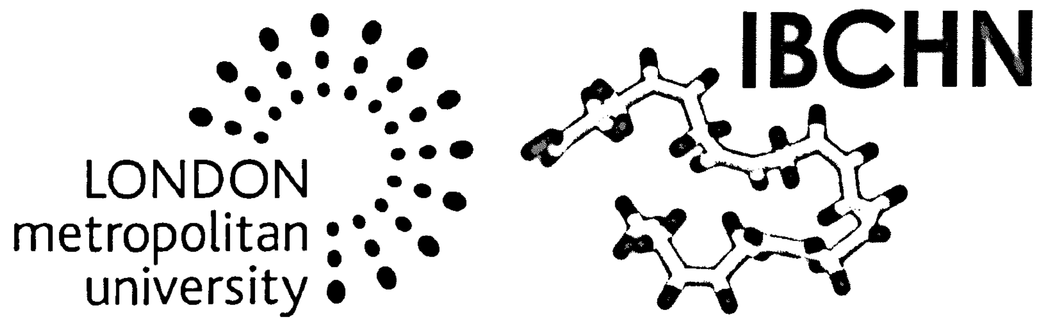

The influence of dietary fatty acids and antioxidant micronutrients on plasma, red blood cell and platelet fatty acids in healthy men and women

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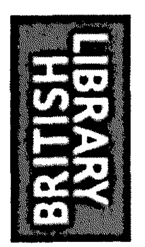

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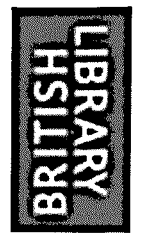

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This thesis is dedicated with all my love to my husband Jim - I couldn't have done it without you xxx

List of Publications Associated with PhD

Manuscripts

- Erythrocyte phospholipid molecular species and fatty acids of Down syndrome children compared to non-affected siblings (2011) Allain A Bueno A, Brand A, Neville M, Lehane C, Brierley N, Crawford MA (In press)
- Geppert J, Min Y, Neville M, et al., (2010) Gender-specific fatty acid profiles in platelet phosphatidyl-choline and -ethanolamine. Prostaglandins Leukot Essent Fatty Acids. 82(1):51-6. (Study 3)
- Manuscript peer-reviewed for the Journal of Human Nutrition and Dietetics: Neville M, Geppert J, Min Y, Crawford M, Ghebremeskel K, (2010) Dietary Fat Intake, Body Composition and Blood Lipids of University Men and Women. (Study 1)
- Manuscript currently being prepared for the British Journal of Nutrition: Neville M, Geppert J, Lowy C, Min Y, Crawford M and Ghebremeskel K (2010) Comparison of fatty acids in diet, plasma and red blood membranes of healthy men and women. (Study 2)

Abstracts

- Neville M, Geppert J, Min Y, Ghebremeskel K (2009) Dietary omega 9 and omega 3 fat, body composition and lipid parameters in healthy Caucasian men and women. ESPEN
- Geppert J, Neville M, Min Y, Lowy C, Ghebremeskel K, Crawford M (2008) Does gender influence polyunsaturated fatty acid composition of mononuclear cells and platelets? 8th Biennial International Scientific Meeting of ISSFAL, Kansas City, Missouri (USA), May 17-22.

- Neville M, Geppert J, Min Y, Lowy C, Ghebremeskel K, Crawford M (2008) Influence of dietary lipids on plasma fatty acid composition in men and women. 8th Biennial International Scientific Meeting of ISSFAL, Kansas City, Missouri (USA), May 17-22.
- Neville M, Geppert J, Min Y, Ghebremeskel K (2007) Macro- and micronutrient intake in University staff and students. 8th National Nutrition & Health Conference (23-24 Nov), London
- Brierley N, Bueno AA, Lehane C, Neville M, Brand A, Crawford M. (2009) Is Fatty Acid intake and metabolism in children with Down's Syndrome different when compared to their non-affected siblings? 10th World Down's Syndrome Congress, Dublin, Ireland. Proceedings of the 10th World Down's Syndrome Congress.

Abstract

Cardiovascular disease rates (CVD) are high in the UK, particularly in men. National surveys report a dietary imbalance of fats and antioxidant micronutrients, which may adversely influence blood fatty acid profile and risk for CVD. We investigated relationships between saturated and unsaturated dietary fat, and antioxidant (retinol, alpha-tocopherol, beta-carotene, iron, zinc, selenium, magnesium and copper) intakes, and blood glucose, lipoproteins and cell membrane fatty acids in a homogenous group of educated, healthy Caucasian men and women.

Volunteers (20 to 50 years, men 52, women 52) were recruited from staff and students of London Metropolitan University. Following correction for under-reporting 40 women and 31 men were included in the dietary analysis. Dietary intake, body composition, blood pressure, fasting blood glucose and lipids, and fatty acid composition of plasma and blood cell membranes (red blood cells, and platelets) were analysed.

The men consumed more saturated fat (11.0 vs 9.8 %energy intake, $p<0.05$), and exhibited elevated glucose (5.41 vs 4.81 mmol/l, $p<0.001$), systolic blood pressure (126.4 vs 112.0 mm/Hg, $p<0.001$), triglycerides (1.22 vs 0.98 mmol/l, $p<0.05$), LDL-cholesterol (2.90 vs 2.55 mmol/l, $p<0.05$), LDL:HDL (2.09 vs 1.44, $p<0.001$) and lower HDL-cholesterol (1.47 vs 1.87 mmol/l, $p< 0.001$) levels compared to the women, despite higher total body and trunk fat in the women (26.5 vs 15.6 %, and 23.3 vs 16.8 %, $p<0.001$ for both). In addition, the men consumed more zinc (11.7 vs 8.8 mg/d, $p<0.001$), and selenium (84 vs 54 µg/d, $p<0.01$), while women consumed more beta-carotene (2.9 vs 2.3 mg/d, $p<0.05$), and vitamin C (138 vs 108 mg/d, $p<0.05$). In addition, gender comparisons of micronutrient densities demonstrated higher copper, magnesium, iron and vitamin E densities in the women's' diets, indicating more balanced nutritional intakes. Despite these findings no differences

were found in plasma micronutrient levels (copper, iron, magnesium, retinol and alpha-tocopherol) between the men and women.

Furthermore, despite similar total n-3 intakes, women displayed higher proportions of DHA in plasma (PC: 2.8 vs 3.2 area%, $p<0.01$), RBCs (PE: 7.8 vs 6.8 area%, $p<0.01$), and in platelets (PC: 1.19 vs 1.05 area%, $p<0.05$; PE: 3.62 vs 3.21 area%, $p<0.05$), and a greater DHA: DPAn-3 (PC: 1.91 vs 1.67 area%, <0.05; PE: 1.15 vs 0.98 area%, $p<0.05$) compared to the men, suggesting enhanced bioconversion rates in this age group of women. In addition, proportionally, lower total n-6 levels and n-6:n-3 fatty acids were found in the platelets of women (PC: 23.9 vs 25.0 area%, $p<0.01$; 10.0 vs 11.7, $p<0.05$) providing further evidence for greater cardio-protection in these women of child-bearing age.

Further analysis suggested that in the women DHA levels appeared to be unrelated to dietary intake, while in contrast in the men, there appeared to be a greater reliance on pre-formed dietary intake, and LDl cholesterol was more responsive to the lowering effect of dietary n-6 fat (Beta -0.382, R^2 0.214, $p<0.01$). In contrast, many associations were found between dietary antioxidants, in particular zinc, and plasma and RBC EPA and DHA levels especially in the women.

The data of this study indicate gender-related differences in fat and micronutrient intakes, and associations with lipoproteins and blood fatty acids. Further research should investigate whether requirements for dietary LC-PUFAs particularly DHA differ for men and women.

1. General Introduction

1.1. The global problem of Non-communicable Chronic Diseases (NCDs)

Non-communicable chronic diseases (NCDs) are the leading cause of mortality in the UK and worldwide. NCDs encompass a broad range of conditions including diabetes, cardiovascular disease, cancer, mental ill-health, chronic respiratory disease, and musculoskeletal disorders (Unwin 2006). Of the NCDs, the greatest prevalence is for CVD, which contributes to 25% of early mortality deaths worldwide in economically and socially-productive age range 15-59 years, particularly in low and middle income countries (WHO 2005). Although the development of CVD may differ from other NCDs, they all share a number of behavioural risk factors including physical inactivity, tobacco smoking, excess dietary energy, unbalanced fat intakes, and micronutrient deficiencies. Evidence for these risk factors is clearly illustrated in the developing countries, alongside an increasing burden of CVD incidence, hence analysis of the health transition in for example Eastern European, and Asian countries, can contribute to an understanding of the aetiology of CVD in the UK.

1.2. Factors influencing CVD risk

Decades of research in CVD incidence in the developed world, have shown that although there may be a genetic element in the development of CVDs, much of the basis is attributable to environmental and behavioural/lifestyle risk factors such as smoking, physical inactivity and poor diet. Variations in CVD rates between developed and developing countries, as well as changes in trends over time, suggest that changes in diet may be associated with reduced risk (WHO 2005).

The recent UN summit on NCDs (Sept 2011) and WHO global forum Addressing the challenge of non-communicable diseases (Apr 2011), put tackling these risk factors at the forefront of government targets, and brought together stakeholders from many different multinational organisations including from the food and drinks companies, and health sectors.

1.2.1. Physical inactivity

It is estimated that, globally, the contribution of physical inactivity to the development of CHD such as ischaemic heart disease is approximately 22% (WHO 2005). Through effects on the circulatory system, skeletal muscle metabolism, development of body fat, and hormonal and immune systems, inverse relationships have been found between physical activity and hyperlipoproteinaemia, hypertension, cardiovascular diseases such as CHD and stroke, impaired glucose metabolism and likelihood of developing NIDDM, osteoporosis, and certain cancers (US Dept of Health and Human Services 1996). Although imprecision and large measurement errors in quantifying exercise from questionnaires are known difficulties in this type of research, two meta-analyses report low incidence of stroke associated with exercise. The first reported results from case-controlled and cohort studies which suggested that moderately active and highly active people respectively have a 20% and 27% lower risk of stroke incidence and mortality compared to low activity subjects (Lee et al., 2003). Similar results have also been reported in a meta-analysis of cohort studies investigating activity at work and during leisure time (Wendel-Vos et al., 2004).

There are several plausible ways by which physical activity might reduce risk of stroke. Physical activity increases energy expenditure and & indirectly lowers body weight, blood pressure, improves lipoprotein profile, glucose

metabolism (Lakka et al., 2007) and may reduce inflammation (Friedenreich et al., 2011). Furthermore, it enhances endothelial and vascular function (Moyna et al., 2004), decreases fibrinogen levels, blood viscosity (Wolfgang et al., 1997) and platelet aggregability (Rauramaa et al., 1986), and increases fibrinolysis (Boman et al., 1994). Taken together, these protective effects may reduce risk of cardiovascular events (Wilund et al 2007) and mortality (Jolliffe et al., 2000) and possibly inflammatory conditions such as diabetes (Helmrich et al., 1991; Manson et al., 1991). Furthermore, exercise appears to decrease age-associated changes in arterial elasticity and vascular endothelial dysfunction, via antioxidant effects ie decreasing NADH oxidase and increasing NO and SOD (Seals et al., 2009).

1.2.2. Smoking

Smoking is a major cause of cardiovascular diseases in that it increases risk of thrombosis and atherosclerosis, and substantially increases mortality risk. The net effects of smoking include decreasing oxygen carrying potential of haemoglobin due to binding with CO, increase in oxidising chemicals, trace metals, and a reactive aldehyde, acrolein, from smoke, and decrease in antioxidants, which results in inflammation, endothelial dysfunction, lipid abnormalities such as oxidation of low-density lipoprotein (LDL), and platelet activation (Burke and Fitzgerald 2003). Furthermore, acrolein binds covalently with apoliprotein A1 found in HDL to form adducts, which are implicated in atherogenesis (Szadkowski and Myers 2008), induction of COX-2 (Park et al., 2007), arterial hypercontraction (Conklin et al., 2006), and inhibition of anti-thrombin (Gugliucci 2007; Review US Dept HHS 2004).

Smoking also increases triglycerides, and insulin resistance, and decreases HDL. In addition, cigarette smoke contains diverse carcinogens such as PAH, N-nitrosamines, aromatic amines, 1,3-butadiene, benzene, aldehydes, and

ethylene oxide which induce cancers via formation of DNA adducts. Reactive intermediate agents critical in forming DNA adducts include diol epoxides of PAH, diazonium ions generated by α-hydroxylation of nitrosamines, nitrenium ions formed from esters of N-hydroxylated aromatic amines, and epoxides such as ethylene oxide. Glutathione and glucuronide conjugation play major roles in detoxification of carcinogens in cigarette smoke. Adducts lead to mutations that drive the process of tumour formation and progression through additional genetic alterations (HHS 2010 Tobacco and Cancer Surgeon General). Although the contribution of smoking in the development of CVDs is beyond doubt, the risk reduction in secondary prevention of non-fatal and fatal MIs was only recently reported in a Cochrane review to be 32% and 36% respectively (Critchley and Capewell 2003). Increasing rates of smoking in countries such as Russia and China, in women particularly, compounded by high saturated fat intakes, is a significant factor explaining the mortality rates in these countries (Zohoori et al., 2001; Gu et al., 2004; FAO 1980, 1990, 2000).

1.2.3. Diet

1.2.3.1. General Overview

CVD is likely to have been uncommon until the advent of the agricultural era, when dramatic changes in diet and lifestyle practices signalled the advent of the so-called diseases of affluence. Increased knowledge and skills in crop production and life-stock husbandry, allowed communities to settle in one place, become self-sufficient in food provision, and ultimately become better nourished. This contrasted with the nomadic lifestyles of previous generations, who relied on hunting wild animals, and foraging wild fruits and vegetables for sustenance and survival. Although, it is likely that the nutritional composition of the nomadic diet had remained similar

for thousands of years, the shift from nomadic to settled lifestyles resulted in significant changes in the nutritional content and types of foods consumed. While food became more plentiful, risk of starvation decreased, and despite rises in infectious disease rates, on the whole, life expectancy increased.

Since the agricultural era, food production techniques have continued to evolve, resulting in the highly processed diet observed today in Western countries such as the UK. The Western diet, characterised by highly refined, energy dense foods, which are high in fat and sugar and low in fibre and micronutrients, is identified as a risk factor for obesity, diabetes, and cardiovascular disease. More recent changes in diet and CVD incidence from industrialised and developing countries such as China, South America, Russia and India provide substantial evidence for a correlation between the two. There is clear evidence that the introduction of a free and globalised market, and subsequent rapid economic change in many developing countries has resulted in societal "Westernisation", typified by adoption of the Western lifestyle and diet in preference for traditional fare and values (Unwin and Alberti 2006). Concurrent with dietary change, paralleled increases in CVD rates are observed, hence it is possible that studies of the recent phenomenons of nutrition transition and CVD incidence in technologically less advanced cultures may be a surrogate for investigating the putative cardiovascular-promoting aspects of the Western diet.

1.2.3.2. Background – dietary importance of Micronutrients and Fat

Centuries of evolutionary progression have been dependent on availability of certain minerals (eg. carbon, nitrogen, phosphorous, sulphur, iodine

and iron), and natural selection of higher species was related to usage of alternative minerals (iron and zinc), which were superior in certain critical physiological processes. Furthermore, development of sophisticated homeostatic mechanisms designed to conserve stores of essential minerals for vital functions in times of dearth decreased reliance on direct mineral sources, and allowed migration inland (Nielsen 2000).

In similarity, natural selection would have also involved differential incorporation of fatty acids in the brain in utero and in the early neonatal period, since differences in brain fatty acid profile and capacity are observed between hominids and apes (Crawford 2002, Cunnane et al,. 1993). Fat as a limiting factor in evolutionary brain development would have been associated with ready access to sea foods rich in omega 3 fats and iodine, thus promoting greater neural function and cell signalling mechanisms in shore-based hominines compared to fast-growing terrestrial animals (Broadhurst et al., 2002).

In contrast, current marginal intakes of micronutrients and essential (omega 3) fatty acids in many global populations, may pose a direct and serious challenge to these evolutionary milestones, since evidence already exists for a detrimental association between certain dietary intakes and CVD outcomes.

A brief overview of the dietary changes from ancestral to the modern times may help to identify some of the dietary components which may be important for maintaining health.

http://primalmovers.wordpress.com/category/paleo-2/

1.2.3.3. Palaeolithic diet and NCD

Since there has been little requirement for natural selection in the past 10,000yrs following the advent of agriculture, in particular since the industrial revolution, there has been little genetic change since Palaeolithic times. Consequently, present-day humans remain genetically similar to their hunter-gatherer ancestors and therefore, hypothetically, at the current time there may be a mismatch between the genome and the modern diet. For this reason, it is relevant to compare constituents and possible health impact of dietary intakes from both periods.

In summary it is estimated that the Palaeolithic diet comprising wild game and uncultivated plant foods would have been comparably higher in micronutrient levels including folic acid and phytochemicals owing to fresh and un-cooked consumption of nuts, legumes and fruits; low in sodium; moderate to high (45-50% of total energy intake) in carbohydrate (CHO) from wild honey as well as fibre from unrefined fruits and vegetables, in addition to legumes and nuts, but low in phytic acid due to low cereal intake; high in protein (approximately 30% of total energy intake); high in cholesterol (approx 480mgd) but low saturated fats, myristic and palmitic acids (low in game but high in today's meats); and with a n-6:n-3 ratio of between 1:1 and 4:1 (Review: Eaton et al., 1997; Cordain et al., 2002).

Furthermore, exploitation of marine foods by coastal and island communities would have supplied protein and lipid, as well as LC-PUFAs, fat soluble vitamins and iodine, not only to the male hunter-gatherers, but equally to the women and children (Broadhurst et al., 2002).

In contrast the current Westernised diet differs from this diet as a consequence of the agricultural and industrialised revolutions, and more recently the fast-food revolution. In summary, this has resulted in a diet differing considerably from the Palaeolithic one, and containing foods which were previously virtually absent such as refined cereal grains and their products, non-human milk and its products, energy dense nutrient poor foods such as refined CHO and sugar, and a wide variety of oils. The main difference between the contemporary and Palaeolithic diets is in the marginalisation of non-cereal CHO sources, and fruits and vegetables, due to current taste demands for sugar and fat. It is argued that the discordance between the Palaeolithic and the Westernised diet is in large part responsible for the pathogenesis of so-called diseases of civilization: obesity, type 2 diabetes, cardiovascular diseases, and certain cancers. Indeed studies have investigated whether a change from a modern to a Palaeolithic diet might positively influence risk factors for CHD. Lindeberg et al., (2009) reported improved glucose control in subjects with impaired glucose tolerance and IHD following adoption of the Palaeolithic diet, while improved blood pressure and weight loss was noted by Osterdahl (2007). Furthermore, Frassetto et al., (2009) reported that insulin sensitivity, and lipoprotein levels were improved without weight loss in nine overweight adults consuming a Palaeolithic diet for 3 weeks, and glycaemic control was improved in diabetics (Jönsson 2009).

1.2.3.4. Nutrition transition and NCD

In contrast, adoption of a processed and refined diet in communities who previously ate a more natural diet is associated with a negative impact on health.

Globalisation, a term coined to apply to integration of isolated communities into the global village, relates to diet (termed "nutrition transition") and lifestyle as well as to economic change. For example economic and dietary changes seen recently in developing countries such as India, Russia, Brazil, and Africa, mirror those observed in 19th century Western countries during the Industrial revolution. Improvements in health via improved sanitation and infectious disease rates are paradoxically accompanied by increased rates for CVD. The devastating impact of globalisation may be particularly well illustrated in the disadvantaged indigenous communities of developed nations such as the Pacific Island communities where diabetes, unknown before the Second World War, now affects 30% of the population (Zimmet et al., 1999, 1997). A similar picture of obesity and chronic ill-health is seen in Australian aborigines, New Zealand Maori, and indigenous tribes of Africa (O'Dea 1991; Delisle 2010).

Nutrition transition is articulated as the adoption of the globalised food system in place of the traditional diet (Popkin et al., 2002, 2006). The result is increased consumption of cheap, readily available, often supermarket-bought foods including snacks, or take-away foods, as observed in countries such as the Philippines, South Africa and Latin America (Popkin 2006). These foods which are highly processed, high in fat, sugar and salt, and low in fibre, vitamins and minerals, promote over-nutrition and sub clinical micronutrient deficiencies (Kennedy et al., 2004). Furthermore a dramatic increase in consumption of meat and milk and

edible oils is observed worldwide, in particular in countries such as Japan, China (Zhai et al., 2009), India and Russia (Popkin 2006; Vlasoff et al., 2008), at growth rates which are concurrent with increased income (Du et al., 2004; Guo et al., 2000).

Some of the explanation for increasing rates of cardiovascular disease in particular in the developing countries (Beaglehole 2003), may have its origins in foetal development (Barker 1992). One theory proposes that undernutrition during pregnancy gives rise to a thrifty phenotype, characterised by changes in foetal energy metabolism, and endocrine and organ functions, which is designed to prepare the organism for a nutritionally depleted environment after birth and into adulthood (Goldberg and Prentice 1994; Barker 1997; Fowden 2001). If nutritional abundance (such as may accompany economic change) replaces chronic hunger, the thrifty phenotype model suggests that the individual may be susceptible to childhood "catch- up obesity" which increases risk of developing diseases of affluence such as the NCDs (Moore 1998; Misra et al., 2009) (Figure 1). This appears to be mediated via adaptive modification of insulin metabolism and a metabolic preference for insulin resistance, which increases the propensity for development of DM in later life (Hale and Barker 1992; Patel et al., 2004). Other proposed theories include the thrifty genotype, which describes an evolutionary genotype which favours thriftiness borne out of natural selection process during repeated exposures to famine (Neel 1962). More recently, this hypothesis has been disputed, and indeed several other genotypes have also been suggested which may not be mutually exclusive.

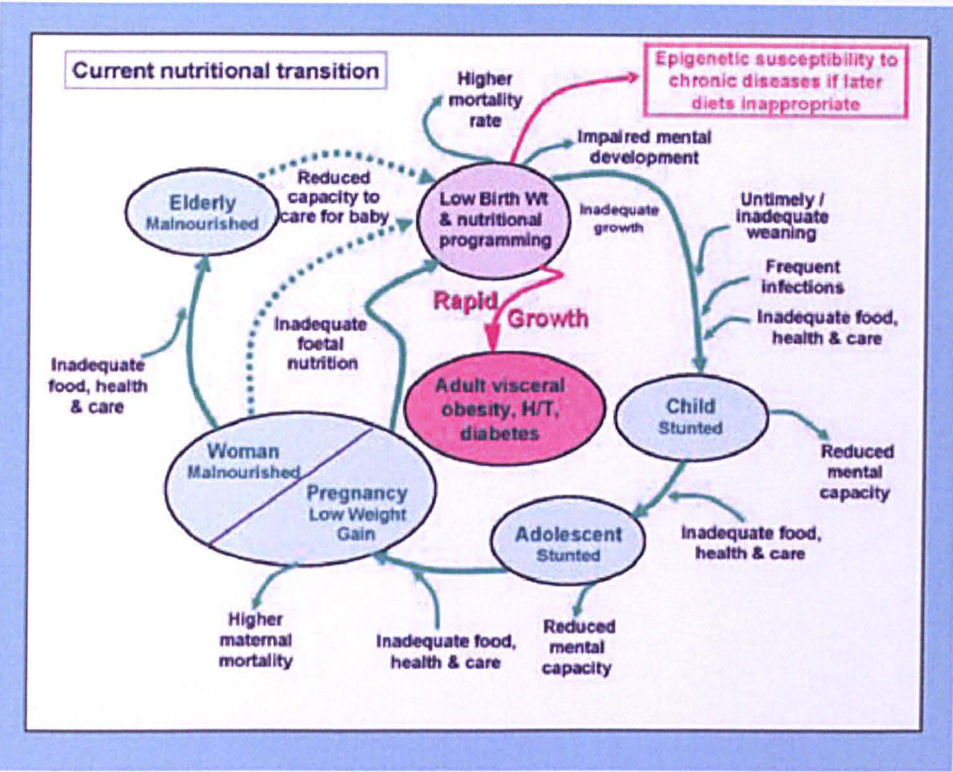

Figure 1: The intergenerational lifecycle: the proposed causal links of the current international nutritional transition Adapted from James et al., (Taken from James et al., 2008)

1.2.3.5. The Westernised diet

As alluded to before, the advent of the agricultural and animal-husbandry era, signalled the start in the development of the Westernised diet currently in evidence across the globe. Domestication of plants and vegetables led to a change in nutritional content, novel foods previously unknown to the hunter-gatherer were introduced, and food processing procedures were developed, which resulted in production of sugar and refined oils. At the same time, domestication of animals introduced milk and dairy to the diet, while confinement and rapidly fattened livestock, invariably with grain feeds, resulted in fatty meats high in SFA with lower proportions of omega-3 (n-3) and more omega-6 (n-6) (Cordain 2005),

Nevertheless, dietary quality (including micronutrient and n-3 intakes) among working class men and women (such as miners who survived on

an equivalent wage of £30,000 - £35,000) during the Victorian era (1850-1880), a period which is synonymously linked with poor health and low survival rates, potentially far exceeded that of today's. Furthermore, despite greater total energy intakes of between 50 and 100%, incidence of overweight and obesity was rare, in particular due to significantly higher levels of physical activity. A wide variety of organically grown fruits, and vegetables were available all year around, and Victorians commonly consumed 8-10 portions per day, in addition to fresh and dried legumes, wholegrain cereals, and imported or seasonally available nuts. Fresh fish, in particular herring, was sold fresh in the markets every week day, or was available dried and salted or pickled. Meat was consumed less often, was always free-range and was frequently offal, which is higher in micronutrients than skeletal muscle consumed today. Furthermore, meat "dripping" was used for bread instead of manufactured margarines, chickens were often kept in back yards for free-range eggs, and milk was often watered down (hence lower in fat). Beer too was frequently watered down and had a lower alcohol content (of between 1 and 3%) than modern beers, while one third of households were teetotal partly owing to the sustained efforts of the anti-alcohol movement. Salt intake was much lower, and smoking among the working class was intermittent, and rare in women until the advent of marketing in the early part of the twentieth century. In short, qualitatively, the Victorian diet was low energy and high nutrient dense, high in fibre and with a lower sodium/potassium ratio, which more closely resembled the Mediterranean or even Palaeolithic diet than the current westernised diet (Clayton and Rowbotham 2009).

In contrast, up to 85.3% of cereals in the modern diet may be highly refined, a recent phenomenon of the past two centuries since the invention of machinery for milling. In addition, the annual per capita demand of sugar has exponentially gone from 6.8 kg intake in 1815 to 54.5 kg in 1970 (Cleave 1974), while the food-processing industry has been primarily responsible for the significant increase in fructose consumption (Cordain 2005), as well as introduction to the diet of oils previously unheard of in conventional foods eg cottonseed, and hydrogenated trans-fats such as elaidic acid (Cordain 2005, Emken 1984, McGovern et al., 1996). Furthermore, alcohol and salt which were practically negligible in the Palaeolithic diet, are now a predominant constituent in the Western diet.

I. Effect of Western Food Production Practices on Nutrient Content of Foods

Following on from the rationing of the war years, government sponsored initiatives encouraged the advent of intensive animal production systems designed to increase production to meet the country's requirements. Unfortunately there was a trade off between quantity and quality, associated with a subsequent decrease in n-3 and micronutrient quality of foods. Resultant changes to livestock production have led to the finding that meat is now a high fat rather than a high protein food, and its composition from high PUFA in particular n-3 to high saturated fat and high n-6 PUFA. This has resulted from selection and confinement of fast growing livestock and use of high-energy grain based feeding systems. For example in the mid Victorian era, fat/protein ratio, and energy density of broiler chickens was approximately 0.4, and 118 kcals per 100g (Letheby

1872), whereas now it is more likely to be 3.2 and 273kcal/100g (FSA). Hence, previous recommendations from the Royal College of Physicians and the British Cardiac Society (Shaper 1976) which included consumption of less red meat and more white meat and poultry due to lower fat content, may no longer be valid. In addition, there is a considerable variation between n-6 to n-3 ratio of currently produced domestic animals and free-living fowl such as pheasant (6.99 vs 3.83 respectively). Even organically produced meat is not a guarantee of nutritional quality with n6:n3 ratios often higher than farmed birds (Wang et al., 2010). Ultimately, this means that the only naturally occurring land-based DHA food source is no longer available. Similarly, due to current conventional feeding systems, the fatty acid content of intensively farmed fish exhibits higher saturated and n6:n3 fatty acid ratio compared to wild fish. This is likely to have a significant impact on usefulness of fish in supplying long chain PUFAs for cardiovascular health and foetal development (Weaver et al., 2008; van Vliet et al., 1990).

ii. Micronutrient Composition of Organic Produce

Evidence indicates that in addition to alterations in fat content, food production practices also influence micronutrient composition. A comparison of micronutrient data from the Composition of Foods Tables revealed that micronutrient data of fruit and vegetable, meat and cheeses has changed considerably from 1940 to 2002. Of particular note, weighted average/combined results highlight the dramatic decreases in copper, iron and magnesium contents of 62% and 37% and 19% respectively (Thomas 2007). Therefore in addition to the observed precipitous changes in food consumption patterns

consisting of high nutrient dense foods of low nutritional value, there are simultaneous reductions in micronutrient content of the foods, which might be considered to be part of a healthy diet, due to food production practices.

Unlike intensive conventional farming methods, the principles of organic farming involve maintenance of soil quality and the diverse

population of micro and macro organisms within the ecosystem, and avoidance of chemical fertilizer applications. The organic practice of reapplying organic materials from plant and animals back into cultivated soils dates back to the beginning of agriculture, but the last century has seen the intensification of farming due to development of technologies for fertilization and weed/pest control, as well as mechanisation and plant breeding. Mineral fertilizers and liming of soils alters the soils natural mineral balance, and hence uptake of minerals by plants. This increases the potential for trace element deficiencies or toxicities in ruminant animals (Reid and Jung 1974; Sillanpaa 1972; Fleming 1963). Nevertheless, more recently it was reported that preferential use of fast-growing, high-yielding cultivars of wheat and other cereals has resulted in decreasing concentrations of minerals, such as zinc and iron, and increasing phytate content in wheat grain, findings which were unrelated to mineral content of the soil (Fan et al., 2008). In contrast, analysis of UK archived soil and wheat grain samples spanning 160 years revealed that Se concentrations in grain over this period have remained low and are related to soil Se concentration (under the influence of fertilizers and atmospheric deposition) and not to plant breeding (Fan et al., 2008). Furthermore it is proposed that the dramatic 50% decrease in Se

dietary intakes between the 1970s and 1990s (Raymen 2000), may be related to the replacement of imported wheat grown in high Se soils from the USA to that produced in the UK where Se soil concentration is low (Broadley 2006)

Nevertheless, there continues to be a lack of consensus on nutritional superiority of organic versus conventional produce. A recent systematic review concluded that out of 55 studies chosen from a possible 52,470 using specific inclusion criteria, there were no differences in nutritional quality of organic foods (Dangour et al., 2009). However, a rebuttal from the Organic Ctr (USA) indicated that organic foods were higher in polyphenols, vitamin C, iron and magnesium. Similarly, a review of studies by Crinnion et al., (2010) reported that iron, riboflavin, vitamin C and calcium were higher in organic produce in addition to antioxidant phytochemicals including carotenoids. Although minerals are dependant on forage type and quality, studies have shown that meat from pasture fed cattle is higher in Ca, Mg and K, B vitamins and vitamin A, compared to a standard corn based grain diet (Duckett et al., 2009; Leheska et al., 2008).

In contrast, lower levels of beta-carotene, B vitamins, vitamins A and E, iron, zinc and selenium are evident in conventionally produced meat, associated with changes in animal husbandry and confinement of animals indoors (Wang et al., 2010; Ponte et al., 2008a; Robertson et al., 1966). There is currently no legislation for nutrient composition of feedstuffs for indoor or outdoor reared livestock, which is at the discretion of manufacturers (FSA 2010). Food fortification has been proposed as a means to target dietary deficiencies of certain micronutrients whilst maintaining traditional dietary patterns. For

example, selenium supplementation of livestock feeds may be used to increase selenium content in meat and eggs (Fisinin et al., 2009; Hintze et al., 2002). Similarly, in order to increase nutritional content of fruits and vegetables, trace elements and metal minerals such as Cu, Mg Se, and Zn can be added to inorganic fertilizers, or breed genotype may be manipulated to assimilate greater concentrations of minerals, and decrease phytate content to enhance micronutrient bioavailability (White and Broadley 2009).

Unfortunately, however, the difficulty with these practices is that in the case of certain trace elements such as vitamins A, D, Ca, Mg Se and iron, the gap between recommended amount and safe upper level intake may be marginal, and thus fortification has the potential to both correct deficiencies and risk high intakes in the same population (SACN 2005). For example, despite significant improvements associated with a worldwide program of Universal Salt Iodisation (USI), deficiency remains high in 31.6% of school-age children across all continents including Europe, while concurrently risk of iodine excess has increased in 34 countries, (Andersson et al., 2010, WHO 2007). In contrast, evidence indicates that iron fortification of foods such as breakfast cereals has resulted in improved micronutrient intakes in at risk populations (Gregory & Lowe, 2000 NDNS), and is not associated with excessive intakes (Fletcher 2004). Similarly, folic acid food fortification designed to meet 30-70% of the needs of the pre-menopausal female population successfully decreased incidence of neural tube defects by 46% (De Wals et al., 2007), while avoiding safety risks in a proportion of the population (Morris and Tangey 2007).

1.2.3.6. The Westernised diet and NCD

Prospective studies report that dietary protein food sources, and hence associated fat intake, which typify the Westernised diet have a major impact on risk for CVD. The Nurses health Study, USA, carried out over 26yrs and involving 84,136 women aged 30 to 55 years reported that red meat and fatty dairy foods were significantly associated with increased risk for CHD while in contrast poultry and fish were significantly associated with a decreased risk. Furthermore, evidence indicates that substitution of red meat with one serving of nuts, low fat dairy, poultry or fish per day may result in 30%, 13%, 19% and 24% reduction respectively in CHD risk (Bernstein et al., 2010). Similar results have been reported in the National Institutes of Health-AARP (formerly known as the American Association of Retired Persons) Diet and Health Study cohort of half a million people aged 50 to 71.

Although prospective studies showing association between unrefined carbohydrate intake and CVD are difficult to find, more data is available for an inverse relationship with wholegrains. For example data from the Greek arm of the EPIC study carried out over 10 years and involving 22,944 healthy adults demonstrated that consumption of a high protein and low carbohydrate diet was related to a higher incidence of total mortality, as well as mortality from cardiovascular diseases (Trichopoulou et al., 2007). In similarity, low intakes of fruits and vegetables were found to correlate strongly with increased risk for CVD mortality in a Finnish study of previously healthy middle-aged men (Rissanen et al., 2003) while several large prospective studies confirm that high fruit and vegetable intakes are inversely related (Nöthlings et al., 2008; Liu et al., 2000; Rissanen et al., 2003).

1.2.3.7. Regional variations in diet and CVD

The Seven countries study highlighted the European wide regional differences in dietary intakes and associated incidence of CVD, while the MONICA project confirmed the differences in CHD related deaths between Northern and Southern Europe (Keys et al., 1986; Tunstall-Pedoe et al., 1994). Despite total fat intakes of over 35% in East Finland, Zutphen, the Netherlands, and Crete in Greece, high saturated fat intakes in Northern countries are in contrast to high monounsaturated fat intakes in Southern countries, and appear to correspond with European-wide differences in death rates from CHD.

Similarly, differences in dietary intakes between Scotland and England may explain North-South variations in CVD rates. For example higher intakes of fried foods, processed and red meat, and lower consumption of fruit, wholemeal bread, low fat dairy foods and poultry were reported in Scotland and the North of England in contrast to intakes in the South-East (Whichelow et al., 1991). A study in Scottish teenagers revealed similar findings, namely intakes of vegetables was lower, and white bread, sugar containing carbonated drinks and chips higher than other parts of the UK. In fact, one in five male Scottish teenagers reported no intake of vegetables over the 4 day dietary collection period (Crawley et al., 1997). The NDNS has reported a similar north-south divide: namely higher intakes of B vitamins, carotenoids, and fibre in the South and higher sodium in the north (Bates et al., 2001) which may contribute to observed regional differences in vascular diseases (Fulton et al., 1978).

1.2.3.8. The Mediterranean diet

The protective effect of the Mediterranean diet was first highlighted in the Seven Countries study, following observations that lower incidence of

CVD was observed in Southern European countries (Keys 1986). This diet consumed among populations bordering the Mediterranean sea, represents a model of healthy eating which is reputed to offer favourable health status into old age, and confer cardioprotection. It is characterised by a pattern comprising high intakes of wholegrains, fish, olive oil, fruit and vegetables, nuts and seeds. It contains moderate amounts of eggs, poultry and dairy, and low intake of sugar and red and processed meat. In addition, wine is consumed in moderate amounts, and is associated with lower incidence of cardiovascular disease. The health benefits are usually associated with the Mediterranean diet as a whole (Iqbal et al., 2008), although any one or all of the individual components may contain the bioactive properties. However, it is proposed that additional protection may arise via synergistic and antagonistic interaction between dietary components.

Since the Seven Countries study, benefits of the Mediterranean diet have been subsequently reported in both observational and interventional studies. The American Association of Retired Persons Diet and Health Study, which included almost 400,000 men and women between 50-71 years, reported a reduced risk of CHD in those with the highest Mediterranean diet score versus those with the lowest, especially in smokers (Mitrou et al., 2007). Those in the highest quintile for consumption of the Mediterranean diet in the Nurses Health Study were less likely to be at risk of CHD and stroke, and die from CVD compared to those in the lowest quintile (Fung et al., 2009). Furthermore, adherence to the Mediterranean diet was shown in a subset of the EPIC study to improve prognosis of subjects who had experienced myocardial infarctions (MIs) (Trichopoulou et al., 2007). In similarity, the Lyon

interventional study reported a consistently lowered incidence of MI and cardiac death at 27 months which was maintained at 4 years, due to continued dietary adherence by subjects despite completion of the study (de Logeril et al., 1999).

I. The Mediterranean diet and risk factors for CVD.

The EPIC study indicated that adherence to the Mediterranean diet was associated with a reduced risk of obesity (Mendez et al., 2006), while several of its components were inversely related to adiposity. Both cross-sectional and prospective studies have clearly shown the positive relationship between high dietary fibre and lower central adiposity (Liese et al., 2004; Wirfalt et al., 2001; Koh-Banerjeet et al., 2004), although RCTs involving high fibre diets have been unable to show a similar benefits on adiposity (Salas-Salvado et al., 2008). Despite its high fat content, the type of fat predominantly is unsaturated fat from liberal use of olive oil particularly in dressings, which also encourages the high intake of vegetables. Although one large cohort study, the Nurses study, showed a weak positive association between total fat intake and weight gain, several more studies report that type of fat is more important. This was clearly demonstrated in the Health Professions study, whereby substitution of PUFA with SFA or TFA resulted in weight gain (Koh-Bannerjee et al., 2003), which may be explained by more ready oxidation (and hence decreased storage) of unsaturated compared to saturated fats (Leyton et al., 1987). Furthermore, fruit and vegetable intake prevalent in the Mediterranean diet may also be protective against obesity. 24% lower risk of obesity was shown in those consuming the highest as

compared to the lowest intakes of fruit and vegetables in the Nurses Health study (He et al., 2004).

In addition, aspects of the Mediterranean diet are associated with improvements in insulin sensitivity. Although there are some inconsistencies in the literature, type of dietary fat appears to be important in insulin resistance. Higher plasma SFA and lower PUFA (biomarkers for dietary intakes of these fats) have been correlated with greater risk of insulin resistance in men (Perez-Jimenez 2001), and dietary intervention studies substituting SFA with MUFA have shown improved insulin sensitivity in epidemiological and prospective studies involving diabetics (Brunner, Wunsch et al., 2001; Mayer et al., 1993; Feskens et al., 1991, 1995). However short and long-term interventional studies have failed to show any benefit (Feskens et al., 1991, 1995; Rivellese et al., 1996).

Similarly type of dietary fat influences hyperlipidaemia. Although a decrease in saturated fat and increase CHO is known to result in adverse effects on triglycerides (TGs) and high density lipoprotein cholesterol (HDL) (Mensink et al., 1992; Meksawan et al., 2004), high n-3 PUFAs from fish are associated with decreased TGs with no effect on HDL in obese, hypertriglyceridaemic and Inuit populations (Skulas-Ray et al., 2007; Feskens et al., 1993).

In addition, although advice regarding altering the glycaemic load of carbohydrates by substituting refined for unrefined cereals and grains has been shown to have some effect on raised lipids, results comparing high or low glycaemic index of carbohydrate diets and dyslipidaemia are inconsistent (Aston et al., 2006).

1.2.3.9. The high PUFA diet

Research in the middle of the 20th century implicated intakes of total fat and cholesterol in the pathogenesis of CVD (Keys 1952), while at the same time there were incriminations that saturated fat may be harmful and early suggestions that EFAs may be beneficial (Sinclair 1968 a, b). Research in indigenous marine-food eating populations in Greenland, gave further indications that high polyunsaturated especially n-3 fatty acids may be instrumental in observed low incidence of atherogenesis. This surprising finding overturned previous opinion, because at the same time, Inuit total dietary fat intakes were approximately 40% of dietary energy, and cholesterol intakes were double those of populations with high rates of CHD and high obesity rates (Bang et al., 1980). In contrast to the Inuit diet, the Danish diet contained significantly lower n-3 PUFAs, and monounsaturated fats, and higher n-6 PUFAs (Bang et al., 1980). Differences in dietary habits between Danes, Greenland Inuits, and Inuits who were living in Denmark, were implicated in more atherogenic plasma lipid profiles in Danes and Inuit emigrants (Dyerberg and Bang 1975). In addition, observations of a decrease in ischaemic heart disease in Oslo during the Second World War, associated with a decrease in meat fat and increase in polyunsaturated fats from fish, prompted suggestions of a beneficial platelet antiaggregatory effect (Dyerberg and Bang 1981). Nevertheless, despite these promising earlier observations, effectiveness of dietary fish and fish oil intakes have at times been unclear. For example, while fish intakes in the Zutphen study were associated with a reduction in deaths from stroke and ischaemic heart disease (Keli et al., 1994; Streppel et al., 2008) and a reduced incidence of fatal MI in 18,244 men in Shanghai (Yuan et al., 2001), data from the Seven Countries study

suggested that beneficial results were not independent of saturated fat and flavonoid intakes (Kromhout et al., 1996). Furthermore, although the DART trial reported that 2033 Welsh men consuming more fatty fish had a 29% reduction in 2 year all-cause mortality especially from CHD (Burr et al., 1989), the follow up study reported a significant increase in risk from sudden cardiac death (Ness et al., 2002). .In contrast, the large Italian GISSI study (Hopper et al., 1999) involving supplementation of 1mg n-3 polyunsaturated fatty acids (PUFAs), in 11,324 post-MI subjects for up to 3.5 years, reduced the risk of death, non-fatal myocardial infarction, and non-fatal stroke by 10%, and the cumulative rate of cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke by 15%.

A similar confusing picture has emerged from a number of recent clinical trials. The Jelis study involving 14,980 hypercholesterolaemic men receiving statins but no evidence of cardiovascular disease, reported that EPA supplementation (1800mg) decreased risk of CVD by 19% over 4.6 years compared to those taking statins alone (Yokoyama et al., 2007). In contrast the Alpha trial involving 4837 patients, 60 through 80 years of age found no significant beneficial effect of EPA and DHA from consumption of margarine on 226 mg of EPA combined with 150 mg of DHA on major cardiovascular events compared to margarine supplemented with ALA or placebo (Kromhout et al., 2010). It is likely that discordance of results from RTCs involving fish oil supplementation and cardiovascular end points are in part due to due to variations in clinical conditions, intakes/doses, study endpoints, as well as type of LC-PUFA contained in the fish or supplement.

Differential effects of EPA and DHA have been shown in studies. Buckley et al., showed that in the short term (~4 weeks duration) supplementation

with DHA may have a more potent hypotriglyceridaemic effect compared to EPA (Buckley et al., 2004), however no differences have been shown in the longer term (Egert et al., 2009, Mori et al., 2000a). Unlike EPA, DHA has been reported to raise HDL levels in normolipidaemic subjects, and also increase LDL in hyperlipidaemic subjects (Egert et al., 2009, Mori et al., 2000a). Furthermore, DHA appears to have more of an effect on systolic blood pressure after 6 weeks of supplementation (Mori et al., 1999) and on heart rate (Grimsgaard et al., 1998). An explanation for this appears to be that compared to EPA, DHA is preferentially incorporated into the myocardial membrane (McLennan 2001). In addition, further evidence is available for a superior effect of DHA over EPA on endothelial function (Mori et al., 2000b). However, although a greater anti-inflammatory effect of DHA has been shown in vitro, insufficient evidence exists for potency in humans.

Nevertheless, human studies indicate that DHA, may have a greater platelet anti-aggregatory effect than EPA, despite both being readily incorporated into the platelet membrane where they influence fluidity via competition with AA to decrease the pro-thrombotic 2-series (TXA2) and increase the 3-series (TXA3) thromboxanes. In summary, while differential beneficial effects of EPA and DHA on risk factors for CVD are apparent, evidence from human supplementation studies of DHA and EPA may be emerging for a more potent effect of DHA (Cottin et al., 2011).

In contrast, there is insufficient evidence to indicate that intakes of ALA are cardioprotective, and benefit is solely associated with its replacement of excess linoleic acid, saturated or trans fatty acids, since conversion of ALA to EPA and DHA, is limited in adult humans (Brenna et al., 2009).

There is particular interest in the greater capacity for elongation of ALA and synthesis of DHA in vegans, and in women of child-bearing age who have higher requirements for foetal demands in utero, particularly as fish intakes in the general population may not be supplying adequate dietary levels of preformed EPA and DHA. The greater ability of females to synthesise long chain polyunsaturated fatty acids (LC-PUFAs) prior to the menopause may potentially confer higher protection against CVD compared to men.

1.3. Risk factors for development of CVD

1.3.1. Introduction

Figure 2 comprehensively summarizes how, in Western developed countries, the development of CVD as with several other NCDs, may originate at several points in the lifecycle and be particularly related to poor quality nutrition at critical periods in the lifecycle (in utero, childhood, adolescence, childbearing years and older age). The model summarizes the intergenerational effects of inadequate diets (characterised by high energy density, unbalanced fat intake and low micronutrient intake) and inactive lifestyle on development of NCD conditions such as cardiovascular disease, as well as obesity, diabetes, and cancer. Although a U or J shaped curve exists for decreasing CVD risk associated with increasing birth weight, a maternal diet containing excess fat or inadequate micronutrients is associated with high or low birthweight babies respectively, who share the finding that they have altered body fat. This important aspect predisposes offspring for hypertension, raised lipids and diabetes during adulthood (Lillycrop 2011; Elahi et al., 2009; Muthayya 2009). Furthermore, studies have found that compared to females, males may be more prone to negative effects of maternal under-nutrition shown by impaired

insulin sensitivity possibly related to faster growth of tissues in utero (Sugden and Holness 2002).

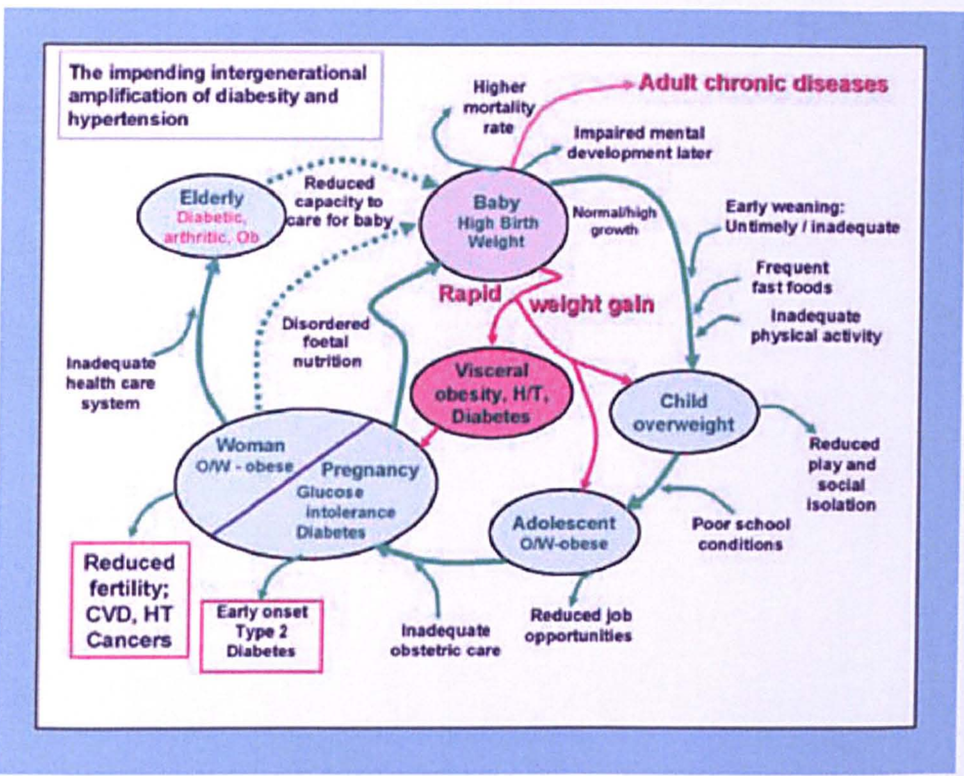

Figure 2: The intergenerational lifecycle: the proposed causal links of the current international nutritional transition but already fat adolescents are entering pregnancy to produce diabesity and hypertension prone children (James et al., 2008)

Moreover, it is likely that increased susceptibility for CVD attributed to sub-optimal peri-natal nutrition would be compounded by adult lifestyle factors including poor diet. Evidence is available to link dietary fat and/or low micronutrient intakes with risk factors for CVD including hyperlipidaemia, and changes in fatty acid composition of plasma, red blood cells and platelets.

1.3.2. Hyperlipidaemia

Substantial evidence is available for a direct effect of dietary fat on blood lipoproteins. We now know that dietary advice to lower saturated fat and cholesterol is ineffective in primary prevention of coronary heart disease, since it results in unfavourable changes to lipoprotein levels. Low saturated

fat intake is associated with a decrease in total, LDL and HDL cholesterol concentrations, while having a negligible effect on proportion of small dense LDL particles (Jebb et al., 2010), and does not improve mortality rates (Corr et al., 1997). In addition, replacement with a high carbohydrate intake is associated with an increase in triglycerides (Ma et al., 2006; Mensink et al., 1992; Meksawan et al., 2004). The first dietary study to show an effect of a change in fat intake on overall mortality was the DART study which included advice to increase oily fish intake along with a low fat diet (Burr et al., 1989) Compared to the low saturated fat diet alone, and the low saturated fat and high cereal diet, the oily fish exhibited a significant reduction in coronary hear disease deaths and 29% reduction in overall mortality after 2 years, despite no change in cholesterol levels. Similar results were reported in the Lyon study (de Logeril et al., 1994) which was based on a Mediterranean type diet: 70% reduction in MI, coronary mortality and total mortality was reported after 2 years, while cholesterol levels remained similar between the groups. These studies were first to propose that reduction in deaths from CVD were not solely related to dietary saturated fat content, and total cholesterol level. Furthermore, results advocated the importance of including n-3 in the diets, for beneficial effects on triglyceride levels and thrombosis (Calzolari et al., 2009). Supplementation with n-3 fatty acids is known to reduce triglycerides by 25% in normolipidaemic subjects, and by 50% in hypertriglyceridaemic subjects (Zuliani et al., 2009). Hence supplementation is now recommended by the American Heart Association at levels of 1g and 0.5g/d for those with and without evidence of CVD, while 4g/d is recommended by the Food and Drug Administration for the treatment of hypertriglyceridaemia.

Nevertheless, because n-3 fat intakes in the general UK population are known to fall short of recommendations, investigation of dietary fat and

micronutrient intakes in a homogenous university sample of men and women, who are better educated and may have greater nutritional awareness, would help to give a clearer understanding of the influence of habitual diet on lipoprotein profile which may indicate greater risk for CVD.

1.3.3. Plasma, red blood cell and platelet fatty acids

Many studies have shown that high intake of preformed EPA and DHA leads to a dose dependent modification of fatty acid composition of plasma lipid pools, cells and tissues, partly at the expense of very long chain n-6 fatty acids eg arachidonic acid (Harris et al., 1991; Marsen et al., 1992; Blonk et al., 1990; Sanders et al., 1983; Rees et al., 2006) although time course and incorporation and clearance rates differs according to cell and tissue type, as well as fatty acid type (Katan et al., 1997). Evidence is available for the association between diets high in PUFAs and fatty acid changes in plasma neutral lipids including cholesterol esters (CE), triglycerides (TG), and phospholipid (PL) fractions, phosphatidyl choline (PC) in plasma, platelets, and red blood cells, as well as phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and sphingomyelin, (SM) (Astorg et al., 2008; van Houlingen et al., 1989). It is known that fatty acid incorporation and clearance rates, as well as plateau concentrations are dependent on tissue type. In plasma, DHA is preferentially incorporated into TG, and PLs, while EPA is incorporated into PLs and CE. Changes in fatty acid levels are seen in TG within hours/days, plasma PC and CE within weeks, and RBC PLs within weeks/months. In addition, DHA is incorporated to a higher level in RBC especially in PE and PS, and turnover is slower than from plasma CE and PC (Prisco et al., 1996; Aterburn et al., 2006; Nikkari et al.,1995; Katan et al., 1997). Therefore, fatty acid data analysis from neutral lipids and phospholipids in plasma and RBCs will provide more comprehensive indication of dietary n-3 LC-PUFAs intake.

Uptake of fatty acids from lipoproteins to platelets, especially PE, occurs rapidly over hours (Englemann et al., 1996), and data may indicate risk for platelet aggrebility and hence CVD (Ahmed and Holub 1984).

Changes in cell and tissue fatty acid content alter their function, and consequently influence their physiological actions. The mechanisms by which cell function may be affected by a change in fatty acid composition include alteration of the fluidity of the lipid bilayer membrane (Rabini et al., 1993), and the structure of the lipid rafts, liquid-ordered areas containing cholesterol, glycosphingolipids (gangliosides), sphingomyelin, and proteins (Schley et al., 2007). In turn this influences the membrane proteins and hence cell signalling and transduction processes, in addition to activation of transcription factors, and consequently gene expression (Harder 2004, Katagiri et al., 2001). Furthermore, changes in fatty acid profile affect eicosanoid production also modifying cell signalling processes, and thus biological activities, which may ultimately influence the pathogenesis of diseases such as cardiovascular disease (Calder et al., 2006).

Further comprehensive data on intakes and food sources of individual dietary n-3 fats as well as a range of antioxidant intakes, which may be protective against oxidative destruction of stored fats, and levels of n-3 and n-6 fatty acids in plasma, platelet and RBCs in a homogenous group of men and women would provide further evidence for associations between dietary intakes and blood levels, and may help to partially explain differences in rates of CVD in populations with differing background diets, and in men and women.

1.4. Patterns and trends in UK dietary intakes

1.4.1. UK Dietary Recommendations for fat and micronutrient intakes

Current dietary recommendations for fats were determined by COMA (1991) and state that no more than the following percentages of dietary energy intake should be derived from the following fats: 35% total fat, 11% (10%) saturated fat, and 6% PUFA intake (n-3 intake should average 0.2g/d), 12% MUFA, 2% TFA. Dietary advice for n-3 fatty acids and micronutrient intakes recommends 0.2g n-3 PUFA per day which equates to approximately 2 portions of fish per week, of which one should be oily. This is based on a scientific review of evidence relating benefit of fish to coronary heart disease (COMA 1994).

Micronutrient requirements are met via an adequate variety of macronutrient intakes as well as fruits and vegetables. Currently it is recommended that in addition to recommendations for fat as outlined above, which in particular are sources for fat soluble vitamins, 50-55% of energy intake should come from a range of carbohydrates, in particular those which are high in fibre, and 15% of energy intake from a variety of protein foods, or 0.75g protein/kg body weight (RNI) (COMA 1994). In addition, the Department of Health (DH) advises that 5 x 80g portions of fruit and vegetables per day are consumed to reduce risk of some cancers, cardiovascular disease and some other chronic diseases (SACN 2007). Following results from the 2002 NDNS reporting low consumption of fruits and vegetables in all age groups, the 5-a day DH campaign was set up to encourage the public to increase intakes.

1.4.2. Dietary intakes in the UK

1.4.2.1. Introduction

Previous national recommendations to change saturated fat intakes (RCP 1976) resulted in a significant increase in linoleic acid, in large part arising from the now more common use of vegetable and seed-oil margarines and oils in place of butter and animal fats by the catering industry (Sanders 2000). Nevertheless, saturated fat intakes continue to remain high (NDNS 2008/9). In contrast, typical intakes of alpha-linolenic in the Western diet remain low at levels between 0.5 and 2g/d (BNF 1999), and UK intakes of n-3 LC-PUFA fail to meet recommendations (Henderson et al., 2002), due to low fish consumption (100g white and 50g oily fish per week, SACNE 2004). In the Palaeolithic diet, n6:n3 ratio was between 2-3 and 1, whereas now, the balance has changed in favour of n-6, with 10-20 fold intakes more commonly seen (BNF 1999). In recent years, a combination of decreasing dietary DHA and increasing dietary linoleic acid has accentuated the competition between LA and ALA.

In addition, present day diets are frequently low in micronutrients (Bowman and Vinyard 2004; Paeratakul et al., 2003). Diets which are high in saturated fat, with an imbalance of essential fatty acids (EFAs), and low in micronutrients, compound the risk for development of NCDs, as previously discussed, and contribute to poorer pregnancy outcomes such as low birthweight and increased risk of preterm delivery in women of childbearing age (Alam 2009; Scholl et al., 2008; Haggarty et al., 2009). An examination of data from national surveys reveals suboptimal dietary intakes in sections of the UK population, which contrast starkly with intakes in other European countries.

1.4.2.2. National trends in fat intake

Although energy intakes in UK men and women have decreased or stayed the same, total fat intake in both men and women has remained similar at approximately 33% (Henderson et al., 2002; Gregory et al., 1990). Data reported from a survey of Irish men and women corroborates these findings (Henderson et al., 2002). In contrast the quality of fat eaten has changed. Both men and women report a lower consumption of saturated fat (although still higher than recommendations), similar MUFA intakes, and higher intake of n-3 PUFAs (1% of total energy intake), while percentage energy intakes from n-6 PUFAs remain at approximately 5% (Bates et al., 2008/9).

1.4.2.3. Micronutrient intakes in the UK

Although the two most recent surveys reveal similarities in vitamin A intakes, previous surveys reported that intakes had increased, likely due to revised data from retinol levels in liver and milk becoming available since the previous survey (Henderson et al., 2002). Similarly intakes of vitamin E appear to have increased over recent years.

Mean iron intakes are similar in men and women between the two most recent surveys. Although percentage of men failing to meet minimum nutritional requirements has not changed from 3%, percentage of women failing to meet requirements fell from 26% in 2000/1 to 20% 2008/09. Similarly, magnesium intakes in men and women have increased, and percentages of people with inadequate intakes decreased. Zinc intakes appear to have remained the same, however in men only, percentages of men not meeting the LRNI has increased in recent years (8% 2008/9 vs 6% 2000/1), albeit non-significantly. In relation to copper, intakes fell

significantly between 1986/7 and 2000/1, and more recently have remained at the lower level in both men and women.

While intakes between more recent surveys (2008/9 and 2000/1) were similar, differences in several micronutrients were evident between 2000/1 and 1986/7 surveys. It is possible, however, that this may reflect updated nutrient data between editions of the Composition of Foods (5th edition 1991, 6th Edition 2002), related to changes in food fortification, or analytical methods, rather than solely variations in consumption patterns (Henderson et al., 2002). Nevertheless, findings that only 30% of adults are meeting the requirements for five-a-day, and 30% of energy intake nationally is being met via energy dense, micronutrient deficient foods such as alcohol, soft drinks, crisps and chips are of serious concern (Bates et al., 2010; Henderson et al., 2002).

1.4.3. Comparison of fat and micronutrient intakes among European Countries and the UK

1.4.3.1. Fat

Analysis of dietary fat data across 10 European centres involved in the EPIC study reveals considerable differences in amount and quality of fat intake between the UK and other countries. Highest total fat intakes of between 40-42% were reported in Greece while lowest intakes were in some Italian centres (<30%), and in the UK, fat intakes (in particular fat quality) varied between the general population and health conscious individuals. In health conscious individuals intakes of PUFA (mainly n-6) were higher in both men and women (7.8% men, 7.3% women), even when compared to other European EPIC centres.

As reported previously (Keys et al., 1986) intakes of saturated fat were highest in Northern and Central European centres, while MUFA intakes were highest in Greece and Spain. The greatest dietary contribution towards MUFA was from olive oil in Greece, Italy and Spain (>50%), whereas in the remainder of centres the main contributors were meat and dairy foods, and nuts and seeds in the UK health-conscious cohort (Ocke et al., 2009). In all EPIC centres, the contribution of red meat to energy was lower in women compared to men (Ocke 2009). Cholesterol intakes were highest in the Spanish cohort from meat, eggs and dairy, and lowest in the UK health-conscious subjects, while particularly high intake of cholesterol originated from butter consumption in Germany (Linseisen et al., 2009).

Highest total intakes of fish and fish products were in Spain and northern Norway, while lowest intake was in UK health conscious men and women, Germans and Dutch, although a north-south divide was evident for fatty fish consumption (Welch et al., 2002). Plasma EPA and DHA levels were higher in Denmark than Spain, associated with a preference for fatty fish intakes (Saadatian-Elahi et al., 2009). In the UK general population, mean intake of fish is 28 to 33g per day of which 69-73% of intake is from white fish, hence dietary n-3 fatty acid levels fall way short of those of fatty fish eating populations.

1.4.3.2. Micronutrients

Comparisons of micronutrient intakes in men and women across Europe indicate significant differences in intakes which are likely to be dependent on many factors such as cultural food preferences, climate, proximity to the sea and local/regional availability of fruits and vegetables, fish, and meat. There appears to be a clear North-South divide of fruit and

vegetable intake in men (not such a clear divide in women) with highest intakes in Greece and lowest in Sweden. In fact, on the whole across all centres, the contribution of fruit to energy intake was higher in women compared to men. The lowest percentage contribution was reported in Swedish and Danish men, while over twice that was reported in UK health conscious and some Spanish centres (Ocke et al., 2009).

When dietary intakes from the EPIC cohorts were analysed for micronutrient content, there was an increasing gradient in intakes of retinol between Southern and Northern countries. Intakes in Sweden, were over three times higher than in parts of Spain, and a similar ratio was seen in women. Meat, added fats and dairy contributed mainly towards retinol in men who had higher intakes across all age groups and in all centres (Jenab et al., 2009; Agudo et al., 2002; Welch et al., 2002; Linseisen et al., 2002, 2009; Slimani et al., 2002)

In relation to beta-carotene, central European countries had higher intakes than Northern, while intermediate intakes were observed in Southern countries. Umea in Sweden had the lowest intakes of beta-carotene compared to the health conscious UK cohorts. In the women, those living in Asturias, Spain had the lowest intake and in North-west France had the highest. Higher intakes of beta-carotene were found in women in the UK general and health conscious cohorts, and indeed across all centres, compared to men. In contrast, intakes of vitamin E and A were higher in men than in women, and in Southern and central countries compared to Northern and the UK (Jenab et al., 2009).

Magnesium intakes were lowest in men in several Mediterranean countries. Of the women, North-West France had the highest intakes, at twice the level of that in Greece, and moderate intakes of magnesium

were consumed in UK men and women. In relation to iron, intakes were higher in southern European countries (Spain Italy and France), although these were non-haem sources, compared to in Northern countries such as Sweden which derive most of their iron as haem from meat sources. Lowest percentage contribution of haem foods to iron intake was found in UK men and women (Welch et al., 2009). Likewise, mean intakes of copper were reported to be lowest in UK women, at almost half the amount consumed in Germany. Selenium intakes were lowest in women and men in Denmark, and highest in Finland, while zinc intakes were lowest in Ireland and highest in German women (Flynn et al., 2009).

1.4.4. Influence of socio-economic factors on dietary quality.

Although results from national dietary surveys present a generalised overview of intakes within a population, there is evidence that socio-economic factors may influence data in both national and European wide studies. For example a systematic review of data from seven countries showed that education level had a significant influence on consumption of fruits and vegetables (Irala-Estévez et al., 2000). In similarity, women with higher educational attainment were found to have diets which were more in line with dietary recommendations (Barker et al., 2009), and data from the EPIC-Norfolk study indicated strong independent effects of social class, educational level, and residential area deprivation on fruit and vegetable consumption (Shohaimi et al., 2004). Therefore, although dietary data from a homogenous population of university men and women may indicate a higher level of nutritional awareness, results may help delineate more clearly the influence of fat and micronutrient intakes on blood glucose, lipoprotein and fatty acid levels.

1.5. Overview

National and European dietary surveys reveal that there are significant regional differences (roughly based on a North-South divide) in fat, shown by higher saturated fat and often lower PUFA in particular n-3, and micronutrient intakes in Northern countries including the UK compared to Mediterranean countries. These findings appear to correlate with observed trends for CVD. Furthermore, gender differences in fat and micronutrient intakes are evident, not least because men eat more (due to their higher body mass and metabolic needs), but also because men and women appear to have preferences for certain foods/food groups. Since we know that men are more at risk for cardiovascular disease compared to women of a similar age (Lawlor et al 2001) prior to the onset of menopause, we were primarily interested in exploring whether dietary variations may partially explain this finding via associations with blood glucose and fats. Although data is available for the UK general population, it is known that food choice and health are related to socio-economic factors. For this reason, we chose a homogenous young University sample of comparably higher socioeconomic and educational status, and investigated differences in dietary fat and micronutrient intakes, and blood glucose, lipoprotein and fatty acid levels between the genders.

1.6. Aims

The strategic aim of the study was to investigate gender differences in n-3 fatty acid intakes and composition of plasma, red blood cells and platelets.

1.7. Hypotheses

- Men and women will eat different amounts of n-3 fatty acids.
- There will be gender differences in antioxidant intakes and associations with n-3 fatty acid status.

- Gender differences will be found in n-3 fatty acid levels in plasma and red blood cells, and associations with n-3 fatty acid intakes.
- Men and women will display variations in fatty acid content of platelets, and differences in associations with dietary n-3 intakes.

1.8. Objectives (specific aims)

The specific aims of the programme were:

- To compare dietary fat intakes in the men and women and investigate whether there are relationships between quality of dietary fat and blood glucose and lipoprotein (HDL, LDL, total cholesterol, TGs) levels.
- To compare micronutrient intakes in the men and women, and explore associations with plasma and red blood cell membrane EPA and DHA.
- To compare plasma and red blood cell membrane n-3 fatty acids in the men and women, and investigate relationships between dietary n-3 fat intakes, and levels of EPA and DHA in plasma and RBCs.
- To compare gender differences in platelet levels of EPA and DHA, and correlations with n-3 dietary fat intakes

2. Methods

2.1. Subject Recruitment

One hundred and four healthy Caucasian volunteers (52 men and 52 women aged 20 to 50 years) aged between 20 and 50 years were recruited from staff and students at London Metropolitan University using posters and presentations (Appendices 9.1). Interested volunteers were E mailed an invitation to participate in the study (Appendix 9.2). Eligibility was assessed via a self-completed questionnaire (Appendix 9.4). The exclusion criteria were: pregnancy, breastfeeding, genetic (cystic fibrosis, sickle cell anaemia, etc.) and non-communicable chronic (diabetes, hyperlipidaemia, inflammatory bowel disease, multiple sclerosis etc) medical conditions, and regular consumption of recreational drugs and excessive alcohol. The eligible subjects were invited to a meeting where additional information on the study (Appendix 9.3), and detailed instructions on how to fill in the food diaries were provided. Camden and Islington Community Research Ethics Committee (09/H072281) approved the study, and written consent (Appendix 9.5) was obtained from the volunteers.

2.2. Dietary assessment

2.2.1. Food diaries

Estimated food diaries were used to improve reliability and validity of dietary assessment. It has been shown previously that although weighed food diaries may increase reliability and validity in the short-term, ≤ 4 days is not representative of habitual intakes (OrtizAndrellucchi et al., 2009), while longer term intakes ≥ 7 days may be associated with lower reliability due to reporting-fatigue. The estimated food diary has been shown to be more reliable in estimation of habitual intakes based on assessment of dietary intakes over 1

week, compared to the FFQ (Brunner et al., 2001; Bingham and Day 1997). Volunteers were asked to record details of all foods and fluids consumed during the 7 consecutive days prior to blood sampling and body composition measurements. Seven day estimated food diaries (Appendix 9.6) were used by volunteers to collect dietary information. Within the diaries, instructions for recording information were provided. A separate page was recommended for information relating to each day's intake. Pages contained columns for type of food, amount and description. Subjects were advised to only record the amount of food that was eaten.

2.2.2. Portion size photographs

Volunteers were given written and oral instructions on how to complete the diary using household measures but were also provided with black and white food portion photographs for commonly eaten foods to assist in description of food portion sizes. The group excluded foods such as bread, eggs, biscuits etc which are easy to describe. A series of 8 photographs representing a range of portion sizes from very small to very large was provided for each food. Each portion size had a unique reference number for identification in food diaries.

In addition, photographs representing typical sizes for a range of glasses, crockery and cutlery were given. Volunteers were asked to keep food wrappers and containers, so that weights and descriptions of pre-packaged foods eaten could be evaluated. Recipes of frequently consumed dishes, which were not already in the original database, were analysed and added to the database.

Studies suggest that food portion size photographs may increase accuracy associated with estimation of food portion sizes but they may be subject to error especially related to age (older people over-estimate), gender (different perceptions of portion size in men and women), weight and BMI (both

associated with underestimation), and occupation (those working with food may have more accurate estimations) (Nelson et al., 1998).

2.2.3. FOODBASE

The energy and nutrient content of the diets were calculated using FOODBASE (Institute of Brain Chemistry and Human Nutrition, London Metropolitan University, London, UK). This is a nutritional analysis program and database based on the sixth edition of McCance and Widdowson's the Composition of Foods (Food Standards Agency, 2002) and its 10 accompanying supplements, plus enhanced fatty acid data produced by the Institute of Brain Chemistry and Human Nutrition. The Institute of Brain Chemistry and Human Nutrition undertook laboratory analyses of foods (Leighfield et al., 1993) to support the database. Many foods especially those high in fat or likely to make a significant contribution to fat intake were given values for each of 50 fatty acids. Unfortunately, however, fatty acid analysis has not been carried out in recent years, and alterations in fat content of fat containing foods such as meat and fish may have changed (Daley et al., 2010; Wang et al., 2009; Bourre 2005). This may inevitably affect the accuracy of our dietary analysis. Nevertheless, without an ongoing program of fatty acid analysis for commonly eaten fat containing foods, this limitation may also be associated with other nutritional databases. It was assumed that using Foodbase with its supplementary fatty acid information, more accurate data may be produced compared to that from other databases. A similar but smaller study carried out at Southampton also used Foodbase to analyse their FFQ n-3 fatty acid data (Bakewell et al., 2006).

The food diary and Foodbase nutrition software has been validated in previous studies (Doyle et al., 1992). To eliminate interpersonal variability in assessment of portion sizes, the same experienced individual (Dietitian) carried out the analysis and interpretation of the food diaries.

2.3. Data Exclusion

Four subjects were excluded at the outset due to incomplete diaries or failure to return diaries (3 men and 1 woman). This left 51 women and 49 men.

2.3.1. Under-reporting

Further adjustments to the data were made based on analysis for under-reporting. Under-reporting, which can be as high 40% in some studies (Voss et al 1997, Macdiarmid et al 1996), is common in dietary studies and can significantly influence validity of results. PAL-values established by FAO/WHO/UNU (1985) and using data from indirect calorimetry and doubly labelled water are used as a measure of daily total energy expenditure (TEE) adjusted for basal metabolic rate (BMR) over 24hrs (SACN 2011). A PAL <1.2 has been calculated by the FAO/WHO/UNU group to represent minimum energy required to support life, and may be used as a means to identify under-reporting in subjects of normal weight who are maintaining their weight. Rennie and colleagues (2007) assessed the level of under-reporting in the most recent NDNS (2001/1) survey and found that at PAL <1.54, 62% of men (mean 1.29) and 72% of women (mean 1.19) under-reported. Similar higher levels of under-reporting in women compared to men using cut-offs have been found in other studies (Price 1997).

We used the minimal cut off cut off of PAL<1.2, which excluded a further 11 women and 18 men (21.6% vs 36.7%, $p<0.05$) from the analysis. 40 women and 31 men were included in the initial dietary analysis (study 1, chapter 3). Of the corrected group, subject occupations were as follows: 13 female, 1 male nutrition students; 4 female, 2 male sports students; 4 female, 2 male other students; 17 female; 19 male staff; 2 female, 7 male other workers who had associations with the university. Similar numbers of men and women exercised (29 women and 23 men), 4 women and 4 men smoked.

Although in general under-reporting of energy intake occurs (Braitman et al 1985, Kromhout et al 1983, Ravussin et al 1982), macronutrient specific under-reporting may also occur simultaneously especially in fat (Voss et al., 1997, Pryer et al., 1997). This suggests that subjects may report dietary information which they feel may reflect healthy eating principles. In our study, no differences were found in percentage energy intakes from macronutrients between the sample groups corrected and uncorrected for under-reporting. Hence it might be assumed that under-reporting did not occur in one particular macronutrient only, but in over-all dietary reporting eg perhaps in estimation of portion sizes. In addition, studies have found that 46% of people under-reported due to feeling embarrassed or guilty about eating certain foods or because amounts to weighing and recording all foods constituted too much effort, difficulty or inconvenience (Macdiarmid et al 1996; Herbert et al., 1997). Bingham and co-workers (1987; 1991) reported that extent of under-reporting was dependent on dietary assessment method, and length of recording period. Interestingly, Rennie and colleagues (2007) found that under-reporting relating to size may have serious implications for interpretation of results, following reanalysis of data from the 2000/1 and 1986/7 NDNS. Mean PAL and fat intakes were found to be lower in obese subjects compared to lean, and to obese subjects in the 1986/7 survey, yet weights and BMIs were higher.

Increased accuracy of reporting based on improved assessment of portion sizes using photographs have been found in both men and women (n=100) (Robinson et al 1997) despite an earlier report that men under-estimate portion sizes more than women when using photographs (Nelson 1994). However in our study the higher level of under-reporting at PAL <1.2 in men may have indicated a lesser ability to estimate food portion sizes using food portion photographs compared to women. Use of food frequency questionnaires, and food portion size photos still involved under-reporting in 40% of subjects in the EPIC-Potsdam study

(Voss et al 1998), although it is possible that in this study, under-reporting was attributed to the use of the food frequency questionnaire, as much as to difficulties in assessing portion sizes using photographs.

2.3.2. Exclusion of subjects due to vitamin and mineral supplements

In chapter 4, in order to investigate micronutrient intakes from the diet alone, we excluded subjects who reported taking vitamin and/or mineral supplements (n=12 ie. 3 women and 9 men) from the original sample group (minus the incomplete diaries), which left 48 women and 40 men. A further 11 women and 15 men were excluded in this study (study 2) due to under-reporting (PAL<1.2) as this has been shown to adverse affect accuracy of micronutrient assessment in the diet (Mirmiran et al., 2006). However, we also compared micronutrient densities (amount/1000kcals) between the genders, which may also help adjust data for under-reporting (Mirmiran et al., 2006). The final sample group for analysis of micronutrient intakes consisted of 38 women and 25 men.

Subject occupations were as follows: 12 female nutrition students, 1 male nutrition student; 3 female sports students, 2 male sports students; 4 female other students, 2 male other students; 17 female staff, 13 male staff; 2 female, 7 male other workers with associations with the university. Of the total sample 3 men and 3 women were smokers, and 28 women (74%) and 19 (76%) men exercised.

2.3.3. Exclusion of subjects due to missing plasma, RBC and platelet n-3 fatty acid data

In chapters 5 and 6, 31 subjects (12 males, 19 females) were excluded due to missing n-3 FA data resulting from loss or spoilage of samples during analysis. Results were available for 73 subjects (40 males, 33 females). No correction

was made for under-reporting as there were no significant differences in mean n-3 fatty acid intakes between corrected and uncorrected sample groups.

Subject occupations were as follows: 10 female nutrition students, 2 male nutrition student; 4 female sports students, 2 male sports students; 5 female other students, 3 male other students; 13 female staff, 28 male staff; 1 female, 5 male other workers with associations with the university. Of the total sample 4 men and 2 women were smokers, and 8 women (24%) and 8 men (20%) exercised.

2.4. Blood collection and processing

Whole blood, 20 mls, was collected from the volunteers into 2 x 10ml tubes containing the anticoagulant, ethylenediaminetetraacetic acid (EDTA). In addition, 5 mls of whole blood was collected into 1 x fluoride tube for glucose measurement. This was centrifuged for 10 minutes at 1200 to 1500 x g, and the upper serum layer removed to labelled eppendorphs and frozen. The blood in the EDTA tubes was kept at room temperature, and separated into its components (plasma, red cells platelets and mononuclear cells) by centrifugation within 2 hours of venesection.

2.4.1. Red cells and plasma separation

The red cells and platelet-leukocyte-rich plasma (PRP) were separated by centrifugation of the EDTA tubes at 110 x g for 10mins at room temperature. Approximately 3mls of the upper PRP layer was removed from each EDTA tube, pooled together in a 15ml conical tube labelled PRP, and processed as described in 2.1.2. The loosely packed red cells were centrifuged at 1500 x g for 10 minutes at room temperature to obtain a red cell pellet and plasma. The upper plasma layer was transferred to a labelled tube, flushed with nitrogen and frozen. An equal volume of saline (0.85% NaCl) was added to the red cells,

mixed gently by inverting the tubes a few times, and the RBC-saline mixture was centrifuged at 1500 x g for 10 minutes. The upper saline layer, the thin layer between the saline and RBCs (white cell "buffy coat") were removed using a pipette and discarded. The process was repeated twice to obtain free red cells. These were divided between 3 labelled storage vials, flushed with nitrogen and frozen.

2.4.2. Separation of platelets and mononuclear cells

3mls of Histopaque-1077 (Sigma-Aldrich, Gillingham, UK) was placed into 2 labelled conical tubes. The PRP was divided between the 2 tubes (approx 3mls in each) and layered on tope of the Histopaque using an electric pipette. The PRP and histopaque were then centrifuged at 400 x g for 30 minutes at room temperature (as per manufacturer's instructions), to form three layers: upper plasma, middle mononuclear cells and platelets, and lower histopaque mixed with RBCs. The upper plasma layer was carefully aspirated using a Pasteur pipette to within 0.5cm of the middle opaque interface and transferred to the labelled plasma vials for freezing. The opaque interface was carefully transferred to a labelled clean conical centrifuge tube, to which was added 5 mls of 0.85% saline. The cells were resuspended in the solution, by aspirating with a Pasteur pipette, and centrifuged at 250 x g for 10 minutes to separate platelets and mononuclear cells (MNCs).

The upper saline layer containing the platelets was removed to another conical centrifuge tube, and centrifuged at 1500 x g for 10-15 minutes. The saline supernatant was removed and discarded, another 5mls of 0.85% saline was added, and the procedure was repeated again. If the upper saline layer was clear, it was removed, and the platelets were left at the bottom of the conical flask covered with enough saline. The cells were resuspended in the saline, removed immediately using a pipette into 4 mls of chloroform-methanol (2:1 with

0.01% of the antioxidant butylated hydroxytoluene or BHT) in a 10 ml vial, flushed with nitrogen and frozen, or extracted immediately.

The MNCs were resuspended in 5mls of 0.85% saline and centrifuged at 250 x g (or 300 x g if not precipitated) for 10 minutes. The upper saline supernatant was removed and enough saline left to cover the cells. As with the platelets, the mononuclear cell pellet was resuspended, removed to 4 mls of chloroform-methanol (2:1 with 0.01% of the antioxidant butylated hydroxytoluene or BHT) in a 10 ml vial, flushed with nitrogen and extracted immediately.

The separated blood fractions – plasma, red cells, and platelets – were stored at –70C until analysis.

2.5. Biochemical analyses

2.5.1. Analysis of blood glucose, total cholesterol, high density cholesterol, low density cholesterol, and triglycerides in plasma.

Blood glucose and lipids were analysed in serum using the Ace Alera Clinical Chemistry System (Alfa Wassermann Diagnostic Technologies B.V. Pompmolenlaan 24, 3447 GK Woerden, The Netherlands; Figure 8).

The Ace Alera is an automated bench-top random access analyzer that is used in the quantitative determination of constituents in blood and other fluids. Analyses are performed using spectrophotometric measurements at 37C.

The machine was calibrated prior to first use and following introduction of a new lot number of test reagents, while quality control tests were run in the morning and at regular intervals during the daily analysis. The calibration report provided the data from the calibration runs for all tests on that requisition, and the quality control report contained identifying information for the control material, test

results, acceptable limits, for those tests and a graphical representation of where the test results fell within the limits.

Glucose, triglycerides, total cholesterol and high density lipoprotein tests were run for each sample, while low density lipoprotein was estimated using the Friedewald equation:

[LDL-chol] = [Total chol] - [HDL-chol] - ([TG]/2.2)
where all concentrations are given in mmol/L

The Friedewald equation was developed in 1972 and is used to estimate LDL due to its simplicity and avoidance of cost, since it relies only on prior knowledge of triglycerides, total cholesterol and high density lipoprotein levels. It has since been validated by Warnick et al., (1990) who confirmed that the equation is valid when triglyceride level is under 400 mg/dl.

Prior to running a test, the reference ranges for each reagent test kit were entered into the Ace Alera software test parameters, and the lot number and analyte concentrations in a calibrator, and the lot number and expected values (mean and SD) for the quality controls were modified in the set up menu. All values were found on the package inserts.

The machine was calibrated for each test, and tests using sample aliquots were run in triplicate.

2.5.1.1. Calibration of Photometric Tests

The Ace Alera constructs a calibration curve by measuring and storing responses to known concentrations of a calibrator. The sample results are calculated by interpolation and curve fitting techniques that are based on the stored calibration curve. In order for samples to be processed, a valid calibration must have been undertaken.

2.5.1.2. Sample Preparation

For some analytes, two reagents were required, one of which was a buffer and was required to activate the second one – not all analytes required 2 reagents. Quality control, level 1 and 2, were assayed at least once daily. These identified the lowest and highest acceptable levels of a lipoprotein or glucose parameter, and were used to determine acceptable system performance. Serum calibrator, Gemcal, was used to calibrate the machine at least once every 14 days or when a new reagent was loaded onto the machine.

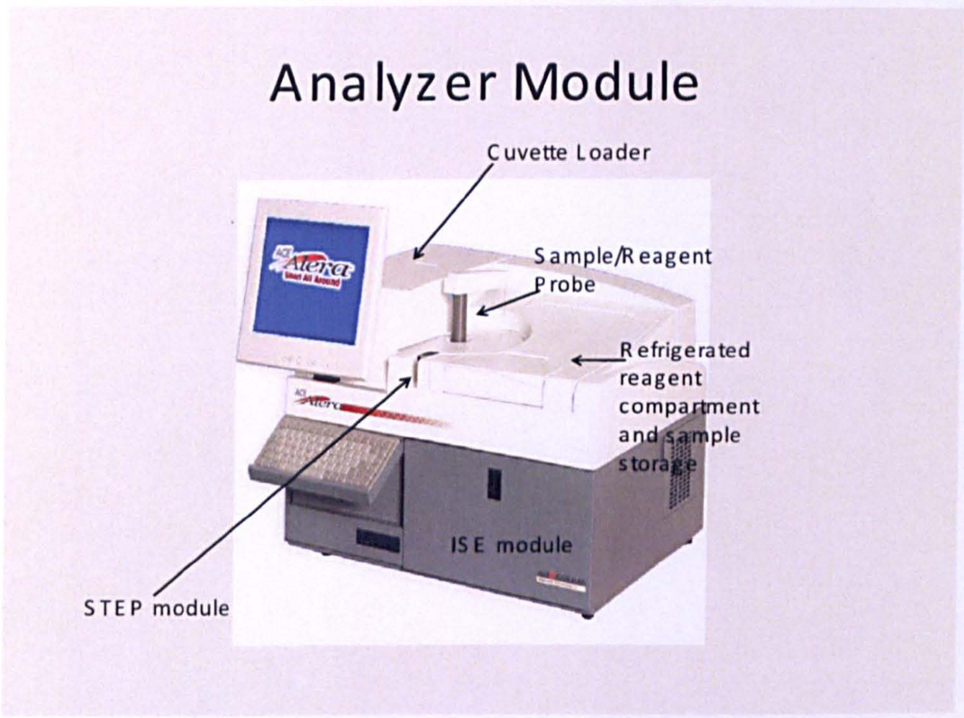

Figure 3 ACE-Alera analyser

Before commencing analysis, the required reagent bottles were opened, labelled and dated, and an evap-cap placed on them to prevent evaporation. The machine reads the dot codes on the base of the reagent to determine expiry date, and stability. They were then placed in the sample tray, the cuvette hopper was filled up, and the waste emptied. Approximately 0.5ml of each calibrator was placed in sample cups using a Pasteur pipette and

these were loaded into segments and placed in the sample tray of the Ace Alera. A load list containing requisitions for each calibration was entered, and the machine was set to run. Once valid calibrations were obtained, a load list containing requisitions for 2 quality controls (QCs) at 2 SD and 3 SD for each analyte, and single samples for each subject was entered into the software. Corresponding sample cups containing controls or samples were assembled in the machine segments. An adequate volume of control or sample to cover the bottom of the sample cup was required. Once the samples and QCs had run, results of calibrations, quality controls and samples were available on screen and in print form. Results were checked to ensure QCs were in range, and to ensure that triplicate sample results did not vary significantly.

The reactions and technical protocols for each test were as follows:

Triglycerides

Colorimetric Method

This method is a fully enzymatic procedure.

Principle

$$\text{Triglycerides} \xrightarrow{\text{LIPASES}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{GK} G\text{-}3\text{-}P + ADP$$

$$G\text{-}3\text{-}P + O_2 \xrightarrow{GPO} \text{dihydroxyacetone-phosphate} + H_2O_2$$

$$2\,H_2O_2 + 4\ \text{Aminoantipyrin} + 4\text{-chorophenol} \xrightarrow{POD} \text{quinoneimine} + HCl + 4H_2O$$

Where:
ATP = Adenosine-5'-triphosphate

GK = Glycerol kinase
G-3-P = Glycerol-3-phosphate
ADP = Adenosine-5'-diphosphate
NAD+ = Nicotinamide adenine dinucleotide
GPO = Glycerol-3-phosphate oxidase

3ul of sample was diluted in a ratio of 1:5 with 0.15 ul of system diluent. 225 ul of Gemcal calibrator was used for each test. The test was carried out three times and an average taken. The Linear calculation method was used. The calibration factor for triglycerides was 11.3 +/- 1.8 mmol/L (1000 +/- 159 mg/dl). The change in absorbance was measured bichromatically at 505 nm/692 nm and was directly proportional to the amount of triglycerides present in the sample.

Quality controls were run whose values were 3 standard deviations (SDs) outside the mean.

NORMAL VALUES (desirable levels)
Risk
Classification
Normal <250 mg/dL
<2.8 mmol/L
Borderline 250 – 500 mg/dL
Hypertriglyceridemia 2.8 – 5.6 mmol/L
Definite >500 mg/dL
Hypertriglyceridemia > 5.6 mmol/L

HDL Cholesterol

The assay consisted of 2 distinct reaction steps:

1. Elimination of chylomicron, VLDL-Cholesterol and LDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase.

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterolesterase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholestenone} + H_2O_2$$

Catalase

$2H_2O_2 \rightarrow 2H_2O + O_2$

2. Specific measurement of HDL-Cholesterol after release of HDL-Cholesterol by detergents in Reagent 2.

Cholesterolesterase

Cholesterol ester → Cholesterol + fatty acid

cholesterol oxidase

Cholesterol + O_2 → Cholestenone + H_2O_2

Peroxidase

$2H_2O_2$+4-AA+HDAOS → Quinone pigment +$4H_2O$

The intensity of the quinone imine dye produced is directly proportional to the cholesterol concentration

HDL cholesterol was analysed in 2 steps using 2 reagents, HDL-R1 and HDL-R2. 3 ul of sample, 225 ul of reagent 1 and 75 ul of reagent 2 were used in the analysis. An incubation period of between 300 and 310 seconds was required. The response calculation was by the Delta method with 2 data points at 43 and 193 seconds. The reaction and bichromatic correction wavelengths were at 592 and 692 nm respectively.

The test was run in triplicate and an average of the results given. The calibrator was specific for measurement of HDL cholesterol and the calibration factor was 1420 +/- 213 mmol/l (36.8 +/- 5.5 mg/dl). The reference range was 1.04 –1.55 mmol/L (40-60mg/dl).

Quality controls run for HDL gave results which were 3 SD outside the range.

Cholesterol

Principle

Cholesterol esters in the serum are completely hydrolyzed to free cholesterol and free fatty acids by pancreatic cholesterol esterase:

Cholesterol esterase
Cholesterol ester +H2O → Cholesterol + Fatty Acids

The cholesterol liberated by the esterase, plus any free cholesterol originally present in the serum, are both oxidized by cholesterol oxidase:

Cholesterol oxidase
Cholesterol + O2 → Cholestene-3-one + H2O2

The liberated peroxide reacts with phenol and 4-aminoantipyrine in a peroxide catalyzed reaction to form a quinoneimine dye which absorbs at 500nm:

Peroxidase
2H2O2 + 4-Aminoantipyrine + phenol → Quinoneimine + 4H2O

The change in absorbance is measured bichromatically at 505 nm/ 692 nm and is directly proportional to the amount of cholesterol present in the sample.

Cholesterol was measured photometrically between 505 and 692 nm. 285 ul of reagent was required for the analysis. 4 ul of sample is mixed with 15 ul of system diluent .The response calculation used the final point method. Data was taken at one data point which is at 243 seconds. The calculation method was calculated using the linear method. 1 calibrator was used

(Gemcal) and the test was run in triplicate. The calibration factor for this test was 18.2 +/- 2.1 mmol/l or 703 +/-81 mg/dl.

The normal range for cholesterol using this test was: 5.2 to 6.24 mmol/l or 200 to 240 mg/dl. Controls were used which give values which were 3 SD outside the normal range.

Glucose

Principle

Glucose Hexokinase (Gluc-HK) catalyzes the oxidation of glucose in the presence of NAD and ATP to give gluconate-6-phosphate and NADH. The total amount of NADH formed is proportional to the initial amount of glucose present. The absorbance of the reaction is bichromatically measured at 340 nm/378 nm.

Colorimetric Method

This is an enzymatic method, using hexokinase.

Samples were diluted with system diluent in a ratio of 1:5. 3 ul of sample were diluted with 15 ul of diluent and 300 ul of reagent.

The Final Point response calculation method was used, and 1 data point at 303 seconds. The reaction wavelength was 340 nm and the bichromatic chromatic wavelength was 378 nm.

The result calculation method was Linear and the result was the mean of 3. The calibration factor was 32 +/- 6.2 mmol/l (577 +/- 117 mg/dl). The reference range was 3.9-5.8 mmol/L or 70-105 mg/dl. Control values gave results which were 3 SD outside the range.

2.5.2. Analysis of retinol, alpha-tocopherol and beta-carotene in plasma using HPLC

2.5.2.1. Preparation of calibration curves for retinol, alpha-tocopherol and beta-carotene

Standard concentrations and dilutions of retinol, alpha-tocopherol and beta-carotene were prepared (Table 1).

Table 1: Standard vitamin concentrations and dilutions used

Substance	Standard concentrate	Standard dilution
Retinol	1mg/ml	100mg/dl
Alpha-tocopherol	10mg/ml	200ug/dl
Beta-carotene	10mg/ml	100ug/dl

2.5.2.2. Preparation of the stock standard

For a new standard curve, standard dilutions were prepared from the stock standard and their absorbance was measured on a spectrophotometer at 325 nm for retinol, 292 nm for alpha-tocopherol and 450 for beta-carotene (Table 2). Lambert-Beer Law was used to determine the exact concentration of the standard dilutions (c) from absorbance (E) (E1% 1cm, extinction of a compound at a certain wavelength; d, thickness of cuvette).

The formula is:

$$E = E1\%\ 1cm\ c\ (g/dl) * d\ (cm)$$

Thus concentration can be calculated by:

$$C\ (g/dl) = E/[E1\%\ 1cm * d\ (cm)]$$

Table 2: Molecular weight, solvent and extinction coefficients for vitamins

Substance	Molecular weight (g/mol)	Solvent	Wavelength (nm)	Extinction coefficient (E1% 1cm)	Source
Retinol	286.5	Ethanol	325	1780	*

Alpha-tocopherol	430.7	Ethanol	292	75.8	*
Beta-carotene	536.88	Chloroform	450	2590	*
Retinol Acetate (internal std)	328.49	Ethanol	324-327	1400	*

*Intraassay und Wiederfindung am 20.3.1995

2.5.2.3. Sample preparation

100 μl of plasma was deproteinised with 500 μl of ethanol in 3 ml vial. The mixture was extracted in 1.5 ml hexane and vortexed for 5 minutes before it was centrifuged at 4000 rpm for a further 8 minutes. The upper organic layer was removed and extracted with 1 ml hexane. This step was repeated twice. The extract was evaporated to dryness under nitrogen, and dissolved in 100 μl of methanol 98%.

2.5.2.4. Separation and identification

The retinol, alpha-tocopherol, and beta-carotene were separated and identified using the Agilent 1100 Series HPLC connected to an autosampler and a diode-array detector (Agilent 1100 G1315B). A column was used (250 mm x 4.6mm 5 μm Hypersil GOLD Thermo Scientific; Part number 25005-254630). The vitamins were eluted using HPLC-grade methanol, at a rate of 1ml/min and a constant temperature of 31C. Retinol, alpha-tocopherol, and beta-carotene were detected at their maximum absorptive wavelengths of 325 nm, 292 nm, and 450 nm respectively. The retention times were: 4.4 minutes for Retinol, 8.4 minutes for alpha-tocopherol, and 22.7 minutes for beta-carotene.

2.5.2.5. Running the standards on the HPLC and preparation of the standard curve

The following settings were entered into the HPLC program:

Pressure: Max 200 bar, min 10 bar

Flow rate: 1ml/min

Injection vol: 25 ul

Temp: 31C

Peak width: >0.1 (min)

Table 3: Vitamin standard concentrations and calibration curves

	17th March	18th March
a-Tocopherol [ug/dl]	Area	Area
0	0	0
156.25	12.2407	11.098
312.5	24.4736	18.526
625	57.9122	41.911
1250	114.856	97.096
2500	241.39	208.459
5000	479.969	446.274
10000	946.382	851.988
Retinol [ug/dl]		
0	0	0
3.125	8.32216	11.344
6.25	16.2631	16.027
12.5	30.935	31.125
25	59.592	60.372
50	115.973	117.573
100	226.814	232.909
200	434.111	430.703
b-Carotene [ug/dl]		
0	0	0
1.5625	0.6986	0.02
3.125	2.5292	0.1
6.25	7.9188	1.11
12.5	21.711	10.42
25	61.7525	43.37
50	165.946	108.39
100	364.61	216.56

Linear regression lines were plot from concentrations versus peak areas using the calibration curves (Table 3). The correlation coefficients of the regression lines were as shown below (Appendix 9.8). Vitamin standard calibration curves were run for the two analysis dates (17th and 18th March)

on subsequent days. Vitamin concentrations in the samples were calculated from their standard curves, using computer software, Agilent Chemstation (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

2.5.2.6. Recoveries and coefficients of variation

The mean percent recoveries calculated from the vitamin calibrants run on 17/03 and 18/03 respectively were, 95.1% and 97.8% for retinol, and 96.6%, and 96.5% for alpha-tocopherol respectively. The coefficients of variations of reproducibility of the assay for 17/03 and 18/03 were 0.4% and 1.2% respectively for retinol, and 0.3% and 1.1% respectively for alpha-tocopherol.

The recoveries and coefficients of variation, and calibration curves for the standard dilutions for beta-carotene indicated that the results were unreliable. Therefore the volunteer beta-carotene results were also deemed unreliable for use in the study, and hence were excluded from any further analysis.

2.6. Determination of plasma minerals and trace elements

2.6.1. Preparation of standards and plasma samples

Clean glassware was soaked in 4% nitric acid for 1hr prior to use in order to remove all organic and inorganic matter. Glassware was then rinsed 10 times with deionised water and dried in the oven at 180C.

A multielement standard (Alfa Aesar, 042602) was used which contained minerals and trace elements. Standards for Zn, Fe, Mg, Cu, Se were prepared in the following dilutions (Table 4) (stock solution 10mg/l in 2% HNO3) using double deionised water.

Table 4: Preparation of calibration curve for mineral standards

No of vial	Conc	Double deionised water	Standard solution
1	4mg/l	7.5ml	5ml
2	2mg/l	6ml	6ml of above solution
3	1mg/l	6ml	6ml of above solution
4	0.5mg/l	6ml	6ml of above solution
5	0.25mg/l	6ml	6ml of above solution
6	0.125mg/l	6ml	6ml of above solution
7	0.0625mg/l	6ml	6ml of above solution
8	0.03125mg/l	6ml	6ml of above solution
9	0.015625mg/l	6ml	6ml of above solution
10	blank	6	0

In order to precipitate protein in the samples, plasma was centrifuged at 2000 x g 10 minutes. Plasma samples were then diluted x100. 100ul of plasma was mixed with 9.9ml of deionised water. Three replicates were run for each sample.

Results were given in mg/l and were multiplied by a factor of 100,000 to correct for the dilution factor and to give results in ng/ml.

2.6.1.1. Identification

The ICP-OES was used to run plasma samples (Figure 4).The top 3 preferred emission wavelengths were chosen for each analyte. Peaks were integrated in order to correct results. The result with the lowest residual standard deviation (RSD) at each wavelength was chosen as this indicated the lowest variability of results for that analyte.

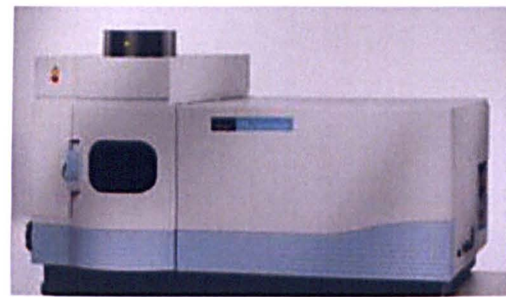

Figure 4: ICP-OES

2.6.1.2. Quantification

Sample analysis and quantification of trace elements was by Winlab32 for ICP.

2.6.2. Exclusion of outliers

Samples of subjects taking nutritional supplements containing minerals or trace elements were excluded. Heterogeneity and normality of variation of results were checked using Radar analysis in Excel. Samples with results which were 2 SD outside the mean were excluded, as were samples where there was a pattern which suggested there may have been a needle blockage.

2.7. Analysis of lipids and fatty acids

Total lipids were extracted from plasma by the method of Folch et al., (1957) with the use of a 2:1 ratio of chloroform and methanol mixture. Both solvents contained 0.01% of the antioxidant butylated hydroxytoluene (BHT, 0.01%) to prevent oxidation of unsaturated fatty acids. The sample volume was 1ml and the ratio of sample to extracting solvent mixture was 1:45 in all cases. All glassware was washed down with appropriate solvent prior to use.

2.7.1. Lipid Extraction

I ml of plasma was pipetted to 45 mls of chloroform and methanol solvent mixture in a 50 ml labelled extraction tube (Figure 5). For red blood cells, 1 ml of sample was added to 15 mls of methanol, to which was added 30 mls of chloroform (Figure 6). Extraction tubes were stoppered and shaken well. A blank containing solvent mixture only was extracted with every batch in order to monitor contaminants from solvents. Extraction tubes containing samples were flushed using nitrogen for approximately 20 seconds, and placed in the fridge for 2 hrs, or overnight for RBCs.

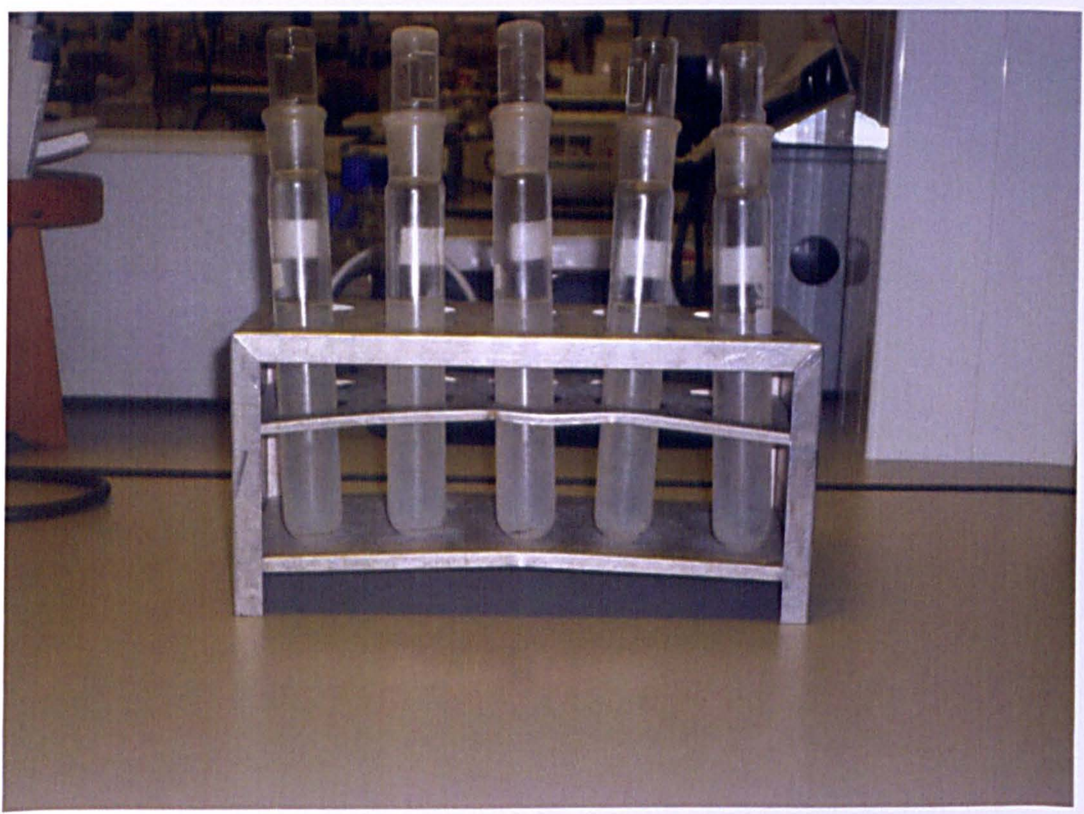

Figure 5: Lipid extraction – plasma

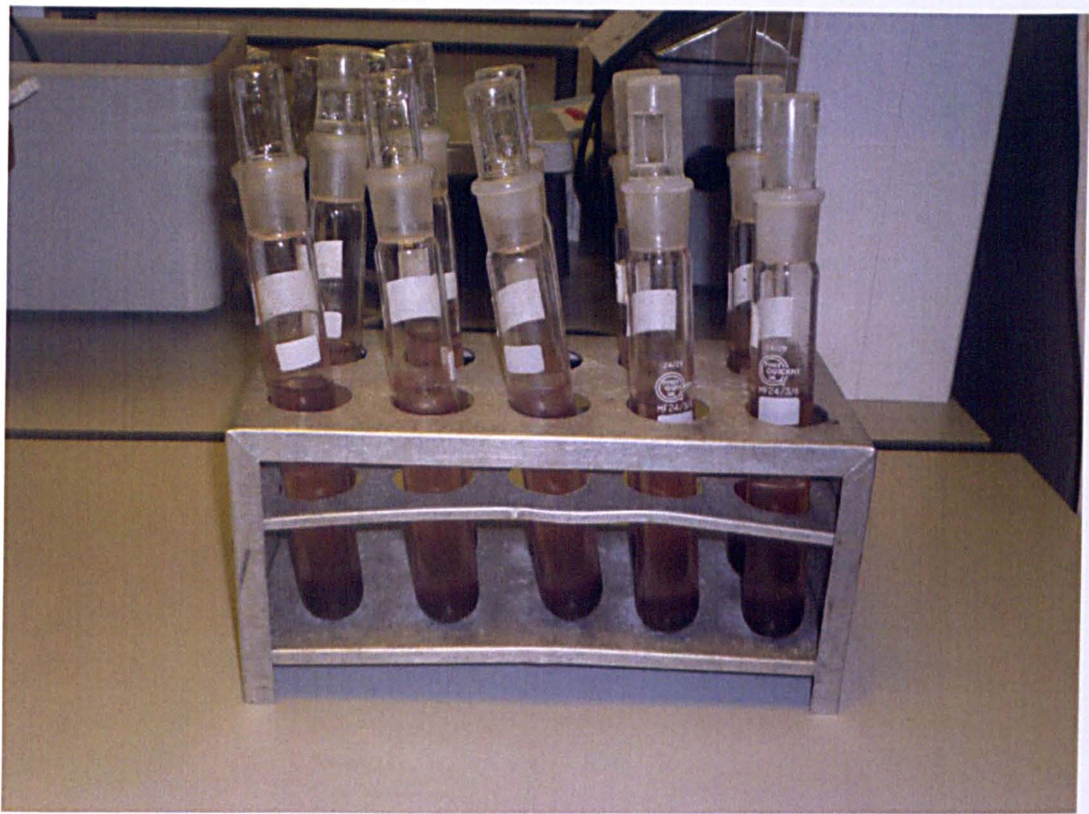

Figure 6: Lipid extraction - red blood cells

2.7.2. Partitioning – phase separation of lipid and non-lipid components

Extraction tubes were removed from the fridge and allowed to return to room temperature. A five clamp stand was set up in the fume cupboard with labelled 100ml partitioning funnels (with well fitting opening valves), and silver filter funnels lined with type 1 filter paper for each sample. Samples were transferred into the filter funnels and extraction tubes washed with 15 ml of solvent (Figure 7). A pipette was used to wash the insides of the extraction tubes with solvent to remove the RBCs. The filter funnels were removed and 25% by volume (45 + 15 mls = 60 mls, 25% = 15mls) of 0.85% saline was added to the samples in the partitioning funnels. The funnels were stoppered, and the solution in the funnels was degassed well with nitrogen for a minute each. The funnels were stored in the fridge at 4C overnight.

Figure 7: Partitioning

2.7.3. Recovery of total lipids

The separating funnels containing the filtrate were removed from the fridge, and stored in a darkened room for 30 minutes to prevent condensation and allow equilibration. They were then returned to the clamp stand, and the stoppers removed. The valves were removed one at a time, and the bottom organic layer drained to the meniscus into 250 ml labelled round bottom flasks. The extracting solvent mixture was evaporated off using a rotary evaporator (BuCHI UK Ltd, Chadderton, Oldham, UK) under a reduced pressure at 37C (Figure 8). 4mls of methanol was added to each sample, rinsed around the sides of the flask and dried off again. This step was repeated twice. The dried lipid was dissolved in chloroform:methanol solvent mixture. In a step that was repeated 2 to 3 times, 2-3 mls of the CM solvent was rinsed around the sides of the flask and removed using a Pasteur pipette to labelled 10 ml storage vials. The storage vials were

dried down with nitrogen to approx 1 ml, flushed with nitrogen and stored at 4C prior to TLC.

Figure 8: Rotary evaporation

2.7.4. Thin-layer chromatography – Separation of lipids

TLC is normally carried out for phospholipids prior to running neutral lipids.

i. Thin-layer chromatography plates

Ready prepared 20 cm square TLC plates of 25 mm thickness, coated with silica gel were used for TLC. They were conditioned (dried) at 100 C for 1 hr in an oven and stored in a desiccators until used.

ii. Separation of plasma phospholipids

a) Equilibration of developing chambers

A rectangular tank containing thick, heavy glass and a close fitting lid, suitable for 2 plates, was used for thin layer chromatography. The tank was washed, rinsed with solvent and allowed to dry before use.

The outer walls of the tank were lined with filter paper. The solvent for phospholipid separation contains chloroform, methanol, and methylamine 65:35:15 and BHT (100mg/l). 150-200mls of solvent was poured into the tank and allowed to equilibriate, with the lid on, for 30 mins before use (the solvent should have reached the top of the filter paper).

b) Application of sample to TLC plates

Once cool, using a plastic guide plate, the silica was scored from top to bottom, approximately 3 cm from each side and from left to right at the top of the plate. Using a soft pencil, pencil lines were drawn from left to right, 2 cm from the bottom of the plate and from top to bottom of the plate to separate the samples from the standards. Each plate held approximately 1 standard and 2 samples. The total lipid extract was dried down and dissolved in approximately 4 drops of CM solvent. Micropipettes were used to draw up, and carefully draw a narrow and evenly distributed line with the standard and samples along the pencil line at the bottom of the plate. 2-3 more drops of CM solution were used to rinse the sides of the vial, and were applied to the plate (Figure 9).

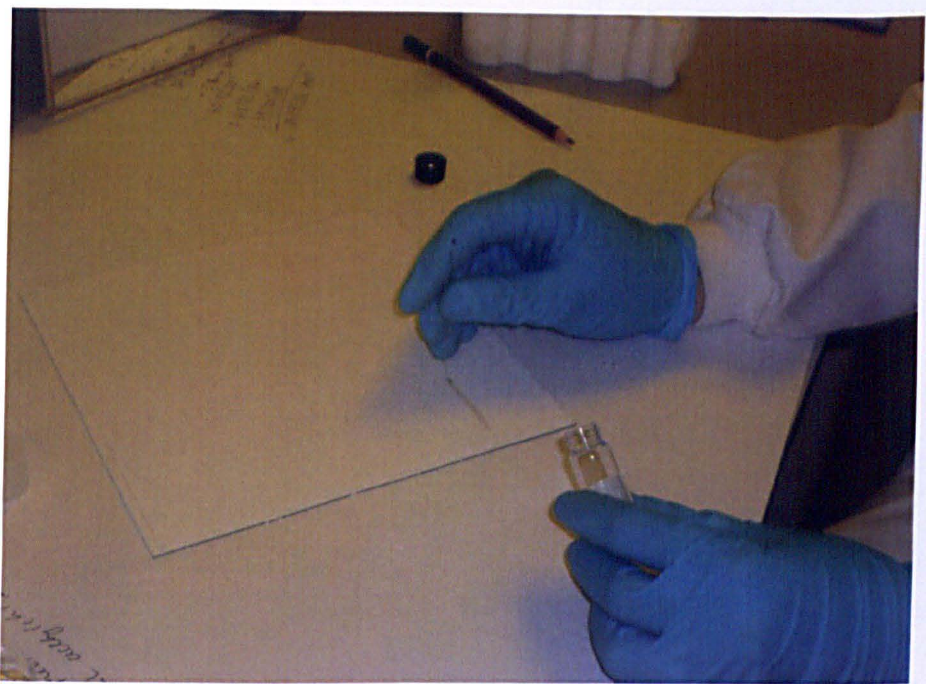

Figure 9: Application of sample to TLC plates

iii. Development of TLC plates

The plates were carefully placed in the TLC tanks while trying not to disturb the equilibrium of the atmosphere, and the lids replaced, ensuring a good seal. They were left to develop for 90 minutes, or until the solvent front had reached the line scored at the top of the plate. Following this, the plates were left in the tank for another 10 mins to improve the definition of the bands. The plates were subsequently removed and allowed to dry in a darkened room.

iv. Visualisation and identification

The plates were removed from the tank, dried in a stream of cold air, and sprayed with 0.1% solution of 2,7-dichlorofluoroscein in methanol in the fume cupboard until the plate was lightly and evenly covered. The plates were dried again under a stream of cold air. The bands were visualised under UV light (Figures 10 & 11 – plasma and RBCs), and the perimeter of the band areas marked with a soft pencil. The bands were identified

using the phospholipid standards. The appropriate bands were scraped onto filter paper using a flat, blunt spatula, and the dust and flakes transferred to labelled methylating tubes via a filter funnel.

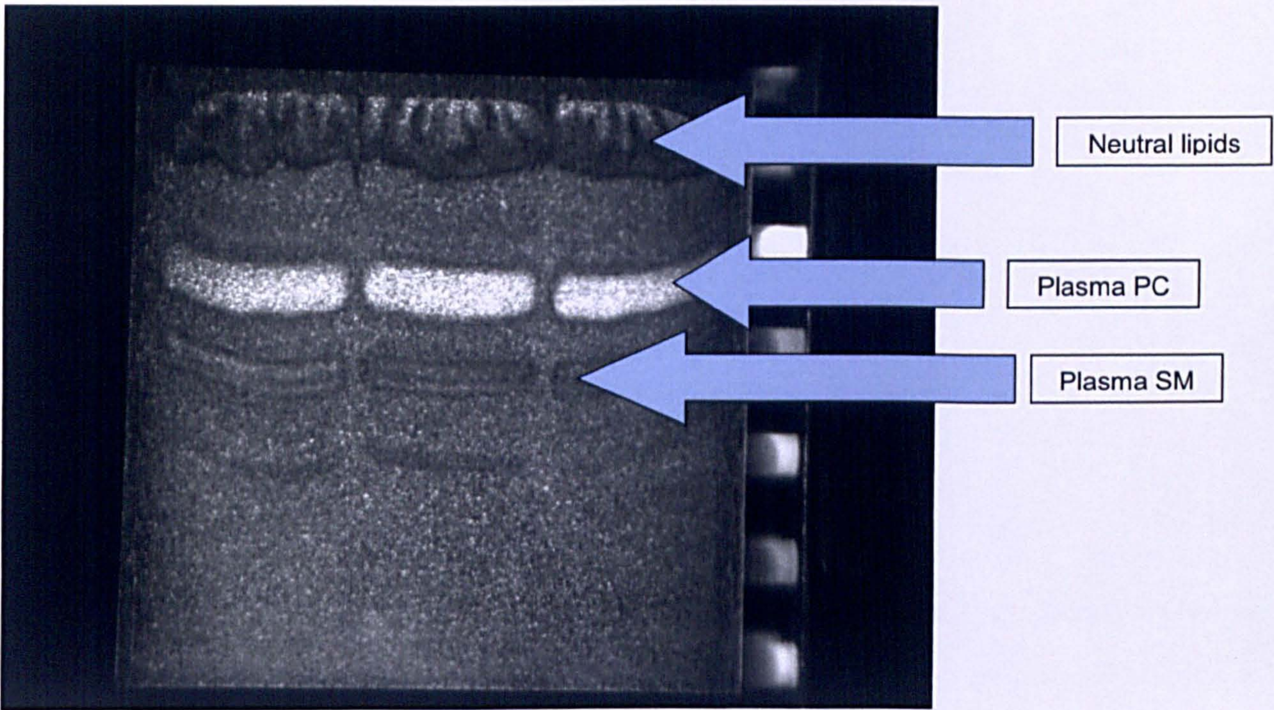

Figure 10: Visualisation of plasma NL and PL bands

PC: phosphatidyl-choline, SM:sphingomyelin

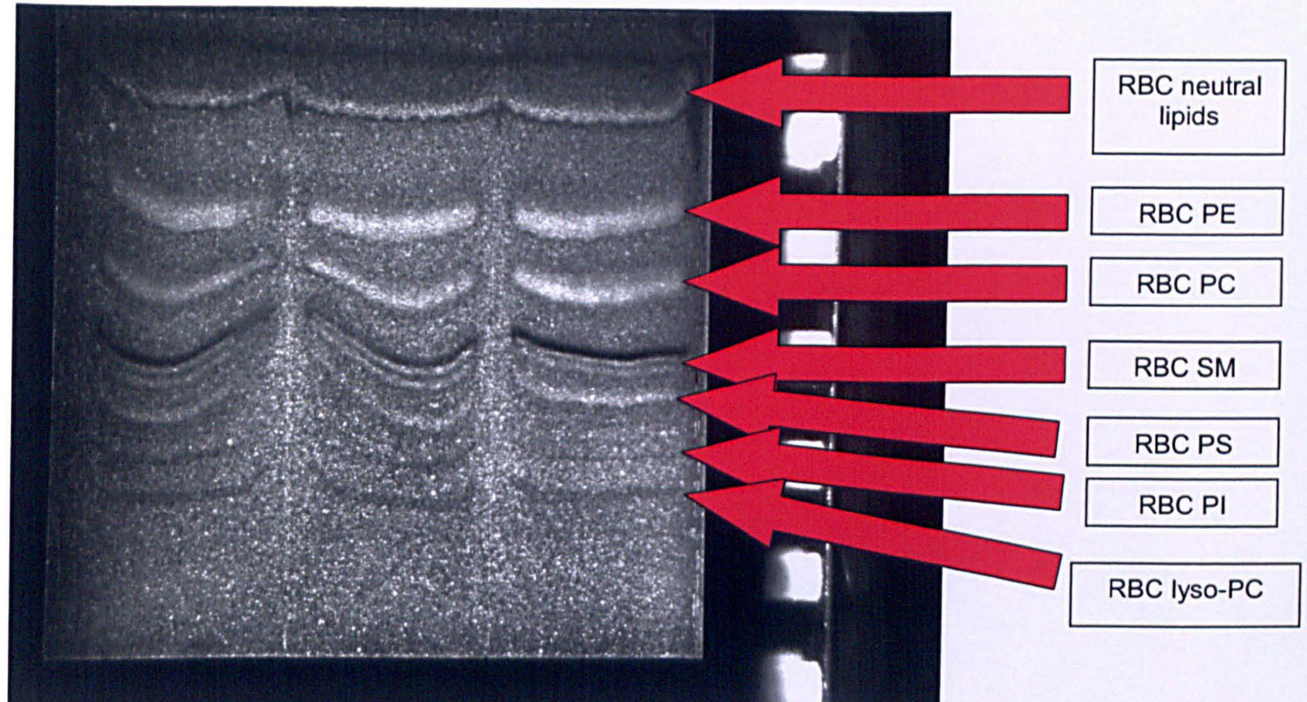

Figure 11: Visualisation of red blood cell PL bands

PE:phosphatidylethanolamine, PC:phosphatidylcholine, SM:sphingomyelin, PS:phosphatidylserine, phosphatidyl-inositol, lyso-PC:Lysophosphatidylcholine

v. Separation of plasma neutral lipid fractions

The neutral lipid (NL) bands are scraped from the phospholipid plate into stoppered centrifuge tubes. 5-10 mls of petrol spirit/diethyl ether (50:50) is added and the tubes shaken well. The tubes are centrifuged at 3000 rmp for 5 mins. The supernatant was removed to a 10 ml vial and the petrol/ether evaporated under a stream of nitrogen. The petrol/ether wash was repeated, and the extract was pooled with the first extract and dried down. 1-2 ml chloroform/methanol and BHT was mixed with the dried lipid, and the solution flushed and stored at 4C until ready for TLC, or TLC'd immediately. The TLC plates were prepared as described previously. The NL solvent used was petrol/ether/formic acid/methanol, 85:15:2.5:1 and BHT at 100mg/l. The NL bands were scraped into methylating tubes as described before. The cholesterol ester band was

located at the top of the plate, and the triglyceride band approximately ¾ way up the plate (Figure 12).

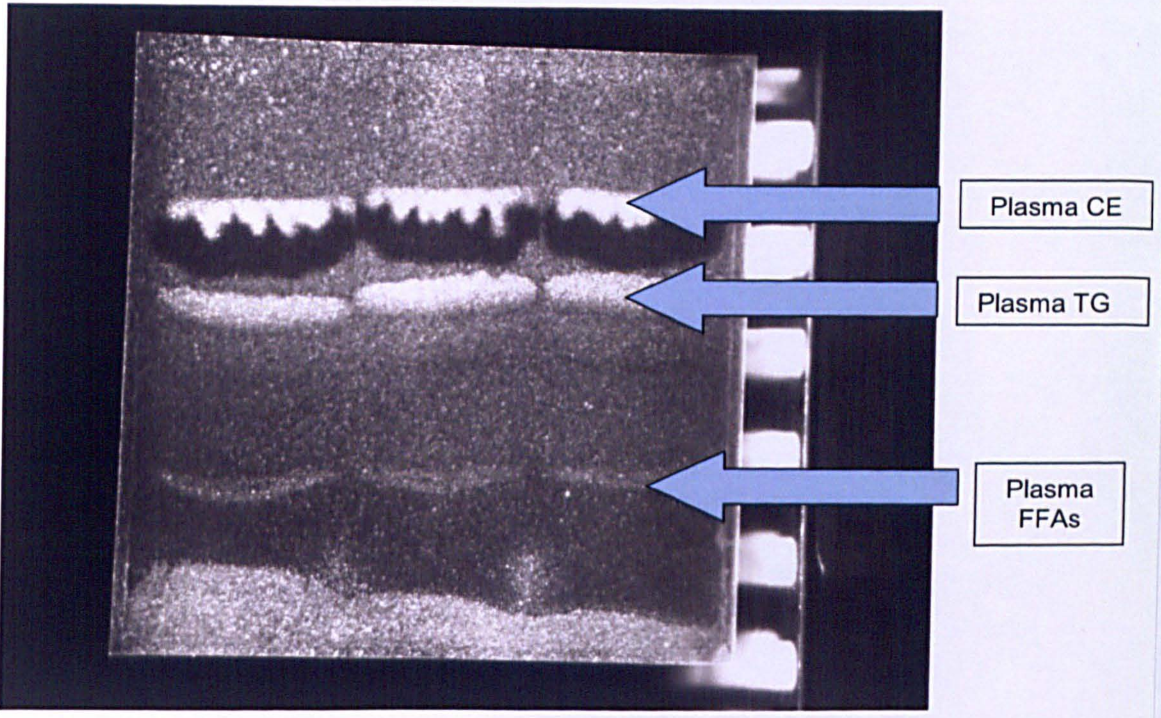

Figure 12: Visualisation of plasma NL bands

2.7.5. Preparation of fatty acid methyl ester

i. Transmethylation

The methylating reagent, 15% acetyl chloride in methanol, must be prepared fresh, and should be no more than 2 days old. 15 ml acetyl chloride was added drop wise to 100 ml dry methanol in a 500ml conical flask, while swirling the flask in the fume cupboard, being careful not to let it boil. The mixture was transferred to a stoppered bottle. 4 ml of the methylating reagent was transferred using a Pasteur pipette, to the bands scraped into the methylating tubes. The solution in the methylating tubes was flushed with nitrogen, vortexed well and the level of the liquid in the tube marked with pen, prior to placing them in the oven. Samples were methylated at 70C for 3 hrs. At 1 and 2 hrs, the

level of the methylating mixture was checked to ensure it had not decreased. Tubes were vortexed and returned to the oven. At the end of the 3 hrs, the tubes were removed from the oven and allowed to cool to room temperature.

ii. Extraction of fatty acid methyl esters

Three rows of tubes were arranged, with 2 test tubes behind each methylated sample. 4 ml of 5% saline and 2 ml petrol spirit and BHT were added to each sample. Methylated tubes were capped and shaken vigorously. Any emulsion formed was broken with a few drops of methanol. The upper petrol layer was carefully removed to a test tube containing 2 ml 2% potassium bicarbonate to neutralise the acid transferred. 1 ml of petrol was added to methylating tube, mixed, removed and pooled with the previous petrol extract in the test tube containing the potassium bicarbonate. This step was repeated again. The test tube containing petrol extract and potassium bicarbonate was vortexed. The upper layer which was formed was removed to a test tube containing 100-200 mg sodium hydroxide, to remove any water in the sample. The solution of fatty acid methyl esters in petrol was carefully removed to a 3 ml vial. The petrol was removed under a stream of nitrogen, and the sample was taken up in 1 ml of heptane, before flushing with nitrogen and storage at 4C or –20 prior to running on the GLC.

2.7.6. Analysis of fatty acid methyl esters by Gas-Liquid Chromatography

i. Background

The concept of Gas chromatography as an analytical tool was first suggested by A.J.P. Martin and R.L.M. Synge (1941) in a paper on liquid

chromatography, as a means of improving speed and efficiency of chromatographic separation. The concept was developed in practice by Martin and A.Y James when they published their epic paper describing the first gas chromatograph, and its use in separation and estimation of volatile fatty acids in 1951.

Gas chromatography is a technique used to separate volatile organic compounds dependent on differences in their partitioning behaviour between the mobile phase and the stationary phase in the column. A gas chromatograph consists of a flowing mobile phase (an inert gas), an injection port, a separation column containing the stationary phase, a detector, and a data recording system (Figure 13).

A sample of the mixture to be analysed, is introduced by a syringe, via an injector port, into the gas mobile phase before it encounters the stationary phase. The components are separated by elution and are detected at different times, dependent on their retention or the time it takes them to travel through the column. The areas under the chromatographic peaks are proportional to the amount of the components (fatty acids) present in the sample, and are calculated by a computer linked to the detector.

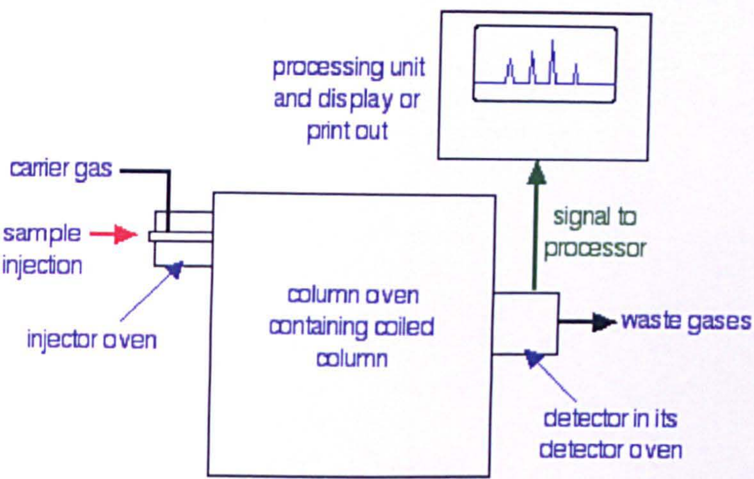

Figure 13: Diagrammatic representation of the GC

http://www.chemguide.co.uk/analysis/chromatography/gas.html

ii. Analysis of fatty acid methyl esters

Fatty acid methyl esters were transferred to brown autosampler vials for GC analysis. 15 drops of sample were used to measure CE, 9 drops were used to measure PC and 15 drops were used to measure TG (where 1 drop equals a nominal volume of 10ul). These were dried down and 1-2 drops of heptane + BHT were added. This was flushed with nitrogen and sealed with a labelled, tight fitting cap. Samples were then loaded onto the autosampler. GC analysis of fatty acids for all individual phospholipids samples was carried out during the same run.

FAME were separated by a gas liquid chromatograph (HRGC MEGA 2 series, Fisons Instruments, Milan, Italy) fitted with a capillary column (30mx0.32mm ID-BP20 0.25µm from SGE, Milton Keynes, UK) with an injection volume of 1.5 µl and a split ratio of 17:1. Hydrogen was used as carrier gas, and the injector and detector temperatures were 240°C and 260°C, respectively. Oven temperature programme started at 150°C for 1 minute, followed by an increase of 3.0°C/min to 205°C and, after 5 minutes at 205°C, a final increase of 2.0°C/min to 220°C. Flame ionization detector signals were evaluated with the software EZChrom

Elite version 3.2 (Scientific Software, Pleasanton, CA, USA). FAME peaks were identified by comparison with standards (Sigma-Aldrich, Gillingham, UK). Values were calculated as area percentages (Area%) of all identified fatty acids (8 - 24 carbon atoms). Additionally, the sums of total PUFA, n-3 PUFA, n-6 PUFA, SFA and MUFA as well as the ratios of DHA to DPAn-3, DHA to ALA, AA to dihomo-gamma linolenic acid (DGLA, 20:3n-6), AA to EPA, AA to DHA and n-6 PUFA to n-3 PUFA were calculated.

iii. Recoveries and co-efficients of variation

Recoveries for all sample runs were >95% of fatty acids, and low coefficients of variation were observed for controls which were run every 10 samples. Low intra-assay fatty acid coefficients of variation confirmed consistency of results, an acceptable level of variability, and correct operation of the instrument. For example, 96.7% recovery, and the following coefficients of variation were observed for plasma PC: 3.7% for Oleic acid, 3.04% for LA, 0.08% for ALA, 2.5% for EPA, and 1.1% for DHA.

2.8. Body composition

All anthropometric measurements were undertaken by the same trained individual.

2.8.1. Height and weight

Height was measured to the nearest 0.5cm using a portable stadiometer. Weight was measured with participants wearing light clothing and no shoes. One kg was subtracted from weight for remaining clothes. Body mass index (BMI) was calculated. Weight and body composition data were determined using the same instrument (Tanita Segmental Body Composition Analyser, BC-

418MA, Tanita Corporation14-2, 1-Chome, Maeno-Cho Itabashi-Ku, Tokyo, Japan T174).

2.8.2. Body fat

Proportions of body fat and lean body mass were determined by bioelectrical impedance analysis (Nunez et al., 1997, Tan et al., 1997).

2.8.3. Tanita

The Tanita BC-418 segmental body composition analyser was used to assess body composition. The segmental reader separates results into fat %, fat mass (kg), and fat free mass (kg), and predicted muscle mass for right arm, right leg, left arm, left leg, and trunk. It gives a print out for weight, BMI, BMR, fat %, fat mass, fat free mass, total body water, desirable body fat ranges, segmental body fat information.

A sample print out is found on the following page (Figure 14). The BC-418 body composition analyser has been validated in a study involving healthy individuals and results correlated highly ($R^2 = 0.94$, $p<0.001$) with reference estimates of %fat and appendicular lean soft tissue by dual-energy x-ray absorptiometry (DXA; Lunar DPX, M Madison, W1) (Pietrobelli et al., 2003).

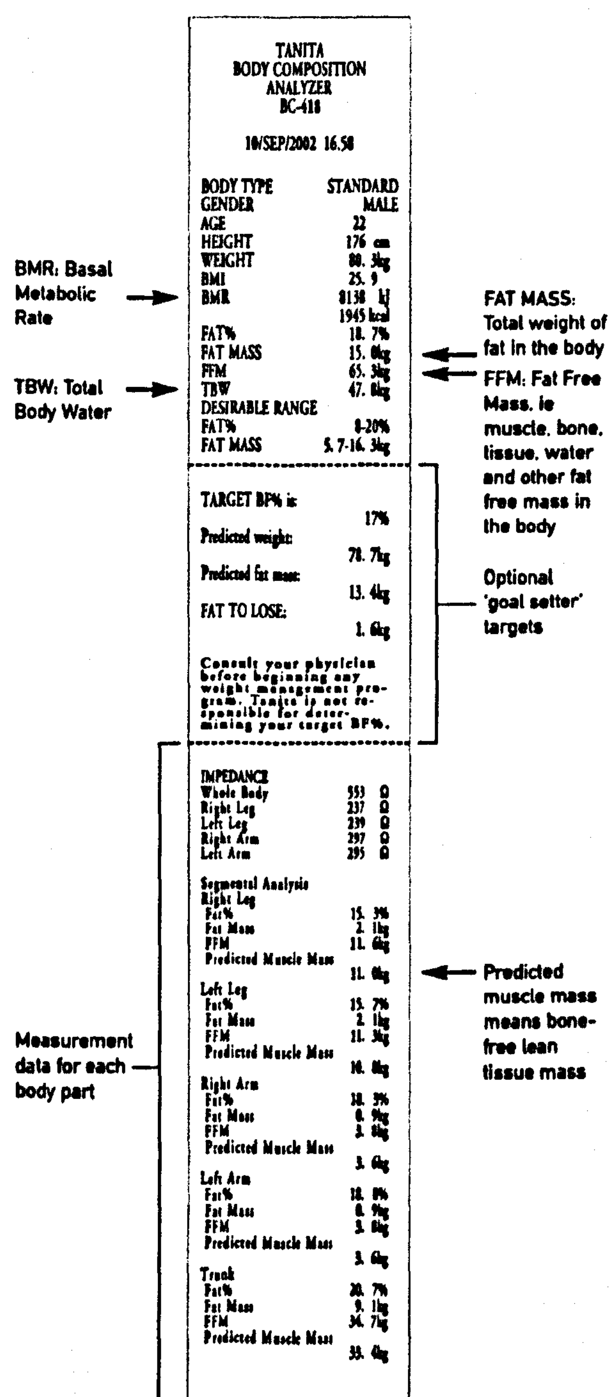

Figure 14: Printout of body composition results from Tanita analyser

2.8.4. Blood pressure

Blood pressure was measured (at least twice and an average taken, same arm) with subjects in the sitting position, feet uncrossed on the floor, and remaining silent, using a digital upper arm blood pressure monitor (Omron 705IT, Shiokoji Horikawa, Shimogyo-ku, Kyoto 600-8530Japan).

2.9. Statistical analyses

Results are presented as means and standard deviations. Data were checked for normality by Shapiro-Wilk normality test and visual inspection of histograms. Differences between variables in men and women were compared by Independent t tests (for normally distributed variables) and by Mann Whitney U tests (for non-normally distributed variables). The log transformation was used as necessary to normalise skewed variables and associations between variables were by (multiple) linear regression (Stepwise method) or Pearson's bivariate correlations (for non-normally distributed variables). For bivariate tabular analysis the chi-square test was used. In cases of expected values smaller than 5, a Fisher exact test was used instead. Exact significances were calculated for all nonparametric tests. Unless otherwise stated, results are given as mean values with corresponding standard deviations (SD). P values below 5% were considered significant.

All the calculations were performed by use of the statistical package SPSS for Windows, Release 16, and R (2.9.1, www.r-project.org). R is an open source online environment for statistical computing. R allows variables and factors to be entered simultaneously into a regression equation.

3. Dietary Fat Intake and Blood Lipids of University Men and Women (Study 1)

3.1. Introduction

The prevalence of obesity continues to rise in the UK, as in many other Western and industrialised countries (Smith, 2007) and is associated with cardiovascular disease (CVD), mediated through the metabolic syndrome (Reaven, 2005). However, this may not be an inevitable consequence of obesity, because CVD is absent in certain obese, high fat consuming populations (Fujimoto, 1996; Mavoa & McCabe 2008), and has led researchers to consider the quality, rather than the quantity of dietary fat (Mozaffarian, 2005; Willett, 2008).

Much is known about the effects of saturated and polyunsaturated fats on lipoproteins, insulin resistance and obesity (Balk et al., 2006; Ma et al., 2006; Arefhosseini et al., 2009). Nevertheless, differences are seen in body composition, and blood glucose and lipid profiles of pre-menopausal women and men of a similar age (Couchepin et al., 2008) despite apparent similar percentage energy intakes from these fats (Henderson et al., 2002; Bakewell et al., 2006). Compared to women of child-bearing age, men appear to be more at risk for CVD. There is a scarcity of comprehensive published data examining dietary factors relating to this gender disparity.

Current dietary fat recommendations are similar for men and women (DH, 1991), and evaluation of dietary cardioprotection is based on a balance of individual fats. In the UK population, saturated and n-6 fat intakes are higher than recommendations (Henderson et al., 2002) and dietary n-3 fatty acids lower than Northern European and Mediterranean countries (Welch et al., 2002). While these surveys recruited heterogeneous socioeconomic and anthropometric populations, with varying dietary

intakes, the current study compared dietary fat intakes in a homogenous Caucasian group of young men and women, and investigated whether there are relationships between quality of dietary fat and blood glucose and lipoprotein (HDL, LDL, total cholesterol, TGs) levels.

3.2. Subjects, Materials and Methods

These are described in full in chapter 2.

3.3. Results

Due to incomplete food diaries, four subjects (3 males, 1 female) were initially excluded from the analysis. Dietary, body composition and blood parameter results were available for 100 subjects (49 males, 51 females).

When data was corrected for under-reporting (corrected group: PAL<1.2), a further 11 women and 18 men were excluded from the analysis. 40 women and 31 men were included in the final analysis. Results were compared with data from the original total sample group (uncorrected group: 51 women, 49 men).

Of the corrected group, subject occupations were as follows: 13 female, 1 male nutrition students; 4 female, 2 male sports students; 4 female, 2 male other students; 17 female; 19 male staff; 2 female, 7 male other workers who had associations with the university. Similar numbers of men and women exercised (29 women and 23 men), 4 women and 4 men smoked.

Gender differences in the results from the corrected sample are discussed below, and comparisons made between the data in the corrected and uncorrected sample groups.

3.3.1. Anthropometric, clinical and demographic data

Anthropometric, clinical and demographic data are presented in Table 5. The men had higher weight, height, fat free mass, basal metabolic rate and systolic blood pressure ($p<0.001$) and lower trunk and body fat ($p<0.001$) compared with the women. There were no differences in mean age, diastolic blood pressure, and weekly exercise taken between the two groups ($p>0.05$), however mean alcohol intake was higher in the men ($p<0.01$).

BMI differed more in the men and women in the uncorrected group compared to the corrected group ($p<0.001$ vs $p<0.01$).

3.3.2. Energy and macronutrients

Dietary energy and macronutrient intakes of the volunteers are shown in Table 6. The men consumed more energy, and absolute carbohydrate, protein and fat than the women (CHO: $p<0.01$, Others: $p<0.001$). However, there were no significant differences in protein and fat consumption between the men and women when intakes were expressed as a percentage of total energy. Energy intake was higher in men, both when it was expressed as absolute intakes ($p<0.001$), and per kilogram of body weight ($p<0.05$), but energy intakes were similar between the genders when expressed per kilogram of fat free mass.

When comparing corrected with uncorrected groups, greater differences were found between men and women in the following: kcals/kg body weight ($p<0.05$ vs NS); carbohydrate intake (g/d) ($p<0.01$ vs 0.001), and %energy ($p<0.05$ vs 0.01). Similar gender differences in dietary intakes of the other macronutrients were found between the corrected and uncorrected groups.

3.3.3. Dietary Fatty Acids

Dietary intake of the fatty acid groups is presented in Table 7. The consumption of saturated (p<0.001), total polyunsaturated (p<0.05), n-6 (p<0.05) and n-3 (p<0.05) fatty acids was higher in the men than in the women. However, when the intakes were expressed as percentage energy intakes, only the intake of saturated fatty acids was significantly higher in the men (p<0.05).

There were no differences in individual fat intakes between the men and women in the corrected and uncorrected groups except for total polyunsaturated (g/d) and n-6 fat intakes (p<0.05 vs p<0.01; p<0.05 vs p<0.01 respectively).

3.3.4. Blood glucose and lipoprotein parameters

The levels of blood glucose and lipids are shown in Table 8.

Plasma glucose (p<0.001), TGs (p<0.05), LDL-cholesterol (p<0.05), and LDL:HDL ratio (p<0.001) were higher, and HDL cholesterol lower (p<0.001) in the men compared with the women. There was no difference in total cholesterol level between the two genders (p>0.05).

Gender differences in results were comparable between the uncorrected and corrected groups, except that in the corrected group only, triglycerides differed significantly between the men and the women (p<0.05)

3.4. Results Tables

Table 5: Subject characteristics – Study 1

	Women (n=40)		Men (n=31)		
	Mean ± SD	Median	Mean ± SD	Median	*P*
Age (yrs)	31.9 ± 6.6	30.5	32.7 ± 7.7	33	NS
Weight (kg)	60.5 ± 6.6	59.6	75.3 ± 10.6	75.2	<0.001
Height (m^2)	166.3 ± 5.9	166	178 ± 7.3	178	<0.001
BMI (wt/ht^2)	21.9 ± 2.7	21.4	23.7 ± 2.6	23.3	<0.01

Body fat (%)	26.5 ± 5.7	25.8	15.6 ± 5.3	16.2	<0.001
Trunk fat (%)	23.3 ± 6.9	22.2	16.8 ± 7.1	17.6	<0.001
Fat free mass (kg)	44.2 ± 2.7	43.7	63.1 ± 6.7	62.5	<0.001
Systolic BP (mm/Hg)	112.0 ± 18.4	114.0	126.4 ± 11.0	128.5	<0.001
Diastolic BP (mm/Hg)	72.5 ± 5.6	72.0	76.2 ± 8.6	75.0	<0.05
BMR (kcal)	1338 ± 79.6	1326	1841 ± 212	1832	<0.001
Exercise (hrs/wk)	2.52 ± 1.97	2.5	3.45 ± 2.88	3.0	NS
Alcohol (units/wk)	11.1 ± 12.2	7.0	24.6 ± 19.0	27.5	<0.01

BMI: Body mass index (wt/ht^2), BP:Blood pressure, BMR:Basal metabolic rate

Table 6: Energy and macronutrient intakes – Study 1

	Women (n=40)		Men (n=31)		
	Mean ± SD	Median	Mean ± SD	Median	*P*
Energy (kcal/d)	2020 ± 362	1959	2702 ± 470	2642	<0.001
(kcal/kg body weight)	33.7 ± 6.7	32	36.2 ± 6.1	35.9	<0.05
(kcal/ffm)	45.7 ± 7.9	44.2	42.7 ± 5.7	41.8	NS
Carbohydrates (g/d)	244 ± 52.3	242	294 ± 78.4	275	<0.01
(%energy)	45.6 ± 6.6	45.5	40.7 ± 7.5	39.0	<0.01
Protein (g/d)	75.4 ± 17.6	70.0	99.5 ± 26.6	94.0	<0.001
(%energy)	15.0 ± 2.8	15.0	14.8 ± 3.2	14.0	NS
Fat (g/d)	79.0 ± 22.0	74.0	105.0 ± 25.0	108.0	<0.001
(%energy)	35.1 ± 6.3	34.0	35.1 ± 6.2	35.0	NS
Fibre (g/d)	14.9 ± 4.7	15.5	16.2 ± 4.7	16.0	NS

Table 7: Individual fat intakes - Study 1

	Women (n=40)		Men (n=31)		
	Mean ± SD	Median	Mean ± SD	Median	*P*
Total saturated fat (g/day)	22.3 ± 8.1	21.0	32.8 ± 8.6	32.0	<0.001
(%energy)	9.8 ± 3.1	10.0	11.0 ± 2.0	11.0	<0.05
Polyunsaturated fat (g/day)	13.5 ± 6.0	11.0	16.1 ± 6.4	15.4	<0.05
(%energy)	6.1 ± 2.1	5.0	5.6 ± 2.1	5.0	NS
Total omega-6 fat (g/day)	11.5 ± 5.6	9.5	13.6 ± 5.8	12.6	<0.05
(%energy)	5.1 ± 1.9	4.5	4.5 ± 1.9	4.0	NS
Total omega-3 fat (g/day)	1.9 ± 1.0	1.7	2.5 ± 1.1	2.2	<0.05
(%energy)	0.9 ± 0.5	0.8	0.8 ± 0.3	0.7	NS
omega-6:omega-3 PUFA	6.9 ± 3.5	6.7	6.0 ± 2.2	6.0	NS
EPA&DHA (g)	0.36 ± 0.36	0.25	0.39 ± 0.44	0.21	NS

Table 8: Mean glucose and lipid parameters (mean ± SD) - Study 1

	Women (n=40)		Men (n=31)		
	Mean SD	Median	Mean SD	Median	P
Blood glucose (mmol/l)	4.81 ± 0.50	4.90	5.41 ± 0.69	5.30	<0.001
Triglycerides (mmol/l)	0.98 ± 0.35	0.90	1.22 ± 0.45	1.20	<0.05
Total cholesterol (mmol/l)	4.61 ± 0.86	4.40	4.79 ± 1.35	4.75	NS
HDL-cholesterol (mmol/l)	1.87 ± 0.45	1.80	1.47 ± 0.35	1.45	<0.001
LDL-cholesterol (mmol/l)	2.55 ± 0.67	2.50	2.90 ± 0.62	3.00	<0.05
LDL:HDL	1.44 ± 0.55	1.29	2.09 ± 0.54	2.06	<0.001

HDL:high density lipoprotein, LDL:low density lipoprotein

Table 9: Linear regression model for blood glucose and lipoprotein parameters and dietary fat intakes – Study 1

		Women (n=40)				Men (n=31)			
Dependent Variables	**Independent variables**	R^2 adjusted	Beta	SE	p	R^2 adjusted	Beta	SE	p
LDL-chol						0.214			
	pctEln-6						-0.382	0.138	<0.01

All variables transformed except for HDL-cholesterol in all subjects, and systolic blood pressure in women, all included fatty acids expressed as percentage of energy intakes

Excluded variables:

- Independent: totkcals, kcal/kgbw, pctElcho, pctEltotfat, pctElprot, pctElsatfat, pctElmufa, pctEIPUFA, pctEIALA, pctEIEPA, pctEIDHA, pctEln-3

- Dependent: totchol, TGs, BG, HDL, SBP,

totkcals: total energy intake; kcal/kgbw: energy intake per kg body weight; pctEl:percentage of total energy intake, cho: carbohydrate; satfat:saturated fat; mufa:monounsaturated fat; PUFA:polyunsaturated fat; ALA:alpha-linolenic acid, EPA:eicosapentaenoic acid; DHA:docosahexaenoic acid; n-3: omega 3; n-6: omega 6; TG triglycerides; BG: blood glucose; HDL: HDL cholesterol; SBP: systolic blood pressure

A linear regression for blood glucose and lipoproteins involving dietary macronutrient and total and individual fat intakes (Tanle 9) indicated a negative association between omega 6 intake and LDL-cholesterol (p<0.01) in men only.

In the uncorrected sample, positive and negative associations were found between omega 3 ($p<0.01$), and omega 6 intake ($p<0.01$), and LDL-cholesterol in men only.

3.5. Discussion

We found that in this Caucasian population who were younger and more educated than the general population (Henderson et al., 2002; [1]), the men consumed more energy, and absolute but not proportional amounts of protein, carbohydrate and fat than the women. The contribution of polyunsaturated fatty acids to total energy intake was close to population recommendations of 6% (DH, 1991), but the combined intake of EPA and DHA was lower than UK recommendations of 0.45g/d (SACN 2004) and European recommendations of 0.5g/day (ISSFAL, 2004).

The assessment of nutrient intakes by estimated food diary is not as robust as other methods (Bingham et al., 1995), hence food portion size photographs were used for greater accuracy. Our uncorrected findings are broadly in line with results of weighed intakes from the National Diet and Nutrition Survey (Henderson et al., 2002) suggesting alignment with the general population: similar percentages of men and women achieved the estimated average requirements for energy, and proportional energy intakes from carbohydrate, protein and fat were comparable between the datasets. Nevertheless, mean percentage energy intakes for carbohydrate; and fibre were below recommendations in our subjects (DH, 1991).

When we corrected data for under-reporting (PAL<1.2), similar differences were found in anthropometric measurements, blood glucose and lipoprotein levels, and dietary intakes of the men and women in this study. Although total energy intakes

[1] http://www.ons.gov.uk/ons/guide-method/census/2011/index.html

differed between the genders, they did not appear to be due to specific under-reporting in one macronutrient, because percentage energy intakes from macronutrients were similar in the uncorrected, and corrected groups. More men than women (36.7% vs 21.6%, $p<0.05$) were found to either under-report or under-estimate dietary intake. This has been found previously (Gnardellis 1998), but not exclusively (Ferrari et al., 2011). Despite this a comparison of energy intakes with NDNS data revealed more similarity with our data when it was uncorrected for under-reporting, probably because data in the NDNS survey was not corrected for under-reporting. The NDNS feasibility study which compared results from diet diaries and the doubly labelled water method, suggested that under-reporting was in the region of 25% and similar in men and women (Henderson et al., 2002).

Further comparisons with NDNS data revealed that anthropometric measurements (weight and BMI) were higher in the NDNS population compared to our subjects (weight: men 84kg vs 75.3kg, and women 69kg vs 60.5kg; BMI: men 27.2 vs 23.7, and women 26.4 vs 21.9). In addition, men and women in our study reported a higher mean weekly number of hours spent in activity of at least moderate intensity compared to subjects in the NDNS (men: 3.52 vs 2.2hrs, and women 2.45 vs 1.2 hrs). Taken together, this indicates that subjects in our study may be more health conscious, although alcohol intakes were higher in our volunteers, possibly due to numbers of students (and university staff) in our population. Nevertheless, it came as a surprise to find that daily percentage intakes of n-3 fatty acids in our sample group (0.8% in men, 0.9% in women) were below national averages (NDNS men and women both 1%, Henderson et al., 2002).

Consistent with previous reports (Hazzard, 1985; Magkos et al., 2008), we found gender variations in some parameters, despite similar alcohol and exercise levels: triglycerides, LDL cholesterol and systolic blood pressure were higher in men and

HDL cholesterol level was higher in women. Surprisingly although the women had higher trunk and total body fat than then men, female systolic blood pressure was lower. This has been reported before (Gupta and Kapoor 2010), and may be because of a protective effect of one of the sex hormones, perhaps oestrogen (Mendelsohn & Karras 1999), or because in women a greater degree of adiposity and higher lipid risk profile is required before adverse effects on blood pressure are observed (Després et al., 1985).

We were interested in examining statistical associations between quantity and quality of fat intake and blood glucose and lipoprotein levels in the men and women in our study, and a linear regression analysis indicated that n-6 fat intake was inversely associated with LDL-cholesterol in men only. In contrast, no associations were observed in the women, or between dietary fat and any other lipoprotein parameter in either gender. We have been unable to find previous evidence from the literature to support this gender discrepancy. While this may be explained by a statistical coincidence, the significance level of 1% indicates the higher strength of the finding and suggests that it may be a valid finding.

In fact, research in hamsters and other rodents suggests the possibility of a gender-diet interaction, and this could offer a feasible explanation for differences in lipoprotein levels in males and females (Morise et al 2004, 2006). Furthermore, there is interesting data in humans regarding differential effectiveness of LC n-3 FAs on platelet aggregation between the genders (Phang et al., 2009). Only EPA has been found to be effective in reducing platelet aggregation in men, whereas EPA, DPA and DHA appear to be equally effective in women. In considering the mechanism for the differential effect between the genders, both sets of researchers suggested that oestrogen may play a role via effects on hepatic lipid metabolism and platelet size/reactivity respectively (Morise et al 2009, Phang et al 2009).

Unfortunately, however, since we did not collect data on oestrogen/sex hormone levels in our subjects, we may only speculate on this statistical finding in our young volunteers. Furthermore, previous studies examining health effects of oestrogen have produced conflicting results (Bhupathy et al., 2010) and suggest that the interaction between diet, gender and health is likely to be the synergistic effect of a plethora of additional intrinsic and extrinsic factors such as genes, lifestyle and environment.

3.6. Conclusion

In summary, dietary intakes in this younger, more educated Metropolitan microcosm were similar to results from the NDNS. There were gender differences in energy and macronutrient intakes including total omega 3, however most differences, except for saturated fat, disappeared when they were adjusted for weight or energy intake. Nevertheless, it is troubling to note that both genders failed to meet recommendations for n-3 fat intakes. Results suggest that even in this healthy educated young population, gender differences exist in blood glucose and lipoprotein levels, and in their response to dietary fat. Further research should be carried out to determine appropriateness of dietary fat recommendations for this age range, which currently do not account for gender.

4. Dietary micronutrient intakes and associations with blood lipoproteins and fatty acids in healthy men and women. (Study 2)

4.1. Introduction

Cigarette smoking, obesity and certain dietary patterns are well-known risk factors for chronic non-communicable illnesses such as cardiovascular disease and cancer. Yet in recent years, increasing evidence points to the role of reactive oxygen species in these pathological processes, and the antioxidant involvement of a host of dietary nutrients. In low/moderate amounts, reactive by-products of oxidative and physiological processes are useful in intracellular signalling, defence against infectious agents and induction of the mitogenic response. However, deleterious overproduction of highly reactive molecules can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA, and may contribute to the development of atherosclerosis.

A complex antioxidant system exists, involving dietary nutrients such as magnesium, zinc, iron, copper, selenium, alpha-tocopherol, retinol and beta-carotene, to maintain the balance between damaging oxidative agents and antioxidant defence (Evans and Halliwell 2001). For example dietary zinc tempers the amplified atherogenic effect of copper and iron; copper and selenium are involved in production of antioxidants nitric oxide and glutathione peroxidase respectively (Yang and Wo 1987; Wo and Yang 1986; Ghayour-Mobarhan et al., 2005); magnesium protects against hypertension and insulin resistance (Houston and Harper 2008; Larsson and Wolk 2007); and vitamins A and E protect lipoproteins and lipid membranes from oxidative damage (Steiner 1981; Gey and Puska 1989; Islam et al., 2000; Hong et al., 2004; Ye and Song 2008).

Nevertheless, despite gender equality in access to protective dietary antioxidants, men in Western countries are more at risk of developing chronic and fatal non-communicable diseases, and of dying at a younger age, compared to women. Evidence from surveys of UK adults, and children (Zive et al., 1996; Henderson et al., 2002; Glynn et al., 2005) indicates that gender disparities in food choice, which originate in childhood, may be responsible for variations in micronutrient intakes in men and women. Previous reports suggest that endorsement of healthy eating is related to gender socialisation (Gough et al., 2006; Courtenay 2000), and this may influence observed inequalities in health and longevity in men and women. The aim of this study was to compare the dietary intake of men and women in a homogenous educated young Caucasian population, with particular reference to 7 key antioxidants (micronutrients), explore gender differences in dietary food choices, and associations between micronutrient intakes/plasma levels, blood glucose and lipoprotein parameters, and plasma and red blood cell fatty acid levels.

4.2. Subjects, Materials and Methods

These are described in detail in chapter 2.

4.3. Results

Subjects who reported taking vitamin and/or mineral supplements (n=12: 3 women and 9 men) were excluded from the original sample group (minus the incomplete diaries) ie. A further 11 women and 15 men were excluded due to under-reporting (PAL<1.2).

The final corrected sample group consisted of 38 women and 25 men. Subject occupations were as follows: 12 female nutrition students, 1 male nutrition student; 3 female sports students, 2 male sports students; 4 female other students, 2 male other students; 17 female staff, 13 male staff; 2 female, 7 male other workers with

associations with the university. Of the total sample 3 men and 3 women were smokers, and 28 women (74%) and 19 (76%) men exercised.

Plasma results were examined for heterogeneity using Excel Radar analysis, and compared with reference ranges for a normal healthy population. For each antioxidant micronutrient, results which were 2 SDs outside the mean were excluded.

4.4. Dietary Antioxidants (micronutrients)

Thirty eight women, and twenty five men were included in the dietary analysis.

4.5. Plasma Antioxidants

Plasma Copper

Twenty five women and seventeen men were included in the analysis for plasma copper.

Plasma Iron

Twenty four women and eighteen men were included in the analysis for plasma iron.

Plasma Magnesium

Thirty women and sixteen men were included in the analysis for plasma magnesium.

Plasma Zinc & Selenium

These results were excluded due to issues re sample collection. Possible reasons for specific effects on selenium and zinc levels may have been: use of unsuitable EDTA tubes for trace element analysis (Moyer et al., 1991; use of butterfly winged infusion needle for blood sampling (Hodnett et al., 2012); or haemolysis during blood separation.

Plasma Retinol and Alpha-tocopherol

Twenty four women and twenty two men were included in the analysis for plasma retinol and twenty six women and twenty two men for alpha-tocopherol.

4.6. Anthropometrics and clinical data

Table 10 shows the mean anthropometric and clinical data. Compared to the women, the men were taller, heavier, and had a slightly higher BMI but they had a higher fat free and, percentage wise, a lower fat mass ($p<0.01$ for BMI and trunk fat, $p<0.001$ for all others) . Despite the women being proportionately fatter than the men, their mean systolic blood pressure, blood glucose ($p<0.001$ for both), LDL and TGs ($p<0.01$, $p<0.05$) were lower, and their HDL ($p<0.01$) was higher.

Significance of gender differences in blood glucose and lipoprotein levels differed between the corrected and uncorrected sample groups respectively as follows: BMI: $p<0.01$ vs $p<0.001$; trunk fat: $p<0.01$ vs $p<0.001$; TGs: $p<0.05$ vs $p>0.05$; HDL: $p<0.01$ vs $p<0.001$.

4.7. Energy and macronutrient intakes

In the corrected group, Men had higher energy and absolute macronutrient intakes (CHO: $p<0.05$, Others: $p<0.001$ for all). They also had higher intakes of saturated and polyunsaturated fats ($p<0.05$ for both). When expressed as a percentage of energy intake, only saturated fat intakes were higher in men compared to women ($p<0.05$).

4.7.1. Micronutrient intakes

Table 11 shows the micronutrient intakes in the men and women. There was a considerable variation of micronutrient intakes in both genders. Zinc and selenium ($p<0.001$, $p<0.01$ respectively) were higher in men compared to women, while in the women beta-carotene and vitamin C ($p<0.05$ for both) was

higher than in the men. Mean vitamin A (retinol equivalents) in men was higher than the 75th centiles due to liver consumption in a few men. Similarly in the men Cu intake was similar to the 75th centile due to liver intake in a sample subset.

In comparing the uncorrected sample group compared with the corrected group, more significant differences were found between men and women in intakes of: selenium ($p<0.001$ vs $p<0.01$), beta-carotene ($p<0.001$ vs $p<0.05$) and vitamin C (NS vs $p<0.05$).

When we compared micronutrient intakes expressed as micronutrient densities between the genders (see appendix 9.7), women were found to have higher intakes of copper (0.88 ± 0.38 vs 0.86 ± 0.61 µg/d, $p<0.05$), iron (7.09 ± 7.01 vs 6.12 ± 6.04 mg/d, $p<0.01$), magnesium (183.6 ± 174.9 vs 149.1 ± 138.2 mg/d, $p<0.01$), beta-carotene (1.52 ± 1.47 vs 0.87 0.73 mg/d , $p<0.001$), vitamin C (72.1 ± 66.3 vs 40.8 ± 35.9 mg/d, $p<0.001$) and vitamin E (5.2 ± 1.92 vs 3.6 ± 3.5, $p<0.001$), although men had higher retinol intake (552.3 ± 251.6 vs 468.8 ± 392.3 µg/d, $p<0.05$), and no gender differences were seen in zinc and selenium intakes.

The average daily contribution of the different food groups towards micronutrient intakes (percentage of total daily intakes) in men and women is outlined in Table 12. Differences in food preferences of men and women were evident.

Vitamin A

Meat, dairy and fats and oils were major contributors to vitamin A intakes. Men appeared to derive a significantly higher amount of vitamin A from meat compared to women. Of the food groups, the food subgroups supplying the greatest dietary amounts of vitamin A in men were offal and products (640.5µg, 60.8%), meat products (99.07 µg, 9.4%), cheese (78.9 µg, 7.5%), and butter (62

μg, 5.9%) and in women were: meat products (96.4 μg, 25.2%), offal (65.4 μg, 17.1%), cheese (56.6 μg, 14.7%) and butter (37.4 μg, 9.8%).

Beta-carotene

Vegetables accounted for most of the dietary intake of carotene in men and women, with fruit, non-alcoholic beverages, dairy, fats and oils, and soups & sauces contributing small amounts. In comparing food sources in men and women, greater intakes from vegetables and fruit were observed in women compared to men. In particular, women derived dietary carotene from root vegetables (908 μg, 33.5%), leafy green vegetables (270 μg, 10%), raw salad vegetables (199 μg, 7.3%), and fresh fruit (160 μg, 6%); while the food subgroups which contributed most to carotene in men included: root vegetables (390 μg, 28%), leafy green vegetables (252 μg, 18%) and raw salad vegetables (114 μg, 8.1%). A few subjects with the highest intakes of beta-carotene reported consumption of carrot juice.

Vitamin E

In women higher vitamin E was supplied by nuts and seeds, and fruit, and in men, more vitamin E in the diet came from cereals and soups and sauces. On examination of the contribution made by different food subgroups, women derived vitamin E in greatest amounts from the following food subgroups: nuts and seeds (1.61 mg, 19.6%), vegetable oils (1.19 mg, 13.3%), and margarine (0.67 mg, 8.2%), while supply of vitamin E in men was from: breakfast cereals (0.96 mg, 13.2%), margarine (0.71 mg, 9.7%), vegetable oils (0.63 mg, 8.6%), and nuts and seeds (0.53 mg, 7.3%).

Iron

Cereals contributed most to dietary iron intakes in men and women, followed by meat, although intake of iron from meat in men was approximately twice that in women. The main food subgroup contributors in women were: breakfast cereals

(1.83 mg, 15%), brown and granary bread and rolls (0.82 mg, 6.7%), and in men highest contribution was from breakfast cereals (2.78 mg, 19.6%), brown and granary bread and rolls (0.97 mg, 6.8%), and alcohol (0.82 mg, 6.05%).

Copper

Cereals supplied the greatest proportion of dietary copper in the diets of both genders, with a small amount being supplied by vegetables. Meat also accounted for much of the dietary copper in men, while fruit was another food source in women. Of the food subgroups, in women 0.28 µg (20%) came from non-citrus fruits, nuts and seeds provided 0.15 µg (11%), 0.09 µg (6.4%) came from powdered drinks; and in men offal provided 0.61 µg (32%), fresh fruit not including citrus (0.11 µg, 5.8%), and nuts and seeds (0.11 µg, 5.8%) .

Zinc

Cereals and meat provided the greatest amounts of zinc in both men and women, although men consumed higher amounts of zinc in these food sources. Lesser amounts of zinc were supplied by dairy foods, nuts and seeds, and vegetables. Many food subgroups contributed to dietary zinc in both genders, however in women breakfast cereals (0.49 mg, 6.5%), nuts and seeds (0.45 mg, 5.9%), brown and granary bread and rolls (0.45 mg, 5.9%), and cheese (0.42 mg, 5.5%), provided the highest amounts. In men, the greatest food subgroup contributions were from: beef and veal (0.87 mg, 8.5%), breakfast cereals (0.8 mg, 7.8%), chocolate biscuits (0.68 mg, 6.6%), and meat products (0.53 mg, 5.2%).

Magnesium

In both genders, cereals, dairy, fruit, nuts and seeds and vegetables accounted for the majority of magnesium intake. Men appeared to derive a greater amount from alcoholic beverages, and meat, and women consumed more magnesium from fruits, and non-alcoholic beverages. The greatest amounts of magnesium

supplied in the food subgroups in women were: 46.21 mg (14%) from powdered drinks, essences and infusions (which included coffee), 33.54 mg (10.4%) from non-citrus fruits, 27.84 mg (8.6%) from nuts and seeds, 24.08 mg (7.5%) from breakfast cereals.

Vitamin C

Fruits, vegetables and non-alcoholic beverages contributed most to vitamin C intakes in both men and women. In particular in women, the subgroups supplying the greatest amounts of vitamin C were: fresh non-citrus fruit (31.22 mg, 29.4%), fruit juice (17.39 mg, 16.4%), fresh citrus fruits (13.52 mg, 12.7%), and leafy green vegetables (12.95 mg, 12.1%). In contrast, in men more vitamin C was supplied by fruit juice (30.33 mg, 38.5%), along with contributions from non-citrus fruits (11 mg, 14%), and leafy green vegetables (5.95 mg, 7.6%).

Selenium

Cereals, fish and nuts and seeds contributed most to selenium intake in men and women, with dairy meat, and poultry supplying lesser amounts. In women the greatest amounts of selenium came from the following sub-groups: white bread and rolls (8.78 µg, 17.8%), white fish (6.65 µg, 13.5%), nuts and seeds (5.28 µg, 10.7%), wholemeal bread and rolls (5.15 µg, 10.4%), , while in men, highest amounts came from: nuts and seeds (17.24 µg, 21.4%), white bread and rolls (12.21 µg, 15.2%), wholemeal bread and rolls (10.35 µg, 13%), and white fish (10.15 µg, 12.6%).

4.8. Plasma micronutrients

Figure 15 shows the mean plasma micronutrient levels in men and women. There were no significant gender differences in plasma micronutrients.

4.9. Correlations between dietary antioxidant micronutrients and plasma and RBC n-3 fatty acids ($p<0.01$)

These correlations are shown in Table 13.

Magnesium

RBCs

Dietary magnesium was associated with PC, PE and PS DHA in the women ($p<0.01$, $p<0.001$, $p<0.001$), while no associations were found in the men.

Selenium

RBCs

Dietary selenium was correlated with PS DHA in the men only ($p<0.01$).

Zinc

Plasma

In the women, dietary zinc was positively associated with CE and PC DHA ($p<0.001$ for each).

RBCs

There were many associations between dietary zinc and FAs in RBCs in the women only (RBC PC DHA $p<0.01$; PE EPA, PE DHA, PS EPA, PS DHA all $p<0.001$).

Iron

RBCs

In the women only, dietary iron was associated with PE and PS DHA ($p<0.01$, $p<0.001$ respectively).

Vitamin E

RBCs

Vitamin E was associated with PS DHA in the men only ($p<0.001$)

4.10. Correlations between plasma micronutrients, and lipoproteins or plasma and RBC fatty acids

The only correlation (inverse) with plasma micronutrients was between diastolic blood pressure and plasma magnesium in the women (-0.569, $p<0.01$).

The only correlation (inverse) found with plasma micronutrients and plasma and RBC fatty acids was between iron and RBC SM EPA level in men (-0.916, $p<0.001$).

4.11. Results Tables

Table 10: Anthropometric and clinical data – Study 2

Characteristic	Women (n=38)			Men (n=25)			
	Mean	SD	Median	Mean	SD	Median	P
Age (yrs)	31.9	6.9	30.5	31.8	7.2	31	NS
Weight kg	60.8	6.6	60.0	75.7	10.2	75.2	<0.001
Height m	166.4	5.9	166.0	177.7	6.8	177.0	<0.001
BMI (kg/ m^2)	21.9	2.7	21.5	23.9	2.8	24.4	<0.01
Body fat %	26.7	5.8	26.2	15.7	5.8	17.1	<0.001
Trunk fat %	23.2	7.5	22.3	16.7	7.3	18.5	<0.01
FFM (kg)	44.3	2.7	43.9	63.4	6.1	62.3	<0.001
SBP mm/Hg	111.8	18.6	113.5	127.1	12.4	127.0	<0.001
DBP mm/Hg	72.6	5.6	72.0	75.5	8.8	75.0	NS
Pulse mm/Hg	70.0	10.8	67.0	69.5	6.8	68.0	NS
BG (mmol/l)	4.8	0.5	4.9	5.4	0.7	5.3	<0.001
Chol (mmol/l)	4.6	0.9	4.4	4.4	1.4	4.8	NS
TG (mmol/l)	1.0	0.4	0.9	1.3	0.5	1.2	<0.05
HDL (mmol/l)	1.8	0.4	1.7	1.5	0.3	1.6	<0.01
LDL (mmol/l)	2.6	0.7	2.5	3.2	1.2	3.0	<0.01

BMI: body mass index, FFM: fat free mass, SBP: systolic blood pressure, DBP: diastolic blood pressure, BG: blood glucose, Chol: total cholesterol, TG: triglycerides, HDL: high density lipoprotein cholesterol, LDL: low density lipoprotein cholesterol

Table 11: Micronutrient intakes – Study 2

Characteristic	Women (n=38)					Men (n=25)					
	Mean	SD	Median	25th	75th	Mean	SD	Median	25th	75th	P
Energy (kcals)	2008	400	1916	1625	2106	2633	461	2587	2119	2718	<0.001
Protein	76	18	70	60	80	99	24	98	75	106	<0.001
Carbohydrate	241	57	239	1812	257	287	86	260	222	313	<0.05
Fat	80	24	75	60	87	101	23	95	73	114	<0.001
Alcohol (mean daily intake)	10.97	12.2	7	2.25	14.7 5	27.1	21.2	29.5	7.5	42.5	<0.01
Exercise (approx hrs/wk)	2.6	2	2.5	1.12	3.0	3.8	2.9	4.0	1.0	6 .0	NS
Cu (µg/d)	1.8	0.9	1.5	1.05	1.9	2.3	2.0	1.5	1.0	1.9	NS
Fe (mg/d)	14.2	4.4	13.8	10.8	15.7	16.1	4.6	15.8	10.9	17.8	NS
Mg mg/d	366	148	337	264	387	385	92	361	296	436	NS
Se (µg/d)	54	22	53	33.3	62.1	84	60	68	49.0	81.6	<0.01
Zn (mg/d)	8.8	2.1	8.9	6.9	9.9	11.7	3.4	11.0	8.6	13.3	<0.001
Vit A (Retinol Equivalents) (µg/d)	935	620	757	608	911	1116	1472	612	486	861	NS

Beta-caro (mg/d)	2.9	2.0	2.8	1.91	3.57	2.3	1.8	2.0	0.92	2.8	<0.05
Vit C (mg/d)	138	56	133	86.5	182	108	71	104	56.5	187	<0.05
Vit E (mg/d)	10.4	4.3	9.5	6.6	12	9.4	3.8	9.9	6.2	10.9	NS

Percentiles by weighted average

Mg: magnesium, Fe: iron, Zn: zinc, Cu: copper, Se: selenium, vit A: vitamin A, Beta-caro: beta-carotene, vit C: vitamin C, vit E: vitamin E

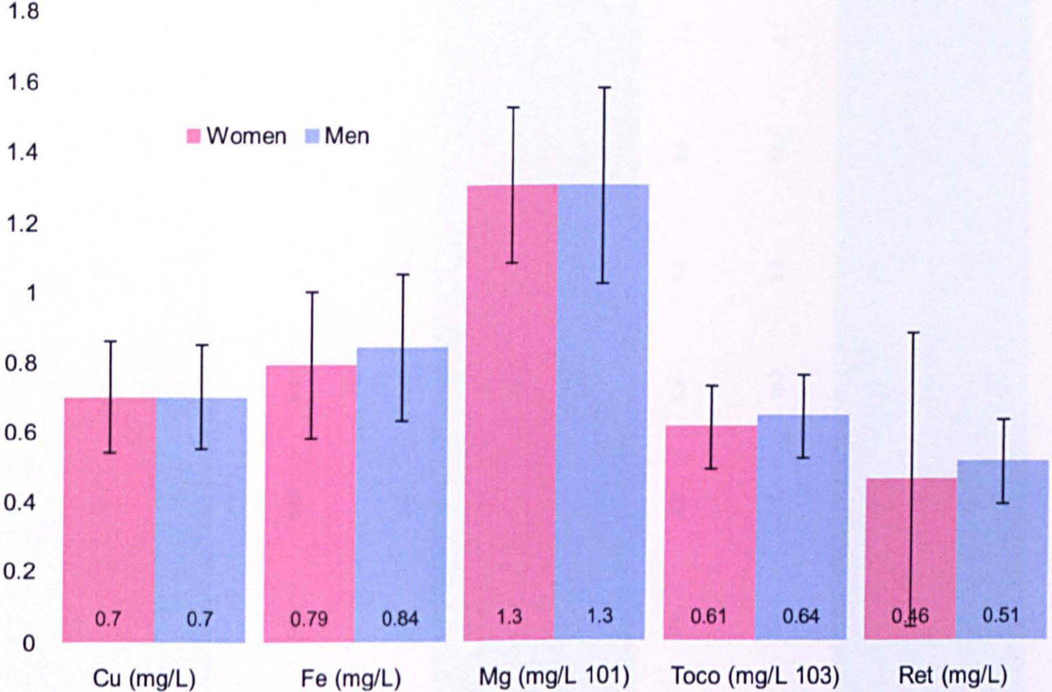

Figure 15: Mean plasma antioxidant levels: copper (25 ♀, 17 ♂), iron (24 ♀, 18 ♂), magnesium (30 ♀, 16 ♂), alpha-tocopherol (26 ♀, 22 ♂), and retinol (24 ♀, 22 ♂) (mg/L) in men and women

Table 12: Contribution of food groups to micronutrient intakes (micronutrient amount in food groups/total amount in the diet %) – Study 2

	Gender	Alcoholic beverages	Cereals	Dairy	Eggs	Fats and Oils	Fish	Fruit	Meat	Non-alcoholic beverages	Nuts and seeds	Patisserie	Poultry	Soups, sauces, herbs and condiments	Sugars, preserves, confectionary, savoury snacks	Vegetables
Copper (mg)	M	2.6	20.0	0.9	0.4	0.4	10.0	6.1	30.9	2.6	6.1	2.2	1.3	2.2	3.5	8.3
	W	2.4	22.4	2.4	0.6	0.6	6.5	18.2	6.5	5.9	9.4	4.1	1.8	2.4	4.1	14.1
Iron (mg)	M	5.4	40.1	1.8	1.4	0.3	4.7	3.9	12.1	4.1	3.0	3.5	2.0	2.7	3.2	12.0
	W	4.7	37.0	2.6	1.3	0.3	2.5	5.6	6.6	5.8	4.5	4.9	1.5	2.9	3.4	16.2
Magnesium (mg)	M	11.6	26.0	7.9	0.4	0.1	4.3	5.9	5.9	8.6	7.0	2.5	3.0	1.8	4.0	10.9
	W	3.0	23.8	8.7	0.4	0.1	2.8	10.7	3.4	14.2	8.1	3.2	2.4	1.9	3.8	13.5
Selenium (µg)	M	0.0	31.0	4.6	1.5	0.0	19.4	1.2	9.4	0.9	20.0	0.8	6.2	0.4	0.3	4.1
	W	0.0	30.7	6.1	2.2	0.0	22.7	2.1	7.1	2.1	9.8	2.2	7.4	0.4	0.6	5.5

	Gender	Alcoholic beverages	Cereals	Dairy	Eggs	Fats and Oils	Fish	Fruit	Meat	Non-alcoholic beverages	Nuts and seeds	Patisserie	Poultry	Soups, sauces, herbs and condiments	Sugars, preserves, confectionary, savoury snacks	Vegetables
Zinc (mg)	M	0.9	23.9	11.9	1.3	0.1	4.5	1.7	31.1	1.0	4.0	2.5	3.7	1.5	2.6	9.3
	W	0.6	25.5	14.0	1.7	0.1	3.5	3.8	18.3	1.9	5.8	4.1	4.5	1.7	2.9	12.0
Vit A (Retinol equivalents) (µg)	M	0.2	0.7	9.7	1.8	7.8	0.4	1.0	50.6	1.6	0.0	3.9	0.7	2.5	0.8	18.5
	W	0.0	0.9	11.4	2.1	11.3	0.5	3.2	17.8	2.4	0.1	4.1	0.3	4.4	1.5	40.1
Pre-formed Retinol (µg)	M	0.2	0.3	11.9	2.4	9.3	0.5	0.0	67.1	0.2	0.0	4.8	0.7	1.1	0.5	0.9
	W	0.1	1.2	21.7	4.3	20.8	0.9	0.0	36.5	0.4	0.1	7.7	0.5	1.5	2.2	2.1
Beta-carotene (µg)	M	0.0	1.9	3.2	0.1	3.4	0.1	3.7	3.6	5.6	0.1	1.3	0.6	6.3	1.4	68.7
	W	0.0	0.6	1.9	0.0	2.6	0.1	6.2	0.9	4.1	0.1	0.7	0.1	7.1	0.9	74.7
Vit C (mg)	M	0.0	0.8	1.7	0.0	0.0	0.2	14.6	1.7	40.9	0.0	0.6	0.1	1.6	1.3	36.6

	Gender	Alcoholic beverages	Cereals	Dairy	Eggs	Fats and Oils	Fish	Fruit	Meat	Non-alcoholic beverages	Nuts and seeds	Patisserie	Poultry	Soups, sauces, herbs and condiments	Sugars, preserves, confectionary, savoury snacks	Vegetables
	W	0.0	0.5	1.0	0.0	0.0	0.1	33.5	0.6	21.8	0.0	0.3	0.0	1.0	0.6	41.1
Vit E (mg)	M	0.1	14.7	3.1	1.5	16.0	4.1	5.2	4.0	2.0	6.6	6.2	1.7	12.1	8.2	15.0
	W	0.0	8.7	4.3	1.1	21.0	3.3	10.6	2.0	1.0	15.6	5.5	1.3	6.6	3.9	15.6

Mg: magnesium, Fe: iron, Zn: zinc, Cu: copper, Se: selenium, vit A: vitamin A, Beta-caro: beta-carotene, vit C: vitamin C, vit E: vitamin E

Table 13: Correlations between dietary antioxidant micronutrients and plasma and RBC EPA and DHA levels in men and women at 0.01 significance level – Study 2

	Independent variables					
(women=38, men=25)	Mg	Se	Zn	Fe	Vit E	Vit A (ret equiv)
Response variables	F	M	F	F	M	F
Plasma						
CE-DHA			0.531 p<0.001			
PC-DHA			0.499 p<0.01			
Red Blood Cells						
PC-DHA	0.472 p<0.01		0.472 p<0.01			
PE-EPA			0.553 p<0.001			
PE-DHA	0.515 p<0.001		0.589 p<0.001	0.487 p<0.01		
PS-EPA			0.549 p<0.001			0.459 p<0.01
PS-DHA	0.531 p<0.001	0.505 p<0.01	0.642 p<0.001	0.577 p<0.001	0.650 p<0.001	

4.12. Discussion

The current results indicate that although total dietary intakes for many micronutrients were broadly similar between the men and women in this young homogenous Caucasian population, gender disparities in food preferences were evident. Intakes of zinc and selenium were significantly higher in the men compared to the women due to a greater contribution from meat, cereals and certain types of nuts, whilst dietary beta-carotene was significantly higher in the women due to higher fruit and vegetable intake.

As a general comment, it was of interest to note that dietary intakes in this sample group could be loosely divided into two different and opposing diets: one that was based on the Mediterranean diet, containing olive oil, nuts and seeds, high fibre foods, fruit vegetables and fish; and a "Western"-type diet containing supermarket

ready made foods, sauces, takeaways etc. Although this aspect wasn't investigated in detail, it appeared that those consuming a Mediterranean-type diet mainly comprised students of nutrition, or sports science, ex-patriots of Mediterranean countries, and females.

Differences in dietary micronutrient levels in men and women

Gender differences in certain dietary micronutrients were evident. In similarity with previous reports (McDaid et al., 2007), the men in this study had greater zinc intakes, most likely from red meat dietary sources, although no significant difference was found when zinc was expressed as micronutrient density (ie. per 1000kcals, see Appendix 9.7). Of interest, iron density in the diet was higher in the women than in the men, although dietary sources were more likely to be of the non-haem variety. In addition, copper, magnesium, and vitamin E density in the women's' diets exceeded the men's, and both absolute amounts and densities of dietary beta-carotene and vitamin C were also higher in the women. These results indicate greater nutritional balance in the women in our study, although absolute intakes of zinc and selenium were lower than in the men due to lower amounts of food sources eaten. Higher nutrient density of micronutrients in the women's' diets may also indicate a greater degree of under-reporting in the women which may not have been corrected at PAL <1.2.

Such gender differences in absolute micronutrient intakes have been reported previously, nevertheless intakes in our subjects were higher than national statistics (Henderson 2002). Although the sample size was inadequate for a detailed comparison with national DRVs, we noted that a third of women in our study did not meet the minimum requirements (LRNI) for dietary selenium. Inadequate selenium intakes are seen in over 50% of women nationally (Mason and Ruxton 2010), and may increase risk for heart disease (Flores-Mateo et al., 2006) and cancer (Rayman

2005). Finally plasma retinol was similar in both genders, although density was higher in the men, as reported previously (Comstock et al., 1988).

Associations between antioxidant micronutrients, blood lipoproteins and plasma and RBC fatty acids.

Our results suggested that there were more associations between dietary micronutrients and plasma and RBC fatty acids in the women than in the men. In particular dietary zinc, which was lower than in the men, appeared to be closely associated with EPA and DHA in many plasma and RBC lipid fractions in the women. In addition, dietary magnesium appeared to have a positive interaction with DHA in RBC PC, PE and PS of women, and an inverse association was found with diastolic blood pressure. Previous evidence for the antioxidant and antihypertensive actions of magnesium are well-documented (Montoyama et al., 1989).

In the men, selenium and vitamin E, both well-known protective antioxidants of cell membrane fatty acids, appeared to have positive associations with RBC PC DHA, while plasma iron seemed to have a strong negative association with RBC SM EPA, possibly indicating a larger pro-oxidant effect, in the men.

Although evidence exists for a higher potential redox capacity in women compared to men (Nadal-Casselas et al., 2011; Gómez-Pérez et al., 2011), we could not find similar studies in healthy subjects to support our findings that men and women utilise antioxidants to a greater extent. The SUVIMAX study found that supplementation with beta-carotene was associated with a reduction in total cancer incidence and mortality in men and not women, although a suggested explanation may have been prior lower antioxidant status, especially of beta-carotene, in the men (Hercberg et al., 2004). Further analysis of the SUVIMAX data, which recruited 12,000 adults over 7.5 years, corroborated this finding. Men who developed cancer had significantly lower baseline levels of beta-carotene, vitamins E and C compared to those who didn't. In contrast, baseline levels of vitamins C, E, Zn and Se did not

differ between the women who did and didn't develop cancer, which may explain lack of relationship between supplementation and cancer (Galan et al., 2005). Taken together these findings indicate that compared to in the men, antioxidant protection of the long chain n-3 fatty acids of plasma and RBCs in the women, may be more effective, more widespread in lipid fractions, and involve different micronutrients.

Gender differences in food choice

Further nutritional analysis revealed that variations in food preferences of men and women may underlie dietary results. For example, nuts, white fish, and bread contributed most to selenium intakes in men and women. Gender disparities were apparent in choice of nuts ie men who ate nuts chose brazil nuts which are an excellent source of selenium, but are also high in fat, while women chose lower energy containing walnuts, which are also low in selenium. Other gender disparities included higher male consumption of offal and red meat, both good sources of selenium and iron. Although iron intake were similar between the genders, it is likely that haem iron intake was higher in the men, unlike in the women who relied more on cereals and green vegetables for the less bio-available non-haem iron. Higher meat consumption in men also explained the significant variance in zinc intakes between the genders. Women on the other hand tended to rely more on zinc-containing foods such as breakfast cereals, nuts and seeds and bread. In contrast, higher total beta-carotene intakes associated with greater consumption of vegetables were observed in the women compared to the men, and this also contributed more to vitamin C intakes. Interestingly compared to women, men obtained more of their beta-carotene and vitamin C from non-alcoholic beverages including fruit juice, while the women obtained more of these vitamins from actual fruit.

Of the remaining micronutrients, although total intakes did not differ significantly between the genders, gender differences in contributions from different foods were evident. In similarity, men appeared to derive more of their vitamin A and copper from meat including offal, while nuts and seeds appeared to contribute more to vitamin E, copper and magnesium in women. Interestingly, alcoholic beverages, in particular beer, appeared to contribute a large proportion of magnesium in the diet of the men, whereas these didn't feature as a particularly high source of magnesium in the women's diets. Previous reports have correlated beer drinking with increased magnesium status (Gorinstein et al., 1998), and this may explain a recent report of its protective effect against development of type 2 diabetes (Estruch et al., 2011).Variations in food choice between men and women have been observed in other surveys (Cronin et al., 1982; Bates et al., 2008/9) and may stem from gender stereotyping and feminine and masculine beliefs and behaviours relating to perceived healthy food choices (Courtenay 2000).

Our data indicate that there were several associations between dietary antioxidants and plasma and RBC cell membrane n-3 fatty acid levels in our sample group, particularly in the women even where intakes were lower than in the men. In contrast, the only association between dietary or plasma micronutrients and blood glucose and lipoproteins was in the women between plasma magnesium and blood pressure, as shown previously (Motovama et al., 1989). Gender differences in influence of antioxidant status (namely zinc) on immune (lymphocyte) response have previously been reported (Finamore et al., 2005), however we have been unable to find evidence to support the differential effect of dietary antioxidants on plasma and RBC cell n-3 status in men and women indicated in our study.

Nevertheless it has been previously observed in certain chronic inflammatory diseases that men with lower antioxidant status for example selenium (Waters et al., 2004), have a greater risk of chronic disease compared to women with similar low

antioxidant levels (Higdon and Frei 2005). Proposed explanations for this include a lower dose-response relationship in women, or gender differences in tissue storage, and metabolism or interaction with other lifestyle factors such as smoking or alcohol intake which may differ in men and women (Waters et al., 2004). In addition, there are reports of an upregulation of antioxidant activities in females compared to males during periods of increased oxidative stress (Kamper et al., 2009; Schuessel et al., 2004), which may be related to an agonistic influence of oestrogen (Barp et al 2002).

Unlike in other studies, we did not find that dietary antioxidant intakes correlated with plasma levels, possibly due to inadequate choice of biomarkers or analysis methods (Hooper et al., 2009). However, what was surprising, was that irrespective of differences in intakes between the men and women, there appeared to be significantly more associations in the women between antioxidant intakes and cell membrane fatty acids, particularly DHA. Antioxidant protection of omega 3 fatty acids may help protect them against lipid peroxidation, and maintain levels in the cell membranes hence reducing risk for lethal cardiac arrythmias (Leaf et al., 2003). This data in the women suggests an oestrogenic or other sex hormone effect. Further research should investigate this putative antioxidant protection and higher DHA levels in plasma and RBC lipid fractions in the women, as well as their more favourable cardioprotective blood glucose and lipoprotein profile.

4.13. Conclusion

Although intakes of most micronutrients were broadly similar, amounts of zinc, selenium, beta carotene and vitamin C consumed differed between the men and women in this study, possibly due to different food preferences. Despite this, more associations were noted between antioxidant micronutrients and cell membrane

fatty acids in the female subjects. This may indicate a greater antioxidant-mediated cardioprotection in the women compared to in the men.

5. Comparison of fatty acids in diet, plasma and red blood membranes of healthy men and women. (Study 3)

5.1. Introduction

Thirty years after initial suggestions that total fat was responsible for development of atherosclerosis (Keys 1952), findings from the Seven Countries study indicated that it was quality of dietary fat which explained varied rates of coronary heart disease in the US, Southern Europe and Japan (Keys 1980). The debate between quality and quantity of fat has continued until recently, despite an early controversial suggestion that coronary heart disease (CHD) was related to an essential fatty acid deficiency (Sinclair 1956), and observations of low CHD incidence in Northern Inuit populations with high n-3 intakes (Dyerberg et al., 1975).

Evidence is now widely available for the potency of long-chain n-3 fats, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), in modifying cardiac risk factors such as platelet aggregation and blood pressure, and serum markers: triglycerides, atherogenic low density lipoprotein (LDL) and high density lipoprotein cholesterol (Bays et al., 2008). Recommended intake for cardiovascular protection from EPA combined with DHA in the UK is 0.45g/d (SACN 2004), while international and US recommendations state minimum intakes of 0.5g/d (FAO/WHO, ISSFAL 2004), increasing to 1g/d in those with CHD, while US safety intake guidelines are currently set at 3g/d (FDA 2004). Despite this, national surveys reveal that intakes of LC n-3 PUFAs in the normal population are falling below recommendations (Henderson 2002).

Previous studies have tended to assess n-3 fatty acid levels in dietary intakes using food frequency questionnaires (FFQs), and correlate findings with levels in blood fractions such as plasma CE (Giltay et al 2004b; Bakewell et al., 2006; Crowe et al.,

2008; Nikkari et al., 1995). However, FFQs may not reliably reflect habitual dietary intakes (Bingham, Gill et al., 1994), and plasma and RBCs differ in their FA incorporation and clearance (Cao et al., 2006). Results of dietary n-3 PUFA intakes, sources and plasma levels from epidemiological data collected in the nineties have been recently reported (Welch et al., 2010). Only a handful of papers report effect of dietary fat on both plasma and RBC fatty acid levels in the same sample group (Sun et al., 2008, Dougherty et al., 1987); although they do not compare differences in individual lipid fractions between the genders. We have recently reported fatty acid composition in platelet PC and PE and differences between men and women from the same cohort (Geppert et al., 2010).

To our knowledge, no other study has comprehensively examined habitual dietary fatty acid intakes and food sources of a homogenous cohort of healthy, educated young to middle-aged men and women, and compared fatty acid consumption with levels in individual lipid fractions of both red blood cells and plasma. The aims of the present study were to compare food intake of a local population with respect of the quality and quantity of fats separately for men and women, and to relate fatty acids in the diet with fatty acid levels in plasma and red blood cells.

5.2. Subjects, Materials and Methods

These are described in detail in chapter 2.

5.3. Results

31 subjects (12 males, 19 females) were excluded due to missing data (incomplete food diaries and missing/spoiled samples). Results were available for 73 subjects (40 males, 33 females).

5.3.1. Anthropometric, clinical and demographic data

Anthropometric, clinical and demographic data are presented in Table 14. There was no difference in the mean ages of men and women. Men were heavier, taller and had a higher BMI than women ($p<0.001$ for height and weight, $p<0.01$ for BMI). Similar numbers of men and women took exercise (32 men, 25 women), had a family history of chronic diseases (17 men and 13 women), and were smokers (4 men and 2 women).

Blood glucose ($p<0.001$), triglycerides ($p<0.05$) and LDL cholesterol were higher in men compared to women ($p<0.01$), and HDL cholesterol was higher in women ($p<0.001$).

5.3.2. Energy and macronutrients

Men had higher absolute intakes of energy (kcals), all macronutrients (g) than women ($p<0.001$), but not of alcohol (g) (mean 24.43, median 14.5, SD 26.97 for men; mean 12.00, SD 13.89, median 6.00 for women, $p=0.138$). When macronutrients and alcohol were expressed as a percentage of energy intake (%energy), there were no significant differences between the genders.

5.3.3. Food Sources of Fatty Acids

Table 15 presents the average derivation of dietary fatty acids by food type separately for men and women. In general, men and women derived fatty acids from similar food sources. Dairy foods including milk, and cheese were major contributors to myristic, palmitic and stearic acids. Fats and oils were also important sources of myristic, palmitic, stearic and palmitoleic acids, in addition to being the primary sources for oleic, linoleic and alpha-linolenic acids. Cereals, and nuts and seeds also contributed to linoleic acid intake. Fish was the major contributor to EPA and DHA as well as being an important source of DPAn3. Meat accounted for most of the arachidonic acid and DPAn3, in addition to being an important contributor to stearic and palmitoleic acids.

Women appeared to consume more oleic and linoleic acids from nuts and seeds, while men consumed more linoleic acid from cereals, and more arachidonic acid and DPAn3 from meat.

5.3.4. Dietary Fatty Acids and Alcohol

Mean daily intakes of total, saturated, monounsaturated, and polyunsaturated fatty acids are listed in Table 16. The proportional dietary composition of the major saturated and unsaturated fats were similar in both genders; the largest proportion of fat intake came from monounsaturated fatty acids, in particular oleic acid; followed by saturated fatty acid intake, especially palmitic acid; and finally polyunsaturated fatty acids, especially linoleic acid. Intake of n-3 polyunsaturated fats was considerably lower than other fats, with mean percentage intakes of between 2.1 ± 0.9 g for men and 1.6 ± 0.8 g for women, the largest proportion coming from alpha-linolenic acid.

Compared to women, men consumed more individual and Σ saturated fatty acids (myristic acid 3.0 ± 1 vs 2.2 ± 1.3 g/d; palmitic acid 14.4 ± 3.6 vs 11.1 ± 4.8 g/d; stearic acid 6.6 ± 1.8 vs 5.1 ± 2.3 g/d, $p<0.001$ for all); individual (palmitoleic acid 1.1 ± 0.4 vs 0.8 ± 0.3 g/d, $p<0.01$; oleic acid 25.0 ± 8.0 vs 21.1 ± 10.5 g/d, $p<0.05$) and Σ monounsaturates (26.2 ± 8.3 vs 22.0 ± 10.8 g/d, $p<0.05$); LA (12.5 ± 5.5 vs 10.2 ± 6.2 g/d, $p<0.05$) and Σ n-6 polyunsaturates (18.0 ± 6.5 vs 14.1 ± 7.4 g/d, $p<0.01$); ALA (1.6 ± 0.6 vs 1.2 ± 0.6 g/d, $p<0.01$) and Σ n-3 polyunsaturates (2.1 ± 0.9 vs 1.6 ± 0.8 g/d, $p<0.05$) (see Table 3). Both genders had comparable intakes of EPA, DPAn3 and DHA when expressed in absolute amounts, percentage of total energy intake or per kg body weight.

There was no difference in alcohol intakes between the genders.

5.3.5. Plasma Fatty Acids

The percentages of selected fatty acids (FA) in the plasma PC, TG and CE fractions are shown in Table 17. There was a higher proportion of stearic acid in the PC (12.7 ± 1.3 vs 11.2 ± 1.7 area%, $p<0.001$) and CE (0.7 ± 0.3 vs 0.6 ± 0.4 area%,, $p<0.01$) fractions, and Σ saturates (43.1 ± 2.0 vs 42.4 ± 1.6 0 area%, $p<0.05$), Σ n-6 polyunsaturates (35.5 ± 2.3 ± 34.2 ± 2.1 area%, $p<0.05$), and DPAn3 (0.7 ± 0.2 vs 0.6 ± 0.2 area%, $p<0.05$) in PC of men compared with women. On the other hand, significantly less DHA (2.8 ± 1.0 vs 3.2 ± 0.7 area%, $p<0.01$) and palmitoleic acid was detected in the PC (0.7 ± 0.2 vs 0.9 ± 0.4 area%, $p<0.001$) and CE (2.1 ± 1.0 vs 2.6 ± 1.0 area%, $p<0.05$) fraction in men compared with women.

The table also shows that proportionately more oleic acid (12.3 ± 1.7 vs 11.3 ± 1.5 area%, $p<0.05$), Σ monounsaturates (15.4 ± 2.2 vs 14.0 ± 1.8 area%, $p<0.01$), and Σ n-3 FA (5.5 ± 1.3 vs 5.0 ± 1.6 area%, $p<0.05$) were in the PC fraction of women compared with men.

5.3.6. Red blood cell fatty acids

The percentages of the main FA in the erythrocyte PC, PE, PS and SM fractions are shown in Table 18. Palmitic acid was significantly higher in all fractions (PC 34.4 ± 2.0 vs 33.0 ± 1.4 area%, $p<0.001$; PE 13.4 ± 1.2 vs 12.5 ± 0.8 area%, $p<0.01$; PS 5.8 ± 0.6 vs 5.5 ± 0.6 area%, $p<0.01$; SM 23.9 ± 2.2 vs 22.4 ± 2.2 area%, $p<0.05$) in women compared with men; in contrast stearic acid was significantly lower in PC (10.5 ± 1.4 vs 11.7 ± 1.0 area%, $p<0.001$) and PE (5.4 ± 0.4 vs 5.7 ± 0.5 area%, $p<0.01$) of women compared to men. Σ saturates were lower in the PE fraction (18.6 ± 0.9 vs 19.3 ± 1.2 area%, $p<0.05$), palmitoleic acid was lower in PC (0.4 ± 0.1 vs 0.5 ± 0.2 area%, $p<0.01$), PE (0.2 ± 0.1 vs 0.3 ± 0.1 area%, $p<0.001$) and PS (0.0 ± 0.0 vs 0.1 ± 0.0 area%, $p<0.05$)

fractions, and oleic acid was lower in PS (5.7 ± 0.8 vs 6.0 ± 0.7, $p<0.05$) in men compared with women. All n-6 FAs were similar in men and women, except for higher Σ n-6 FAs in PE in men (33.1 ± 2.2 vs 32.0 ± 1.6 area%, $p<0.05$). The n-3 FA, DPAn3 was significantly higher in the PC (0.6 ± 0.1 vs 0.5 ± 0.1 area%, $p<0.01$) and PS (3.7 ± 0.6 vs 3.4 ± 0.5 area%, $p<0.05$) fraction in men compared with women and there were similar trends for PE and SM. In contrast, DHA was significantly higher in the PE fraction (7.8 ± 1.3 vs 6.8 ± 1.5 area%, $p<0.01$) in women compared with men and again showed a similar pattern for PS and PC, while SM levels were similar in both genders.

5.3.7. Associations between dietary fatty acids, lifestyle factors (age, BMI, exercise, alcohol intake, smoking status) and plasma and RBC n-3 fatty acids

Table 19 shows the associations between dietary ALA, EPA, DPAn3 and DHA intake, age, BMI, exercise, alcohol intake, smoking status, and DHA concentrations in plasma and red blood cells.

1. Plasma

In men, dietary DHA was associated with DHA level in CE, PC and TG ($p<0.001$ for all), whereas in women it was associated with PC DHA ($p<0.05$) only.

There were no correlations between plasma palmitic acid (a surrogate marker for beta-oxidation) and DHA levels in men or women (data not shown).

2. Red blood cells

Dietary DHA was associated with DHA concentration in all RBC fractions (PC, PE and PS $p<0.001$, SM $p<0.05$) in men, and only with PC DHA in women. Exercise had a positive effect on DHA level in PC ($p<0.05$), PS ($p<0.05$) and PE ($p<0.001$) of women, and age had a positive association with DHA level in PE

($p<0.05$) of men. Finally dietary ALA, and alcohol intake had a negative effect on DHA ($p<0.05$, $p<0.01$ respectively) in SM of men.

Further analysis in female subjects, revealed significant differences between exercising (n=8) and non-exercising (n=25) women in mean DHA level (data not shown) in plasma CE ($p=0.01$), and RBC PC ($p=0.017$), PE ($p=0.002$), and PS ($p=0.02$). No differences were found between systolic blood pressure or alcohol intake dependent on exercise status. There were no correlations between plasma or RBC palmitic acid (a surrogate marker for beta-oxidation) and DHA levels.

5.4. Results Tables

Table 14: Subject characteristics – Study 3

	Men (n=40)		Women (n=33)		p
	Mean	SD	Mean	SD	
Age (yrs)	32.62	7.97	32.94	7.18	ns
Height (cm)	177.71	7.51	166.82	5.84	<0.001
Weight (kg)	76.51	10.12	63.72	12.88	<0.001
BMI (kg/m^2)	24.25	3.09	22.96	4.79	<0.01
Systolic BP (mm/Hg)	125.30	11.26	113.21	9.67	<0.001
Diastolic BP (mm/Hg)	75.15	9.38	72.58	6.19	ns
Fat free mass (kg)	64.24	7.11	44.95	3.49	<0.001
Fat mass (kg)	15.79	5.77	28.08	8.07	<0.001

SD standard deviation, BP blood pressure, BMI body mass index

Table 15: Contribution of food groups to individual fatty acids (mg) – Study 3

(n=40 men, n=33 women)

Fatty acid	Gender	Cereals	Dairy	Eggs	Fats and Oils	Fish	Meat	Nuts and seeds	Poultry	Vegetables
C14:0	M	148.8	1470.9	53.3	750.0	56.7	293.7	71.3	9.2	223.3
	W	93.5	809.7	8.6	509.7	50.9	135.9	23.8	9.9	131.7
C16:0	M	1348.1	3565.4	391.6	3416.5	297.3	2972.3	1080.2	261.6	891.6
	W	683.9	1969.1	272.9	2482.3	251.5	1519.4	905.7	292.0	613.7
C18:0	M	337.9	1608.1	129.2	1580.4	58.7	1858.9	594.9	67.9	389.6
	W	179.9	879.4	83.7	1071.3	48.5	1009.3	495.6	75.6	253.1
C16:1n7	M	86.0	210.3	54.8	330.8	76.2	339.2	23.7	57.4	47.8
	W	48.7	115.9	45.6	165.6	57.6	151.8	32.2	59.1	27.2
C18:1n9	M	1649.4	2735.9	602.6	10685.3	250.9	4104.5	3585.7	517.7	1412.3
	W	839.7	1523.2	529.6	8412.6	224.9	2107.8	4757.5	539.4	1023.6
C18:2n6	M	2813.2	183.6	116.7	4245.7	46.2	969.5	079.5	137.4	1008.1
	W	1246.5	136.3	121.2	3720.5	60.6	554.5	6624.1	293.9	823.2
C20:4n6	M	4.9	0	15.4	0		124.2	0	3.5	1.0
	W	2.5	0	13.9	0	7.8	69.4	0	4.9	0.4
C18:3n3	M	209.7	87.5	8.01	621.9	15.4	112.9	119.2	6.9	260.4
	W	88.5	53.2	9.9	443.5	17.6	55.6	86.7	29.9	228.3
C20:5n3	M	0.3	0	1.2	0	130.8	35.6	0	0.3	0.8
	W	0.2	0	1.1	0	104.5	22.1	0	0.6	0.3
C22:5n3	M	1.0	0.	3.4	0	27.2	55.2	0	0.4	0.5
	W	0.6	0	3.1	0	23.9	30.6	0	0.6	0.2
C22:6n3	M	1.0	0	6.8	0	199.0	25.7	0	4.1	0.1
	W	0.7	0	6.1	0	161.1	18.4	0	6.2	0.1

C14:myristic, C16:palmitic, C18:stearic, C16:1n7:palmitoleic, C18:1n9:oleic, C18:2n6:linoleic, c20:4n6:arachidonic, c18:3n3:alpha-linolenic, C20:5n3:eicosapentaenoic, C22:5n3:docosapentaenoic, C22:6n3:docosahexaenoic

Table 16: Dietary fatty acids and alcohol – Study 3

	Men (n=40)					Women (n=33)					p
Fatty acids (g/d)	**mean**	**sd**	**median**	**25th**	**75th**	**mean**	**sd**	**median**	**25th**	**75th**	
14:0	3.0***	1.0	2.8	2.4	3.4	2.2	1.3	2.0	1.4	2.8	<0.001
16:0	14.4***	3.6	14.3	11.4	17.0	11.1	4.8	10.0	8.2	13.1	<0.001
18:0	6.6**	1.8	6.4	5.2	8.2	5.1	2.3	4.8	3.5	6.0	<0.001
Σ Saturates	24.0***	6.1	23.6	20.0	28.8	18.4	8.2	17.2	13.4	22.5	<0.001
16:1	1.1**	0.4	1.1	0.8	1.4	0.8	0.3	0.8	0.6	1.1	<0.01
18:1	25.0*	8.0	24.7	19.0	29.5	21.1	10.5	18.3	15.5	26.0	p<0.05
Σ Monounsaturates	26.2*	8.3	26.1	19.8	30.7	22.0	10.8	18.8	16.2	27.1	p<0.05
18:2n6	12.5*	5.5	11.8	7.9	16.4	10.2	6.2	9.2	6.5	1.1	p<0.05
20:4n6	1.4	1.0	1.2	1.0	1.6	1.1	0.5	1.1	0.7	1.6	ns
Σ n-6	18.0**	6.5	17.5	12.9	21.6	14.1	7.4	12.5	9.8	15.6	p<0.01
18:3n3	1.6**	0.6	1.6	1.1	2.0	1.2	0.6	1.1	0.8	1.5	<0.01
20:5n3	0.2	0.2	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.2	ns
22:5n3	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.1	ns
22:6n3	0.2	0.3	0.1	0.1	0.4	0.2	0.2	0.2	0.0	0.3	ns
Σ n-3	2.1*	0.9	1.9	1.5	2.6	1.6	0.8	1.6	1.0	2.0	<0.05
Σ Dietary Fat	70.02**	19.46	69.28	55.33	81.49	56.08	24.85	53.26	41.63	68.40	<0.01
Alcohol	24.43	26.97	14.5	0.00	42.50	12.00	13.89	6.00	1.00	17.50	ns

*p<0.05, **p<0.01, ***p<0.001 (difference between means)
sd:standard deviation, Σ:total
Percentiles by weighted average, 25th: 25th percentile, 75th: 75th percentile

Table 17: Plasma fatty acids – Study 3

(Men = 40, Women = 33)												
	PC				TG				CE			
	M		W		M		W		M		W	
Fatty acids (area%)	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
16:0	29.3	2.2	30.0	2.4	24.9	4.0	23.5	3.7	10.5	0.9	10.7	1.9
18:0	12.7***	1.3	11.2	1.7	3.2	1.2	2.7	0.9	0.7**	0.3	0.6	0.4
Σ Saturates	43.1*	1.9	42.4	1.6	31.3	5.5	28.6	4.7	12.0	1.1	12.1	2.4
16:1	0.7***	0.2	0.9	0.4	3.0	1.1	3.4	1.0	2.1*	0.9	2.6	1.0
18:1	11.3*	1.5	12.3	1.7	36.4	4.4	35.3	4.6	17.5	1.8	18.1	4.2
Σ Monounsaturates	13.9**	1.8	15.4	2.2	43.2	4.0	42.3	5.2	20.8	2.5	22.1	5.0
18:2n6	23.6	2.5	22.7	2.4	15.7	3.9	18.1	7.8	53.3	4.2	51.8	7.3
20:4n6	8.4	1.4	8.2	1.8	1.1	0.3	1.5	0.9	6.4	1.4	6.3	1.9
22:4n6	0.2	0.1	0.2	0.1								
22:5n6	0.1	0.1	0.2	0.1								
Σ n-6	35.5*	2.3	34.2	2.1	18.2	4.4	20.1	8.7	61.0	4.3	59.3	7.5
18:3n3	0.3	0.1	0.3	0.1	1.1	0.4	1.1	0.4	0.5	0.2	0.6	0.2
20:5n3	1.1	0.8	1.2	0.8	0.4	0.2	0.5	0.7	1.1	0.7	1.1	0.6
22:5n3	0.7*	0.2	0.6	0.2	0.4	0.2	0.4	0.2				
22:6n3	2.8**	1.0	3.2	0.7	0.68***	0.79	1.21	1.33	0.6*	0.2	0.6	0.2
Σ n-3	5.0*	1.6	5.5	1.3	2.9	1.5	3.8	2.6	2.4	1.5	2.4	0.8

*$p<0.05$, **$p<0.01$, ***$p<0.001$
M:men, W:women, PC:phosphatidylcholine, TG:triglycerides, CE:cholesterol esters, sd:standard deviation, Σ:total

Table 181 Red cell fatty acids- Study 3

(Men = 40, women = 33)																
	PC				PE				PS				SM			
	M		W		M		W		M		W		M		W	
Fatty acids (area%)	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
16:0	33.0***	1.4	34.4	1.9	12.5**	0.8	13.4	1.2	5.5**	0.6	5.8	0.6	22.4*	2.2	23.9	2.2
18:0	11.7***	1.0	10.5	1.4	5.7**	0.5	5.4	0.4	40.6	0.8	40.4	0.7	9.8	1.4	10.1	1.3
Σ Saturates	45.6	0.8	45.8	0.8	18.6*	0.9	19.3	1.2	47.1	0.6	47.1	0.5	63.8	2.1	63.8	1.6
16:1	0.4**	0.1	0.5	0.2	0.2***	0.1	0.3	0.1	0.0*	0.0	0.1	0.0	0.1	0.0	0.1	0.0
18:1	15.9	1.1	15.3	1.5	14.5	0.9	14.3	1.1	5.7*	0.8	6.0	0.7	2.4	0.4	2.2	0.3
Σ Monounsaturates	18.22	1.2	17.9	1.4	16.0	1.0	15.9	1.1	7.0	0.9	7.3	0.7	24.3	1.8	24.7	2.4
18:2n6	20.5	1.8	20.6	2.0	5.2	0.7	5.0	0.8	3.7	1.1	3.5	1.2	2.3	0.4	2.2	0.3
20:4n6	6.5	1.0	6.3	1.1	20.2	1.3	19.6	1.2	21.5	2.2	21.9	2.1	0.1	0.6	3.0	0.6
22:4n6	0.3	0.1	0.3	0.1	5.7	1.1	5.4	0.9	3.0	0.6	2.8	0.6	0.5	0.2	0.5	0.2
22:5n6	0.1	0.1	0.1	0.1	0.6	0.2	0.6	0.2	1.0	0.2	1.0	0.2	0.3	0.1	0.3	0.1
Σ n-6	30.1	1.5	30.0	1.4	33.1*	2.2	32.0	1.6	32.1	2.5	31.6	1.6	6.7	1.3	6.4	1.0
18:3n3	0.2	0.1	0.2	0.1	0.1*	0.0	0.2	0.0	0.0	0.0	0.0	0.0				
20:5n3	0.8	0.5	0.8	0.4	1.4	0.5	1.5	0.5	0.5	0.2	0.5	0.2	0.1	0.0	0.1	0.0
22:5n3	0.6**	0.1	0.5	0.1	4.3	0.5	4.1	0.6	3.7*	0.6	3.4	0.5	0.8	0.3	0.7	0.2
22:6n3	2.1	0.7	2.3	0.5	6.8**	1.5	7.8	1.3	8.1	1.8	8.6	1.6	2.6	1.0	2.7	0.8
Σ n-3	3.7	1.2	3.9	0.8	12.6	2.1	13.5	1.8	12.4	2.2	12.6	1.9	3.5	1.3	3.5	1.0

*p<0.05, **p<0.01, ***p<0.001
M:men, W:women, PC:phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, SM:sphingomyelin, sd:standard deviation, Σ:total

Table 19: Multi-linear regression (stepwise) between dietary n-3 fatty acids, selected lifestyle variables and plasma and RBC DHA levels – Study 3

		Men (n=40)				Women (n=33)			
Dependent Variables	**Independent variables**	**R^2 adjusted**	**Beta**	**SE**	**p**	**R^2 adjusted**	**Beta**	**SE**	**p**
log pl DHA CE		0.293							
	log DHA diet		0.177	0.043	p<0.001				
log pl DHA PC		0.306				0.172			
	log DHA diet		0.159	0.037	p<0.001		0.083	0.029	p<0.05
log pl DHA TG		0.242							
	log DHA diet		0.323	0.088	p<0.001				
log RBC DHA PC		0.281				0.334			
	Exercise (y)						0.180	0.075	p<0.05
	log DHA diet		0.149	0.037	p<0.001		0.082	0.028	p<0.01
log RBC DHA PE		0.356				0.091			
	log Age		0.269	0.122	p<0.05				
	Exercise (y)						0.218	0.058	p<0.001
	log DHA diet		0.092	0.024	p<0.001				
log RBC DHA PS		0.293				0.136			
	Exercise (y)						0.185	0.075	p<0.05
	log DHA diet		0.108	0.026	p<0.001				
Log RBC DHA SM		0.284							
	log Alcohol		-0.081	0.029	p<0.01				
	log ALA diet		-0.240	0.108	p<0.05				
	log DHA diet		0.101	0.039	p<0.05				

Excluded variables: BMI, Smoking status, dietary EPA
Coefficients and SEs in brackets, * p<0.05, **p<0.01, ***p<0.001

5.5. Discussion

To our knowledge, this study provides the most comprehensive results for dietary, plasma and red blood cell membrane n-3 fatty acid levels in a homogenous sample of healthy, educated men and women. We have shown that despite similarities in proportional intakes (when expressed as percentage energy) of long chain n-3 fatty acids in both genders, women had higher levels of DHA, and lower DPAn3 levels in plasma and erythrocyte phospholipids, compared to men. These results are consistent with data from other studies, independent of carefully controlled (Giltay et al., 2004b) or habitual dietary intakes (Bakewell et al., 2006), even where intakes of EPA and DPAn3 were greater in male subjects (Crowe et al., 2008).

Proportional dietary intakes for energy and macronutrients in our study are consistent with a large national UK survey (Henderson et al., 2002), although our nutritionally aware volunteers had lower total and n-6 polyunsaturated fat intakes, and higher n-3 fat intakes. Indeed, mean percentage intakes of saturated and monounsaturated fats met current recommendations, namely <11%, and 13% of daily energy intake respectively, and intake of LA and n-3 fats accounted for <6%, and 0.2% of dietary energy respectively (DOH 1991; BNF 1992) but fell below more recent ones (ISSFAL 2004). It is likely that usage of 7-day diaries and food portion size photographs (Nelson et al., 1997) in this study provided more reliable results of habitual dietary intakes (Bingham and Day 1997), compared to food frequency questionnaires or 24-hr recalls used in previous studies (Bakewell et al., 2006; Crowe et al., 2008). Moreover, using a specialised fatty acid database (Foodbase, IBCHN) we were able to examine differences in derivation of dietary fatty acids by food type in men and women. There were widespread gender similarities in amounts and food sources of n-3 fatty acids. However, despite a relatively greater contribution of DPAn3 from meat in men,

which may partially explain their higher plasma and red cell DPAn3 levels, comparable seafood intakes and plasma and red cell EPA in both genders could not explain higher DHA % levels in female plasma and red blood cell fractions.

RBCs are unable to produce phospholipids de novo, or saturate or elongate fatty acids, and so are reliant on plasma lipoproteins for direct exchange of PC or uptake of lysophospholipids for acylation in the RBC membrane (Brossard et al., 1997). Striking differences in fatty acid content were found between plasma and RBC fractions in our subjects, most notably in stearic acid. As reported in the current and previous studies, n-3 fatty acid levels, in particular EPA and DHA in plasma PC and CE correlated highly with RBC PC (data not shown), indicating the interrelationships between blood fractions (Sun et al., 2008). Supplementation studies report that increasing DHA concentration in plasma and RBCs occurs within a similar timeframe, although to different extents (Katan et al., 1997, Sun et al., 2008, Dougherty et al., 1987), and reflects dietary intakes over preceding weeks (Hodson et al., 2008). We found that DHA levels in all plasma fractions of women were higher than in men, while only PE RBC DHA level was higher in females. This suggests that compared to men, women may incorporate DHA to a greater extent in plasma, which is then transferred into red cells via uptake of plasma phospholipids, especially PC. Nevertheless, similar regression coefficients for dietary DHA in plasma and RBC PC in both genders, indicate transfer of dietary DHA between the two blood compartments.

Furthermore, in addition to their preformed supply in the diet, it is generally accepted that a biosynthetic pathway exists for EPA and DHA from essential fatty acid precursor, ALA, which is shared with second EFA, linoleic acid. An abundant LA compared to ALA diet, significantly reduces biosynthesis of DHA via desaturation and beta-oxidation due to competition for rate-limiting desaturase enzyme, Δ6 (Tu et al., 2010, Chan et al., 1993). Dietary ALA increases

phospholipid EPA in dose-dependent manner (Burdge et al., 2006), however influence on DHA status is less clear (Arterburn et al., 2006) especially in men (Burdge et al., 2003). In the current study, male subjects consumed more dietary ALA, and a negative association was found with RBC SM DHA. This may be explained by ALA substrate competition for Δ6 desaturase with tetracosapentaenoic acid (24:5n3) in the final conversion steps between DPAn3 and DHA (Portolesi et al., 2007, Cleland et al., 2005, Childs et al., 2010). Our results therefore provide further indications that the metabolic fate of ALA differs in men, which is understood to include beta-oxidation, carbon recycling and direct incorporation into structural lipids (Burdge et al., 2003). On the other hand, higher DHA results seen in our female subjects, suggest a greater endogenous conversion from n-3 fatty acid precursor, ALA and/or DPAn3 related to higher relative Δ6 desaturase activity (Childs et al., 2010). This may explain lower DPAn3 levels found in female plasma PC, and RBC PC and PS. Moreover, further analysis revealed that in men, dietary DHA was associated with DHA level in all plasma and RBC fractions, whereas in women, it was associated with plasma and RBC PC DHA only. Taken together with data from our previous research in platelet PC and PE (Geppert et al., 2010), these results provide further evidence for a greater reliance on preformed DHA in men, compared to women. Enhanced ability for up-regulation of the desaturation and elongation pathway in young women, purportedly under oestrogenic control, may be designed to prepare them physiologically for the demands of the foetus during pregnancy (Innis and Friesen 2008).

In similarity with our findings in men, several studies report an increase in blood n-3 levels in both genders associated with age (Crowe et al., 2008). Unlike in our male subjects (data not shown) however, age paralleled higher dietary LC-n-3 fat intakes (Crowe et al., 2008, Kuriki et al., 2002) and lower LA consumption (de

Groot et al., 2009). Interestingly in women, exercise was associated with an increase in RBC DHA level (despite similar total energy intakes - data not shown). Previous studies report conflicting results (Yates et al., 2009, Tanaka et al., 2003, Conquer et al., 2002) and differences in results may relate to type and intensity of exercise. The negative association of alcohol with DHA concentration in RBC SM in men, unlike an earlier study in moderate-wine drinkers (di Giuseppe et al., 2009), may be explained by preference for beer in our subjects. Di Giuseppi et al., (2009) reported that beneficial effects on plasma DHA level were observed in women, appeared to be only partially related to alcohol intake, and may have involved interaction of n-3 FA metabolism with polyphenols in wine.

5.6. Conclusion

In conclusion, this study shows that despite similar dietary intakes in men and women, gender differences exist in plasma and erythrocyte fatty acids, especially in n-3 fatty acids. Moreover, there may be a further impact of lifestyle factors on DHA concentrations in men and women. Of particular note, it appears that of all dietary n-3 fatty acids, DHA has the strongest and most consistent effect on plasma and erythrocyte DHA levels compared to any of its precursors, particularly in men.

6. Gender specific n-6 and n-3 fatty acids profile in platelet phosphatidyl-choline and –ethanolamine. (Study 4)

6.1. Introduction

The essential fatty acids, linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) and their long-chain polyunsaturated derivatives (LC-PUFA) are indispensable for human development and optimum health. Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are considered the most important functional LC-PUFA. DHA and AA are found in high concentrations in structural lipids of the central nervous system and have been shown to be important for brain development and function (Innis 1991; Uauy et al., 2001; Clandinin et al., 1991). Adequate accretion of both DHA and AA in brain and retina is particularly important during the rapid brain growth, which takes place in the perinatal period (Clandinin et al., 1991). Moreover, n-3 LC-PUFA contribute significantly to the biophysical properties of the phospholipid bilayer by providing an appropriate environment for the activities of membrane-associated proteins (Mitchell et al., 2003). The incorporation of n-3 LC-PUFA in cell membranes also modifies eicosanoid production resulting in altered platelet and leukocyte reactivities (Lewis et al., 1990; Tilley et al., 2001). The accumulation of eicosapentaenoic acid (EPA, 20:5n-3) and DHA in circulating blood platelets provides for a partial replacement of AA in membrane phospholipids resulting in less production of pro-aggregatory and vaso-constrictive thromboxane (TX) A_2 upon platelet stimulation due to the competitive inhibitory effects of EPA and/or DHA on cyclo-oxygenase activity (Leaf and Weber 1988; Kristensen et al., 1989).

This could result in less platelet aggregation and thrombosis and may contribute to the beneficial effects of n-3 LC-PUFA on cardiovascular risk.

For optimal cardiovascular health, the International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommends a minimum combined intake of EPA and DHA of 500 mg/day for healthy adults (Cunnan et al., 2004).. Dietary sources of preformed EPA and DHA are primarily fish and seafood (Meyer et al., 2003). In the UK and other Western countries dietary intakes of EPA and DHA are low compared with ALA or LA intakes. In the British diet, estimated average daily intakes are 1.55 g ALA, 60 mg EPA and 100 mg DHA (MAFF 1997). This suggests that the conversion of ALA to EPA and DHA may be important for providing n-3 LC-PUFA for cell membranes. The de novo synthesis of AA, EPA and DHA from their precursors involves a series of desaturation, elongation and β-oxidation steps (Sprecher 2002). The ability of humans to convert ALA into n-3 LC-PUFA has been studied using two approaches: increased dietary ALA consumption or use of stable isotope-labelled ALA. In men, both study approaches showed conversion to EPA and docosapentaenoic acid (DPAn-3, 22:5n-3), but limited conversion to DHA (reviewed in Burdge & Calder 2005). Using a stable-isotope tracer technique, Burdge and coworkers found that women of reproductive age had a much greater capacity to convert ALA to DHA compared with men of a similar age (Burdge et al 2002a; Burdge et al., 2002b). In agreement with these results, Pawlosky *et al.,* (2003) reported that, when subjects subsisted on a beef-based diet, the rate constant coefficient for the conversion of DPAn-3 to DHA was much greater in women than in men (Pawlosky et al., 2003). The larger rate constant coefficient in women led to a nearly 3-fold higher amount of DPAn-3 utilised for DHA synthesis compared with men. The implication of these findings is that conversion of DPAn-3 to DHA, which requires both Δ6-desaturase activity and peroxisomal β-oxidation, is

modified by gender and may be regulated independently from the activity of earlier steps in the pathway. Furthermore, partitioning of ALA towards β-oxidation and carbon recycling into saturated (SFA) and monounsaturated fatty acids (MUFA) appears to be lower in women than in men (Burdge et al 2002a; Burdge et al., 2002b). This means that women would have a greater availability to convert ALA to longer-chain n-3 PUFA than men who use fatty acids as an energy source to a greater extent. It is plausible that oestrogen may act as an agonist of DHA synthesis, as women taking an oral contraceptive pill had higher concentrations of labeled DHA in their circulation than those who did not (Burdge et al 2002b; Giltay et al., 2004b). In addition, treatment of postmenopausal women with the selective oestrogen receptor modulator raloxifene or hormone replacement therapy (using conjugated equine oestrogens plus medroxy-progesterone acetate) increased plasma cholesterol ester DHA concentrations (Giltay et al., 2004a).

So far, four studies have been undertaken in human subjects to investigate if the assumed gender-related differences in n-3 PUFA metabolism are reflected in circulating plasma levels of n-3 LC-PUFA (reviewed in Childs *et al.,*. 2008). Although these studies vary in sample size, extent of dietary control and blood lipids analysed, they all found that women had higher plasma/serum DHA concentrations compared with men (Giltay et al., 2004b; Bakewell et al., 2006; Crowe et al., 2008; Nikkari et al., 1995). Two studies also reported lower circulating concentrations of EPA and DPAn-3 in women compared with men (Bakewell et al., 2006; Crowe et al., 208) which may indicate either a greater conversion of EPA and DPAn-3 into DHA or their displacement from blood lipids by DHA.

To our knowledge, no study has directly compared n-3 LC-PUFA composition of platelet membrane in men and women of similar age consuming their habitual

diets. Hence, the present study investigated if there is gender difference in the n-6 and n-3 fatty acid composition of platelet phospholipids.

6.2. Subjects, Materials and Methods

These are described in detail in chapter 2.

6.3. Results

Results were available for seventy-four volunteers (34 female, 40 male). 1 more female was included in study 4, who wasn't in study 3 due to missing plasma and RBC data.

6.3.1. Subjects

The male and female volunteers did not differ in age, diastolic blood pressure and number of smokers (Table 20). Men had higher height, weight, body mass index (BMI), fat free mass and total body water as well as lower proportions of total body fat compared with women. The systolic blood pressure was significantly higher in men than in women. Fourteen of the thirty-four women (41%) used an oral contraceptive pill.

6.3.2. Energy, macronutrient and fatty acid intakes

Energy, macronutrient and selected individual fatty acid intakes from the diet are summarised in Table 21. Men had higher consumption of energy and absolute intakes of fat, protein and carbohydrates than female volunteers. However, when it was expressed as percentage of total energy intake (%energy), there were no significant differences between the men and women in fat, protein and carbohydrate intakes. Alcohol consumption (absolute and %energy) did not differ between the male and female subjects. Absolute intakes of total saturated fatty acids, total PUFA and total n-6 PUFA were higher in men compared with women, whilst intakes expressed as

%energy showed no gender difference. Absolute and %energy consumption of total n-3 PUFA were similar between genders.

Regarding the dietary intake of individual fatty acids, men had higher absolute intakes of oleic, LA and ALA compared with women, whereas the intakes of these fatty acids as %energy showed no gender difference. There were no significant differences in the intakes (absolute and %energy) of AA, EPA, and DHA between genders. Combined daily EPA and DHA intake showed a huge variation in both women and men (10th percentile: 61mg in women and 50mg in men; 90th percentile: 875 mg in women and 971 mg in men). The ratios of total n-6:n-3 PUFA and LA:ALA in the diet were similar in both genders.

6.3.3. Fatty acid composition of platelet phospholipids

Analysis of the fatty acid composition of platelet phospholipids showed specific differences in individual PUFA between men and women (Tables 22 and 23). Women had higher DHA in platelet PC (1.19 vs. 1.05 area%, $p<0.05$) and PE (3.62 vs. 3.21 area%, $p<0.05$) compared with men. The proportion of EPA in platelet PE was significantly higher in women than men (1.10 vs. 0.93 area%, $p<0.05$). The mean ratio of DHA:DPAn-3 was higher in platelet PC (+14%, $p<0.05$) and PE (+17%, $p<0.05$) in women compared with men. The percentage of AA and total n-6 PUFA in platelet PC were significantly lower in women than men. In comparison with the male subjects, the female volunteers had a lower AA:EPA ratio in both platelet PE (-21%, $p<0.05$) and PC (-25%, $p<0.05$). Women also had a lower AA:DHA ratio than men in both platelet PE (-14%, $p<0.05$) and PC (-22%, $p<0.01$). The mean ratio of n-6 to n-3 PUFA in platelet PC was 15% lower in women, but did not differ in platelet PE. Women also had lower total SFA levels in platelet PE. There were no significant gender differences in any of the other investigated PUFA. Proportions of individual n-3 LC-PUFA were each strongly correlated

between platelet PE and PC in both men and women (EPA r=0.85, DPAn-3 r=0.72, DHA r=0.86, p<0.001 for all).

6.3.4. Correlations between dietary PUFA intake and platelet fatty acid composition

The correlations between dietary n-3 PUFA intakes (mg/kg body weight) and platelet n-3 LC-PUFA are shown separately for men and women in Table 24. In both genders, dietary EPA and DHA intake showed a positive correlation with EPA in platelet PC and PE. Dietary EPA was correlated with DHA in platelet PE in men only. Platelet DHA was not correlated with dietary DHA intake in women, whereas a positive correlation was found in men. No correlations were found between dietary ALA intake and EPA or DHA percentage in platelet PE or PC in either gender (data not shown).

6.4. Results tables.

Table 20: Subject characteristics – Study 4

	Women (*n*=34)		Men (*n*=40)		*P*[a]
	Mean	SD	Mean	SD	
Age (years)	32.7	7.3	32.6	8.0	NS
Height (cm)	167	6	178	8	<0.001
Weight (kg)	63.8	12.7	76.5	10.1	<0.001
BMI (kg/m^2)	22.9	4.7	24.3	3.1	<0.01
Body Fat (%)	28.1	7.9	15.8	5.8	<0.001
Fat Free Mass (kg)	45.0	3.5	64.2	7.1	<0.001
Total Body Water (kg)	33.0	2.5	46.9	5.0	<0.001
Blood pressure (mm Hg)					
Systolic	113	10	125	11	<0.001
Diastolic	72	6	75	9	NS
Smokers (*n*)	2		4		NS
Use of oral contraceptives (*n*)	14		-		-

BMI, body mass index; NS, not significant.
[a] Between-group differences were analysed using Independent-Samples T test (age, height, diastolic blood pressure), Mann-Whitney U test (all other numerical variables) and Fisher's Exact Test (smokers).

Table 21: Energy, macronutrient and selected fatty acid intakes – Study 4

	Women (*n*=34)			**Men (*n*=40)**			**P**
	Median	**Mean**	**SD**	**Median**	**Mean**	**SD**	
Energy (kcal/d)	1806	1775	498	2365	2405	597	<0.001
Fat (g/d)	70	72	28	100	94	27	0.001
(%energy)	36	36	7	37	35	6	NS
Protein (g/d)	65	66	18	89	90	27	<0.001
(%energy)	14.5	15.3	3.6	14.5	15.2	2.9	NS
Carbohydrates (g/d)	205	206	64	260	274	82	<0.001
(%energy)	46	47	9	45	46	8	NS
Alcohol (g/d)	7.0	12.1	13.7	14.5	24.4	27.0	NS
(% energy)	0.03	0.05	0.05	0.04	0.07	0.07	NS
Total SFA (g/d)	20.0	21.5	9.0	28.5	28.9	8.6	0.001
(%energy)	10.5	10.7	2.8	11.0	11.0	2.2	NS
Oleic acid (g/d)	18.9	21.3	10.2	24.5	25.4	8.8	<0.05
(%energy)	9.8	10.6	3.4	9.4	9.5	2.4	NS
Linoleic (g/d)	9.1	9.9	6.2	11.5	12.0	5.9	0.030
(%energy)	4.5	4.8	2.1	4.0	4.5	2.0	NS
Arachidonic (mg/d)	108	112	61	120	146	98	NS
(%energy)	0.06	0.06	0.03	0.05	0.05	0.03	NS
Total n-6 PUFA (g/d)	9.3	10.1	6.2	11.8	12.3	6.0	<0.05
(%energy)	4.5	4.9	2.1	4.1	4.6	2.0	NS
α-Linolenic (g/d)	1.13	1.24	0.65	1.59	1.58	0.67	<0.05
(%energy)	0.58	0.62	0.30	0.56	0.60	0.25	NS
EPA (mg/d)	97	145	138	82	164	198	NS
(%energy)	0.06	0.08	0.07	0.03	0.06	0.06	NS
DHA (mg/d)	154	202	198	115	228	267	NS
(%energy)	0.09	0.11	0.10	0.04	0.09	0.09	NS
Total n-3 PUFA (g/d)	1.6	1.7	0.8	2.0	2.1	1.0	NS
(%energy)	0.80	0.85	0.39	0.72	0.79	0.33	NS
Total PUFA (g/d)	10.9	12.0	6.7	14.4	14.6	6.7	<0.05
(%energy)	5.3	5.9	2.3	4.9	5.5	2.2	NS
n-6 : n-3 PUFA	6.2	6.7	3.7	5.6	6.2	2.3	NS
Linoleic : α-linolenic	8.5	8.5	3.7	7.3	7.9	2.3	NS

Table 22: N-6 and n-3 fatty acid composition of platelet phosphatidylcholine – Study 4

	Women (*n*=34)		Men (*n*=40)		*P*
	Mean	SD	Mean	SD	
Area%					
Linoleic	8.5	1.1	8.5	1.1	NS
γ-Linolenic	0.051	0.009	0.058	0.020	NS
Dihomo-γ-linolenic	1.73	0.35	1.79	0.29	NS
Arachidonic	11.9	1.1	12.9	1.4	<0.01
Total n-6 PUFA	23.9	1.3	25.0	1.8	<0.01
α-Linolenic	0.077	0.023	0.083	0.055	NS
EPA	0.56	0.27	0.49	0.31	NS
DPAn-3	0.64	0.14	0.65	0.14	NS
DHA	1.19	0.26	1.05	0.32	<0.05
Total n-3 PUFA	2.50	0.55	2.32	0.65	NS
Total SFA	47.6	1.9	47.2	1.4	NS
Total MUFA	24.8	1.2	24.3	1.3	NS
Ratios					
DHA: DPAn-3	1.91	0.45	1.67	0.52	<0.05
DHA: α-Linolenic	16.5	5.2	14.9	7.0	NS
AA : DHGLA	7.2	1.6	7.4	1.2	NS
AA : EPA	26.9	14.2	35.7	25.1	<0.05
AA : DHA	10.5	2.8	13.5	4.7	<0.01
n-6 : n-3 PUFA	10.0	2.2	11.7	3.7	<0.05

Table 23: N-6 and n-3 fatty acid composition of platelet phosphatidylethanolamine – Study 4

	Women (*n*=34)		**Men (*n*=40)**		**P**
	Mean	**SD**	**Mean**	**SD**	
Area%					
Linoleic	3.4	0.9	3.3	0.6	NS
γ-Linolenic	0.027	0.011	0.031	0.009	NS
Dihomo-γ-linolenic	0.84	0.22	0.82	0.16	NS
Arachidonic	39.3	1.8	39.7	1.7	NS
Total n-6 PUFA	48.7	1.7	49.1	1.9	NS
α-Linolenic	0.061	0.023	0.070	0.034	NS
EPA	1.10	0.47	0.93	0.52	<0.05
DPAn-3	3.21	0.55	3.38	0.71	NS
DHA	3.62	0.83	3.21	0.82	<0.05
Total n-3 PUFA	8.0	1.3	7.6	1.4	NS
Total SFA	20.4	1.5	21.3	1.7	<0.05
Total MUFA	8.0	0.8	7.8	1.1	NS
Ratios					
DHA: DPAn-3	1.15	0.27	0.98	0.29	<0.05
DHA: ALA	67.0	28.4	55.3	29.9	NS
AA : DHGLA	50.0	12.7	50.5	10.0	NS
AA : EPA	42.4	18.5	53.4	23.2	<0.05
AA : DHA	11.5	3.1	13.4	4.4	<0.05
n-6 : n-3 PUFA	6.3	1.3	6.7	1.5	NS

Table 24: Spearman correlations between selected dietary n-3 LC-PUFA and platelet PC and PE – Study 4

	Dietary intake (mg/kg body weight)			
	EPA		DHA	
	♀ (*n*=34)	♂ (*n*=40)	♀ (*n*=34)	♂ (*n*=40)
Platelet PC				
EPA	0.578***	0.457**	0.577**	0.514**
DHA	NS	NS	NS	NS
Platelet PE				
EPA	0.431*	0.379*	0.449**	0.394*
DHA	NS	0.448**	NS	0.461**

♀, women; ♂, men.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.5. Discussion

Previously, studies (Giltay et al., 2004b; Bakewell et al., 2006; Crowe et al., 2008; Nikkari et al., 1995) have shown higher or equal plasma or serum DHA levels in women compared with men. This study examined if platelet PC and PE reflect similar difference in men and women. We found that women had higher platelet DHA and lower ratios of AA:EPA and AA:DHA compared with men despite consuming comparable diets; these differences were observed in both platelet PC and PE. There was no difference in macronutrient intake including n-6 or n-3 fatty acids when expressed as %energy between genders. This suggests that the observed differences in platelet fatty acid composition are likely to reflect gender difference in de novo synthesis of LC-PUFA. Overall, the type of fat consumed in our volunteers who had special interests in nutrition or sport science, was closer to dietary recommendations than that of the British population (Marriott and Buttriss 2003). However, even in this homogenous population, the dietary EPA and DHA intake showed a huge variation from less than 20mg to more than 1g per day.

Greater circulating plasma DHA concentration in women than men would increase the DHA supply for incorporation into circulating blood cells, which could

explain the observed higher DHA proportion in platelet phospholipids in women in this study. The exchange of FA from plasma to circulating blood cells can occur by several mechanisms: uptake of albumin-bound FA (NEFA or lyso-PC) by platelets/leukocytes and erythrocytes (Brossard et al., 1997; Lemaitre-Delaunay et al., 1999), direct transfer of phospholipids from circulating lipoproteins (LDL and HDL) to blood cell membranes (Engelmann et al., 1996) or lipoprotein lipase-favoured transfer of VLDL-associated phospholipids (Ibrahim et al., 2006). Additionally, there is also an incorporation of fatty acids into maturing blood cells at stages preceding their appearance in the circulation.

Studies using stable isotope-labelled fatty acid demonstrated that conversion of DPAn-3 to DHA, which requires both Δ6-desaturase activity and peroxisomal β-oxidation, is up-regulated in women compared with men (Pawlosky et al., 2003). A greater conversion of DPAn-3 to DHA in women than men may also explain women's higher ratio of DHA to DPAn-3 in platelet phospholipids in this study as well as the lower DPAn-3 concentrations in plasma or serum phospholipids in women reported by Bakewell et al., (2006) and Crowe et al., (2008).

Mean proportions of AA and total n-6 PUFA in platelet PC were significantly lower in women than men (AA -8%; total n-6 PUFA -5%). This is in line with the 5% lower proportion of total n-6 PUFA in women than men in plasma PC reported by Bakewell and co-workers (2006). Explanations for this finding could be that there is a competition between n-3 and n-6 PUFA for incorporation into phospholipids, or a different selectivity for FA incorporation in men and women.

The observed lower platelet AA relative to EPA and DHA as well as the lower n-6:n-3 PUFA ratio in women might contribute to the lower cardiovascular risk in young women compared with men of the same age. Both EPA and DHA compete with AA for the 2-acyl position of platelet membrane phospholipids and as substrate for phospholipase A2, cyclo-oxygenase and lipoxygenase enzymes

(Das 2008). As a result, when stimulated, such platelets produce less TXA2 and more TXA3, which is less potent in inducing platelet aggregation and thrombosis.

The present study found that platelet EPA correlate positively with dietary intake of both EPA and DHA. This suggests that platelets are highly susceptible to changes in dietary fat intake. Previous studies demonstrated that strict vegans with no intake of preformed n-3 LC-PUFA had lower EPA and DHA in platelet lipids compared to omnivore controls (Agren et al., 1995; Sanders et al., 1992). However, in our study platelet DHA were associated with the dietary intake of preformed EPA and DHA in the PE in men only, while no correlation was observed in women.

6.6. Conclusion

Taken together, these results suggest that maintenance of DHA concentrations in tissues in men might depend on dietary sources to a greater extend than in women who seemed to have a greater capacity for endogenous DHA synthesis. Thus, dietary guidelines may need to recommend higher intake of preformed n-3 LC-PUFA for men than for women to achieve the same tissue levels. Increasing the low EPA and DHA relative to AA concentration in platelets of men by increasing the proportions of n-3 LC-PUFA may eventually have beneficial effects on cardiovascular risk in men. In conclusion, this study shows that platelet LC-PUFA composition differs between women and men consuming a similar diet with a more beneficial FA profile in women.

7. Overall Study Conclusions

7.1. Overview

The ever increasing non-communicable disease burden, which includes CVD, is closely related to obesity and the clustering of metabolic risk factors which includes hypertension, hyperglycaemia and hyperlipidaemia. Although the exact aetiology is unclear and likely to be a complex relationship between genes and environment, it is closely related to dietary habits, and dietary treatment may be considered to be the cornerstone in its management and prevention. Previous recommendations involved reduction of weight, an increase in activity level and anti-atherogenic dietary advice, which focused on reduction of saturated fat and macronutrient substitution.

However, contrary to dietary treatment goals, replacement of saturated fat with carbohydrate does not improve CVD outcome. Instead, increased intake of refined carbohydrate can exacerbate atherogenic dyslipidemia associated with higher insulin resistance, which includes elevated triglycerides, and small LDL particles, and reduced HDL cholesterol. In contrast, replacement of saturated fat by PUFA or MUFA is largely associated with benefit, shown by a decrease in LDL and HDL. Moreover, the beneficial properties of n-3 PUFAs in protecting against CVD via decrease in triglyceride synthesis, platelet reactivity and aggregation, and modulation of inflammation, arrhythmia and hypertension are now generally well recognised.

In the UK, saturated and polyunsaturated fat intakes differ from fish-eating populations such as those living near the Mediterranean who experience lower CVD rates. National dietary surveys reveal that saturated fat intake is higher than recommendations, while n-6 to n-3 ratio exceeds required level for beneficial health-promoting effects. Due to competition for metabolism by Δ6-desaturase, a

higher LA to ALA intake increases formation of AA, with a subsequent reduction in available EPA for incorporation into cell membrane phospholipids. This promotes pro-inflammatory and pro-aggregatory eicosanoid production, eg thromboxane A2, and hence CVD risk.

Moreover, there is an apparent gender disparity in biologic risk markers for CVD. Young men display higher LDL-cholesterol, triglycerides, and systolic blood pressure, glucose and lower HDL-cholesterol, and plasma and cell membrane DHA levels than young women, which places them at higher risk for CVD episodes. It is unclear whether this is related to dietary differences between the genders.

We have conducted a pilot study **(Chapter III (Study 1)** to investigate relationships between saturated and unsaturated dietary fat intakes, and blood glucose and lipid parameters in Caucasian University men and women. Volunteers (20 to 50 years, men 52, women 52) were recruited from staff and students of London Metropolitan University. Dietary intake was assessed, and body composition, blood pressure and fasting blood glucose and lipids measured. Results were corrected for under-reporting, although this did not appear to significantly affect differences in dietary intakes between the men and women. Men consumed more saturated fat (32.8g vs 22.3 g/d, $p<0.001$) although proportional intakes from the macronutrients and other individual fatty acids were similar. Nevertheless, despite comparable dietary and lifestyle data between the genders, which indicated an awareness of health-promoting dietary principles and activity levels, the men had elevated levels of glucose (5.41 vs 4.81 mmol/l, $p<0.001$), LDL-cholesterol (2.09 vs 1.44 mmol/l, $p<0.001$), triglycerides (1.22 vs 0.98 mmol/l) systolic blood pressure (126.4 vs 112.0 mm/Hg, $p<0.001$), and lower HDL-cholesterol (1.47 vs 1.87 mmol/l, $p<0.001$) compared with their female counterparts. The only association between dietary fat and blood glucose or lipid

paramaters was in the men ie between dietary n-6 intake and LDL -cholesterol (R^2 -0.382, $p<0.01$). The data of this study indicate widespread low compliance with n-3 fatty acid recommendations and a possible gender-related difference in response to dietary fat. Despite being highly health conscious and physically active, blood lipid levels in the men are indicative of a risk for CVD. In addition to enhanced nutritional education to increase seafood intakes in this age-group of men and women, customised dietary and lifestyle advice may be required in the men.

Furthermore it is known that adequate antioxidant factors to prevent losses from plasma and cell membranes are important to maintain tissue stores, since EFA and LC-n-3 FAs are rapidly oxidised in the presence of oxygen to peroxides and toxic free radicals. It is reported that dietary antioxidant intakes and plasma levels differ in men and women, and this may have a negative effect on plasma and RBC levels of cardio-protective LC n-3 Fas.

In **Chapter IV (Study 3)** we assessed the dietary intake with particular reference to 7 key antioxidant micronutrients (Fe, Cu, Zn, Se, Mg, retinol, alpha-tocopherol and beta-carotene) and investigated associations with blood lipids. Dietary intakes of selenium and zinc, were higher in men (84 vs 54 µg/d, $p<0.01$; 11.7 vs 8.8 mg/d, $p<0.001$ respectively) while dietary beta-carotene and vitamin C were higher in women (2.9 vs 2.3 µg/d, $p<0.05$; 138 vs 108 mg/d, $p<0.05$). It was noted during the study that although men consumed more food than women, proportional energy and macronutrient intakes (except saturated fat) were similar, however micronutrient intakes differed between the genders who demonstrated variations in food choice. For example in the men, a preference for meat including offal, cereals and brazil nuts explained higher intakes of zinc, retinol, iron and selenium (retinol and iron non-significantly), while in the women, a preference for fruits and vegetables was associated with higher intakes of vitamin

C and beta-carotene. Nevertheless, irrespective of levels in the diet, more associations were found between dietary antioxidants and plasma and RBC cell membrane n-3 fatty acid levels in the women. This may suggest a greater antioxidant defence against lipid peroxidation which may conserve blood levels of EPA and DHA in the women.

Moreover, studies indicate that young premenopausal women are better able to synthesise long-chain n-3 fatty acid, docosahexaenoic acid, from its precursors, compared to men of a similar age. Although dietary ALA intake may be higher in men than in women, its metabolic fate differs. Women exhibit elevated ALA to LC-n-3 FA conversion rates via elongation, desaturation and beta-oxidation, while men appear to partition a significant proportion of ALA towards beta-oxidation for energy production, or synthesis of SFA or MUFA (Burdge et al 2002 a, b). A tenable explanation for greater synthesis of DHA in women relates to putative action of the sex hormones, seemingly designed to prepare them for pregnancy, to meet the high DHA requirements of the foetus and neonate (Innis et al., 2008), since levels decrease after the menopause.

Several studies report higher plasma DHA levels in women than in men, and compare dietary intakes of omega 3 fatty acids with plasma levels in an effort to explain this gender discrepancy. However, there are indications that diet may not be the only factor influencing DHA levels since irrespective of carefully controlled or freely chosen diets, plasma DHA levels are higher in premenopausal women, especially those taking the oestrogen containing contraceptive (Giltay et al 2004a), in male to female transsexuals taking oestrogen supplements (Giltay et al 2004b), and in oestrogen treated postmenopausal women compared to men (Giltay et al 2004a). Furthermore, data from 4900 randomly selected UK men and women recruited from the general population consuming fatty fish intakes of 12-40g per day, revealed that circulating plasma DHA levels were higher in women

than in men (Welch et al., 2006, 2010). Similar results were reported in a small university population (Bakewell et al., 2006), and a large New Zealand study. These two studies however used arguably less reliable methods (FFQ and 24hr recall) to assess habitual intakes (Bingham 1997), which may have differed in preformed dietary DHA. Furthermore, in the NZ study a comprehensive dietary analysis was not possible due to an incomplete n-3 FA database (Crowe et al., 2008).

Further analysis of the EPIC Norfolk data revealed that the ratio between long chain plasma n-3 FAs and the precursor, dietary ALA was higher in the young women of childbearing age, suggesting enhanced conversion rates (Welch et al., 2010). In addition, Welch and co-workers (2010) reported higher potential conversion rates of dietary ALA to LC n-3 FAs in non-fish eaters versus fish-eaters, reflecting results from a small previous metabolic study (Pavlosky et al., 2003).

Previous similar studies have investigated FA levels in plasma only, although this is a marker of short-term intake (days-weeks), while data is limited in red blood cells, which reflect intakes over months (Katan et al., 1997) and correlate more closely with cardiac n-3 FA levels (Harris et al., 2004). Furthermore, FA levels in platelets which are more indicative of cardiovascular risk (Vognild et al., 1998) are seldom reported.

We carried out a comprehensive investigation of habitual dietary n-3 fatty acid intakes and compared results with n-3 FA levels in plasma, red blood cells and platelets. In addition, we chose a homogenous group of educated young university men and women who were likely to have healthier diets and higher intakes of n-3 fatty acids than the general population.

Chapter V (Study 3) investigated gender differences in dietary intakes, and plasma and red blood cell (RBC) fatty acids. Dietary intake was assessed using a 7-day food diary, and fatty acid concentrations of plasma phosphatidyl-choline

(PC), triacylglycerol (TG), and cholesteryl esters (CE), and red blood cell PC, phosphatidyl-serine (PS), phosphatidyl-ethanolamine (PE) and sphingomyelin (SM) were determined. Despite similar percentage energy intakes from long-chain n-3 fats, DHA concentrations in plasma PC ($p<0.01$), TG ($p<0.001$) and CE ($p<0.05$), and RBC PE ($p<0.01$) were higher in women compared to men. In women, dietary DHA was associated with plasma ($p<0.05$) and RBC PC ($p<0.01$) DHA only, whereas in men, it was related to DHA level in plasma PC ($p<0.001$), TG ($p<0.001$) and CE ($p<0.001$), and in RBC PC ($p<0.001$), PS ($p<0.05$), PE ($p<0.001$) and SM ($p<0.05$). Interestingly in women, exercise had a positive interaction with DHA concentration in RBC PC ($p<0.05$), PE (0.001) and PS ($p<0.05$). These results provide further evidence for a greater reliance on preformed DHA in the men, compared to in the women.

Following on from this, **Chapter VI (Study 4)** study investigated the relationship between diet, platelet phospholipid fatty acids and gender. Dietary intake and platelet phosphatidyl-choline (PC) and phosphatidylethanolamine (PE) fatty acids were determined. Absolute and %energy intakes of arachidonic acid (AA), eicosapentaenoic acid (EPA), and DHA, and the ratios of total n-6/n-3 PUFA and linoleic/alpha-linolenic acids did not differ between the sexes. However, women had higher platelet DHA in PC and PE than men. Also EPA was higher in women's PE. Conversely, men had elevated AA and total n-6 fatty acids in PC. The higher platelet DHA levels and lower platelet AA/EPA and AA/DHA ratios in women of childbearing age compared with men, may lead to less platelet aggregation and vaso-occlusion.

7.2. Implications

The implications from these studies of young to middle-aged Caucasian University men and women include the following:

1. Both genders had comparable proportional dietary intakes of most macronutrients, although the men had a higher consumption of saturated fat.
2. Compared to women, LDL cholesterol in men may be more affected (inversely associated) by dietary omega 6 fatty acids.
3. The men displayed higher LDL cholesterol, higher triglycerides, lower HDL cholesterol and higher systolic blood pressure levels (as has been previously reported)
4. Even in educated subjects who volunteer to participate in a dietary study, under-reporting occurs at a similar level to studies involving other populations.
5. Gender differences in food preferences may be responsible for differences in levels of dietary micronutrients in the men and the women.
6. More associations between dietary antioxidants and cell membrane EPA and DHA were found in the women. This may confer greater protection against lipid peroxidation of LC n-3 FAs, and hence positively influence cardio-protection in the women
7. Intakes of LC-PUFAs were lower than recommendations, and average national intakes, in both men and women.
8. Despite similar proportional dietary intakes of all long-chain omega-3 fats, higher levels of DHA were found in the plasma, red blood cell membrane and platelet phospholipids of women. This may suggest higher conversion rates from ALA and EPA compared to men (as previously reported).

9. DHA level in RBC PLs was positively associated with dietary DHA in the men. This may indicate that men have a greater reliance on dietary intakes of pre-formed DHA compared to women.
10. Lower n-6 fatty acids were found in platelet PC of the women. This may indicate a competition between n-3 and n-6 PUFA for incorporation into phospholipids or a greater/different selectivity for FA incorporation in the men and women.
11. Higher level of AA was found in platelets in the men. Consequently this may increase susceptibility for production of pro-aggregatory and vaso-constrictive eicosanoids, and hence CVD risk in the men.
12. A positive interaction between exercise and DHA level was found in the women.

7.3. Future Work

Further investigation would be useful to:

1. Repeat this study in a homogenous sample of post-menopausal or pre-pubertal females and males of a similar age, consuming similar dietary intakes, to provide further data for the influence of sex hormones on blood fatty acid levels.
2. Examine dietary and cell membrane fatty acids in low socio-economic groups with very low fish intakes to explore possible conversion rates, and in non-Caucasian ethnic groups.
3. Understand why fish consumption continues to be lower in all sectors of the UK population than is observed in northern and southern countries, despite the UK being surrounded by sea, and endowed with a wealth of rivers.
4. Explore further the effect of exercise on blood n-3 fatty acids

5. Address the social/cultural factors which may contribute to differential food preferences (meat vs fruit and vegetables, usage of ready meals) in men and women.
6. Investigate further possible gender differences in dietary fat and antioxidant requirements.

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9. Appendices

9.1. PPT presentation slides – The effect of dietary fat

A 3-Countries Study to Investigate:

The Effect of Dietary Fat on Cell Membrane Omega-6 and Omega-3 and the Increased Incidence of Chronic Disease

Marita Neville MSc, BSc, SRD
Julia Geppert PhD, MSc, BSc.

Background

- Dietary intake of total & sat fat is higher than UK recommendations (NDNS 2001/2)
- ⇑ obesity, and ⇑ chronic diseases (DM, CHD, Cancers etc) (DH 2006)
- Related to type of dietary fat - sat fat, n-6, n-3 PUFA (Simopoulos 2006, Nielson et al 2005, Hagfors et al 2005)
- May be mediated via changes in lipid membranes
- ? Age and gender effects
- ? Ethnic effect

What do we need for the study?

- 100 people (50 men, 50 women, 20-50 yrs) to participate
- Subjects to:
  - Fill in a simple lifestyle questionnaire
  - Complete a food diary of all food and drink taken over 7 days
  - Provide a 30ml blood sample

What you will get in return?

- Opportunity to participate in an original study being undertaken in the UK, Norway and Israel
- Nutritional analysis including fatty acids
- (Healthy Eating guidelines)
- Membrane fatty acid profile
- Entry into a fantastic draw

Study Assurances:

- Ethical approval for the study was granted in Feb 2007
- All information will be kept **strictly confidential**
- Freedom to change your mind at any stage

9.2. Invitation to participate in the study

INVITATION TO PARTICIPATE IN A NUTRITIONAL STUDY

TITLE: **BACKGROUND DIETARY LIPID AND MEMBRANE LIPID MAP**

INVESTIGATORS: **MARITA NEVILLE, JULIA GEPPERT, PROFESSOR KEBREAB GHEBREMESKEL RESEARCHERS, INSTITUTE OF BRAIN CHEMISTRY & HUMAN NUTRITION, LONDON METROPOLITAN UNIVERSITY**

We invite you to take part in a nutritional study. The information given explains the purpose of the study. Please make sure you understand the purpose before agreeing to take part in the study. If you have any questions, or want further information please do not hesitate to ask

Background: It is now accepted that qualitative and quantitative changes in consumption of dietary fat is primarily responsible for the rise in cardio-vascular disease, type 2 diabetes, high blood pressure, and some types of cancers in the developed Western Countries since the second half of the last century. According to recent dietary surveys, the habitual diets of Israel, Norway and UK populations are high in saturated fatty acids. Although there are various postulations, the mechanism by which diets high in saturated fat, and an imbalance in the n6:n3 ratio precipitate chronic diseases is not well understood. Nevertheless, it is suggested that diet-induced alterations in membrane phospholipids and the consequential abnormal signaling in gene and protein expression, plays a pivotal role in the genesis and progress of these chronic diseases.

Aim: To elucidate the effect of contrasting background dietary lipids on membrane lipid map in order to help understand the link between dietary lipid, membrane lipid composition and contrasting incidence of chronic diseases.

Protocol: One hundred healthy volunteers aged 20-50 (50 male and 50 female) will be randomly recruited from University students and staff. Our findings will be compared with those of parallel studies in Norway and Israel. In order to compare the UK data with that from the two other countries and ensure homogeneity, all subjects recruited will be Caucasian. **Exclusions**: Membrane lipid composition can be influenced by pregnancy and lactation, genetic (sickle cell, cystic fibrosis) and non-communicable chronic (diabetes, high blood pressure, inflammatory bowel, multiple sclerosis) diseases, and regular use of prohibited drugs and alcohol, as a result such individuals will be excluded from the study.

If you agree to participate in the study

- You will be asked to complete a simple lifestyle questionnaire.
- Keep a detailed diary of what you eat and drink for seven days.
- We will take 30ml of blood within 2-3 days following collection of dietary information.

The results of the study will help us to understand whether background dietary lipid has an effect on membrane lipid map.

Personal data will be treated in the strictest confidence and not passed on to others without the written consent of the participant. You are free not to participate in the study and may withdraw from the study at any time and are not obliged to give any reason.

Marita Neville m.neville@londonmet.ac.uk
Julia Geppert j.geppert@londonmet.ac.uk

9.3. Information leaflet

INVITATION TO PARTICIPATE IN A NUTRITIONAL STUDY

TITLE: **DIETARY FAT AND BLOOD CELL MEMBRANE FATTY ACIDS**

INVESTIGATORS: **Marita Neville, Julia Geppert, Professor Kebreab Ghebremeskel,**
Institute of Brain Chemistry & Human Nutrition,
London Metropolitan University

We would like to invite you to take part in a nutritional study. This information leaflet explains the purpose of the study. It is important that you understand the purpose of the study before agreeing to participate in it. Please feel free to contact Marita or Julia if you have any questions or wish to discuss the study further.

Background:
We know from recent dietary surveys that current intakes of total and ***saturated*** fat (fats generally in foods of animal origin) are higher than UK recommendations. In addition, the numbers of overweight and obese people in the UK are rising, and there is an increase in obesity-related diseases such as heart disease and diabetes.

Surveys in other countries, such as Norway and Israel, have found that national total fat intake is high. However, relative to UK diets, Norwegian and Israeli diets tend to contain higher amounts of a different type of fat called ***polyunsaturated*** fat (containing polyunsaturated fatty acids or *PUFAs*).

Norway and Israel, however, differ from each other in the source and type of PUFAs eaten: a higher proportion of the PUFAs in Norwegian diets come from oily fish, which contain omega-3 PUFAs, while in the Israeli diet, a higher proportion of PUFAs come from seed and vegetable oils, which contain omega-6 PUFAs.

The researchers in this study are interested in how the amount and type of fat in the normal diet of individuals' affects the way in which saturated fats, omega-6 and omega-3 PUFAs are stored in the body's cell walls or *cell membranes.* This is because changes in the fatty acids of cell membranes can affect the way in which the cell functions, and furthermore, may affect a person's risk of developing certain diseases such as those mentioned above.

In addition to possible effects of different diets on cell membrane fatty acids, a person's age and gender may also influence the way in which his or her diet affects their cell membrane fatty acids.

Therefore, we will also compare findings between men and women, as well as people of different ages.

Aim:

To investigate the effect of normal diet on cell membrane fatty acids (saturated fatty acids, omega-3 and omega-6 PUFAs) in order to help understand the link between dietary fat, cell membrane fatty acids and possible risk of certain diseases.

Protocol:
One hundred healthy volunteers aged 20-50 (50 male and 50 female) will be randomly recruited from University students and staff. Our findings will be compared with those of studies being undertaken at the same time in Norway and Israel.
In order to compare the UK data with that from the two other countries, all subjects recruited initially will be Caucasian. However people from other ethnic backgrounds may also be recruited, since we hope to investigate influence of ethnicity at a later stage in the study.

Exclusions:
Cell membrane fatty acids can be influenced by pregnancy and lactation, genetic (sickle cell, cystic fibrosis) and non-communicable chronic (diabetes, high blood pressure, inflammatory bowel, multiple sclerosis) diseases, and regular use of prohibited drugs and alcohol. Therefore these people will not be eligible for the study.

If you agree to participate in the study

- You will be asked to complete a simple lifestyle questionnaire.
- Keep a detailed diary of what you eat and drink for seven days.
- We will take 30ml of blood within 2-3 days following collection of dietary information.

The results of the study will help us to understand whether dietary fat has an effect on cell membrane fatty acids.

Personal data will be treated in the strictest confidence and will be not passed on to others without the written consent of the participant. You are free not to participate in the study, and may withdraw from the study at any time without giving any reason.

Marita Neville Contact details: m.neville@londonmet.ac.uk
and

Julia Geppert Contact details: j.geppert@londonmet.ac.uk

9.4. Eligibility form

Personal Data

Please E mail completed forms to m.neville@londonmet.ac.uk or j.geppert@londonmet.ac.uk or return to room T6-01, 6th floor tower block London Metropolitan University, Holloway Rd. N7 1DB

Surname: First Name:

Gender & Age: Ethnic background:

Address:

Phone: E mail:

Health Status and Lifestyle Questionnaire:

Please delete whichever does not apply to you and fill in any additional information:

Pregnant or breastfeeding: Yes/No

Diabetes: Yes/No

Raised blood lipids: Yes/No

Inflammatory bowel disease: Yes/No

Multiple Sclerosis: Yes/No

Cystic Fibrosis: Yes/No

Sickle cell anaemia: Yes/No

Other medical conditions? Yes/No

Do you drink alcohol? Yes/No

Approx how many units per week? units per week
(1 unit = 1glass wine, 1 pub measure spirits, ½ pint beer)

Do you smoke? Yes/No

Do you take any recreational drugs? Yes/No

THIS FORM WILL BE KEPT STRICTLY CONFIDENTIAL
Thank you very much for your co-operation

9.5. Consent Form

WRITTEN CONSENT FORM

Title of Project: **Dietary Fatty Acid and Blood Cell Membrane Fatty Acid Study**
Name of Subject:

Address:

I have read the attached information on the research in which I have been asked to participate and have been given a copy to keep. I have had the opportunity to discuss the details and asked questions about this information.

The Investigator has explained the nature and purpose of the research and I believe that I understand what is being proposed. For example, I understand that this study is part of a research project designed to understand the link between dietary fat, membrane fatty acid composition and contrasting incidence of chronic diseases in 3 different countries (UK, Norway and Israel), and that it has been approved by the London Metropolitan University's Research Ethics Committee.

I have been informed that the proposed study involves monitoring of food intake and blood fatty acids, which have been explained to me, together with possible risk involved.

I understand that my personal involvement and my particular data from this trial will remain strictly confidential. Only researchers involved in the study will have access to the data, or where applicable, the sponsor which funded the research.

I hereby fully and freely consent to participate in the study, which has been fully explained to me and understand that I can withdraw from participation at any time without giving any reason.

VOLUNTEER'S NAME: (BLOCK CAPITALS) ....

VOLUNTEER'S NAME: SIGNATURE ....

VOLUNTEER'S WITNESS' NAME: ....

WITNESS' SIGNATURE: ....

DATE: ....

As the Investigator responsible for this research (or a designated deputy), I confirm that I have explained to the volunteer named above the nature and purpose of the research to be undertaken.

INVESTIGATOR'S NAME: ....

INVESTIGATOR'S SIGNATURE : ....

DATE: ....

9.6. 7-day Food Diary

Name ______________________ **Code No.** __________

Address ______________________

____

____

Tel No. ______________________ **Date** ______________

NUTRITION ASSESSMENT

FOOD DIARY

QUESTIONNAIRE

The following personal data is required to complete an assessment of your nutritional status. All information will be strictly confidential. Please answer all the questions

1. **Height (cms)** ____________________

2. **Weight (kg)** ____________________

3. Age ____________________________

4. Are you on any special diet such as vegetarian or weight reducing?
Yes/No

If yes, please describe ____

5. Are you currently taking oral contraceptives or having a contraceptive injection or implant?

Yes/No

6. Do you currently smoke?
Yes/No

If yes, how many cigarettes/day ______________________________

7 Do you exercise (eg jogging, cycling, swimming) once a week or more?
Yes/No

If yes, please describe ______________________________

8 Do you consider yourself to be under more than usual work-related or
Yes/No
emotional stress?

9.Do you have any family history of chronic disease (eg. Heart disease, diabetes, or high blood pressure)

Yes/No

10. Do you regularly take any vitamin/mineral supplements?
Yes/No

If yes, please state the name and if possible, the contents ____________________

____

Date ____________

COMPLETING YOUR 7 DAY FOOD DIARY

The purpose of keeping a food diary is to allow us to assess your dietary intake, both in terms of quantity
and quality. The degree of accuracy relies on the completeness of your record.

Choose a week that you expect will be representative of your usual eating pattern. A seven day record is generally assumed to give more valid results; if however, this is not convenient, you may record for a minimum of 5 days, providing you include a weekend.

Write down **EVERYTHING** that you eat and drink during the seven days, remembering to add anything
you may have eaten while away from home. Try to do this after each meal while the information is still
fresh in your mind.

Please give a DETAILED description of foods eaten, for example .....

BREAD	was it white, brown, wholemeal, granary etc?
MARGARINE	please give the brand name eg Flora, Stork SB etc
MEAT	was it lamb, beef, pork, chicken etc?

	was it lean only or did you eat both lean and fat? was it leg, shoulder, chop etc? was it roast, fried, grilled, stewed etc?
POTATO	was it boiled, roast, sautéed, chipped or mashed. If mashed was milk and butter/margarine added?
FRIED FOOD	name the type of oil or fat used eg Sainsburys corn oil

Quantities of food eaten should be described as accurately as possible, for example
1 ham & salad sandwich ....

white bread, 2 slices of large loaf (medium sliced)
margarine, Tescos Soya, thinly spread
lettuce, 3 leaves
tomato, 1 medium
ham, lean only, 1 slice

If you have kitchen weighing scales, do use them when possible. Where weights are given on bars of chocolate, crisp packets, cans of drink etc, please state them.

We have put together an example page opposite. This cannot cover the many foods we all eat. When completing the diary, please identify the foods you eat in your own words, remembering that weights are important, where you can please provide them eg.

fish stew	salt cod, ackee, fried onions & tomatoes in corn oil	10oz

Remember to write everything down. The more accurate your diary is, the more valid the assessment will be. Good luck!

Please return to:
Institute of Brain Chemistry & Human Nutrition
TB 9/4, London Metropolitan University
166-220 Holloway Road, London, N7 8DB
Telephone: 0207 133 2446

EXAMPLE OF A DAY'S RECORD

DAY	TIME	FOOD ITEM	DESCRIPTION	AMOUNT
TUES	8.00 am	TEA	TYPHOO	1 MUG
		MILK	SEMI-SKIMMED	1 TABLESPOON
		SUGAR	WHITE, GRANULATED	2 TEASPOONS
		CORNFLAKES	TESCO BRAND	1 MEDIUM BOWL
		SUGAR	WHITE, GRANULATED	1 TEASPOON
		MILK	SEMI-SKIMMED	¼ PINT
		TOAST	WHITE MEDIUM SLICED LOAF	2 SLICES
		MARGARINE	STORK SB	THINLY SPREAD
		JAM	STRAWBERRY	2 TEASPOONS
	11.00 am	KIT KAT	ROWNTREES	4 FINGERS
		ORANGE JUICE	FRESH (CARTON)	250 ml
	1.00 pm	VEGETABLE CURRY	POTATO, CAULIFLOWER, CHICKPEAS,	2 TABLESPOONS
			ONION, TOMATOES, CORN OIL	
		FISH CURRY	½ MACKEREL, FRIED ONION IN CORN	3 TABLESPOONS
			OIL, TOMATO	
		RICE	BOILED WHITE	4 TABLESPOONS
		WATER	EVIAN MINERAL	1 LARGE GLASS
	4.00 pm	TEA	AS AT 8.00am (WITH MILK & SUGAR)	1 MUG
		BISCUIT	McVITIES CHOCOLATE DIGESTIVE	3
	7.00 pm	EGG	FRIED IN SAFEWAYS VEG. OIL	1 EGG
		SAUSAGES	LARGE PORK, SAFEWAYS, GRILLED	2
		CHIPS	McCAINS OVEN CHIPS	¼ 454g PKT
		BAKED BEANS	HP	½ 420g TIN
		SATSUMAS	FRESH	2 MEDIUM
		COFFEE (BLACK)	INSTANT	1 MUG
		ICE CREAM	VANILLA	2 SCOOPS
	9.00 pm	LAGER	HARP	½ PINT
		CRISPS	WALKERS, SALT & VINEGAR	1 PACKET
	11.00 pm	CREAM CRACKERS	JACOBS	2
		MARGARINE	STORK SB	MEDIUM SPREAD
		CHEESE	CHEDDAR	MATCH BOX SIZE

		MILK	SEMI SKIMMED	1 MUG

REMARKS

DAY	TIME	FOOD ITEM	DESCRIPTION	AMOUNT

REMARKS

9.7. Micronutrient Density

Table 25: Micronutrient density – Study 2

Characteristic	Women (n=38)			Men (n=25)			
	Mean	SD	Median	Mean	SD	Median	P
Cu (µg/d)	0.88	0.39	0.75	0.86	0.76	0.61	<0.05
Fe (mg/d)	7.09	1.76	7.01	6.12	1.39	6.04	<0.05
Mg mg/d	183.6	66.5	174.9	149.1	40.9	138.2	<0.01
Se (µg/d)	26.4	10.1	25.5	32.9	26.3	25.5	NS
Zn (mg/d)	4.41	0.78	4.31	4.45	1.09	4.25	NS
Vit A (Retinol Equivalents) (µg/d)	468.8	255.6	392.3	552.3	684.7	251.6	<0.05
Beta-caro (mg/d)	1.52	1.06	1.47	0.87	0.73	0.73	<0.001
Vit C (mg/d)	72.1	29.07	66.3	40.8	23.7	35.9	<0.001
Vit E (mg/d)	5.2	1.92	4.9	3.6	1.4	3.5	<0.001

9.8. Vitamin Calibration Curves

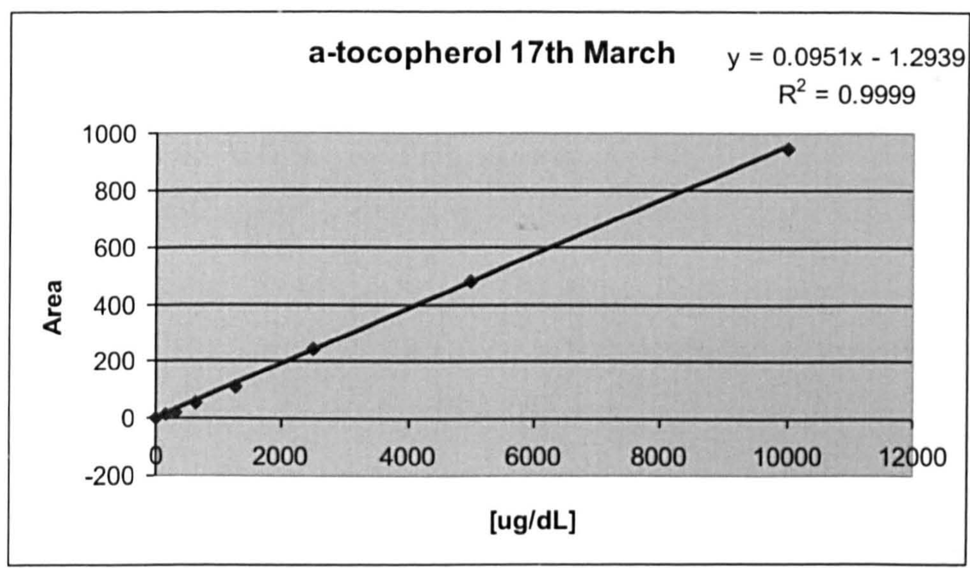

Figure 16: Alpha-Tocopherol standard calibration curve for 17th March

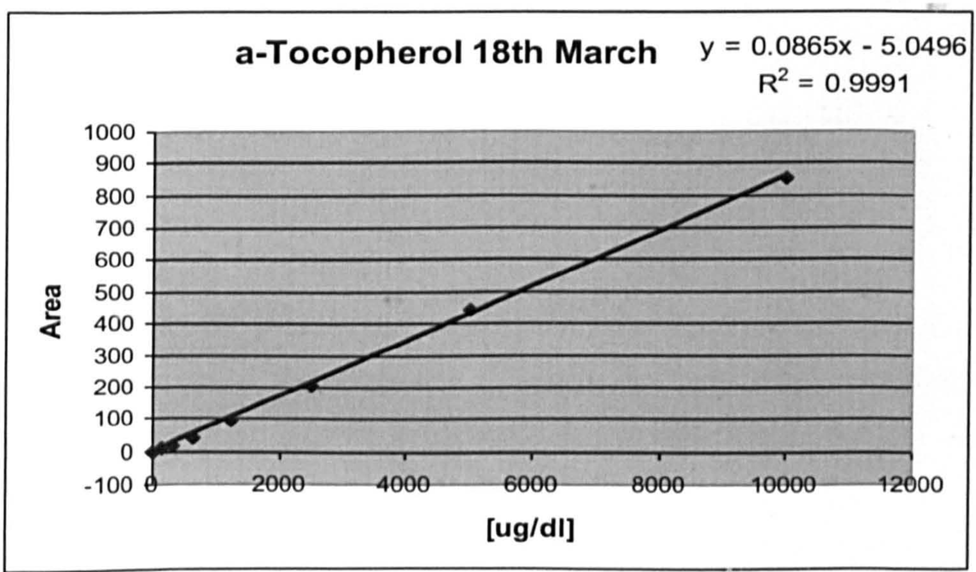

Figure 17: Alpha-Tocopherol standard calibration curve for 18th March

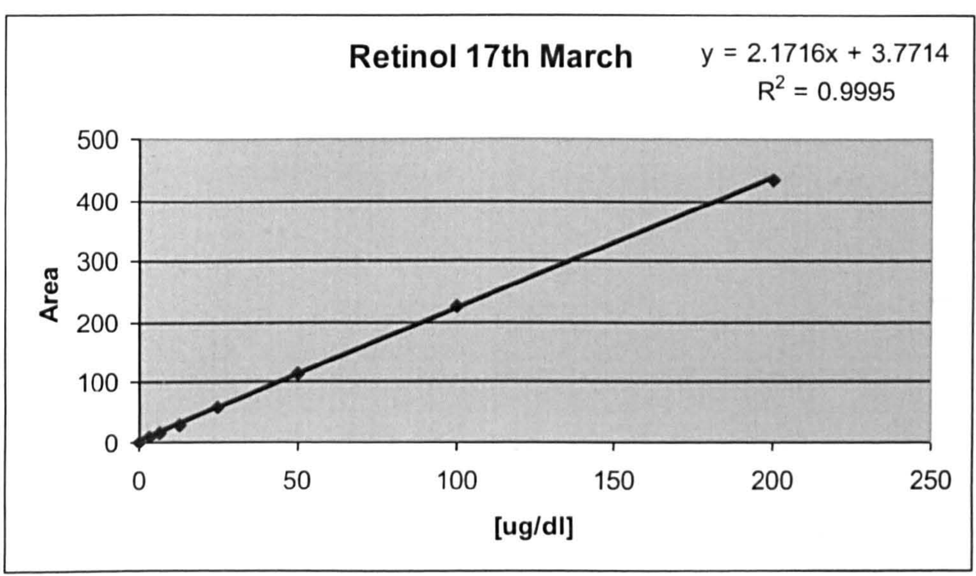

Figure 18: Retinol standard calibration curve for 17th March

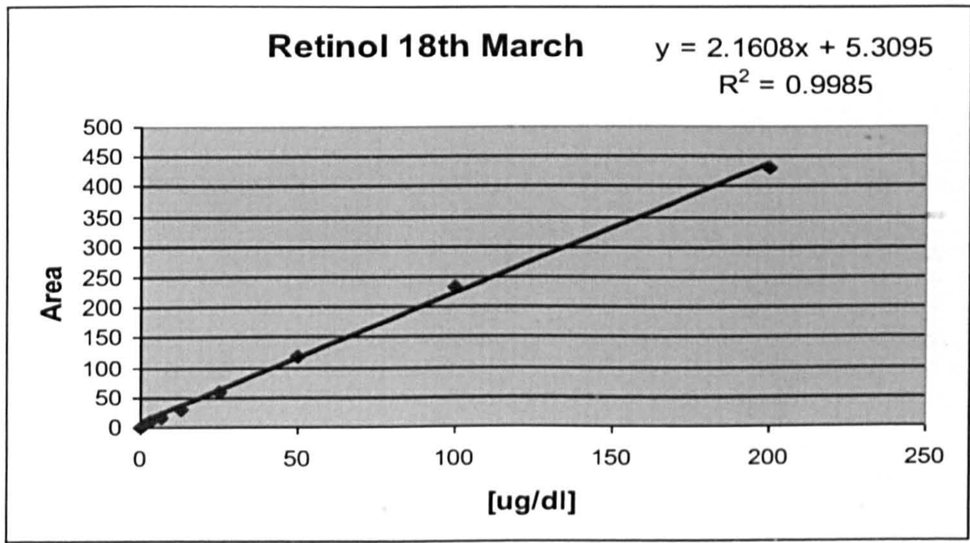

Figure 19: Retinol standard calibration curve for 18th March