what constitutes cardiovascular risk is reviewed in chapter one and the methods used to conduct the studies presented in this thesis are described in chapter two.

Chapter three evaluates the effects of social deprivation in lipid management in a community setting. This study analysed lipid measurements and lipid lowering agents prescriptions across 34 electoral wards in Kingston Upon Hull and East Riding of Yorkshire with different deprivation scores. A ward is a primary territorial unit which represent a part of an electoral district. It showed that both lipid measurements and prescriptions were similar in deprived and wealthier wards (correlation values: $r=0.18$ and $r=0.50$ respectively) and this might represent service under provision in poorer areas.

The effect of isoflavones on insulin resistance and cardiovascular risk factors in postmenopausal women with diet controlled type 2 diabetes is studied in chapter four. Oral supplementation with 132 mg/day for a three months period has no significant effects modulating glycaemic control (glucose, $p=0.59$; HbA1c, $p=0.58$), insulin

resistance (HOMA-IR method, p=0.24) or cardiovascular risk factors (total cholesterol, p=0.96; HDL cholesterol, p=0.93; LDL cholesterol, p=0.97; triglycerides, p=0.74; body mass index, p=0.97; systolic blood pressure, p=0.35 and diastolic blood pressure, p=0.38). This implies that either a combination of soy protein and isoflavones or soy protein on their own are needed to induce the beneficial effect reported in the literature.

Chapter five evaluates the combined effect of a hypocaloric diet (1500 kcal/day) and metformin (500mg three times a day) on cardiovascular risk factors and on the biological variation of lipids in obese patients with PCOS. This intervention improved central obesity (waist/hip ratio, p=0.02; body mass index, p=0.008), blood pressure (systolic blood pressure=0.02; diastolic blood pressure, p=0.009) and LDL levels (p=0.03). However, the biological variation of lipids is similar to that in health and remained unchanged with this intervention. Therefore, reference value changes obtained from healthy individuals could be used to monitor serial lipids levels i.e. in response to a therapeutic intervention.

The effects of subcutaneous recombinant growth hormone (rGH) in patients with adult onset growth hormone deficiency on exercise capacity, quality of life and general cardiovascular risk factors is explored in chapter six. Three months treatment with a fixed, low dose of rGH (dose=0.4 mg/day) improved total body fat (p=0.05) and normalised plasma IGF1 (p=0.0001) without a significant effect on cardiovascular risk factors, exercise capacity or quality of life. Mitochondrial succinate dehydrogenase (SDH) increased by a 2.7 fold in both the active and placebo phases when compared with baseline, possibly related to an increase in patient's daily activities.

Finally, chapter seven analyses the biological variation of N terminal probrain natriuretic peptide (NT-proBNP) in postmenopausal women with and without type 2 diabetes. Type 2 diabetes appears to have little influence in the biological variation of this natriuretic peptide. This implies that current guidelines on the use of NT-proBNP for the screening, diagnosis and evaluation of serial results in hearth failure would be applicable in type 2 diabetes. The reference change values obtained in this study could be used to determine if two NT-proBNP levels are different due to the effect of treatment rather than their biological variation.

Chapter 1

Introduction

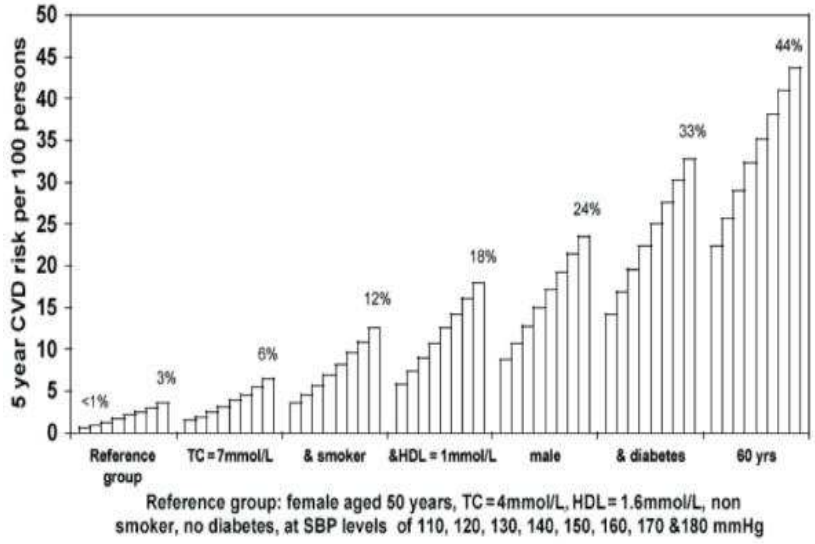
1.1. What constitutes increased cardiovascular risk?

Cardiovascular disease (CVD) is the major cause of premature death in the UK and Wales[1] and an important contributor to the escalating cost in health care[2]. In most cases, atherosclerosis is the underlying pathology that develops insidiously over many years and it is usually advanced by the time the patient presents clinically. The risk of experiencing a cardiovascular event is associated with several factors that can be broadly divided in those that are unmodifiable such as increasing age, male gender, inheritance and ethnicity but also with others that can be corrected. This second group includes hypertension, hyperlipidaemia, smoking, obesity, diabetes mellitus, left ventricular hypertrophy, sedentary lifestyle, social deprivation and certain behavioural patterns. In addition some emerging biomarkers such as fibrinogen, plasma activator inhibitor-1 (PAI-1), homocysteine, C-reactive protein (CRP), brain natriuretic peptide (BNP) and asymmetric dimethylarginine might reflect higher risk of CVD.

It has become apparent during clinical practice and in epidemiological studies that these risk factors tend to cluster which translates in progressively higher cardiovascular absolute risk (the probability of developing CVD over a given time period). Assessment of this cardiovascular risk is important to instigate preventative or therapeutic strategies both at an individual or population level to reduce the burden of cardiovascular disease. Depending upon the co-existence of none, one or more risk factors, the level of added risk rises from low to moderate or high, determining the global risk of cardiovascular events in an individual (Figure 1.1.) which is needed to identify those subjects at higher risk who will require the higher priority for treatment.

Figure 1.1.

Absolute risk of cardiovascular disease over 5 years in patients by systolic blood pressure at specific levels of other risk factors. Derived from Anderson et al.[3]



The relative risk estimate represents the ratio of the incidence of the exposed versus the non exposed population. Higher relative risk in young adults indicates the long term risk accompanying the risk factors, which in turn may help to design a long term strategy for the individual, i.e. changes in lifestyle. The 10 year absolute risk rises with age and provides the opportunity to reduce absolute short term risk by immediately targeting risk factors in the clinical setting and it is usually favoured in treatment guidelines[4].

Therefore, an early preventative strategy aimed to modify cardiovascular risk factors and lifestyle may impact upon mortality and morbidity from CVD[5].

1.1.1. Cardiovascular risk and hyperlipidaemia in socially deprived areas

Social inequalities, socioeconomic status and ethnic differences are powerful predictors of cardiovascular morbidity and mortality, with an increased prevalence of CVD in deprived areas[6]. There is a geographical variation in the incidence of CVD in the United Kingdom with a gradient north-south being cardiovascular mortality higher in the north, inner city areas and in manual workers[7]. Poverty has an important effect on the risk of having a first myocardial infarction, the chance of reaching the hospital alive and the probability of survival in the first month[8]. The use of interventional cardiology services in deprived communities is variable[9, 10] and it is influenced by the proximity to specialist centres i.e. higher coronary artery bypass and coronary angioplasty intervention rates for those closer to referral centres[11]. Overall, social deprivation carries a substantial independent adverse effect on hospital/mid-term survival and quality of life in those patients undergoing coronary artery revascularization [12, 13].

This relationship between social deprivation and coronary heart disease is likely to be complex and multifactorial clustering defined cardiovascular risk factors such as unhealthy lifestyles, smoking, hypertension, lipid disorders, diabetes, other co morbid conditions and/or reduced service provision or access to medical care. However, a socially or economically disadvantaged population might have differences in health expectations, health-seeking responses or in changing unhealthy behavioural patterns that may account for some of the apparent under provision in service[14, 15].

Dyslipidaemia is an important and reversible risk factor contributing to accelerated

CVD in deprived areas. The World Health Report 2002 estimated that 56% of global ischemic heart disease and around 18% of global cerebrovascular disease are due to raised total cholesterol above the theoretical minimum of 3.8 mmol/L[16]. Abnormal lipid profiles can be responsible for up to 45% of myocardial infarctions in Europe, increasing the risk of heart attacks over three times in those with raised levels as compared to those with normal profiles[17]. Epidemiological studies have shown that cholesterol is log-linearly related to coronary heart disease (CHD) mortality and that a long term reduction in serum concentrations of total cholesterol of 0.6mmol/L lowers the risk of ischaemic heart disease by 25-30% in people aged 55-64 years[18]. Furthermore, a reduction in LDL cholesterol of 1.6 mmol/L halves the risk of CHD events after two years[19]. Lipid modification also results in 37% reduction in acute major coronary events in both in men and women without clinical evidence of CHD who have an average total cholesterol levels and below average HDL levels[20].

Therefore, interventions aimed to improve hyperlipidaemia in both primary and secondary prevention of ischemic heart disease are essential if reductions in fatal and non fatal CHD events are to be achieved. The initial step is to identify all patients with established cardiovascular disease (or CHD risk equivalent) and all asymptomatic people with multiple risk factors and a 10 year risk CHD of ≥20% since current guidelines recommend that they should be therapeutically targeted first[21-23]. This can be evaluated with the use of risk assessment tools such as the Framingham score, Sheffield table or British Joint Society charts. The limitations of these tools are that they tend to underestimate the CVD risk in certain group of patients, especially those living in socially deprived areas with lower incomes, those of South Asian ethnic backgrounds [24]

and those with inherited hyperlipidaemias, type 2 diabetes mellitus, morbid obesity, severe hypertension, metabolic syndrome or with a family history of premature CHD. New risk prediction algorithms such as QRISK2 which includes social deprivation and ethnicity, might be therefore more accurate in CVD risk assessment than traditional tools in these subgroups [25].

Different therapeutic strategies can be applied to reduce this cardiovascular risk that include lifestyle and dietary modification and the use of pharmacological agents such as statins and fibrates, targeting those populations with the highest need for them to reduce health inequalities. Dietary management alone usually results in small reductions in cholesterol levels but it maybe be very effective in a small number of well motivated patients[26]. Those patients, who have adequately responded to life style modifications after three month trial, should be encouraged to maintain it. However, if after three months the effect of lifestyle modification has failed to lower cholesterol levels below the recommended target, lipid lowering therapy should be initiated.

1.1.2. Cardiovascular risk in type 2 Diabetes

Recent estimates suggest that the global prevalence of diabetes was in excess of 171 million in 2000 with an projected rise to 366 million in 2030[27]. The majority of these patients will develop type 2 diabetes, a chronic and progressive disorder characterised by hyperglycaemia in the context of insulin resistance and relative insulin deficiency. Although type 2 diabetes is traditionally a disease of adulthood, it has been increasingly diagnosed in children due to the higher obesity rates in younger generations[28].

Cardiovascular complications are responsible for the high mortality and morbidity in patients with diabetes, in particular an excess of coronary heart disease and stroke. Traditionally, it was estimated that type 2 diabetes increased the risk of a fatal cardiovascular event by approximately twofold[29,30]. A recent metaanalysis of 37 prospective cohort studies indicates that the overall estimate of the relative risk (rr) for fatal CHD associated with diabetes was 2.06 in men [rr: 2.06 (95% confidence interval: 1.81 to 2.34)] and 3.50 in women [rr:3.50(2.70 to 4.53)][31].

Patients with type 2 diabetes without previous myocardial infarction (MI) have as high risk of suffering a MI as those without diabetes who have already had a previous MI[32]. Furthermore, the twenty eight days and one year mortality rate of hospitalised patients for a first MI is higher in type 2 diabetes as reported in the FINMONICA study[33], particularly in women[34]. The prospective Hoorn Dutch population based cohort study also showed this gender difference since women with diabetes but without previous CVD disease appeared to have higher fatal CVD events than those women without diabetes but previous CVD. In contrast, men with diabetes and no previous CVD disease, had lower fatal CVD events as compared to those men without diabetes and previous CVD[35]. Patients with type 2 diabetes also tend to have more extensive coronary disease with greater and more rapid progression of the percentage of atheroma plaque volume[36], more multivessel, multilesion and small vessel disease than non-diabetic patients[37].

The pathophysiology of cardiovascular disease in type 2 diabetes is complex and influenced by the presence of multiple risk factors (Table 1.1.).

Table 1.1. Cardiovascular risk factors in type 2 diabetes

Modifiable risk factors	**Predisposing risk factors**
Hypertension	Family history
Atherogenic dyslipidaemia	Disease duration
Low HDL cholesterol levels	Age
Hyperglycaemia	
Hyperinsulinaemia	
Albuminuria	
Smoking/Sedentarism	
Obesity/central fat distribution	

One of the earliest atherogenic changes that precedes clinical and morphological manifestations of CVD by decades is a progressive endothelial dysfunction or "functional atherosclerosis"[38] which tend to present early in type 2 diabetes[39]. This is characterised by reduced endothelium dependent vasodilatation, vascular smooth muscle proliferation and pro-thrombotic state[40] which creates an homeostatic imbalance that can lead to a fatty streak formation, by inducing an endothelial inflammatory response due to the vascular wall invasion by lipids and leukocytes. Hyperglycaemia, dyslipidaemia and insulin resistance are closely related to endothelial dysfunction. Hyperglycaemia promotes glycosylation and inactivation of antioxidant proteins thus increasing intracellular oxidative stress, leads to the migration and proliferation of smooth muscle cells by activating cytokines and growth factors through advanced glycation end products[41] and induces altered vascular permeability by increasing adhesion molecules ICAM and VCAM[42]. Insulin resistance impairs NO-dependant vasodilatation even in normotensive, non obese type 2 diabetes patients[43]. The role of

dyslipidaemia is discussed in section 1.2.2. This endothelial dysfunction worsens over time, with significantly impaired vasodilatation in coronary arteries that exhibit angiographic evidence of atherosclerosis[44].

Of particular interest is the finding that whereas oestrogens enhance vascular reactivity in premenopausal, healthy women, this is impaired in the postmenopausal status and in type 2 diabetes[45]. Rossi et al conducted a prospective cohort study with 840 apparently healthy, non diabetic, non obese, postmenopausal women who were followed up for approximately 4 years. They observed that for each unit decrease in flow mediated brachial artery dilatation (a surrogate method to assess endothelial function) there was a significant 32% increase in the relative ratio of incident diabetes suggesting that impaired endothelial function may play an important role in diabetogenesis in postmenopausal women[46]. Furthermore, following menopause HDL cholesterol falls and LDL cholesterol, triglycerides and more dense lipoprotein sub fraction levels increase[47]. Oestrogen may also prevent the oxidation of LDL within the arterial wall[48].

1.1.3. Cardiovascular risk in Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is a common, heterogeneous disorder that combines metabolic manifestations, chronic anovulation and androgen excess in women of reproductive age with an estimated prevalence of 4-6%.

PCOS is associated with impaired glucose tolerance, diabetes[49, 50] and insulin resistance in both obese and lean women[51]. Additional cardiovascular risk factors present in this condition are obesity, hypertension, atherogenic dyslipidaemia[52] and abnormalities in the coagulation pathways. Paradisi et al. found that obese women with PCOS exhibit

50% reduction in endothelium dependant vasodilatation, diminished response to the vasodilating action of insulin and that this endothelial dysfunction was strongly and inversely correlated with levels of free testosterone[53]. They also have evidence of premature carotid atherosclerosis[54] and more extensive atherosclerosis on cardiac catheterisation than women without PCOS[55].

This high risk profile would theoretically predict greater cardiovascular events, however, this remains controversial. Pierpoint et al followed up a total of 786 women with PCOS for an average of 30 years and concluded that the rate of death from ischaemic heart disease was not significantly increased when compared with expected rates in the population[56]. Similarly, Wild et al reported, in a retrospective cohort study, that coronary heart disease was equally common in women with PCOS when compared with the general population but the crude odd ratio for cerebrovascular disease was increased at 2.8[57]. Shaw et al[58] compared postmenopausal women with and without clinical features of PCOS when evaluated for suspected cardiac ischaemia and observed an increased prevalence of more multivessel CAD and worsening cardiovascular event-free survival in the PCOS arm.

1.1.4. Cardiovascular risk in Adult Growth Hormone deficiency

Human growth hormone (GH), a polypeptide synthesised by the anterior pituitary gland and secreted in a highly pulsatile fashion, has multiple metabolic actions that regulate body composition, fluid homeostasis, glucose, lipid and bone metabolism and cardiovascular function. Its deficiency in adults is commonly caused by pituitary tumours, either as a consequence of mass invasion or its treatment, particularly after

radiotherapy. Other less common aetiologies are trauma, granulomatous or metastatic diseases, post partum pituitary necrosis, lymphocytic hypophysitis and idiopathic.

Five large retrospective epidemiological studies have reported that long term mortality in patients with hypopituitarism is higher than the expected in the general population, with standard total mortality ratios (SMRs) between 1.20-2.17 and it have been postulated that GH deficiency might contribute to this excess mortality. Three of these studies[59-61] suggest that this may be caused by a 1.6 to 1.9 fold increase in cardiovascular mortality, mainly cerebrovascular but this excess vascular mortality was less clear in the other two cohorts[62, 63]. The West Midlands prospective hypopituitary study[64] tried to address this question: a group of 1014 patients with hypopituitarism were followed up for 8 years and they confirmed an increased total mortality [SMR: 1.87 (99% CI 1.62-2.16, p<0.0001], mainly attributed to respiratory [2.66(1.72-4.11), p<0.0001), cerebrovascular [2.44 (1.58-4.18), p<0.0001) and cardiovascular causes [1·82 (1·3-2·54), p<0·0001]. However, only untreated gonadotropin deficiency rather than GH deficiency was associated with higher mortality. Therefore, it remains unclear if GH deficiency per se significantly contributes to the excess of cardiovascular mortality observed in hypopituitarism.

Nevertheless, patients with growth hormone deficiency also have a cluster of significant cardiovascular risk factors. Their body composition is abnormal with an excess of total fat mass, mainly truncally distributed, increased skinfold thickness and reduced lean body mass in both adults with GH deficiency[65-67] and GH insufficiency[68]. Dyslipidaemia is mainly characterised by abnormalities in lipid metabolism with an enhanced VLDL secretion and down regulation of LDL receptors, resulting in high plasma LDL and triglycerides levels, low HDL levels and abnormal postprandial

lipoprotein clearance despite normal lipoprotein lipase activity[69]. The interaction of these atherogenic lipoprotein particles with the endothelium can trigger an inflammatory response and multiple homeostatic factors abnormalities such as increased fibrinogen levels, plasminogen activator-1 inhibitor (PAI-1), tissue plasminogen activator antigen (tpa), CRP and cytoadhesive molecules[70, 71] although some of these changes are subtle if GHD patients are weight matched with controls[72]. Furthermore, endothelial dysfunction with impaired endothelium dependant vasodilatation probably due to a diminished nitric oxide generation mediated by low IGF-1[73], a significant increase in plaques and in carotid intima-media thickness in both carotid and femoral arteries[74], indicates the development of premature atherosclerosis which is not only limited to older patients but also extended to those with childhood onset GHD[75]. In addition, cardiac performance on peak exercise also appears to be reduced[76] .

The effects of GH on carbohydrate metabolism is intricate and both severe or partial GH deficiency can lead to impaired insulin sensitivity[77, 78]. Finally, blood pressure in adult GH deficiency positively correlates with age, particularly in females[79].

1.2. Soy supplementation, hyperlipidaemia and insulin resistance in type 2 diabetes and postmenopausal women

Oestrogen deficiency has been implicated in the higher risk of CVD and possible increased risk of type 2 diabetes observed in postmenopausal women as mentioned in section 1.1.2. Therefore, oestrogen replacement in the form of HRT, has been used in an attempt to reduce this cardiovascular risk, because of its positive effects on lipids, LDL oxidation[48] and vascular reactivity[45]. Observational data derived from prospective cohort studies initially supported the concept that oestrogen replacement may prevent heart disease[80-83]. However, randomised, placebo-control trials such as HERS (Heart

and estrogen/progestin intervention trial) and WHI (Women's Health Initiative trial) have questioned oestrogen efficacy and safety for primary and secondary prevention of CVD. The HERS trial found that opposed conjugated oestrogens did not reduce the overall cardiovascular events in postmenopausal women with uterus and established CVD after 4y follow up but increased thromboembolic events and gallbladder disease[84]. The WHI study looked at the effects of opposed conjugated oestrogens in postmenopausal women with uterus in primary prevention for CVD. It was planned to have a duration of 8.5 years however, it was discontinued 3 years earlier after all cause mortality was not affected but the risk of breast cancer exceed the designated boundary along with some increase in myocardial infarction, stroke and pulmonary embolism/deep venous thrombosis[85]. A parallel arm of the WHI trial that recruited postmenopausal women with prior hysterectomy and received only conjugated oestrogens (instead of a combination of conjugated oestrogens and methylprogesterone) was also stopped earlier, after 6.8 years, following a 39% increase incidence of strokes with no significant effect in the CHD incidence [86].

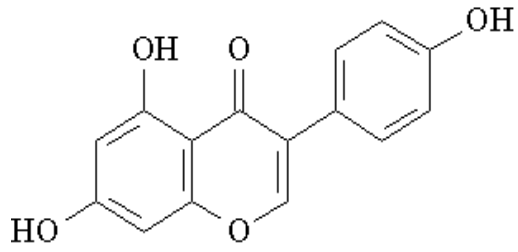
In view of the above results, there has been increasing interest in finding alternative, safer treatments such as phytoestrogens to reduce CVD, especially in postmenopausal women and type 2 diabetes.

1.2.1. What are phytoestrogens?

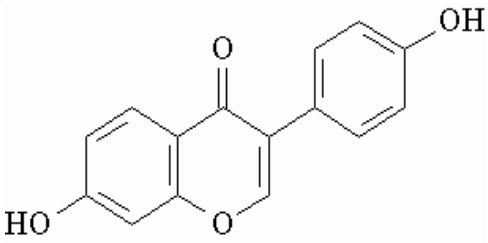
Phytoestrogens are a group of plant-derived, non steroidal compounds, with a heterocyclic diphenolic chemical structure very similar to 17β oestradiol (Figure 1.2.). They behave like selective oestrogen receptor modulators (SERMS) with both oestrogenic and antiestrogenic effects depending on the target tissue they act[87]. Three main groups of phytoestrogens have been identified: isoflavanoids, coumestans and

lignans (Figure 1.3.) and perhaps one of the most studied are isoflavones. Isoflavones, in their glycosilated form found in soy bean containing food, are inactive and require to be activated into an aglycone form by the intestinal flora. They are absorbed by the intestinal epithelium and approximately 50% circulate bound to protein, being the remaining 50% free to competitively bind to oestrogen receptors with higher affinity for β than for α receptors. Their half life is variable, usually between 3 and 7 hours (8h genistein, 5 daidzein) and they undergo enteropathic circulation, common to many steroids.

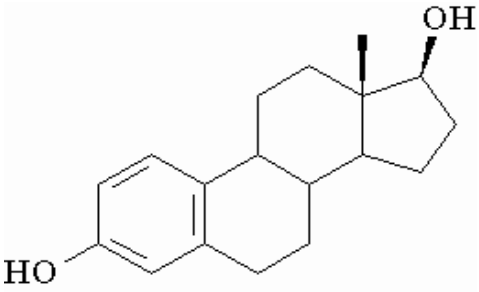
Figure 1.2. Chemical structures: beta oestradiol, genistein and daidzein.



Genistein



Daidzein



17 β Oestradiol

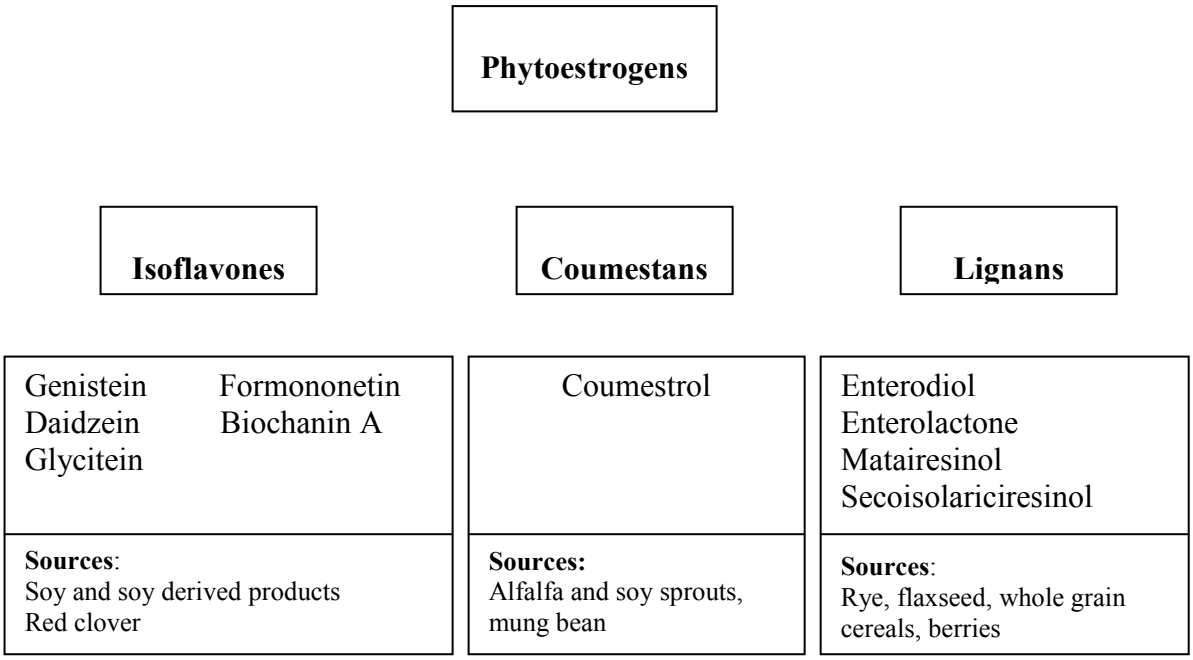
Phytoestrogens act as weak oestrogen agonists when bound to β oestrogen receptors, which are mainly located in the vascular system, bone, urogenital tract and central nervous system. Their affinity for α oestrogen receptors, distributed predominately in

breast, endometrial and liver tissue, is low i.e. genistein 5% for α receptors and 36% for β receptors[88].

Oestrogen receptors (ER) have an intrinsic plasticity, shifting their structure depending on the type of substance that binds to them. Once bound by oestrogens or isoflavones, the ER undergoes through conformational changes that modulate the transcription of the target genes. Isoflavones, especially genistein, competitively bind to oestrogen receptors, as effectively as 17β oestradiol but the concentration required to induce transcription is 10^4 greater[89]. What ultimately determines the extent of agonist/antagonist action of isoflavones i.e. genistein is the position of the several helices of ER, in particular the folding of helix 12[90].

However, the biological effects of isoflavones are far more complex than the ones explained by their interaction with oestrogen receptors. They also have antioxidant properties acting as free radical scavengers[91], can induce enzymatic inhibition, in particular of tyrosine kinase, topoisomerase I and II and other enzymes implicated in cellular proliferation and oncogene expression[92 93], inhibit angiogenesis in vitro[94], and they stimulate SHBG synthesis[95].

Figure 1.3. Types of phytoestrogens and main food sources.



Some of these properties indicate that isoflavones might have a potential role preventing or delaying cardiovascular disease by intervening at several steps in the atherosclerotic plaque formation. Proposed mechanism include: reduction in LDL cholesterol[96] and in its susceptibility to oxidation[97], enhancement of nitric oxide dependant vasodilatation[98], improvement in arterial compliance[99] and inhibition of pro-inflammatory cytokines [100], cell adhesion proteins[101] and platelet aggregation [102].

These could be especially advantageous in postmenopausal women and type 2 diabetes. Firstly, some studies suggest that isoflavones might help to relieve postmenopausal hot flushes and improve bone health which is particularly important in this subgroup of patients. Secondly, phytoestrogens have very little affinity for α oestrogen receptors, mainly located in breast, endometrium and liver, hence it is unlikely they increase the risk of uterine or breast neoplasia seen in the HERS cohort following HRT treatment.

To date, there are no reports of increased tumorigenesis in humans or thromboembolic events. Finally, isoflavones might have a beneficial effect lowering fasting blood glucose, fasting insulin and HbA1c levels, particularly when consumed in combination with soy protein but it remains uncertain if this is a direct effect of purified isoflavones alone[103].

However, caution should be exerted regarding this functional food since there are no large, consistent randomised trials about all these potential beneficial effect and there is much controversy in the literature regarding their activity.

1.2.2. Hyperlipidaemia in type 2 diabetes

Type 2 diabetes is associated with an interrelated group of lipid abnormalities known as atherogenic dyslipidaemia that differs to the pattern found in the general population in that patients with diabetes have twice the prevalence of low HDL and high triglycerides with similar levels of total cholesterol when compared to non diabetics [3, 104].

Hence, hypertriglyceridaemia, reduced HDL and raised small, dense LDL particles are the main components[105] of this profile that predispose patients to develop cardiovascular disease[105] (Table 1.2.). Additional features of diabetic dyslipidaemia are the presence of compositional changes in VLDL, LDL and HDL, postprandial lipaemia and increased coagulant factors (fibrinogen, factor VII and plasminogen activating factor) [106, 107].

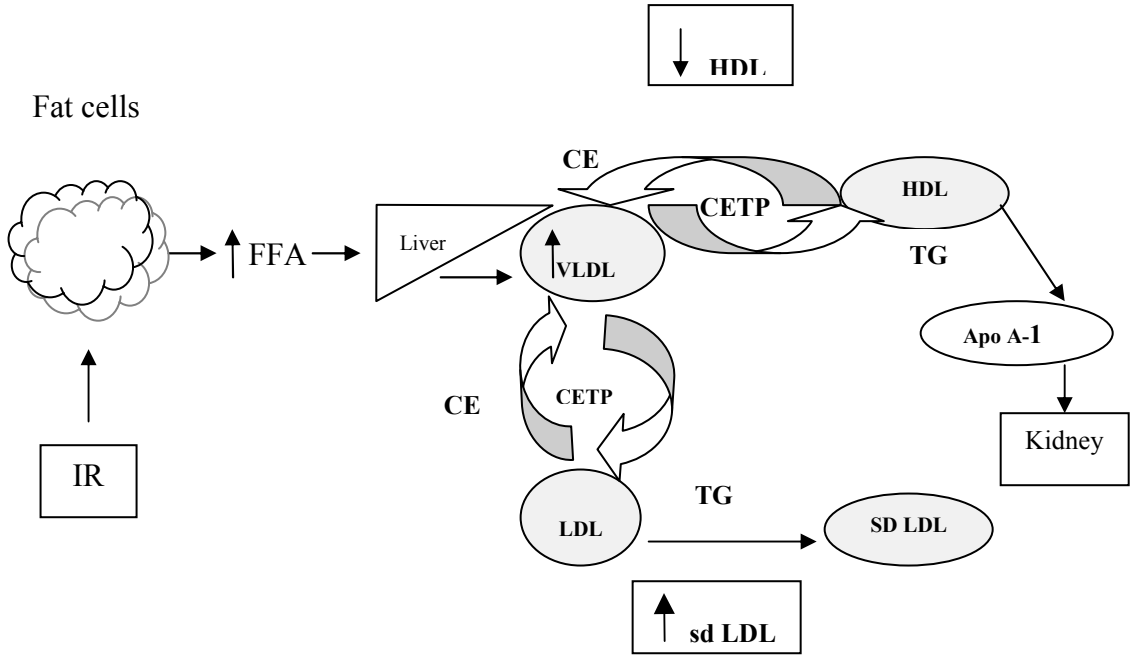
Table 1.2. Dyslipidaemia in type 2 diabetes

Increased	Decreased
Triglycerides	HDL
VLDL	Apo A-I
Small, dense LDL particles	
Apo B	

There is strong correlation between hypertriglyceridaemia and insulin resistance[108] and low HDL is also associated with hyperinsulinaemia in both obese and non obese subjects[109]. Therefore, the role of insulin resistance in the physiopathology of diabetic dyslipidaemia is central being hypertriglyceridaemia the primary abnormality[110, 111]. The fasting and postprandial hyperinsulinaemia found in these individuals, is inadequate to suppress the release of free fatty acids (FFA) from the insulin resistant adipose tissue into the circulation. The liver increases the synthesis of endogenous triglycerides with the additional FFA leading to an excess production of triglycerides rich in very low lipoproteins (VLDL) (Figure 1.4.).

The VLDL excess acts as an additional substrate for the cholesteryl ester transfer protein (CETP) which catalyses the exchange of triglycerides and cholesteryl esters between VLDL, HDL and LDL particles[112] leading to the formation of cholesterol rich VLDL remnant particles, that are atherogenic,[113] and triglyceride rich HDL and LDL. These triglyceride enriched particles undergo hydrolysis through lipolytic pathways (i.e. hepatic lipase) to form small, dense, lipid depleted LDL particles and lower levels of cholesterol depleted HDL, since the apo A-I dissociated during lipolysis has a faster renal clearance than the apo A-I associated with HDL.

Figure 1.4. Development of atherogenic dyslipidaemia: metabolic pathway.



IR: insulin resistance FFA: free fatty acids; VLDL: very low density lipoprotein; LDL: low density lipoprotein; sd-LDL: small, dense LDL particles; HDL: high density lipoprotein; CETP: cholesteryl ester transfer protein; CE: cholesteryl ester; TG: triglycerides. Adapted from Ginsberg[110].

Hence, although LDL cholesterol levels tend to remain within normality, qualitative changes increase its atherogenic potential. The LDL cholesteryl ester core surrounded by apoB100 diminishes as the proportion of triglycerides increases leading to a lower number of cholesterol molecules per apoB100[114]. Additionally, the rate of glycation of apoB is increased[115] in diabetic patients and this impairs the affinity for the apoB receptor that prolongs their circulation time and within the arterial wall where they are more prone to oxidation[116]. This increases the likelihood of up regulating the macrophages scavenger receptors, particularly type A (SR-A) expressed mainly in macrophages surface, inducing phagocytosis, foam cell formation and adhesion to

atherosclerotic lesions[117, 118]. The presence of advanced glycation end products (AGE) could also have a potential role in the diabetic dyslipidaemia since they can bind to HDL receptors inhibiting selective uptake of HDL cholesterol ester and cholesterol efflux[119].

Hyperinsulinaemia and hypertriglyceridaemia are also associated with elevated plasminogen activator inhibitor type 1 (PAI-1) levels[120] that together with the increased fibrinogen and platelet aggregability predispose to thromboembolic phenomena in diabetic patients[121].

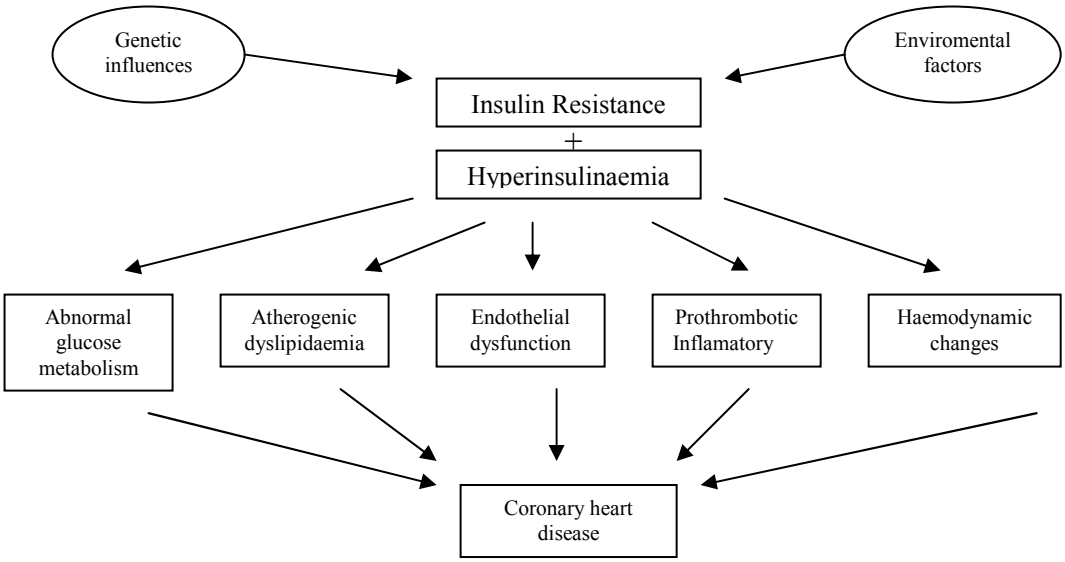
Since there is a strong linear relationship between the level of LDL and CHD risk, as discussed in section 1.1.1, several intervention studies have evaluated if lowering LDL improves cardiovascular risk in type 2 diabetes. The post hoc analysis of the early statin trials revealed that lowering elevated[122] and moderately elevated/average[123] LDL cholesterol levels in secondary prevention reduced total mortality and major coronary events in the diabetic subgroups comparable to non diabetics. Likewise, the diabetic subgroup treated with gemfibrozil in the Helsinki Heart Study for primary prevention (HHS) and veterans affairs high density lipoprotein intervention trial (VA-HIT) for secondary prevention had fewer cardiac events than those in the control group[124, 125]. The large multicenter, randomised, placebo controlled MRC/BHF Heart Protection Study[126], CARDS[127] and ASCOT-LLA[128] trials were specifically designed to assess the effects of statins in type 2 diabetes and they confirmed that treatment with statins in primary prevention reduces death rate, acute coronary events, coronary revascularizations and stroke in patients with average LDL levels. Based on this evidence, current guidelines recommend to lower LDL as a first priority [21-23] and then consider treatment to normalise HDL and triglycerides since residual low HDL and high

triglycerides appear to contribute to residual CHD even when LDL levels have been corrected[129].

1.2.3. Insulin resistance in type 2 diabetes

Insulin resistance (IR), an early feature of the natural history of type 2 diabetes, is the failure of endogenous insulin to produce its usual biological effects in peripheral target tissues. Although the basic defect responsible for the development of IR remains unknown, the interaction between the pancreatic β cells, the liver, skeletal muscle and adipose tissue give rise to several metabolic, endothelial and inflammatory abnormalities that increase the cardiovascular risk (Figure 1.5.).

Figure 1.5. Proposed role of insulin resistance and hyperinsulinaemia in coronary heart disease. Adapted from Reaven G[130].



The maintenance of glucose homeostasis is dependent on a balanced, dynamic interaction between target tissue sensitivity (i.e. skeletal muscle, liver) and insulin secretion that is appropriate for the prandial/fasting state. In insulin resistance, insulin function is defective, and despite an initial hyperinsulinaemia aimed to correct the suboptimal peripheral glucose utilization, in genetically predisposed, high risk individuals this will eventually lead to pancreatic β cell exhaustion and consequently, type 2 diabetes.

One of the primary defects in insulin resistant individuals is the chronic plasma FFA elevations due to the diminished visceral fat sensitivity to the antilipolytic insulin action following a mixed meal or a glucose load[131]. The increased FFA concentration contributes to insulin resistance in muscle and liver and this will be discussed below. Obesity itself plays an important role in IR since large, hypertrophied adipocytes are unable to increase their lipid storage capacity and the excess triglycerides accumulate instead in skeletal muscle, liver and β cells, exacerbating IR. Additionally, the adipose tissue secretes several proinflammatory cytokines such as interleukin 6 (IL-6), tumour necrosis factor alpha (TNFα)[132] and PAI-1[133] that are associated with endothelial dysfunction and thrombosis.

Insulin resistance in muscle is induced by multiple mechanisms including elevated plasma FFA concentrations, intramyocellar fat accumulation and reduced fat oxidation independent of FFA levels[134]. The onset of insulin action in myocytes is delayed, reducing glucose uptake and glycogen synthesis. Downregulation of the GLUT 4 cellular receptors induced by hyperinsulinaemia[135], insulin postreceptor defects and mitochondrial dysfunction in oxidative phosphorylation pathways occur that are in concert responsible for the diminished insulin sensitivity[134].

In the basal state, the liver represents a major site of IR with an accelerated rate of glucose output despite the presence of hyperinsulinaemia and hyperglycaemia. In type 2 diabetic subjects, the fasting plasma FFA concentration and lipid oxidation rate are increased and the elevated plasma FFA induce hepatic insulin resistance by inhibiting the insulin signal transduction system.

1.3. The biological variation of lipids

1.3.1. The nature of biological variability and its clinical relevance

Clinical laboratory tests are widely used to analyse biomarkers since they can objectively reflect biological changes in an individual. Their usefulness in epidemiological studies, screening, diagnosis and monitoring different pathological conditions is influenced by different sources of variation such as preanalytical (specimen collection), analytical (imprecision and bias), postanalytical (reporting of the result) and biological. The biological variation is the natural fluctuation of these analytes around a homeostatic setting point, either in a random fashion and/or in a more predictable daily, monthly or seasonal circadian rhythm. Critical periods in life such as puberty or menopause and illnesses can also introduce very rapidly variations in an analyte. Awareness of these fluctuations is essential to appropriately obtain specimens that reflect the clinical scenario studied[136], for example the timing of troponin collection following an acute myocardial infarction. This biological variation comprises two components: the within-person (the variation around the homeostatic setting point in an

individual) and between-person variation (the homeostatic setting point among different subjects). Both are mainly used to set analytical specifications, to evaluate serial changes in an analyte and to assess the clinical utility of population based reference intervals[137].

The interpretation of a given test to screen/support a clinical diagnosis is usually aided by comparing the obtained value with population based reference limits although clinical fixed cut-off points derived from locally agreed protocols or consensus clinical guidelines can also be used for this purpose. However, an analyte such as creatinine for example, could have a small, individual intrinsic variation, and a change in its value, although unusual for that person, might remain within the reference limits and it could be interpreted as being "normal". This concept is represented by the Index of Individuality (IoI)[138] which is the ratio between the within subject and the between subject biological variations. If this index is low, especially <0.6, population reference intervals are of limited utility deciding if a significant change has occurred and for these analytes with marked individuality, comparison with previous values could provide more useful information. Conversely, if the IoI is high, especially >1.4, the analyte has little individuality and therefore population intervals can be used to assess change.

It is also essential that the differences in values observed in a set of serial results, used for example, to monitor a condition or its response to therapy, truly reflect changes in the pathological process (i.e. the patient is improving or worsening) rather than the influence of preanalytical, analytical or biological variations. This can be evaluated with the reference change value (RCV) or critical difference that is dependent on the biological variation data (both in health and in disease)[139]. Assuming the preanalytical

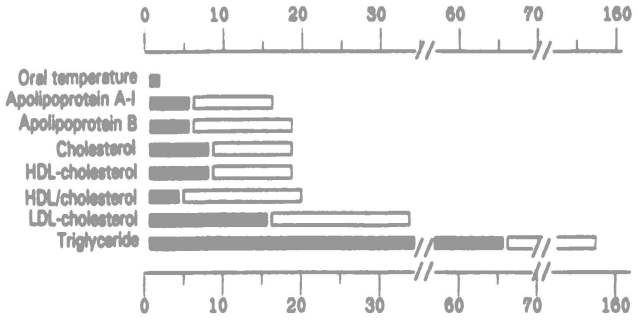
and analytical variations have been minimised, the difference between two values should be greater than the combined intrinsic variation in the two results.

The biological variation data can also be used to calculate the number of samples needed to estimate the homeostatic setting point within certain percentage of the true value, to select the best test for a clinical setting, to calculate the reliability coefficient used in epidemiology and in the development of a new test procedure[140].

1.3.2 The biological variation of lipids

Lipid measurement is one of the essential components in the risk stratification and management of cardiovascular disease in both primary and secondary prevention. Control of pre analytical and analytical factors together with awareness of their biological variation are required to obtain reliable levels that permit appropriate therapeutic intervention. Several factors influence the variability of serum lipids in an individual such as prandial status, menstrual cycle, pregnancy, menopause, seasons, ageing, dehydration and certain illnesses and medications[141-143] which have to be taken into account when measuring these analytes. Moreover, they appear to have a daily rhythmic variation ranging between 5% for Apo A1 to 65% for triglycerides[144] (Figure 1.6.).

Figure 1.6. Daily maximum variation of lipids expressed as a percentage of daily mean. Adapted from Rivera-Coll A[144] .



Ricos et al[145] published a database that included the within biological variation for lipids and lipoproteins (Table 1.3.). Triglycerides (TG) have the greatest biological variation which limits their utility in risk assessment and introduces variability in the calculation of LDLc with the Friedewald equation which provides optimum results only if TG<2.3 and acceptable results up to TG=4.5[146].

Table 1.3. Influence of within and between subject variation of lipids and lipoproteins. Adapted from Ricos C. [145]

	Total-c	**HDL-c**	**LDL-c**	**TG**	**Apo A1**	**Apo B**
CVi (%)	5.4	7.1	8.3	21.0	6.5	6.9
CVg (%)	15.2	19.7	25.7	37.2	13.4	22.8

CVi: within subject variation; CVg: between subject variation; Total-c: total cholesterol; HDL-c: HDL cholesterol; LDL-c: LDL cholesterol; TG: triglycerides; Apo A1: apolipoprotein A1; Apo B: apolipoprotein B.

This together with the need to obtain a fasting specimen to determine TG levels makes more valuable to initially screen for HDL and total cholesterol in the general population. However, including TG is essential when considering genetic, secondary causes of dyslipidaemia and decision on therapeutic intervention.

1.3.3. Dyslipidaemia in Polycystic Ovarian Syndrome

Lipids disturbances have been observed in approximately 70% of the patients with PCOS[147] being hypertriglyceridaemia[148, 149], low HDL and/or HDL2 and Apo AI[150-152] and raised small, dense LDL particle the abnormalities more commonly reported[153] although the presence of this atherogenic lipid profile is variable depending on the PCOS phenotype studied[154].

Different interrelated pathological processes appear to contribute to the dyslipidaemia found in this condition being obesity, insulin resistance and hyperandrogenism the most widely described. Android type obesity is a major contributor to the dyslipidaemia observed in PCOS[151, 155]. Centrally distributed adipocytes are more insulin resistant than peripheral ones[156] and even lean patients with PCOS may show an excess of intraabdominal visceral fat[157]. Legro et al[158] found that 81% of insulin resistant PCOS patients had lipid abnormalities as compared to 65% in those with normal insulin sensitivity therefore, the magnitude of insulin resistance appears to correlate with the degree of dyslipidaemia[159]. It has been postulated that hyperinsulinaemia and hyperandrogenemia cause a release of free fatty acids into the circulation, following an increased catacholamine induced lipolysis in the adipocytes, that stimulate the secretion of very low density lipoprotein(VLDL) in the liver and this ultimately leads to hypertriglyceridaemia[160].

In addition, insulin resistance[161] and high androgen levels[162] have been associated with increased hepatic lipase activity which has a role in the catabolism of HDL, depleting HDL from lipids. Raised atherogenic, small dense LDL subfractions (LDL III and IV) have also been reported even in subgroups of patients with PCOS who otherwise have normal plasma lipids[163 164].

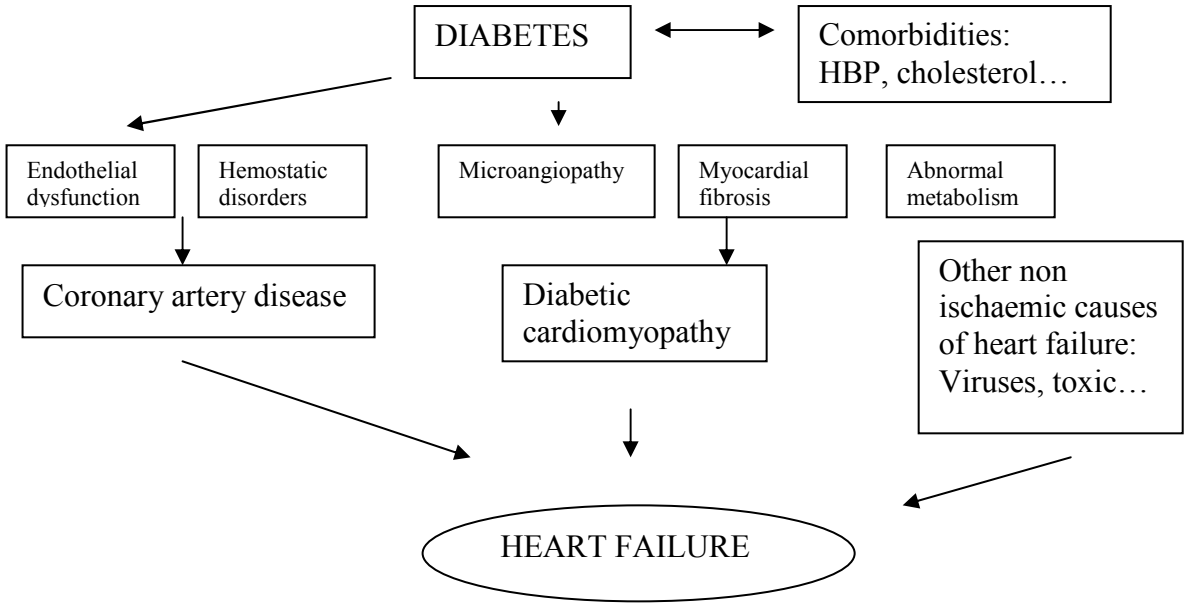
1.4. Brain Natriuretic peptide as a marker of cardiac dysfunction in type 2 diabetes

Traditionally, heart failure was thought to have an incidence ranging between two and fivefold excess in patients with type 2 diabetes as compared with the general population[165]. However, more contemporary studies point towards a higher incidence of up to 11 times depending on the subgroup studied[166-168].

Different plausible mechanisms have been proposed to explain this high incidence of heart failure in this group of patients. It is likely that the cluster of cardiovascular risk factors and the accelerated coronary atherosclerosis seen in type 2 diabetes play an important role. In addition, some authors suggest the possible existence of a specific diabetic cardiomyopathy[169] related to underlying microangiopathy[170-172], myocardial fibrosis[173, 174] and metabolic factors such as hyperglycaemia, impaired myocardial glucose uptake, increased turnover of free fatty acids and abnormalities in calcium homeostasis[175, 176] (Figure 1.7.).

Current views recommend to follow standard guidelines for investigating and managing heart failure in type 2 diabetes since there are no specific trials to address therapy in this group of patients[177, 178]. However, it is particularly important to identify the early stages since heart failure has poor prognosis in patients with diabetes[179].

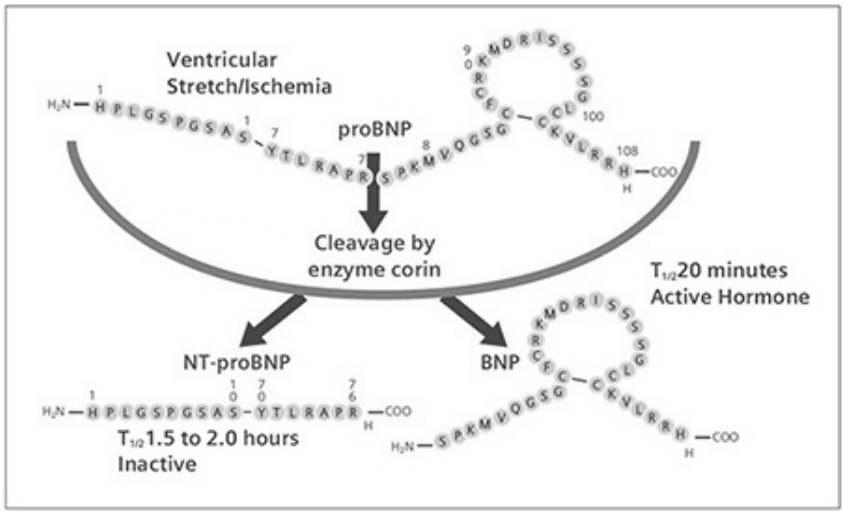
Figure 1.7. Potential mechanisms linking type 2 diabetes to heart failure. Adapted from Bauters C.[176]



Biological markers, such as natriuretic peptides, have emerged as potential tools to assist in screening and early diagnosis of heart failure[178].

The family of natriuretic peptides comprises brain natriuretic peptide (BNP), atrial natriuretic peptide, C-type nautriuretic peptide and urodilatin. BNP is a 32 amino acid polypeptide secreted by the ventricular myocytes as a precursor, proBNP, in response to tension and stretch, and it acts as a counter regulatory hormone to the renin angiotensin-system (RAS), promoting balanced vasodilatation. It is activated by a protease which splits it in two fractions: the biologically active BNP and the inactive N-terminal proBNP (NT-proBNP) (Figure 1.8.). Both fractions circulate in plasma, and can be quantified by immunoassay.

Figure 1.8. Secretion of BNP and Pro BNP



ProBNP molecule cleaved to BNP and NT-proBNP.

Measurement of BNP and NT-proBNP is a useful marker of both systolic and diastolic heart failure since their plasma levels are proportional to the severity of heart failure and correlate with New York Heart Association function class [180 181 182]. They seem to have prognostic, screening and diagnostic value in emergency[183] and primary care settings[184-186]. The prospective, multicentre breathing not properly study (the BNP study)[187] measured BNP levels in 1586 subjects who presented with acute dyspnoea. The reference standard of heart failure was adjudicated by two cardiologists blinded to the BNP levels. BNP was more accurate identifying heart failure as the cause of dyspnoea than clinical judgement alone. A level of 100 pg/ml had a diagnostic accuracy of 83.4% and using a cut point of 50 pg/ml, the negative predictive value was 96%. The PRIDE study (N-terminal PRo-BNP Investigation of Dyspnoea in the Emergency department study) [188] aimed to compare the diagnostic accuracy between NT-proBNP levels and the admiting physician (blinded to the analyte levels) identifying acute heart failure in 600 patients. A NT-proBNP level < 300 pg/ml was optimal to exclude heart failure with a negative predictive value of 99%. Again, it was superior to clinical

judgement alone. The purpose of the prospective, randomized, controlled Community Study[185] was to determine if BNP improved diagnostic accuracy in patients presenting with dyspnoea and/or peripheral oedema in a primary care setting. A total of 305 patients was included, GPs made a preliminary diagnosis based on clinical presentation and then the patients underwent a full cardiological evaluation that included echocardiograms and BNP levels. The patients were then randomised to control group (GP was not aware of BNP levels) or BNP group (GP received BNP levels). The diagnostic accuracy made by GP improved 21% in the BNP group, helping the exclusion of heart failure in those with normal/low levels of BNP. Therefore, BNP and/or NT-proBNP measurements could be advantageous tools in selecting patients with dyspnoea for echocardiography which may be cost effective particularly in primary care.

BNP and NT-proBNP could be used as surrogate markers in heart failure to assess prognosis since they have a strong association with total mortality [189, 190] and can predict further cardiac events after an acute episode[191]. Therefore these natriuretic peptides may assist in the decision making to when hospitalise a patient following an acute episode[192, 193] and improve clinical outcomes following hospitalization since they might guide therapy, reducing the length of hospital stay and the rate of first rehospitalisation [194].

There are a limited number of studies about the use of these biomarkers in patients with diabetes reporting conflicting results. NT-proBNP levels appear to be raised in type 2 diabetes without overt cardiovascular disease[195] and with/without microalbuminuria[196] as compared with the general population. However, the diabetic subgroup from the BNP study had similar BNP levels when compared with non diabetics regardless of their heart failure status (no heart failure, heart failure and previous history of heart

failure)[197]. Pre-screening with BNP might allow early identification of those diabetic patients likely to have ventricular dysfunction[198] and Epshteyn et al[199] suggested that a BNP cut off level of 40pg/ml (29.5pmol/L) may be used to exclude systolic and diastolic ventricular dysfunction in asymptomatic, diabetic patients. This finding is conflicting with the results obtained by Fang et al[200] since BNP did not appear to be sufficiently sensitive to identify subclinical ventricular dysfunction in their cohort and Liew et al [201] who compared a random sample of 100 type 2 diabetes patients without hypertension or overt cardiovascular disease with subjects matched by age and sex with normal glucose tolerance and impaired glucose tolerance, found that NT-proBNP levels were similar across the groups suggesting that screening for early left ventricular dysfunction in this group of diabetic patients has little utility. Nevertheless, BNP could be used as prognostic tool to aid risk stratification since it appear to be a predictor in cardiac and all cause mortality in patients with diabetes[202].

BNP and NT- proBNP have potential limitations in clinical practice. Firstly, there are conditions other than heart failure that can lead to increased levels such as atrial fibrillation[203], acute coronary syndrome, pulmonary embolism, hypertension, renal dysfunction[204] and anaemia[205, 206] . Secondly, advanced age and female sex appear to increase both levels[207] while obesity reduces them[208]. And finally, BNP and NT-proBNP levels are influenced by their intraindividual biological variation.

1.5. The aims of undertaken research

- To determine the influence of social deprivation in lipid measurements and lipid lowering drug prescriptions in Kingston upon Hull and East Riding of Yorkshire.

- To determine the effects of dietary supplementation with isolated soy isoflavones on insulin resistance and cardiovascular risk factors in postmenopausal women with type 2 diabetes.

- To determine the effects of metformin and a hypocaloric diet on the biological variation of lipids and on cardiovascular risk factors in patients with PCOS.

- To determine the effects of low dose of rGH on exercise capacity, cardiovascular risk factors, quality of life and mitochondrial oxidative activity in adult growth hormone deficiency.

- To determine the biological variation of NT-proBNP in postmenopausal women with type 2 diabetes and compare it to the biological variation seen in age and weight matched controls.

Chapter 2

Materials and Methods

2.1. Laboratory Methods and Reagents

2.1.1. Biological Variability Studies

Venous blood samples were collected after twelve hours overnight fast, following a ten minutes period of rest, into serum gel tubes (Beckton Dickinson) and one fluoride oxalate tube between 8.00-9.00 am, on 10 consecutive occasions at 4 days intervals. Samples were separated by centrifugation at 2000g for 15 minutes at 4 ^{0}C and the serum obtained was stored in two aliquots at –20 ^{0}C within one hour of collection. The serum samples were split before the assay.

All the serum samples were thawed and thoroughly mixed before the analysis. The duplicate samples (i.e. two per visit) were randomized and then analysed in a single continuous batch using a single batch of reagents. Total cholesterol, triglycerides and HDL cholesterol were measured enzymatically using a Synchron LX20 analyser (Beckman-Coulter, High Wycombe, UK) using the manufacturers recommended protocol. LDL was calculated with the Friedewald equation ((LDL cholesterol)= (total cholesterol)-(HDL cholesterol)-(triglycerides)/5).

NT-proBNP plasma levels were quantified with an electrochemiluminescence immunoassay used on the Roche Elecsys® Systems 1010/2010. The analytical sensitivity was 0.6pmol/L with a measuring range between 0.6 and 4130 pmol/L .The within run precision with this method is 1.8%. The assay is unaffected by bilirubin, haemolysis or lipaemia with no stated cross-reactions with ANP/NT-proANP, BNP, CNP, aldosterone, angiotensin (I, II, III) or renin.

2.1.2. Intervention Studies

2.1.2.1. Biometric parameters

The subjects were weighted between 8-9am with calibrated scales and in light clothes. Sitting blood pressure was measured after a five minutes of rest period using an automated device (NPB-3900, Nellcor Puritan Bennett, Pleasanton, California).

Waist measurements were taken at the narrowest waist level, or if this was not apparent, at the midpoint between the lowest rib and the top of the iliac crest. Hip measurements were taken at the level of the greatest protrusion of the gluteal muscles. The percentage of body fat was estimated with a monitor using Bioelectrical Impedance Analysis (BIA, Tanita®). This sends a low, safe electrical signal that passes freely through lean muscle, but meets resistance when it comes into contact with body fat. Body composition is calculated mathematically, based upon the speed at which the signal passes through the body.

2.1.2.2. Blood samples

They were collected after a twelve hour fast, following a 10 minutes period of rest, into EDTA, serum gel and fluoride oxalate tubes. Samples were separated by centrifugation at 2000g for 15 minutes at 4 ^{0}C. The aliquots were stored at –20 ^{0}C within one hour of collection. Plasma glucose was measured using a Synchron LX 20 analyser (Beckman-Coulter, High Wycombe, UK), using the manufacturer´s recommended protocol. The coefficient of variation for this assay was 1.2% at a mean glucose value of 5.3 mmol/L. Lipid levels (total cholesterol, triglycerides and HDL cholesterol) were also measured using a Synchron LX 20 analyser. LDL levels were calculated using the Friedewald equation with concentrations given in mg/dL. Serum insulin was assayed using an immunometric assay performed on the DPC Immulite 2000 analyser (Euro/DPC,

Llanberis, UK). The coefficient of variation of this method was 8% calculated using duplicate study samples. The analytical sensitivity was 2 μU/ml with no stated cross-reactivity with proinsulin. The insulin resistance was calculated using the Homeostasis Model Assessment (HOMA) method (HOMA-IR = (insulin × glucose)/ 22.5)[209]. Glycated haemoglobin (HbA1c) was measured on a DCCT aligned HA-8140 analyser (A.Menarini diagnostic, Berkshire, UK) using the manufacturers recommended protocol. NT-proBNP was measured with an electrochemiluminescence immunoassay used on the Roche Elecsys® Systems 1010/2010.

2.1.2.3. Muscle biopsies and SDH assay

Quadriceps muscle biopsies were frozen immediately after excision, mounted onto cork discs, frozen in 2-methylbutane at -196°C and finally 12μm sections were cut using a cryostat and mounted onto Polysine (VWR) microscope slides which were stored at -25°C until use. All samples were analysed in a single batch. Thawed sections were incubated for one hour at 37°C in 0.5M Sorenson's buffer containing 0.5M sodium succinate and 1mg ml-1 nitroblue tetrazolium. After a water rinse, the sections were fixed in formal saline and mounted in glycergel for optical density analysis using a light microscope equipped with a Cool-Snap digital camera and Image Pro plus image analysis software (Media Cybernetics, Wokingham, UK). Four random fields of view at 100x magnification were used from each section. Black and white images were captured, background corrected and calibrated for incident light. An average optical density value for each was measured.

2.2. Statistical Analysis

Statistical analysis was performed using SPSS for windows, version 15.0 (SPSS Inc., Chicago, Illinois) or stata statistical computer package (StataCorp, 2007), in the growth hormone study. The results obtained were considered statistically significant if the two-tailed p value was less than 0.05. Details of sample size calculations for the biological variability studies are detailed below.

The sample size calculation for the intervention studies are described in the relevant chapters.

2.2.1. Biological Variability Studies

Biovariability data was analysed by calculating analytical, within subject, and between subject variances (SD_A^2, SD_I^2, SD_G^2, respectively) according to the methods of Fraser and Co- workers [137 210]. By this technique, analytical variance (SD_A^2) was calculated from the difference between duplicate results for each specimen ($SD_A^2 = \Sigma d^2/2N$, where d is the difference between duplicates, and N is the number of paired results). The variance of the first set of duplicate results for each subject on the ten assessment days was used to calculate the average biological intra-individual variance (SD_I^2) by subtraction of SD_A^2 from the observed dispersion (equal to $SD_I^2 + SD_A^2$). Subtracting $SD_I^2 + SD_A^2$ from the overall variance of the set of first results determined the inter-individual variance (SD_G^2). The intra-individual (SD_I) and inter-individual (SD_G) variations were estimated as square roots of the respective variance component estimates. The reference change value or critical difference between two consecutive samples in an individual subject (i.e. the smallest percentage change unlikely to be due to biological variability)was calculated using the formula $RCV= 2.77*(CV_A^2 + CV_I^2)^{½}$ where CV_I is the within subject biological coefficient of variation and CV_A is the

analytical coefficient of variation[137]. The index of individuality was derived from the ratio of intra- and inter individual variation (SD_I/SD_G)[137, 138]. As explained in section 1.3.1, when the IoI for a particular test is ≤ 0.6, conventional population based reference intervals are of limited value in the detection of unusual results for a particular individual. When the IoI is ≥ 1.4, the variation of an individual will fit populations reference limits more closely so being suitable as a screening test. The sample size and number of repeated measures used were based on previous studies investigating biological variation in measured analytes[140, 211].

2.2.2. Intervention Studies

The two treatment groups were compared using the paired t test for biochemical data and the Wilcoxon signed rank test for the clinical observations and biochemical data that was not normally distributed when tested using the Kolmogorov-Smirnov test.

2.3. Ethics

All subjects gave their informed written consent prior to entering the studies that had been approved by the Hull and East Riding and York Local Research Ethics committees.

Chapter 3

The influence of social deprivation on lipid measurements and lipid lowering agents prescriptions in Hull in East Yorkshire region

3.1. Introduction

Cardiovascular disease (CVD) represents a major cause of premature mortality in the Western world, as described in section 1.1. and measures to reduce chronic cardiovascular risk factors such as hyperlipidaemia are important to improve clinical outcomes. Lipid lowering drugs are an effective treatment in primary prevention for patients with moderate to high risk of CVD, reducing the relative risk of major coronary and cerebrovascular events by approximately 29% and 14% respectively and revascularization rates by 34% [212]. Furthermore, significant reductions in fatal and non fatal CHD events can be achieved in patients with high and average cholesterol levels if they are used in secondary prevention [122, 123, 213].

However, lipid lowering drug prescribing varies between health authorities and between patients on the basis of age, gender, demographics [214-216], ethnicity[217] and deprivation[218 219]. Since social deprivation is associated with increased prevalence of CVD, targeting at risk, deprived areas would be advantageous and theoretically they would require higher rates of prescriptions to reduce that risk. Several surveys in different regions in the UK have reported variable results in statin prescription according to deprivation indexes: either positively correlated [220], negatively correlated [218, 219] or no association with levels of deprivation [221-223] probably reflecting local policies and re-distribution of resources.

My aim was to study the relationship between social deprivation and the rate of lipid measurements and the lipid lowering agent prescriptions in Kingston upon Hull and East Riding of Yorkshire (KUH and ERY), a heterogeneous county with a mixture of urban and rural areas with different indexes of social deprivation. The largest ethnic group is Caucasian (98.8%) with only small percentage of ethnic minorities and low emigrational movements giving rise to a highly stable population[224]. Multiple deprivation is more marked in Hull, with over half of its wards within the most deprived 10% of wards in England [225]. We hypothesised that the rate of lipid measurements and the lipid lowering agent prescriptions in KUH and ERY would vary according to levels of social deprivation.

3.2. Methods

This is an observational, cross-sectional, retrospective study. Prescribing analysis and lipid measurement data were obtained from 96 different GP surgeries distributed amongst 4 different PCTs in KUH and ERY between January 2002 and December 2002. Postal codes were used to locate and distribute the data across the different electoral wards according to the 1996 geographical boundaries.

The index of multiple deprivation 2000 (IDM 2000) [225] designed by Oxford University and based in an interim Census analysis compiled in 1998 was the source used to evaluate social deprivation. The IDM 2000 is a ward level index, made up of six main domain Indexes (Income, Employment, Health deprivation and disability, Education and Training, Housing and Geographical access to services) and a supplementary index based in Child Poverty. These different domain indexes have a specific weight when added to create an overall index being 25% income and employment respectively, 15% Health and Education each, and 10% geographical access and housing each. The overall index is represented as a score and the higher the score, the higher the percentage of the population in a given ward is deprived.

Reference demographic data (total population per ward and age-group distribution per ward) was extracted from the 1998 Oxford Census population estimates although sex distribution across the different wards was not available, and therefore not included.

3.3. Statistical analysis

Statistical analysis was performed using the SPSS program version 15. The index of multiple deprivation was correlated with the number of lipid prescriptions and lipid requests in each of the selected electoral wards, adjusting for number of surgery patients and age (>60 y old).

3.4. Results

A total number of 46700 lipid requests and 181850 lipid lowering agent prescriptions were analysed across 34 wards (20 within Kingston Upon Hull and 14 within East Riding) with different scores for social deprivation (Table 3.1.). Eleven of those wards were located in rural areas being the rest city based wards.

There was no significant linear relationship between the level of total deprivation and the number of lipid prescriptions (r=0.50) or lipid requests (r=0.18) amongst the electoral wards (Figure 3.1.).

Figure 3.1. Correlation between total deprivation scores and lipid requests /lipid lowering agents prescriptions in Hull and East Riding of Yorkshire.

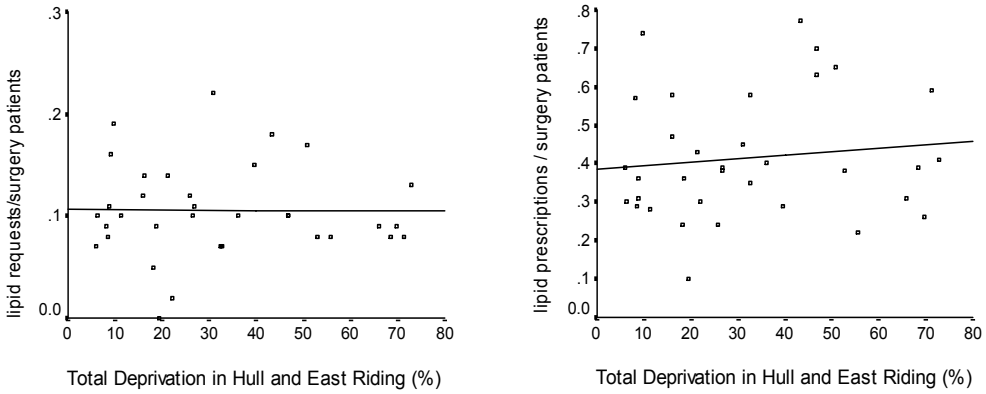


Table 3.1. Electoral wards with associated Index of multiple deprivation score (IMDS).

Wards	Urban/ Rural	TLR	TLP	IMDS	Wards	Urban/ Rural	TLR	TLP	IMDS
1	Urban	2922	12149	36.31	18	Urban	780	4915	46.80
2	Urban	1700	3535	30.91	19	Urban	2661	9026	65.91
3	Urban	1050	4600	43.39	20	Urban	1470	3772	55.60
4	Urban	2462	9526	50.82	21	Rural	425	1687	9.68
5	Urban	1564	7407	68.41	22	Rural	1224	3348	11.35
6	Urban	3483	10759	72.91	23	Rural	1170	6593	6.07
7	Urban	276	1890	46.59	24	Urban	1413	5492	18.65
8	Urban	583	1125	39.58	25	Rural	949	2984	21.29
9	Urban	670	3165	32.66	26	Rural	805	2320	6.32
10	Urban	2588	9428	26.52	27	Rural	2901	9929	8.51
11	Urban	728	2458	26.78	28	Urban	350	1020	8.71
12	Urban	103	1884	22.01	29	Urban	1147	2591	8.91
13	Urban	1386	2703	25.86	30	Rural	916	5728	8.02
14	Urban	1407	4232	69.64	31	Rural	3271	13716	16.08
15	Urban	1055	5172	52.86	32	Rural	92	1843	19.43
16	Urban	1049	5488	18.14	33	Rural	876	7011	32.49
17	Urban	494	3732	71.17	34	Rural	2730	10622	15.93

TLR: total lipids request TLP: total lipids prescriptions.

3.5. Discussion

Blood lipid measurement and lipid lowering agent prescriptions were similar across the wards irrespectively of social deprivation. This indicates that healthcare needs are equally met and, superficially, this is reassuring. However, this data may obscure the fact that social deprivation areas should consume more healthcare resources since their CVD prevalence is higher. Therefore, if high deprivation areas are being treated appropriately it means low deprivation areas are being over investigated and treated. Alternatively, if the low deprivation areas are being treated appropriately then these results indicate that high deprivation areas are being under treated and under investigated.

The data collection period for this study predated the implementation of the new GMS contract for general practitioners, which gives incentives to treat all patients who have established (or are at high risk of developing) CHD. Therefore, it will be interesting to observe if these findings change the discrimination between high and low deprivation areas.

This study has a number of limitations. Although the majority of the data was available through a computerised central network, values from the most outward wards, mainly in ER, were stored in a parallel system which was not readily accessible and this might have caused data collection bias. Secondly, the IDM-2000 uses electoral wards as the unit to measure deprivation. Electoral wards are groups of an average of 5500 people linked together by the electoral distribution[226]. These geographical boundaries tend to be variable and prone to change. They indeed changed during the study period and the new electoral wards available on Census 2001(C-2001), which had the latest and more

complete demographic information, did not correspond with those of IDM-2000 and therefore, a direct comparison between C-2001 and deprivation indexes could not be performed. Instead, we used the interim Census analysis compiled in 1998 by Oxford University which did not include sex distribution on the wards. Alternatively, Jarman scores could have been used as they are equated to the more stable enumeration districts (ED: units of 250-500 people). However, the current Geographic Areas system employed to correlate ED and deprivation only allows 1991 ED to be linked to a defined areas, and these ED are based on 1981 Census information which is outdated. Towsend score has been used in previous studies but is also 10 years old [227, 228].

Geographical location of surgeries is also important when electoral wards are used as basic unit to classify deprived areas, particularly if they are close to boundaries in between wards. This is particularly important because the patient register within a given surgery could include a mixed type of population with different risks of CHD as they would travel from different wards with different indexes of deprivation. Finally, we could not obtain consultation data and therefore, it is difficult to know if the lack of differences observed in high and low deprivation areas are due to primary or secondary prevention identification/prescription factors.

In conclusion, the measurement of lipid profiles and lipid lowering agent's prescriptions were not correlated with social deprivation.

Chapter 4

Effects of Soy Isoflavones Intake in Postmenopausal Women with Type 2 Diabetes

4.1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in women in developed countries [229] and women with diabetes are four times more likely to die from CVD than men [34]. Amongst others, postmenopausal oestrogen depletion, greater insulin resistance and dyslipidaemia [230, 231] may contribute to their high risk of accelerated CVD. Modification of lifestyle is important to reduce CVD risk factors and delay progression of type 2 diabetes associated complications. In particular, the addition of oral supplements, such as soy products as part of a healthy diet, has attracted recent interest because their beneficial effects on the lipid profiles [232-237]. However, scant information is available on the effects of soy in individuals with type 2 diabetes [238-240], who are at higher risk due to hyperlipidemia, lower HDL level and abnormalities in LDL/lipoprotein composition [241]. It also remains unclear whether a beneficial effect can be attributed to the soy protein or isoflavones.

A previous study showed that soy protein combined with isoflavones could improve glycaemic control, insulin resistance and lipids in patients with type 2 diabetes [238]. Therefore, my aim was to determine if this effect was due to the isoflavone component alone.

4.2. Subjects

A total of thirty two Caucasian postmenopausal women with type 2 diabetes were recruited for the study. They met the following inclusion criteria: diagnosis of type 2 diabetes (according to WHO criteria [242]) controlled with diet alone and absent menstrual periods for more than one year. Subjects were excluded if they had a secondary cause for hyperglycaemia, previous history of breast or uterine cancer, alcohol abuse, untreated or uncontrolled hypothyroidism or if they received oral hypoglycaemic agents, insulin,

oestrogen supplementation (in the preceding 6 months) or newly introduced statins (less than 4 months prior recruitment) as a part of their treatment.

The baseline characteristics of the subjects are included in table 4.1. They were obese (BMI= 31 ± 6), with a mean age of 65 (range: 52-76 years old), mean duration of diabetes of 3 years (range: 3 months to 10 years) and mild elevation of total cholesterol (5.4 mmol/l ± 1) and LDL levels (3.4 mmol/l ± 0.9).

4.3. Methods

Study design

This was a randomized, double blind, placebo controlled, cross over study of 28 week duration. The placebo and active phases lasted 12 weeks each and were separated by a 4 week wash out period. Randomization was performed by the pharmacy department on site (Hull Royal Infirmary) by a random number generating table, and the code numbers were only available in case of an emergency. This study was approved by the Hull and East Riding Local Research Ethics Committee and all subjects provided written and informed consent.

Intervention

The patients received an identical looking tablet containing either isoflavones or placebo and then crossed over to the alternative supplement that was taken once a day.

The isoflavone preparation (Essential Nutrition, Brough, U.K) contained a mixture of genistein, daidzein and glycitein (53%, 37% and 10% respectively) present as their glucoside conjugate in 95%. The dose used was 132 mg/day and the product was completely devoid of soluble fiber.

The placebo supplement consisted in an identical tablet containing pure microcrystalline cellulose (Essential Nutrition, Brough, and U.K) with no calorie content.

Dietary advice was provided from a registered dietician prior recruitment, and the patients were instructed to maintain their diabetes diet and levels of physical activity throughout the study. An information leaflet, containing a list of soy and soy based products, was also provided in order to minimise their ingestion. Compliance was monitored by counting the returned tablets. In addition, dietary reinforcement was performed at each visit.

Study Measurements

Baseline biometric parameters (weight and blood pressure) were obtained prior blood sampling as described in section 2.1.2.1.

Venous blood samples were collected after a 12 hour overnight fasting at screening and after each study visit: baseline, 12 weeks after each treatment phase and following the washout period. Glucose, lipids, serum insulin, CRP and HOMA-IR[209] were calculated as described in section 2.1.2.2.

4.4. Sample Size Calculation and statistical analysis

Sample Size Calculation

A sample of 32 patients was needed, assuming a 20% drop out rate, to detect with a probability of 80% and using a two sided 5% significance level, a 13% true difference in

LDL between treatments (assuming a common SD of 16) and 20% true difference in fasting insulin with a common SD of 25.

Statistical analysis

Mean percentage changes obtained at the end of isoflavone treatment were compared to those at the end of the placebo phase using the paired Student´s t test if they followed a Gaussian distribution or the Wilcoxon´s signed-rank test if those changes violated the assumption of normality when tested using the Kolmogorov-Smirnov test (CRP and HOMA-IR data). The period effect (calculated by comparing the mean difference between placebo and isoflavone treatment in the group starting on placebo with the group starting on isoflavones) and the carry-over effect (comparing baseline values for each treatment group) were tested using the Student´s t-test. The results were considered statistically significant if the two-tailed P value was <0.05. Statistical analysis was performed using SPSS (version 15). P values are included in Table 4.1.

4.5. Results

Six subjects withdrew from the study: subject 1 (S1) required a cholecystectomy, S2 a coronary angioplasty, S3 had an acute attack of polymyalgia rheumatica requiring steroids and S4-6 were unable to comply with study requirements. Therefore, a total of 26 patients completed the study and no period effect or carryover effects were detected. Both study preparations were well tolerated with >90% compliance. Similar side effects were reported: flatulence (six subjects on both treatments) and heartburn (one subject with placebo, two subjects with isoflavones).

Effects on glycaemic control

There were no significant differences in glucose (p=0.59), HbA1c (p=0.58), insulin resistance (HOMA-IR method, p=0.24) between soy and placebo phases.

Effects on lipids

No significant changes were seen in either total cholesterol (p=0.96), HDL (p=0.93), LDL (p=0.97) or triglyceride levels (p=0.74) between the two treatment phases.

Other effects

There was no difference in either BMI (p=0.97), blood pressure (systolic, p=0.35; diastolic, p=0.38) or CRP (p=0.40) between treatment and placebo phases.

4.6. Discussion

This study showed that soy isoflavones alone do not confer significant cardiovascular protection or positive effects on glycaemic control in this group of patients. This is in accord with studies with red clover isoflavones in either postmenopausal women without evidence of CVD or postmenopausal women with diabetes treated with diet and/or oral hypoglycaemic agents that observed no change of plasma lipoproteins or glycated haemoglobin, although basal endothelial function[243] and arterial compliance[244] did alter. Similarly, in a small cohort of postmenopausal women with diabetic retinopathy, isolated isoflavones alone had little effect on their lipid or glycaemic parameters when ingested for twelve weeks[245]. However, it is possible that effects mediated by isoflavones are too modest to be detected over the 3 month study period.

Epidemiological studies[246, 247] have indicated that there is no significant association between the standard western dietary intake of isoflavones (0.369 – 0.770 mg/day) and the reduction of cardiovascular events or lipid levels in different cohorts of

postmenopausal women over prolonged periods of time (4-6 years). Our subjects received more than 150 times this amount, yet no significant changes were observed over a 3 month period. The dose of isoflavones given was the same used previously within 30g soy protein that reduced these cardiovascular and glycaemic parameters within the same timeframe[238]. In addition, supplementation with isoflavones alone (40-150mg/day) in subjects without diabetes showed that there is no change in lipid profile in peri/postmenopausal women, both healthy and mildly hypercholesterolaemic[99 248, 249].

Isolated soy protein has been shown to reduce total cholesterol (9.3%), LDL cholesterol(12.9%) and triglycerides (10.5%) [250] though this was limited as the preparation was not truly isoflavone free. A more contemporary metaanalysis [251] evaluated 23 randomized controlled trials and reported that the combination of soy protein with isoflavones intact was associated with significant reductions, but less pronounced, in total cholesterol (3.77%), LDL cholesterol (5.25%) and triglycerides (7.27%). This effect was more apparent within a short period of time, more in men and premenopausal women than in postmenopausal women and if the isoflavone intake was >80mg/day. Significant, although mild, increases of HDL cholesterol (3.03%) were also noticed but only in trials of >12 weeks duration. These different reductions in lipid levels found by Anderson et al [250] and Zhan et al [251] appear to be related to the initial lipid concentration i.e. the lipid lowering effect is more marked if the initial levels are higher. Taku et al [252] analysed 11 randomized, control studies and concluded that soy protein enriched with isoflavones significantly reduced total and LDL cholesterol compared with the same amounts of isoflavone-depleted soy protein and that 102mg of isoflavones/day for 1-3 months, independently of the amount of soy protein ingested, would lower total cholesterol by 1.77% and LDL by 3.58%.

Two recent studies in subjects with diet controlled type 2 diabetes treated with protein soy isolate containing 80mg isoflavones/day [253]and in patients with type 2 diabetes and nephropathy following 4 years supplementation with a similar supplement [254] also point towards a reduction in lipid parameters with this combination therapy.

The FDA recommends that 25g soy protein/day may reduce CVD[255] since beneficial effects have been observed with different combinations of soy protein and isoflavones in healthy or mildly hypercholesterolaemic postmenopausal / perimenopausal women [232, 234, 256] and in men and postmenopausal women with type 2 diabetes and no diabetic complications[238, 239]. However, the American Heart association (AHA) Nutrition Committee [257] recently reevaluated the evidence on soy protein and CVD disease and concluded that the reductions in LDL cholesterol concentrations (3%) were small relative to the amount of soy protein tested in the studies (on average 50g/day) that represents approximately half of the total daily protein intake. Additionally, the use of isoflavones is not recommended since the effect on lipids is minimal.

In conclusion, oral supplementation with 132 mg/day of isoflavones alone in diet controlled, postmenopausal women with type 2 diabetes did not alter CVD markers over a 3 month period. This suggests that either the soy protein component alone or a synergistic effect between the protein with the isoflavones may be responsible for the modification of glycaemic control and lipid concentrations.

Table 4.1. Subjects' characteristics and effects on cardiovascular risk at the start of the trial and after treatment (mean **± SD).** % change: percent difference in changes between isoflavones and placebo phases ((mean, (95% confidence**))**

	Placebo			**Isoflavones**			**Treatment difference (P value)**	**Period Effect (p value)**	**Cross Over Effect (p value)**
	Baseline	**3 months**	**% change mean (95% CI)**	**Baseline**	**3 months**	**%change mean (95% CI)**			
BMI (Kg/m^2)	31 ± 6.4	30.7±5.5	0.01 (-0.55, 0.59)	30.7 ± 5.5	30.7 ± 5.5	0.02 (-0.69,0.75)	p=0.97	p=0.1	p=0.7
S. BP (mmHg)	133 ±15	137 ±16	4.26 (-1.28, 9.8)	130 ± 16	129 ± 12	0.69 (-3.44,4.8)	p=0.35	p=0.5	p=0.9
D. BP (mmHg)	75 ± 10	76 ± 8	2.79 (-2.99, 8.57)	73 ± 9	74 ± 9	-0.24(-4.23,3.76)	p=0.38	p=0.5	p=0.7
HbA1c (%)	6.7 ± 0.6	6.8 ± 0.7	1.00 (-0.20, 2.2)	6.7 ± 0.7	6.8 ± 0.6	1.56 (-0.43,3.5)	p=0.58	p=0.5	p=0.2
Gluc(mmol/L)	7.0 ±1.4	6.9 ± 1.3	-0.34 (-3.6,2.9)	6.9 ± 1.3	6.8 ± 1.2	-1.6 (-4.3,1.13)	p=0.59	p=0.1	p=0.1
Insul. (µlU/ml)	14.1±10.9	13±6.9	-0.39 (-12,11.2)	12.8±8.1	14.1±9.2	-13.4(-27.3,0.38)	p=0.15	p=0.4	p=0.5
HOMA-IR	4.6 ± 4.5	4.5± 2.5	-4.49 (-15.58,6.6)	4.03± 3.3	4.5 ± 3.8	-15.95 (-31.1,-0.77)	p=0.17	p=0.5	p=0.7
Chol (mmol/L)	5.4 ± 1	5.4 ± 0.9	2.14 (-7.54,3.25)	5.4 ± 1	5.5 ± 0.9	2.01 (-6.4,2.4)	p=0.96	p=0.3	p=0.9
LDL(mmol/L)	3.4 ± 0.9	3.4 ± 0.8	3.6 (-10.5,3.1)	3.4 ± 0.8	3.5 ± 0.9	3.85 (-9.9,2.2)	p=0.97	p=0.2	p=0.7
HDL(mmol/L)	1.2 ± 0.3	1.2 ± 0.3	0.37 (-3.69,4.4)	1.2 ± 0.3	1.2 ± 0.3	0.16 (-3.4,3.6)	p=0.93	p=0.8	p=0.4
TG(mmol/L)	1.8 ± 0.8	1.7 ± 0.7	-4.16 (-16.2, -7.9)	1.9 ± 0.9	1.8 ± 0.9	-1.19 (-13,3,10.9)	p=0.74	p=0.8	p=0.2
CRP	5.1 ± 6.7	6.4 ± 10.1	24.4 (-46.5,2.21)	5.4 ± 8	6.2 ± 8	44(-43.5,133)	p=0.40	p=0.3	p=0.4

BMI: body mass index; S.BP: systolic blood pressure; D.BP: diastolic blood pressure; Gluc: plasma glucose; Insul: Insulin;HOMA-IR: HOMA-Insulin resistance; Chol: total cholesterol;TG: triglycerides; LDL: low density lipoproteins; HDL: high density lipoproteins; CRP:C-reactive protein, SD: standard deviation.

Chapter 5

Effects of metformin and hypocaloric diet on lipids and their biological variation in polycystic ovarian syndrome

5.1. Introduction

Atherogenic dyslipidaemia is one of the risk factors for cardiovascular disease associated with polycystic ovarian syndrome (PCOS). Although there is currently limited evidence to conclude that this abnormal lipid profile is linked to a greater number of cardiovascular events in this group of patients[56], nevertheless attempts have been made to use dyslipidaemia as a surrogate marker to evaluate that risk and to consider therapeutic interventions if detected.

As discussed in section 1.3.1, the reference change value (RCV) is useful to assess the significance of serial changes in an analyte and it is dependent on the components of the biological variation. These components have been calculated in health for several analytes, including lipids and lipoproteins[145] but this quantitative data is limited in disease. A pathological process, such as PCOS, might modify the intrinsic set point and the fluctuations around that set point and therefore, influence the RCV since this is dependent on the intrinsic variation in an individual. This is clinically relevant if a physician needs to calculate, for example, the patient's risk for cardiovascular disease and the response to diet and drug therapy, particularly if the biological variation of lipids found in health is lower than in PCOS since the calculation of RCV in this case scenario might lead to a false positive result and influence for example, the initiation of therapy when is not required. The within subject biological variation of lipids have been estimated in individuals with type 1 diabetes [258], mild hypercholesterolaemia [259], lipid disorders [260], hypertension [261], chronic renal failure [262], chronic liver disease [263]and coronary artery disease [264]. However, no data is available for patients with PCOS.

Therefore, the aim of this study was to evaluate the lipid biological variation in PCOS, both in untreated and treated patients. Secondary outcomes were to assess if therapy can significantly modify waist-hip ratio, % of body fat, body mass index, insulin resistance, plasma glucose levels and blood pressure.

5.2. Subjects

Eleven obese, Caucasian women with PCOS according to Rotterdam criteria[265]were enrolled in the study. The exclusion criteria included impaired glucose tolerance, diabetes mellitus, uncontrolled hypothyroidism and patients on hormonal treatment and antihyperlipidaemic medication. In addition, non classical 21-hydroxylase deficiency, hyperprolactinaemia and androgen secreting tumours were excluded by appropriate testing [266].

Table 5.1. includes baseline characteristics. The subjects were obese (mean ± SD: BMI 33.71 ± 6) premenopausal women with a mean age of 26 (range: 20-38 years old). They had an increased waist-hip ratio (0.92 cm ± 0.05, range: 0.81- 1) and % of total body fat (47.8 ± 4.4, range: 40-51). The patients were normotensive, mildly hypercholesterolaemic (5.2 mmol/L ± 1) with mildly raised LDL levels (3.20 mmol/L ± 0.8).

5.3. Methods

Study design

This was a 24 weeks, open labelled trial divided in three phases: phase1 (6 weeks, pre-treatment), phase2 (3 months intervention phase) and phase3 (6 weeks, post-treatment).

This study followed the declaration of Helsinki guidelines and it was approved by the Hull and East Riding Local Research Ethics Committee. The clinical trial registration number is ISRCTN65353256. All subjects provided written and informed consent.

Intervention

All the subjects were on an unrestrictive diet at the beginning of the trial and were instructed not to modify their usual eating patterns during the pre-treatment phase. The patients received a combination of metformin (dose: 500mg three times/day) and a mildly hypocaloric diet (1500 kcal/day) during the intervention period. Dietary recommendations and a booklet produced by the British Heart Foundation[267] were provided that advised them about the daily portions of the different food groups required to achieve an intake of 1,500 kcal/day and also included examples of a day's eating plan. Compliance was monitored by counting the returned tablets and reviewing food diaries. In addition, dietary reinforcement was performed at each visit.

Study Measurements

Biometric parameters and blood samples to measure glucose and insulin to calculate HOMA-IR were obtained as described in section 2.1.2.1 and 2.1.2.2. The samples used in the biological variation were collected and processed as detailed in section 2.1.1.

5.4. Sample Size Calculation and statistical analysis

Sample Size Calculation

The sample size and number of repeated measures used were based on previous studies investigating biological variation in measured analytes[140, 211, 268].

Statistical analysis

Biovariability data was analysed as described in section 2.2.1.

The two treatment groups were compared using the paired t test for biochemical data and the Wilcoxon signed rank test for the clinical observations using SPSS version 15, and statistical significance was reached if $p<0.05$. Biochemical data that followed a non Gaussian distribution when tested using Kolmogorov-Smirnov test were also analysed using Wilcoxon signed rank test.

5.5. Results

Table 5.1 includes baseline characteristics and biological variation data. Compliance with treatment was >90% and 40% developed some gastrointestinal side effects (nausea, flatulence, and loose stools) when treated with metformin but these were short lived. All the patients completed the trial.

Effects on lipids and biological variation data

LDL levels obtained following 3 months treatment with metformin and a hypocaloric diet significantly improved when compared with baseline levels (mean ± SD, 3.2±0.8 versus 3.0±0.8, $p=0.03$).The intraindividual and interindividual variance contributed

19% and 79% to the total variance respectively. After 3 months of treatment, they contributed 15% and 83% to the total variance respectively.

The critical difference between two consecutive LDL samples was 1.05 mmol/L i.e. 33% of the mean value in biguanide naive PCOS patients and 0.87 mmol/L i.e. 29% of the mean value if those patients were treated with hypocaloric diet and metformin. The IoI was 0.49 and 0.42 before and after treatment respectively.

No significant changes were observed in mean values of total cholesterol, HDL or triglyceride levels and the biological variation in all these analytes was similar before and after treatment.

Effects on insulin resistance

There were no significant differences in glucose (p=0.42), fasting plasma insulin (p=0.15) or insulin resistance (HOMA-IR method, p=0.21) before and after treatment.

Other effects

Treatment with metformin and hypocaloric diet significantly reduced BMI by 4.2 % (p=0.008), systolic and diastolic BP by 9.9% and 8.7% respectively (p=0.02 and p=0.009) and waist-hip ratio by 1.8% (p=0.02). There was a trend to reduce total body fat by 2.9% although it did not reached statistical significance (p=0.06).

5.6. Discussion

This study shows that the biological variation of triglycerides, total, HDL and LDL cholesterol in obese patients with PCOS is similar to that reported in health by Ricos et al[145] although LDL and total cholesterol have slightly wider fluctuations (Table 5.2.). Furthermore, a combined treatment with a hypocaloric diet and metformin appears to have little effect in the intrinsic fluctuations of lipids levels. The clinical implication of these findings is that using a RCV derived from healthy subjects to monitor lipids levels before and after treatment in patients with PCOS would be reasonable. The low index of individuality found in these patients also indicates that population reference values are less useful determining if a given result is abnormal in an individual patient and observation of serial results might yield more information.

Treatment with hypocaloric diet and metformin significantly improved body mass index with a trend to reduced total body fat although the latter did not reach statistical significance. However, there was an improvement in central obesity as indicated by the reduction in waist hip ratio although it was insufficient to ameliorate insulin resistance in our cohort. To what extend these findings are related to a synergistic effect of both interventions or to each one individually needs to be further evaluated. Some insight can be gained from the Cochrane metaanalysis on metformin use in PCOS[269] and previous data from our group [270] that suggest that metformin alone is not associated with significant changes in BMI or waist-hip ratio. However, other authors report weight loss with metformin use thought to be related to diminished appetite and consequently, food intake reduction[271]. A low calorie diet intake (1200-1400kcal/day) reduces BMI, waist and hip circumference in PCOS and these changes are more pronounced after 6 months of calorie restriction [272-274]. However, if this hypocaloric diet is combined with metformin 850mg bd for 6 months the improvement in these parameters is more

marked [272] and this effect could be noticeable within 3 months as seen in our subjects. .If diet and metformin are continued for up to four years, a stable 8% weight loss can be achieved for that period of time [275].

With regards to the effects on mean lipid levels, we observed only a modest reduction in total LDL concentration. Limited amount of data are available in PCOS patients treated with a similar combination therapy but it appears to have little short term effect in patients with mildly elevated total cholesterol, LDL or triglyceride levels [276, 277]. A recent study looking at the long term effects of metformin and diet over 4 years reported that LDL levels improve by 6% -7% by the end of years 1, 2 and 3 and 11% at the end of year 4 suggesting that perhaps longer periods of therapeutic intervention are required if a significant change in lipids is to be obtained [275]. However, since the pattern of dyslipidaemia found in PCOS patients is associated with an increased small, dense atherogenic particles with a nearly normal or minimally elevated LDL[161, 164] this modest change might be misleading if there is a significant reduction in LDL III particles when obese PCOS are treated with a hypocaloric diet and metformin and to date, this remains unknown.

A surprising finding was that insulin resistance was not significantly modified by the addition of metformin to a hypoglycaemic diet especially when metformin administration appears to improve insulin sensitivity in PCOS as demonstrated by the euglycaemic hyperinsulinaemic clamp [278]. One possible explanation is that either the dose or the length of treatment with metformin were insufficient to cause an improvement in HOMA-IR, others have shown a higher dose of metformin (2550 mg/day) for 8 months was needed to make a difference[279].

In conclusion, PCOS has little significant impact on the intrinsic biological variation of serum lipids that remains within a similar range following treatment with a hypocaloric diet and metformin and therefore, RVC lipid values could be extrapolated from healthy subjects. This combination therapy improved blood pressure and central obesity without a significant effect in insulin resistance and only a modest effect on total LDL levels.

Table 5.1. A. Baseline characteristics of the subjects (means±SD)
B. Baseline characteristics of lipids (means±SD) and biological variation data

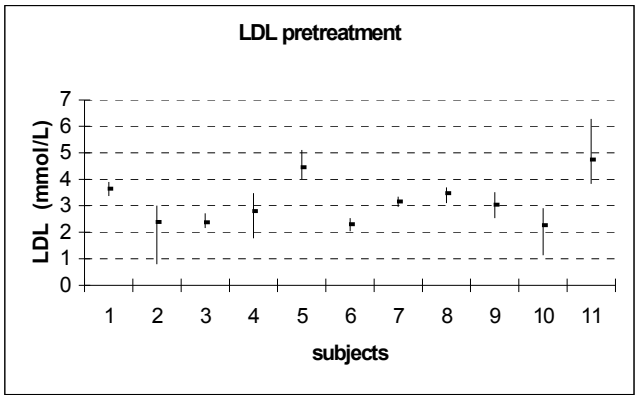
A	**Baseline (N=11)**					**After 3 months treatment with METFORMIN (N=11)**					**P value**
Age (years)	26.8±5.1										
BMI	33.71±6					32.5±6					p=0.008*
Waist_hip ratio (cm)	0.92±0,05					0.90±0.05					p=0.02*
Tanita (% fat)	47.8±4.4					46.8±4.4					p=0.06
BPsystolic (mmHg)	121±9.6					116±6					p=0.02*
BPdiastolic (mmHg)	71±3.9					68±3.5					p=0.009*
F. Insulin (μU/ml)	18.2±8.1					15±8.7					p= 0.15
F. Glucose (mmol/L)	4.9±0.5					4.7±0.5					p=0.42
HOMA I-R	3.9					3.1					p=0.21
B	mean±SD	CVa	CVi	CVg	IoI	mean±SD	CVa	CVi	CVg	IoI	
T.cholesterol (mmol/L)	5.2±1	2.4	8.4	19.8	0.42	5.01±0.9	2.1	8.4	16.9	0.49	p=0.10
LDL (mmol/L)	3.2±0.8	3.6	12.0	24.5	0.49	3±0.8	3.8	10.6	25.0	0.42	p=0.03*
TG (mmol/L)	1.9±1.2	3.8	19.1	61.2	0.31	1.75±1	3.7	21.5	59.4	0.36	p=0.24
HDL (mmol/L)	1.2±0.2	2.6	7.9	18.1	0.43	1.17±0.2	2.6	8.12	17.8	0.45	p=0.72

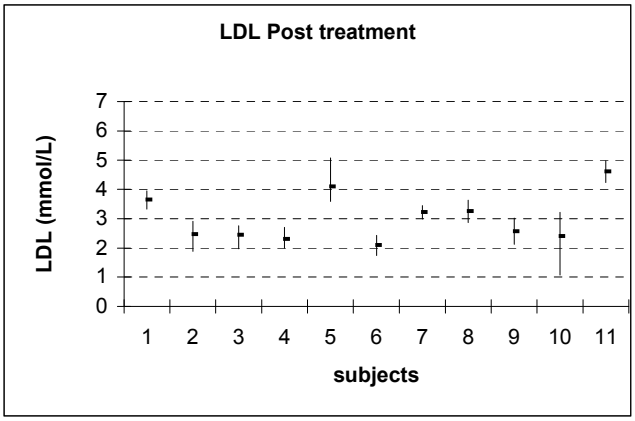
BMI: body mass index; BP: blood pressure; T.Cholesterol: total cholesterol; LDL: low density lipoprotein; TG: triglycerides; HDL: high density lipoprotein; F. Insulin: fasting insulin; F. Glucose: fasting glucose; SD: standard deviation; CVa: analytical coefficient of variation; CVi: within subject biological coefficient of variation; CVg: between subject coefficient of variation; IoI: index of Individuality.

Table 5.2. Within subject (CVi) and Between subject (CVg) biological variation in health and PCOS. Data in health(1): extracted from Ricos database [145].

	Health (1)	PCOS	Health (1)	PCOS
	CVi (%)	CVi (%)	CVg(%)	CVg(%)
Total cholesterol	5.4	8.4	15.2	19.8
LDL	8.3	12.0	25.7	24.5
HDL	7.1	7.9	19.7	18.1
TG	21.0	19.1	37.2	61.2

Figure 5.1. Means and range of values (unadjusted for analytical variation) for LDL before and after treatment.





Chapter 6

Effects of low dose of growth hormone replacement on peripheral muscle and cardiovascular risk factors in severe growth hormone deficiency

6. 1. Introduction

Patients with hypopituitarism have reduced life expectancy than the anticipated in the general population, mainly attributed to cerebrovascular and cardiovascular disease [64]. The exact mechanism responsible for this excess mortality remains uncertain but it has been postulated that growth hormone deficiency maybe contributory due to its association with a clustering of cardiovascular risk factors such as central adiposity, reduced lean mass, insulin resistance, disturbances in lipid and lipoprotein metabolism, premature atherosclerosis, left ventricular dysfunction and reduced ventricular mass[74, 280-282].

An additional risk factor for cardiovascular disease found in AGHD is a low level of aerobic capacity and this together with the above abnormalities could contribute to their increased fatigue and impaired quality of life. One way to objectively measure cardiovascular fitness and aerobic power is to calculate the peak oxygen consumption (VO2max) that represents the maximum capacity of an individual's body to use oxygen during incremental exercise. Studies assessing VO2max in AGDH have reported a low aerobic capacity with diminished VO2max up to 28% [283, 284] compared with predicted values [285]. This is relevant in these group of patients since submaximal aerobic capacity in subjects without documented coronary artery disease has been identified as an independent risk factor for all cause and cardiovascular mortality [286].Therefore, measures of aerobic performance could be used to objectively assess the functional response and fatigue following GH replacement.

Altered muscle metabolism [287] and mitochondrial dysfunction have been proposed as possible factors associated with low aerobic capacity [288]. The mitochondria play an essential role in the regulation of oxidative cell metabolism and might contribute to cardiac dysfunction and myocyte injury via loss of metabolic energy. GH receptors have

been identified in this organelle [289] through which GH could increase mitochondrial oxidative capacity in cardiac and skeletal muscle following its administration as seen in healthy subjects who received a GH infusion for 14 days [290] and in older women when it is combined with aerobic exercise [291] and therefore, this could be theoretically a mechanism by which GH replacement could improve muscle function in patients with AGHD. One of the crucial antioxidant enzymes involved in oxidative cell metabolism is the succinate dehydrogenase (SDH). It is embedded in the inner mitochondrial membrane as a part of a multiprotein complex (complex II) and catalyses the oxidation of succinate to fumarate in the Krebs cycle and feeds electrons to the respiratory chain ubiquinone pool[292]. Succinate dehydrogenase deficiency is associated with mitochondrial disorders which mainly affect organs dependent on oxidative metabolism such as brain, skeletal and cardiac muscle [293] but it is unknown if AGHD is associated with muscle mitochondrial dysfunction. Therefore, the aim of this study was to evaluate the effects of low dose and short term administration of GH on peripheral muscle oxidative capacity, exercise capacity, cardiovascular risk markers and quality of life.

6.2. Subjects

Seventeen patients (10 males, 7 females, mean age 48 ±14 years, range 19-74 years) with hypopituitarism resulting from pituitary tumours that were treated with surgery, radiotherapy or both were recruited. Severe GH deficiency was confirmed by a peak GH response to insulin induced hypoglycaemia of less than 9mU/L (3ng/ml). The average time from diagnosis to inclusion in the trial was 33 months. Subjects received stable hormone replacement doses for thyroid, adrenal and testosterone deficiencies throughout the study and none of them had been previously treated with rGH. During the study period they were instructed to follow their usual diet and activity. Subject characteristics are highlighted in Table 6.1. All subjects provided written consent.

6.3. Methods

Design and Intervention

This was a six month, double blind, randomized, cross-over, placebo controlled trial of subcutaneous recombinant GH (Lilly rGH®, 0.4mg/day for 12 weeks) versus placebo (sterile diluent containing glycerol and m-cresol, for 12 weeks). Randomisation was performed by the pharmaceutical department with a random number generating table. Daily rGH or placebo injections were prepared by a pharmacist who was independent from the trial, and patients were randomised to either placebo or rGH for 3 months before being crossed over to the second arm of the study. To maintain the blinding of the study it was not possible to escalate the GH dose. The study followed the declaration of Helsinki guidelines and was approved by our local ethics committee. The trial registration number is ISRCTN94165486.

Study measurements

The following measurements were obtained at baseline, 3 and 6 months using standard methods described in section 2.1.2.:

- Baseline observations included body mass index (BMI), systolic and diastolic blood pressure, waist-hip ratio and total body fat. Cardiovascular risk markers such as total cholesterol, triglycerides, glucose and HbA1c together with IGF1 were also measured. QoL-AGHDA questionnaire was used to assess their quality of life.

- Quadriceps muscle biopsies were taken to assess changes in succinate dehydrogenase activity that was measured as a surrogate marker of muscle mitochondrial function at each clinical visit.

- Exercise capacity was assessed calculating the maximal oxygen consumption (VO2max) following cardiopulmonary exercise test, using a modified Bruce protocol. During the test, patients wore a tightly fitting facemask to which was connected a capnograph and a sample tube enabling online measurement of ventilation and metabolic gas exchange. A respiratory exchange ratio (RER) >1 was taken to suffest a maximal effort together with an attainment of at least 85% of maximal heart rate.

Table 6.1.: Baseline characteristics of the study population.

Patient	Sex	Age	Diagnosis	Time from diagnosis to entry into trial (months)	Therapy
1	M	51	Post Sx: Pituitary adenoma	50	-
2	M	64	Post Sx: Microprolactinoma	13	A
3	F	48	Pituitary adenoma	22	A, B
4	M	46	Post Sx: Pituitary macroadenoma	23	A, E
5	F	54	Post Sx: Pituitary macroadenoma	9	A, B
6	F	28	Post Pituitary Sx	3	B
7	F	19	Cystic prolactinoma	59	C
8	M	50	Post Sx: Acromegaly	25	-
9	F	58	Post Sx: Pituitary macroadenoma	40	B
10	F	36	Post Sx: Prolactinoma	2	B
11	M	30	Post Sx: Craniopharyngioma	27	A, B, F
12	F	44	Empty sella	20	C
13	M	44	Post Sx: Prolactinoma	27	A, D, E
14	M	69	Post Sx & RTX Pituitary adenoma	145	A, B, E
15	M	62	Pituitary macroadenoma	22	A, E
16	M	49	Cranial surgery	68	A, B, E
17	M	74	Post Sx: Pituitary Macroadenoma	18	

Sx: surgery; A: thyroxine; B: hydrocortisone; C: quinagolide; D: carbegoline; E: sustanon; F: human chorionic gonadotrophin.

Compliance was checked and reinforced at each visit following randomization. It was monitored based on counting the returned empty vials of the study medication. The patients were instructed not to modify their usual diet or exercise load.

6.4. Sample size calculation and statistical analysis

Sample size calculation

A sample of 16 patients was needed to detect with a probability of 80% and using a two sided 5% significance level, a 17% true difference in VO2max between treatments (assuming a common SD of 16). The 17% difference in VO2max following rGH treatment is based on the improvement observed in Cuneo et al study [287].

Statistical analysis

Mean changes obtained at the end of rGH treatment were compared with those at the end of the placebo phase, using the paired Student's t test. The data was normally distributed when tested using Kolmogorov-Smirnov test. Adjusting for period effect was carried out by the Hills-Armitage method [294]. Statistical analysis was performed using the Stata Statistical Computer package (StataCorp, 2007) and the results were considered statistically significant if the two-tailed p value was <0.05.

6.5. Results

All the patients completed the study. Baseline characteristics of the subjects are included in Table 6.1. All subjects were compliant with rGH subcutaneous injections and they did not have any significant side effects other than transitory local discomfort following muscle biopsies.

Effects on muscle oxidative capacity

Succinate dehydrogenase levels (SDH) increased by a 2.7 fold after 3 months therapy with rGH when compared with baseline (0.08 ± 0.02 versus 0.03± 0.01) (Figure 6.1.). However, this change did not differ to the response of SDH in the placebo phase (p=0.13) and nor was there differences in SDH between sexes.

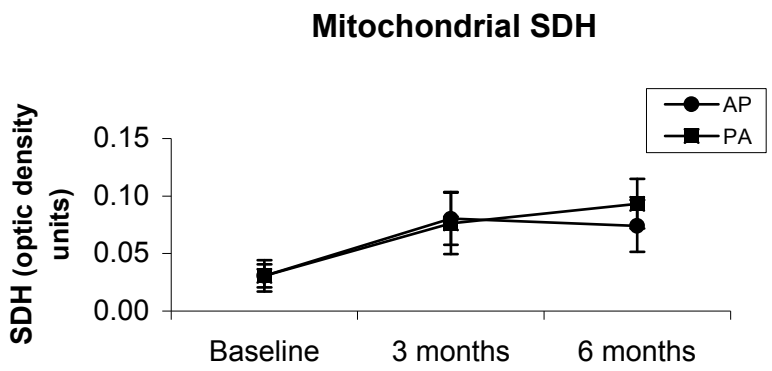
Effects on quality of life

Following 3 months of rGH therapy, the mean change in quality of life scores was no significant when compared with placebo therapy (p=0.38) and the SDH increase did not relate to Qol-AGHDA scores.

Effects on exercise capacity

No significant effects in peak oxygen consumption (peak VO2), slope of the relation between ventilation and carbon dioxide production (VE/VCO2), anaerobic threshold (AT), respiratory exchange ratio (RER), exercise time or pulse at maximal exercise were noticeable (Table 6.2.).

Figure 6.1. A schematic representation of the changes seen in mitochondrial SDH.



AP: active phase followed by placebo phase PA: placebo phase followed by active phase

Effects on IGF1 and cardiovascular markers.

IGF1 levels obtained after rGH replacement were significantly higher and within the reference range than those seen in the placebo arm (mean±SD: 189±71 microgr/L versus 121±50 microgr/L, $p<0.0001$). Total body fat was significantly reduced by 3.2% following rGH administration (36.2±10.4 versus 37.6±10.7, $p=0.05$). We also observed an increase in systolic blood pressure that was significantly higher in the rGH treatment arm (136 mmHg ±16 versus 129 mmHg±15, $p=0.02$).

No significant changes were observed in BMI, waist/hip ratio, diastolic blood pressure, blood glucose, HbA1c, total cholesterol or triglycerides.

Table 6.2. Effects of rGH and placebo on exercise capacity.

	Baseline	**Placebo**	**Active**	**p value**
Peak VO_2 (ml/kg. min)	26.2 ±7.6	24.3± 6.2	24.1± 7.5	0.51
VE/VCO_2	27.6 ±3.9	26.9 ±4.5	28.3 ±5.65	0.86
AT (l/min)	15.4 ±4.2	15.2 ±4.8	15.6 ±5.7	0.95
Peak RER	1.11 ±0.07	1.08 ±0.14	1.06 ±0.14	0.06
Exercise time (min)	11.5±4.5	12.1 ±4.1	11 ±4	0.64
Pulse at maximal exercise (beats/min)	152± 30	154 ±30	147 ±26	0.9

Group mean scores after GH therapy period, placebo period GH therapy. Values are expressed as a mean (+/-) standard deviation. VO_2: peak oxygen consumption; VE/VCO_2: slope of the relation between ventilation and carbon dioxide production; AT: anaerobic threshold; RER: respiratory exchange ratio.

Table 6.3. Effects of rGH and placebo on IGF1, SDH, cardiovascular markers and quality of life.

	Baseline	Placebo (3 months)	rGH (3 months)	Paired difference (95% CI)	p value
Age (years, range)	48 ±14 (19-74)				
Tanita (%)	37.4 ± 9.8	37.6± 10.7	36.2±10.4	-1.5 (-2.9,0.0)	0.05*
BMI (Kg/m^2)	33.9 ± 5.8	34.1±6.18	33.9± 6.2	-0.14 (-0.55,0.27)	0.48
W-H ratio	0.97 ± 0.23	0.94±0.07	0.93±0.05	-0.01 (-0.04,0.18)	0.41
SBP (mmHg)	134 ± 14	129±15	136±16	6.8 (1.2,12.4)	0.02*
DBP (mmHg)	83 ± 10.8	77±8.6	79±13	2.3 (-3.4,7.9)	0.40
Glucose(mmol/L)	5.5 ± 0.7	5.1±1.05	5.1±0.9	0.0(-0.41,0.41)	0.99
HbA1c (%)	6.08 ± 1.02	5.6±0.4	5.8±0.8	0.2 (-0.07,0.49)	0.12
IGF1 ug/L	115.5 ± 47	121±50	189±71	67.9 (40,94)	<0.0001**
T.Chol. (mmol/L)	5.5 ± 0.7	5.6±0.8	5.4±0.7	-0.28(-0.5,0.02)	0.06
TG (mmol/L)	1.6 ± 1.1	1.5±0.9	1.4±0.7	-0.2 (-0.4,0.04)	0.09
SDH (OD)	0.03 ± 0.01	0.07±0.02	0.08±0.02	0.01 (0.0,0.3)	0.13
QoL-AGHDA	15 ± 5	16 ± 6	15 ± 8	-0.9 (-3.1,1.2)	0.38

Values represent mean ± SD during the cross over trial (adjusted for period effect).
Abbreviations: SD: standard deviation; BMI: body mass index; W-H: waist hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; IGF1: Insulin Growth factor 1; T.Chol: total cholesterol; TG: triglycerides; SDH: succinate dehydrogenase; OD units: optic density; QoL-AGHDA: quality of life questionnaire.

6.6. Discussion

Mitochondrial oxidative activity in peripheral muscle was enhanced in our patients irrespectively of the treatment received. One possible explanation is that the self reported increase in the usual daily activities rather than rGH replacement perse, was responsible for the increment in SDH levels. Similar findings have been observed in

young, healthy adults who showed a positive association between SDH levels and their habitual physical activity in daily life independently of the intensity of the activities performed [295]. SDH is also influenced by the level of physical training i.e. lower in subjects who lead a sedentary life as compared to those who exercise [296] either intermittently, continuously or following endurance training [297, 298]. Furthermore, the introduction of moderate exercise training in patients with AGHD can mimic the effects of GH replacement on physical capacity [299].

However, this enhanced mitochondrial oxidative activity did not translate in a significant improvement in the subjects' quality of life nor their aerobic capacity. Changes in the latter appear to be influenced by the length of rGH replacement as concluded in a recent metaanalysis reporting positive effects on overall exercise capacity in patients with AGHD following 6 to 18 months of rGH replacement [300]. In this context, similar increases in VO2max can be achieved either individualising rGH dose or using higher doses based on body weight [301]. Shorter treatment periods lasting between 3 to 6 months are not sufficient to significantly improve the maximum oxygen uptake (VO2max) in accord with our results [299, 302, 303].

Although it is currently recommended to treat AGHD with low dose of rGH to minimise side effects [304], the overall cardiovascular benefit achieved with this approach is less clear especially in long term outcomes. Modest reductions of LDL, total cholesterol and fat mass seem to persist with smaller doses with a dose dependant effect on fat (i.e. higher fat losses on higher doses of rGH) [305]. In our cohort, three months replacement with 0.4mg/day of rGH normalised IGF1 levels and reduced the % of body fat without impairing glycaemic control or diastolic blood pressure. However, this dose and/or the length of treatment were inadequate to improve central obesity and

hypercholesterolaemia. It has been suggested that patients with the most adverse lipid profile benefit the most following rGH replacement [306] and this could explain the lack of response in our patients. A slight but significant increase in systolic blood pressure was also noticed, possibly due to fluid retention.

The length of rGH replacement also appears to have an impact on cardiovascular risk factors associated with AGHD although there is some variability in the data reported. Modification of total cholesterol and LDL levels and/or body composition may occur as early as two months [307] with the most rate of change between 6 months and a year [308, 309] [305, 310], and continue up to 10 years, particularly in the case of LDL[306] [309, 311].

Our study has a number of limitations. All of the patients normalised their IGF1 levels on the single dose of rGH given, but because of the design of the study it was not possible to further titrate the rGH dose higher. Secondly, the length of GH deficiency may have been too short to have allowed more of a GH deficient spectrum to develop.

In conclusion, a fixed, low dose of rGH administered in a double blind controlled cross over trial for 3 months in AGHD secondary to hypopituitarism favourably alters body fat mass and levels of IGF1 without a significant effect in cardiovascular risk factors or exercise capacity. The increment of mitochondrial SDH observed in both treatments from baseline precedes changes in aerobic capacity and it is possibly related to a gentle increase in daily activities though this did not correlate with an improvement in QoL.

Chapter 7

Biological Variation of N-Terminal pro-Brain Natriuretic peptide in postmenopausal women with Type 2 Diabetes

7.1. Introduction

Brain natriuretic peptide (BNP) and N terminal probrain natriuretic peptide (NT-proBNP) are useful both as prognostic biomarkers in patients with heart failure and screening tools to rule out heart failure in patients presenting with acute dyspnoea as discussed in chapter 1.4. Their utility could be especially advantageous in postmenopausal women since they lose their cardiovascular protection when menopause is attained placing them at higher risk of developing cardiovascular disease and heart failure [312] and in type 2 diabetes since the incidence of heart failure is high, it is associated with a worse prognosis and routine screening and follow up with echocardiography is time consuming, expensive and not readily accessible. Epshteyn et al showed that BNP levels could be useful to screen diabetic patients for the presence or absence of left ventricular dysfunction [199].

However, their value to monitor disease progression and/or response to treatment has been questioned due to their marked biological variation reported both in health [313] and in chronic heart failure[314, 315]. There are also differences in the intrinsic variability between natriuretic peptides, being NT-proBNP more stable than BNP, likely related to their physiological release and clearance. NT-proBNP has a longer half life than BNP (between 1 and 2 hours as opposite to 20 minutes) and greater reliance on glomerular filtration because it lacks a clearance receptor that metabolizes it [316]. Hence, NT-proBNP has been proposed as screening method in patients with diabetes to identify early left ventricular dysfunction and possibly to monitor disease progression and/or response to treatment. It has been noticed that NT-proBNP levels appear to be raised in type 2 diabetes without overt cardiovascular disease[195] and without microalbuminuria[196] when compared to those of healthy subjects and therefore, it is

possible that the biological variation of NT-proBNP may differ in type 2 diabetes as a result of the increased cardiovascular risk markers seen in this condition.

This study was undertaken to establish whether NT-proBNP levels in type 2 diabetes remain within narrow biological limits or vary more widely over time when compared with the general population. This information is used to derive the critical difference values for NT-proBNP in postmenopausal type 2 diabetic women that are essential for the accurate interpretation of any change between serial NT-proBNP measurements.

7.2. Subjects

Twelve obese (body mass index greater than 30) Caucasian postmenopausal women without known heart disease and diet-controlled type 2 diabetes diagnosed according to WHO criteria[242] were weight-matched with eleven healthy Caucasian postmenopausal women with normal fasting blood glucose (fasting blood glucose range: 3.9-5.5mmol/L). Clinical findings and echocardiography excluded heart failure.

Women were considered postmenopausal if they had amenorrhoea for >1 year and FSH levels >20 IU/l. Exclusion criteria included secondary causes of hyperglycaemia, oestrogen or antihypertensive therapy, untreated hypothyroidism, history of drug, alcohol abuse or smoking and nephropathy.

7. 3. Methods

Sample collection, laboratory methods and reagents used are as described in section 2.1.1.

7. 4. Statistical analysis

Statistical analysis is described in section 2.2.1. The mean values of NT-proBNP were found to be non Gaussian (by Kolmogorov test) in both subgroups and therefore data was log transformed before analysis with SPSS. Statistical significance was reached if $p<0.05$.

7.5. Results

All the patients completed the study. Table 7.1 summarizes the clinical and biochemical characteristics of the subjects.

Table 7.1. Clinical and biochemical features of study subjects.

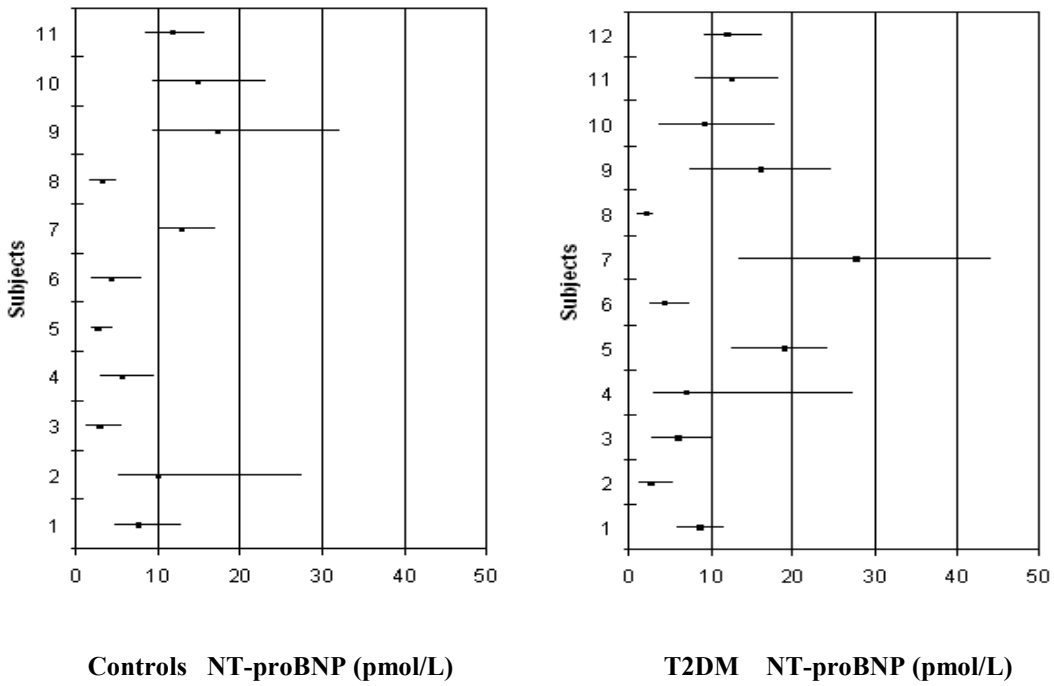
Parameter	**Type 2 Diabetes (n=12)**	**Controls (n=11)**	**P value**
Age (years)	61.7±7.0	56.2±6.1	0.06
Weight (Kg)	77.5±8.1	79.9±13.4	0.60
BMI (Kg/m^2)	31.1±3.3	32.4±5.3	0.49
Fasting glucose (mmol/L)	7.6±2.3	5.0±0.5	<0.001*
NT-proBNP(pmol/L)	10.7±8.5	8.49±6.0	0.42

Data expressed as mean± SD. BMI: body mass index.

The mean NT-proBNP level was similar in both groups (Type 2 DM, 10.7pmol/L ± 8.5 versus control, 8.49±6.0 pmol/L, p=0.42). In the control group, the analytical coefficient of variation (CVa) contributed 7.9% of the total variance. After accounting for the analytical variation, the intraindividual (CVi) and interindividual (CVg) coefficients of variation contributed 21.4% and 29% respectively. The critical difference for sequential NT-proBNP values was between –67% and +204% and the index of individuality was 0.74.

In the group with type 2 diabetes, the CVa contributed 7.07% of the total variance and after adjusting for the analytical variance, the CVi was 18.3% and CVg contributed 26.6%. The critical difference was between –70% and +236% and the index of individuality was 0.69.

Figure 7.1. Means and range of values for NT-proBNP (unadjusted for analytical variation) in controls and subjects with type 2 diabetes (T2DM).



7.6. Discussion

This is the first study to show that the absolute NT-proBNP value and its biological variation are as wide in postmenopausal women with diet controlled type 2 diabetes as in healthy postmenopausal women, suggesting that type 2 diabetes perse, at least in the early stages, does not significantly alter NT-proBNP levels in this group of patients. This wide natural variation needs to be accounted for the interpretation of serial NT-proBNP levels in postmenopausal women with and without type 2 diabetes and the critical difference values provided here could be used to assess the significance of any change between two consecutive measurements. These values suggests that small changes between two single measurements of NT-proBNP in the same subject with type 2 diabetes maybe more a consequence of the biological variation rather than the reflection of therapeutic interventions or disease progression because they must rise by more than 236% or fall by greater than 70% before any critical difference can be assured.

The wide spectrum of severity in heart failure and the expense and limited access to echocardiography, have prompted consideration of NT-proBNP as screening tool, particularly in high risk subgroups such as patients with type 2 diabetes who can be asymptomatic and yet have underlying left ventricular dysfunction[317]. If NT-proBNP was to be considered as a suitable screening test in postmenopausal women with type 2 diabetes, its intraindividual variation should be similar to the variation of the population as a whole. This would allow detecting a rise of NT-proBNP levels, in the majority of the subjects, above 2 or 3 SD as they will then lie above the upper limit of normal. This is estimated with the "index of individuality" (IoI) that assess the suitability of a biological test for disease screening when the separation between disease and health is not marked [137, 138]. A test with an IoI of ≥1.4 has the most potential for screening, while

one of <0.6 will be less valuable. The low index of individuality in postmenopausal women with/without type 2 diabetes suggests that when used in isolation, NT-proBNP has little potential to detect the presence of early heart failure, and therefore, more traditional and well validated methods such as echocardiography in addition to the clinical picture are preferable.

Previous studies have evaluated the biological variation of NT-proBNP in health and in stable chronic cardiac failure. Although NT-proBNP secretion does not appear to follow a circadian rhythm [318], it has a substantial intraindividual variation in health with suggested reference change values ranging between 26% [313] and 90% [319]. In chronic heart failure, this high intraindividual variation remains and changes in values from week to week of up to 98% are required to indicate a change in clinical status[314, 315]. The cohorts included in these studies are mixed, with a variable male/female ratio and age range and may partially explain the different percentage of change required. It is known that NT-proBNP is affected by gender, age and obesity although the exact physiological mechanisms responsible for this remain unclear. Our postmenopausal subjects were age, gender and BMI matched to avoid these confounding factors and indeed, the biological variation was wide with large reference change values both in health and in type 2 diabetes.

Asakawa et al reported that BNP levels in normotensive diabetics without clinical heart disease were not affected by microalbuminuria, peripheral vascular disease or cerebrovascular disease and therefore, raised levels should prompt to investigate for cardiac diasease[320].

In conclusion, the biological variability for NT-proBNP is as wide in healthy postmenopausal women as in those with diet controlled type 2 diabetes indicating that

type 2 diabetes perse does not appear to modify the intraindividual variability. NT-proBNP has a high degree of individuality and therefore, population based reference intervals have limited value screening abnormal results. The reference change values reported here could be used to identify changes in serial NT-proBNP measurements due to pathological changes rather than related to its biological variation.

Chapter 8

Summary discussion

This thesis has examined three separate but related aspects in patients who are at risk of developing cardiovascular disease. Firstly, the impact of social deprivation in lipid measurement and lipid lowering agents prescriptions. Secondly, the role of different interventions such as isoflavones supplementation, a combination therapy with metformin and hypocaloric diet and rGH replacement on cardiovascular risk factors and/or glycaemic control and insulin resistance in different pathological conditions. Additionally, the effects of rGH on exercise capacity have been evaluated. Finally, the biological variation of NT-proBNP in postmenopausal women with type 2 diabetes and the biological variation of lipids in both untreated and treated PCOS patients.

A literature review focusing on cardiovascular risk factors identified in socially deprived areas, type 2 diabetes mellitus, PCOS and adult GH deficiency has been presented with a particular emphasis on dyslipidaemia in both PCOS and type 2 diabetes and insulin resistance in type 2 diabetes. Studies that have contributed to gain knowledge in this field point that these three pathological conditions share a common ground of cardiovascular risk factors. Interventions aimed to modify those in patients with type 2 diabetes improve the poor cardiovascular outcomes associated with this condition[29-35]. However, it remains unknown if targeting these cardiovascular risk factors in PCOS or AGHD could have an impact in cardiovascular end points since these are not well described in these groups of patients. Therefore, long term, prospective, studies specifically designed to evaluate the effects of risk factors modification on cardiovascular outcomes in PCOS and AGHD are required.

The influence of social deprivation on lipid measurements and lipid lowering agents prescriptions described in this thesis points towards discordance between the higher need to reduce cardiovascular risk factors in impoverish local areas and current practice. Individual behaviours, poor awareness about the relevance of reducing risk factors to improve cardiovascular health, inequality in geographical distribution of GP practices and mismatched resources are possible contributors to these findings.

The General Medical Service (GMS) contract of 1990 introduced extra capitated payments for each patient who lived in a deprived electoral ward (based on the Jarman index) to compensate for the additional workload incurred by GPs working in deprived areas. Therefore, theoretically those wards with higher level of deprivation should have been funded appropriately. However, the GMS contract was criticised because the Jarman index was based on census data only updated every 10 years and the score at which deprivation payment was awarded was higher than intended[321]. It would be interesting to re-evaluate my observations following the introduction of the new GMC contract in 2004[322]. This new contract has replaced the Jarman index for the Carr-Hill formula that adjusts the global sum received by a practice. This formula takes into consideration differences in the population served by a given GP surgery i.e. sex, age, morbidity, mortality, list turnover, rurality and the cost of living in some areas. Additional payments are rewarded if practices achieve certain clinical quality indicators (QOF points), for example % of total cholesterol measurements and % of total cholesterol levels <5mmol/L in patients with coronary heart disease or diabetes in the previous 15 months (QOF: DM16, DM17, CHD7 and CHD8). Theoretically, this would encourage general practitioners to measure lipid profiles and to treat accordingly if elevated, especially in areas of deprivation where the prevalence of diabetes and cardiovascular disease is high.

The use of phytoestrogens to improve an unfavourable cardiovascular profile in postmenopausal women with type 2 diabetes seems an attractive option in view of the safety concerns raised in prospective, randomised trials[84, 85] following HRT treatment.

Although a combination of soy protein and isoflavones has been shown to have beneficial effects in these group of women, it remains unclear which specific component is responsible. This question has been addressed in my study: a combination of three main isoflavones on their own have very little impact modifying cardiovascular risk factors, finding in line with current literature, and therefore they would be unsuitable as a therapeutic alternative to more conventional treatments used to improve lipid and glycaemic control.

It is unclear why isoflavones in association with soy protein should be more effective than isoflavones alone. There are three possibilities that need further evaluation. Firstly, that there is a matrix effect of the isoflavones in the soy protein: isoflavone dose response studies are needed (with the amount of soy protein as a constant) to clarify that question. A second possibility is that soy protein is the active component rather than isoflavones: studies with isoflavone free soy protein (not currently available) would be required to address this. Thirdly, there is a synergistic effect between both soy protein and isoflavones and therefore both of them need to be administered together.

However, even the combination of isoflavones and soy protein only has a modest effect improving lipid parameters, possibly dependant on the initial concentration of cholesterol levels and menopausal status and only following supplementation with 50g of soy protein that represents approximately half of the recommended daily protein intake. Although a diet rich in soy protein could be beneficial from the cardiovascular

point of view due to the low content in saturated fats [257], current evidence does not support its use as an alternative lipid lowering agent.

PCOS is a common disorder seen in both gynaecology and endocrinology clinics due to its fertility and metabolic abnormalities. The presence of central adiposity has been described not only in the classic, overweight PCOS patient but also in the thin subgroup and this contributes to increase the risk of insulin resistance, and consequently, lipids abnormalities. Diet modification and insulin sensitizing agents, such as metformin, could improve central obesity and insulin sensitivity following short term administration, hypothesis explored in my third study. A combination of both appears to reduce BMI and central obesity following three months treatment with a tendency to diminish total body fat. In clinical practice, especially in overweight PCOS patients, lifestyle modification with emphasis on the need for healthy diet/calorie restriction and regular aerobic exercise is an essential yet frequently overlooked treatment due to compliance issues. The addition of metformin in this group of patients could be advantageous as it appears that metformin and hypocaloric diet have a synergistic effect on body weight. However, if insulin resistance were to be improved with this combination therapy it is likely that higher doses of metformin, administered for longer periods of time would be necessary, in view of the lack of response with 1.5 g of metformin a day. Likewise, I observed very little effect on lipid levels or their biological variation, and only total LDL improved slightly. However, a normal or marginally raised LDL might be misleading since there are different atherogenic LDL phenotypes. The predominance of the small, dense LDL (LDL-III) or very small, dense LDL (LDL-IV) subfractions have been accepted as emerging CV risk factor[147]. Patients with PCOS have a higher proportion of LDL-III and IV relative to controls [161, 164] and therefore, they could be at risk of subclinical endothelial damage. Metformin appears to

reduce the subfraction LDL-III[323] in patients with type 2 diabetes with very modest effect in their total LDL cholesterol [324] but to date, this remains unknown in PCOS, and further studies to clarify this are required. To what extend hyperlipidaemia is secondary to the central obesity found in these patients or secondary to PCOS perse remains also unclear and needs addressing. Furthermore, there is limited data available to determine the relative risk of suffering a cardiovascular event in patients with well established PCOS, particularly in women who transition to their postmenopausal state and therefore, long term prospective epidemiological trials are needed.

AGHD is associated with reduced aerobic capacity, a cardiovascular risk factor that can impair quality of life and contribute to the fatigue commonly found in this condition. It has been postulated that mitochondrial dysfunction could play a role in this aerobic dysfunction and this has been explored in chapter six. Short term, low dose of rGH normalised IGF1 levels and reduced total body fat, however, it did not appear to improve aerobic capacity or quality of life, and the changes in enzimatic succinate dehydrogenase activity observed are more likely to be related to gentle changes in physical activity rather than GH replacement. The overall cardiovascular benefit observed in my study was very modest, possibly related to the dose and length of therapy since positive effects on lean and fat body mass, LDL, total cholesterol and diastolic blood pressure have been described, sometimes with a reduction of insulin sensitivity[305]. Therefore, longer, prospective studies with clinical end points are warranted to determine the global cardiovascular benefit following rGH replacement. In this study, I focused on a single measure of antioxidant mitochondrial capacity, SDH but it would be interesting to determine the potential effects of rGH supplementation on other antioxidants/enzymes such as glutathione, catalase and superoxide dismutase.

The prognosis of heart failure in patients with type 2 diabetes is poor[325] and if coronary heart disease is present, type 2 diabetes is an independent factor for the progression of heart failure[179]. Therefore, the use of B type cardiac natriuretic peptides as an alternative to echocardiography to screen and diagnose early heart failure in type 2 diabetes could be advantageous due to its simplicity and comparative low cost. Current general guidelines advice to restrict the use of BNP or NT-proBNP to acute or chronic settings to exclude the diagnosis of heart failure if there is uncertainty in the clinical presentation and it is not recommended to screen large, asymptomatic cohorts of population nor replace echocardiography [326]. Another potential application would be to monitor response to therapy but this remains controversial in view to their marked biological variation and the possibility that type 2 diabetes could raise NT-proBNP levels in patients without heart failure [195, 199] hypothesis explored in my fourth study. Based on the findings presented, type 2 diabetes per se does not appear to significantly influence the marked biological variation of NT-proBNP in postmenopausal women and therefore, current guidelines on the use of natriuretic peptides could be extrapolated.

The critical difference values obtained in my study could be used to titrate or monitor response to therapy although the change required in between samples is wide and this might limit its utility. Nevertheless, recent prospective randomised trials in patients with symptomatic heart failure (approximately 19% with diabetes) suggests that intensive management guided by serial levels of NT-proBNP improves mortality at 1 year and it is associated with lower mortality year at 3 years, especially in those <75 year old [327, 328]. Similar results were obtained measuring BNP in patients with established heart failure at 15 months mainly due to adjustment of ACEI and β blocker doses[329]. Therefore, NT-proBNP could be potentially used to monitor response but further

research is required to clarity the frequency of testing needed and the "target" NT-proBNP levels to be achieved.

The results obtained from the studies presented in this thesis add on information to further our understanding on how interventions and novel therapies such as isoflavones can influence the management and/or treatment of conditions associated with increased cardiovascular risk. Large scale studies with cardiovascular end points will determine if the cardiovascular risk factors associated with such as PCOS or AGHD require an early and aggressive treatment to prevent cardiovascular events.

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