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Trans Fatty Acids in Adipose Tissue and Erythrocytes of Irish Adults

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Declaration

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Abbreviations Used

AMI	Acute myocardial infarction
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
EDTA	Ethylene-diamine-tetra-acetic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FASEB	Federation of American Societies for Experimental Biology
FDA	Food and Drug Administration
FFA	Free fatty acid
FFQ	Food frequency questionnaire
FID	Flame Ionisation Detector
FIQ	Fat intake Questionnaire
FTIR	Fourier Transform Infra-red Spectrophotometry
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectroscopy
HCL	Hydrogen chloride
HDL	High density lipoprotein
IFST	Institute of Food Science & Technology
IR	Infra-red Spectrophotometry
LDL	Low density lipoprotein
MAFF	Ministry of Agriculture Fisheries and Foods
MI	Myocardial infarction
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RBCM	Red blood cell membrane
RPM	Revolutions per minute
TFA	Trans fatty acid
UK	United Kingdom
US	United States
VLDL	Very low density lipoprotein

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Abstract

Trans fatty acids (TFA) are produced by the partial hydrogenation of vegetable and marine oils. TFA are not synthesized in the human body. The fatty acid composition of adipose tissue reflects the habitual intake of TFA over the previous 1-2 years. Recent studies associate TFA with an increased serum level of low density lipoproteins (LDL) and therefore an increased risk of coronary heart disease (CHD).

The objective of the present study was to assess the level of TFA in the subcutaneous fat and erythrocyte membranes of subjects from an Irish population. One fat aspirate and one blood sample were taken from each subject (n=122).

Results for this population were: mean TFA 4.22 (SD 0.8) g/100 g adipose tissue lipid, mean serum HDL 1.07 (sd 0.4) mmol/l, LDL 3.54 (SD 1.5) mmol/l and total cholesterol 5.53(1.49) mmol/l.

The 18:1*t* TFA content of erythrocyte membranes was: mean 0.85 (SD 0.39) g/100 g erythrocyte lipid.

A positive (*r* 0.13) non-significant (*P*=0.24) relationship was observed between adipose tissue 18:1*t* and serum LDL levels. A significantly stronger relationship (*r* 0.33, *P*=0.002) was observed, however between adipose tissue 16:1*t* and serum LDL levels. These results suggest that 16:1*t* may, in fact, be the more offending isomer with respect to CHD risk. There was a significant correlation (*r* 0.32, P=0.01) between 18:1*t* (adipose tissue) and 18:1*t* (erythrocyte membranes) suggesting that, although levels of TFA in adipose tissue and erythrocyte membranes are different in magnitude, there is a relationship between them.

These, the first such data for an Irish population, in agreement with published data, show some evidence that increased levels of TFA in adipose tissue (and thus the diet) are associated with raised serum LDL.

CHAPTER 1

Introduction to *trans* fatty acids

1.1 The origination of Trans fatty Acids

Trans fatty acids (TFA) occur naturally in small amounts (approx. 5-6%) in the meat and dairy products of ruminant animals (Thomas *et al*, 1981) and in varied amounts in commercially prepared partially hydrogenated vegetable and marine oils. TFA cannot be made in the human body - i.e. there is no endogenous conversion associated with TFA, like that which exists for other polyunsaturated fatty acids (PUFA) as shown in figure 1.1.

1.1.1 Naturally occurring TFA - Biohydrogenation

TFA occur naturally in ruminant animals by a process known as biohydrogenation which is a result of the action of particular micro-organisms present in the rumen of the animal. This biochemical process induces the isomerisation of carbon double bonds in a molecule from the *cis* to the *trans* configuration using various biological catalysts in the process. The predominant *trans* isomer in ruminant fat is vaccenic acid (C18:1-t11). Migration of double bonds also occurs at this time, which may result in many geometrical isomers (Parodi *et al*, 1976). The amount of TFA in the fat of a ruminant animal will depend on the type of animal and the seasonal variation in their diet. The total concentration of TFA in bovine fat for example, ranges from 2-7% depending on the breed of animal, the climate and the feed supplied.

N-3	N-6	N-9	N-7
		Stearic acid	**Palmitic acid**
		18:0	**16:0**
α-linolenic acid	**Linoleic acid**	↓ Δ9	↓ Δ9
18:3n-3	**18:2n-6**		
↓ *Δ6*	↓ *Δ6*	**Oleic acid**	**Palmitoleic acid**
		18:1n-9	**16:1n-7**
18:4n-3	**γ-linolenic acid**	↓ *Δ6*	↓ *Δ6*
	18:3n-6		
↓ *Elongase*	↓ *Elongase*	**18:2n-9**	**16:2n-7**
20:4n-3	**Dihomo γ-linolenic**	↓ *Elongase*	↓ *Elongase*
	20:3n-6		
↓ *Δ5*	↓ *Δ5*	**20:2n-9**	**18:2n-7**
Eicosapentaenoic acid	**Arachidonic acid**	↓ *Δ5*	↓ *Δ5*
20:5n-3	**20:4n-6**		
↓ *Elongase*	↓ *Elongase*	**20:3n-9**	**18:3n-7**
Docosapentaenoic acid	**22:4n-6**	↓ *Elongase*	↓ *Elongase*
22:5n-3			
↓ *Elongase*	↓↑ *Δ4*	**22:3n-9**	**20:3 n-7**
24:5n-3	**22:5n-6**		
↓ *Δ6*		↓↑ *Δ4*	↓↑ *Δ4*
24:6n-3			
		22:4n-9	**20:4n-7**
↓*retroconversion*			
Docosahexaenoic acid			
22:6n-3			

Figure 1.1 Pathways of metabolic synthesis of fatty acids. Δ4, Δ5, Δ6, Δ9, Δ12 and Δ15, respective desaturase enzymes including newly accepted pathway to docosahexaenoic acid (Périchon *et al*, 1998; Gurr *et al*, 1999)

1.1.2 Industrially produced TFA: Hydrogenation

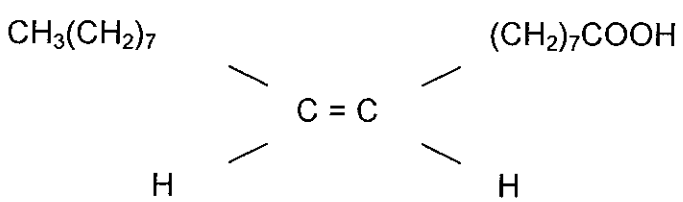
The use of highly unsaturated fats i.e. vegetable and marine fats in the food industry can be quite problematic due to their actual physical properties e.g., their low melting point and their susceptibility to oxidation. Essentially, partially hydrogenated monounsaturated and/or polyunsaturated oils used in many food formulations will exhibit the physical characteristics of a solid or saturated fat (which are normally avoided due to the health risks associated with saturated fat and cholesterol in animal foods) due to the higher melting point of *trans* fatty acids (produced by the hydrogenation process) which will contribute substantially to the solidity of the finished product. This ultimately increases the economic viability of the fat. For example, stearic acid (C18:0) is solid at room temperature, oleic acid (C18:1*c*9) which has the same number of carbon atoms, but one unsaturated bond, is liquid at room temperature. The switching of the unsaturated bond in oleic acid from the *cis* to the *trans* configuration (via hydrogenation) increases the solidity of the fat e.g. elaidic acid (C18:1*t*9) also has one unsaturated bond (similar to oleic acid), but is now in the *trans* configuration, and is solid at room temperature.

The hydrogenation of edible oils has been a well accepted technique since the 1940's. The economic importance of the process can be seen in the fact that, in the United States alone, of the 4.5 million tonnes of 'visible' fats and oils consumed annually, almost 2.72 million tonnes (60%) are subject to hydrogenation, i.e. will be in the form of hard vegetable and marine spreads and cooking fats. These hydrogenated oils were developed mainly as an alternative to animal fats and tropical oils used in baking and spreads (Kris-Etherton, 1995).

Industrial hydrogenation is carried out at a very high temperature (~180°C) using hydrogen gas and a nickel catalyst, the majority of which is recovered at the end of the reaction. In effect, the process 'adds' hydrogen atoms to some or maybe all of the unsaturated carbon double bonds in a fatty acid molecule, thus partially hydrogenating it. The addition of the hydrogen atom(s) makes the fatty acid more saturated.

As a result of hydrogenation, however some degree of isomerisation will occur (as in bio-hydrogenation). Carbon double bonds in naturally occurring fats and oils are mostly of the *cis* configuration that is the hydrogen atoms of the unsaturated C=C bond are on the same side. Hydrogenation promotes the 'switching over' of these hydrogen atoms to form a geometrical (*trans*) isomer. These *trans* bonds have the same molecular weight at their *cis* counterparts, but the hydrogen atoms are on opposite sides (figure1.2) of the double bond.

(a) Oleic acid (C18:1*c*9)
cis configuration



(b) Elaidic acid (C18:1t9)
trans configuration

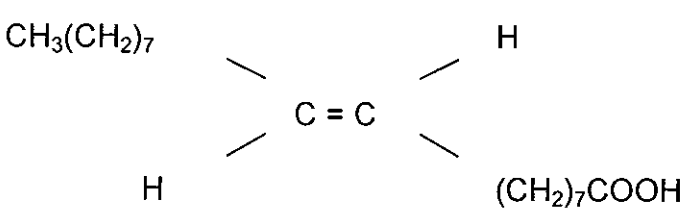


Figure 1.2 Geometrical isomers of C18:1 fatty acid

The different arrangement around the C=C bond alters the shape of the molecule. C=C bonds of the *cis* configuration are bent at 120°, i.e. four of these bonds in a row would give a horseshoe shape along the fatty acid molecule. *Trans* C=C double bonds, however, 'cancel' each other out so that the resulting molecule is straighter than one which contains all *cis* bonds. This consequence of hydrogenation may have profound effects on molecule packaging, and may be especially important, for example in membranes, which are made up predominantly of phospholipids. The predominant *trans* isomers in partially hydrogenated vegetable oils are C18:1*t*9, 10 and 11 (Gurr, 1996)

1.2 *Trans* fatty acids in the diet

1.2.1 Origins of TFA in the diet

Approximately 30% of the TFA in the diet originates from ruminant sources, 60% from industrially hydrogenated fat and the remainder from animal fat (non-ruminants consuming TFA in their diets) and some plant lipids (Bolton-Smith *et al* 1995).

Recent studies show the main provider of total (and industrially hydrogenated) *trans* fatty acids to be the '*Pudding*' group of foods comprising of cakes, biscuits, pies etc., containing vegetable shortening (Bolton-Smith *et al*, 1995; Hulshof, 1999), followed by margarines, red meat, butter and milk (Willet *et al*, 1993; Hulshof, 1999). The level to which margarines contribute will depend, of course on the amount of *trans* fatty acids present. Some margarines have been shown to contain over 30% of the total fat as *trans* isomers (Enig *et al*,

1984) yet some may contain negligible amounts e.g. 0.5% (Michels and Sacks 1995).

1.2.2 Intakes of TFA

Although there are not a great number of studies measuring the daily TFA intakes in populations, recent data show the average intake to be 4-6g per capita (UK), corresponding to approximately 6% of total fat in the diet (BNF, 1995).

European intake studies show similar results. US data, however, do show slightly higher average intakes (see table 1.1).

Country / Study	**Total TFA (g/day)**
USA - Enig *et al*, 1990	12.8
- Hunter & Applewhite, 1991	8.1
- Troisi *et al*, 1992	3.4
- Hudgins *et al*, 1991	7.6
Denmark - Stender *et al*, 1995	5.0
UK - Gregory *et al*, 1990	4.9
- Bolton-Smith *et al*, 1995	7.1 (males) 6.4 (females)

Table 1.1 Estimated intakes of TFA in different populations

A study of over ten thousand Scottish men and women in the mid 1980's was conducted to measure the TFA in the diet (Bolton-Smith *et al*, 1995). A food frequency questionnaire (FFQ) was used and results showed the average intake to be 7.1g and 6.4g per capita for males and females respectively (as a

percentage of total fat or energy, the level was higher for women than for men), but levels were as high as 48g per capita. One American report showed how an individual with a reasonably normal diet can have quite a high intake of TFA (Litin and Sacks, 1993) (see table 1.2) below

Meal/Part of meal	***Trans* content (g)**
Breakfast - one average doughnut	3.19
Lunch - one small portion French fries	3.43
2 teaspoons stick margarine	1.24
2 cookies	1.72
total	**9.58**

Table 1.2 Sample daily diet and possible *trans* fatty acid content

A diet that may resemble the Irish diet somewhat more closely was used in a more recent study (Hulshof *et al*, 1999). Average per capita intakes of TFA in the UK were estimated to be 2.8g (1.3% total energy) which were designated *moderate* intakes. The main contributors of TFA in the diet were milk and cheese 18.8%; TFA resulting from hydrogenation (biscuits cakes and pies) 20%; and natural oils and fats 35.5%. In this study the lowest intakes of TFA were found in the Mediterranean countries, moderate intakes (as noted above), in The UK, The Netherlands, Norway and Belgium, and the highest in Iceland (table 1.3). (NB TFA intakes were estimated in each country using the most up to date food consumption tables and not individual food frequency questionnaires)

Gender	Range g/capita/day	Range % total energy intake
Males	1.2 - 6.7	0.5 - 2.1
Females	1.7 - 4.1	0.8 -1.9

Table 1.3 The range of intakes of TFA in 14 different western European countries (Hulshof *et al*, 1999)

1.2.3 Measurement of the intake of *trans* fatty acids

The fatty acid composition of subcutaneous adipose tissue has long been established as an accurate marker for the intake of specific fatty acids (that are not endogenously produced) over the previous 600 days approximately (Benyen *et al*, 1980). The sampling of adipose tissue however is quite an invasive procedure to use for a dietary assessment which could be carried out (albeit with under or over estimations) in another, less invasive way. Also, if the diet has been changed over the course of the 600 days, a fat biopsy may not be an accurate reflection of the diet.

The Diet History method has long been regarded as the gold standard for dietary analysis. It is a time-consuming method, however, and can take up to an hour for a detailed analysis, and there is always some degree of under or over reporting by subjects.

Twenty-four hour recall is another method of dietary analysis and has been used to measure the fatty acid contents of diets. This method is interview-assisted and involves retrospective recording of the dietary intake of a subject over the previous 24 hours. This method was employed in one study to estimate the intake of a particular fatty acid in one population, and involved

taking the mean of 19 twenty-four hour recalls over a two year period (Van-Staveren *et al*, 1986), which again is very time-consuming.

Subjects can keep dietary records/diaries. These are advantageous with respect to the researchers' time in that they are not interview-assisted, but they depend on a high degree of dedication on the part of the subject, which cannot always be relied upon. Furthermore, the actual maintenance of a diary can often affect food preferences and choices.

Food frequency questionnaires (FFQs) have been validated against linoleic acid (Van-Staveren *et al*, 1986; London *et al*, 1991; Bolton-Smith *et al*, 1995), eicosapentaenoic acid and other n-3 and n-6 polyunsaturated fatty acids (Hunter *et al*, 1992), which are produced only to a limited extent in the human body (figure 1.1). FFQs have also been shown to exhibit similar validity in the measurement of polyunsaturated fat intake to the diet history method (van-Staveren *et al*, 1986). FFQs can be administered both quickly and easily, and can also be tailored to a particular nutrient e.g. a Fat Intake Questionnaire (FIQ). The TFA content of foods must be known in order to enable the researcher to quantitate the TFA in the diet of an individual. TFA levels in food can be obtained from direct analysis, industrial product information, and levels reported in literature and existing published documents (e.g. by The Ministry of Agriculture Fisheries and Foods in the UK (MAFF)).

1.3 *Trans* fatty acids and health

1.3.1 Animal Studies

Many studies investigating the metabolic effects of TFA on the body are carried out using rat models, as it is quite difficult to regulate the diet of a human unless they are actually observed in the laboratory/controlled environment. Animal studies have shown that when fat containing *trans* isomers of fatty acid are included as part of the fat content of the diet, no detrimental growth effects are observed. Some studies show, however, that where there is an EFA deficiency, TFA can be potentially detrimental by interfering with the metabolism of linoleic and alpha-linolenic acids (Privett et al, 1997; Sugano and Ikeda, 1996).

1.3.2 TFA and Coronary Heart Disease (CHD)

Extensive reviews carried out by health authorities in the 1980's failed to draw any conclusive evidence regarding the detrimental aspects of TFA (FASEB, 1985; BNF 1987).
More recent studies, however, examining the effects of TFA on blood lipids (Mensink and Katan 1990; Troisi *et al*, 1992; Judd *et al*, 1994; Hu *et al*, 1997; Lichtenstein *et al*, 1999) and the consequential risk of coronary heart disease (Willet *et al*, 1993; Ascherio *et al*, 1994; Pietinen *et al*, 1997; Lichtenstein, 2000) have caused great concern. These studies present real evidence that TFA have detrimental health effects.

In a prospective study carried out in 1993, Willet *et al* studied the relationship between TFA intake and the development of CHD. Dietary data from 85,095 women was analysed. An eight year follow-up showed 431 new cases of CHD, and, having adjusted for age and total energy intake, the intake of *trans* fatty acids was found to be directly correlated to the development of CHD.

Controlling for other parameters such as cigarette smoking etc. did not change the association observed.

Willet and colleagues also noted that a regular intake of confectionery type foods (such as cakes, biscuits, pies etc.) was also significantly associated with development of CHD. Controlling for other parameters, by eliminating those subjects who may have changed their habitual dietary intake because of an early or previous diagnosis of CHD, enhanced the strength of the correlation.

Another interesting observation was that, having analysed the dietary content of *trans* fatty acids originating from ruminant animals and from vegetable origin separately, there was a significant correlation between the vegetable- *trans* (C18:1*t*9; elaidic acid) and the development of CHD but not between the ruminant-*trans(*C18:1*t*11; vaccenic acid*)* and CHD (the lack of this correlation however could be due to the fact that most of the *trans* intake actually originates from vegetable sources (Ascherio *et al*, 1994)).

As a closing quote to their findings Willet *et al* (1993) said that the production of TFA for use as solid fats -

"May have reduced the anticipated benefits of substituting these oils for highly saturated fats" and instead *"contributed to the occurrence of coronary heart disease".*

Further reports, however, having weighed the respective effects of TFA and saturated fat on blood lipids recommend that the fats high in *trans* should not be replaced with fats such as butter or lard but instead with those fats exhibiting little or no detrimental effects on blood lipids e.g. oils and spreads containing less than 1% or no *trans* fatty acids. A prospective study (Hu *et al*, 1997) based on more than 80,000 women enrolled in The Nurses' Health

Study concluded that replacing saturated and *trans* unsaturated fat with unhydrogenated mono- and polyunsaturated fat is more effective in preventing CHD even than reducing overall fat intake.

Another case-control study concurred with the findings of Willet *et al*, and reporting a positive relationship between margarine (containing TFA) and the development of myocardial infarction (MI) or angina (Ascherio *et al*, 1994). This study looked at both men and women with no previous history of MI or angina (both indicators of the presence of CHD). 197 matched pairs (cases and controls) were used, and other coronary risk factors were recorded. The dietary intake of subjects was measured using a pre-validated food frequency questionnaire (quicker method than that of the gold-standard dietary history method) which included a detailed description of fats / margarines used on a regular basis.

Adjusted for age and sex, the intakes of TFA were positively associated with an increased risk of CHD. The intakes of TFA were divided into quintiles, the range of which was 3.05g - 6.47g/ day. Adjusted for any changes that may have occurred in the dietary pattern of subjects and affected results, the relative risk then became stronger. In this study, Ascherio *et al* also noted that at higher intakes of TFA (i.e. in the upper quintiles) the risk of CHD was further enhanced, even when the effect this would have on blood lipids was disregarded. This finding in itself, maybe most important, as blood lipid levels can essentially be lowered by means of medication etc., but it is this finding that suggests that it may be other metabolic effects of TFA the are most detrimental.

Other studies (Aro *et al*, 1995) showed inconclusive results with respect to increased risk of acute myocardial infarction (AMI) and C18:1*t* intake (based on levels measured in adipose tissue samples).

Other studies which looked at the levels of TFA in the adipose tissue (which reflects long term dietary TFA status) of subjects and compared them to the serum lipid profiles found some correlation between these parameters. Troisi *et al*, in 1992 examined this correlation and found a significant but weakly positive correlation between the level of TFA in the adipose tissue of subjects and their corresponding LDL(low density lipoprotein)-cholesterol levels (n=748, r=0.07, P=0.04). He also reported a negative correlation between HDL cholesterol and TFA in adipose tissue, i.e. the more TFA that was present, the lower the HDL(high density lipoprotein)-cholesterol level. Statistically, this corresponded to a 27% increase in the risk of CHD. More reliance can ultimately be placed upon studies of this nature, as studies which record intake of TFA through means of dietary questionnaire can be unreliable.

1.3.2.1 The effect of TFA on Low and High Density Lipoprotein

Much evidence has been presented regarding the effect of TFA on serum LDL - cholesterol levels. In 1992, Nestle and colleagues reported a significant increase in LDL levels following a diet containing 7% energy as TFA, relative to an oleic acid rich diet. This study involved feeding four different diets at random to each of 27 mildly hypercholesterolaemic male subjects for a period of three weeks for each experimental diet. The diets were as follows:

- Butter fat rich (lauric acid, myristic acid, palmitic acid) diet
- Oleic rich diet
- Elaidic rich (TFA) diet
- Palmitic diet

Compared to baseline lipid levels, a decrease in serum LDL concentrations was shown in subjects having followed the oleic rich diet. Changes in serum HDL cholesterol levels were similar for both oleic and elaidic rich diets (more instability exists regarding changes in HDL levels with TFA in the diet). The elaidic rich diet produced a significant increase in lipoprotein$_{[a]}$ compared to all other diets (also shown by Mensink and Katan (1992) although not by Judd *et al* (1994)). The elaidic rich diet, however, containing 7% of total energy as TFA and representing approximately 20.2g of TFA in the test diet, was higher than the average intakes e.g. in a US population (n=341) TFA intake was 3.05 - 6.47 (Ascherio *et al*, 1993).

Zock and Katan, in 1992 also carried out a similar feeding study. They showed a significant increase in LDL cholesterol on moving from a linoleic acid rich diet to a stearic rich diet and an even more significant increase in LDL cholesterol on moving from baseline to an elaidic acid rich diet. This diet however also contained 7.7% of total energy as TFA. Other studies show similar findings (Mensink and Katan, 1990; Judd *et al*, 1994; Almendigan *et al*, 1995).

To compare the relative effects of butter fat, TFA and soft margarine on serum lipid levels, Wood *et al* (1993) used the following butters / spreads in a feeding trial (table 1.3).

Test fat	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1c	C18:1t	C18:2
Butter	2.9	3.9	11.9	31.4	14.6	20.6	5.3	3.6
Butter-sunflower	0.9	1.5	5.5	18.6	10.9	18.5	2.6	36.5
Butter-olive	1.4	1.7	6.2	24.7	10.1	41.9	2.6	10.5
Hard margarine		0.5	0.7	13.9	6.6	43.4	29.0	3.5
soft margarine	0.2	3.6	1.6	12.4	4.5	13.8	0	61.3

Table 1.3 Fatty acid composition of the test fats used (Wood *et al*, 1993)

Each subject used each of the test fats for a period of six weeks. Results showed total serum and LDL-cholesterol was significantly increased using butter and reduced using soft margarine relative to baseline cholesterol levels. Butter-sunflower, butter-olive and hard margarine produced similar but non-significant changes from baseline values. HDL cholesterol levels were highest following the butter and butter-olive test fats but not significantly so. The soft margarine produced LDL cholesterol levels significantly lower than all other test fats (showing the protective effect of C18:2*c* (linoleic acid)). The butter test fat produced the highest LDL cholesterol levels (C16:0 - the main saturate). With respect to other lipoproteins e.g. apolipoprotein A-1 and B, the soft margarine test fat produced the most favourable results.
In contrast with this finding, however, in a more recent study Sundram *et al* (1997) found that a diet rich in TFA(5.5% energy) raised total cholesterol and LDL significantly compared to diets rich in C18:1c and C16:0 respectively (as similarly reported by Aro *et al*, 1997) - suggesting that TFA can be more atherogenic than naturally occurring saturated fatty acids.

It must be remembered, however, that these test diets represent the consumption of particular fatty acids at individual levels that would not normally be consumed. Levels of this caliber are used so as to maximise the effect that a particular fat will have on the serum lipids thereby enhancing the overall effect and making it more measurable. Apart from the test fats allocated in this study, however, subjects were allowed to follow self selected diets which would introduce a wider range of fatty acids into the overall picture.

From previous studies it is yet unclear as to the effect TFA have on high density lipoprotein (HDL) but most of the evidence seems to show decreased HDL levels.

Some studies report significant (unfavourable) decreases in HDL (Mensink and Katan, 1992; Zock and Katan, 1992; Judd *et al*, 1994) when diets containing reasonably high levels of TFA (11%, 8%, 7% respectively) are fed to subjects. Whereas other studies (Laine *et al*, 1982; Lichtenstein *et al*, 1993; Judd *et al*, 1994) present conflicting evidence having fed subjects lower levels of TFA (3%, 4%, 4% respectively) reflecting more realistic intakes.

In agreement with the deleterious effect of TFA on HDL reported above, however, Sundram *et al* (1997) reported significantly lower HDL levels during the consumption of a diet rich in TFA (5.5% energy) compared to diets rich in either C18:1*c* or C16:0. Supporting this finding, Lichtenstein *et al* (1999) observed the following: a gradual increase in LDL levels (5%, 7%, 9%, 11% and 12% respectively) and decrease in HDL levels (6%, 4%, 4%, 4% and 3% respectively) whilst consuming the following diets (having initially following a butter rich diet);

- Soybean oil enriched (<0.5% TFA)
- Semi-liquid margarine (<0.5% TFA)
- Soft Margarine (7.4% TFA)
- Shortening (9.9% TFA)
- Stick Margarine (20.1% TFA)

(All of the above test diets contained the same food, and percentage energy from fat (30%) and were each followed for a period of 35 days.)

Another recent study, however (Judd *et al,* 1998) showed lower total cholesterol and LDL levels following the use of a margarine (TFA and PUFA rich) versus the use of butter, over a five week period; there was no observed difference in HDL levels between the diets.

Overall, these feeding studies have established the link between TFA and an increase in LDL levels but opinions still divided as to the effects on HDL. Again, these studies mostly do not reflect normal dietary habits - the level fed being often quite high in comparison to realistic intakes of TFA on a self-selected western diet.

1.3.2.2 Effect on Lipoprotein $_{[a]}$

Lipoprotein$_{[a]}$ ($L_{[a]}$) has long been regarded as an independent risk factor for heart disease when present at elevated levels in serum. The metabolic effects of TFA on serum $L_{[a]}$ levels have been examined by a small number of studies only, most of which report that TFA increases $L_{[a]}$ levels (Mensink and Katan, 1992; Nestle *et al*, 1993; Aro *et al*, 1997) but others which report no significant increase in levels (Lichtenstein *et al*, 1993; Clevidence *et al* 1997). Having reviewed these studies, the evidence is unclear as to the true effects TFA may have on serum $L_{[a]}$ at normal levels of intake. One of these studies (Clevidence *et al*, 1997) reports that a subset of subjects whose serum $L_{[a]}$ levels were high to begin with, responded to a high TFA test diet (6.6% energy as TFA) with a further 5% increase in serum $L_{[a]}$ levels. It may be these individuals, therefore, with elevated serum $L_{[a]}$ levels (which is largely dependant on genetic polymorphism (Lahoz *et al*, 1998)) who are at a higher risk (with respect to coronary heart disease) from high intake of TFA.

1.3.2.3 Interactions with essential fatty Acids

Interactions between TFA and essential fatty acid (EFA) metabolism have been observed using in-vitro models. Svensson *et al* (1983) showed that partially hydrogenated fish oils in the diet of rats reduced desaturase enzyme activity - i.e. interferes with the conversion of linoleic acid (C18:2n-6) to arachidonic acid (C20:4n-6), which manifests as an increase in C18:2n-6 and decrease in C20:4n-6 levels in phospholipids. Studies investigating the effect of TFA on desaturase activity in human subjects postulate that a low EFA status is also a causative factor in the down-grading of this enzyme activity (Gurr *et al*, 1983). It is very difficult for an individual to be lacking in EFAs, due to their wide availability in the diet. Therefore this effect will not usually manifest itself in a real life situation.

Premature infants however, can have borderline EFA status (Uauy and Hoffman, 2000) and TFA may compete with EFA for the enzyme systems involved in the normal development of the nervous system and eyesight. The infant's intake of TFA from breast milk will be dependent on the mother's dietary intake of TFA (Innis and King, 1999)

1.3.2.4 Changes in Cholesterol Ester *Trans*fer Protein

Cholesterol ester transfer protein (CETP) catalyses the movement of cholesterol esters from HDL to lipoproteins of lower density (LDL, VLDL). Lagrost *et al* (1992) showed that the rate of transfer of cholesterol esters from HDL to LDL was increased by elaidic acid (C18:1*t*9) but not by the corresponding *cis* isomer oleic acid (C18:1*c*9).

The potential detrimental effect that TFA may have on CETP activity in human subjects was investigated by van -Tol *et al* in 1995. 55 volunteers were randomly assigned each of three different test diets to consume over a defined period. This study showed CETP activity levels at 114 (% of reference serum) following the TFA rich diet as opposed to 96 and 97 following the linoleic and stearic acid rich diets respectively.

1.3.2.5 Proposed effect on thrombogenesis

One in vitro study has shown that collagen-induced platelet aggregation is inhibited less by *trans* isomers than *cis* isomers in the diets of porcine models. Therefore, thrombogenesis may be increased (Peacock and Wahle, 1988).

1.3.3 TFA and cancer

Literature regarding a possible association between TFA and cancer is scarce to date. Early studies have attempted to show a link between the use of industrially hydrogenated fat and the development of cancer of the breast, colon and prostate (Enig *et al*, 1978). This study however failed to control for other factors which may ameliorate the risk of cancer e.g. lifestyle factors, smoking etc. In animal models TFA have been found to increase tumour growth (Hogan *et al*, 1984). Further studies however failed to find any adverse effects of TFA on tumourgenesis in rat models (FASEB, 1985).
One study which looked at the *trans* stores in adipose tissue of American women found an increase in cancer risk where there were higher levels of *trans* fatty acids in adipose tissue stores, in some cases twice that of similar European subjects (London *et al*, 1993).

Whilst examining the biomarkers of fatty acid exposure and breast cancer risk, Kohlmeier (1999) found an indication that TFA may be associated with increased risk of breast cancer. This study was based on the association between adipose tissue stores of TFA in 698 American women and the presence or absence of breast cancer. Kohlmeier acknowledged however, that the study of individual fatty acids and their link with cancer is very much in its infancy.

Holmes *et al* (1999) using data from the Nurses' Health Study, however, found no evidence that a reduced intake of total fat or any particular fat was associated with a decreased risk of breast cancer.

Clearly the evidence supporting a link between TFA and the development of cancer is quite weak and further investigations would be required to strengthen or disprove this hypothesis.

From the current published literature therefore, it can be deduced that the most established metabolic effect TFA have on the human body is that they raise serum levels of LDL cholesterol. Most of the studies (but not all) showing this however are based on using test diets which contain rather high levels of TFA (i.e. >7% of total energy).

1.4 *Trans* fatty acids in adipose tissue.

The lipids in adipose tissue stores are slowly but continually being turned over. This allows a fine degree of metabolic control in a dynamic system. Lipid turnover is sufficiently slow so that the fatty acid composition of adipose stores in the human will only change every 600 days approximately, given that there

are no fluctuations in the body weight (Benyen *et al*, 1980; Benyen and Katan, 1985).

The analysis of the fatty acid profiles of subcutaneous adipose tissue samples therefore will give a reasonably accurate picture of the habitual intake, both quantitatively and qualitatively of fat in the diet of an individual over the previous two years approximately (Hirsch *et al*, 1960).

Similar to linoleic acid (C18:2*c*n-6) there is no bio-formation of *trans* fatty acid isomers in the human body. Hence, it can be assumed that all the *trans* fatty acids in the adipose tissue of an individual are present as a direct result of the diet of that individual (Johnston *et al*, 1957)

The analysis of the fatty acid content of adipose tissue therefore proves an objective marker of the long-term intake of those fatty acids that are not synthesised in the body. For example, a positive correlation (r=0.7) has been shown between the dietary intake and the adipose tissue content of essential fatty acids such as linoleic acid (C18:2*c*) (van-Staveren *et al*, 1986) whereas a weaker correlation (r=0.45, P=0.01) has been shown between monounsaturated and saturated fatty acid contents in the diet and adipose tissue (Hunter *et al* 1992)

To date much research has been carried out in the UK, the US and the Netherlands with respect to the TFA content of adipose tissue. The studies report a wide range (0.99-8.2%) in the proportions of *trans* isomers in the subcutaneous adipose tissue of populations (Enig *et al*, 1984; London *et al*, 1991; Hudgins *et al*, 1991).

three months (average life of erythrocytes) if, when compared, the levels in erythrocytes bear a relationship to the levels in the adipose tissue of the same subjects.

1.6 Determination of *trans* fatty acids

This section deals with the different analytical methods that can be used to determine the level of *trans* unsaturation and/or the individual *trans* isomers in a sample, concentrating on the particular analytical methods used in this study.

1.6.1 Total *Trans* Content - Fourier Transform Infra-red Spectrophotometry

The analysis of the total *trans* bonds in a sample is dependent on the use of Infrared (IR) spectrophotometry. Isolated *trans* double bonds show absorption at approximately 967cm-1 (10.3mm) under controlled conditions using an appropriate reference standard. Measurement of the intensity of this absorbance is the basis for this method (International Union of Pure and Applied Chemistry, 1987) i.e. the intensity of this absorbance will be a function of the number of *trans* bonds. Triglycerides or fatty acids are converted to methyl esters before making IR measurements.
Fourier *Trans*form Infra-red Spectrophotometry (FTIR) employs computing techniques to assist the calculations and integration necessary to process the percentage isolated *trans* bonds in a sample. Computing techniques also allow the selection of certain regions of a spectrum for further analysis if necessary (Lanser and Emken, 1988).

1.6.2 Individual *trans* isomers - Gas Chromatography

The analysis of the individual *trans* isomers of fatty acids in a sample e.g. the level of C18:1*t* (elaidic acid) in a sample depends on the use of chromatography techniques, most popularly capillary gas chromatography (GC).

Chromatography is used primarily for the separation of compounds in a sample, which are distributed between two phases, one of which is stationary (stationary phase) and one that moves (mobile phase). In the case of GC, the carrier gas used is the mobile phase and the stationary phase in one which is coated onto the inside of the column.

Analyses of TFA by GC requires the conversion of the fatty acids in a sample to the corresponding methyl esters, which are more volatile and therefore suitable for use in the GC system (figure 1.3 for diagrammatic representation)

1.6.2.1 Sample Injection

The main principal of GC involves pushing solutes through a column using an inert carrier gas (usually nitrogen or helium). The compounds enter the column as a 'plug' of vapour which is formed by the injection of a small amount (usually 1-5μl) of liquid through a rubber septum into a vapourisation chamber using a micro syringe (figure 1.4)

1.6.2.2 Column Type

There are many different types of columns (e.g. packed and stationary phases). The type of column selected will depend on the analysis being carried out and the resolution required by the analyst. Chromatographic columns can be up to 100m in length. Optimal conditions for analysis will depend amongst other things on the column temperature. An analysis can be carried out isothermally i.e. at the same temperature from start to finish, or temperature programming can be used where the column temperature is increased either continually or in a stepwise fashion as the separation proceeds. Temperature programming can vastly improve a chromatogram, with respect to shorter duration and improved resolution.

1.6.2.3 Detection

Many detectors are available for use with GC. These include Electron Capture, Nitrogen-Phosphorous and Flame Photometric Detectors but the most widely used is the Flame Ionisation Detector (FID) (figure 1.5).
The effluent from the distal end of the column passes into the detector block and mixes with hydrogen and air and is then ignited electrically. Organic compounds when burned in this way will usually produce ions or electrons that conduct electricity through the flame. The resulting current is measured and amplification allows the integration of the signal for recording on the chart. FIDs are easy to use, quite durable and can be used for most organic compounds. The only drawback with using FIDs is that the sample is unrecoverable upon completion of analysis.

At present, the most widely used capillary column type for the separation of positional *cis* and *trans* isomers of fatty acids is a fused silica capillary column using a suitable polar stationary phase. Previous studies carried out on foods and adipose tissue have all used similar types of column with slightly different stationary phases (Ratnayake, 1990; Grandgirard *et al*, 1987; Wijesundera *et al*, 1989; Joseph and Ackman, 1991). Specially prepared stationary phases, i.e. for the specific separation of *cis* and *trans* isomers are also available (e.g. a 100m x 0.25mm I.D., 20mm fused silica capillary column coated with SP-2560 stationary phase) (Lanser and Emken, 1988; London *et al* 1991; Hudgins *et al*, 1991; Hunter *et al*, 1992; Ratnayake *et al*, 1995). SP-2560 columns are valuable for analysing complex fatty acid methyl ester samples. Critical parameters involved in the optimal use of this column include oven temperature, carrier gas linear velocity, and sample concentration. For example, when the oven temperature is increased, *cis* isomers are better resolved from *trans* isomers in a complex mixture. Decreasing the oven temperate improves the resolution of the *trans* isomers.

To determine the optimal temperature for a particular analysis, a series of samples must be analysed at temperature increments of 2°C until optimal resolution is obtained. Similarly, care must be taken when choosing the linear gas flow rate for a particular analysis e.g. resolution of *cis* and *trans* isomers is lost by increasing the carrier flow rate from 20 to 30 cm^3/second (although run time is reduced substantially).

Resolution will also be affected by the sample concentration, which oftentimes is often difficult to control. In general, better separation will be achieved when the concentration is less than 0.5mg/ml. Ultimately, the overall concentration of sample can be adjusted to give the best response.

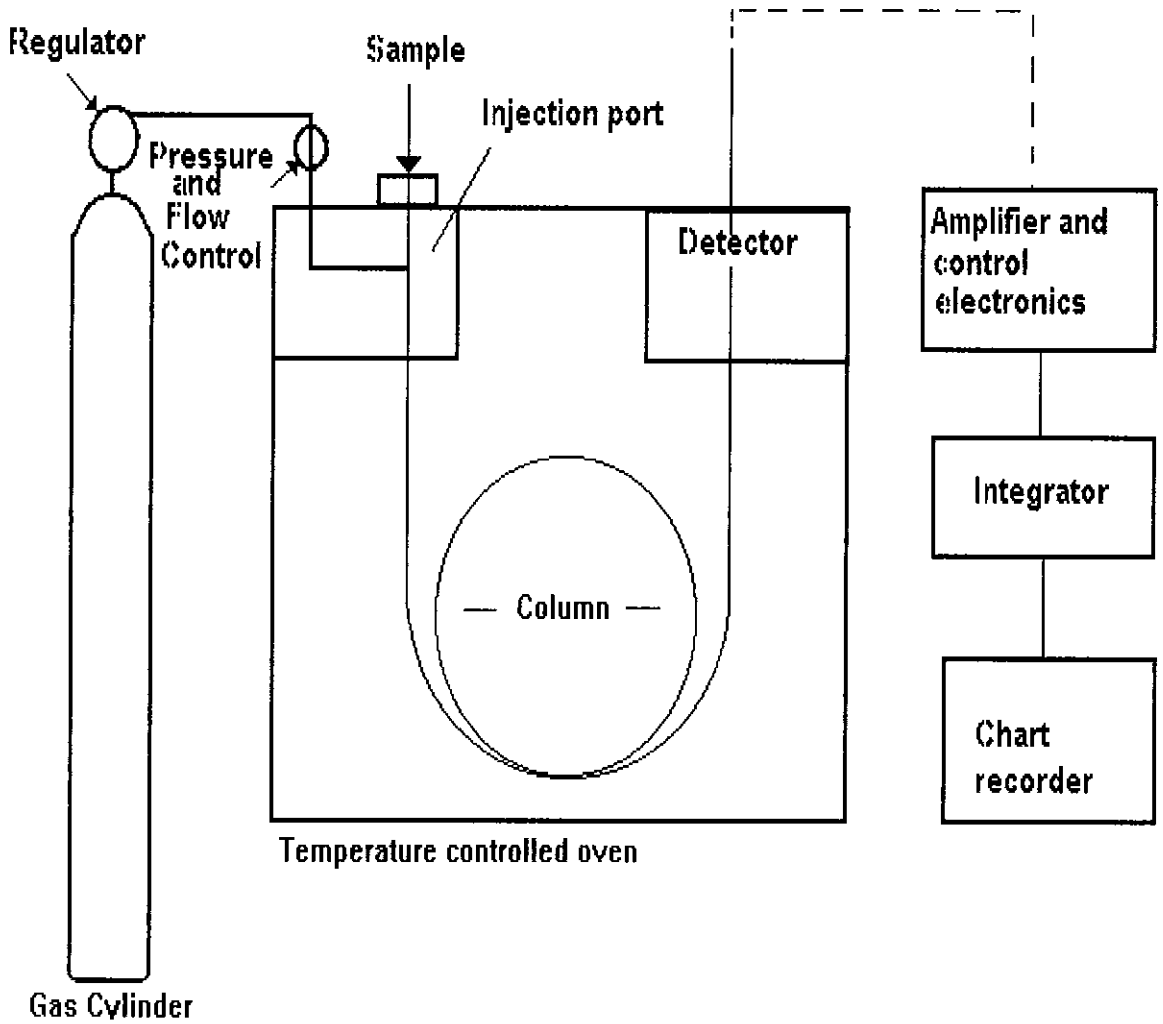


Figure 1.3 A diagrammatic representation of a Gas Chromatograph

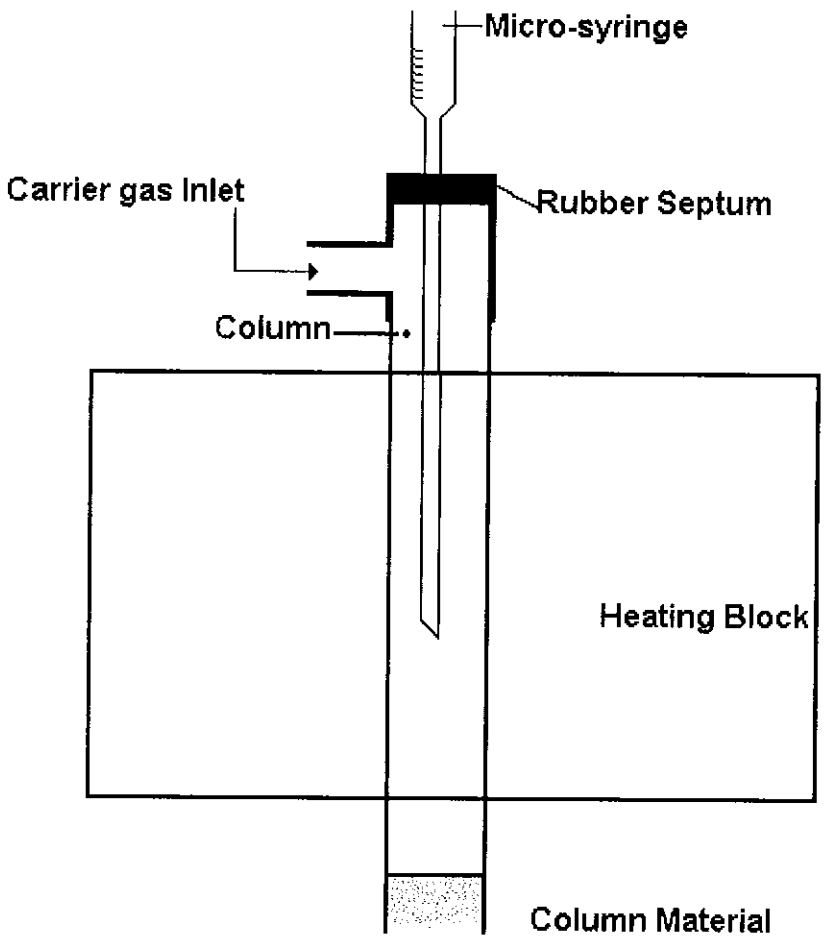


Figure1.4 A diagrammatic representation of the sample injection system.

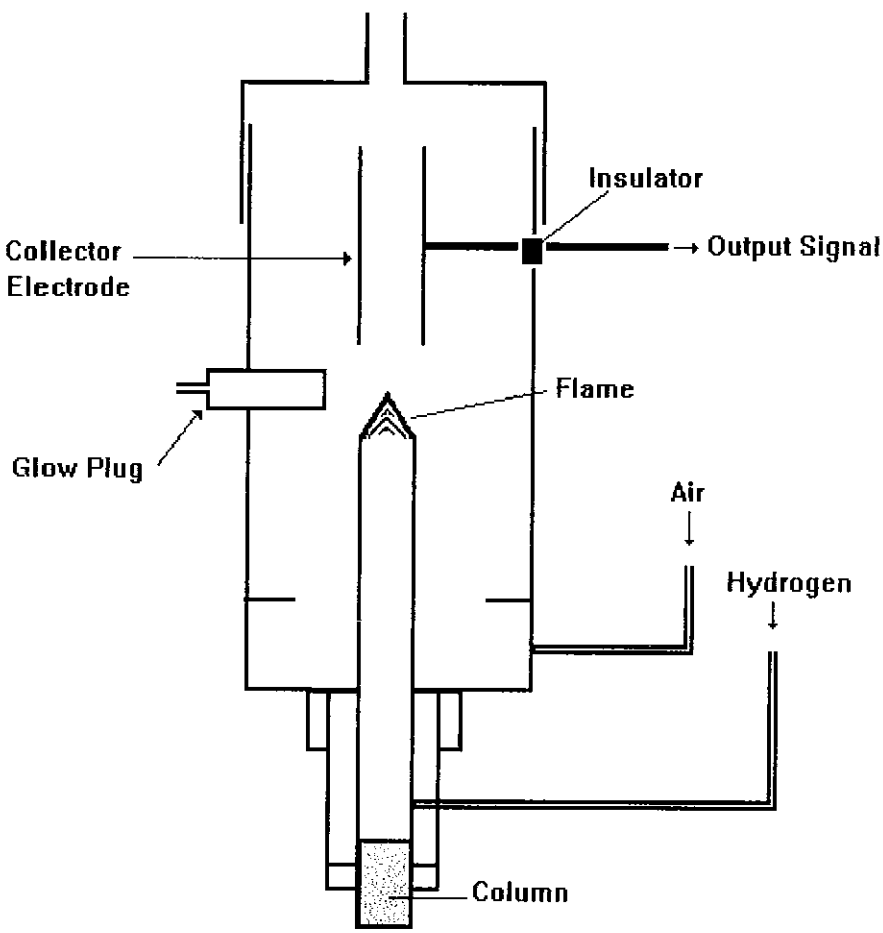

Insulator
Collector
Electrode
Output Signal
Flame
Glow Plug
Air
Hydrogen
Column

Figure 1.5 Diagrammatic representation of Flame Ionisation Detector

1.6.3 Gas Chromatography - Mass Spectroscopy (GC-MS)

This method identifies a mixture of gaseous ions according to their mass-charge ratio. A sample injection onto the capillary column in the GC travels through the column under appropriate optimal conditions for the particular analysis, but instead of a detector at the distal end of the column there is an inlet into the MS system. As the compound eluted from the capillary column passes into the MS system, it travels through an ionic source (there are many different forms of this ionisation) which splits each molecule into ion fragments as it passes through. The fragments then pass through a mass analyser that separates molecular ions, fragments and fragment radicals into groups according to their mass/charge ratio. These in turn generate an ionic current, the strength of which is proportional to the relative abundance (intensity) of these ion fragments analysed. A mass spectrum is a plot of this intensity versus the mass:charge ratio (Skoog and Leary, 1992).
The spectra are then stored in a database for subsequent processing.
The use of GC-MS to analyse *trans* fatty acids does not bestow any advantages over and above the actual identification of the fatty acids in a sample compared to regular GC. It is, however, a clean, fast, easy to use and reproducible method of analysis.

CHAPTER 2

Materials and Methods

2.1 Ethical approval

Ethical approval for the study was granted by the Ethics Committee at St. James' Hospital, Dublin 8.

2.2 Subjects

122 healthy adults (59 females, 62 males) were recruited from Aer Rianta Irish Airports and Team Aer Lingus, at the Medical Centre, Dublin Airport with the help of a notice in a weekly internal magazine and a circulated notice (Appendices I and II respectively).

With respect to their participation in the study, each subject was briefed in either of two ways; (a) as part of a group briefing session or (b) by means of a patient information letter (appendix IX). Each subject was then required to complete and return an 'Eligibility Questionnaire' (appendix IV). On the basis of this questionnaire, subjects were accepted or not into the study. Having been accepted as a participant in the study, each subject was then sent an appointment date and time at which to attend the Medical Centre (appendix V). On arrival at the time of their appointment, each subject was initially re-briefed as to what was expected from them; i.e. the tissue samples that they were required to give. The subject, the researcher and the nurse who was taking the samples then signed a standard consent form issued by St James' Hospital and Federated Dublin Voluntary Hospitals joint research ethics committee (appendix II).

One blood sample and one adipose tissue sample were then taken. All tissue samples were taken by a fully qualified Registered General Nurse who was

trained in the area of phlebotomy and specific subcutaneous adipose tissue aspiration for the purpose of the study.

2.3 Dietary analysis

On subsequent occasions, subjects were interviewed with respect to their dietary intake. Firstly, by means of a detailed Dietary History (the standard method for analysing the diet) and secondly by an interview-assisted Fat Frequency Questionnaire. A detailed Dietary History involves questioning the subject as to all the different foods which may be present in their diet at any given time, i.e. is based around the individuals meal patterns. Food atlas and portion sizes are used to assist the analyst. This method takes approximately one hour to complete. The FIQ focuses mainly on fat-containing foods and how often the individual may consume this food for example once a week, twice a week, more often or never. This questionnaire takes approximately 20 minutes to administer.

The fatty acid profiles of the diets were analysed using a nutrient data analysis computer application called FOODBASE (version 3.1). The data used by FOODBASE is from the 5th Edition of McCance and Widdowson's *The Composition of Foods*.

For the purpose of this study, carried out using an Irish subject population, 240 Irish foodstuffs which would be main sources of hydrogenated fatty acids in Irish diets (e.g. various brands of margarines, cooking fats, spreads and confectionery products such as biscuits) were analysed using a method developed specifically for this study, for their fatty acid content (including the TFA content (elaidic acid (C18:1*t*9)) (O'Neill, 1998). This fatty acids profiles of these foodstuffs was then substituted for the corresponding UK data in

FOODBASE, so that the resulting TFA measured by the dietary methods would be representative of the Irish diet.

2.4 Adipose tissue samples

2.4.1 Aspiration of adipose tissue samples

Subcutaneous adipose tissue samples were aspirated using a modification of the method originally described by Hirsch (1960). Using this method, a 19g needle (16G1" Microlance 3 x 1.6 x 40mm) connected to a luer adapter (Vacutainer Brand, Becton Dickinson) and yellow holder (Vacutainer Brand, 16 x 38mm, Becton Dickinson) is inserted into a fold of skin pinched at the area of the abdomen proximal to the naval. To apply suction, an evacuated tube (Vacutainer Brand, Becton Dickinson) was connected, and the whole apparatus then moved back and forth in close but varied directions to maximise the yield of tissue obtained (Beynen and Katan, 1985).
After removal, the needle and Luer adapter are unscrewed from the yellow holder, and both were placed into the vacutainer tube. Samples taken on site were stored in the vacutainer at -18°C for a maximum of 9 hours and then transferred to <-70°C until further analysis.

2.4.2 Extraction of fat

Samples removed from freezing were allowed to thaw at room temperature (this took approximately 10-15 minutes depending on the size of the sample). If the adipose tissue in the luer adapter visibly contained no blood, i.e. was a creamy yellow colour with no trace of red), then the sample was directly

transferred to a pre-weighed, graduated 1ml glass vial (Reactivial™ small reaction vials, total capacity 1ml, Pierce, Rockford, Illinois) and manually dissolved in hexane, using a small metal rod. Most samples also contained a certain amount of skin, which could be removed from the vial after the fat had dissolved.

If, however, the sample did contain blood, it had to be filtered (Figure 2.1). The blood was easily separated from the fat by heating. By using a hot air gun, heat was applied to the Luer adapter and this hardened any clot or blood present and also melted the actual fat in the sample. Filtration involved the use of a short piece (7cm x 4mm i.d.) of glass tubing containing fused glass beads at one end, which retained skin particles and hardened blood, allowing the heated lipid solution to pass through into the glass vial. The hexane was then evaporated under nitrogen, and the reacti-vial was then re-weighed so as to calculate the total weight of the fat extracted from that particular adipose tissue sample.

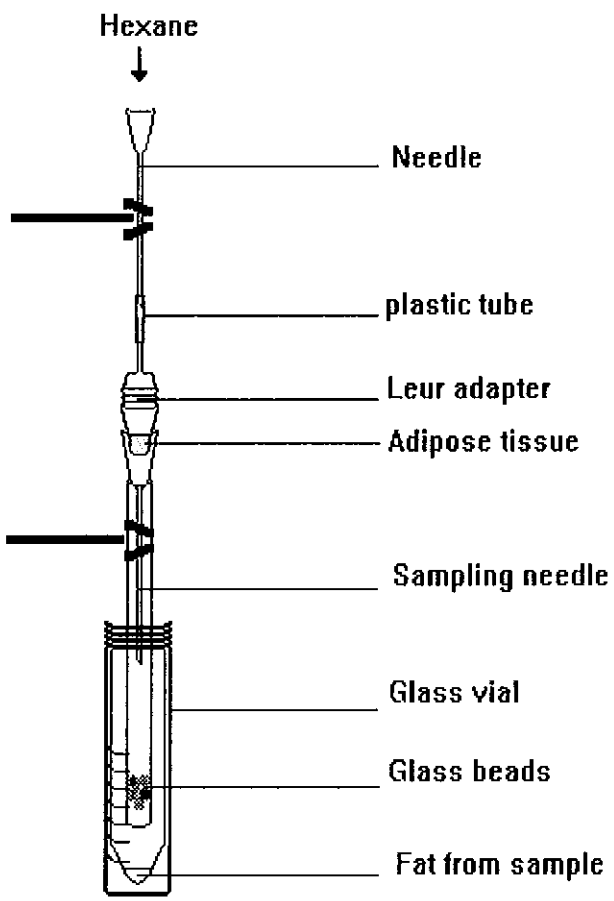


Figure 2.1 Apparatus used for transferring lipid from adipose tissue sample into glass vial

2.4.3 Preparation of adipose tissue fatty acid methyl esters.

Because of the small amount of extracted adipose fat (30mg or less in most cases) available and a requirement by collaborators on the project to measure *trans* content using both infrared spectrophotometric and gas chromatographic methods, the following procedure (O'Neill, 1998) was used to prepare fatty acid methyl ester (FAME) extracts suitable for both purposes.

Accurately weighed samples of approximately 10mg of adipose lipid was dissolved in 0.1ml of isopropyl ether in a 1ml Pierce Reactivial and were transesterified at ambient temperature with 0.1ml of 1M sodium methoxide for 20 minutes. Methylation of any free fatty acids present in the original adipose sample was completed using 0.2ml of 5% methanolic hydrogen chloride (HCL) (prepared by the addition of 1ml of pure acetyl chloride to 10ml of methanol, cooled in an ice bath; Christie (1982)). The vials were sealed with teflon-lined screw caps and the methylation was carried out at 90^{o}C for 1hr. After cooling, samples were evaporated to dryness under nitrogen and then further dried for 5-10 minutes at 70^{o}C until no odour of HCL could be detected. The FAMEs were dissolved in bromochloromethane to give a concentration of exactly 2% w/v based on the weight of the sample taken. Before sealing, approximately 20mg of anhydrous sodium sulphate was added to each vial to remove any residual traces of moisture from the extracts.

2.5 Analysis of adipose tissue *trans* fatty acid content

2.5.1 Fourier Transform Infrared Spectrophotometry (FTIR)

The analysis of the total *TFA* content of lipid extracted from adipose tissue samples was measured by FTIR (ATI Matson Research series Fourier Transform Infrared Spectrophotometry). The method used was developed (and carried out) specifically for this study by research partners in The Department of Food Science, University College, Dublin.

2.5.2 Gas Chromatography

FAMEs were analysed by capillary gas chromatography using an SP-2560 Flexible Fused Silica Capillary Column (100m x 0.25mm i.d., 20mm, Supelco UK, Poole, Dorset) on a Pye Unicam 610 series gas chromatograph equipped with a flame ionisation detector. To optimise resolution of *cis* and *trans* isomers the operating conditions shown in table 2.1 were employed. Peak areas were integrated using a Spectraphysics SP4290 computing integrator. Confirmation of identities of separated FAMEs was obtained by comparison of retention times of authentic standards run under the same conditions. A sample GC trace can be seen in figure 2.2.

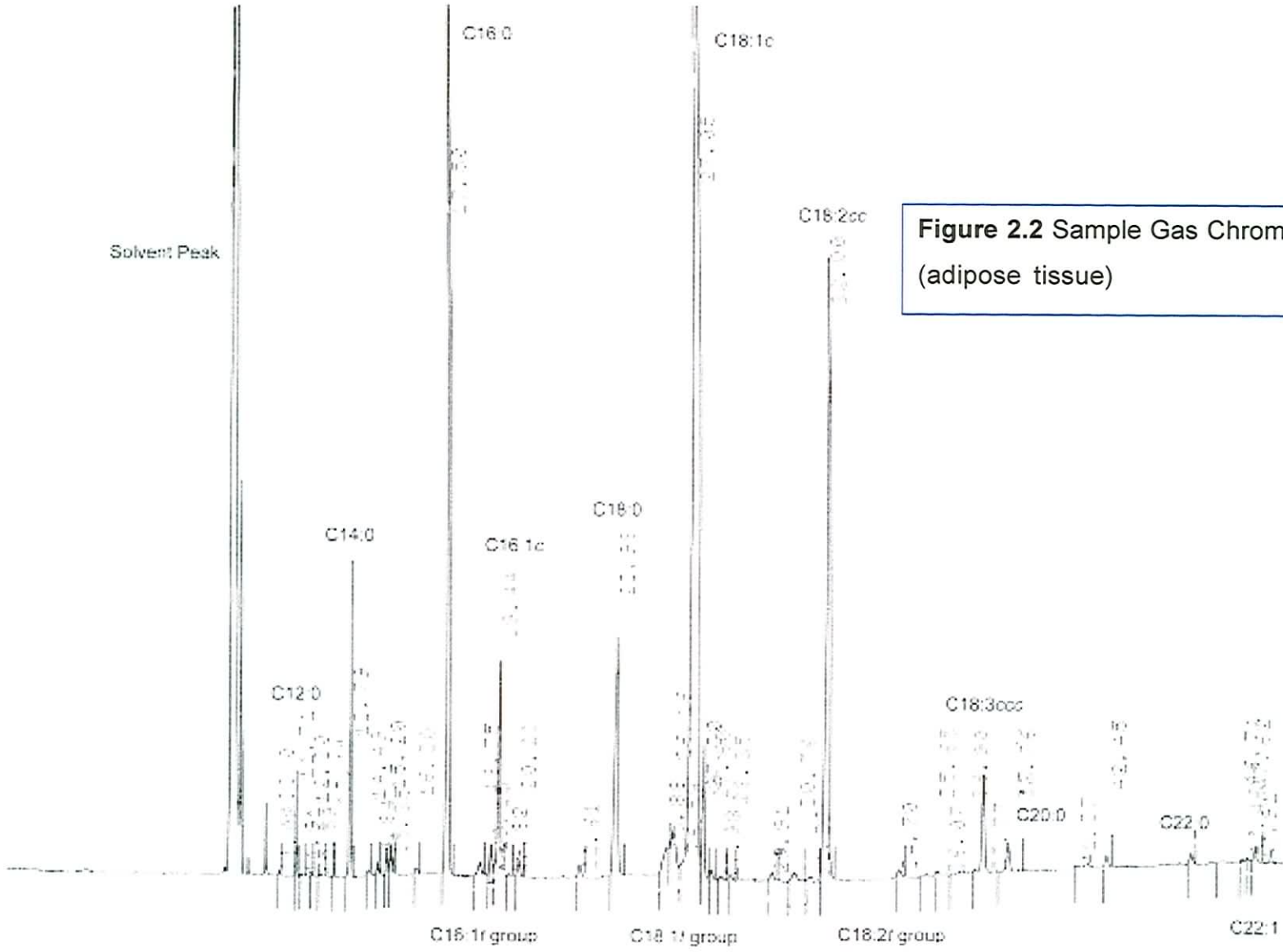


Figure 2.2 Sample Gas Chromatograph (adipose tissue)

Parameter	Value
Carrier gas (hydrogen)	Flow rate 0.8ml/minute
Column initial temperature	175 °C
Initial time	30 minutes
Program rate	2 °C/minute
Final temperature	210°C
Injector temperature	240°C
Sample volume	1μl
Split ratio	50:1
Total run time	48 minutes approx.

Table 2.2 Temperature program used for the optimal analysis of *trans* isomers of fatty acids in adipose tissue using Gas Chromatography.

2.6 Blood samples

2.6.1 Sampling technique

Blood samples were taken from the antecubital vein of each subject:
After a tourniquet was applied to the upper arm, the area over the selected vein was wiped with an alcohol swab (Fannin Healthcare Ltd, Dublin). A needle (PrecisionGlide, 21G 1.5", Becton Dickinson) was attached to a Luer adapter (as before) and a yellow holder (as before) and the vein was punctured. An evacuated vacutainer (as before) containing ethylene-diamine-tetra-acetic acid (EDTA) as an anti-coagulant was then connected to the apparatus and the blood was collected. As soon as the vacutainer was full, the tourniquet was loosened and the vacutainer removed. A small piece of cotton wool was then

applied to the puncture site and fixed with surgical tape (Fannin Healthcare Ltd, Dublin).

Blood samples were centrifuged immediately at 2000 rpm for 10 minutes. The serum was removed and stored in 400µl aliquots at <-70°C for further analysis. The buffy coat was removed by suction using a pasteur pipette, and then discarded. The remaining erythrocytes were washed three times with two volumes of 0.9% NaCl, and stored at <-70°C until further analysis.

2.6.2 Preparation of erythrocyte fatty acids methyl esters

FAMEs of erythrocyte fatty acids were prepared according to a method described by Taylor *et al* (1987) as follows: 800µl of erythrocyte suspension (containing red cells and an equal volume of isotonic sodium chloride solution) was added to a 20ml teflon lined screw capped tube. Transesterification was carried out using 2ml of methanol/6 mmol/L hydrochloric acid (prepared using 50ml concentrated HCL and 250ml of methanol) at 90°C for 2.5 hours. Lipids were then extracted into 4ml of hexane and 100ml of amyl alcohol, followed by centrifugation for 5 minutes at 1500 rpm. The top layer in the tube, containing the lipid, was then removed into a clean tube and the solvents evaporated under a stream of nitrogen. Fatty acids were converted to fatty acid methyl esters by the addition of 500ml of Boron Trifluoride /Methanol (14%) at 60°C for 10 minutes. FAMEs were then extracted into 5ml of hexane, washed twice with saturated sodium chloride solution and finally dried with sufficient sodium sulphate to remove any aqueous material present. The hexane present was then dried off, again under a stream of nitrogen, and the FAMEs finally re-dissolved in 50ml of hexane, ready for injection onto the gas chromatograph.

2.6.3 Analysis of *trans* fatty acid content of erythrocytes

Methyl esters of erythrocyte fatty acids were analysed on a HP 6890 MSD Chemstation system. This system integrates the operation of a mass spectrometer with a gas chromatograph. The Chemstation software allows the analyst to acquire, process and report data from the GC/MS instrument. The column used in the chromatograph was the same as that used for the analysis of the adipose tissue FAMEs - a 100m x 0.25mm SP-2560.
Column conditions for analysis optimising the resolution of *cis* and *trans* isomers are shown in Table 2.3 below. A sample GC-MS trace can be seen in figure 2.3.

Parameter	**Value**
Carrier gas (Helium)	Flow rate - 0.6ml/min
Column initial temperature	180 °C
Initial time	15 minutes
Program rate 1	2 °C/minute
Column temperature 1	195 °C
Final time 1	10 minutes
Program rate 2	2 °C/minute
Column temperature 2	210 °C
Final time 2	20 minutes
Injector temperature	240
Sample volume	1µl
Total run time	60 minutes approx.

Table 2.3 Temperature program used for the optimal analysis of *trans* isomers of fatty acids in erythrocytes using Gas Chromatography.

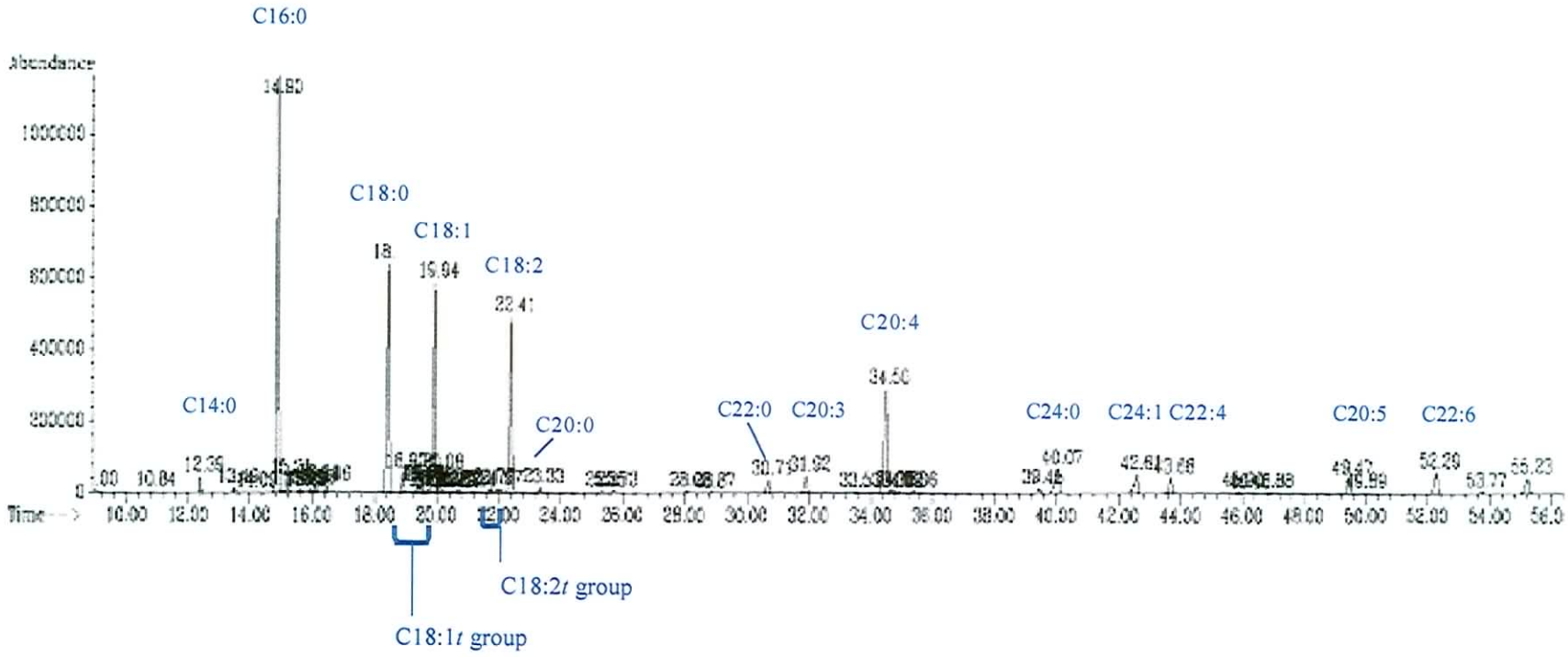


Figure 2.3 Sample GC-MS trace (Red Blood Cell (RBC) fatty acids)

On traces which resolved C18:3 fatty acid methyl esters, this occurred at approximately 31 minutes

2.7 Analysis of total serum cholesterol and lipoproteins

Serum low density lipoprotein (LDL), high density lipoprotein (HDL) and total cholesterol (Chol) were quantified in triplicate from aliquots of serum samples using specific enzyme assay kits: LDL-C (No. 726 290), HDL-C (No. 543 004) and Chod-Pap (No. 1442 350) respectively (Boehringer Mannheim, Germany). The test principals involved can be seen in table 2.4. Quality controls used for the individual tests are detailed in table 2.5.

Lipid fraction	**Test principal**
Cholesterol Siedal *et al*, 1983 Katterman *et al*,1984	*cholesterol esterase* Cholesterol + H_2O ⟶ Cholesterol + RCOOH *cholesterol oxidase* Cholesterol + O_2 ⟶ Δ^4-cholestenone + H_2O_2 *POD* $2H_2O_2$ + 4-aminophenazone + phenol ⟶ 4-(*p*-benzoquinone-monoimino)-phenazone + $4H_2O$
HDL Burstein *et al*, 1970 Lopes-Virella et al, 1977	Chylomicrons, VLDL and LDL are precipitated by adding phosphotungistic acid and magnesium ions to the sample. Centrifugation leaves only the HDL in the supernatant; their cholesterol content is determined enzymatically
LDL As per cholesterol	LDL are precipitated by adding polyvinylsulphate to the sample. Their concentration is calculated from the difference between the serum total cholesterol and the cholesterol in the supernatant after centrifugation.

Table 2.4 Test principal for analysis of serum lipids fractions

Lipid fraction	Quality control for accuracy
Cholesterol	Precinorm® U, Precinorm® L, Precipath® U, Precipath® L
HDL	Precinorm® L, Precipath® L
LDL	Precinorm® L, Precipath® L

Table 2.5 Quality controls used for accuracy in lipid fraction assays

2.8 Statistical Analysis

Statistical analyses were carried out using SPSS (Statistical Package for the Social Sciences) for Windows 95 version 8.0. Product-moment correlation coefficients (Pearson *r*) (with significance levels (*P*) calculated) were used to determine the extent to which values of sets of variables were proportional, or bore a relationship to one another.
Data subsets (e.g. Chol:HDL etc) were similarly tested.

2.9 Study participant feedback

Upon completion of the analysis of the study, participants received each of the following:

(a) The results of the detailed dietary assessment carried out by the study run in conjunction with this present study, and dietary advice based on these results.

(b) The results of the analysis of adipose tissue and blood samples (appendix VIII (a) and (b)).

Details included the total *trans* fatty acid level analysed in the adipose tissue, the total, LDL and HDL serum cholesterol levels, a brief explanation of each of the above parameters and advice based on results obtained - e.g. if cholesterol levels were noted to be higher than normal levels, the subject was advised to have their serum total cholesterol levels re-checked by their own General Practitioner.

CHAPTER 3

Results

3.1 Subject data

Subject information is shown in table 3.1. Samples for both adipose tissue and blood were not attainable from every subject. For this reason, and with respect to statistical analysis, the number (n) of subjects or samples and the number of pairs of data is detailed for each test.

Appendix III shows the full complement of correlations carried out.

3.2 Fatty Acid Analysis - Adipose Tissue and Erythrocyte Membranes

Table 3.2 gives a comprehensive overview - allowing comparisons - of the fatty acid profiles analysed by gas chromatography in both the adipose tissue and erythrocyte membranes of subjects.

3.2.1 *Trans* Fatty Acids - Statistical Analysis

With respect to TFA, a positive (n=54, r=0.32), significant (P=0.01) relationship was observed between C18:1*t* in adipose tissue and C18:1*t* in erythrocytes (see figure 3.1), even though C18:1*t* is present in the red cells to a much lesser extent (see table 3.2). Figure 3.2 shows the relevant inter-correlations between adipose tissue, erythrocyte and dietary TFA. Correlations were not observed between other non endogenous fatty acids (eg C18:2*t*) in adipose tissue and RBCs.

C18:1*t* levels in adipose tissue of male subjects (mean 3.15, SD 0.92) was significantly higher (p=0.002) than levels measured in females (mean 2.95, SD 0.6) (n=28). There was no significant difference in C18:2*c* levels between males and females.

A positive correlation was drawn (n=74, r=0.56, p<0.05) between erythrocyte C18:2*cc* and erythrocyte C20:4 (figure 3.3), and also between erythrocyte C18:1*c* and erythrocyte C18:1*t* (n=74, r=0.27, p<0.05).

3.2.2 Dietary Fatty Acids

Diet history and food intake questionnaire TFA levels in foods were analysed using infrared spectrophotometry.

TFA levels measured in the diet (i.e. diet history method) can be seen in table 3.3. This table shows also the total TFA as analysed by gas chromatography and infrared spectrophotometry. There is a difference in adipose tissue levels of TFA measured using the two methods because the Infrared method gives the total *trans* isomers present in a sample, where as the GC method gives the individual *trans* isomer groups (as can be detected) and the level expressed here is the sum of those individual isomers.

There was good correlation between the two dietary methods (n=82, r=0.67, p<0.05) (figure 3.4). The relationship between the diet History TFA and total adipose tissue TFA as analysed by GC was positive (n=87, r=0.16) but not significantly so (p=0.18). However a positive (n=87, r=0.22) more significant (p=0.06) relationship resulted when examining adipose tissue C18:1*t* and diet history. A stronger relationship was seen when C18:2*c* levels in adipose

tissue and dietary (FIQ) data were examined (n=84, r=0.51, p<0.05) (figure 3.5). From table 3.4 it also evident that the analysis of adipose tissue TFA by GC gives less variable results than the analysis of TFA by IR or the dietary measurements, since the magnitude of the standard deviation (0.85) from the mean is much smaller than that for the diet history method (2.53) or Food Intake Questionnaire method (2.53).

3.3 Total Serum Cholesterol and Lipoproteins

Mean, range and standard deviation of total cholesterol, serum HDL and LDL for all subjects and female and male subjects can be seen in table 3.4. HDL levels in females were found to be significantly higher (P=0.004) than those in males. The cholesterol:HDL ratio of males (mean 6.42, SD 2.05) was found to be significantly higher (P=0.005) than that of females (mean 4.54, SD 1.62). With respect to serum total cholesterol and LDL, there was no significant difference between the sexes.

In these subjects, a positive (r=0.35) significant (P=0.002) relationship was found between BMI and cholesterol:HDL ratio (n=87) (figure 3.6).

No relationship was observed between total TFA in adipose tissue analysed by GC and serum LDL. Examining the relationship between serum LDL and adipose tissue C18:1t of subjects (n=87) however, a positive (r=0.13) but non significant (P=0.24) relationship was observed (a non-significant negative relationship was observed between erythrocyte C18:1t and serum LDL).

When the tertile of subjects with the highest C18:1*t* levels in their adipose tissue were examined however, a stronger (n=27, *r*=0.3, *P*=0.13) but non significant relationship was observed.

Adipose tissue C16:1*t* also correlated (n=87, *r*=0.33) significantly (*P*=0.002) with LDL (figure 3.7), this correlation however, did not strengthen when the top tertile for C16:1*t* (n=27) was examined. There was no significant relationship observed between diet history total TFA and serum LDL.

Other significant relationships between serum lipoprotein/cholesterol and adipose tissue fatty acids include the following: C16:1*t* correlated positively with cholesterol (*r*=0.25, *P*<0.05), and also with cholesterol:HDL (*r*=0.27, *P*<0.05), and negatively (*r*=-0.19) but not significantly (*P*=0.4) with HDL.

There was no significant relationship observed between adipose tissue C14:0 (n=87) and cholesterol:HDL levels, but erythrocyte C14:0 and cholesterol:HDL were significantly correlated (n= 63, *r*= 0.3, P= 0.04).

A significant, negative (*r*=-0.23, *P*<0.05) correlation was found between serum HDL and erythrocyte C20:5. Although non significant, this negative correlation was also observed between erythrocyte C22:6 and HDL (*r*=-0.12, *P*=0.35) (These long chain PUFAs were not detected in adipose tissue samples).

Adipose Tissue

	C12:0‡	C14:0	C16:0	C16:1*t* (total)	C16:1c	C18:0	C18:1*t* (total)
mean	0.32	2.55	19.85	0.65	3.59	5.18	3.06
SD	0.12	0.42	1.45	0.13	0.8	0.97	0.8

Erythrocytes

mean	/	0.33	24.72	/	0.25	16.28	0.85
SD	/	0.11	4.82	/	1.6	3.47	0.39

Adipose Tissue

	C18:1*c*	C18:2*tt* (total)	C18:2	C18:3	C20:0	C22:0	C22:1
mean	42.21	0.51	13.87	1.76	0.55	0.18	0.3
SD	2.08	0.43	2.49	0.52	0.15	0.09	0.11

Erythrocytes

mean	12.5	0.43	10.29	0.1	0.22	/	/
SD	1.8	0.22	2.4	0.17	0.1	/	/

Erythrocytes - (none of the following fatty acids identified in Adipose Tissue)

	C20:3	C20:4	C24:0	C24:1	C22:4	C20:5	C22:6
mean	1	11.62	2.45	2.23	1.48	2.23	3
SD	0.45	2.6	1.18	1	0.4	0.47	1.16

Table 3.2_The mean and standard deviation (SD) % of fatty acids (g/100g lipid) analysed by Gas Chromatography in Adipose Tissue (n=101) and Erythrocyte Membranes (n=72) of subjects

‡ For nomenclature of fatty acids see Appendix VII

/ Denotes no peak detected

	Diet History Method Total TFA	Food Intake Questionnaire Total TFA	Adipose Tissue (GC) Total C16:1*t*, C18:1*t*, C18:2 *t*	Adipose Tissue (FTIR) Total TFA	Erythrocyte (RBC) (GC-MS) Total C18:1*t*, C18:2 *t*
N	82	84	101	97	72
Mean	5.58	5.9	4.26	5.07	1.26
Standard Deviation	2.53	2.53	0.85	1.12	0.3

Table 3.3 The mean and standard deviation of total *Trans* fatty Acids levels measured (g/100g of lipid)

N Number of subjects
GC Gas Chromatography
FTIR Fourier Transform Infrared Spectrophotometry

	N	Cholesterol (mmol/l)	HDL(mmol/l)	LDL (mmol/l)
Both Sexes	109	5.53(1.49) range 1.68-8.87	1.08(0.4) range 0.46-2.09	3.54(1.5) range 0.47-7.32
Females Only	46	5.29(1.27) range 2.67-8.42	1.22(0.47) range 0.46-2.07	3.19(1.39) range 0.51-7.09
Males Only	63	5.71(1.62) range 1.68-8.87	0.94(0.31) range 0.5-2.09	3.8(1.5) range 0.47-7.3

Table 3.4 The average (standard deviation) and range of values for serum total cholesterol and lipoproteins.

HDL (High Density Lipoprotein)
LDL (Low Density Lipoprotein)

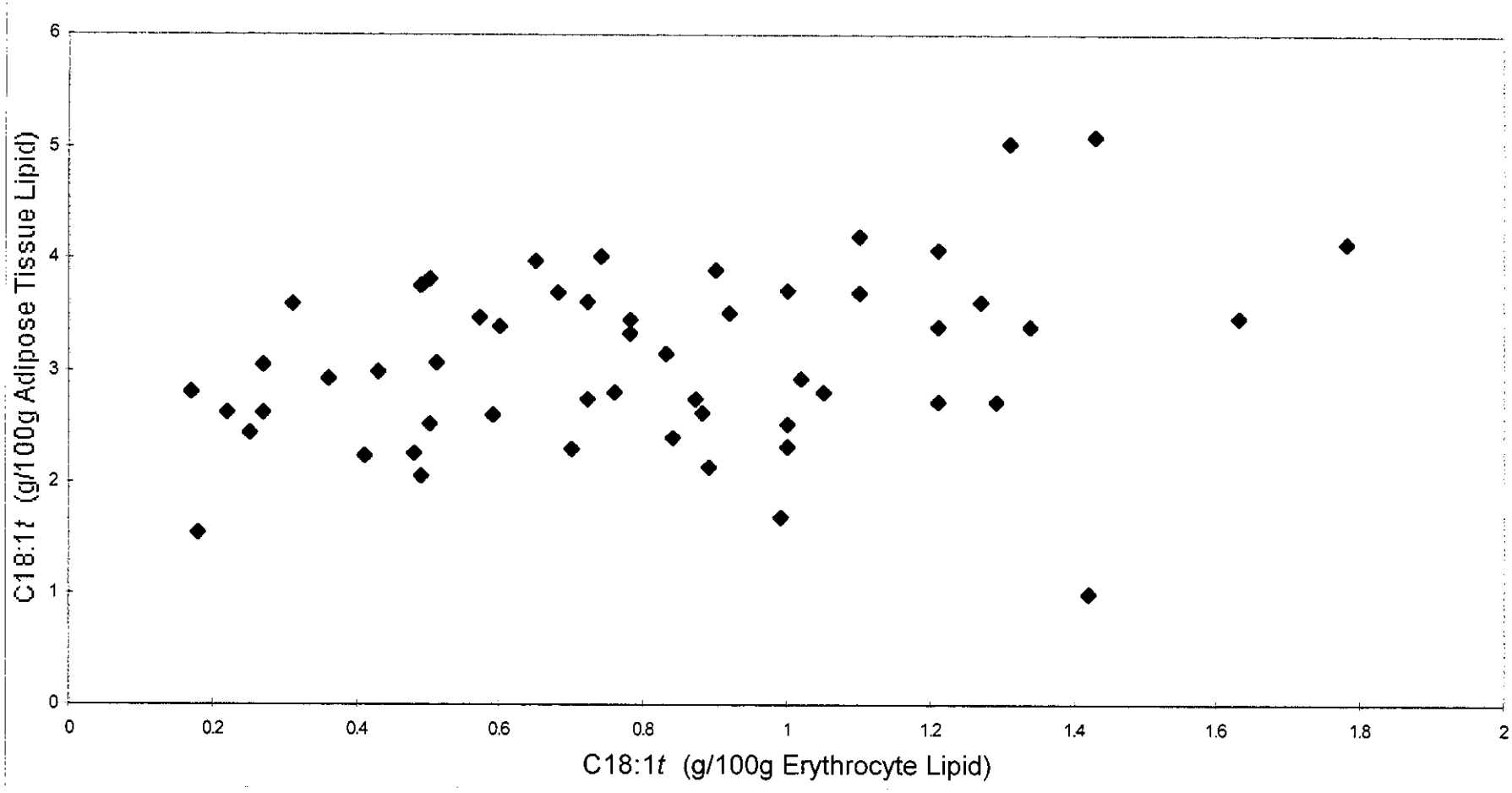


Figure 3.1 Adipose Tissue C18:1t v Erythrocyte C18:1t(n=54)

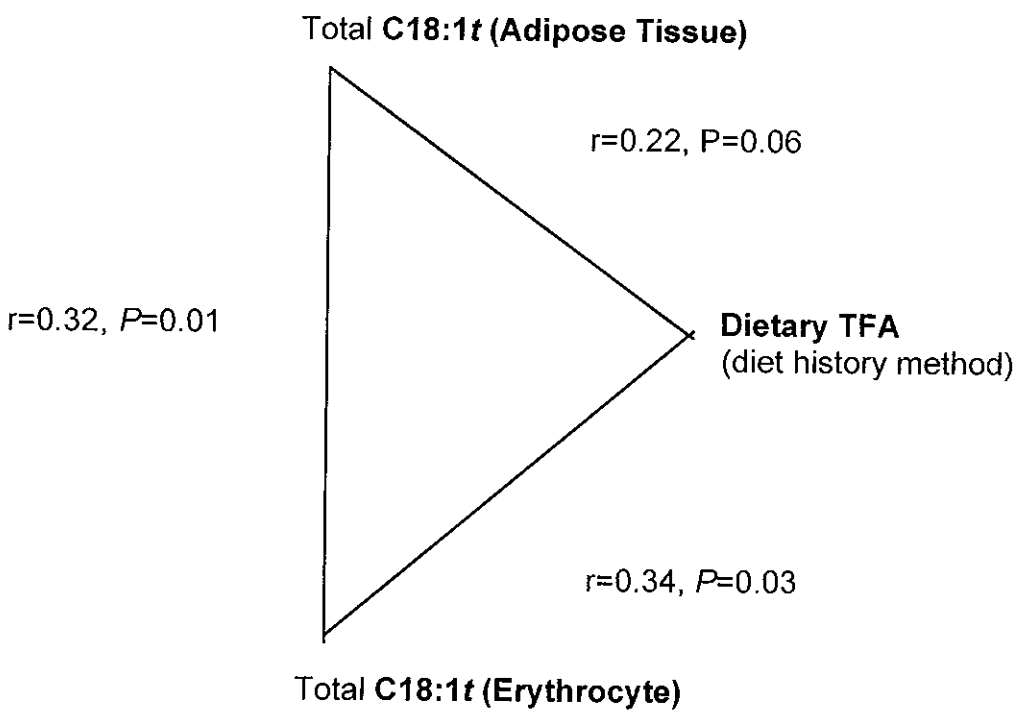


Figure3.2 The inter-correlations between some significant parameters measured in the study.

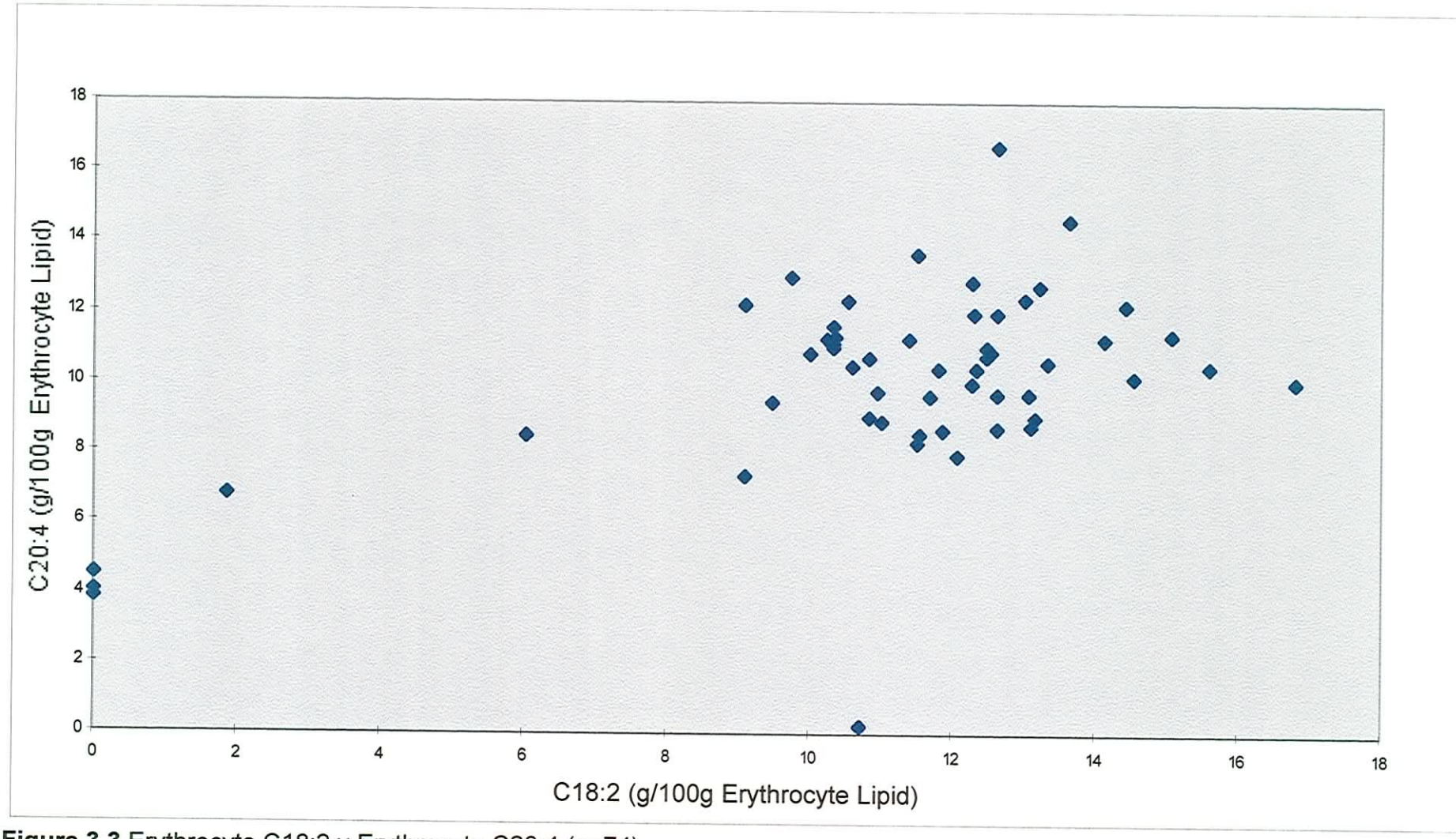


Figure 3.3 Erythrocyte C18:2 v Erythrocyte C20:4 (n=74)

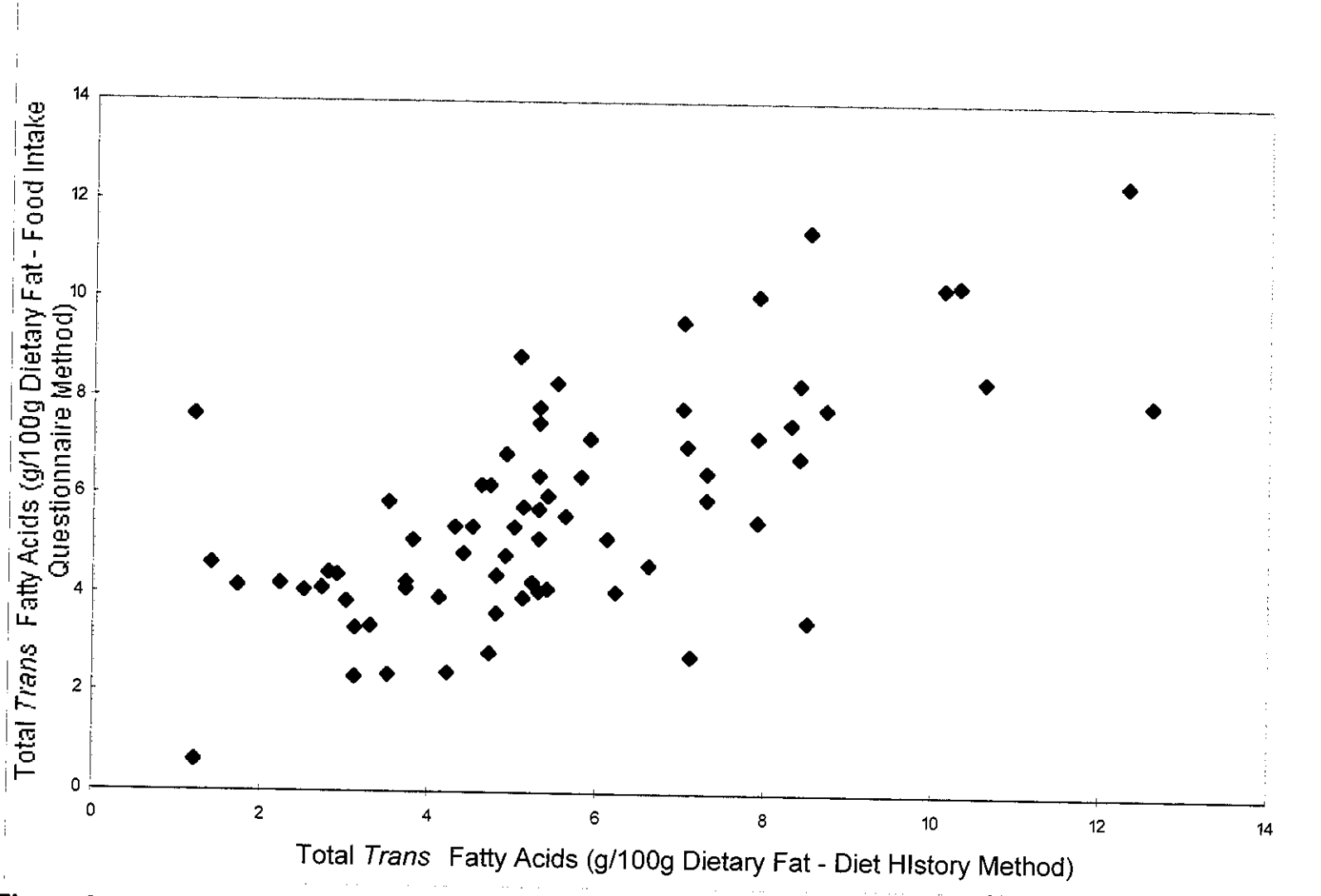


Figure 3.4 Diet History TFA v Food Intake Questionnaire TFA (n=82)

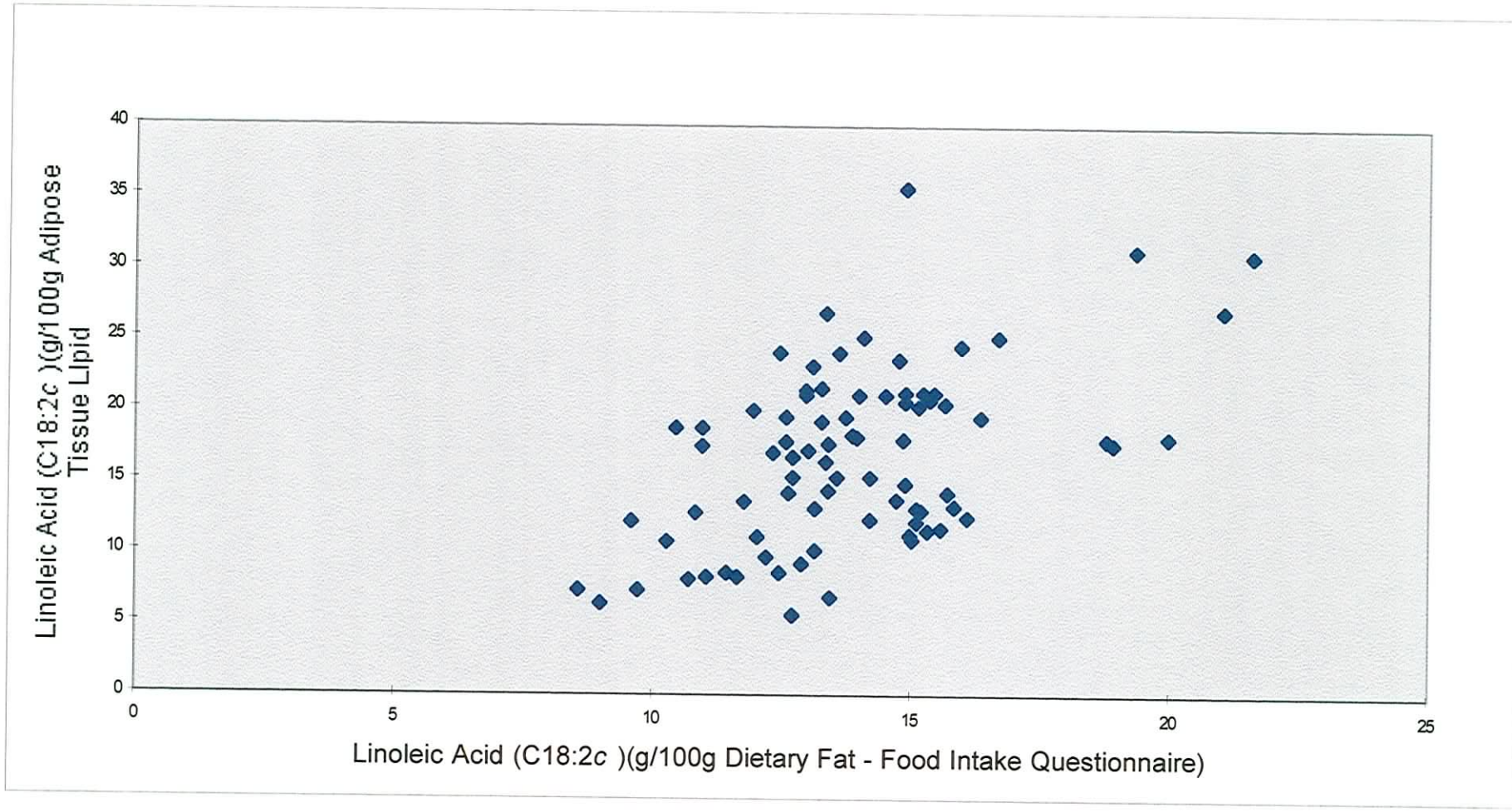


Figure 3.5 Linoleic Acid Adipose Tissue v Linoleic Acid (Food Intake Questionnaire) (n=84)

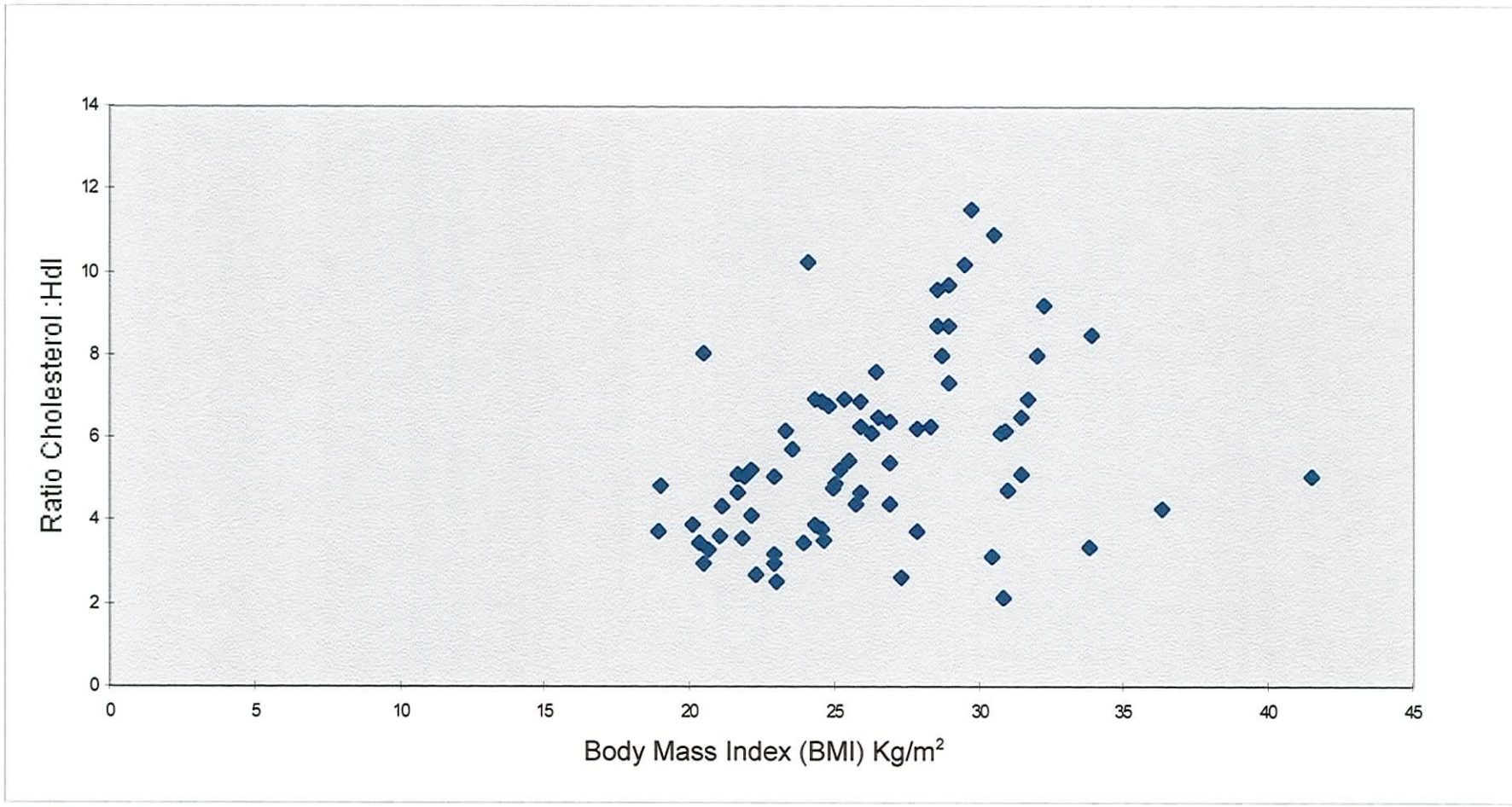


Figure 3.6 Body Mass Index v Cholesterol:HDL (n=87)

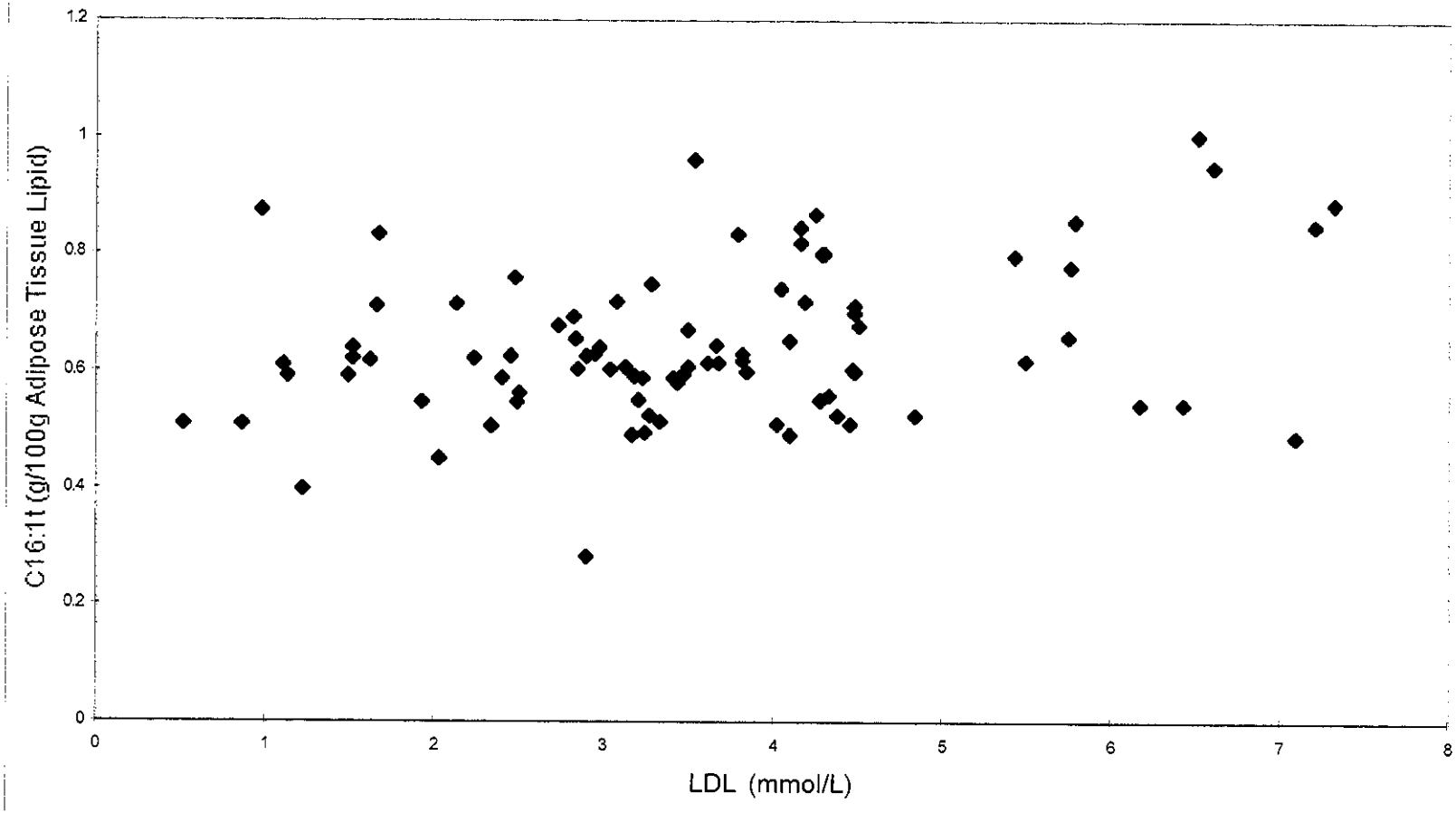


Figure 3.7 Total Serum Low Density Lipoprotein (LDL) v C16:1*t* (Adipose Tissue) n=87

CHAPTER 4

Discussion

4.1 Analysis of *trans* fatty acids

The data presented in this study (with respect to TFA) are the first to be generated from an Irish population.

In the present study, the analysis of fatty acids in adipose tissue was carried using two methods - Fourier Transform Infrared Spectrophotometry (FTIR) and Gas Chromatography (GC). FTIR (as described in chapter 2, section 2.5.1) returns the **total** number of *trans* bonds in a sample. The FTIR method for the very small adipose tissue sample size used in this study is, as yet, under development. For this reason, the total adipose tissue TFA analysed by FTIR may not give a true result. GC on the other hand will separate *cis* and *trans* isomers but it is possible only to identify those isomers for which authentic standards are available, or those on which previous work has been carried out so that results are available from the literature for comparison of patterns or retention times as appropriate. The GC total result is therefore an underestimate because many longer chain TFA isomers (many of which will be present as a result of the dietary consumption of partially hydrogenated fish oil) cannot be identified, largely due to the overlap between *cis* and *trans* isomers (Ratnayake et al, 1990). For this reason, and for the purpose of this study, emphasis has been placed more upon the results of those individual

groups of *trans* isomers which are identifiable, namely total C16:1*t*, total C18:1*t* and total C18:2*t* upon which a greater degree of confidence can be placed. However, the total values obtained by both the FTIR and GC methods are useful for comparison with previous studies that have used similar GC methods. The existence of a relationship(r=0.41, P<0.001) between total *trans* analysed by the two methods and between the total C18:1*t* by GC and total *trans* by IR (r=0.65, P<.001) allows some confidence in the results.

4.2 Adipose tissue data

The total *trans* and total C18:1*t* measured in the adipose tissue of subjects was similar to those levels reported for other populations (tables 4.1 and 4.2). These studies were chosen for comparison as they use very similar methods of analyses including exactly the same type of GC column as the present study. Emphasis has been placed upon total C18:1*t* in this table (4.1) as it is the major *trans* constituent in the adipose tissue of subjects following self selected diets.

Levels of C22:6n-3 in adipose tissue were consistently less than 0.1% and C20:5n-3 levels were negligible. These longer chain PUFA are not present in great amounts in adipose tissue because actual subcutaneous fat tissue is relatively solid, compared, for example, with the very fluid membranes of erythrocytes which conversely incorporate long chain PUFA to a much greater extent.

4.3 Erythrocyte fatty acids

Table 4.3 shows the five major fatty acids in membranes of subjects in the present study as compared with the rather few other contemporary studies involving different populations but using similar methods. This table highlights the differences that exist in the erythrocyte fatty acid profiles of different populations which, in turn, reflects their respective diets. The results for the Italian population show the effect on the red cell lipids of the cardiovascular-protective Mediterranean diet (Sanders *et al*, 1990) - the large proportion of C18:1*c* present may be indicative of intakes of olive oil. The subjects in the present study show high levels of erythrocyte C16:0, an offending saturate with respect to heart disease (Grundy and Denke, 1990) . The results also demonstrate significant links between total C18:2*c* in the diet and erythrocytes. With respect to TFA in erythrocyte membranes, levels correlated with, but were not present to the same extent (figure 4.1) as in adipose tissue; a possible explanation for this is discussed in section 4.5. Total C16:1*t* levels were negligible in some and absent in most of the erythrocyte samples analysed. Total C18:2*t* levels were present in very small amounts, and again, were not detected in all samples.

4.4 Dietary data

The results of this study indicate the validity of the FIQ with respect to the

non-endogenously produced fatty acids , as previously shown in other studies (van-Staveren *et al*, 1986; London *et al*, 1991; Lemaitre *et al*, 1998).

The positive relationship between total C18:1*t* in adipose tissue and total dietary TFA (diet history method) (r=0.22, P=0.06) (an identical match is not expected, Summers *et al*, 2000), the significant relationship between red cell total C18:1*t* and total dietary TFA (diet history method) (r=0.34, P=0.03), and the strong relationship (r=0.67,P<0.001) between total dietary TFA by diet history and FIQ suggests that the FIQ is a reliable method of estimating dietary non-endogenous fatty acids.

To validate his FFQ, van-Staveren *et al* (1986) showed a significant relationship between total C18:2*c* in the diet and adipose tissue (r=0.7) - his dietary method involved the mean of nineteen 24 hour dietary recalls which is considered to be quite an accurate measure of self-selected diets. Using a less arduous method (FIQ) the present study returned a significant correlation between total dietary C18:2*c* and total adipose tissue C18:2*c* (r=0.51, P<0.001).

Other studies have correlated saturated fatty acids in the diet with levels in adipose tissue (Field *et al*, 1985). These saturates, however, as they may be synthesised endogenously, may not be present in adipose tissue as a direct result of the diet, and so these associations were not focused upon here.

4.4.1 The 'Fat Busters' Club

As dietary histories and FIQ's were being documented, it was brought to the notice of the researcher that a number of subjects may have been participating in the study to facilitate a weight reduction program. These subjects were denoted '*The Fat Busters Club*' The estimated number of the subjects was n=30. The inclusion of these subjects' data in the study may affect some of the results (i.e. of the adipose tissue and/or dietary results), but this is difficult to assess as there are no previous data (i.e. prior to study) and no data were collected after the study.

4.5 Relationship between TFA in adipose tissue and erythrocytes

The positive relationship observed between total C18:1*t* in adipose tissue and erythrocyte membranes (r=0.31, P=0.01) shows the connection between them with respect to the individual. No correlations were observed for C18:2*c* and C18:3*c* between adipose tissue and erythrocytes, which was somewhat unexpected as both are essential fatty acids i.e. originate only from the diet, as do TFA (Neoptolemous, 1988; Farquhar and Ahrens 1983; Wood *et al*, 1984). This observation could possibly be due to the faster turnover of erythrocytes (approximately 120 days) that would result in alteration of membrane composition due to seasonal changes in the diet which would not be observed in the adipose tissue because its turnover is so much slower (approximately 600 days).

As previously discussed, correlations were found between TFA in the diet and adipose tissue as expected. It is quite apparent however (figure 4.1) that the TFA in erythrocyte membranes, although they may bear a significant relationship to that of the diet and adipose tissue, are present at lower levels. Few studies have examined the incorporation of TFA into different body tissues. One study, however, upon examination of body tissue from rats who had been fed diets rich in TFA found that the incorporation of C18:1*t* into erythrocyte lipid was 60% lower than that which was incorporated into adipose tissue. Interestingly, the present study shows C18:1*t* levels in erythrocytes to be 70% lower than that in adipose tissue. A possible explanation this may be that there is selective incorporation of fatty acids into different body tissues. Clearly, however, this proposal requires further investigation using human subjects.

4.6 Cholesterol and lipoproteins

Total serum cholesterol, LDL and HDL were analysed as described in chapter 2 section 2.7. Blood samples were taken in the post-prandial state as an individual will be in this state for the majority of the day and therefore will present a more natural picture than when fasting.

As has been previously reported (Heiss *et al*, 1980), HDL levels were significantly higher in females than in males. The opposite was the case for the cholesterol:HDL ratio. Both of these demonstrate the protective effect of the female gender against coronary heart disease. It in unclear as to the exact reason for the difference in the levels of cholesterol and lipoproteins between

men and women, but it is thought that the effect may be as a result of hormonal influences. It is interesting also at this stage to note the fact that C18:1*t* levels were significantly higher in males than in females (chapter 3, section 3.2.1). This, based on previous studies and the evidence subsequently provided in this chapter, could also contribute to the protective effect of the female gender.

4.7 TFA in adipose tissue and coronary heart disease risk

As previously described (chapter1, section 1.3.2.1) many studies have shown a positive association between TFA in the diet and lipoproteins in the blood. Most of these studies show that TFA in the diet can raise LDL levels (Judd *et al*, 1994; Nestle *et al*, 1992) and also affect HDL levels (more controversial) (Judd *et al*, 1992; Wood *et al*, 1993). The present study shows a correlation between blood lipids and the major *trans* isomer group in the diet of the study population, total C18:1*t*. The same, however, was true for total C16:1*t* in the adipose tissue, which correlated positively and even more significantly than total C18:1*t* with LDL, cholesterol:HDL ratio (and negatively but not significantly with HDL). Total C16:1*t* also correlated significantly with total serum cholesterol (r=0.25, P<0.05) in contrast with the findings of previous studies (Nestle *et al*, 1992; Mattson, 1975) which observed no effect of TFA on total cholesterol levels. However, it has been reported that fatal CHD cases have significantly higher levels of C16:1*t* in their adipose tissue than that of controls (Thomas *et al*, 1983; Thomas and Winter, 1987). These findings suggest the

C16:1*t* may be potentially more damaging with respect to CHD risk than C18:1*t*, upon which much emphasis has been previously placed.

It has been previously proposed that partially hydrogenated fat or oils will act and have a similar effect to that of saturates (Mensink and Katan, 1990). Saturates C12:0, C14:0 and C16:0 have been shown to be the most 'atherogenic' with respect to CHD (Denke and Grundy, 1991). A possible explanation for the stronger association between C16:1*t* and LDL than C18:1*t* and LDL could be that C16:1*t* acts more like its corresponding saturate than does C18:1*t*, and hence mimics the detrimental effects and contributes to risk factors for CHD.

The relationship between both C16:1*t* and C18:1*t* in adipose tissue and total cholesterol, LDL, and cholesterol:HDL ratio are similar in their positivity but not in their magnitude.

In agreement with the present study, some studies measuring individual TFA isomers do suggest that C16:1*t* is the more offending *trans* isomer. In an extensive review on TFA and CHD, Allison (1995) shows a statistically significant association ($P<0.001$) between C16:1*t* and *'the presence of CHD or electrocardiogram risk factors'*

The overall implications of these findings point to the necessity of investigating the individual trans isomers that can be identified, and looking at their possible implications for CHD.

Also of interest is the observation that when the upper tertiles of TFA are examined, the relationship between total C18:1*t* and LDL becomes more significantly positive, indicating that there may possibly be a threshold above

which TFA may become more offending. Mann (1994) suggested a threshold of 10g TFA per day, above which impairment of sterol metabolism occurred. This work, however, involved studying a Maasai population with an average energy intake of 4000-5000kcal/day, who exist almost exclusively on milk and beef and who consistently had cholesterol levels lower than 4.40mmol/l, with a daily intake of approximately 160g fat per day (of which 3% (4-7g) was TFA, bovine in origin).

The results of examining the upper tertiles also concur with feeding studies that use high 'unnatural' amounts of TFA in test diets to maximise the effects of TFA in order to make them more measurable. The importance of looking at the higher TFA intakes was also suggested by Ascherio *et al* (1993) who proposed that higher intakes of TFA may increase the risk of myocardial infarction independent of the effect on lipoproteins. This was important with respect to C16:1*t* which had a potent effect on lipoproteins until the upper tertiles were examined when, in contrast with the present study the correlations disappeared almost completely.

4.8 C16:1*t* versus C18:1*t* as offending isomers (with respect to CHD)

It is clear from published data that the main source of TFA in the diet and subsequently the adipose tissue originates from partially hydrogenated vegetable oil, the main isomer group being C18:1*t* (approximately 75% of 'total'

trans isomers in a sample) For this reason, it is the CHD risk associated with C18:1*t* that have been the focus of much attention, especially when considering that the higher TFA tertiles gave a stronger correlation. C16:1*t* is present in adipose tissue in very small amounts - in the present study average C16:1*t* being 0.65% (of total lipid), (ranging from 0.2-1%) so it in understandable why less research emphasis is placed on this isomer. When the total adipose tissue *trans* isomer content can be examined - that is when all isomers are identifiable - this will encompass all the *trans* in the diet and will therefore contribute to the whole picture. All the individual isomers can then be examined separately with respect to their contribution to disease, as has been done with the identifiable isomers in the present study.

Taking into consideration that C16:1*t* isomers are largely animal in origin (Sommerfield, 1983), an area which should be further investigated is the extent of the effect of C18:1*t*-11 (bovine in origin) versus C18:1*t*-9 (vegetable in origin) to examine if there is a difference in their potential detrimental effect. The outcome may point to non-vegetable sources in general as the main offenders with respect to CHD. This suggestion is further supported by the fact that there were no significant correlations observed between C18:2*t* (mostly vegetable in origin) and lipid profiles in the present study (Although Willet *et al*, 1993 observed a significant risk associated with vegetable-originating C18:1*t*9 (elaidic) and CHD but not between C18:1*t*11(vaccenic) and CHD).

4.9 Erythrocyte fatty acids and CHD risk

Polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 families are necessary for proper growth and body functions. PUFAs make up the majority of fatty acids in erythrocyte membranes.

Fish oils are rich in long chain PUFA especially C20:5n-3(EPA) and C22:6n-3(DHA). Intakes of fish oils (rich in n-3 polyunsaturated fatty acids) in the diet have been shown to reduce the risk of CHD (Kromhout *et al*, 1985; Kromhout *et al*, 1995; Kang and Leaf, 2000; Siscovick *et al*, 2000; Stampfer *et al*, 2000; von Schacky, 2000).

N-3 fatty acids have been shown to reduce levels of triglyceride (Roche and Gibney, 2000) and very low density lipoprotein (VLDL) (Harris, 1999) and most, but not all studies report a favourable increase in HDL levels (Harris, 1989). However, some studies report an increase in LDL (Sullivan *et al*, 1990; Gerhard, 1991; Suzukawa *et al*, 1995).

In the present study, erythrocyte EPA did not correlate significantly with LDL or total serum cholesterol. This was also the case for DHA. The absence of correlation may be due to the fact that these fatty acids were present only in small quantities (2-3%), and it has been previously reported that only high doses of fish oils rich in EPA and DHA (resulting in higher levels in tissues) had beneficial effects (Failor *et al*, 1988).

The strongest correlation in the present study (with respect to PUFA and lipoproteins) was between C20:5n-6 and HDL - that being a significantly negative one (r=-0.23, P<0.005). Most studies have proposed the beneficial

effects of n-3 (as above) and possible detrimental effects of n-6 PUFA (Eritsland, 2000; Simopoulos, 1999; present study).

The finding of the present study, as can be judged from the correlations (appendix III) does not strengthen or weaken the hypothesis regarding PUFA and health either way.

	Van- Stavern *et al*, 1986 mean (standard deviation) n=19	Present Study mean (standard deviation) n=110
Total C16:1*t*	0.88 (0.21)	0.65 (0.13)
Total C18:1*t*	3.07 (0.70)	3.06 (0.80)
Total C18:2*t*	0.42 (0.08)	0.51 (0.43)

Table 4.1 Comparison of the individual trans isomers detected in adipose tissue of subjects with that of a previous study.

Study	N	Mean	Standard Deviation	Range
Present Study	***117***	***4.27***	***0.81***	***1.72-6.4****
Israeli	8	4.5	1.5	1.90-6.6
Population[1]				
US Women[2]	118	4.3	1.13	2.32-8.2*
US Data[3]	76	4.14	0.97	0.99-6.2*

Table 4.2 Percentage *trans* fatty acid levels in adipose tissue lipid as reported by various studies, using Gas Chromatography

[1] Enig *et al*, 1984
[2] London *et al*, 1991
[3] Hudgins *et al*, 1991
*Using SP-2560 Fused Silica Capillary Column (100m x 0.25mm i.d.)

Study/Subjects	C16:0	C18:0	C18:1c	C18:2c	C20:4
Present Study (Irish Subjects)	*24.72(4.8)*	*16.28(3.4)*	*12.5(1.8)*	*10.2(2.4)*	*11.6(2.6)*
Italian Population[1]	25.5(4.9)	10.29(3.1)	17.2(3.0)	8.3(1.9)	6.7(2.2)
Mexican Population[2]	17.15(0.6)	29.67(1.3)	13.09(1.7)	0.54(0.3)	18.2(1.2)
English Population[3]	19.28(0.9)	13.41(0.6)	13.59(0.2)	8.85(0.3)	11.16(0.3)
Canadian Population[4]	20.68(0.3)	14.71(0.2)	14.83(0.2)	9.78(0.2)	15.13(0.3)

Table 4.3 Comparison of the average (standard deviation) major fatty acids in the erythrocyte membranes from various studies in different countries

[1] Angelico *et al*, 1980

[2] Cuevas-Covarrabius *et al*, 1993

[3] Lea *et al*, 1982

[4] Manku *et al*, 1983

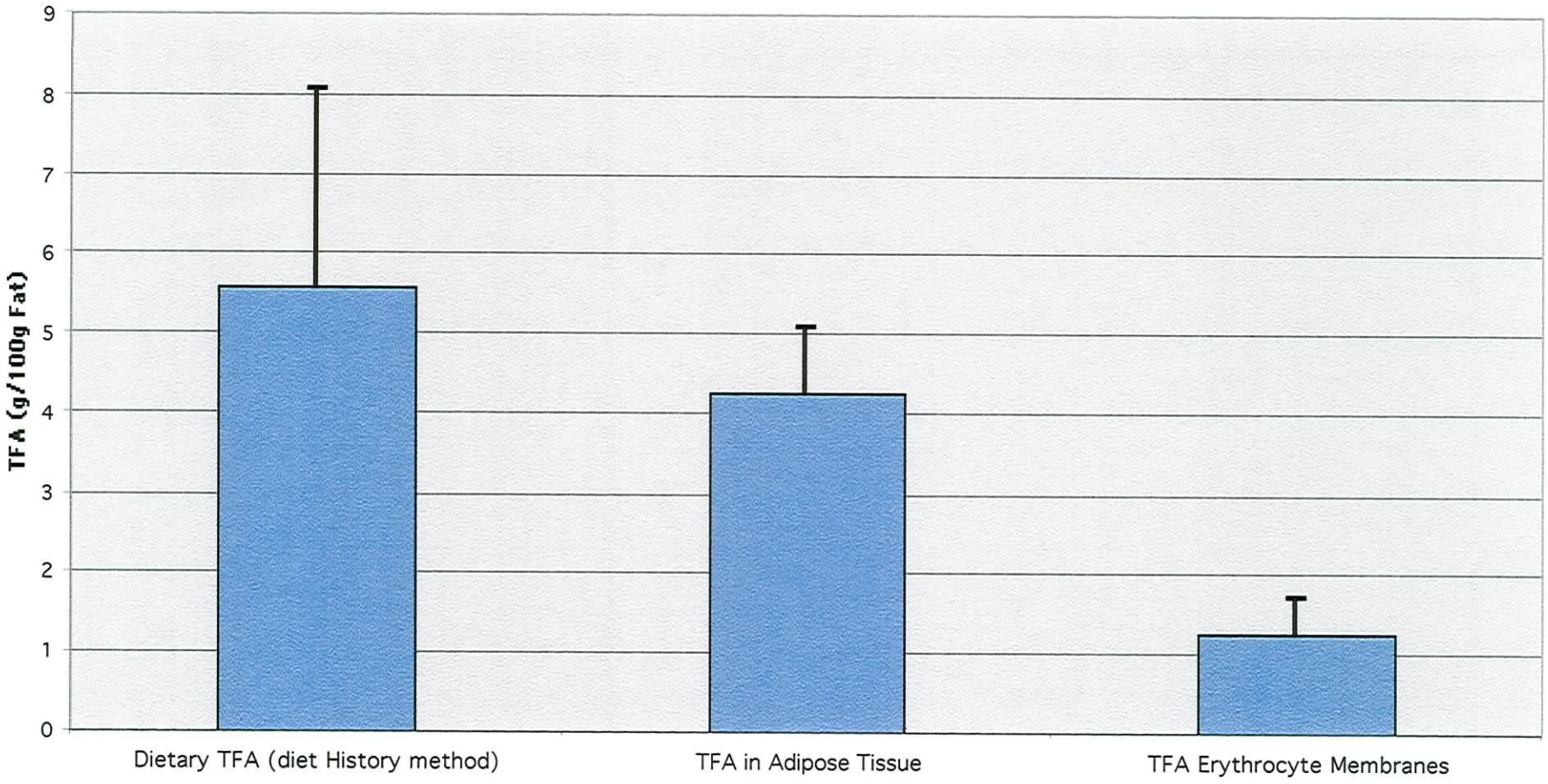


Figure 4.1 The % TFA (g/100g fat) (analysed by gas chromatography) in the various lipids measured in the study.

CHAPTER 5

Conclusions

5.1 TFA levels

From the results in this study one can conclude that the TFA levels in the adipose tissue of healthy Irish adults do not differ significantly from those reported in other studies carried out in other countries using similar methods.

TFA are present in erythrocyte membranes of a population whose adipose tissue contains TFA. The level of adipose tissue TFA reflects the level of TFA in the diet, as assessed by the dietary history method.

The results obtained from the correlations between Dietary History, Fat Intake Questionnaire and adipose tissue analysis indicate that a FIQ is a valid method of estimating the TFA content of diets.

5.2 Incorporation of TFA into body tissues

Further studies investigating the selectivity of incorporation of TFA into body tissues would also be useful in determining the potential risk TFA poses to membranes if present in the diet at high levels. A study of this nature would also prove interesting with respect to neonates - for example the displacement of EFA C18:2c by C18:2t could subsequently interfere with the formation of C20:4 which is present in concentrated levels in the brain and retina of developing neonates (Koletzko, 1992).

'that the amount of trans fatty acid present in a food, including dietary supplements, be included in the amount and percent daily value (%DV) declared for saturated fatty acids'.

The FDA also proposes that wherever saturated fat limits are placed on nutrient claims, health claims, or disclosure and disqualifying levels, that the amount of TFA must also be limited (United states Food and Drug Administration, 1999).

The Institute of Food Science & Technology (IFST) (UK) supports the WHO recommendation that:

'the manufacturers should reduce the levels of TFA arising from hydrogenation'

and while no proposals have been made for the provision of food labelling, the IFST:

'continues to support the need for continuing research in this whole area' (IFST, 1999).

5.4 Further Studies

A most useful study with respect to TFA would be a prospective study in which TFA are fed to subjects for a period of time and then removed

completely from the diet to observe the actual effects on lipoproteins and cholesterol in the blood. A study of this nature however would exclude the use of self-selected diets and would therefore be difficult to perform.

As a final statement, it is fair to say from the findings of this study that the relationship between TFA and CHD is not a simple one, and further investigations are needed to clarify the role of TFA in this important disease.

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Appendices

Chemicals Used

Acetyl Chloride

Amyl Alcohol

Boron Triflouride

Bromochloromethane

Hexane

Hydrochloric acid

Isopropyl Ether

Methanol

Sodium Chloride (anhydrous)

Sodium Methoxide

Sodium Sulphate

Authentic Standards

Cis-6-Octadecenoic methyl ester

Trans-6-Octadecenoic methyl ester

Cis-7-Octadecenoic methyl ester

Trans-7-Octadecenoic methyl ester

Cis-9-Octadecenoic methyl ester

Trans-9-Octadecenoic methyl ester

Cis-11-Octadecenoic methyl ester

Trans-11-Octadecenoic methyl ester

Cis-12-Octadecenoic methyl ester

Trans-12-Octadecenoic methyl ester

Cis-13-Octadecenoic methyl ester

Trans-13-Octadecenoic methyl ester

Linoleic acid methyl ester cis/trans isomers

Linoleic acid methyl ester isomer mix

Appendix I Chemicals and authentic standards used

St James's Hospital and Federated Dublin Voluntary Hospitals
Joint research ethics committee

CONSENT FORM

Title of research study:

An assessment of the current dietary intakes of fatty acids, with particular focus on *trans* fatty acids of Irish adults..

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.
I have read or had read to me, the patient information leaflet and this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement.

PARTICIPANTS NAME:

PATICIPANTS SIGNATURE:

DATE:

Statement of investigator's responsibility:

I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physicians Signature:
Date:

(Keep the original of this form in the patient's medical record, give one copy to the participant, and keep one copy in the investigators records,)

Appendix II Consent Form

Appendix III

Correlations

Abbreviations

BMI	Body Mass Index
DH	Diet History
FIQ	Fat Intake Questionnaire
RBC	Red Blood Cell
AT	Adipose Tissue

Parameters	correlation coefficient *r*	significance level *P*
BMI and LDL	-0.01	0.9
BMI and CHOL	-0.14	0.24
BMI and C16:0	0.07	0.6
BMI and TFA (GC)	0.02	0.85
BMI and CHOL:HDL	0.35	0.000
C14:0 (AT) and LDL	0.11	0.33
C14:0 (AT) and HDL	0.09	0.43
C14:0 (AT) and CHOL	0.14	0.2
C14:0 (RBC) and CHOL	0.19	0.14
C14:0 (RBC) and LDL	0.11	0.4
C14:0 (RBC) and CHOL:HDL	0.3	0.04
C16:0 (AT) and CHOL	0.26	0.04
C16:1*t* (AT) and CHOL	0.25	0.01
C16:1*t* (AT) and LDL	0.33	0.002
C16:1*t* (AT) and HDL	-0.09	0.39
C16:1*t* (AT) and CHOL:HDL	0.27	0.01
C18:0 (AT) and CHOL:HDL	-0.16	0.15
C18:0 (AT) and LDL	-0.09	0.41
C18:0 (AT) and HDL	0.04	0.7
C18:0 (AT) and C18:0 (RBC)	0.03	0.83
C18:1 (AT) and HDL	-0.04	0.71

APPENDIX III CORRELATIONS

C18:1 (RBC) and C18:1*t* (RBC)	0.27	0.05
C18:1*t* (RBC) and LDL	-0.14	0.29
C18:1*t* (RBC) and HDL	-0.2	0.13
C18:1*t* (RBC) and C18:2 (RBC)	-0.06	0.62
C18:1*t* (RBC) and C18:3 (RBC)	0.31	0.01
C18:1*t* (AT) and CHOL	0.06	0.58
C18:1*t* (AT) and C18:1*t* (RBC)	0.32	0.01
C18:1*t* (AT) and C18:2 (RBC)	-0.05	0.7
C18:1*t* (AT) and C20:4 (RBC)	-0.18	0.2
C18:1*t* (AT) and C18:1 (AT)	-0.26	0.02
C18:1*t* (AT) and HDL	-0.1	0.41
C18:2 (AT) and FIQ C18:2	0.51	0.000
C18:2 (AT) and C18:1*t* (AT)	-0.08	0.44
C18:2 (AT) and C16:1*t*	-0.002	0.98
C18:2 (AT) and LDL	-0.13	0.23
C18:2 (AT) and C18:2 RBC	0.04	0.77
C18:2 FIQ and RBC C18:2	-0.01	0.94
C18:2 FIQ and C20:4 RBC	-0.05	0.75
C18:2*t* (RBC) and LDL	-0.16	0.22
C18:2*t* (RBC) and HDL	0.004	0.97
C18:2*t* (RBC) and CHOL:HDL	-0.07	0.57
C18:2*t* (RBC) and HDL:LDL	-0.11	0.41
C18:2*t* (AT) and C18:2 (AT)	-0.004	0.96

APPENDIX III CORRELATIONS

C18:2*t* (AT) and C18:3 (AT)	0.02	0.8
C18:2*t* (RBC) and C18:2 (AT)	0.16	0.24
C18:2*t* (RBC) and C18:3 (AT)	-0.14	0.31
C18:2*t* (RBC) and C18:2 (RBC)	0.4	0.001
C18:2*t* (RBC) and C18:3 (RBC)	0.08	0.6
C18:3 (AT) and C18:3 RBC	-0.07	0.5
C18:3 RBC and HDL	-0.11	0.39
C18:3 (RBC) and C22:6 (RBC)	0.04	0.76
C18:2 (RBC) and C20:5 (RBC)	0.05	0.70
C20:0 (AT) and CHOL	0.13	0.23
C20:0 (AT) and C20:0 RBC	-0.1	0.44
C20:4 RBC and C18:2 RBC	0.56	0.000
C22:1 (AT) and C16:1c (AT)	0.19	0.08
C20:5 (RBC) and C16:1 RBC	0.22	0.1
C20:5 (RBC) and LDL	-0.12	0.34
C20:5 (RBC) and HDL	-0.23	0.000
C22:6 (RBC) and LDL	0.03	0.83
C22:6 and CHOL:HDL	0.05	0.6
C22:6 (RBC) and HDL	-0.12	0.35
CHOL:HDL and C18:1t (AT)	0.08	0.42
DHTFA and C18:1t (RBC)	0.34	0.03
CHOL and C18:0 (RBC)	-0.08	0.47

APPENDIX III CORRELATIONS

DH TFA and GC TFA	0.16	0.18
DH TFA and C18:1t (AT)	0.22	0.06
DH TFA and LDL	0.14	0.23
DH TFA and CHOL:HDL	0.19	0.11
DH TFA and FIQ TFA	0.67	0.000
LDL and C18:1t (AT)	0.13	0.24
LDL and C18:1t (AT) [top third]	0.3	0.13
LDL and C16:1t (AT)	0.33	0.002
LDL and C16:1t (AT) [top third]	-0.09	0.64
LDL:HDL and GC TFA	0.04	0.75
LDL and C18:1t (RBC)	-0.13	0.29
LDL:HDL and C18:3 (RBC)	-0.05	0.6
LDL and GC TFA	0.05	0.65
TFA (GC) and TFA (IR)	0.41	0.000
TFA (IR) and C18:1t	0.65	0.000

APPENDIX III CORRELATIONS

ELIGIBILITY QUESTIONNAIRE

Name : .....

Work Address: .....

Department .....

Please tick the appropriate boxes and fill in the blanks where necessary

	Yes	No
1. Are you aged between 20 and 60 years	☐	☐
2. Are you trying to lose weight at the moment	☐	☐
3. Are you trying to increase your weight at the moment?	☐	☐
4. Are you currently following any special diet?	☐	☐

If yes please give details

_____

_____

5. Do you suffer from any medical condition?	☐	☐

If yes please give details

_____

_____

6. Are you taking any medication at the moment?	☐	☐

If yes please give details

_____

_____

7. If you are female, are you, or do you expect to become pregnant in the next six months?	☐	☐

Please give details of any other information which you may think is relevant

_____

_____

Thank you for your co-operation

Appendix IV Eligibility Questionnaire

Lab 329

Ph: 01 4024656

Fax: 01 4024999

October 1996

Dear

Your identity number is: _____

An appointment has been scheduled for you to attend the medical centre (old vaccination room, on the left as you enter), on _____ at _____.

If this time does not suit you, please contact me at the above number as soon as possible, and another appointment can be arranged for you.

Thank you.

Yours sincerely

Linda Hogan

Appendix V Identity number and appointment letter

A CLINICAL NUTRITION STUDY

IN CONJUNCTION WITH

DUBLIN INSTITUTE OF TECHNOLOGY,

UNIVERSITY COLLEGE DUBLIN

AND

UNIVERSITY OF DUBLIN, TRINITY COLLEGE

- CORONARY HEART DISEASE IS A MAJOR PROBLEM IN IRELAND

- THE AMOUNT AND TYPE OF FAT THE YOU EAT ON A DAILY BASIS HAS BEEN SHOWN TO BE DIRECTLY RELATED TO YOUR CHANCE OF DEVELOPING THIS DISEASE

- WE ARE TRYING TO CREATE A NEW WAY OF LOOKING AT YOUR DAILY EATING HABITS, WHICH WILL ENABLE US TO DETECT QUICKLY AND RELIABLY IF YOU NEED TO CHANGE YOUR EATING HABITS, SO AS TO MINIMISE YOUR RISK OF DEVELOPING HEART DISEASE.

- TO DO THIS, WE NEED **120 HEALTHY VOLUNTEERS** TO PARTICIPATE IN OUR STUDY

- IF YOU DO PARTICPIATE, YOU WILL RECEIVE A FULL DIETARY ASSESSMENT, AND ADVICE FROM QUALKIFIED CLINICAL NUTRITIONISTS/DIETITIANS, BASED ON THE INFORMTION OBTAINED FROM YOU, DURING THE COURSE OF THE STUDY.

THANK YOU FOR YOUR SUPPORT

DATES YET TO BE CONFIRMED

Appendix VI Notice Circulated to Aer Rianta Staff, May 1996

C12:0	Dodecanoic
C14:0	Tetradecanoic acid
C16:0	Hexadecanoic acid
C16:1*t*	*Trans*-Hexadecenoic acid
C16:1*c*	*Trans*-Hexadecenoic acid
C18:0	Octadecanoic acid
C18:1*t*	*Trans*-Octadecaenoic acid
C18:1*c*	*Cis*-Octadecaenoic acid
C18:2*tt*	*Trans*-Octadecadienoic acid
C18:2*cc*	*Cis*-Octadecadienoic acid
C18:3	Octadecatrienoic acid
C20:0	Eicosanoic acid
C22:0	Docosanoic acid
C22:1	Docosaenoic acid
C20:3	Eicosatrienoic acid
C20:4	Eicosatetraenoic
C24:0	Tetracosanoic acid
C24:1	Tetracosenoic acid
C22:4	Docosatetraenoic acid
C20:5	Eicosapentaenoic acid
C22:6	Docosahexaenoic acid

Appendix VII Nomenclature of fatty acids

NUTRITION STUDY 1996/97

In conjunction with

Dublin Institute of Technology,

University College Dublin,

and

University of Dublin, Trinity College,

at

The Medical Centre, Dublin Airport

PARTICIPANT FEEDBACK FORM (BIOCHEMISTRY)

NAME	
SEX	
ID NUMBER	

PARAMETER	NORMAL RANGE	RESULT
TOTAL CHOLESTEROL	**5.2 - 6.0 mmol/l**	
HDL CHOLESTEROL	**>0.9mmol/l**	
LDL CHOLESTEROL	**<3.88mmol/l**	
TRANS FATTY ACIDS	**2.21 - 8.75%**	

COMMENTS

Thank you very much for your invaluable time and patience during the course of this study, we hope that you have enjoyed and benefited from your participation

Appendix VIII (a) Participant biochemistry feedback form September 12 1997

Dear

Enclosed are the results of the analysis carried out on your blood/fat sample.
The parameters included are:

1. **Total Cholesterol**: This measurement is the cholesterol measurement you will be most familiar with. It is the measurement of all the cholesterol types in you blood and the test that would be carried out if you were to have a general 'cholesterol test'. The normal ranges are listed - but please remember that your blood sample was not taken after an over night fats so may be slightly raised.

2. **HDL Cholesterol**: This is the so-called 'good' cholesterol. It is healthy to have a high HDL measurement i.e. >0.9mmol/l

3. **LDL Cholesterol**: This is known as the 'bad' cholesterol. It is, therefore more favourable to have a level close to or lower than 3.88mmol/l

4. **Trans fatty acid %**: This is the percentage of trans fatty acids (TFA) in your fat stores, which reflects the amount of TFA in your diet. It has recently been shown that a high intake of TFSA may lead to an increase in LDL cholesterol. TFA are found in most hard margarines and most confectionery.

The information enclosed should, along with the dietary advice in the package explain in enough detail the results of your participation in the study. If you do have any questions, however please telephone (01) 4024656, and I will be happy to answer any questions, or to return your call regarding same.

Our research so far has been a great success, and could not have proceeded without your participation. I would like to take this opportunity to thank you for your valuable time and patience throughout the course of the study.

Thanking you

Yours sincerely

Linda Hogan

Appendix VIII (b) Participant Feedback detail letter

June 1996

Dear Participant

Thank you for volunteering to take part in our study. The purpose of this letter is to inform you as to what exactly will be expected from you as a participant in the study.

First of all, we would like to assure you that all information obtained from you is particular to this study and will be kept strictly confidential.

On two separate occasions, you will be interviewed by a Clinical Nutritionist and details of your daily eating habits will be recorded.

We will need from you one small sample of blood and one very small sample of fat tissue (about the same size as 1/4 of a matchstick). The fat sample will be collected in the same way as a blood test. All samples will be taken b y a specially trained, State registered Nurse.

At the end of the study, you will receive a report form with details of the results of your diet and samples. If you complete the study, you will be invited to a private consultation with a clinical Nutritionist who will advise you based on your won results.

Please complete the Eligibility Questionnaire attached to this letter, and return it to us using the labelled envelope provided.

When we receive the questionnaire back, we will send you an appointment to attend at The Medical centre. We hope to begin sampling in mid September.

Thank you again for agreeing to participate.

Yours sincerely,

Linda Hogan (BSc) Marie Cantwell (BSc)

Appendix IX Patient Information Letter

Volunteers Required

Approximately 120 healthy volunteers are required to participate in a health promotion study.
Clinical Nutritionist & Dietitian Linda Hogan, Dublin is carrying out this study as part of a Strategic Development Programme run by the DIT in conjunction with TCD and UCD. If selected you will be interviewed regarding your diet by a qualified Nutritionist, and will have both a blood and a fat sample taken (each by syringe needle - with minimal pain), in the Medical Centre, by a qualified nurse.
Ethical approval for the study has been obtained from The Ethics Committee at St James' Hospital. The purpose of this project is to study an individuals diet and compare it to his/her blood fats/tissue fats.
Apart from the obvious scientific benefit accruing to society as a whole from the above study, it carries benefits also to the volunteers, in the form of reassurance if the studies are normal, or in the form of specialised dietary advice if prompted by an individuals results. This project has been approved and supported by the head of the Medical Centre, Dr. Lee and by the company.
If you are interested could you please write to Harry Galvin, Safety & Security Advisor, MD 118 with the following details:

Name, staff no and contact number on or before Thursday 23rd May 1996.

Appendix X Text from Internal Team Aer Lingus Staff Newsletter 17th May, 1996