

## Dietary lipids and vascular function: UK Food Standards Agency workshop report

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The UK Food Standards Agency convened a group of expert scientists to review current research investigating the effect of dietary lipids on vascular function. The workshop highlighted the need for intervention studies to be sufficiently powered for these measures and that they should be corroborated with other, more validated, risk factors for CVD. Work presented at the workshop suggested a beneficial effect of long-chain *n*-3 PUFA and a detrimental effect of *trans* fatty acids. The workshop also considered the importance of the choice of study population in dietary intervention studies and that 'at risk' subgroups within the general population may be more appropriate than subjects that are unrepresentatively healthy.

**Vascular function: Dietary lipids: *n*-3 Polyunsaturated fatty acids: Fish oils: *trans* Fatty acids: Saturated fatty acids: Food Standards Agency**

On 3 March 2003, the UK Food Standards Agency (FSA) convened a workshop on the use of measures of vascular function in dietary intervention studies and the effect of dietary lipids upon them. The results from recently completed studies (both FSA and non-FSA funded) were presented, and the workshop was chaired by Professor Joseph Vita (Boston University). The aim of the workshop was to determine where this work has taken us and where further work should be concentrated, as well as acting as a vehicle for dissemination. The research recommendations will feed into the future direction of FSA-funded nutrition research, and may also be of value in guiding other funders.

The vascular endothelium is an active tissue and possesses numerous anti-atherogenic functions in a normal healthy state. These include regulation of blood flow in response to metabolic demands, inhibition of blood clotting and prevention of inflammatory cell adhesion to and subsequent migration through the endothelium. Endothelial 'dysfunction' can be considered to be present when the

properties of the endothelium, either in a basal state or after stimulation, have altered in a way that is inappropriate with regard to preservation of normal function. Endothelial dysfunction is characterised by an alteration in phenotype, including decreased bioavailability of NO, a shift in the relative balance of pro- and anticoagulant factors, and increased expression of pro-inflammatory molecules. These changes may increase the risk for vasoconstriction, thrombosis, vascular inflammation and plaque activation, the processes that contribute to the development and clinical expression of CVD. Impaired endothelium-dependent vasodilation has emerged as a readily measurable endothelial response that appears to correlate with a number of diverse endothelial functions.

Evidence that endothelial dysfunction contributes to the atherogenic process is provided by clinical studies showing impaired vasodilator responses in patients with CVD risk factors. These abnormalities are often present before the development of clinically or angiographically evident atherosclerosis. For example, classical risk factors

including advancing age, dyslipidaemia, hypertension, diabetes mellitus and cigarette smoking have been associated with impaired endothelium-dependent vasodilation. Similarly, endothelial dysfunction has been reported in the setting of novel risk factors, such as hyperhomocysteinaemia, elevated lipoprotein(a) concentration, obesity, insulin resistance and systemic inflammation and/or infection. Patients with advanced atherosclerosis also display endothelial dysfunction. Further evidence for the relevance of endothelial dysfunction is provided by the observation that diverse therapies proven to reduce cardiovascular risk also have the ability to improve endothelial function. Thus, lipid-lowering therapy, angiotensin-converting enzyme inhibitors, smoking cessation and exercise all improve endothelial function and reduce cardiovascular risk. On the basis of these studies, endothelial function has been proposed as a clinically useful barometer of the combined impact of risk factors on vascular health (Vita & Keane, 2002).

The strongest evidence for the clinical importance of endothelial function is provided by prospective studies that demonstrate the prognostic value of endothelial dysfunction for CVD. This issue has been investigated in patients with established atherosclerosis (Neunteufl *et al.* 2000; Schächinger *et al.* 2000; Suwaidi *et al.* 2000; Heitzer *et al.* 2001; Gokce *et al.* 2002, 2003; Halcox *et al.* 2002) and in subjects with elevated CVD risk factors (Perticone *et al.* 2001; Modena *et al.* 2002; Schindler *et al.* 2003). To date, however, no study has specifically examined the question of whether reversing endothelial dysfunction can be equated with a reduction in cardiovascular risk. Such information is critical before endothelial function can be completely accepted as a true surrogate marker for cardiovascular risk.

### Measurement of vascular function

A number of methods have been used to examine endothelial function. Most examine endothelium-dependent vasodilation. Vasodilator responses to endothelium-dependent stimuli have been examined in the coronary circulation using quantitative angiography, the forearm microcirculation using venous occlusion plethysmography, and the conduit brachial artery using high-resolution ultrasound (flow-mediated dilatation; FMD) (Vita, 2002). FMD involves a series of ultrasonographic measurements of blood vessel diameter and flow. A blood pressure cuff is then placed around the arm and inflated to a suprasystolic pressure for approximately 4 min. On release of the cuff after 5 min, reactive hyperaemia is induced and shear stress promotes endothelium-dependent vasorelaxation.

Alternatively, small vessel endothelial function can be measured by using the skin circulation as a surrogate marker of generalised microvascular endothelial function. In this process, vasoactive chemicals (e.g. acetylcholine (endothelium-dependent) and sodium nitroprusside (endothelium-independent)) can be delivered transdermally to the skin microvessels using a small electric current (iontophoresis). The subsequent microvascular response can be measured by laser Doppler imaging, which measures perfusion in the top 1.0–1.5 mm of skin. Although the

prognostic value of small vessel endothelial function has yet to be demonstrated, changes in the microcirculation have been shown to parallel changes in the macrocirculation (Ramsay *et al.* 2002).

Arterial 'stiffness' measures (based on measurements of the material properties of the vessel wall or the capacitance function of the vessel as a whole) can also be assessed: pulse pressure, pulse wave analysis, pulse wave velocity, digital volume pulse and pulse contour analyses. This is a measure of the elasticity of the vessel (the relative volume change for a given pressure increment) and appears, in part, to depend on the vascular endothelium. Measures of arterial 'stiffness' have the advantage of being fast and simple and have been shown to have prognostic value for cardiovascular events (Boutouyrie *et al.* 2002; London & Cohn, 2002; Safar *et al.* 2002).

Finally, circulating markers of endothelial function may be assessed in blood. In subjects without known CVD, soluble intracellular adhesion molecule-1 (Ridker *et al.* 1998) and tissue plasminogen activator (Thogersen *et al.* 1998) have been shown to predict future cardiovascular events. In patients with known CVD, soluble intracellular adhesion molecule-1 (Haim *et al.* 2002), von Willebrand factor (Thompson *et al.* 1995; Wiman *et al.* 2000), tissue plasminogen activator (Thompson *et al.* 1995) and plasminogen activator inhibitor-1 (Hamsten *et al.* 1987) are independent predictors of future events. These studies are consistent with studies suggesting that non-endothelium-derived markers of inflammation, such as C-reactive protein, identify high-risk individuals (Pearson *et al.* 2003).

Most recently, endothelial progenitor cells have been measured in blood and their function was shown to relate to risk factors and endothelium-dependent vasodilation (Hill *et al.* 2003). An important issue in this field is the lack of uniform standards for measurement of endothelium-dependent vasodilation or other aspects of endothelial function, and the lack of a consensus in regard to the best approach for specific types of study.

### Dietary factors

The strength of evidence for dietary factors affecting endothelial function differs for differing dietary constituents. Replacement of a saturated fat-enriched diet with a high-fat high-MUFA diet, but not a low-fat high-carbohydrate diet, was shown to improve FMD in hypercholesterolaemic patients (Fuentes *et al.* 2001); furthermore, in healthy subjects, a high-fat high-MUFA diet was also shown to decrease circulating markers of endothelial activation (plasminogen activator inhibitor-1, von Willebrand factor and tissue factor pathway inhibitor), whereas a low-fat high-carbohydrate or high-fat high-saturated fat diet did not (Perez-Jimenez *et al.* 1999). Other studies, however, have shown no difference in either FMD (de Roos *et al.* 2001b) or arterial elasticity (Ashton *et al.* 2000) when comparing high-fat high-MUFA diets with low-fat high-carbohydrate diets in healthy subjects.

High doses of fish oils (4–5 g EPA + docosahexaenoic acid (DHA)/d) have been shown to improve FMD in hypercholesterolaemic subjects (Goodfellow *et al.* 2000), forearm blood flow in healthy subjects (Chin *et al.* 1993)

and endothelium-dependent vasodilator responses to intracoronary acetylcholine infusion in heart-transplant recipients (Fleischhauer *et al.* 1993). Mori *et al.* (2000) demonstrated that DHA (4 g/d), but not EPA (4 g/d), enhanced forearm blood flow.

High doses of folic acid (5–10 mg/d) have also been shown to improve FMD in coronary-artery disease patients (Title *et al.* 2000; Doshi *et al.* 2001) and smokers (O'Grady *et al.* 2002), forearm blood flow, but not arterial elasticity, in smokers (Mangoni *et al.* 2002), and volumetric coronary blood flow in hyperhomocysteinaemic patients with coronary artery disease (Willems *et al.* 2002). This effect was shown to be independent of a plasma homocysteine-lowering effect (Doshi *et al.* 2002). Lower doses of folic acid (e.g. 0.4 mg/d), which are attainable through the diet and result in maximal homocysteine reductions, have no effect on FMD (Pullin *et al.* 2001; Hirsch *et al.* 2002).

Flavonoid-rich beverages have been demonstrated to improve endothelial function with acute and long-term intake of flavonoid-containing beverages including tea (Duffy *et al.* 2001), grape juice (Stein *et al.* 1999) and de-alcoholised red wine (Agewall *et al.* 2000).

The results from studies investigating the effect of high doses of vitamin E (typically  $\geq 590$  mg  $\alpha$ -tocopherol/d) and vitamin C ( $\geq 1$  g/d) on endothelial function are equivocal (for review, see Duffy *et al.* 1999). Although one study suggested that patients with multiple risk factors, particularly cigarette smoking, derive benefit from vitamin E supplementation (Heitzer *et al.* 1999), most other studies have failed to show a beneficial effect in patients with coronary artery disease or type 2 diabetes mellitus (Elliott *et al.* 1995; Chowienzyk *et al.* 1998; Gazis *et al.* 1999; Title *et al.* 2000). Studies of combinations of antioxidants, typically vitamin C, vitamin E and  $\beta$ -carotene, have also provided disappointing results (Gilligan *et al.* 1994; MacKechnie *et al.* 2002). The findings with vitamin E and combination antioxidant therapy are consistent with large-scale clinical studies that demonstrated no benefit of antioxidant treatment on CHD (GISSI-Prevenzione Investigators, 1999; Yusuf *et al.* 2000; Heart Protection Study Collaborative Group, 2002).

A large number of studies have demonstrated a beneficial effect of acute, high-dose vitamin C (ascorbic acid) administration on endothelium-dependent vasodilation in patients with CVD and other disease states (Duffy *et al.* 1999). These studies provide insight into the pathophysiological mechanisms of vascular dysfunction and support a role of increased oxidative stress; however, the applicability of these studies to chronic vitamin C treatment on clinical endpoints remains uncertain.

It has been hypothesised that high-fat meals act as a trigger for acute coronary syndromes in populations with established atherosclerosis, by inducing a hypercoagulable and a vasoconstrictor state (Anderson *et al.* 2001). Several studies have demonstrated that high-fat meals, but not high-carbohydrate low-fat meals, induce acute impairment of FMD postprandially in healthy subjects (Plotnick *et al.* 1997, 2003; Vogel *et al.* 1997; Ong *et al.* 1999; Bae *et al.* 2001, 2003; Marchesi *et al.* 2000), diabetics (Fard *et al.* 2000) and, to a greater extent, patients with CHD (Ling *et al.* 2002). This impairment was attenuated by supplementing

the test meal with either vitamins C (1–2 g) and E (590 mg) (Plotnick *et al.* 1997, 2003; Katz *et al.* 2001; Ling *et al.* 2002; Bae *et al.* 2003) or foods rich in these nutrients (fruits and vegetables concentrate (Plotnick *et al.* 2003), balsamic vinegar and salad (Vogel *et al.* 2000)). High-fat meals have also been shown to postprandially impair the coronary microcirculation (Hozumi *et al.* 2002), systemic arterial compliance (Nestel *et al.* 2001) and the rheologic response to L-arginine, which was attenuated by the addition of vegetables rich in vitamins C and E to the test meal (Esposito *et al.* 2003). Most studies also observed postprandial impairment of vascular function following a high-fat meal to be associated with postprandial hypertriacylglycerolaemia; as background diet has also been shown to affect the postprandial triacylglycerol response following a high-fat meal (Roche *et al.* 1998; Wolever & Mehling, 2003) this may also be a factor.

Not all studies, however, have demonstrated postprandial impairment of FMD following a high-fat meal (Djousse *et al.* 1999; Raitakari *et al.* 2000; Gokce *et al.* 2001; Sejda *et al.* 2002). The reason for this discrepancy is unclear, and could be related to differences in the patient population studied or in the specific type of fat consumed. For example, Williams *et al.* (1999) demonstrated a meal high in oxidised fat, but not uncooked fat, impaired FMD; however, in a subsequent study no effect of heat modified fat was observed (Williams *et al.* 2001).

### Measure variability

Dr Margreet Olthof presented results on studies investigating the variability in FMD of the brachial artery in young healthy subjects. The variability of FMD is large and this is important for the design of trials with FMD as an end point. The within-subject CV of the FMD measured off-line after images had been recorded on videotape (B-mode) is 50% (de Roos *et al.* 2003). In addition, the within-subject variability of the FMD measured with the wall-track method (M-mode) was determined: fourteen healthy, non-smoking subjects (seven men, seven women; age range 20–25 years) were measured on six occasions after an overnight fast.

The main findings from the study were:

- the mean FMD was 7.8 (SD 2.6) % baseline diameter. Within-subject CV of FMD measured with the wall-track method was 46.4 %.

Thus, the within-subject variability of FMD measured with the wall-track method was similar to that of the B-mode. The consequences for the number of subjects needed in intervention trials is as follows: to detect a 2% difference in FMD (power 0.80,  $\alpha$  0.05), thirty to fifty subjects are needed in a crossover design, and sixty to one hundred subjects per group in a parallel design. Variability can be reduced by taking duplicate measurements. For B-mode ultrasound, duplicate reading of the videotapes is recommended, since the variation in reading of videotapes is large (CV 34%; de Roos *et al.* 2003). The variation of 50% for single measurements was in accord with values obtained by other workers present.

### Dietary modification of vascular function

Dr Bruce Griffin presented preliminary findings (BA Griffin, MD Griffin, IR Davies, P Chowienczyk, GAA Ferns, DJ Millward and TAB Sanders, unpublished results) on indices of vascular function from an FSA-funded project (the OPTILIP study) that attempts to identify the optimum proportions of  $\alpha$ -linolenic and long-chain *n*-3 PUFA in the UK diet with regard to cardiovascular risk factors. Healthy middle-aged men and women (forty men, thirty women, aged 45–65 years) were randomised to diets: a control diet with an intake of *n*-6 and *n*-3 fatty acids similar to that in the UK diet; a diet enriched with long-chain *n*-3 PUFA; a diet enriched with a similar amount of  $\alpha$ -linolenic acid; a diet enriched with long-chain *n*-3 PUFA and  $\alpha$ -linolenic acid; a diet with a more moderate  $\alpha$ -linolenic acid enrichment (the target *n*-6:*n*-3 fatty acid ratios were 10:1, 3:1, 3:1, 3:1 and 5:1 respectively). The dietary intervention period was 6 months.

FMD was measured in the brachial artery at rest and using a forearm occlusion cuff (5 min; endothelium-dependent vasodilatation) and sublingual glycerol trinitrate (300  $\mu$ g per tablet, 3 min; endothelium-independent vasodilatation) to induce reactive hyperaemia. Arterial diameters were measured and averaged from four points in the two-dimensional ultrasound image of the vessel wall at 30, 90 and 180 s post-cuff, and 3.5 and 5.0 min after the administration of glycerol trinitrate (30 and 90 s post-glycerol trinitrate). FMD was assessed immediately before and after 6 months of dietary intervention.

These preliminary results suggest show no significant dietary effects on maximal FMD or pulse wave velocity; however, the duration of FMD may be less in subjects on *n*-3 fatty acid-rich diets. The clinical relevance of this finding, if confirmed, is unknown.

Dr Nicole de Roos presented the results of long-term and postprandial studies of saturated and *trans* fatty acids on vascular function. A high intake of *trans* fat increases the risk of CHD and some investigators believe that the effect of *trans* fatty acids is stronger than that of saturated fatty acids (Ascherio *et al.* 1999). The changes in serum cholesterol concentrations seen in metabolic studies after feeding *trans* fatty acids for a few weeks cannot fully explain the increased risk of CHD (Mensink & Katan, 1992; Zock *et al.* 1995). Other effects, therefore, may contribute to the risk associated with high intakes of *trans* fats. All studies were performed in healthy volunteers between 18 and 70 years of age. The long-term dietary controlled study was performed in a crossover design with twenty-nine men and women (de Roos *et al.* 2001a), the postprandial study with twenty-one men (de Roos *et al.* 2002).

The main results were:

- replacement of 9.2 % energy from saturated fatty acids by *trans* fatty acids significantly decreased serum HDL-cholesterol after 4 weeks by 0.39 mmol/l (95 % CI 0.28, 0.50) and impaired FMD from 6.2 to 4.4 %, a decrease of 1.8 %-points (95 % CI 0.4, 3.2);
- this long-term adverse effect of *trans* fatty acids was not observed in the postprandial study: FMD after an

oral fat load of 1 g/kg bodyweight was 3.1 v. 2.6 % before, with *trans* fatty acids and saturated fatty acids having similar effects.

This work suggests, therefore, that only long-term replacement of saturated fatty acids by *trans* fatty acids impairs vascular function. This may explain why *trans* fatty acids relate more strongly to risk of CVD than saturated fatty acids. The absence of an impairment in postprandial FMD was in contrast to some other, although not all, studies described in the literature.

Dr David Muller presented results from projects funded by the FSA, the Medical Research Council and the British Heart Foundation: they investigated the effects of genetic and other risk factors on the relationship between long-chain *n*-3 PUFA, antioxidant status and vascular function (Leeson *et al.* 2002a,b,c). Increased fish consumption (resulting in increased *n*-3 PUFA consumption) and increased vitamin E intake are associated with a decreased risk of CVD, whereas a common variant of the endothelial NO synthase gene (Glu298Asp) has been shown to increase the risk of CVD (Shimasaki *et al.* 1998). These factors alone or in combination with each other and other known risk factors (e.g. smoking and blood lipids) have been related to measures of vascular function in early adulthood and thereby the development of CVD.

Endothelial function was assessed using FMD in 326 normal subjects aged 20–28 years. Arterial distensibility was also measured. These measures of vascular function were related to *n*-3 PUFA concentrations in plasma and erythrocyte membranes, plasma vitamin E concentrations and total antioxidant status, the genotype of the endothelial NO synthase glutamate–aspartate polymorphism, cardiovascular risk factors and diet.

The principal findings were:

- FMD was significantly reduced in smokers ( $P < 0.05$ ) and there was a significant positive relationship between *n*-3 PUFA status and FMD in smokers ( $P < 0.01$ ), which was not present in non-smokers (Leeson *et al.* 2002c);
- there were significant positive relationships between FMD and *n*-3 PUFA status in those subjects in the top one-third for fasting glucose, insulin and triacylglycerol levels with no relationships in those with lower levels (Leeson *et al.* 2002c);
- neither plasma vitamin E concentrations (alone or adjusted for lipid concentrations) nor total antioxidant status were related to any of the measures of vascular function (Leeson *et al.* 2002b);
- in the group as a whole, FMD was similar for the different endothelial NO synthase genotypes (Leeson *et al.* 2002a);
- there were no significant differences in FMD between smokers and non-smokers with the Glu/Glu genotype, whereas in subjects with Glu/Asp and Asp/Asp genotypes the smokers had a significantly reduced FMD ( $P < 0.01$ ; Leeson *et al.* 2002a);
- there was a significant relationship between *n*-3 PUFA status and FMD in Glu/Asp and Asp/Asp subjects ( $P < 0.05$ ), but not in those with the Glu/Glu genotype (Leeson *et al.* 2002a).

These effects were seen with either DHA alone or DHA + EPA but not with EPA alone. The results show particularly: (1) the positive relationship between *n*-3 PUFA status and FMD in smokers and the subjects in the top one-third for fasting glucose, insulin and triacylglycerol levels; (2) the positive relationship between *n*-3 PUFA status and FMD in the endothelial NO synthase genotypes with an Asp allele, suggest the possibility of prevention strategies with increased *n*-3 PUFA intake targeted to specific groups at risk of CVD.

Professor Jill Belch presented results from two FSA-funded projects investigating the effects of dietary lipids on vascular function. In the first project (Khan *et al.* 2003), a total of 210 healthy subjects (men and women aged 40–65 years, non-smokers) were randomised to one of six groups (*n* 35) supplemented for 8 months with either (g/d): placebo oil 10, olive oil 10, evening primrose (*Oenothera biennis*) oil 5 + placebo oil 5, soyabean oil 5 + placebo oil 5; tuna oil 5 (0.30 g EPA, 1.35 g DHA) + placebo oil 5, evening primrose oil 5 + tuna oil 5. The placebo oil comprised 250 g soyabean oil and 750 g fractionated coconut oil/kg; this was designed to reflect the average fatty acid composition of the Scottish diet.

Subjects were assessed for fatty acid status, biochemical and cellular markers of endothelial behaviour, haemostatic function and endothelial cell function and vascular tone, as determined by iontophoresis and laser Doppler imaging.

The main results were:

- tuna oil improved endothelial-dependent vascular responses; furthermore, there was a positive correlation between endothelial-dependent vascular responses and total plasma *n*-3 PUFA concentration;
- none of the other supplemented diets affected this measure of endothelial cell function;
- platelet aggregation was decreased after tuna oil + evening primrose oil and after olive-oil supplementation, but increased after evening primrose-oil supplementation.

These results suggests that fish-oil supplementation, at levels attainable through the diet, enhances both endothelial cell and platelet behaviour.

In the second study (JJF Belch, A Anderson, F Daly, J Dick, M McClaren, F Khan, A Hill and K Barton, unpublished results), 105 study healthy subjects (men and women, non-smokers, age  $\geq$  40 years) were randomised, following a 1-month run-in, to one of three dietary groups (*n* 34): palmate, stearate or control. All subjects were maintained on their habitual total, saturated and unsaturated fat intakes. The intervention period lasted 4 months. The palmate and stearate groups received 50–60% total saturated fat intake (6–10% total dietary energy) exchanged with either palmitic or stearic acid respectively. Professor Annie Anderson presented details of how this was achieved using margarines and biscuits of the appropriate saturated fat composition provided by Unilever (Vlaardingen, The Netherlands). Subjects were assessed for saturated fatty acid status, endothelial cell function and vascular tone (using iontophoresis and laser Doppler imaging), arterial compliance (pulse wave

augmentation measured at the radial artery using applanation tonometry) and haemostatic function.

The main results were:

- no changes in any of the measures of vascular function were observed either before or after any of the dietary interventions;
- analysis of the biochemical and cellular markers showed no significant changes between the experimental groups and the control group. Analysis pre- and post-intervention, however, showed no significant changes in the control group, but the exchange that increased stearic acid seemed to produce what might be considered as beneficial changes, whereas the palmitic acid exchange did not. In the stearic group platelet NO production increased, P-selectin, E-selectin and fibrinogen levels fell, and in the palmitic acid group prothrombin time increased;
- in the palmitic acid group the proportion of oleic acid was decreased in the esterified forms of fatty acids present in plasma relative to the stearic acid and control groups.

In conclusion, therefore, in the first of these studies vasodilator changes were achieved in endothelial cell function with tuna-oil supplementation. In the second study, stearic acid appears to have some advantages over palmitic acid in terms of potential CV effects, but neither affected vascular function.

Professor Philip Calder presented results from an FSA-funded project investigating the association of *n*-3 PUFA with stability of advanced atherosclerotic plaques (Thies *et al.* 2003). Patients awaiting carotid endarterectomy were randomly assigned to consume placebo, sunflower-oil or fish-oil capsules until surgery, when the atherosclerotic plaque was removed (*n* > 59 per treatment group). Patients in the fish-oil group consumed an extra 1.4 g EPA and DHA/d. The fatty acid compositions of LDL and carotid plaque were determined. Plaques were classified morphologically. The presence of T lymphocytes and macrophages in the plaques was determined by immunohistochemistry. The mean duration of supplementation was 50 (range 7–189) d and did not differ between the groups.

The main results were:

- plasma triacylglycerol concentration was significantly decreased (mean decrease 28%) in patients receiving fish oil;
- the proportions of EPA and DHA increased significantly in LDL-lipid fractions in patients receiving fish oil. The proportions of EPA and DHA were higher in carotid plaque phospholipids, cholesteryl esters and triacylglycerols in patients receiving fish oil compared with patients in the placebo group;
- in the fish-oil group, fewer plaques had thin fibrous caps and signs of inflammation and more plaques had thick fibrous caps and fewer signs of inflammation, compared with the other two groups;
- the number of macrophages in the plaques from patients receiving fish oil was lower than in the other two groups.

This study shows that advanced atherosclerotic plaques are dynamic and readily incorporate *n*-3 PUFA. Incorporation of *n*-3 PUFA into carotid plaques is associated with a reduced number of macrophages and fewer signs of inflammation. These observations suggest that *n*-3 PUFA induce changes that may increase the stability of atherosclerotic plaques. If this is so, it represents a novel mechanism by which fish oil might prevent non-fatal and fatal cardiovascular events (for discussion, see Thies *et al.* 2003). The mechanism by which fish oil decreases the number of macrophages in plaques is not clear; it may involve decreased infiltration, increased loss (increased movement out of plaques or increased apoptosis) or a combination of such effects. The study did not investigate the activation state of the macrophages or the presence of inflammatory mediators within the plaques. These will be examined in a new study.

Professor Robert Grimble presented results from an extension of the previous FSA project to determine whether genetic variation in cytokine genes influences the effect of PUFA on atherosclerotic plaques (RF Grimble, WM Howell, PC Calder, CP Sheraman, P Gallagher, K Rekasem and F Thies, unpublished results). Inflammation plays a pivotal role in the development and stability of atherosclerotic plaques. Cytokines, key mediators of the inflammatory process, have been demonstrated to be present in atherosclerotic plaques (Ross, 1999). Individuals can be classed as habitually high-, medium-, or low-producers of certain cytokines (Jacob *et al.* 1990). Single nucleotide polymorphisms in cytokine genes, usually in the promoter region, result in altered levels of cytokine production, e.g. TNF- $\alpha$ . Single nucleotide polymorphisms in pro-inflammatory cytokine genes have been linked with increased mortality during infection and after surgery (McGuire *et al.* 1994; Stuber *et al.* 1996) and with increased susceptibility to inflammatory disease (Schaaf *et al.* 2001). The ability of fish oil to exert an anti-inflammatory effect in healthy subjects, by suppression of TNF production, was influenced by the pre-supplementation level of cytokine production, and by single nucleotide polymorphisms at +252 in the TNF- $\beta$  gene (Grimble *et al.* 2002).

Preliminary studies indicate that:

- TNFB22 variant of the TNF- $\beta$  single nucleotide polymorphism may influence the soft lipid content of plaques;
- plaque characteristics, assessed according to the modified American Heart Association score, indicate that fish-oil supplementation results in plaques with a lower score in subjects possessing the TNF2 allele of the single nucleotide polymorphism in the TNF gene than patients homozygous for the TNF1 allele.

Thus, genotypes associated with a raised inflammatory status may be influenced to a greater extent by PUFA supplementation than those associated with a lower status. It should be noted, however, that due to the varying lengths of PUFA supplementation time and the absence of any effect on clinical end points, the results should be treated with some caution.

Currently, Biotechnology and Biological Sciences Research Council-funded studies are investigating the

genomic nature of differing sensitivities of cytokine production to the anti-inflammatory influence of fish oil and vitamin E. Further work on gene nutrient influences on atherosclerosis requires clear demonstrations of clinical benefits resulting from nutritional intervention.

## Discussion

Endothelial function appears to reflect the integrated effects of risk factors on the vasculature, and the development of endothelial dysfunction is an early event in the atherogenic process. Measures of endothelial function may provide a useful non-invasive methodology for assessing a clinically relevant surrogate end point; however, there is much methodological and physiological variability and this requires that studies are sufficiently powered in terms of sample size. Further, these issues make a cross-over study design particularly useful.

Whether effecting a change in endothelial function results in a corresponding change in CVD risk remains to be determined. Measures of endothelial function, therefore, should be corroborated with other, more validated, risk factors for CVD, both conventional (fasting lipids, blood pressure, glucose concentration), and novel (high-sensitivity C-reactive protein, soluble intercellular adhesion molecule-1, von Willebrand factor, tissue plasminogen activator, plasminogen activator inhibitor-1), and measures of insulin sensitivity. As different study populations may display different risk factors, the choice of corroborating measures may depend on the specific study population.

Work presented at the workshop showed that in healthy subjects moderate fish-oil supplementation, at levels attainable through the diet, had a beneficial effect on endothelial function. Other ongoing FSA studies also address this issue and confirmation of an effect of dietary levels of *n*-3 PUFA on endothelial function will be important. In subjects at risk of CVD, higher circulating levels of DHA and EPA were associated with improved endothelial function. In patients awaiting carotid endarterectomy, moderate fish-oil supplementation appeared to stabilise advanced atherosclerotic plaques making them less likely to rupture. Overall, these studies lend further support for population dietary recommendations to increase intake of oily fish. Other work presented showed that replacement of dietary saturated fatty acids with *trans* fatty acids impairs endothelial function and further supports population dietary recommendations to reduce *trans* fatty acid intake.

Another issue that was highlighted at the workshop is the choice of study population. The remit of FSA-funded research is not to study disease *per se*, but the impact of diet on the prevention of disease. The extrapolation of data from a disease population to a 'normal' population is difficult; however, if too many conditions of 'normality' are stipulated in the study inclusion criteria (e.g. blood pressure, BMI, plasma lipids) the study population can be unrepresentatively healthy: almost 30% of the English population have been diagnosed with some form of CVD (Department of Health, 1999). In addition, it is unlikely that relatively short-term interventions with modest changes in dietary intake can adequately mimic the effects of a lifetime exposure in individuals who are 'healthy'.

Study populations exhibiting one or more risk factors for CVD, therefore, may be a more appropriate model and would also highlight vulnerable groups within the general population.

### Recommendations

- Measures of endothelial function may provide a useful non-invasive methodology for assessing a clinically relevant surrogate endpoint, but at present such measures should be used in the context of studies that use other more traditional risk factors and other surrogate endpoints for CVD.
- For dietary intervention studies it is important to provide enough statistical power to account for both measure and physiological variation.
- The choice of study population in dietary intervention studies is important: 'at risk' subgroups within the general population may be more appropriate, e.g. as defined by CVD risk factors, genotype or lifestyle factors.

### Attendees

Professor Joe Vita, Boston University; Professor Jill Belch and Professor Annie Anderson, Ninewells Hospital and Medical School, Dundee, Scotland, UK; Professor Bob Grimble, University of Southampton; Dr David Muller, Institute of Child Health, London, UK; Dr Naveed Sattar, University of Glasgow; Dr Nicole de Roos, University Medical Centre, Utrecht, The Netherlands; Professor Christine Williams, Dr James Latham, Dr Julie Lovegrove and Dr Chris Armah, University of Reading; Professor Tom Sanders, King's College, London; Dr Bruce Griffin, Dr Margaret Griffin, Professor Joe Millward and Professor Gordon Ferns, University of Surrey; Professor Philip Calder, University of Southampton; Dr Philip Chowienczyk, King's College, London; Dr Frank Thies, University of Aberdeen; Professor Rudolph Riemersma, University of Edinburgh; Professor Klaus Wahle, Rowett Research Institute, Aberdeen, Scotland, UK; Dr Margreet Olthof, Wageningen Centre for Food Sciences, Nutrition and Health Program, The Netherlands; Dr Judy Buttriss, British Nutrition Foundation, London, UK; Dr Richard Draijer and Dr Christine Kroner, Unilever Research, Vlaardingen, The Netherlands; Dr Ray Rice, International Society for the Study of Fatty Acids and Lipids, Tiverton, UK; Mr Ben Walters, Dr Alison Tedstone, Ms Mamta Singh and Dr Peter Sanderson, Food Standards Agency, London, UK.

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