Vitamin E, Vitamin E/Lipid Ratios And Their Monitoring in Human Disease.

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Abstract

This thesis investigates the role of vitamin E in protection of lipids and phospholipids against free radical damage in three studies on distinct patient groups. Levels of vitamin A, cholesterol, triglyceride and HDL-cholesterol were also assessed.

The first study assesses nutritional aspects of vitamin E levels in hospital in-patients, patients on nutritional supplements and patients from general practices located in areas of differing deprivation categories. Plasma vitamin A levels were assessed to review this vitamin's status as levels below 0.7 μmol/L may indicate inadequacy. It was expected that the vitamin E when corrected for lipid would be lower in the intensive care and high dependency unit patient groups. However this was not case as the vitamin E/cholesterol was highest in patients in the intensive care unit. This observation challenges the convention of correcting vitamin E with cholesterol or cholesterol and triglyceride when assessing depletion in acutely ill patients due to the effects of the acute phase response on the lowering of cholesterol levels. The study also indicates no difference in vitamin E levels between deprivation categories 1 & 2 and categories 6 & 7.

The second study assesses vitamins A and E as well as cholesterol, triglyceride and HDL-cholesterol against cardiac troponin I (a specific marker of myocardial muscle damage). The findings of this study indicate no correlation between the vitamin E/cholesterol ratio or vitamin E and myocardial damage, as assessed by elevated levels of troponin I.

The third study assessed vitamins A and E as well as cholesterol and triglyceride in schizophrenia and tardive dyskinesia. These two groups were studied because lipid peroxidation and therefore vitamin E depletion may be associated with schizophrenia and tardive dyskinesia. In this study the vitamin E/cholesterol ratio showed no difference between the schizophrenic patients

and normal controls. There was also no difference between patients with tardive dyskinesia and normal controls or schizophrenic controls. However in all schizophrenic groups vitamin E alone was significantly lower than the respective control groups but still well within the normal range.

The results from these studies suggest that low levels of plasma vitamin E are a rare finding. The concept of correcting vitamin E with lipid may be appropriate in the normal healthy population, out patients and the general ward population but this correction should not be used when assessing acutely ill inpatients. In these acutely ill patients levels of vitamin E should be assessed with indicators of acute phase response (C-reactive protein), liver function and lipid status.

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Introduction.

The thesis reviews the various processes involved in free radical action in biological process, particularly those associated with oxygen and considers defence mechanisms available as they apply in man. The study focuses on the role of vitamin E as the principle defence against free radical damage of lipids and plasma lipids in the cell membrane structure.

Certain nutritional and disease states can challenge these free radical defences. The studies of patient groups examine how this antioxidant vitamin responds qualitatively to such a challenge.

This thesis is based on three studies on distinct patient groups:

- Nutritional aspects of hospital in-patients, nutritionally supported outpatients and patients from general practices located in areas of differing deprivation categories.
- Patients with chest pain in the intensive care unit.
- Schizophrenic patients with or without Tardive Dyskinaesia

Each study group will be treated separately, having their own introduction including the role of antioxidants and lipid peroxidation relevant to the study.

The following section of the thesis will give a general outline of the mechanisms of free radical production, their affects on the lipid components of biological systems and their control by antioxidants.

1.1 Free Radicals.

Atoms consist of a nucleus (made up of uncharged neutrons and positively charge protons) and negatively charged electrons, which spin around the nucleus in defined shells. In most biological molecules electrons exist in pairs, as this is the stable configuration. Given this requirement for pairing, any situation in which a species is generated with an unpaired electron will result in a potentially reactive entity known as a free radical. They can be highly reactive atoms or molecules containing an odd number of electrons, such that one or more is unpaired [1]. Free radicals can be formed in three ways (Figure 1):

a) When a covalent bond is broken if one electron from each of the shared pair remains with each atom, i.e. homolytic fission occurs.

b) By the loss of a single electron from a normal molecule.

c) The addition of a single electron to a normal molecule.

Radical Formation;

a) By homolytic fission: $X:Y \rightarrow X^{\bullet} + Y^{\bullet}$

b) By electron transfer: $A \rightarrow A^{\bullet+} + e^{-}$

c) By electron transfer: $A + e^{-} \rightarrow A^{\bullet-}$

The unpaired electron and its radical nature are conventionally indicated by a heavy superscript dot.

Figure 1.

Electron transfer by addition is far more common in biological systems than is homolytic fission, the latter requiring high-energy input such as UV light, high temperatures or ionising radiation.

The most reactive free radicals in biological systems are radical derivatives of oxygen. A superoxide free radical anion ('superoxide') will be produced by the reduction of oxygen caused by the transfer of a single electron (Figure 2)

Superoxide Production:

$$O_2 + e^- \rightarrow O_2^{-\bullet}$$

Figure 2.

A two-electron reduction with hydrogen ion would produce hydrogen peroxide (Figure 3).

Hydrogen Peroxide Production:

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

Figure 3.

In biological systems, hydrogen peroxide is often formed when two superoxide molecules react together (Figure 4).

Hydrogen Peroxide Formation Via Superoxide Production:

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

Figure 4.

The reaction shown in figure 4 is known as a dismutation reaction as the radical reactants produce non-radical products. This reaction occurs as a slow spontaneous reaction or can be catalysed by the enzyme superoxide dismutase.[2] Hydrogen peroxide is not a free radical as such but is known as 'a reactive oxygen species' (ROS). This group of ROS includes not only oxygen free radicals but also non-radical oxygen derivatives that are involved in oxygen free radical production.

Hydrogen peroxide has a tendency to break down spontaneously, especially in the presence of transition ions (such as iron or copper). On break down it forms the most reactive and damaging of oxygen free radicals, the hydroxyl radical[3] and because of this it is an important compound in radical biochemistry (Figure 5).

Hydrogen Peroxide Break Down:

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

Figure 5.

The reaction shown in figure 5 is often referred to as the iron catalysed Haber-Weiss reaction. A non-catalysed form of this reaction can occur when the superoxide radical reacts directly with the hydrogen peroxide. However this spontaneous reaction is less likely to occur in biological systems because of the low steady state concentrations of the reactants. The iron-catalysed reaction can still be considered to be dependent on superoxide as both the source of hydrogen peroxide (Figure 4) and as the reductant of the transition metal ions (Figure 6).

Reduction of Transition Metal Ions by Superoxide:

$$O_2^{-\bullet} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$O_2^{-\bullet} + Cu^{2+} \rightarrow Cu^{+} + O_2$$

Figure 6.

The ferrous (Fe^{2+}) and cuprous (Cu^{+}) ions are much more reactive with hydrogen peroxide than their oxidised counterparts. The autoxidation of these reduced transition metals can also generate superoxide (Figure 7).

Autoxidation of Reduced Transition Metals

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-\bullet}$$

Figure 7.

These reversible redox reactions between the transition metal ions and oxygen are extremely important in the promotion of free radical reactions[4].

1.2 Common Sources of Free Radicals

There are a limited number of free radical species that appear to play a central role in biological and pathological processes. These radicals are important because they possess redox potentials such that they aggressively react with other cellular molecules or, as more commonly occurs, they participate in chemical reactions that subsequently yield highly reactive free radical species.

Common sources of biologically important free radicals include:

1: Mitochondrial Electron Transport.

2: Polymorphonuclear leukocytes - NADPH oxidase.

3: Prostaglandin synthesis.

4: Oxyhaemoglobin to methaemoglobin reaction.

5: Xanthine oxidase.

6: Reactions involving transition metals - Haber-Weiss.

7: Reactions of toxins, e.g., paracetamol, carbon tetrachloride, paraquat, ethanol.

8: Radiation injury.

Under normal circumstances, the major source of free radicals in cells is electron 'leakage' from electron transport chains generating superoxide. Other enzymes can also produce superoxide or hydrogen peroxide, such as the range of flavin oxidases located in peroxisomes. Other potential sources of superoxide in animal cells include ascorbic acid (Vitamin C), thiols (e.g. glutathione,

cysteine), adrenaline and flavin co-enzymes. These pro-oxidation reactions can be greatly enhanced by the involvement of transition metal ions such as iron.

The major biological process leading to oxygen derived $O_2^{-\bullet}$ generation is electron transport associated with the mitochondrial and endoplasmic reticulum membranes[5,6,7]. Normally, the reductive conversion of oxygen to H_2O by the oxidase process requires the sequential transfer of electrons, which is accompanied by free radical formation. Mitochondria provide a high rate of $O_2^{-\bullet}$ production when the inner membrane associated respiratory chain carriers are in the reduced state and Adenosine Diphosphate (ADP) is low[8,9]. The current understanding is that the sequence ubiquinone to cytochrome *b* is the most likely site of $O_2^{-\bullet}$ generation[10]. The intramitochondrial steady-state concentration of superoxide has been estimated to be 8×10^{-12} M[10]. Most of the $O_2^{-\bullet}$ produced by mitochondria is converted to H_2O_2 by mitochondrial superoxide dismutase; although some of this H_2O_2 can diffuse into the cytosol. To minimise the amount of H_2O_2 leakage into the surrounding cytoplasm, it would be biologically advantageous for the cell to neutralise $O_2^{-\bullet}$ within the mitochondrial compartment as much as possible by superoxide dismutase.

The microsomes of the endoplasmic reticulum and nuclear membranes also contain electron transport systems and cytochrome *P*-450 and b_5, which may produce free radicals. Cytochrome *P*-450 is the terminal component of electron transport chains found in adrenal mitochondria and liver microsomes. Cytochrome b_5 appears to be a specific electron carrier for the desaturation of fatty acids to cis-unsaturated fatty acids. The role of cytochrome *P*-450 is hydroxylation rather than oxidative phosphorylation. During NADPH oxidation,

liver microsomes generate $O_2^{-\bullet}$ and H_2O_2, both in the absence and presence of mixed function oxidase substrates[11,12,13]. Changes in the isoenzyme status of NADPH-cytochrome *P*-450 reductase may influence the potential to generate free radicals[14].

Numerous catalytic cytosolic enzymes also contribute to $O_2^{-\bullet}$, $OH^{\bullet}$ and particularly H_2O_2 generation. Various oxidases, localised throughout the cells, are present in high concentrations in peroxisomes of the liver[17]. Peroxisomes contain high amounts of catalase, which can neutralise the potentially damaging effects of H_2O_2. The action of catalase is fundamentally the same as that employed by peroxidase (although 10^4 times faster than peroxidase) in that it catalyses the breakdown of hydrogen peroxide to oxygen and water. This will be returned to later.

Other major non-membrane related sites of free radical generation are found in cells engaged in phagocytic activity. A respiratory burst during phagocytic activity generates reactive oxygen radicals essential for the host's defence against invading microbes. During phagocytosis, a membrane-bound oxidase is activated in these cells, leading to an increased O_2 uptake and production of $O_2^{-\bullet}$ and H_2O_2. It is generally believed that the activated phagocytic cells generate substantial amounts of H_2O_2 and that this species is mainly responsible for the cytotoxic potential seen in localised tissue inflammation[16,17]. In addition to H_2O_2 generation $OH^{\bullet}$ may also be generated during phagocytosis. Hydroxyl radical generation could arise from the Haber-Weiss reaction or the iron catalysing Fenton reaction in which H_2O_2 is reduced to $OH^{\bullet}$ by Fe^{2+}.

Xanthine oxidase is another well-known source of $O_2^{-\bullet}$ generation as indicated by its ability to reduce cytochrome *c*[18,19]. The wide distribution of this

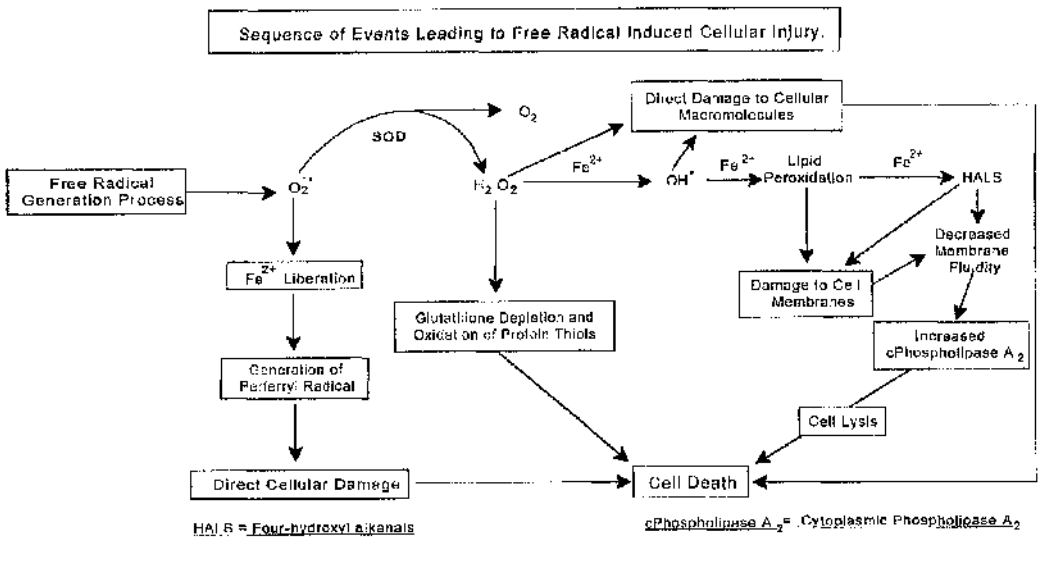
enzyme suggests a potentially important role in pathogenic conditions such as ischaemic injury in a wide variety of organs including myocardium and the intestinal mucosa[20]. Under ischaemic conditions cellular ATP levels decrease, leading to a rise in cellular calcium with activation of a protease responsible for the conversion xanthine dehydrogenase to xanthine oxidase. Upon reperfusion and reoxygenation, xanthine oxidase uses oxygen to metabolise hypoxanthine to uric acid with the production of $O_2^{-\bullet}$ [21].

1.2.1 Superoxide Radical

Superoxide is commonly formed from oxygen and a redox cycling molecule (Figure 9). It is a weak oxidant[22] and a potent reductant[23] with a half-life in the millisecond range[24]. It is unlikely to cause significant cellular injury on its own, as it is a weak oxidant. Once formed the superoxide radical may have one of several fates. It may react with nucleophilic thiol groups[23,25] and may thus either predispose the cell to further oxidative stress by depleting glutathione (GSH), or react with thiol groups on enzymes or other cellular proteins and inactivate them. Given the short-life of superoxide, it will react only with thiols in the immediate vicinity of its formation.

Superoxide can, however, initiate a cascade of events that can ultimately cause significant oxidative damage. A critical component of this cascade can be seen in figure 5, this interaction of the superoxide molecule with hydrogen peroxide in the presence of ferrous (Fe^{2+}) ions produce the highly reactive hydroxyl radical ($OH^{\bullet}$). The $OH^{\bullet}$ radical thus generated can result in significant cellular damage[23,26,27]. Superoxide can mobilise iron from intracellular ferritin stores[28,29]. Ferritin contains ferric iron[30] that can be reduced

by superoxide to the ferrous form and thus liberated to participate in free radical generating reactions or a series of other important events in free radical induced cellular injury. In addition, some groups[31,32] consider that superoxide can react with ferric iron to form the oxidising perferryl radical (Figure 8).



Adapted from J. A. Brent et al : Clinical Toxicology Vol. 31 (1), 139 - 171, 1993.

Figure 8.

This ferrous-oxygen species may initiate lipid peroxidation. The perferryl ion, which is an intermediate species formed during $O_2^{-\bullet}$ reduction of Fe (III), may be formed only if the iron ions are complexed by a chelator (e.g. ADP, ATP). In this case superoxide would be involved in the reduction of Fe^{3+} to Fe^{2+}. Other authors[33] argue that the perferryl ions are unreactive. The exact mechanism of the reaction of a ferrous chelate-oxygen complex with unsaturated fatty acids is unclear. However, most superoxide does not participate in the above reactions for several reasons. Superoxide has a very limited lifespan, forming hydrogen

peroxide and oxygen by a dismutation reaction (Figure 4). Although this last reaction occurs spontaneously, as has been noted it is further promoted by the enzyme superoxide dismutase (SOD)[33].

The superoxide radical is capable of limited cellular damage itself but may initiate a sequence of events (Figures 8 & 9) leading to considerable oxidative injury.

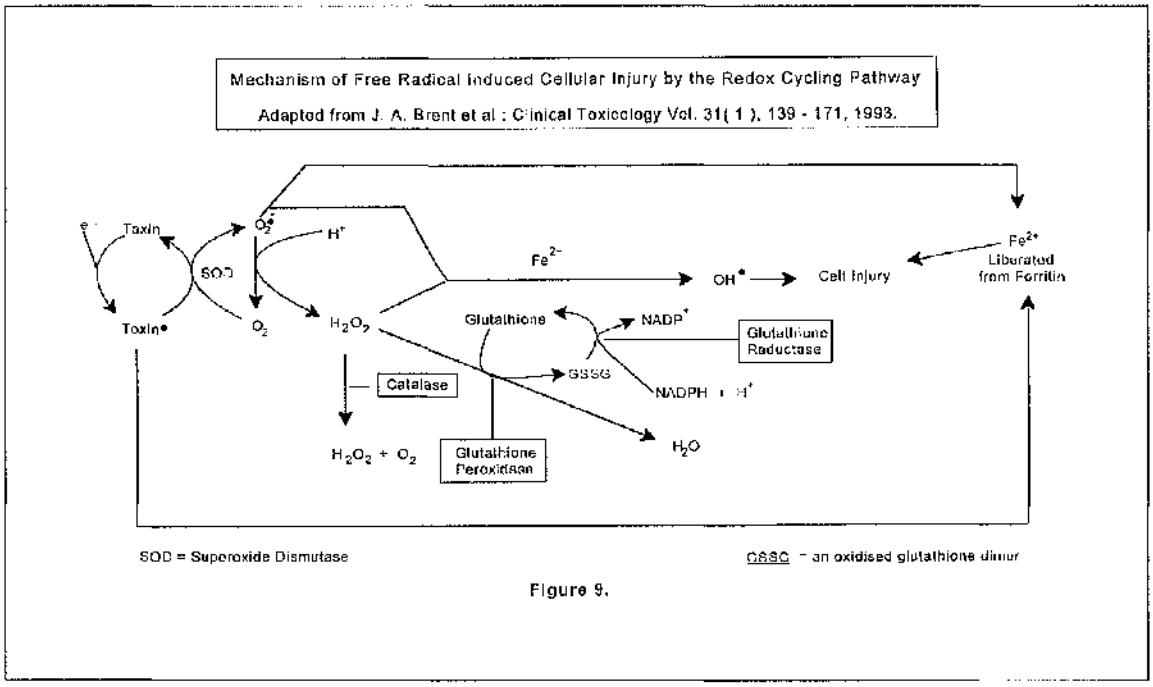


Figure 9.

Endogenous antioxidant cellular defences, coupled with spontaneous dismutation of superoxide, limit its effect. However, under conditions of excessive superoxide formation or GSH depletion, superoxide can be predicted to have a more pronounced effect.

1.2.2 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a potentially strong oxidising agent. It is formed, as previously described in figure 4, by the dismutation of superoxide. Once formed, hydrogen peroxide may either cause oxidative damage directly or, like superoxide, initiate a sequence (Figures 8 & 9) that generates highly reactive free radicals. The direct damage caused by hydrogen peroxide relates to its ability to oxidise protein thiols[23] and deoxyribonucleic acid (DNA), causing strand breakage[33,34]. These effects are attenuated by the tendency for hydrogen peroxide to react slowly[23]. More important is the role of hydrogen peroxide as a substrate for the Haber-Weiss reaction (Figure 5). This reaction forms the powerful hydroxyl radical. The principle effect of hydrogen peroxide, like superoxide, is its participation in a cascade leading to oxidative damage (Figure 9).

The potential for hydrogen peroxide induced oxidative damage is moderated by two major enzyme defences, the action of catalase[35] (Figure 10) and glutathione peroxidase[36] (Figure 11).

Action of Catalase

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Figure 10

Action of Glutathione Peroxidase

$$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$$

GSSG = an oxidised glutathione dimer.

Figure 11

These two enzymes have the capacity to convert hydrogen peroxide to unreactive molecules.

1.2.3 Hydroxyl Radical

The hydroxyl radical ($OH^{\bullet}$) is the most potent free radical found in biological systems[23]. It is indirectly formed from the generation of superoxide and hydrogen peroxide, see section 1.2.2. The hydroxyl radical can react with virtually any cellular molecule, and its effects on lipids[23,27,37,38], proteins[39], and nucleic acids[40] have all been implicated as its sites of action. Its action on the fatty acid component of membrane phospholipids have been suggested as essential events in cellular oxidative damage[23,27,37,41]. As shown simplistically in figure 12, the hydroxyl radical can abstract a methylene hydrogen from a fatty acid(LH) forming a lipid radical ($L^{\bullet}$)[42].

Lipid Radical Formation

$$OH^{\bullet} + LH \rightarrow H_2O + L^{\bullet}$$

Figure 12.

This lipid radical can react with adjacent lipids, creating further radicals, and causing a self-perpetuating sequence known as lipid peroxidation (LPO)[42]. This will be discussed more fully in the section 1.3 on lipid peroxidation. As most free radical scavenging molecules are found in the aqueous phase, with tocopherols (principally α - tocopherol)[43] a notable exception, lipid peroxidation is relatively remote to these defences.

The highly nucleophilic hydroxyl radical can react with thiols and thus damage membrane proteins[39]. It can also react with both the deoxyribose backbone and bases of DNA causing damage to the genome[40].

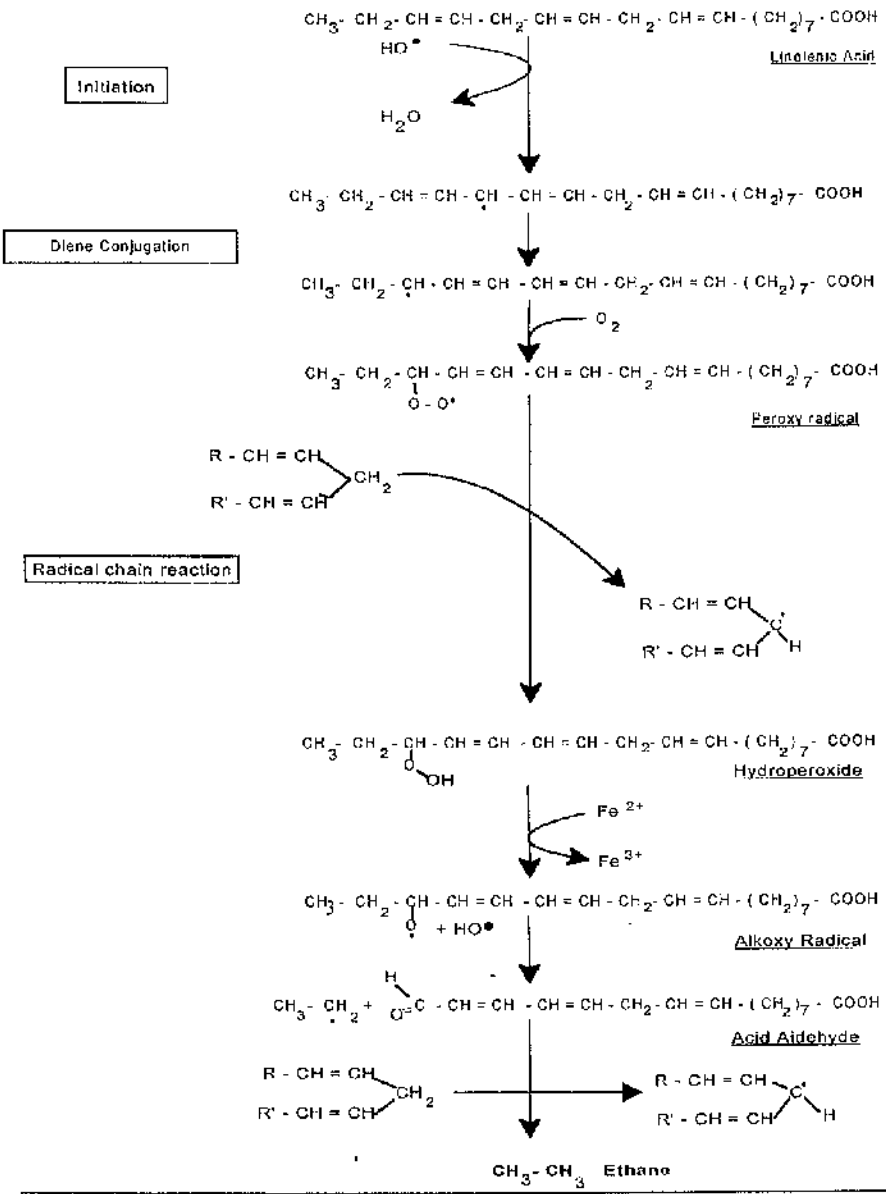
This type of damage could be catastrophic for the cell and because of this animals have evolved enzymatic and non-enzymatic antioxidant defences to deal with the inevitable low-level production of free radicals during normal metabolism.

1.3 Damage caused by free radicals: Lipid Peroxidation

Polyunsaturated lipids are integral to the entire supporting system of cells, including cell membranes, endoplasmic reticulum and mitochondria. Disruption of their structural integrity has dire consequences for cellular function.
The oxidative destruction of polyunsaturated fatty acids (PUFA) is particularly damaging because it proceeds as a self-perpetuating chain-reaction[42,44].

It is generally accepted that the lipid peroxidation reaction process starts after hydrogen abstraction from an unsaturated fatty acid. An example the peroxidation process is given in figure 13. The lipid radical formed reacts with molecular oxygen, if present, to form a peroxy radical. With this step radical chain-reactions can occur[45,46,47,48]. The reaction products are monohydroperoxides of lipids. These will break down spontaneously or with metal catalysts initiating further chain-reactions. The alkoxy radicals formed can undergo cleavage of the C - C bonds. The example given in figure 13 shows the simplest scheme for lipid peroxidation reactions. It demonstrates that the cleavage of the C - C bonds results in the formation of unsaturated fatty acid aldehydes and alkyl radicals, which in turn initiate new radical chain reactions. The formation of the alkanes ethane, *n*-pentane and others during lipid peroxidation occurs by the mechanisms shown in figure 13[49].

Figure 13



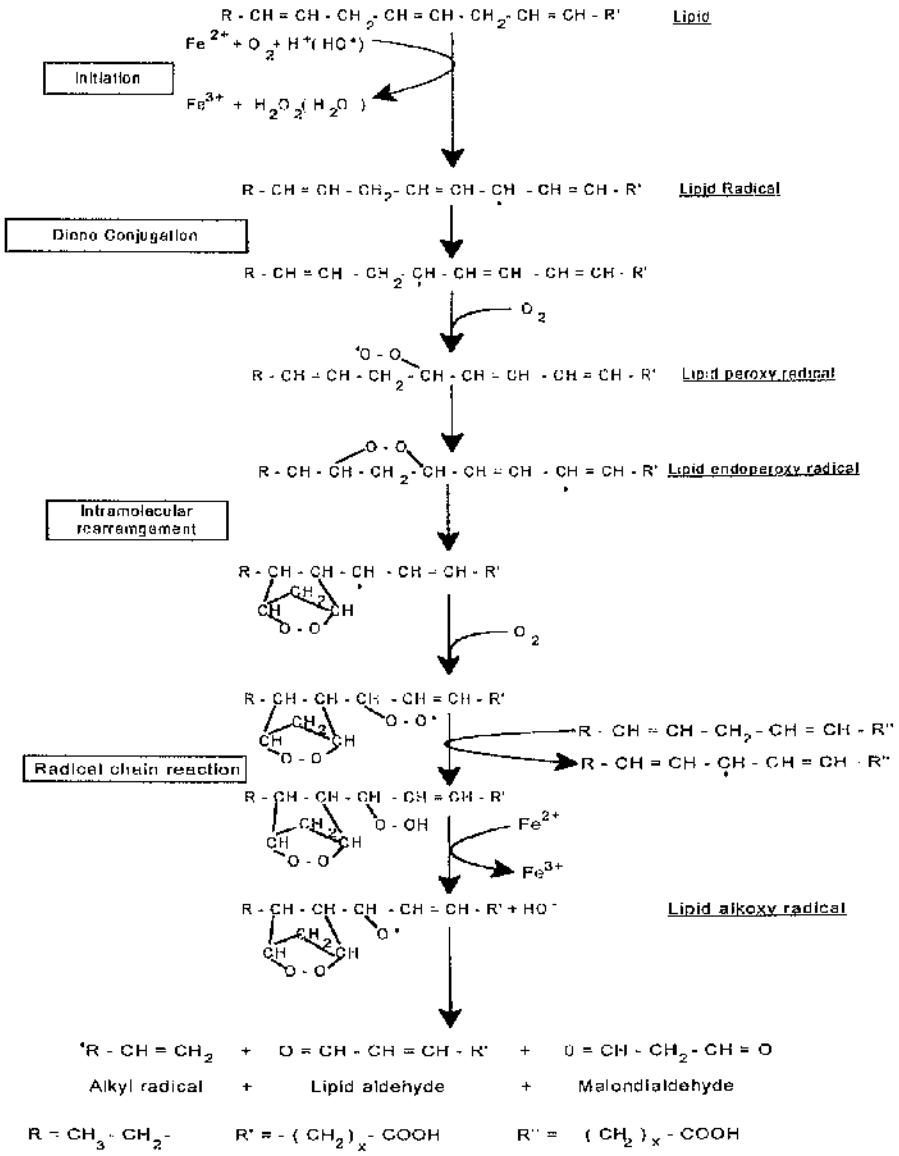
Scheme representing the formation of ethane during lipid peroxidation induced by hydroxyl radicals.

Adapted from H. Kappus: In Oxidative Stress.
Ed. H. Sies: Academic Press, 273 - 309, 1985

The ω3 ($18:3^{9,12,15}$) unsaturated fatty acid (Linolenic acid, 9,12,15 - Octadecatrienoic acid) yield ethane, and the ω6 ($18:2^{9,12}$) unsaturated fatty acid (Linoleic acid, 9,12 - Octadecadienoic acid) yield *n*-pentane. That transition metal ions are involved in lipid hydroperoxide decomposition has been proved *in vitro* experiments[50,51,52,53]. At least two isolated double bonds are needed to form monohydroperoxides of fatty acids to form alkanes. Alkenes can also be formed.

The quantity of monohydroperoxides of lipids formed in biological systems is relatively low, as shown by studies measuring ethane and *n*-pentane. The major part of the lipid peroxidation process in biological systems seems to involve very complicated reaction sequences. One of these is presented in figure 14, although hypothetical[54,55] it is based on reaction products formed. The formation of endoperoxides during lipid peroxidation has long been established. If this endoperoxy radical undergoes intramolecular rearrangement followed by a reaction with oxygen and in the presence of Fe^{2+} a lipid alkoxy radical is formed. Malondialdehyde is an important reaction product of the lipid alkoxy radical and is measured in biological systems that undergo lipid peroxidation. There are some indications that the decomposition of the cyclic lipid endoperoxy-hydroperoxide is catalysed by metal ions[54,56]. The breakdown of cyclic endoperoxy-hydroperoxides would also yield unsaturated lipid aldehydes and alkenyl or alkyl radicals that would also initiate radical chain reactions.

Figure 14



Scheme representing the formation of malondialdehyde during lipid peroxidation induced by ferrous - oxygen complexes or by hydroxyl radicals

Adapted from H. Kappus: In Oxidative Stress.
Ed. H. Sies: Academic Press, 273 - 309, 1985

Reactions schemes as shown in figures 13 and 14 are only parts of very complex reaction sequences. Many more products have been detected from autoxidation experiments with PUFA or phospholipids. These additional compounds, such as hydroxy and epoxy derivatives, ketones, polyhydroperoxides, dimers and polymers of fatty acids, are formed during different radical chain reactions[45,57], but they are all derived from hydroperoxides or endoperoxides. In biological systems only a few of these lipid peroxidation products have been identified e.g. malondialdeyde, 4-hydroxynonenal and the alkanes ethane and *n*-pentane.

The most important events in lipid peroxidation are radical chain reactions. If they are not terminated by the reaction of two radicals they proceed concurrently, destroying all lipid phases, especially lipid membranes that contain many essential unsaturated fatty acids. In addition lipid peroxidation generates diffusible toxic second messengers (Figure 8) such as 4-hydroxyl alkanals (HALS)[58,59,60,61]. An example of a HAL is 4 - hydroxynonenal that is a major by-product of 1,6 - arachidonic acid peroxidation. These molecules have been demonstrated to inhibit enzymes (i.e. glucose-6-phosphatase[62]), increase the permeability of hepatocyte membranes[63], kill hepatocytes[58,59,63], lyse erythrocytes[64], inhibit protein synthesis[65,66], block DNA replication[67], inhibit mitochondrial respiration[68], and decrease membrane fluidity[59]. The latter effect has been implicated in the activation of phospholipase A_2 which catalyses the formation of lysophosphatides[59] and has other important biological effects which will be discussed later. Doubts have been raised about the *in vivo* relevance of HALS[69] as most experimental results are based on levels 100 times higher than would be found physiologically. However it has been postulated that intracellular

HALS produced during hepatocyte lipid peroxidation may have higher concentrations in the microsomes where they were generated, and therefore may be damaging to molecules at that site[59]. Thus the precise role of HALS in the free radical cascade is yet to be fully determined.

1.4 Common Pathway of Cell Death.

It is clear that there are many aspects of cellular function affected by free radical generating systems. As shown in figure 8, as these effects progress and amplify a great number of cellular functions are compromised. Several aspects of cellular function have been singled out as the potential driver of cell death. Prominent among these are alterations in intracellular calcium homeostasis[59,70] and cell lysis induced by cytoplasmic phospholipase A_2[71,72]. Since all these process occur simultaneously in the cascade of toxic cellular injury, it is difficult to isolate a single factor as the specific mediator of cytotoxicity.

Cells normally maintain a large calcium gradient with the extracellular fluid that contains 10^3 to 10^4 times as much of this cation as the cytosol. One response to xenobiotic cellular damage is inhibition of thio-dependent calcium dependent homeostatic enzymes[59,70,71]. Although several free radical-generating xenobiotics cause a loss of calcium homeostasis, this has been best studied following carbon tetrachloride poisoning, in which an increase in cytosolytic calcium occurs within and hour of poisoning[73,74,75]. This calcium influx appears to be more than a non-specific effect on enzymatic mechanisms because inhibition of sodium/potassium ATPase occurs hours later[76,77]. At such time there is a general compromise of cell function, membrane permeability non-specifically increases and there is a large secondary calcium influx[76,78] that

marks a pre-terminal event for the cell[78]. However calcium influx has been questioned as the cause of cell death as a 8-fold increase in cytosolic calcium with ionophores does not seem to adversely affect the cell[79].

The enzyme phospholipase A_2 (Type IVA)[80] has been suggested as an important component of free radical effects, and there is good evidence to support this idea[59,72]. Lipid peroxidation[59], HALS[81, 82, 83], and an increase in intracellular calcium have all been demonstrated to decrease membrane fluidity and increase membrane permeability which stimulates phospholipase A_2. In addition, a rise in intracellular calcium directly activates phospholipase A_2[72,83,84]. This enzyme catalyses the release of arachidonate and to a lesser extent eicosapentanoic fatty acids from the lipid membrane and forms of lysophosphatides, which can result in cellular lysis[59,72]. In addition arachidonate is the source of prostaglandins and biologically active eicosanoids. Whether there is a single or multiple mediators leading to cellular death remains to be elucidated.

1.5 Primary defence systems against oxidative threat.

Living in an oxidative environment, aerobic organisms have evolved a series of endogenous free radical protecting, or scavenging, systems. Antioxidants are the primary defence mechanisms against oxidative threat. As shown in Table 1, such a system encompasses many substances[85,86] that are often called by such generic names as antioxidants, free radical scavengers, chain terminators or reductants. The antioxidant systems responsible for cellular protection against oxidative stress are as diverse as the free radicals themselves. To provide maximum protection, cells contain a variety of substances capable of scavenging many different species of free radicals, including lipid peroxides and organic

carbon-centred free radicals (Table 1). These scavengers are strategically compartmentalised in subcellular organelles within the cell to provide maximum protection. For instance, superoxide dismutase (SOD), catalase (CT) and glutathione peroxidase (GSH-PX) are not only distributed in the cytosol but are also localised in mitochondria, where most of the intracellular free radicals are produced[87,88]. In the red cell as much as 10% of its energy supply is routed through the hexose monophosphate shunt and provide a source of reductants through NADPH. Although considerable progress has been made in identifying and understanding the mode of action of individual enzymes and antioxidant defence components, the complexity of the intracellular network of various antioxidants has impeded understanding of the overall protective efficacy of the cytosolic defence system.

In addition to the integration of intracellular cytosolic defences, the co-operative interaction between the various antioxidants in extracellular fluids including plasma is crucial for maximum suppression of free radical reactions in these compartments.

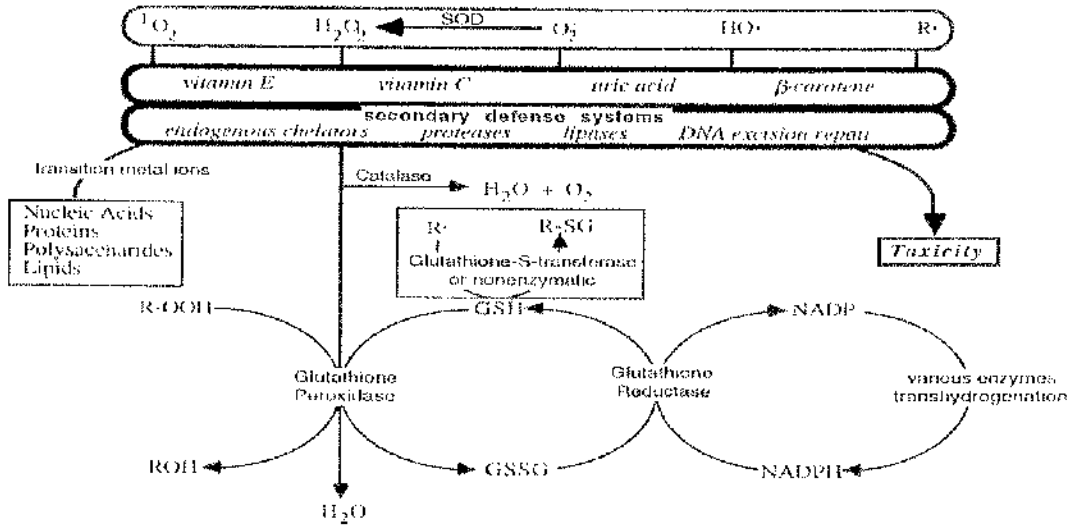
Category	Structure	Tissue Site	Actions
Enzyme Systems			
Superoxide dismutase	Cu/Zn SOD Mn SOD Cu SOD	Primarily cytosol, also nucleus. Primarily mitochondria. Primarily plasma	Catalyse dismutation of O_2^- to H_2O_2.
Catalase	Terameric hemoprotein	Peroxisomes	Catalyses dismutation of H_2O_2, reduces methyl and ethyl hydroperoxides.
GSH redox cycle			
GSH peroxidase	Selenoprotein	Primarily cytosol, also mitochondria.	Catalyses reduction of H_2O_2 and other hydro- peroxides (lipid peroxides, lipoxygenase products).
GSH reductase	Dimeric protein	Primarily cytosol, also mitochondria.	Catalyses reduction of low molecular weight disulphide s.
Fat-soluble Compounds			
Vitamin E	Fat- soluble Vitamin	Lipid membranes, extracellular fluids (including alveolar)	Converts O_2^-, OH, and lipid peroxyl radicals to less reactive forms. Breaks lipid chain reactions.
β-Carotene	Metabolic precursor to Vitamin A.	Membranes of tissues	Scavenges O_2^-, reacts directly with peroxyl radicals
Bilirubin	Product of hemo-protein catabolism.	Blood stream, tissue.	Chain breaking antioxidant. Reacts with ROO..
Water-soluble Compounds			
Vitamin C	Water-soluble Vitamin	Wide distribution in intra- and extra-cellular fluids.	Directly scavenges O_2^- and OH. Neutralises oxidants from stimulated neutrophils. Contributes to regeneration of vitamin E *(in vitro)*.
Uric Acid	Oxidised purine base	Wide distribution.	Scavenges O_2^-, OH, and oxohaem oxidants, and peroxyl radicals. Prevents oxidation of vitamin C.
Cysteine	Amino acid.	Wide distribution.	Reduces various organic compounds by donating electrons from sulphydryl groups.
GSH	Tripeptide.	Largely intracellular, also alveolus.	In addition to role as substrate in GSH redox cycle reacts directly with O_2^-, OH and organic free radicals.

Table 1

Major Antioxidant Scavenging Components

1.5.1 Classification of Antioxidants

The antioxidant defence systems traditionally have been termed "primary" or "secondary" to designate the scavenging action of antioxidants. Primary defences interact with free radicals generated directly from O_2 (namely $O_2^{-\bullet}$): secondary defences scavenge radicals arising from dismutation of $O_2^{-\bullet}$ [89] (Figure 15).



Biological antioxidant defence systems. All aerobic cells contain a spectrum of chemical and enzymatic antioxidants. These work in concert to minimise the initiation and propagation of undesirable oxidative reactions within cells (primary defence) or to repair damage (secondary defence). Adapted from: Natural Antioxidants in Human Health and Disease (1994) Ed., Balz Frei. Academic Press.

Figure 15

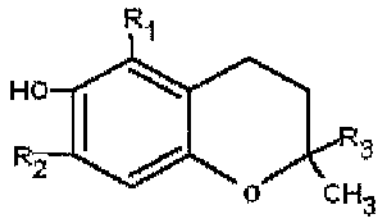
Some investigators more broadly classify the various chemical antioxidants themselves as the primary defence component and scavenging enzymes as the

secondary defence component.

As the knowledge and understanding of free radical biochemistry increases, the scope of the term defence system has also broadened to encompass several major enzymes involved in the removal or repair of damaged cell constituents[90,91,92,93,94]. Davies[89] has proposed the following as a comprehensive classification of antioxidant defence systems. Primary defences include antioxidant compounds such as vitamins A, C, and E, glutathione, and uric acid. Also included under the primary defence classification would be the antioxidant scavenging enzymes such as SOD, CT and peroxidases. For secondary defences, he suggests lipolytic enzymes, phospholipases, proteolytic enzymes, proteases, peptidases, DNA repair enzymes, endonuclease, exonuclease and ligase.

1.5.2 Vitamin E.

Vitamin E is the most widely distributed antioxidant in the plant and animal kingdoms. Vitamin E is a generic term for a group of tocol and tocotrienol derivatives that contribute to some degree to vitamin E activity. It is known to be a mixture of 4 tocopherols (alpha, beta, delta and gamma (Figure 16)).



Vitamin E

	R_1	R_2	R_3
α-Tocopherol	CH_3	CH_3	$C_{16}H_{33}$
β-Tocopherol	CH_3	H	$C_{16}H_{33}$
γ-Tocopherol	H	CH_3	$C_{16}H_{33}$
δ-Tocopherol	H	H	$C_{16}H_{33}$

Figure 16: Structure of Tocopherols

α-Tocopherol is the major form of vitamin E in human plasma and tissues [95] and is the most potent of the four naturally occurring tocopherols [96].

Vitamin E is found in the diet primarily in vegetable oils and also in cereal grains, green plants, egg yolk, milk fat, liver, nuts and vegetables. Based on studies of induced vitamin E deficiency in humans and assessing levels of vitamin E required to prevent hydrogen peroxide induced haemolysis, the new United States Recommended Daily Allowance (RDA) for men and women was set at 15 mg/day[97]. There is no United Kingdom Reference Nutrient Intake (RNI) although it has been suggested that an intake of 5 mg/day and 7 mg/day is appropriate for adult women and men respectively[98].

The absorption of vitamin E is incomplete, varying between 21% and 74%, as dietary factors influence emulsification and solubility in mixed bile salt micelles and thus the uptake of tocopherols by the small intestine. Although plasma carrier proteins have been described for other fat-soluble vitamins, such as the retinol-binding protein [99] no plasma carrier proteins have been discovered for vitamin E. Although vitamin E is present in all of the lipoprotein fractions, it is the low and high density lipoproteins (LDL and HDL) that are the major vehicles for transport in human plasma [100,101,102,103]. Studies have documented that vitamin E can exchange between lipoproteins and red blood cells [104], and between plasma and

red blood cells [105]. α-Tocopherol, which exchanges more slowly than cholesterol but much more rapidly than triglyceride, does not require a transfer protein for exchange to occur [106]. One route by which at least some tissues probably receive significant amounts of vitamin E is via the LDL receptor mechanism. This mechanism would simultaneously deliver to cells peroxidizable lipids, such as linoleic acid and the antioxidant, vitamin E. Tissues with relatively high levels of LDL receptors, such as the adrenal gland, may obtain most of their α-tocopherol in this way. Another mechanism available for vitamin E uptake involves lipoprotein lipase, which is present in the circulation on the endothelial lining of capillary walls and hydrolyses triglycerides in chylomicrons and VLDL to free fatty acids and monoglycerides [107]. In vitro, this enzyme functions as a transfer protein for vitamin E during lipolysis of triglyceride-rich lipoproteins and emulsions [108]. It is likely that tissues with high levels of lipoprotein lipase activity, such as adipose tissue and muscle, might transfer significant amounts of vitamin E by this mechanism. The brain also has been reported to have lipoprotein lipase activity [109], which might serve to mediate some vitamin E transfer into this organ. α-Tocopherol enrichment of plasma and tissues is mediated by the α-tocopherol transfer protein (α-TTP), a cytosolic lipid-transfer protein expressed in the liver [110,111,112,113]. Although the mechanism is unknown[114], α-TTP is believed to selectively transfer α-tocopherol from lipoproteins taken up by hepatocytes via the endocytic pathway to newly secreted lipoproteins, which facilitate its delivery to peripheral tissues[111]. Humans with α-TTP gene defects have extremely low plasma α-tocopherol concentrations and develop severe neurodegenerative disease unless they are treated with high doses of vitamin E[115,116,117] . This protein specifically transfers RRR-α-tocopherol. As vitamin E is transported in plasma within lipoproteins its plasma concentration varies with the total lipid (cholesterol, triglyceride and phospholipid) [118]. α-tocopherol being extremely lipophilic is therefore found predominantly in plasma lipoproteins and membrane fractions where it is a potent chain-breaking antioxidant (Table 1).

Vitamin E scavenges the chain-carrying peroxyl radicals rapidly and interrupts chain propagation. For this reason it is the major lipid-phase antioxidant[119,120]. α-tocopherol has been demonstrated to inhibit hepatocyte lipid peroxidation caused by ethanol[96,121], carbon tetrachloride[122,123,124], and diaquat[96]. When microsomes are challenged by one of these xenobiotics there tends to be a lag phase before initiation of lipid peroxidation. This time period corresponds to the time required for the depletion of microsomal α-tocopherol[125]. α-tocopherol (α-TocH) was previously described as a chain-breaking antioxidant because it reacts with lipid peroxyl radicals (Figure 17).

$$LOO^{\bullet} + \alpha\text{-TocH} \rightarrow \alpha\text{-TocH}^{\bullet} + LOOH$$

Figure 17.

The reaction shown above interferes with the chain-propagating step of lipid peroxidation. One molecule of vitamin E scavenges two molecules of peroxyl radicals as follows:

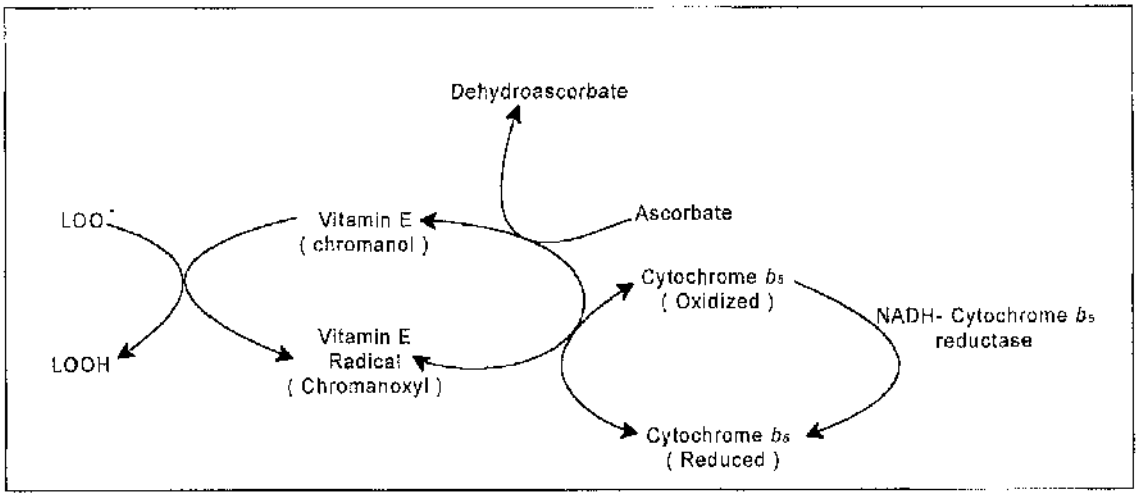
- It first scavenges a peroxyl radical and donates a hydrogen atom to yield lipid hydroperoxide and vitamin E radical.
- Vitamin E radical does not propagate the chain but reacts with another peroxyl radical rapidly to give a stable adduct.
- This adduct may further react with peroxyl radical to give the epoxide and alkoxyl radical.

Alpha- tocopherol is also known to react with lipid radicals in an analogous fashion[26]. Once formed, the alpha-tocopherol radical may be reduced by GSH[126] (Figure 18).

$$2\ \alpha\text{-TocH}^{\bullet} + 2\ GSH \rightarrow 2\ \alpha\text{-TocH} + GS\text{-}SG$$

Figure 18

It has been found, in erythrocyte membranes, that vitamin E can be protected by either NADH-cytochrome b_5 - dependent enzymatic recycling or by a nonenzymic pathway involving ascorbate (Figure 19) [127].



Scheme showing the possible enzymic and nonenzymatic pathways of vitamin E recycling in human erythrocyte membranes. Adapted from Constantinescu et al [127].

Figure 19

The level of vitamin E in the human erythrocyte membrane is relatively low compared with plasma levels, the ratio of these two amounts being approximately 1:3 [128]. Nevertheless, normal erythrocytes from healthy subjects

do not contain any significant amounts of lipid peroxides when freshly isolated [129,130]. This observation could have two possible explanations:

1) oxidised vitamin E is constantly replaced by fresh (reduced) vitamin E from the plasma lipoprotein pool, or

2) oxidised vitamin E is restored to its reduced form in situ by enzymatic and/ or nonenzymatic processes.

The first possibility is still a subject of debate, since neither the diffusion of reduced vitamin E from plasma to membranes occurs rapidly enough to replenish the oxidised molecules and no direct experimental evidences exists to support the hypothesis of a specialised carrier, which replaces oxidised with reduced vitamin E. As for the second possibility, it is now generally accepted that vitamin E radicals generated in various types of membranes can be reduced by compounds naturally present in biological systems. These compounds can be either reducing agents or substrates for redox enzymes contained in the membranes [131,132,133,134].

Among the reducing agents is vitamin C, which functions as a water-soluble chain-breaking antioxidant and acts synergistically with vitamin E to protect both endo- and plasma membranes from oxidative damage. Vitamin C regenerates vitamin E by directly reducing tocopheroxyl radicals generated when vitamin E scavenges reactive free radicals [135]. This interaction between the radicals of these two vitamins can take place in the erythrocyte membrane too, where vitamins C and E reside separately outside and within the membrane, respectively [136,137]. It should be noted that the sparing action of vitamin C for vitamin E does not always indicate a synergistic effect by both vitamins. When radicals are generated initially in the aqueous region and attack

membranes from outside, vitamin C and other water-soluble antioxidants can scavenge the radicals in the aqueous phase before the radicals attack the membranes, hence vitamin E in the membranes may not be consumed initially. Vitamin E traps radicals after most of the antioxidants in the aqueous phase are used up and the radicals reach the membranes. In this case, the inhibitory effect is not synergistic but additive. On the other hand synergistic inhibition takes place when radicals reaching the membrane lipids or radical generation within the membranes leads to the production of the vitamin E radical which is reduced by vitamin C to regenerate vitamin E. There is doubt as to whether this reaction involving vitamin C occurs in humans, at the membrane aqueous interface, *in vivo*[138] as it has not been unequivocally proven. However there are a few publications, which imply that this interaction takes place in biological systems[139, 140]. Enzymatic recycling of vitamin E has been demonstrated in many types of biological systems, including mitochondria[132,141], rat liver microsomes[131,132,142], and human platelets[143]. No data are available, however, for enzymatic vitamin E recycling in human erythrocyte membranes.

In the endomembrane of microsomes and mitochondria, chromanoxyl radicals of vitamin E can be reduced to form chromanols by NAD(P)H-dependent enzymes. Plasma membranes of various cell types (including erythrocytes) also contain NAD(P)H oxidoreductase enzymes, as well as flavins, thiols, *b*-type cytochromes and coenzyme Q_{10}[144], which could play a role in vitamin E recycling. Specifically, the erythrocyte membrane contains a transmembrane NADH-ferricyanide reductase associated with both proton movement out of cells[144,132] and the activation of membrane proteins by phosphorylation[145], as well as NADH-cytochrome *c* reductase on the inner side

of the membrane [146] that is antigenically identical to NADH-cytochrome b_5 reductase of endoplasmic reticulum [147]. The natural substrate for this latter enzyme, which is not known to be involved in any transport function or fatty acid desaturation process [144], is still the subject of debate, since two fractions that seem to have different substrates (a soluble one and a membrane bound one) have been isolated.

The role of vitamin E in the human body is not clearly established, but it is known to be an essential nutrient in more than 20 vertebrate species. The vitamin plays some role in forming red blood cells and muscle and other tissues and in preventing the oxidation of vitamin A and fats.

Vitamin E supplementation is popularly advocated for a wide range of diseases, but no substantial evidence has been found to back these claims. The report of the panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy records that few adverse effects have been reported from doses of vitamin E up to 3200 mg/day although the usual intakes were 3.5 mg to 19.5 mg per day in adult men and slightly lower in women[98]. Although vitamin E is stored in the body, overdoses appear to have lower toxic effects than do overdoses of other fat-soluble vitamins [148].

1.5.3 Vitamin C (Ascorbic Acid).

Vitamin C is important in the formation and maintenance of collagen, the protein that supports many body structures and plays a major role in the formation of bones and teeth. It also enhances the absorption of iron from foods of vegetable origin. It is widely distributed in mammalian tissues, but it is present in relatively high amounts in the adrenal and pituitary glands. Lesser amounts are found in the liver, spleen, pancreas and brain. Although excess ascorbic acid is quickly excreted in the urine, large and prolonged doses can result in the formation of bladder and kidney stones due to increased production of oxalate, the destruction of concomitantly ingested B12, facilitation of too much iron absorption, and the loss of calcium from bones [149]. Sources of vitamin C include citrus fruits, fresh strawberries, cantaloupe, pineapple, and guava. Good vegetable sources are broccoli, Brussels sprouts, tomatoes, spinach, kale, green peppers, cabbage, turnips and because of its importance in the diet, potatoes [150]. The Estimated Average Daily Requirement (EAR) for adult males and females is 25 mg per day with higher requirements probably in smokers. The RNI is defined as two standard deviations (2SD) above the EAR. For Vitamin C this is 40 mg/day[98].

In contrast to vitamin E, vitamin C (Figure 20) is hydrophilic and is therefore more prevalent in an aqueous environment than vitamin E. Its role as an antioxidant is indicated by its known free radical scavenging action. As a reducing and antioxidant agent, it reacts with $O_2^{\cdot -}$ and $OH^{\cdot}$ and various lipid hydroperoxides. As has been shown in section 1.4.2 it has a possible role in the recycling of vitamin E [127].

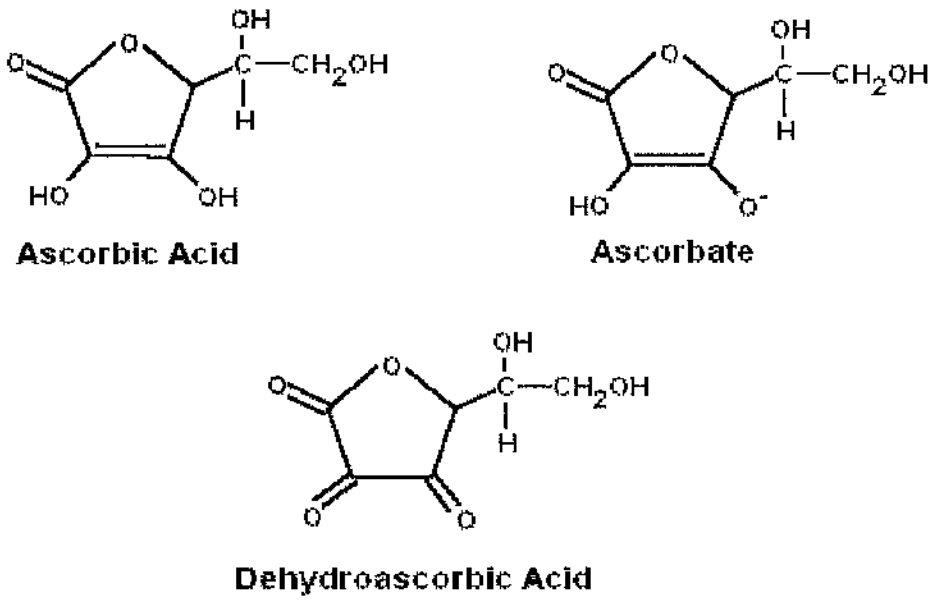


Figure 20

When compared with other water-soluble antioxidants, vitamin C offers the most effective protection against plasma lipid peroxidation[151, 152,153] by scavenging free radicals before they damage plasma lipid components such as low density lipoprotein (LDL). If the previously discussed recycling action of vitamin C on vitamin E is proven then its action would spare vitamin E and allow it to protect against LDL oxidation.

However, vitamin C as well as being an antioxidant can also be a prooxidant[139,154,155]. Vitamin C exerts a sparing effect on the antioxidant effects of vitamin E and selenium. On the other hand, excess amounts (~ 1mM) may act as a prooxidant in the presence of the transition metals Fe^{3+} or Cu^{2+} by

generating cofactors of activated oxygen radicals during the promotion of lipid peroxidation. Several studies[156,157,158] have shown that vitamin C's prooxidant action, which induces lipid peroxidation, resides in its ability to reduce Fe^{3+} to the Fe^{2+} state; Fe^{2+} is known to be a potent free radical producer. A study by Aruoma et al [158] provide good examples for such interactions with metals, thereby inducing the prooxidant property of ascorbate. In this study, oxidative modification of DNA bases was substantially enhanced in the presence of ascorbate. The complexity of a dual role of vitamin C becomes magnified when other antioxidants like vitamin E are present (as in *in vivo* conditions). For instance, lipid peroxidation of microsomes is enhanced by vitamin C in vitamin E deficient animals but suppressed by vitamin C under normal conditions. However it has been pointed out that the concentrations of copper and iron complexes required to accelerate free radical reactions are not generally available in the extracellular fluids of the human body [159]. The antioxidant properties of vitamin C in the human body will normally predominate, except in metal overload disease states [159]. Although more studies are required, it appears that the concentration and subcellular localisation of vitamin C might be important factors contributing to the sharply contrasting actions of this vitamin. As the pK_a of ascorbic acid is 4.25, the ascorbate anion (AH^-) is the predominant form existing at the physiological pH. Ascorbate (AH^-) oxidatively forms dehydroascorbic acid (DHA) through a reversible oxidation with the intermediate formation of the ascorbyl radical ($A^{-\bullet}$). It has been suggested that DHA may be more effective than ascorbate against Cu^{2+}-induced lipid peroxidation in low density lipoproteins [160].

1.5.4 Carotenoids and Retinol (Vitamin A).

Vitamin A is available in the diet, either as preformed vitamin A (retinol) or from carotenoids which are cleaved to retinol in the body. Major sources of carotenoids are vegetables and fruit, e.g. carrot, tomato, grapefruit, broccoli, orange and mango[161]. They are absorbed from the intestine with an absorption efficiency inversely related to caroteniod intake. Absorption is particularly low when intake of dietary fat is low. The main carotenoids are α- and β-carotene and cryptoxanthin. Dietary vitamin A is available in the form of provitamin A precursor compounds cleaved enzymatically in the intestinal mucosa or liver to retinol[162,163] or directly from animal food: liver, milk, egg and fish[164]. Lipoproteins and retinol-binding protein are responsible for the transport and plasma regulation of retinol (vitamin A)[165]. β-Carotene and lycopene (major dietary carotenoids) are transported primarily within low density lipoprotein (LDL)[166]. Structures of these molecules are shown in figure 21.

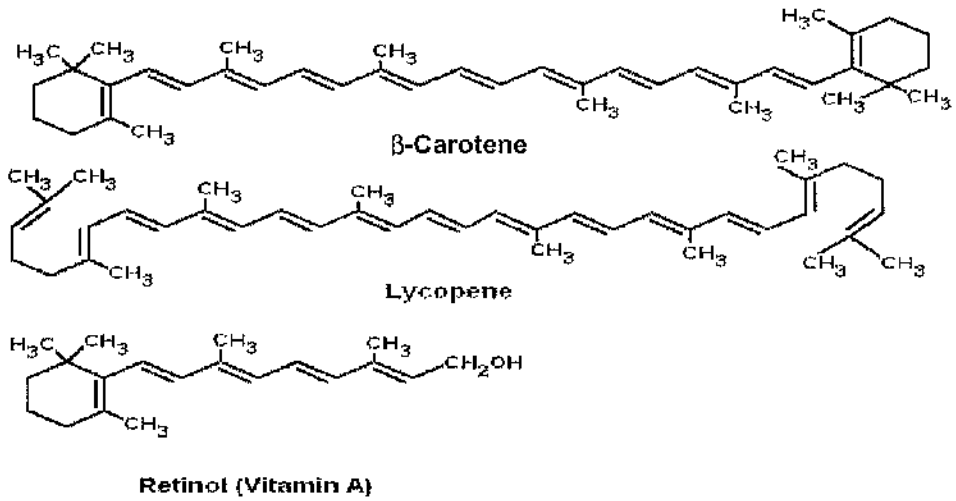


Figure 21

Estimated Average Requirements (EAR) of vitamin A for males and females is 496 μg and 402 μg respectively per day as retinol. The carotenoids have long been considered antioxidants because of their capacity to scavenge free radicals [167,168,169]. Carotenoids protect lipids against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen [170,171,172]. The quenching of singlet molecular oxygen ($^1O_2$) results almost entirely from energy transfer and yields ground state oxygen and a triplet excited carotenoid. The energy is dissipated through the interaction between triplet excited carotenoid and the solvent (Figure 22).

$$^1O_2 + \text{carotenoid} \rightarrow O_2 + \text{carotenoid}^*$$

$$\text{carotenoid}^* \rightarrow \text{carotenoid} + \text{heat.}$$

Figure 22

However the contribution of β-carotene to the quenching of $^1O_2$ is minor *in vivo* and can be ignored [171]. β-Carotene displays an efficient biological radical-trapping antioxidant activity through its inhibition of lipid peroxidation induced by the xanthine oxidase system [169]. The structural arrangement of β-carotene (Figure 21) with their long chains of double bonds suggests that they would make excellent scavengers for reactive free radicals. The bleaching of carotenoid colour upon oxidation [135] supports this idea. The antioxidant action of

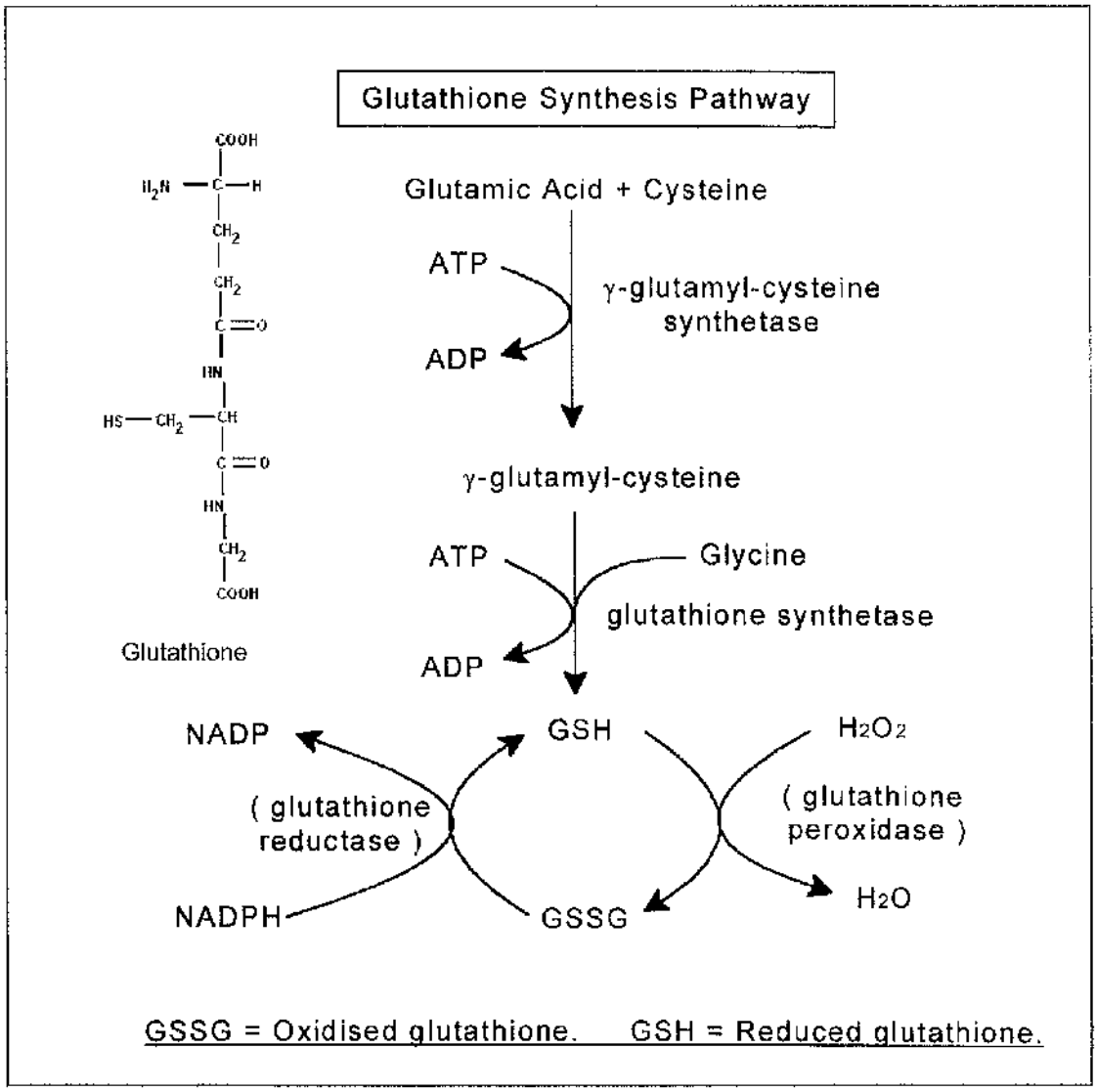
carotenoids has also been demonstrated *in vivo*[173] with regard to lipid peroxidation.

β-Carotene reacts with the superoxide radical and forms a β-carotene-superoxide radical addition complex, whereas lycopene undergoes a reversible electron transfer with the superoxide radical. β-Carotene as well as retinoic acid (widely used in the treatment of skin diseases) and its methyl ester inhibit lipid peroxidation at low oxygen tension, whereas at high oxygen tension they exhibit prooxidant activity. At very low pO_2 of 4 mm Hg, β-carotene inhibits lipid peroxidation even more effectively than α-tocopherol[173]. However it should be noted that normal oxygen tension in human arterial and venous blood is about 83 – 108 mm Hg and 40 – 61 mm Hg respectively and therefore it is unlikely that oxygen tensions of 4 mm Hg would occur in a healthy individual but may do so in peripheral tissue in vascular shut down, or in ischaemic tissue.

Plasma retinol concentrations are insensitive indicators of vitamin A status because a homeostatic mechanism maintains them at a reasonably constant level over a large range of liver reserves. They fall unequivocally below 0.7 μmol/L only in later stages of deficiency[98].

1.5.5. Glutathione

Glutathione (Figure 23) is an important water-soluble antioxidant and reducing agent, synthesised from glutamate, cysteine and glycine in many cell types and present intracellularly in the millimolar range.



Glutathione structure and synthesis pathway

Figure 23

It acts as a substrate or cosubstrate in numerous enzymatic reactions e.g. the glutathione peroxidase reaction.

Reduced glutathione (GSH) is the most abundant low molecular weight thiol present in virtually all cell systems. The intracellular concentration is generally ≅ 0.5 mM but sometimes reaches levels as high as 10 mM [174,175]. Reduced glutathione is characterised by its reactive thio group and its γ-glutamyl bond, which makes it resistant to peptidase attack. Its versatility arises from chemical properties that allow it to serve as both a nucleophile and an effective reductant by interacting with numerous electrophilic and oxidising compounds such as H_2O_2, $O_2^{-\bullet}$ and $^{\bullet}OH$. As an effective reductant, GSH plays an important role in a variety of detoxification processes. This includes the nullification of peroxide damage, as evidenced by GSH depletion, which increases the susceptibility of animals to cytotoxicity and affects drug interventions in neoplastic diseases [175].

Reduced glutathione readily interacts with free radicals, notably hydroxyl and carbon radicals, by donating a hydrogen atom. Such reactions can provide protection by neutralising reactive $^{\bullet}OH$ that is considered a major source of free radical damage. The existence of a GSH-dependent factor that inhibits lipid peroxidation in membranes has been suggested [176,177]. Such inhibition may be related to a catalytic amount of vitamin E that undergoes a series of reaction steps involving the regeneration by GSH (Figure 24)

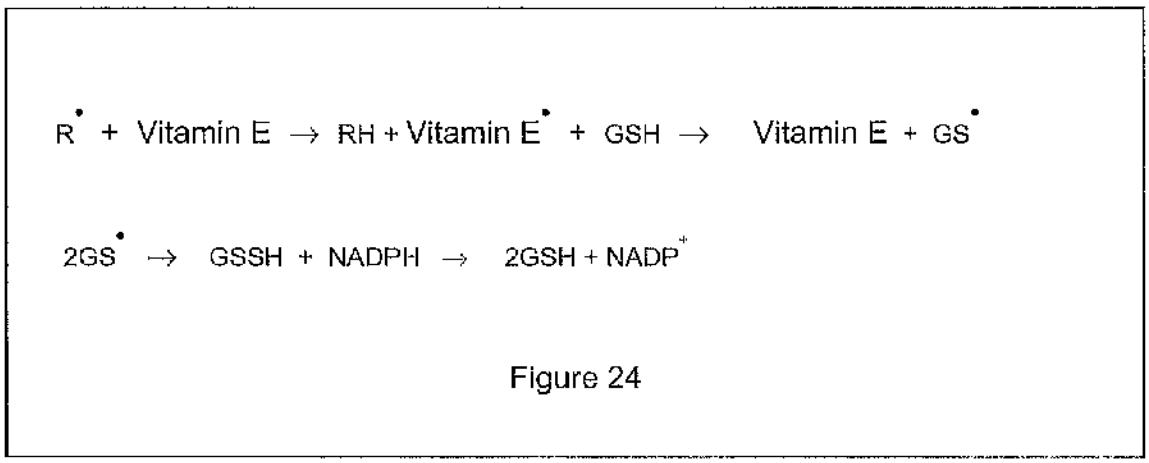


Figure 24

As with most antioxidant defences, the levels of GSH fluctuate under various physiological conditions, including ageing [174,178] and some neoplastic diseases that are usually accompanied by increased lipid peroxides [179]. Whether this inverse correlation between the lower content of GSH and the higher level of peroxides represents a causal relationship is not yet established. Some studies [179] showed that a decrease in GSH levels with age may be due to either an increase in the oxidation rate or to a decrease in overall GSH turnover resulting from an increased degradation and/ or reduced biosynthesis of total GSH. Biosynthesis of GSH (Figure 23) from its constituent amino acids is catalysed by glutamylcysteine synthetase and GSH synthetase. In cells other than erythrocytes, γ-glutamyltransferase (which also degrades GSH conjugates produced by glutathione transferases) can break down GSH. This enzyme is located in the plasma membrane with its active site facing the extracellular space where it acts on GSH and transfers the glutamate residue on to other amino acids such as cysteine, methionine and glutamine.

1.5.6 Other Antioxidants.

Uric acid has traditionally been considered merely as an end product of purine metabolism in humans. However, its function as a biological antioxidant has been increasingly recognised. The *in vitro* scavenging of $^{\bullet}OH$ by urate was first reported in 1960 by Howell and Wyngarden [180]. Its antioxidant action was confirmed by its protection against oxidative damage [85]. Recent renewed interest in uric acid has focused attention on its role as a physiological antioxidant [181.,182]. Although the precise biochemical mechanism of the antioxidant action of urate is not well defined, reports indicate that uric acid may act by preserving (sparing) plasma vitamin C [183], probably by complexing transition metals such as iron and copper [182]. It has been shown that uric acid, located in the cell cytosol, cannot scavenge radicals within the liposomal membranes [184]. Thus it acts against radicals generated in the aqueous phase not lipid derived radicals. It has been shown that aqueous radicals can be trapped by uric acid before they attack membranes.

Catalase (CT) is a major primary antioxidant defence component (figure 9) that primarily works to catalyse the decomposition of H_2O_2 to H_2O [185] (figure10), sharing this function with glutathione peroxidase (GSH-PX). Both enzymes detoxify oxygen-reactive radicals by catalysing the formation of H_2O_2 derived from superoxide. In the presence of low H_2O_2 levels, organic peroxides are preferentially catalysed by glutathione peroxidase. However, at high H_2O_2 concentrations, there are metabolised by catalase. Like SOD, the tissue distribution of CT is widespread. The level of activity varies not only between tissues but also within the cell itself. The liver, kidney and red blood cell possess relatively high levels of CT. In hepatocytes, peroxisomes exhibit an expectedly

high CT activity, although activity was also found in the cytosol [13]. CT is not a circulating enzyme and has a half-life of only minutes [6].

Glutathione Peroxidase (GSH-PX) is located intracellularly in the cytosol and mitochondrial matrix [186]. It catalyses the reduction of H_2O_2 (figure 11) and organic hydroperoxides (figure 25).

Glutathione Peroxidase reduction of Organic Hydroperoxide.

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH$$

Figure 25

Both types of GSH-PX enzymes, selenium dependent and selenium, independent, have been shown to catalyse the reactions in figures 11 and 25 and therefore protect against radical damage by reducing peroxides. However they possess different substrate specificities. The selenium dependent peroxidase is found in the cytosol and exhibits low capacity for the reduction of H_2O_2. The selenium independent peroxidase utilises organic hydroperoxides as preferred substrate over H_2O_2. The involvement of GSH and the possible participation of GSH-PX in the inhibition of lipid peroxidation were first reported by McCay et al [187] and later by Burk [188].

Another form of selenium-dependent GSH-PX associated with membranes was reported by Ursini et al [189, 190]. This cytosolic protein exhibited GSH-PX

activity on degradation of phospholipid hydroperoxide. Further investigations are required to elucidate its physiological function.

As selenium is required for the optimal activity of GSH-PX it has been found that deficiency depresses GSH-PX activity and therefore alters antioxidant defence systems. Selenium deficiency has little effect on SOD or CT activity[87]. This indicates that antioxidant enzymes in liver and muscles are capable of adapting to this deficiency to minimise changes in antioxidant defence capacity. In this regard, the adaptive response to selenium deficiency also includes a greatly increased hepatic glutathione transferase activity[88].

1.6 Secondary Defence Systems Against Oxidative Stress.

As previously suggested in section 1.5 the components for secondary defences against lipid peroxidation are: lipolytic enzymes and phospholipases, The following sections summarise their function.

1.6.1 Lipolytic Enzymes.

The biological importance of phospholipases in the cellular regulatory processes has been firmly established[191,192]. However, their participation in defence mechanisms against free radical damage and cellular repair of damaged membrane structure was not fully explored until recently. Cells are equipped with various enzymes responsible for the reconstruction of damaged or altered membrane constituents. It has been suggested that phospholipase-mediated removal of damaged fatty acid moieties from membrane lipids could be an effective way to maintain membrane integrity and at the same time provide a means to regulate lipid turnover[191]. Investigators have shown that the

peroxidation of membrane lipids can stimulate the lipolytic action of phospholipase A_2[192]. The phospholipase A_2 (PLA_2) enzyme comprises a rapidly growing superfamily of intracellular and secreted proteins, which hydrolyse the acyl-group at the sn-2 position of glycerophospholipids (Figure 26) to release fatty acids and lysophospholipids[80].

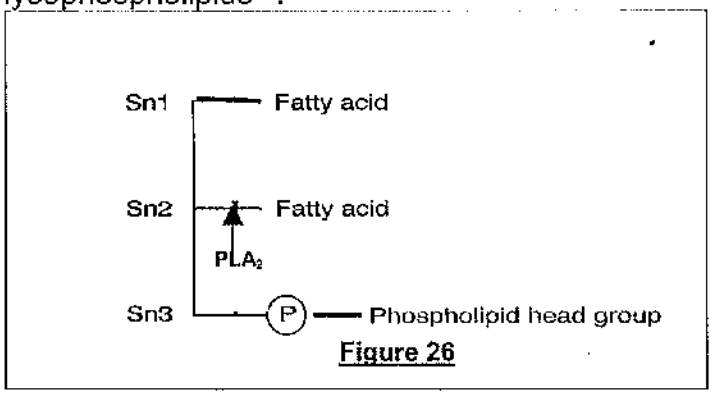


Figure 26

The diverse PLA_2 enzymes have been classified into Groups for I to XI thus far. Recent clinical studies suggest that calcium dependent secretory phospholipase A_2 ($sPLA_2$) of group IIA may contribute to atherosclerosis and that their plasma levels my predict cardiovascular events[193]. Increased levels of calcium dependent cytoplasmic PLA_2 enzyme of group IV has been found in red cells of schizophrenic patients[194]. The predilection of phospholipase A_2 for oxidised lipids could have very significant physiological ramifications with regard to membrane repair or detoxification process because it could provide cells with additional protective mechanisms against lipid peroxidation. It has been demonstrated that there is a correlation between age-related increase in phospholipase A_2 of microsomal membranes and an increase in oxidatively altered lipid hydroperoxides of the same microsomes[195]. It may be that phospholipase A_2 enhances peroxidation or has a protective role in different clinical conditions.

1.7 Disease States Associated with Free Radical Damage

1.7.1 Ischaemic Heart Disease

The pathological changes associated with the development of coronary heart disease take place over many years. These changes can be considered to be:

- initial arterial injury
- development of a fibrous plaque at the site of injury
- blood clot formation
- the myocardial infarction itself.

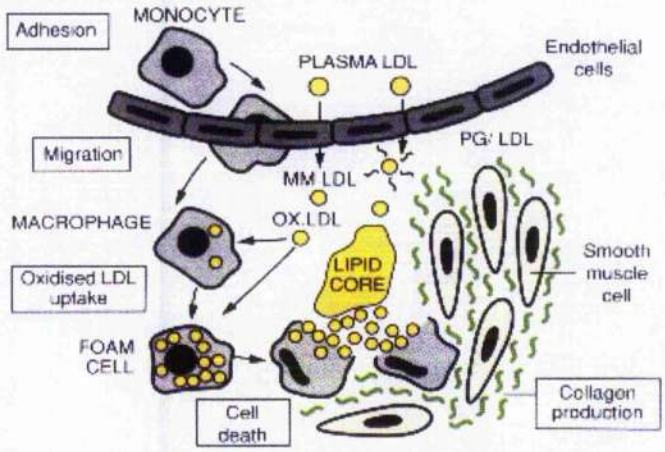
They arise from a series of abnormal physiological responses that are influenced by a wide array of factors[196]. Some of these factors are genetic, some age related, some lifestyle related and some associated with diet. The incidence of coronary heart disease cannot therefore be attributed to a single aspect, let alone a single component of diet. The abnormal physiological conditions which affect the frequency and severity of injury to the coronary arteries include increased blood pressure, increased lipid peroxidation, an increased tendency for platelets to aggregate and an increased inflammatory response.

In turn, these are influenced by components of diet. For example, blood pressure is increased by high intake of salt, the ratio of sodium to potassium intake, alcohol intake and obesity. The potential for lipid peroxidation is affected by the susceptibility of the fatty acids in low density lipoprotein (LDL) particles to oxidation (polyunsaturates are most prone to oxidation) and the balance between prooxidants (e.g. free iron) and antioxidants

(e.g. Vitamins E and C and β-carotene).

The build up of the fibrous plaque in the injured artery wall is determined by a series of factors which affect both lipid and thrombus deposition, e.g. raised plasma cholesterol (influenced by the fat, fatty acid and soluble fibre content of the diet), increased plasma fibrinogen (probably not affected by diet, though influenced by smoking), and increased plasma insulin levels (associated with centrally located fat disposition). The formation of a major thrombus and the severity of the heart attack will be influenced by factors, which affect blood coagulation and the regulation of heartbeat.

A concept that is gaining considerable support, sometimes referred to as the 'antioxidant hypothesis' or 'oxidised LDL hypothesis', helps draw together some of these risk factors and suggests' a mechanism by which atherosclerosis progresses and perhaps even how the initial injury to the artery occurs[197] (Figure 27).



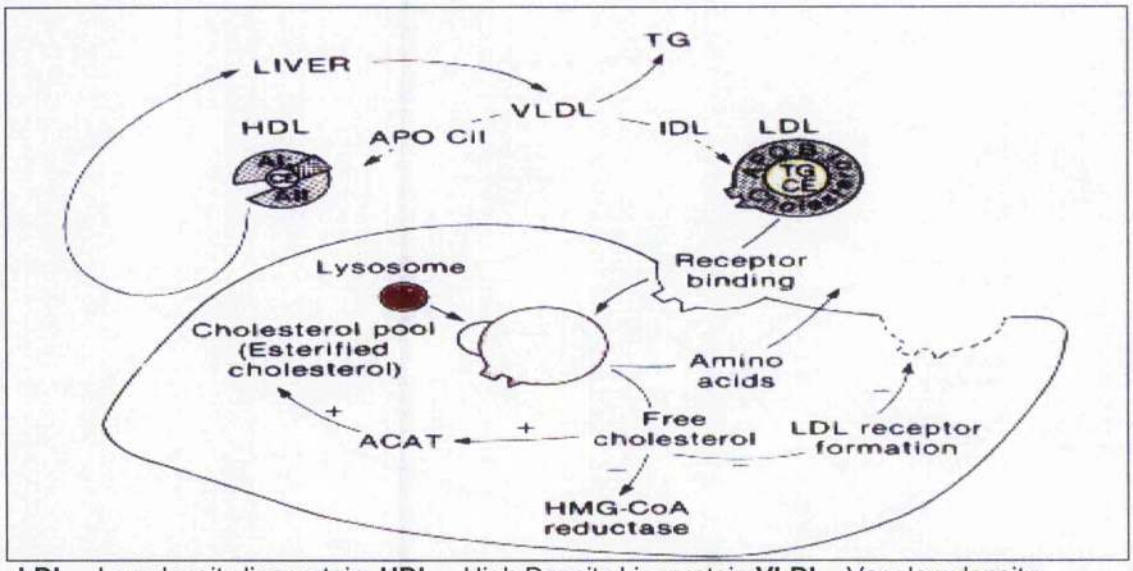
LDL enters the blood vessel intima where it is modified to become an inflammatory stimulant and then oxidized. Monocytes enter the intima, take up oxidized lipid to become foam cells and then die. The cholesterol released creates the lipid core. Smooth muscle cells form collagen to enclose the core.

[mm-LDL – minimally modified LDL, OX. LDL – Oxidised LDL, PG – proteoglycan]

Figure 27: Processes in atherosclerosis plaque formation.
Adapted from; Davies, M. J.; B.J.Card. Vol. 4 Supp. 1, 1997, S4 – S7.

It is this mechanism that may provide the link between the importance of fruit and vegetables, as well as other dietary sources of antioxidants, and the need for a moderation in fat intake.

Although high levels of cholesterol-rich LDL particles in the plasma are strongly associated with an increase of coronary heart disease, these particles are not atherogenic in themselves[197]. Although they can enter the blood vessel wall, by way of classical LDL receptor mechanism (Figure 28), they do not create an inflammatory reaction or tissue damage.



LDL – Low density lipoprotein. **HDL** – High Density Lipoprotein.**VLDL** –Very low density lipoprotein. **IDL** – Intermediate density lipoprotein.**TG** – triglyceride. **APO CII** - Apolipoprotein CII is essential to the catabolism of VLDL and Chylomicrons. **APO B** – the major protein moiety of all lipoproteins other than HDL and has a major role in ensuring lipid delivery to the extrahepatic cells. It allows the recognition and binding of LDL to specific high-affinity receptors on the cell membrane. , **ACAT** – Acyl-cholesterol acyltransferase, required for cholesterol esterification intracellularly. **HMG-CoA Reductase** – 3-hydroxy-3-methylglutaryl-CoA Reductase, required for endogenous cholesterol synthesis. **+** = positive feedback. **-** = negative feedback.

Figure 28: LDL Catabolism Via High Affinity Receptor Pathway found in Liver and Peripheral Cells (Adapted from; Tietz Texbook of Clinical Chemistry 2nd edition, Burtis C. A., AshwoodE. R., Editors (1994) Chapter 23, page 1027.)

In contrast, LDL particles, which have been modified by oxidation, are no longer recognised by the LDL receptors on the cell surface and behave as if it is foreign material, in that they invoke an inflammatory response. This oxidised LDL (Ox-LDL) is taken up more avidly by macrophages than native LDL via the scavenger receptor mechanism that is not subject to regulation by the cellular cholesterol content[198]. The characteristics of oxidised LDL are given in Table 2.

Table 2.

Characteristics of oxidized LDL

Reduction in polyunsaturated fatty acids
Increase in lipid peroxides.
Increase in negative charge of apoB 100*
Increase in oxysterol content.
Increase in lysolecithin content
Fragmentation of apo B-100
Increased uptake by macrophage scavenger receptor.

* Apo B 100 is the major apolipoprotein present in LDL particles

The reduction of polyunsaturated fatty acid in oxidised LDL is accompanied by an increase in lipid peroxides and aldehydes [199, 200]. Extensive oxidative modification of LDL results in derivatization of lysine residues on apolipoprotein B (apo B) by lipid hydroperoxides or aldehydes such as malondialdehyde, 4-hydroxynonenal and hexanal, thus increasing the negative charge on LDL [201]. These changes in apo B lead to increased LDL uptake by the scavenger receptor on the macrophage [202].

It is worthwhile reviewing this complex process. The initiating event for production of Ox-LDL is the peroxidation of poylunsaturated fatty acids on LDL. Figure 27 shows the scheme depicting the relationship between Ox-LDL (a potent chemoattractant for circulating monocytes) and the formation of the lipid accumulation in the artery wall, the fatty streak lesion.

In the early phase of oxidation, mild oxidation of LDL results in the formation of minimally modified LDL (mm-LDL) in the subendothelial space. Once formed, mm-LDL may be able to induce the endothelium to express adhesion molecules for monocytes and to secrete monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF). These events result in the monocyte binding to the endothelium and the subsequent migration of the monocyte into the subendothelial space. There, mm-LDL promotes differentiation into macrophages, via M-CSF, which in turn, modify mm-LDL into a more oxidized form. This Ox-LDL can then be processed by way of the scavenger receptor mechanism, thus leading to accumulation of cholesterol esters [203]. As Ox-LDL is a potent inhibitor of macrophage motility, it may also promote retention of macrophages in the arterial wall. Products of LDL oxidation are cytotoxic and this cytotoxicity may be important in inducing endothelial cell dysfunction and in promoting the evolution of the fatty streak into a more complex and advanced lesion. Ox-LDL may be responsible for reduced fibrinolysis in blood vessels and may also promote pro-coagulant activity by inducing expression of tissue factor (TF)[204], a low molecular weight (45-kD) membrane bound glycoprotein. In addition to its regulation of coagulation it is also a critical determinant of thrombin generation in normal haemostatis and in atherothrombotic disease[205,206]. The binding of TF to factor VII is the first step in the extrinsic coagulation cascade. In human atherosclerotic vessels, TF is abundant, particularly within the acellular lipid core of the plaques and in the lumen where it is expressed by monocyte-derived macrophages[207,208]. The clinical consequences of inappropriately high intravascular expression of TF are often catastrophic. Intraluminal TF activity can trigger the thrombogenic cascade

that underlies the often lethal thrombotic complications of atherosclerosis. It has been shown that plasminogen activator inhibitor–1 (PAI-1) levels are increased in coronary heart disease [209]. Ox-LDL causes endothelial cells in culture to increase PAI-1 synthesis and secretion in a dose-dependent fashion [210]. It also inhibits nitric oxide production and this, in turn, could promote vasoconstriction [211] and platelet adhesiveness [212,213]

Several lines of evidence can be cited in support of the in vivo existence of oxidized LDL (Table 3).

Table 3
Evidence for the *in vivo* existence of oxidised LDL (Ox-LDL).

Antibodies to Ox-LDL immunostain in rabbit atherosclerotic lesions.
LDL isolated from aortic atherosclerotic lesions have properties similar to Ox-LDL.
Autoantibodies in plasma of humans and rabbits react with Ox-LDL.
Antioxidants like butylated hydroxytoluene (BHT), diphenyl phenylenediamine (DPPD), probucol and vitamin E prevent progression of atherosclerosis in animal models. it should be noted that BHT in doses >1% of the diet can cause hepatic and renal dysfunction and that DPPD is a mutagen.

A modified form of LDL that has many of the physical, chemical and biological properties of Ox-LDL occurs in arterial lesions. For example oxidatively modified and fragmented apo B has been isolated form the plasma of normal subjects and from patients with atherosclerosis [214]. Also, antibodies against epitopes on Ox-LDL recognize material in atherosclerotic lesions, but not in normal arteries; circulating antibodies against epitopes on Ox-LDL have been demonstrated in

the plasma of Watanabe heritable hyperlipidaemic (WHHL) rabbits and in humans[215,216]. The presence of autoantibodies against Ox-LDL has been positively correlated[217] with the progression of atherosclerosis, as manifested by carotid artery stenosis. Also, it was recently shown that the susceptibility of LDL to oxidation varied with the severity of coronary atherosclerosis, as evaluated by angiography[218].

It is thought that the oxidation of LDL is more likely to occur in the artery wall than in the circulation because of protective processes, which exist in the blood. The most important elements in the protection of LDL against oxidation are considered to be a combination of vitamin C in plasma and vitamin E in the LDL particle[219]. It was originally believed that the accumulation of cholesterol in the artery wall was the key to atherosclerosis but it is now clear that cholesterol itself is not a major factor during the formation of the lesion[220] but follows later after damage has begun. There is evidence that the fatty streaks formed at the beginning of this process can disappear again, but under appropriate conditions may gradually develop into raised plaques characteristic of atherosclerosis. As damage progresses, ulceration can occur resulting in disruption of the endothelium, attracting platelets and other blood components which aggravate the damage. In addition, small pieces of tissue can become dislodged, thus forming the focus for the development of a thrombus.

Under normal circumstances these vulnerable LDL particles would be protected by antioxidants, such as vitamin E, leading to decreased production of mm-LDL[212,213,221]. The lower production of mm-LDL would reduce release of lysosomal enzymes and lipids, decrease plaque progression, endothelial damage and disturbances of plaque structure. However, when antioxidant

status is compromised either because of poor dietary intake of these nutrients or because of high requirements this protective mechanism will be impaired. The susceptibility of the LDL particle to oxidation depends partly on its fatty acid composition, partly upon its own antioxidant status (the balance of pro- and antioxidants) and also upon its size (small particles are more susceptible). There is now increasing evidence that particles enriched by dietary means with monounsaturated fatty acids (as opposed to polyunsaturates) are relatively resistant to such damage.

It is not clear why there are marked differences in the incidence of coronary heart disease between countries but the major risk factors (smoking, blood cholesterol and hypertension) account for only 50 % of its occurrence. There is a view that some factor associated with fruit and vegetable intake may help explain some of the remainder.

The acknowledged importance of fruit and vegetables is not new but our knowledge of why they are important has been expanding. They collectively provided a wide variety of nutrients, with different sub-groups being rich in particular nutrients and substances not currently recognised as nutrients, such as flavonoids (found in citrus fruit and red wine). A number of these nutrients have antioxidant properties and it is on these that particular interest has focused.

Dietary surveys in various countries have shown strong inverse correlations between mortality and coronary heart disease and the intake of fresh fruit/ vegetables and vitamins C, E, and β-carotene [222].

In the WHO/ MONICA project, which was designed to compare heart disease mortality in a number of countries, data have been collected on plasma antioxidant levels in middle-aged men from 16 European populations, in which

the difference in age-specific mortality from coronary heart disease ranged six-fold. Two of the 'classical' risk factors, total plasma cholesterol and blood pressure, were found to be only weakly correlated with coronary heart disease risk and surprisingly there was no significant correlation with smoking habits[223]. The authors suggest that the latter finding might be explained by the known deleterious effects of smoking balanced in Southern European populations by protective dietary factors such as vitamin E. Indeed a strong inverse correlation was found between plasma vitamin E levels and coronary heart disease, and vitamin C showed moderately strong inverse relationship. Correlations were also found for vitamin A but were weaker, and the correlation was weak or absent for β-carotene and selenium[223]. However studies[224,225] have indicated that selenium might be important in reducing heart disease risk, in at least some circumstances. Furthermore markedly lower selenium levels in patients with severe atherosclerosis have been observed compared with controls[226]. In two major prospective studies, one in men[227] and the other in women[228], high intakes of vitamin E were associated with a reduced incidence of coronary heart disease.

The following studies provide inconclusive evidence regarding vitamin E and reduction in cardiovascular risk. More than thirty-four thousand post-menopausal women with no overt cardiovascular disease were followed for seven years after completing dietary questionnaires, including information on dietary supplements[229]. The risk of death from coronary heart disease was lower in those with a higher intake of vitamin E (diet plus supplements), but there was little to suggest that vitamin E from the diet had any impact. Vitamin A and C intake seemed unimportant in determining heart disease. Although diet may be important this study may simply indicate healthy people with a different lifestyle rather than being protective in its own right. A recent randomised

placebo controlled trial of large doses of vitamin E in 2,002 patients with coronary heart disease followed for a median of 510 days in the Cambridge region (CHAOS), showed a 77 % risk reduction in non-fatal myocardial infarction but with a non-significant higher cardiovascular and all cause mortality in those taking the vitamin [230]. This trial is encouraging but had insufficient power to resolve the disparity between the apparent benefits of myocardial infarction but possible adverse affects of death. It also had methodological problems in that the vitamin E (d-α-tocopherol) supplements were reduced from 800 IU/day to
400 IU/day and there was no attempt at randomization between the two vitamin E dosage groups. The observation of the CHAOS trial has not been reproduced in two larger trials.

The Heart Outcomes Prevention Evaluation (HOPE) Study[231] enrolled 2545 women and 6996 men of 55 years or older who were at high risk for cardiovascular events because they had cardiovascular disease or diabetes in relation to one other risk factor. 16.2%, randomly assigned, received 400 IU of vitamin E daily from natural sources or a matching placebo for 4.5 years. There was no significant difference in the number of deaths from cardiovascular causes between the supplemented group and the placebo group (relative risk, 1.05. p = 0.33). This trial concluded that patients who were at high risk for cardiovascular events, supplementation with vitamin E had no apparent effect on cardiovascular outcomes. The GISSI-Prevenzione trial[232] trial was an open-label, randomised study that evaluated the effects of ω3 PUFA (polyunsaturated fatty acids) and vitamin E on the combined end point of death, nonfatal myocardial infarction, and nonfatal stroke in 11,324 male and female patients who survived a myocardial infarction within 3 months prior to randomisation. Patients were randomised to ω3 PUFA 1 g/day, vitamin E 300 mg/day (approx. 300 IU/day), both ω3 PUPA and vitamin E, or no treatment. Median time between suffering the myocardial infarction and being randomized was 16 days,

and follow-up continued for 3.5 years. The population was relatively low risk; only 16% of patients were 70 years of age or older, 14% had an ejection fraction below 40% as measured by echocardiogram, and 29% had a positive exercise stress test. No significant baseline differences existed between any of the treatment groups. Two-way and four-way intent-to-treat analyses evaluating the effects of vitamin E supplementation showed vitamin E had no clinically significant benefit on the combined end point of all-cause mortality, nonfatal myocardial infarction, or nonfatal stroke. When the secondary outcome measure of cardiovascular death was analyzed, the four-way analysis showed that the vitamin E group had a relative risk of suffering a cardiovascular death of 0.80 (95% CI 0.65-0.99) compared with the control group. The relative risk for ω3 PUFA supplementation group was 0.70 (95% CI 0.56-0.87) compared to the control group and was significantly better than the vitamin E group. When the effect of ω3 PUPA plus vitamin E was evaluated, the benefits on the combined end point and on total mortality were similar to those with ω3 PUFA alone. Adverse effects led to the discontinuation of the study drug in 3.8% of the ω3 PUFA patients and 2.1% of the vitamin E patients. The most frequently reported reactions for vitamin E were gastrointestinal disturbances (2.9%) and nausea (0.4%). Malignancy was diagnosed in 2.2% of the control patients, 2.7% of the ω3 PUFA patients, and 2.6% of the vitamin E patients during the study. Evidence for vitamin E supplementation for secondary prevention of myocardial damage is not convincing and indeed the MRC/BHF Heart Protection Study[233] has not been able to demonstrate any benefits from such supplementation. Other large-scale randomized trials are currently in progress and still to report. Long term follow-up continues in several completed trials.

Further trials:

1: The trial of vitamin E and aspirin in women is part of the Womans Health study, which will end in 2001. This study is being used to assign 40,000 women aged 45 years or older with no history of cardiovascular disease to receive vitamin E or placebo and aspirin or placebo. The primary end point will be reduction in all major vascular events. The results of the vitamin E randomisation for this study have not yet been reported.

2: The Women's Angiographic Vitamin and Oestrogen (WAVE) trial is designed to include 400-450 postmenopausal women aged 75 years or younger with angiographically documented coronary vessel occlusion of 15-75%. The four treatment groups will receive hormone replacement therapy (HRT) and antioxidants (vitamin E and vitamin C), active HRT and anti-oxidant placebo, HRT placebo and antioxidants, or HRT placebo and antioxidant placebo. The primary end point is angiographic change. Recruitment ended in 1999, and subjects will be followed through 2001. The results of this study indicated that combination of vitamin E and vitamin C did not retard the progression of coronary atherosclerosis over a 2.8-year follow-up among 423 postmenopausal women[233a].

3: The Women's Antioxidant and Cardiovascular Study[233b], which prospectively will evaluate the effect of vitamin E, vitamin C, and B-carotene on the risk of major cardiovascular events. Recruitment began in 1993. The trial will continue until 2002. This study is still ongoing and has not yet reported[233c]. However, because of the factorial design used, it will allow for examination of potential interactions between vitamin E and other antioxidants.

1.7.2 Schizophrenia

Schizophrenia is probably the most devastating of the psychiatric syndromes. Characteristic features include hallucinations, delusions, and bizarre behaviour and deterioration from the previous level of occupational and social functioning. It is a common disease with a life time prevalence of 0.5 – 1%[234]. The Diagnostic and Statistical Manual of Mental Disorders [235] gives the current diagnostic criteria for schizophrenia and can be divided into two groups which have characteristic symptoms. A schizophrenic can be described as having predominantly positive symptoms of thought disorder or delusions or hallucinations. Or predominantly negative symptoms of apathy and withdrawn behaviour characteristics. Schizophrenia usually occurs in persons with significant genetic predisposition to the illness. The concordance rate for monozygotic twins has been reported to be between 40 per cent and 65 per cent whereas a 10 per cent to 15 per cent rate has been reported in dizygotic twins[236]. The risk with one affected parent is 5% to 10 %, but it is 45 to 50% if both parents have schizophrenia. Adoption studies now document an increased risk for the development of schizophrenia in children of schizophrenic patients, even if the children are adopted into a healthy family before development of schizophrenia in the biologic parents. Rates for development of schizophrenia are lower if the child is raised into a psychologically healthy family than an unhealthy one, underlining the importance of environment for expression of the apparent inherited vulnerability[236].

The exact mode of inheritance is complex, and most investigators believe a polygenic model best fits the data[237]. Certainly, classic Mendelian models are not sufficient. Additionally, since most of those with relatives who have schizophrenia do not develop illness, multiple environmental have been proposed. These may not be specific but instead involved multiple serious insults, including a traumatic environment, exposure to drugs (phencyclidine,

lysergic acid diethylamide, amphetamines), birth trauma or viral exposure in utero.

The pathological mechanisms of this extremely complex syndrome are unknown. Certainly the most influential theory hypothesizes excessive dopamine activity in the mesolimbic and frontal regions. The potency of almost all of the effective antipsychotic agents is highly correlated with the ability to block central dopamine activity. Albeit indirect, these observations provide the strongest evidence of an etiologic link between schizophrenia and dopamine activity. There is provocative evidence from positron emission tomography (PET) and cerebral blood flow studies that the frontal region in schizophrenics is hypo-functional relative to posterior brain regions, perhaps reflecting an injury to dorsolateral frontal regions that become manifest in early adulthood. This is also consistent with observations of enlarged ventricles in about 30% of patients. Post-mortem dopamine receptor binding studies, as well as positron emission tomographic dopamine receptor-binding studies, showed increased mesolimbic dopamine receptors in schizophrenic patients, even in those who have not received the dopamine blocking neuroleptics. Current theories suggest that this up-regulation of dopamine receptors could be secondary to decreased inhibition of this region by hypofunctional frontal regions. Although these theories have been the most productive and consistent areas of research into the pathophysiology of schizophrenia, they are considered an incomplete explanation of the phenomena involved, and this remains an active area of investigation. A membrane phospholipid hypothesis (Horobin (1994))[238] has been put forward as a more appropriate explanation for the biochemical basis for the neurodevelopment concept of schizophrenia and has now become a more probable hypothesis for the development of schizophrenia.

Studies showing that polyunsaturated fatty acids (PUFA) of the ω3 and ω6 series are depleted in cell membranes of neuroleptic treated schizophrenic patients gave first indications that phospholipids may be involved in this disease

process. Peet et al [239] showed that schizophrenic patients have a bimodal distribution of PUFA and found reduced levels of ω6 PUFA which correlated with plasma levels of thiobarbituric acid reactive substances (TBARS), suggesting that there was increased oxidative breakdown of these PUFA. In schizophrenic patients Glen et al [240] found that a group of patients with the lowest red blood cell (RBC) membrane levels of 20 and 22 carbon PUFA were those who showed predominantly negative symptomatology. Other evidence suggests that breakdown of PUFA is increased in schizophrenia. This includes studies using magnetic resonance spectroscopy, which have shown decreased levels of phosphomonoesters and increased levels of phosphodiesters in the frontal cortex of drug free schizophrenic patients, consistent with decreased synthesis and/or increased breakdown of membrane phospholipids [241]. This index of increased phospholipid breakdown correlates with severity of schizophrenic symptoms [242]. There are two reports that pentane in expired air is significantly increased in schizophrenic patients, again consistent with increased oxidative breakdown of PUFA [243,244].

Increased phospholipase activity is one mechanism, which would promote increased breakdown of membrane phospholipids. It is therefore of great interest that significant elevations of phospholipase A_2 (PLA_2) have been reported in the platelets of schizophrenic patients [245]. This report refers to a non-specific measurement of PLA_2 activity . More recently as has been reported earlier, levels of type IV cytosolic PLA_2 has been shown to be increased in red blood cells of patients with schizophrenia[194]. Arachidonate is released selectively by type IVA phopholipase A_2 and may therefore modify the transmission in dopaminergic synapes (to quote Piomelli (1996) by "arachidonate mobilisation and stimulation of the presynaptic metabotropic receptors")[246]. There have now been two reports of genetic abnormalities in the PLA_2 gene of schizophrenic patients. One group has reported a significant difference in allele distribution between schizophrenic patients and controls at a

polymorphic site on the promoter region of the $cPLA_2$ gene [247]. It is therefore possible that a genetically determined abnormality of phospholipase activity is responsible for at least in part the depletion of PUFA in cell membranes of schizophrenic patients. However the evidence for oxidative damage in this condition remains strong.

The phospholipid hypothesis of schizophrenia is able to integrate neurodevelopment and receptor based hypotheses and also provides new approaches to treatment. A genetically determined predisposition towards depletion of membrane levels of PUFA which could be modulated by dietary intake in infancy and later life could underlie reported neurodevelopmental abnormalities in schizophrenia. In addition, the membrane lipid environment has significant effects upon neuroreceptor function and this may underpin the efficacy of currently available treatment approaches, which are aimed at modulating receptor function

1.7.3 Tardive Dyskinesia

Tardive Dyskinesia (TD) is the name given to a set of abnormal, involuntary movements of the orofacial area or extremities. It is thought to result from prolonged treatment with neuroleptic medications that help control symptoms of severe mental illness, particularly schizophrenia. It literally means 'late movement disorder'. Although the aetiology is unclear, it has been postulated that lipid peroxidation, through the action of free radicals is a possible mechanism for neuronal damage in tardive dyskinesia [248] . Clinical evidence supporting the view that free radicals are involved in this process include elevated levels of lipid peroxidation in the cerebrospinal fluid of dyskinetic patients [249] and a possible action of vitamin E on dyskinetic symptoms. Of a number of studies, examining the therapeutic effect of vitamin E on Tardive Dyskinesia, some but not all have found some benefit [250, 251, 252].

2 Materials and Methods

2.1 Materials and Subjects

2.1.1 Materials

Names and address of suppliers of reagents, analytical systems, hardware and software can be found in Appendix 1.

2.1.2 Nutrition Study Subjects and Controls

Subjects in this study group were recruited from:

1. Patients in the all wards of the Victoria Infirmary, Glasgow who had a baseline screen for nutritional assessment (January 1994 to December 2000). As this sample was a baseline level no patient was on either enteral or parenteral nutritional support. As these samples were being used for nutritional monitoring no ethical permission was required.

2. Patients who were on home enteral nutrition (January 1994 to December 2000). These patients were either wholly or partly on artificial feeding. The enteral feeds contain only the Reference Nutrient Intake (RNI) of vitamins and minerals. As these samples were being used for nutritional monitoring no ethical permission was required.

3. Patients from two General Practices', situated in areas of population with different deprivation categories based on the Carstairs and Morris[253] index of deprivation and related to postal code. As these samples were being used for lipid/nutritional monitoring no ethical permission was required.

4: Normal control subjects were recruited form the staff of Biochemistry Department, Victoria Infirmary, Glasgow.

2.1.3 Intensive Care Unit Myocardial Injury Study Subjects.

Patients admitted to the intensive care unit of the Victoria Infirmary, Glasgow, had an assessment of myocardial injury by measurement of cardiac troponin I throughout their stay in this unit[254]. Measurements for the vitamin levels were undertaken on samples obtained on day 1 of the admission of patients from this study group. None of these patients had commenced supplementary feeding. The Local Medical Ethics Committee approved the original study and deemed informed consent was not required.

2.1.4 Schizophrenia Study and a Study of Tardive Dyskinesia Subjects and Controls

1. Schizophrenia Study

a) Patients with schizophrenia, satisfying DSM-III-R criteria[235] for the diagnosis of schizophrenia, were recruited by the Highland Psychiatric Research Group, and categorised as having either predominantly positive or predominantly negative symptoms. These patients with positive symptoms exhibit persistent symptoms of either thought disorder or hallucinations and delusions while those with negative symptoms show apathy and withdrawn behaviour characteristics. Ethical permission for this study was obtained from the ethics committee of Craig Dunain Hospital.

b) A normal control group from the staff of Craig Dunain Hospital, Inverness, was also supplied for this study.

2. Tardive Dyskinesia Study

a) Subjects were recruited from the long-term wards of Bellsdyke Hospital, Larbert. All patients satisfied DSM-III-R criteria[235] for schizophrenia and had been exposed to antipsychotic medication for a minimum of five years. Dyskinetic movements were assessed using a standardized procedure employing the Abnormal Involuntary Movement Scale (AIMS) [255]. Ethical permission for this study was obtained from the ethics committee of Bellsdyke Hospital.

b) A normal control group was also recruited from hospital staff for this study.

2.1.5 Statistical Analysis and Methods

All statistical analysis was carried out on Analyse-It version 1.44 (Analyse-It Software Limited, UK) a data analysis toolkit for Microsoft Excel. All variables with >20 observations were assessed for normal distribution using the Shapiro-Wilk W test[256], useful for sample sizes up-to 5,000, which is regarded as the most powerful statistical test for normality. A coefficient of 1 indicates normality and a low p-value indicates the sample is non-normally distributed. Non-normally distributed data is shown with the median and inter-quartile range and normally distributed data with the mean and standard deviation. For completeness of the data tables the mean, standard deviation, median and inter-quartile range are shown, although it is appreciated that subsequent statistical tests should be applied appropriately. If variables were not normally distributed then the Mann-Whitney U test[257] was applied. This method formally tests for a difference between medians of two independent samples and is also commonly referred to as the Wilcoxon rank-sum test. It is regarded as a powerful alternative to the independent samples t-test. If variables were normally distributed then an independent samples t-test[257] was applied. This method formally tests for a difference between the mean of one sample against a hypothesised mean, or between the means of two independent samples. Pearson correlation was used to assess an association between 2 related variables[257]. Multiple linear regression analysis is used to predict values for a response variable (Y) based on one or more predictor variables[257].

2.2 Methods

2.2.1 Vitamin A and vitamin E analysis.

Principle

Vitamins A and E were determined simultaneously by a high pressure liquid chromatography (HPLC) technique adapted form a method described by Bieri *et al*[258].

Plasma are mixed with ethanol and internal standard (tocopherol acetate and retinol acetate), spun and extracted with hexane. The hexane layer is separated, evaporated and reconstituted with methanol.

The extracted sample is injected onto a 150 x 3.2 mm column of Ultracarb 5 μ ODS (20) (Phenomenex) with a mobile phase (acetonitrile/methanol, 80/20 by volume) running at 1.5 ml/minute. Retinol is monitored at 325 nm and α-tocopherol at 292 nm.

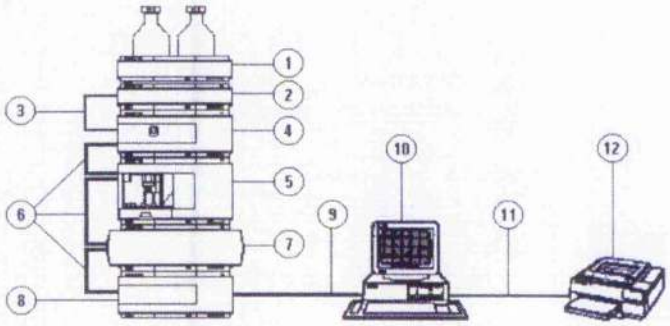
Reagents

α-tocopherol, tocopherol acetate, retinol and retinol acetate all HPLC grade, were obtained from Sigma Chemical Co. HPLC grade acetonitrile, methanol and hexane were obtained from British Drug House (BDH) and absolute alcohol from Hayman Ltd.

Chromatography

The high performance liquid chromatography system was an Agilent Technology HP 1100 series(Figure 29) comprising:

HP 1100 System



1. Solvent Cabinet
2. Vacuum Degasser
3. Remote Cable (5061-3378)
4. Pump
5. Autosampler
6. Controller Area Network (CAN) bus cables (5181-1516) included with each HP 1100 module
7. Column Compartment
8. Detector
9. HP 10833B HPIB cable included with HP ChemStation
10. HP ChemStation
11. Centronics cable included with HP ChemStation
12. Printer

Figure 29

2: HP 1100 Series G1322A Vacuum Degasser,
4: HP 1100 Series G1311A Quaternary Pump
5: HP 1100 Series G1313A Autosampler
7: HP 1100 Series G1316A Thermostatted Column Compartment
8: HP 1100 Series G1314A Variable Wavelength Detector.
10: HP Chemstation (HPLC control and integration software)

The mobile phase was degassed by the vacuum degasser and mixed in a ratio of 80/20 volume/volume (acetonitrile/methanol) by the multi-channel gradient valve in the quaternary pump. The flow rate was 1.5 mLs per minute. The separation was carried out on 30 µl extracts in mobile phase, isocratically, on a 150 x 3.2 mm column of 5 µ particles of Ultracarb ODS (20) (Phenomenex). This column was protected by a 30 x 4.3 mm guard column of 5 µ particles of Ultracarb ODS (20) (Phenomenex). The variable wavelength detector was programmed to monitor at 325 nm from zero to 4.5 minutes and at 292 nm from 4.5 to 10.5 minutes. Retention times for Vitamin A (Retinol) and Vitamin E (α-tocopherol) were 1.9 minutes and 8.2 minutes respectively. Retention times for the Internal standards retinol acetate and tocopherol acetate were 2.3 minutes and 9.5 minutes respectively. Chemstation software monitored the output and then integrated the peaks using an internal standard method.

Standard preparation

Stock standards were stored at –20 °C and working standards were prepared under natural light and stored at –20°C. Table 4 shows stock concentrations and dilutions to produce working standards, formula weights and molar extinction coefficients.

Table 4: Stock and Working Standards

Vitamin	Absorbance Wavelength (nm)	Formula Weight	Molar Extinction Coefficient	Stock Concentration	Working Standard Dilution
Retinol	324 –325	286.5	52572.75	3.49 mmol/L	125 µl stock + 25 ml of ethanol
Retinol Acetate	326	328.8	50917.50	3.04 mmol/L	125 µl stock + 25 ml of ethanol
α-tocopherol	294	430.7	3057.97	4.64 mmol/L	125 µl stock + 25 ml of ethanol
Tocopherol acetate	284	472.8	2033.04	4.23 mmol/L	500 µl stock + 25 ml of ethanol

Working standard concentrations are calculated by use of the Beer-Lambert law, which states that the absorption of light energy at any wavelength is solely dependent on the concentration of the absorbing material and the length of the light path through the absorbing medium. The equation for this relationship is given below:

$$A = \log_{10} {}^{I_0}/_{I} = \varepsilon c l$$

Where A = absorbance, I_o = intensity of incident light, I = intensity of transmitted light, c = concentration, ε = molar extinction coefficient and l = the light path which is usually 1 centimetre.

Given the extinction coefficient of a substance at a wavelength corresponding to maximum absorption, any solution of this substance can have its concentration determined by measuring the absorbance of the unknown solution at the same wavelength, provided there are no interfering substances present.

Working solutions of retinol and α-tocopherol are scanned over a wavelength range (270 nm to 370 nm for retinol and 250 nm to 350 nm for α-tocopherol) on a Perkin-Elmer Lamda 5 UV/VIS spectrophotometer blanking with ethanol. Use of the above equation allows the concentration of the working standards to be determined using the molar extinction coefficient and the maximum absorbance

obtained on the scan which relates to either retinol or α-tocopherol. You do not require to calculate the concentrations of tocopherol acetate or retinol acetate as they are used as internal standards. Both tocopherol acetate and retinol acetate are added to the same ethanol solution to produce a combined internal standard solution.

Sample preparation

200 µl of retinol and 200 µl α-tocopherol are mixed with 200 µl of HPLC grade water (BDH) and 200 µl of internal standard solution. 200 µl of plasma are mixed with 400 µl of ethanol and 200 µl of internal standard solution. The standard/samples are vortex mixed for 1 minute and then 400 µl of hexane is added. The standard/samples are vortex mixed for a further 3 minutes and then spun at 1500 rpm for 5 minutes. 300 µl of the upper (hexane) layer is removed into an eppendorph tube. The hexane is evaporated to dryness using a heating block, set at 50 °C, under a stream of oxygen free nitrogen for 2 minutes. The standard/samples are reconstituted in 100 µl of methanol and vortexed thoroughly for 3 minutes. The sample is then placed into a glass insert in an amber vial and sealed with a butyl rubber seal and screw cap. The vial is placed on the auto-sampler and 30 µl is injected onto the column. The Chemstation software collects and integrates the data. From the method described above the known concentrations of retinol and α-tocopherol standards are typed into the calibration table of the Chemstation software to allow for the calculation of your unknown samples. The internal standard allows for extraction variability.

Quality Control

Each assay run of vitamin analysis has a quality control sample at position 3 after the two standard vials and every 10th position thereafter plus one level at the end of the run.

Sensitivity

The lowest detectable level of was estimated to be 0.1 µmol/L for vitamin A and 1 µmol/L for vitamin E.

Precision:

The precision was assessed using unassayed quality control material form Bio-Rad (Lyphocheck Immunoassay controls 1 & 3).

Vitamin A:

Level (µmol/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
0.44	7.2%	8.8%
1.44	3.8%	8.0%

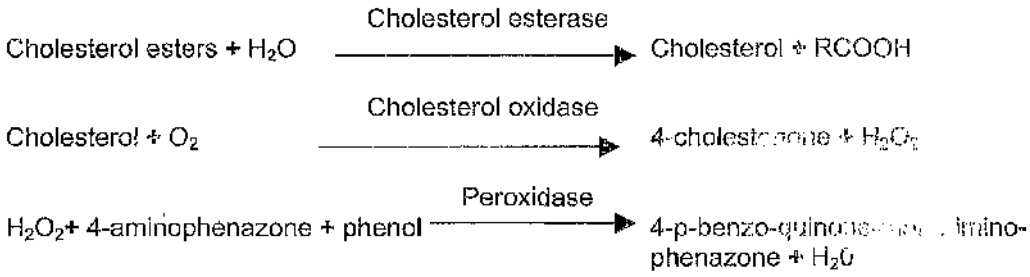
Vitamin E:

Level (µmol/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
8	7.0%	9.2%
15	3.1%	6.0%

2.2.2 Cholesterol analysis

Principle

Cholesterol esters are hydrolysed by cholesterol esterase to form fatty acids and free cholesterol, which is subsequently oxidised to cholestenone and hydrogen peroxide by cholesterol oxidase. The hydrogen peroxide is oxidised by a peroxidase liberating oxygen which is used in the oxidative condensation of phenol and 4-aminophenazone to form 4-(p-benzoquinone-monoimino)-phenazone, a red coloured compound, which is measured bichromatically at 505/700nm. Analysis was carried out on the Hitachi 717 clinical chemistry analyser.



Reagents.

Roche Diagnostics, Kit Catalogue Number: 1489437 10 x 100 mL

Pipes Buffer pH 6.8	75 mmol/L.
Magnesium	10 mmol/L.
4-Aminophenazone	0.15 mmol/L.
Sodium cholate	0.2 mmol/L.
Phenol	4.2 mmol/L.
Fatty acid polyglycolether	0.1 %.
Cholesterol esterase	0.5 U/mL.
Cholesterol oxidase	>0.15 U/mL.
Peroxidase	0.25 U/mL.

Store at 2-8°C

Standard.

Precipath L: Roche Diagnostics , Catalogue Number:1285874. 4 x 3 mL.

Method.

The analyser is calibrated once a week, or as required, using the Precipath L standard.

Internal Quality Control

Diagnostics Scotland Low, Mid and High quality control material are analysed every day

Method Linearity.

0.08 - 20.7 mmol/L

Sensitivity.

0.08 mmol/L

Precision

Precision was determined using low, mid and high quality control material from Diagnostic Scotland.

Level (mmol/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
3.9	0.82%	2.7%
5.9	0.93%	2.0%
7.5	0.74%	1.7%

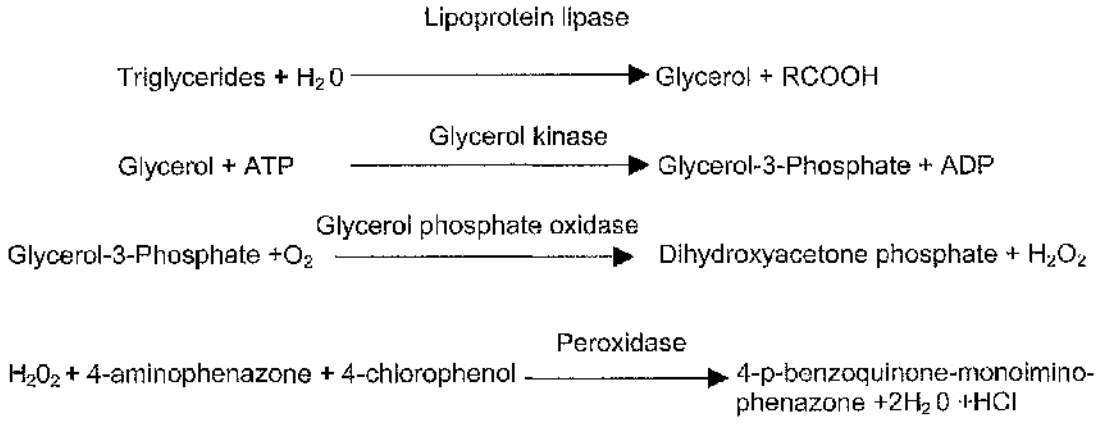
Interference.

Haemoglobin >2g/L.

2.2.3 Triglyceride analysis

Principle

Triglycerides are hydrolysed by lipase to glycerol and fatty acids the former being converted to glycerol -3- phosphate in the presence of ATP by glycerokinase in the first auxiliary reaction. Glycerophosphate oxidase converts glycerol -3-phosphate to dihydroxyacetone phosphate with the formation of hydrogen peroxide. This hydrogen peroxide is used in the final indicator reaction in the oxidative condensation of 4-aminophenazone and 4-chlorophenol via peroxidase to form a red coloured compound which is measured bichromatically as 4-(p-benzoquinone-mono-imino)-phenazone at 505/700 nm. Analysis was carried out on the Hitachi 717 clinical chemistry analyser.



Reagents.

Roche Diagnostics, Kit Catalogue Number: 1488899, 10 x 100 mL

Reagent:

Pipes buffer pH 6.8	50 mmol/L.
Magnesium sulphate.	40 mmol/L.
Disodium EDTA.	10 mmol/L.
Sododium cholate	0.2 mmol/L.
4-chiorophenol	4.7 mmol/L.
Potassium hexacyanoferrate	1 µmol/L.
Fatty alcohol polyglycolether	0.65%.
Adenosine triphosphate	1.4 mmol/L
4-Aminophenazone	0.13 mmol/L
Lipoprotein lipase	5 U/mL.
Glycerol phosphate Oxidase	2.5 U/mL.
Glycerol kinase	0.19 U/mL.
Peroxidase	0.10 U/mL.

Stable up to expiration date at 2-8°C or 14 days refrigerated on analyser opened.

Standard.

Precipath L. Roche Diagnostics, Kit Catalogue Number: 1285874. 4 x 3 mL.

Method.

The analyser is calibrated once a week, or as required, using the Precipath L standard.

Internal Quality Control

Diagnostics Scotland Low, Mid and High quality control material are analysed daily.

Linearity

0.05 - 11.4 mmol/L.

Sensitivity.

0.05 mmol/L

Precision

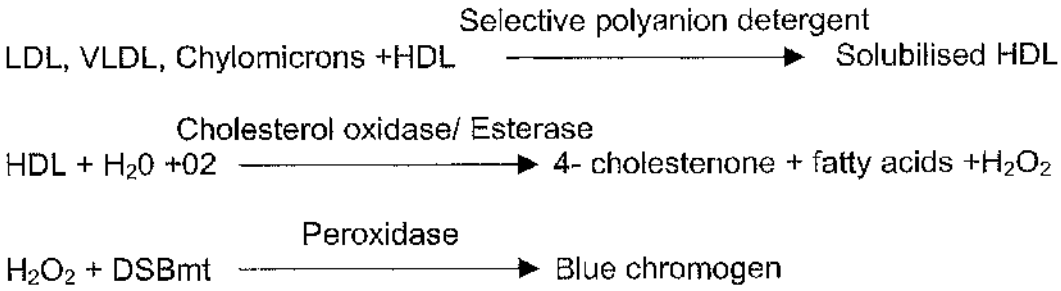
Precision was determined using low, mid and high quality control material from Diagnostic Scotland.

Level (mmol/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
1.10	1.18%	2.4%
1.85	0.86%	2.4%
2.73	0.81%	1.8%

2.2.4 High Density Lipoprotein (HDL) analysis

Principle

A selective detergent solubilises only HDL, the other lipoproteins (LDL and VLDL) and chylomicrons are left inactive against the enzyme reactivity. Hydrogen peroxide formed is oxidised and the chromogen N, N-bis (4-sulphobutyl)-m-toluidine-disodium (DSBmt) combines with 4-aminophenazone in an oxidative condensation reaction to form a blue complex proportional to the HDL-Cholesterol present and is measured at 600/700nm.



Reagents.
Bio-Stat, Kit Catalogue Number: 915090 1 x 60 ml, 1 x 20 ml.

Reagent 1

Buffer	30 mmol/L pH 7.0
4-aminophenazone	0.9 mmol/L
Polyanion detergent contains sodium hydroxide	2.8 mg

Reagent 2

Cholesterol esterase	7200 U/L
Cholesterol oxidase	7200 U/L
Chromogen (DSBmt)	1.1 mmol/L
Sodium hydroxide	2.8 mg.

Store at 4° C.

Standardisation

Bio Stat calibrator Catalogue Number: 915084. Concentration: 1.4 mmol/L CDC referenced.
Store at 2-8 °C. Stable for 7 days after reconstitution at this temperature.

Method.

Analysis was carried out on the Hitachi 717 clinical chemistry analyser along with total cholesterol and triglycerides as part of the lipid profile. The analyser calibrated once a week while blanking the reagent daily

Linearity.

0.08 - 3.12 mmol/L

Internal Quality Control

Diagnostics Scotland Low, Mid and High quality control material are analysed daily.

Sensitivity.

0.08 mmol/L

Precision

Precision was determined using low, mid and high quality control material from Diagnostic Scotland.

Level (mmol/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
1.38	1.3%	3.25%
1.58	1.0%	4.74%
2.21	1.5%	3.63%

Interferences.

Unaffected by bilirubin up to 342 µmol/L, Haemolysis up to 1.6 mmol/L, and Triglyceride up to 13.5 mmol/L

2.2.5 C-Reactive Protein analysis

Principle

Buffered antigen and specific antibody combine to form an immunoturbidmetric solution. Polyethylene glycol was added to increase polymerisation with dithioerytritol to minimise interference from Rheumatoid Factor by denaturing it. The turbidity is measure bichromatically at 340/700 nm. Analysis was carried out on the Hitachi 717 clinical chemistry analyser.

Reagents.

Reagent 1

Dako reaction Buffer. Catalogue Number: S2006. Store at 4°C.

Reagent 2

Dako Rabbit anti-human CRP. Catalogue Number: Q0329 Store at 4° C.

Diluent Buffer Catalogue Number: S2005 Store at 4° C.

Dilute exactly 10 ml of antibody (Reagent 2) with 10 ml of dilution buffer and mix gently to avoid frothing.

Standardisation

Protein Reference Unit (PRU) CRP calibrant SPS-03 175 mg/L WHO Referenced. Store at –20°C.

Method.

Analysis was carried out on the Hitachi 717 clinical chemistry analyser. The analyser is calibrated once a week while blanking the reagent daily

Linearity.

0 – 175 mg/L.

Internal Quality Control

SPS-12 (50 mg /L), SPS-13 (10 mg/L). Supplied from the Protein Reference Unit.
Store at –20^{O}C

Reference Range.

0 – 10 mg/L

2.2.6 Cardiac Troponin I Analysis

Introduction

Cardiac troponin I (cTnI, molecular weight: 24000) is a contractile protein exclusively present in the cardiac muscle[259,260]. Troponin I is one of three subunits of the troponin complex (I,T,C), which with tropomyosin is bound to actin in the thin filament of the myofibril, its physiological role is to inhibit the ATPase activity of the actinmyosin complex in the absence of calcium, and therefore, to prevent muscular contraction[261].

Three troponin I tissue isoforms have been identified:

- Fast troponin I and slow troponin I with molecular weights of 19800 each, expressed in fast twitch and slow twitch skeletal muscle fibers, respectively.
- Cardiac troponin I with an N-terminal site having an additional chain of 31 amino acid residues.

Cardiac troponin I levels in acute myocardial infarction (AMI) exhibit similar rise and fall patterns to those found in CK-MB. Cumulative data from several studies indicate troponin I levels are detectable (above quoted values for non AMI samples) 3-6 hours after the onset of chest pain. Troponin I levels peak at approximately 24 in non-thrombolysed AMI patients and 10 to 12 hours in thrombolysed patients and can remain elevated for 5-6 days post AMI[262,263,264]. Sequencing of cardiac troponin I from mammals has shown important differences between the cardiac[265] and skeletal[266] forms. Skeletal muscle does not express cardiac troponin I, either during development or in response to stimuli[260]. No cross reactivity with skeletal troponin I isoforms allows distinction between cardiac and skeletal injuries, and allows diagnosis of myocardial

infarction even when associated with muscle lesions (rhabdomyolysis, polytraumatism) [264,267,268,269].

Cardiac troponin I appears to be particularly useful in the following areas:

- Exclusion/confirmation of myocardial infarction.
- Risk assessment of patients with unstable angina pectoris and diagnosis of microinfarcts.
- Monitoring of the outcome of thrombolytic therapy.
- Diagnosis of pan-operative myocardial damage (Coronary artery bypass grafting, angioplasty).
- Diagnosis of rejection in heterotropic cardiac transplantation.

Principle

The Beckman Coulter Access troponin I assay is an immunoenzymatic ("sandwich") assay. A sample is added to a reaction vessel along with monoclonal anti-cardiac troponin I antibody conjugated to alkaline phosphatase and paramagnetic particles coated with monoclonal anti-cardiac troponin I antibody. The human cardiac troponin I binds to the anti-cardiac troponin I antibody on the solid phase, while the anti-cardiac troponin I antibody-alkaline phosphatase conjugate reacts with different antigenic sites on the cardiac troponin I molecules. After the incubation, the separation in a magnetic field and washing removes material not bound to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, is added and the light generated by its reaction with the captured alkaline phosphatase is measured with a luminometer. The photon production is proportional to the amount of enzyme conjugate present at the end of the reaction, and therefore, to the concentration of cardiac troponin I in the sample. The amount of analyte in the sample is determined by means of a stored, multi-point calibration curve.

Reagents

Beckman Coulter (UK), Catalogue Number: 33320: 100 determinations, 2 packs, 50 tests/pack

A pack is stable until the expiration date stated on the label when stored at 2 to 10°C. After initial use, the pack is stable at 2 to 10°C for 28 days.

Beckman Coulter Access Troponin I Reagent Pack: R1

R1a: Paramagnetic particles coated with mouse monoclonal anti-human cardiac troponin I suspended in TRIS-NaCl buffer, with bovine serum albumin (BSA), 0.05% sodium azide.

R1b: 0.1N NaOH

R1c: 0.08 M succinic acid

R1d: Purified mouse immunoglobulins, in TRIS-NaCl buffer, with <0.1% sodium azide, 0.06% ProClin** 300.

R1e: Conjugate: Mouse monoclonal anti-human cardiac troponin I coupled to alkaline phosphatase in TRIS-NaCl buffer, with BSA, and <0.1% sodium azide.

Patient Sample

Serum samples collected with Becton Dickson separator gel tubes were used for this assay. Fifty (50) µl of sample is used for each determination. Samples containing up to 110 mg/l bilirubin, 500 IU/L heparin, 50,000 IU/L streptokinase, lipaemic samples containing the equivalent of 6 g/L triglycerides and haemolysed samples containing up to 5 g/L haemoglobin do not affect the concentration of cardiac troponin I assayed.

Quality Control

Beckman Coulter catalogue Number: 33329, Access Troponin I QC: 2.5 ml/vial. 6 x lyophilized vials, 3 levels.

2 x QC1: Human cardiac troponin I at a level of approximately 0.25 µg/L in buffered human serum matrix containing0.1 % Kathon.

2 x QC2: Human cardiac troponin I at a level of approximately 5 µg/L in buffered human serum matrix containing 0.1% Kathon.

2 x QC3: Human cardiac troponin I at a level of approximately 25 µg/L in buffered human serum matrix containing 0.1% Kathon.

Each level is assayed once a day.

Sensitivity

0.03 µg/L

Precision

Precision was determined using quality control material from Beckman Coulter as described above.

Level (µg/L)	Within Batch CV (n = 10)	Between Batch CV (n = 50)
0.25	5.4%	7.0%
5.0	3.0%	5.1%
25.0	3.4%	6.1%

Calibrators

Beckman Coulter Access Troponin I Calibrators: catalogue Number:33325, 1 ml/vial. Lyophilized.

SO: Buffered human serum matrix with 0.1% Kathon. Contains 0.0 µg/l human cardiac troponin I.

S1, S2, S3, 54, S5: Human cardiac troponin I at levels of approximately 0.1, 0.5, 2, 10 and 50 µg/L, respectively, in buffered human serum matrix with 0.1% Kathon.

Details of Calibration

An active calibration curve is required for all tests. For the Troponin I assay, calibration is required every 28 days to maintain an active calibration curve

Results

Patient test results are calculated automatically by the system software using a smoothing spline curve math model. The amount of analyte in the sample is determined from the measured light production by means of the stored calibration curve.

Limitations of the Procedure

1. Samples can be accurately measured within the reportable range of the lower limit of detection and the highest calibrator value (approximately 0.03—50 ng/ml (µg/l)). If a sample contains more troponin I than the stated value of the S5 calibrator, report the result as greater than that value. Alternatively, dilute one volume of sample with an equal volume of Access Troponin I diluent.

2. Human anti-mouse antibodies (HAMA) may be present in samples from patients who have received immunotherapy[270,271]. This assay has been specifically formulated to minimize the effect of these antibodies on the assay. However, carefully evaluate results from patients known to have received such therapy or known to contain other heterophilic antibodies.

3. The Access Troponin I assay has no discernible "hook effect" up to 600 µg/L.

3 Studies: Outline of Studies, Results and Discussion.

3.1 Vitamins A and E In Nutrition Studies

3.1.1 Introduction

Studies have shown that 50% of patients (recruited from five disciplines - general surgery, general medicine, respiratory medicine, orthopaedic surgery and medicine for the elderly) are malnourished on admission to hospital and may deteriorate during their stay[272]. Patients who are undernourished are more likely to have complications of their illness[273], have prolonged wound healing[274] and take longer to recover and to be discharged from hospital[275]. Patients in this study who require either parenteral or enteral nutritional support are more likely to have deficiencies of important antioxidants as well as being subject to possible increased levels of free radical damage. In order to balance their nutritional deficiencies and maintain their nutritional status macronutrients are given as well as micronutrients such as retinol, vitamin E, glutathione and selenium. The antioxidant effects of such micronutrients, described earlier should afford protection against further free radical cellular damage.

As heart disease is the overall major cause of death in Scotland with the Greater Glasgow Health Board area showing one of the highest standardised mortality ratio (SMR) for ischaemic heart disease at 127 (Data supplied by the Registrar General for Scotland, the Office for National Statistics and the Governments Actuary's Department) in areas of highest deprivation. In 1998 Gordon McLaren and Marion Bain published a report (Deprivation and Health in Scotland – Insights form NHS Data), which provided an overview of the relationship between material deprivation and health in Scotland. In the introduction to this report the Chief Medical Officer for Scotland voiced particular

concern as to the differences in levels of health between the most affluent and most deprived. Coronary heart disease was highlighted, as mortality rates form acute myocardial infarction (AMI) in those under 65 are higher in those from more deprived areas. Because of this finding subjects from two general practices, in different areas of deprivation category, are also assessed.

3.1.2 Objectives

The objectives of this study were to measure vitamin E a lipid antioxidant and vitamin A to assess adequacy of status. In assessing subjects' vitamin levels a range of hospital ward environments were selected, general wards, high dependency and intensive care units in the Victoria Infirmary that reflect severity of illness of the subject. It was hoped to assess whether severity of illness (possible low antioxidant status and elevated free radical production) would show different, degrees of depletion of vitamin E. C-reactive protein[276], an acute phase protein produced by the liver, was also measured in the general ward, nutritional support and ICU patient groups as the acute phase is known to reduce the cholesterol content in both low and high-density lipoproteins[277]. Subjects from two general practices in areas of Glasgow of differing deprivation category based on postal code were studied. The deprivation category assessed by Carstairs and Morris[253] were as follows: For Glasgow post code G45 – deprivation category 7, G73 – deprivation category 6, G44 - deprivation category 2, and G76 -deprivation category 1, deprivation category 7 being the most deprived and deprivation category 1 the least deprived. The standardised mortality ratio (SMR) reported in the year 1999 (males and females 0 – 64 years), as assessed by postal code, were as follows: G45 – 127, G73 – 118, G44 – 83 and G76 – 83 . These patients also had their vitamin levels measured to compare them against each area and against the ward subjects. It was hoped to assess whether there is a difference in levels in the general population and the hospital population.

3.1.3 Reference Ranges

Normal ranges were calculated from data obtained from the normal control group (Table 7). The traditional method of describing a reference range, shown below, was used as the vitamin E and vitamin A in this group were normally distributed (Table 6) and is sufficiently consistent with textbook ranges (Tietz, Clinical Chemistry, second edition 1994), vitamin E: 12 – 46 μmol/L and vitamin A: 1.05 – 2.8 μmol/L

Reference range: Mean ± 1.96 standard deviations, which encompasses 95% of the reference sample.

Vitamin A:	1.06 – 3.74 μmol/L
Vitamin E:	13 – 52 μmol/L

As discussed in section 1.4.2 vitamin E is transported in the plasma within lipoproteins and varies with total lipid. In line with other researchers the vitamin E has been corrected for cholesterol and triglyceride. However as vitamin E is predominantly transported within LDL and HDL the vitamin E has also been corrected for cholesterol alone. The vitamin E/cholesterol ratio is normally distributed in control subjects therefore the above method was used to calculated a normal range. However the vitamin E/(cholesterol +triglyceride) ratio is non-normally distributed (table 6) therefore the median and inter-quartile range is used.

Reference Range:

Vitamin E/(Cholesterol + Triglyceride) Ratio:	3.46 – 6.06
Vitamin E/Cholesterol Ratio:	3.01 – 9.17

Sex Difference:

Using the larger general practice study group there was no statistically significant difference of vitamin E, lipid corrected vitamin E or cholesterol corrected vitamin E between males and females. However males appeared to

have a slightly higher vitamin A level than females (p=0.04).

In the clinical context, with cholesterol measurements it is preferable to consider 'action limits' for primary and secondary prevention and for patients 'at risk'. 'At risk' groups defined those at high risk (approximately 3% per year) of a coronary event – non-fatal MI or coronary death. These current guidelines have been adopted by Greater Glasgow Health Board.

The following give details of 'at risk' patients:

- Men aged 55 to 74 who smoke and are hypertensive.
- Men or women who are diabetic (<75 years).
- Men or women (<75 years) with a strong family history of coronary heart disease (CHD).
- Men or women (<75 years) with evidence of atherosclerotic disease. – e.g. intermittent claudication, peripheral vascular disease.
- Strong family history of hyperlipidaemia.

The following action limits which depend on risk status are currently in use.

Primary Prevention action limits:

Cholesterol: Men >8.0 mmol/L, Women >9.0 mmol/L

Secondary Prevention action limits (Men and Women):

Cholesterol: >5.0 mmol/L

'At risk' (Men and Women)

Cholesterol: >5.5 mmol/L

Other Reference values:

HDL-cholesterol: Men >1.0 mmol/L, Females >1.1 mmol/L

Triglyceride: <2.3 mmol/L.

3.1.4 Results

Statistical Analysis of Data from Nutrition Studies

The demographic details of the subjects studied in the six groups are summarised in table 5.

Table 5: Demographic Details

	Normal Controls	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients	GP Patients
N	26	540	70	186	171	99
Sex (M/F)	16/10	301/239	35/35	101/85	92/79	52/47
Mean Age	45.4 [12.0]	63.3 [17.0]	62.7 [15.7]	61.0 [16.4]	54.4 [12.6]	53.2 [14.3]
Age Range	22 to 64	15 to 99	17 to 89	15 to 85	27 to 80	17 to 79

Mean + [Standard Deviation]

The results for the Shapiro-Wilk W tests non-normality for all parameters studied in each subject group are summarised in table 6.

Table 6: Shapiro-Wilk W Test for Non-normality

	Normal Controls	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients	GP Patients
Vitamin A	0.9398 p: NS	0.9157 p: <0.0001	0.7960 p: <0.0001	0.8660 p: <0.0001	0.9542 p: <0.0001	0.9558 p: 0.0022
Vitamin E	0.9616 p: NS	0.8639 p: <0.0001	0.9415 p: 0.0027	0.9192 p: <0.0001	0.9677 p: 0.0005	0.9596 p: 0.0040
Cholesterol	0.9113 p: 0.0283	0.9580 p: <0.0001	0.9613 p: 0.0316	0.9708 p: 0.0007	0.9822 p: 0.0271	0.9818 p: NS
Triglyceride	0.8858 p: 0.0077	0.8656 p: <0.0001	0.7232 p: <0.0001	0.8012 p: <0.0001	0.8264 p: <0.0001	0.8012 p: <0.0001
HDL-Cholesterol	0.9072 p: 0.0228	0.9670 p: <0.0001	0.9671 p: NS	0.9119 p: <0.0001	0.8740 p: <0.0001	0.9721 p: 0.0484
Vitamin E/ Lipid Ratio	0.9079 p: 0.0236	0.9527 p: <0.0001	0.9685 p: NS	0.9913 p: NS	0.9771 p: 0.006	0.9691 p: NS
Vitamin E/ Cholesterol Ratio	0.9277 p: NS	0.9177 p: <0.0001	0.9475 p: 0.0058	0.9481 p: <0.0001	0.8738 p: <0.0001	0.9519 p: 0.0014
C-reactive Protein	NA	0.9440 p: 0.006	0.9660 p: 0.7947	0.9944 p: 0.09988	0.5661 p: <0.0001	NA

Table shows Shapiro-Wilk Coefficient and p value.

A p value < 0.05 indicates significant non-normality of distribution

As expected patient groups show non-normality of distribution for the above analysis. See section 2.1.5 for the application of appropriate statistical tests.

The measurements made in plasma in these nutritional studies are presented in table 7 as mean + standard deviation as well as the median + interquartile range. The vitamin E/lipid ratio is expresses as μmol of vitamin E per mmol of cholesterol and triglyceride in plasma. The vitamin E/cholesterol ratio is expressed as μmol of vitamin E per mmol of cholesterol in plasma.

Table 7: Vitamin, Lipid and CRP Levels

	Normal Controls	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients	GP Patients
Vitamin A (μmol/L)	2.40 [0.68] 2.33 [1.12]	1.25 [0.69] 1.17 [0.84]	0.84 [0.49] 0.71 [0.34]	0.84 [0.48] 0.74 [0.54]	2.02 [0.82] 1.88 [1.05]	2.55 [0.84] 2.50 [1.02]
Vitamin E (μmol/L)	32.6 [12.4] 31.5 [13.5]	26.3 [12.5] 24.0 [12.0]	20.2 [7.4] 19.0 [9.0]	17.7 [9.58] 16.0 [12.8]	35.0 [17.4] 36.0 [30.0]	44.2 [15.7] 38.4 [21.1]
Cholesterol (mmol/L)	5.28 [1.16] 5.45 [1.67]	3.78 [1.28] 3.60 [1.60]	2.93 [0.93] 2.95 [1.50]	2.38 [0.98] 2.30 [1.30]	4.18 [1.26] 4.30 [1.80]	5.90 [1.39] 5.80 [2.10]
Triglyceride (mmol/L)	1.66 [1.10] 1.22 [1.61]	1.48 [0.80] 1.31 [0.91]	1.52 [0.92] 1.40 [0.88]	1.57 [1.12] 1.32 [1.16]	1.47 [0.81] 1.35 [0.81]	1.96 [1.16] 1.69 [1.26]
HDL-Cholesterol (mmol/L)	1.36 [0.42] 1.24 [0.56]	0.96 [0.41] 0.90 [0.50]	0.80 [0.36] 0.80 [0.40]	0.64 [0.40] 0.60 [0.60]	1.28 [0.40] 1.20 [0.40]	1.26 [0.33] 1.20 [0.40]
CRP (mg/L)	LAB	88.7 [65] 78 [95]	206 [91] 204 [76.5]	186 [81] 184 [107]	35.7 [63.5] 13 [42.5]	NA
Vitamin E/ Lipid Ratio (μmol/mmol)	4.73 [1.19] 4.76 [1.30]	5.09 [1.85] 4.81 [2.11]	4.58 [1.32] 4.50 [1.56]	4.54 [1.53] 4.45 [1.96]	6.12 [2.37] 6.44 [3.10]	5.26 [1.24] 5.23 [1.33]
Vitamin E/ Cholesterol Ratio (μmol/mmol)	6.09 [1.57] 6.08 [1.56]	7.14 [2.72] 6.59 [3.11]	7.04 [2.15] 6.86 [2.31]	7.56 [2.58] 7.43 [2.86]	8.46 [4.10] 8.26 [4.02]	6.96 [1.80] 6.67 [2.10]

Mean+ [Standard Deviation] and Median + [Inter-quartile range]

The above data was presented as patient groups show non-normality of distribution.

No C-reactive protein analysis were carried out on the normal control group or the GP patient group.

LAB: For comparison of data the laboratory's normal reference range was used (up to10 mg/L).

Table 8: Correlation Between Vitamin E and Cholesterol

Subject Groups	Pearson Correlation r.	p.
Normal Controls	0.73 [0.48 – 0.87]	<0.0001
Ward Patients	0.52 [0.45 – 0.58]	<0.0001
High Dependency Unit	0.53 [0.33 – 0.68]	<0.0001
Intensive Care Unit	0.73 [0.66 – 0.79]	<0.0001
Home Nutrition Patients	0.58 [0.47 – 0.67]	<0.0001
GP Patients	0.73 [0.62 – 0.81]	<0.0001

Number in brackets represents 95% Confidence Interval

Table 9: Correlation Between Vitamin E and Triglyceride.

Subject Groups	Pearson Correlation r.	p.
Normal Controls	0.53 [0.18 to 0.76]	0.0051
Ward Patients	0.37 [0.29 to 0.44]	<0.0001
High Dependency Unit	0.15 [-0.09 to 0.38]	NS
Intensive Care Unit	-0.23 [-0.37 to –0.09]	<0.0001
Home Nutrition Patients	0.50 [0.38 to 0.60]	<0.0001
GP Patients	0.59 [0.44 to 0.70]	<0.0001

Number in brackets represents 95% Confidence Interval

Table 10: Correlation Between Cholesterol and CRP

Subject Groups	Pearson Correlation r.	p.
Ward Patients	-0.39 [-0.55 to –0.20]	0.0002
High Dependency Unit	-0.12 [-0.60 to –0.42]	0.6686
Intensive Care Unit	-0.14 [-0.47 to 0.23]	0.4652
Home Nutrition Patients	-0.07 [-0.42 to 0.30]	0.7138

Number in brackets represents 95% Confidence Interval

Table 11: Statistical Analysis (Mann-Whitney) CRP Nutrition Study

	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients				
High Dependency Unit	<0.0001↑			
Intensive Care Unit	<0.0001 ↑	NS		
Home Nutrition Patients	<0.0001 ↓	<0.0001 ↓	<0.0001 ↓	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: The HDU subjects have a significantly higher CRP levels than the ward subjects.

Table 12: Statistical Analysis (Mann-Whitney) Vitamin A Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	<0.0001 ↓				
High Dependency Unit	<0.0001 ↓	<0.0001 ↓			
Intensive Care Unit	<0.0001 ↓	<0.0001 ↓	NS		
Home Nutrition Patients	0.0098 ↓	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	
GP Patients	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: The ward subjects have a significantly lower Vitamin A than the normal control group.

Table 13: Statistical Analysis (Mann-Whitney) Vitamin E Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	0.0035 ↓				
High Dependency Unit	<0.0001 ↓	<0.0001 ↓			
Intensive Care Unit	<0.0001 ↓	<0.0001 ↓	0.0100 ↓		
Home Nutrition Patients	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	
GP Patients	0.0113 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	0.0112 ↑

NS = Not statistically significant ↑ Significantly higher. ↓ Significantly lower

Table should be read left to right. E.g.: The ward subjects have a significantly lower Vitamin E than the normal control group.

Table 14: Statistical Analysis (Mann-Whitney): Cholesterol Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	<0.0001 ↓				
High Dependency Unit	<0.0001 ↓	<0.0001 ↓			
Intensive Care Unit	<0.0001 ↓	<0.0001 ↓	<0.0001 ↓		
Home Nutrition Patients	<0.0001 ↓	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	
GP Patients	0.0605	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑

NS = Not statistically significant ↑Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: The ward subjects have a significantly lower cholesterol than the normal control group.

Table 15: Statistical Analysis (Mann-Whitney): Triglyceride Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	NS				
High Dependency Unit	NS	NS			
Intensive Care Unit	NS	NS	NS		
Home Nutrition Patients	NS	NS	NS	NS	
GP Patients	NS	<0.0001 ↑	0.0157 ↑	0.0011 ↑	0.0003 ↑

NS = Not statistically significant ↑Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: There is no statistically significant difference in triglyceride levels between ward patients and the normal control group.

Table 16: Statistical Analysis (Mann-Whitney): HDL-Cholesterol Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	<0.0001 ↓				
High Dependency Unit	<0.0001 ↓	0.0025 ↓			
Intensive Care Unit	<0.0001 ↓	<0.0001 ↓	0.0021 ↓		
Home Nutrition Patients	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	
GP Patients	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	NS

NS = Not statistically significant ↑Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: The ward subjects have a significantly lower HDL-cholesterol than the normal control group.

Table 17: Statistical Analysis (Mann-Whitney)

Vitamin E/ Lipid (Cholesterol + Triglyceride) Ratio: Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	NS				
High Dependency Unit	NS	0.0354 ↓			
Intensive Care Unit	NS	0.0016 ↓	NS		
Home Nutrition Patients	0.0014 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	
GP Patients	0.0262 ↑	0.0520	0.0003 ↑	<0.0001 ↑	0.0002 ↓

NS = Not statistically significant ↑Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: High dependency unit subjects have a significantly lower Vitamin E/lipid ratio than the ward patient group.

Table 18: Statistical Analysis (Mann-Whitney)

Vitamin E/ Cholesterol Ratio: Nutrition Study

	Normal Controls	Ward Patients	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Ward Patients	<0.0001 ↑				
High Dependency Unit	<0.0001 ↑	NS			
Intensive Care Unit	<0.0001 ↑	0.0134 ↑	NS		
Home Nutrition Patients	0.0098 ↑	<0.0001 ↑	0.0035 ↑	0.0240 ↑	
GP Patients	0.0221 ↑	NS	NS	0.0337 ↓	0.0006 ↓

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table should be read left to right. E.g.: The ward patient subjects have a significantly higher Vitamin E/cholesterol ratio than the normal control group.

Table 19: GP Data: Patients of Known Deprivation Category

	Normal Controls	GP Patients Dep. Cat 1 & 2	GP Patients Dep. Cat 6 & 7
n.	26	50	25
Mean Age	45.5 [12.0]	54.9 [11.4]	51.2 [13.8]
Sex (M/F)	16/10	31/19	9/16
Age Range	22 to 64	26 to 75	32 to 73
Vitamin A (μmol/L)	2.40 [0.68] 2.33 [1.12]	2.79 [0.82] 2.66 [0.66]	2.49 [0.62] 2.42 [0.90]
Vitamin E (μmol/L)	32.6 [12.4] 31.5 [13.5]	45.4 [16.4] 41.8 [23.3]	42.5 [13.9] 37.9 [19.5]
Cholesterol (mmol/L)	5.28 [1.16] 5.45 [1.67]	6.29 [1.33] 6.30 [1.68]	5.93 [1.23] 6.00 [2.10]
Triglyceride (mmol/L)	1.66 [1.10] 1.22 [1.61]	2.10 [1.16] 1.86 [1.26]	2.34 [1.40] 2.28 [1.87]
HDL-Cholesterol (mmol/L)	1.36 [0.42] 1.24 [0.56]	1.29 [0.38] 1.25 [0.43]	1.17 [0.28] 1.10 [0.40]
Vitamin E/ Lipid Ratio	4.73 [1.19] 4.76 [1.30]	5.37 [1.30] 5.02 [1.26]	5.18 [1.10] 5.54 [1.21]
Vitamin E/ Cholesterol Ratio	6.09 [1.57] 6.08 [1.56]	7.16 [2.02] 6.46 [2.55]	7.12 [1.56] 7.22 [1.72]

Mean Levels + [Standard Deviation] and Median + [Inter-quartile Range]

Table 20 compares the results found in subjects from least deprived areas (Dep. Cat. 1 & 2) with the other nutrition study groups including subjects from the most deprived areas (Dep. Cat. 6 & 7).

Table 20: Statistical Analysis(Mann-Whitney)

GP Patients of deprivation category 1 & 2 compared to all other nutrition study groups.

Vs.	Normal Controls	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients	GP Patients Deprivation Cat. 6 & 7
Vitamin A (μmol/L)	NS	<0.0001↑	<0.0001 ↑	<0.0001 ↑	<0.0001↑	NS
Vitamin E (μmol/L)	0.0008 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	0.0012 ↑	NS
Cholesterol (mmol/L)	0.0032 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	NS
Triglyceride (mmol/L)	0.0469 ↑	<0.0001 ↑	0.0030 ↑	0.0006 ↑	<0.0001 ↑	NS
HDL-Cholesterol (mmol/L)	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	NS	NS
Vitamin E/ Lipid Ratio	0.0285 ↑	NS	0.0015 ↑	0.0006 ↑	0.0118 ↓	NS
Vitamin E/ Cholesterol Ratio	0.0375 ↑	NS	NS	NS	0.0265 ↓	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

E.g.: GP subjects of deprivation categories 1 & 2 have a significantly higher Vitamin A level than the ward patient subjects

There is no significant difference in any of the parameters between deprivation categories 1 & 2 and deprivation categories 6 & 7.

Table 21 compares the results found in subjects from most deprived areas (Dep. Cat. 6 & 7) with the other nutrition study groups including subjects from the least deprived areas (Dep. Cat. 1 & 2).

Table 21: Statistical Analysis(Mann-Whitney):GP Patients

GP Patients of deprivation category 6 & 7 compared to all other nutrition study groups.

Vs.	Normal Controls	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients	GP Patients Deprivation Cat. 1 & 2
Vitamin A (µmol/L)	NS	<0.0001↑	<0.0001 ↑	<0.0001 ↑	0.0017 ↑	NS
Vitamin E (µmol/L)	0.0115 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	0.0424 ↑	NS
Cholesterol (mmol/L)	NS	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	<0.0001 ↑	NS
Triglyceride (mmol/L)	0.0373 ↑	0.0013 ↑	0.0078 ↑	0.0025 ↑	0.0014 ↑	NS
HDL-Cholesterol (mmol/L)	NS	0.0018 ↑	<0.0001 ↑	<0.0001 ↑	NS	NS
Vitamin E/ Lipid Ratio	0.0438 ↑	NS	0.0051 ↑	0.0120 ↑	0.0125 ↓	NS
Vitamin E/ Cholesterol Ratio	0.0053 ↑	NS	NS	NS	0.0697	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

E.g.: GP subjects of deprivation categories 6 & 7 have a significantly higher Vitamin A level than the ward patient subjects.

There is no significant difference in any of the parameters between deprivation categories 1 & 2 and deprivation categories 6 & 7.

3.1.5 Discussion

The vitamin A measurements were included to reflect the plasma status of another fat soluble vitamin in the groups studied. The order of decreasing mean or median level of vitamin A (table 7) for these groups were:

GP Subjects >Control Subjects >Home Nutrition Subjects >Ward Subjects >High Dependency Unit Subjects and Intensive Care Unit Subjects

This order matches the intuitive expectations of the likely nutritional status for these subject groups. The position of the home nutrition group reflects and is indeed a tribute to the success of their dietary manipulation. However caution should be applied to these data as a plasma level is not an assessment of whole body stores and interpretation of levels may also be complicated by co-existing liver disease and acute phase response. Indeed only a few patients in the ICU and HDU groups showed basal values of < 0.7 µmol/L, a level that has previously been used to assess vitamin A deficiency.

The order of decreasing mean or median level of cholesterol and HDL-cholesterol (table 7) for the six groups were:

Cholesterol:

GP Subjects >Control Subjects >Home Nutrition Subjects >Ward Subjects >High Dependency Unit Subjects > Intensive Care Unit Subjects

HDL-Cholesterol:

Control Subjects > GP Subjects >Home Nutrition Subjects >Ward Subjects >High Dependency Unit Subjects > Intensive Care Unit Subjects

The plasma samples from GP subjects were stated to be fasting samples, however the triglyceride is only higher in this group and this finding may be due to possible dietary effects or non-fasting.

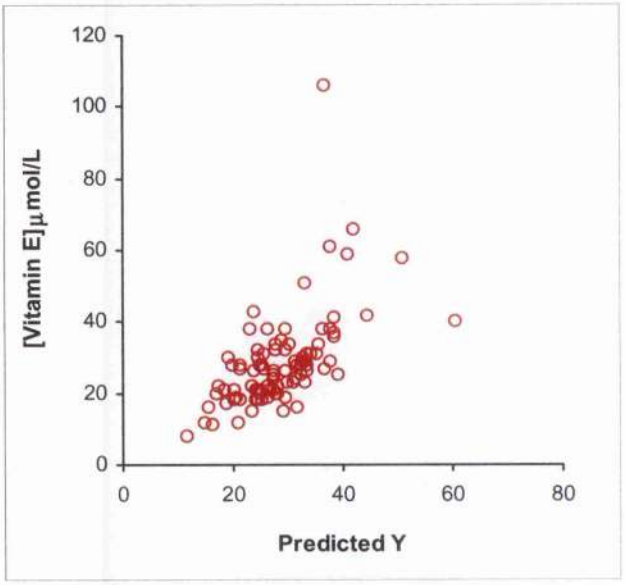
It was not anticipated that the level of plasma vitamin E found in the six subject groups would follow so closely the cholesterol levels with correlations

foremany of the groups as high as r = 0.73. This close correlation suggests some form of homeostatic mechanism in the LDL particle to retain vitamin E within the LDL particle.

As has been previously discussed there is a clear correlation (Table 8) between plasma cholesterol and plasma vitamin E but the correlation between triglyceride and vitamin E (Table 9) is not as close. Some vitamin E cholesterol correlation was expected as vitamin E is transported in the blood mainly in LDL. The correlation coefficient is maximal (0.73) in normal controls and GP subjects and lowest (0.52) in ward patients. The correlation between vitamin E and cholesterol in intensive care unit patients, which remains at 0.73 is surprising. This despite the low levels of cholesterol found in the plasma of these subjects. The median cholesterol of 2.30 mmol/L for intensive care patients (Table 7) is significantly lower (Table14) than the 5.45 mmol/L median found in control subjects. This observation challenges the convention that vitamin E depletion can best be found by examining the vitamin E/cholesterol ratio or even vitamin E/lipid (Cholesterol+Triglyceride) ratio in plasma. The vitamin E/Cholesterol ratio median of 7.43 found for the intensive care subject group is the highest found in all our subject groups, barring the home nutrition patients who all receive supplementation, at RNI level, in their feeding regimen. The intensive care unit subjects, who had been expected to be most challenged in terms of free radical exposure, show no lipid corrected vitamin E deficiency. This striking finding is possibly due to the effects of acute phase response on lowering cholesterol content in LDL and HDL. This cholesterol lowering results either from increased catabolism of cholesterol-rich lipoproteins, or the sequestration and retention of LDL in the endothelial spaces[277].

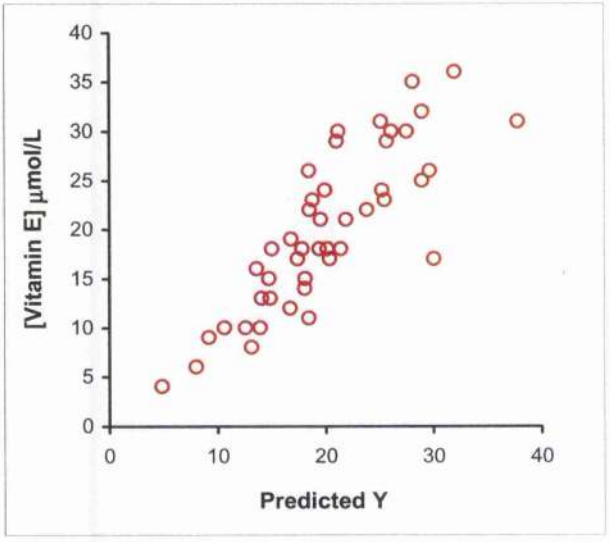
Table 10 indicates that there is a negative correlation between CRP and cholesterol. Figures 30,31 and 32 show multiple linear regression analysis of results from ward patients, acute ill patients in ICU and HDU and nutrition patients respectively. This statistical analysis is used to assess whether the acute phase

response, as indicated by CRP, has any significant effect when correlating vitamin E and cholesterol.



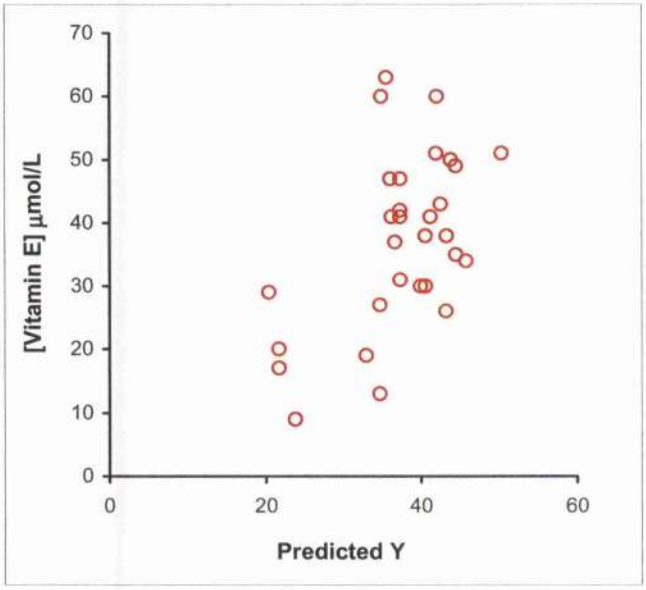
Cholesterol: p <0.0001; CRP: p 0.9141

Figure 30: Multiple Linear Regression. Vitamin E vs. Cholesterol and CRP Ward Patients



Cholesterol: p <0.0001; CRP: p 0.0104

Figure 31: Multiple Linear Regression. Vitamin E vs. Cholesterol and CRP Acutely Ill Patients (HDU and ICU)



Cholesterol: p 0.0030; CRP: p 0.9537

Figure 32: Multiple Linear Regression. Vitamin E vs. Cholesterol and CRP Home Nutrition Patients

The acute phase response has no significant effect on the correlation of vitamin E with cholesterol in either the ward group of patients or the home nutrition patients (Figs 30 and 32). In acutely ill patients in the ICU or HDU (Fig 31) the acute phase response has a significant effect on the correlation of cholesterol and vitamin E. This is an important observation and replicates the findings by Curran et al[291].

The data from tables 20 and 21 suggests that there is no statistical difference in any of the parameters measured between the least deprived GP subjects category 1 & 2 and the most deprived category 6 & 7. GP subjects of deprivation category 1 & 2 have significantly higher levels of cholesterol and triglyceride (table 19 & 20) than normal controls and GP subjects of deprivation category 6 & 7 have significantly higher levels of triglyceride (table 19 & 21) than normal controls. Both of the GP subject groups have higher vitamin E (table 19,20 &21), vitamin E/cholesterol ratios (table 19,20 &21) and vitamin E/lipid ratios

(table 19,20 &21) than normal controls. However it should be noted that there were significantly more females in the deprivation category 6 & 7 group than the other two groups and this may have an effect on the measured parameters.

These findings are at odds with what was expected. As there is a known association between deprivation and ischaemic heart disease, it was assumed that subjects with deprivation categories 6 & 7 would have increased levels of peroxidation and therefore lower levels of vitamin E. On the data from this study this appears not to be so and that vitamin E appears to be relatively well conserved even in deprived populations.

As previously reported there appears to be no sex difference in vitamin E level but there is a well known sex differential in relation to cholesterol.

3.2 Vitamins A and E in Intensive Care Unit Cardiac Injury Study .

3.2.1 Introduction

Specific biochemical markers the cardiac troponins have recently become more generally available to clinical laboratories. These new markers allow the determination of myocardial muscle damage. This protein is part of a regulatory complex (consisting of two subunits of troponin T, one subunit of troponin C and one subunit of troponin I) which lies on the tropomyosin strand along the actin filiment. The low homology between the skeletal muscle and cardiac isoforms, as well as a specific amino acid sequence in the cardiac isoform, has allowed the production of monoclonal antibodies to give a specific assay for the determination of the cardiac isoform. A serum marker which can reflect minor (as well as major) cardiac muscle cell damage provides the opportunity to observe the effects on vitamin E in patients who are facing free radial challenge.

Samples for measurement were taken over a six month period when all patients admitted to ICU, for any reason, were included. The patients had measurements of serum cTnI and were classified on the basis of these results. The points chosen for classification were taken from a study of clinical interpretation of cTnI results which classified levels from 1000 patients admitted to the Victoria Infirmary with acute chest pain[278]. This classification is as follows:

- A cTnI level of ≤ 0.04 μg/L indicates no myocardial injury assuming 6 hours post cardiac event.
- A cTnI level between 0.04 μg/L and 0.09 μg/L is borderline and may suggest developing myocardial injury if < 6 hours post cardiac event or the end point of a cardiac event if >120 hours post cardiac event.
- A cTnI level of ≥ 0.1 μg/L indicates myocardial tissue injury and a level >0.5 μg/L indicates myocardial damage most probably due to myocardial infarction.

Levels of cTnI may also allow a study of cardiac muscle damage in relation to the possible protective effect of vitamin E.

3.2.2 Objectives

To assess levels of vitamin E in a group of critically ill patients, in ICU on day one of their stay. This group of patients also had an assessment of myocardial muscle injury by measurement of cardiac troponin I.

3.2.3 Results

Table 22: Demographic Details for ICU Myocardial Injury Study

	cTnI Levels <0.04 No Myocardial Injury	CTnI levels >0.04 but <0.1 Borderline	CTnI levels >0.1 Myocardial Injury
n	48	15	45
Sex (M/F)	27/21	5/10	23/22
Mean Age	59.0 [18.7]	63.3 [22.0]	62.0 [18.8]
Age Range	19 to 85	26 to 99	19 to 89

The results for the Shapiro-Wilk W tests non-normality for all parameters studied in each subject group are summarised in table 23.

Table 23: Shapiro-Wilk W Test for Non-normality

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.04 but <0.1 Borderline	cTnI levels >0.1 Myocardial Injury
Vitamin A	0.9648 p: NS	0.8636 p: 0.0342	0.9321 p: 0.0124
Vitamin E	0.9560 p: NS	0.8933 p: NS	0.9415 p: 0.0243
Cholesterol	0.9677 p: NS	0.8429 p: 0.0138	0.09135 p: 0.0026
Triglyceride	0.8257 p: <0.0001	0.8142 p: 0.0056	0.08212 p: <0.0001
Vitamin E/ Lipid Ratio	0.9788 p: NS	0.9476 p: NS	0.09740 p: NS
Vitamin E/ Cholesterol Ratio	0.9791 p: NS	0.9641 p: NS	0.9252 p: 0.0064

Table shows Shapiro-Wilk Coefficient and p value.

A p value < 0.05 indicates significant non-normality of distribution

The measurements made in plasma in the ICU myocardial injury study are presented in table 24 as mean + standard deviation as well as the median + interquartile range. The vitamin E/lipid ratio is expressed as μmol of vitamin E per mmol of cholesterol and triglyceride in plasma. The vitamin E/cholesterol ratio is expressed as μmol of vitamin E per mmol of cholesterol in plasma.

Table 24: Vitamins A & E, Cholesterol and Triglyceride.

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.04 but <0.1 Borderline	cTnI levels >0.1 Myocardial Injury
Vitamin A (μmol/L)	1.23 [0.52] 1.12 [0.72]	0.78 [0.36] 0.76 [0.60]	1.06 [0.67] 0.97 [0.92]
Vitamin E (μmol/L)	20.4 [9.6] 18.0 [12.2]	17.3 [9.6] 16.0 [8.0]	19.7 [10.7] 18.0 [13.0]
Cholesterol (mmol/L)	3.14 [1.32] 2.78 [1.88]	2.30 [0.86] 2.28 [0.74]	3.10 [1.67] 2.62 [2.27]
Triglyceride (mmol/L)	1.20 [0.73] 1.05 [0.74]	1.10 [0.74] 0.88 [0.66]	1.55 [1.11] 1.18 [0.99]
Vitamin E/ Lipid Ratio	4.79 [1.38] 4.93 [1.85]	5.01 [1.92] 4.65 [2.08]	4.37 [1.42] 4.48 [1.52]
Vitamin E/ Cholesterol Ratio	6.73 [2.10] 6.75 [2.22]	7.36 [2.60] 7.46 [3.36]	6.62 [2.18] 6.30 [1.75]

Mean Levels + [Standard Deviation] and Median + [Inter-quartile Range]

Table 25: Serum Cardiac Troponin I Levels, ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.04 but <0.1 Borderline	cTnI levels >0.1 Myocardial Injury
Mean Cardiac Troponin I (μg/L)	0.017 [0.012]	0.059 [0.011]	0.808 [1.425]
Cardiac Troponin I (μg/L) Range	0.001 to 0.040	0.05 to 0.087	0.10 to 9.05

Mean Levels + [Standard Deviation]

Table 26: Statistical Analysis (Mann-Whitney): Vitamin A

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	0.0047 ↓	NS
cTnI levels >0.1 Myocardial Injury	0.0891	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Subjects with borderline cTnI levels have significantly lower levels of vitamin A than in subjects with no myocardial injury.

Table 27: Statistical Analysis (Mann-Whitney): Vitamin E

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	NS	NS
cTnI levels >0.1 Myocardial Injury	NS	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table 28: Statistical Analysis (Mann-Whitney): Cholesterol

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	0.0162 ↓	NS
cTnI levels >0.1 Myocardial Injury	NS	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Subjects with borderline cTnI levels have significantly lower levels of cholesterol than in subjects with no myocardial injury.

Table 29: Statistical Analysis (Mann-Whitney): Triglyceride

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	NS	0.0772
cTnI levels >0.1 Myocardial Injury	NS	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table 30: Statistical Analysis (Mann-Whitney).

Vitamin E/Lipid (Cholesterol + Triglyceride) Ratio

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	NS	NS
cTnI levels >0.1 Myocardial Injury	NS	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table 31: Statistical Analysis (Mann-Whitney): Vitamin E/Cholesterol Ratio

ICU Myocardial Injury Study

	cTnI Levels ≤0.04 No Myocardial Injury	cTnI levels >0.1 Myocardial Injury
cTnI levels >0.04 but <0.1 Borderline	NS	NS
cTnI levels >0.1 Myocardial Injury	NS	

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

3.2.4 Discussion

ICU Myocardial Injury Study

This study of vitamin E and lipid levels in patients in intensive care allowed an opportunity to examine vitamin E levels in a clinical situation where their antioxidant defences are likely to undergo challenge. It has already been shown that injury to the myocardium was a feature of these ill patients[254]. Those showing an elevated serum cardiac troponin I could be argued as having the greatest stress.

A surprisingly high proportion of patients (42%) had a level of cardiac troponin I > 0.1 µg/L in this ICU study population on their first day of admission. As the samples analysed were on the patients first day of admission to ICU it suggests that the injury had been initiated before admission to the unit. It is before ICU stabilisation that these patients may be exposed to the dual insults of hypoxia and hypotension. The myocardial injury in the majority of these patients may result from causes other that coronary thrombosis[279]. In fact a retrospective case-note review found that myocardial infarction had been diagnosed in only four cases. Increases in cTnI may be a consequence of membrane leakage and not a result of damage to the myofibrillar structure or cell death. Troponin I exists in a free cytosolic form as well as complexed with troponin T and troponin C[280]. This damage may be initiated by the action of oxygen free radicals on the cell membranes[281,282].

The vitamin A levels of the subjects with no evidence of myocardial injury (table 24) were similar to those found in general wards (table 8) in the previous nutrition study. The borderline group had lower levels of vitamin A (tables 24 & 26) but similar levels to those found in the ICU subjects (table7) in the nutrition study.

The patients with a borderline level of cTnI have the lowest cholesterol levels (tables 24 & 28). These findings may be related to the case mix in this study and may not be replicated in another examination. There is no significant difference in either the vitamin E/cholesterol ratio or vitamin E/lipid ratio between any of the cTnI groups (tables 24,30 & 31). All cTnI groups give similar ratios (table 24) to that found for ICU patients in the nutrition study (table 7). These findings should be treated with caution as cholesterol may be lowered in acute phase response. Unfortunately no CRP data is available for this set of patients. However in view of the findings of the previous study it is more than likely that these patients also have lowered cholesterol due to the acute phase response.

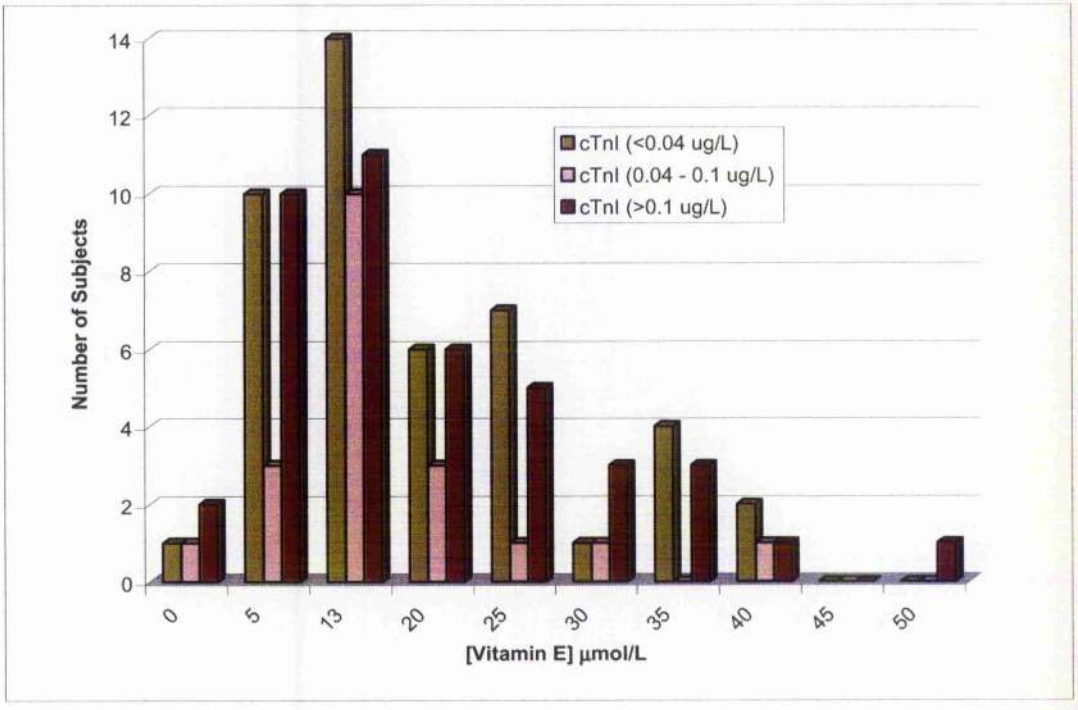


Figure 33: Distribution of Vitamin E in ICU Cardiac Injury Study

Figure 33 shows the distribution of vitamin E levels in the three study groups The distribution of patients with no myocardial injury (represented by cTnI levels of <0.04 ug/L) are of the same order as patients with myocardial injury (represented by cTnI levels of >0.1 ug/L). These data call into question the antioxidant action of vitamin E with regard to ischaemic damage caused by oxygen free radicals. But it is interesting to note that the potential role of vitamin E as an antioxidant has been called into question because there is vitamin E accumulated in the atherosclerotic lesion in humans[283]. It would appear that although vitamin E is an important antioxidant, its biological activity appears to modulate a variety of cellular functions, such as inhibition of smooth muscle cell proliferation[284,285] or modulation of platelet function[286] , which are not a result of its antioxidant activity. Tissues would therefore require an adequate supply of vitamin E if it is to be involved in cellular modulation. Low levels of plasma vitamin E may be insufficient to allow modulation of cellular processes. It was the results of this study, which reinforced the doubts about conventional measures of lipid correcting vitamin E in challenged states.

3.3 Vitamins A and E in Schizophrenia and Tardive Dyskinaesia.

3.3.1 Introduction

If the phospholipid hypothesis, with regard to schizophrenia outlined in 1.7.2. is correct then increased lipid peroxidation may deplete vitamin E as it is the major membrane antioxidant. This may also be the case in Tardive Dyskinesa because prolonged medication with neuroleptic drugs may lead to increased lipid peroxidation which in turn may lead to low levels of vitamin E[248].

3.3.2 Objectives

To measure Vitamins A and E (as well as cholesterol, triglyceride and HDL- Cholesterol) in schizophrenic patients with positive and negative symptoms to assess any difference in level between these groups and normal control subjects. Also to measure Vitamins A and E (as well as cholesterol, triglyceride and HDL- Cholesterol) to assess any difference between schizophrenic patients with and without symptoms of Tardive Dyskinesia and normal control subjects.

3.3.3 Results

Table 32: Demographic Details for Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms	Schizophrenic Patients Negative Symptoms
n	39	30	16
Sex (M/F)	21/18	22/8	13/3
Mean Age	37.2 [14.4]	36.1 [8.4]	49.0 [10.3]
Age Range	16 to 65	23 to 58	27 to 56

From the age data there is no significant difference between the controls and the positives but there are significant differences between the control group and the negatives ($p = 0.0070$) and between the positives and the negatives ($p = 0.0001$).

The results for the Shapiro-Wilk W tests non-normality for all parameters studied in each subject group are summarised in table 33.

Table 33: Shapiro-Wilk W Test for Non-Normality: Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms	Schizophrenic Patients Negative Symptoms
Vitamin A	0.9090 p: 0.0040	0.9644 p: NS	0.8821 p: 0.0418
Vitamin E	0.9554 p: NS	0.9386 p: NS	0.9442 p: NS
Cholesterol	0.9444 p: 0.0532	0.9765 p: NS	0.9553 p: NS
Triglyceride	0.7126 p: <0.0001	0.9422 p: NS	0.9078 p: NS
Vitamin E/ Lipid Ratio	0.9370 p: 0.0303	0.7320 p: <0.0001	0.8930 p: 0.0621
Vitamin E/ Cholesterol Ratio	0.9468 p: NS	0.7710 p: <0.0001	0.9342 p: NS

Table shows Shapiro-Wilk Coefficient and p value.

A p value < 0.05 indicates significant non-normality of distribution

The measurements made in plasma in the schizophrenia patients study are presented in table 32 as mean + standard deviation as well as the median + interquartile range. The vitamin E/lipid ratio is expressed as μmol of vitamin E per mmol of cholesterol and triglyceride in plasma. The vitamin E/cholesterol ratio is expressed as μmol of vitamin E per mmol of cholesterol in plasma.

Table 34: Vitamins A & E, Cholesterol and Triglyceride.

Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms	Schizophrenic Patients Negative Symptoms
Vitamin A (μmol/L)	1.89 [0.48] 1.67 [0.67]	1.73 [0.44] 1.64 [0.46]	1.96 [0.61] 1.82 [0.89]
Vitamin E (μmol/L)	34.7 [11.1] 33.0 [16.4]	33.3 [12.1] 32.0 [12.7]	40.8 [10.8] 40.7 [18.7]
Cholesterol (mmol/L)	4.66 [1.24] 4.40 [1.25]	4.59 [1.04] 4.72 [1.48]	5.42 [0.71] 5.57 [0.74]
Triglyceride (mmol/L)	1.34 [0.98] 1.08 [0.68]	1.97 [1.01] 1.76 [1.61]	2.34 [1.38] 2.18 [2.49]
Vitamin E/ Lipid Ratio	5.83 [1.16] 5.60 [1.36]	5.46 [2.88] 4.80 [1.52]	5.49 [1.82] 4.85 [2.60]
Vitamin E/ Cholesterol Ratio	7.47 [1.58] 7.11 [1.73]	7.73 [4.04] 6.86 [2.60]	7.62 [2.14] 7.80 [3.92]

Mean Levels + [Standard Deviation] and Median + [Inter-quartile Range]

Table 35: Statistical Analysis (Mann-Whitney): Vitamin A

Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	NS	
Schizophrenic Patients Negative Symptoms	NS	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table 36: Statistical Analysis (Mann-Whitney): Vitamin E Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	NS	
Schizophrenic Patients Negative Symptoms	0.0783	0.0338 ↑

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Patients with negative symptoms had a significantly higher vitamin E levels than patients with positive symptoms.

Table 37: Statistical Analysis (Mann-Whitney): Cholesterol Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	NS	
Schizophrenic Patients Negative Symptoms	0.0054 ↑	0.0065 ↑

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Patients with negative symptoms had borderline significantly higher cholesterol levels than either the normal controls or the patients with positive symptoms.

Table 38: Statistical Analysis (Mann-Whitney): Triglyceride Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	0.0016 ↑	
Schizophrenic Patients Negative Symptoms	0.0054 ↑	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Both types of schizophrenic patient had significantly higher levels of triglyceride than the normal control group.

Table 39: Statistical Analysis (Mann-Whitney) Vitamin E/ Lipid (Cholesterol + Triglyceride) Ratio: Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	0.0040 ↓	
Schizophrenic Patients Negative Symptoms	NS	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Only the schizophrenic patients with positive symptoms had a significantly lower vitamin E/lipid ratio than the normal control group

Table 40: Statistical Analysis (Mann-Whitney): Vitamin E/ Cholesterol Ratio Schizophrenia Patients Study

	Normal Controls	Schizophrenic Patients Positive Symptoms
Schizophrenic Patients Positive Symptoms	NS	
Schizophrenic Patients Negative Symptoms	NS	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Table 41: Demographic Details for Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Tardive Dyskinesia	Schizophrenic Patients Controls
n	10	16	16
Sex (M/F)	5/5	5/11	2/14
Mean Age	53.3 [11.5]	63.4 [11.1]	60.4 [12.1]
Age Range	22 to 64	34 to 87	35 to 84

From the age data there is a significant difference between the normal controls and both the Schizophrenic Control group (p: <0.05) and the Tardive Dyskinesia Group (p: <0.05).

Table 41a: Shapiro-Wilk W Test for Non-Normality: Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Tardive Dyskinesia	Schizophrenic Patients Controls
Vitamin A	0.9291 p: NS	0.8983 p: NS	0.9326 p: NS
Vitamin E	0.9553 p: NS	0.8267 p: NS	0.9591 p: NS
Cholesterol	0.7913 p: 0.0114	0.9556 p: NS	0.9346 p: NS
Triglyceride	0.9107 p: NS	0.8285 p: 0.0067	0.8790 p: 0.0459
Vitamin E/ Lipid Ratio	0.9099 p: NS	0.9290 p: NS	0.8930 p: 0.0621
Vitamin E/ Cholesterol Ratio	0.8966 p: NS	0.9788 p: NS	0.9321 p: NS

Table shows Shapiro-Wilk Coefficient and p value.

A p value < 0.05 indicates significant non-normality of distribution

Table 42: Vitamins A & E, Cholesterol and Triglyceride.

Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Tardive Dyskinesia	Schizophrenic Patients Controls
Vitamin A (μmol/L)	2.70 [0.60] 2.86 [0.88]	2.50 [0.93] 2.34 [1.02]	2.17 [0.50] 2.11 [0.76]
Vitamin E (μmol/L)	35.8 [7.4] 36.2 [7.2]	26.6 [11.2] 24.0 [12.2]	26.2 [6.1] 26.0 [9.3]
Cholesterol (mmol/L)	5.74 [0.81] 6.17 [1.24]	4.84 [1.29] 4.77 [1.84]	4.71 [0.95] 4.62 [1.42]
Triglyceride (mmol/L)	2.25 [1.07] 1.99 [1.09]	2.23 [1.32] 1.85 [1.69]	1.46 [0.72] 1.44 [0.78]*
HDL-Cholesterol (mmol/L)	1.27 [0.35] 1.28 [0.44]	0.97 [0.17] 0.97 [0.23]	1.10 [0.14] 1.12 [0.14]*
Vitamin E/ Lipid Ratio	4.50 [0.92] 4.23 [0.98]	3.74 [0.62] 3.94 [1.03]	4.44 [0.91] 4.12 [1.54]*
Vitamin E/ Cholesterol Ratio	6.22 [0.96] 6.54 [1.19]	5.42 [1.17] 5.62 [1.83]	5.76 [1.15] 5.88 [2.08]*

Mean Levels + [Standard Deviation] and Median + [Inter-quartile Range]

Although the majority of analytes for the above subject groups showed a normal distribution (Table 41a) median and Interquartile Ranges are shown for consistency.

* One patient was omitted from the schizophrenic control group as the triglyceride was >3SD from the mean and was therefore regarded as an outlier.

Table 43: Statistical Analysis (Independent Samples t-test): Vitamin A

Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	0.0108 ↓	
Schizophrenic Patients Tardive Dyskinesia	NS	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Schizophrenic controls had significantly lower vitamin A levels than the normal control group

Table 44: Statistical Analysis (Independent Samples t-test): Vitamin E

Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	0.0026 ↓	
Schizophrenic Patients Tardive Dyskinesia	0.0309 ↓	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

Both the schizophrenic controls and Tardive Dyskinesia patients has significantly lower levels of vitamin E than the control group.

Table 45: Statistical Analysis (Independent Samples t-test): Cholesterol

Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	0.0095 ↓	
Schizophrenic Patients Tardive Dyskinesia	0.0591	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

The schizophrenic control group had significantly lower levels of cholesterol than the normal control group.

Table 46: Statistical Analysis (Independent Samples t-test): Triglyceride Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	0.0379 ↓	
Schizophrenic Patients Tardive Dyskinesia	NS	0.0558

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

The schizophrenic control group had significantly lower levels of triglyceride than the normal control group.

Table 47: Statistical Analysis (Independent Samples t-test) HDL-Cholesterol. Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	NS	
Schizophrenic Patients Tardive Dyskinesia	0.0062 ↓	0.0264 ↓

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

The Tardive Dyskinesia patient group had significantly lower levels of HDL-cholesterol than the normal control group and the schizophrenic control group.

Table 48: Statistical Analysis (Independent Samples t-test)

Vitamin E/ Lipid (Cholesterol + Triglyceride) Ratio

Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	NS	
Schizophrenic Patients Tardive Dyskinesia	0.0182 ↓	0.0159 ↓

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

The Tardive Dyskinesia patient group had significantly lower levels of vitamin E/lipid ratio than the normal control group and the schizophrenic control group.

Table 49: Statistical Analysis (Independent Samples t-test)

Vitamin E/ Cholesterol Ratio. Tardive Dyskinesia Study

	Normal Controls	Schizophrenic Patients Controls
Schizophrenic Patients Controls	NS	
Schizophrenic Patients Tardive Dyskinesia	0.0831	NS

NS = Not statistically significant ↑ Significantly Higher. ↓ Significantly lower

3.3.4: Discussion

Schizophrenic Patients with positive or negative symptoms

Tables 32 to 40 show the demographic, descriptive and statistical analysis carried out for the subject groups in the study. The schizophrenic groups had significantly more males than females with the negative group significantly older than the other two groups. There was no difference in the vitamin A levels (table 34 & 35) of the subject groups suggesting adequacy of intake.

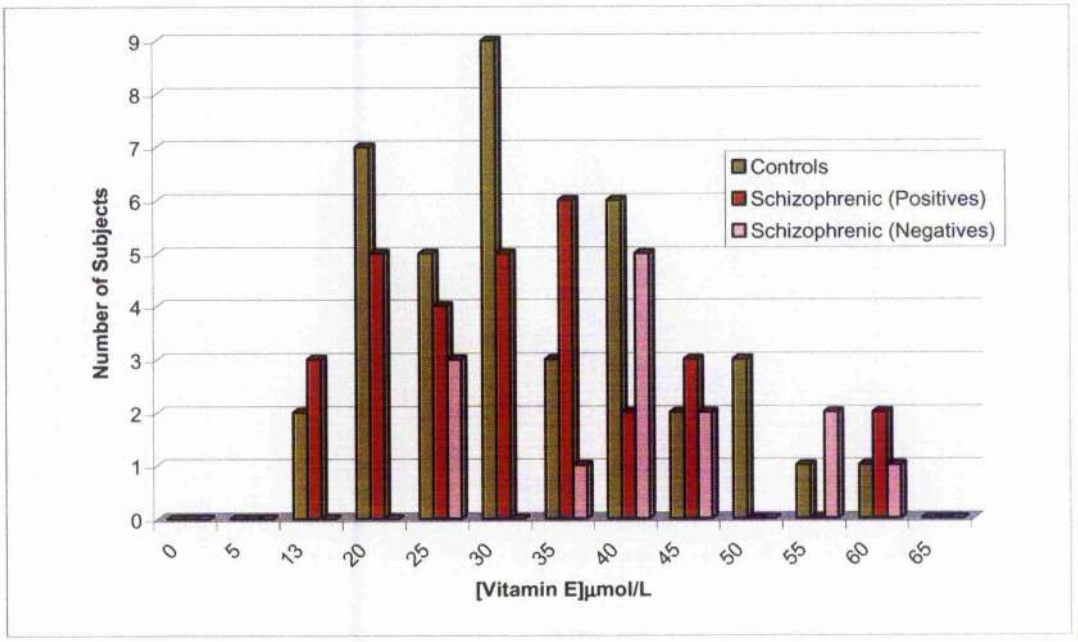


Figure 34: Distribution of Vitamin E levels in Schizophrenic Patients Study

Figure 34 shows the distribution of vitamin E levels in all subjects in this study. It clearly shows no subjects within these three groups had a vitamin E level indicating deficiency. In fact both schizophrenic groups showed distributions which were skewed to higher vitamin E levels than the control group. Patients with negative symptoms showed higher levels of vitamin E (tables 34 & 36), and cholesterol (tables 34 & 37) when compared with the

positive symptoms group. The negative symptoms group also showed higher levels of cholesterol (tables 34 & 37) and triglyceride (tables 34 & 38) than the normal control group. The positive group showed higher levels of triglyceride (table 34 & 38) when compared to the normal controls and was the only group to show a lower level of vitamin E/lipid ratio (tables 34 & 39). However, neither the positive group nor the negative group showed any statistical difference with the normal control group when analysing the vitamin E/cholesterol data (table 40). The lower vitamin E/lipid ratio in the positive symptom group is due to the action of triglyceride, which is higher in this group. The data provides no supporting evidence that vitamin E depletion is a feature of these patients. There are, however, differences between schizophrenic patients with positive and negative symptoms with respect to plasma vitamin E levels

Tardive Dyskinesia Patients.

The schizophrenic patients from this study showed a predominance of female subjects. The schizophrenic groups were also statistically older (table 41). There was no significant difference between the schizophrenic subject groups with regard to vitamin A suggesting adequacy of intake in those two groups. However the schizophrenic control group did have significantly lower levels than the normal control group (tables 42 & 43).

Vitamin E levels were statistically significantly lower in both schizophrenic groups compared to normal controls (tables 42 & 44). Although it visually appears in figure 35 that the tardive dyskinesia group is not normally distributed non-parametric statistical measures, Mann-Whitney, confirm these findings (normal controls vs tardive dyskinesia group (p: 0.131) and normal controls vs schizophrenic controls (p: 0.0044)). However figure 35 shows no group with a vitamin E level indicating deficiency.

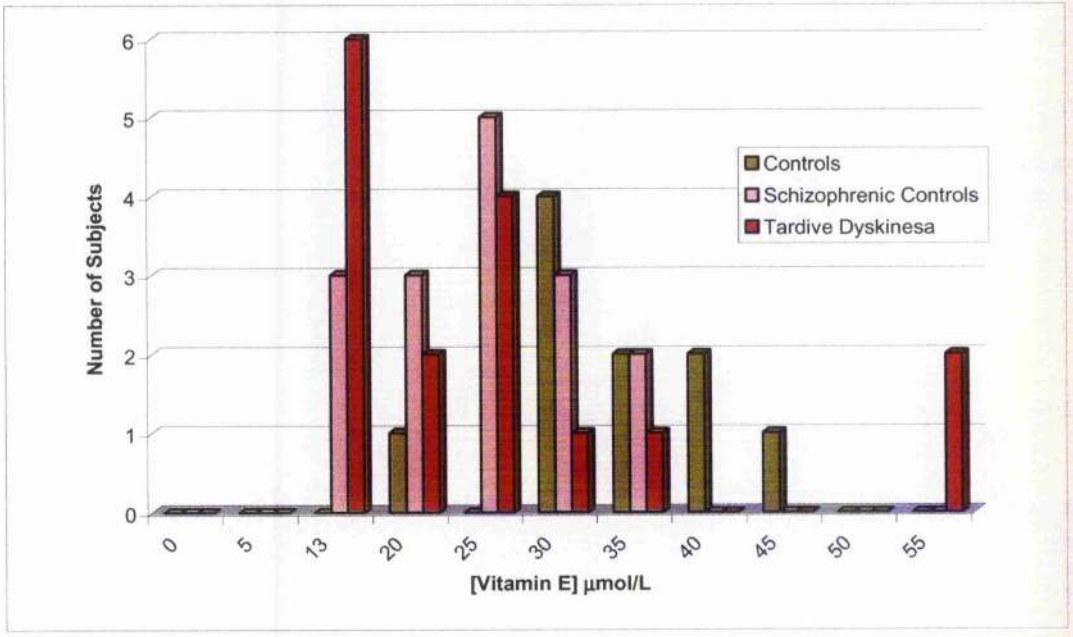


Figure 35: Distribution of Vitamin E levels in Tardive Dyskinesa Study

The cholesterol and triglyceride levels were lower in the schizophrenic control group compared to normal controls (tables 42, 45 & 46). The HDL-cholesterol level was lower in the tardive dyskinesia group compared to both the normal controls and the schizophrenic controls (tables 42 & 47) and may be due to lack of exercise and high levels of smoking[287] in this patient group. The vitamin E lipid ratio levels showed significantly lower levels in the schizophrenic tardive dyskinesia group compared to the control group also a lower level was found in the tardive dyskinesia group compared to the schizophrenic control group (tables 42 & 48).

A chronic and sustained deficiency of vitamin E could conceivably render subjects more susceptible to free radical damage, lipid membrane peroxidation and ultimately tardive dyskinesia. However there is a serious caution to this conclusion as the vitamin E/cholesterol ratio shows no difference between the three groups (tables 42 & 49). These data may be due to the effect of triglyceride on the vitamin E lipid ratio with the tardive dyskinesia group having triglycerides levels similar to the normal controls but higher than the schizophrenic controls. Some of these data have been published (Brown K, Reid A, et al 1998)[288]. This study has limitations the most important being sample size. In addition, the schizophrenic subjects enrolled into the study were atypical in that they were mostly old and chronically institutionalised.

4 General Discussion

In the introduction to this thesis free radical damage, particularly lipid peroxidation, and the use of antioxidants to prevent disease states associated with radical damage was discussed. On reading the literature there is an assumption that antioxidants, such as vitamin E, will be depleted to such an extent as to cause deficiency or low levels of vitamin which are insufficient to defend against radical attack and therefore radical production can then initiate disease processes. The data from studies carried out in this thesis have not added support to this view although the selection of subjects with more severe abnormalities may have shown an effect.

The highest percentage of subjects with vitamin A levels below 0.7 µmol/L were found in the ICU and HDU subjects (table 50). This finding is not surprising as these patients probably have low levels of retinol binding protein due to the acute phase and in some cases liver disease. This may also have implications in studies of vitamin E.

Table 50: Number and Percentage of Nutrition Study Patients with Vitamin A levels less than 0.7 µmol/ L

Nutrition Study	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Vitamin A <0.7 µmol/L	114	33	84	2
Total	540	70	186	171
Percentage	21.1%	47.1%	45.2%	1.2%

No level of vitamin E below 13 µmol/L (lower limit of the calculated normal range, page 88) was found in normal control subjects, general practice subjects or either of the schizophrenic study subjects. Figure 35a shows the distribution of vitamin E levels in all study groups and table 51 indicates the percentage of subjects with vitamin E levels below lower limit of the normal range.

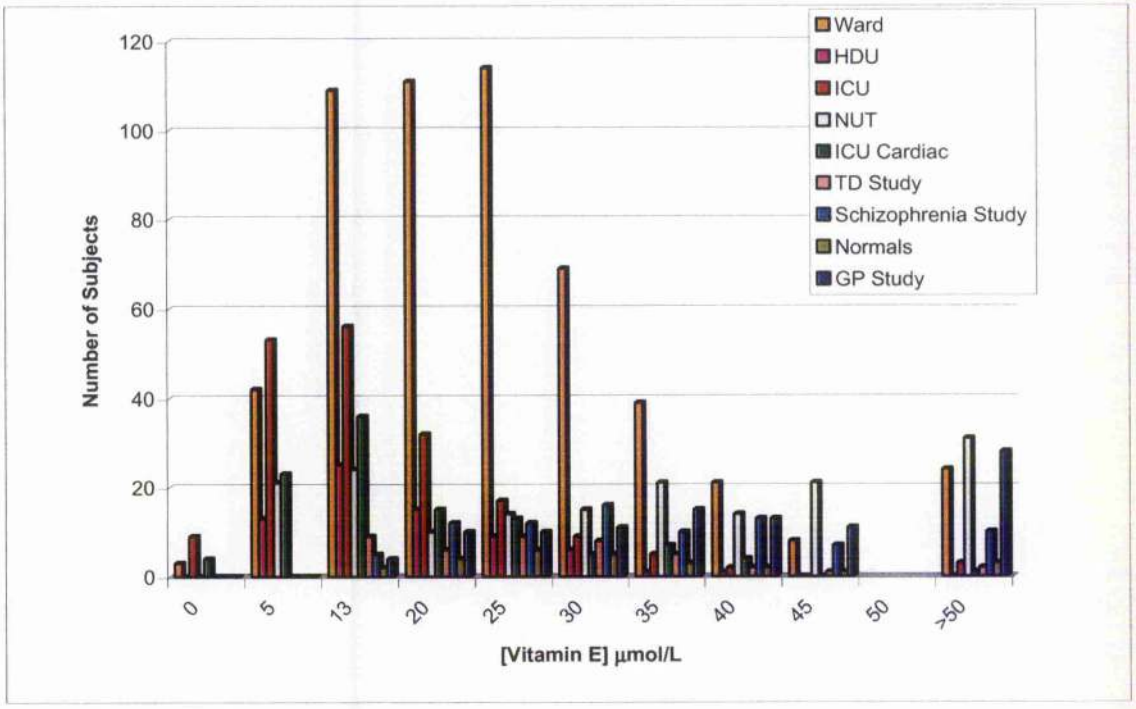


Figure 35a: Distribution of Vitamin E levels in all Studies

Table 51: Number and Percentage of Nutrition Study Patients below the lower limit of normal for Vitamin E

Nutrition Study	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Vitamin E <13 µmoi/L	45	13	62	21
Total	540	70	186	171
Percentage	8.3%	18.6%	33.3%	12.2%

As expected the largest percentage of subjects with low vitamin E levels can be found the ICU ward. However, previous publications[103,118] have suggested that vitamin E should not be conventionally treated in isolation and should be related to lipid either as a ratio of cholesterol or of cholesterol + triglyceride. Table 52 and 53 indicate the percentage of subjects with their vitamin E/cholesterol and vitamin E/ lipid ratios below the lower limit of normal.

Table 52: Number and Percentage of Nutrition Study Patients below the lower limit of normal for Vitamin E/Lipid Ratio

Patients	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Vitamin E/lipid Ratio <2.4	12	2	16	11
Total	540	70	186	171
Percentage	2.2%	2.8%	8.6%	6.4%

Table 53: Number and Percentage of Nutrition Study Patients below the lower limit of normal for Vitamin E/Cholesterol Ratio

Patients	Ward	High Dependency Unit	Intensive Care Unit	Home Nutrition Patients
Vitamin E/cholesterol Ratio <3.01	14	1	4	7
Total	540	70	186	171
Percentage	2.6%	1.4%	2.2%	4.1%

There is substantial literature indicating that patients in intensive care are stressed by free radical damage and yet the observations reported in the studies of intensive care patients shows that subnormal levels of vitamin E, expressed as either vitamin E/lipid ratio (table 52) or vitamin E/cholesterol ratio (table 53), were unusual. This apparent contradiction has resulted in a reconsideration of the correction of vitamin E with lipid in acutely ill patients.

From a whole body point of view it is essential that the dietary source of vitamin E absorbed and transported to the liver is adequately distributed to the point of need, where membrane free radical activity is required to be countered. Although, as seen in figures 36 – 44, there is generally a good correlation between cholesterol and vitamin E this correlation should be treated with caution in the acutely ill patient where a low cholesterol may be due to the effects of the acute phase (figure 31) and the amount of vitamin E transported in the blood is low. Sequestered vitamin E as occurs in the acute phase[277] may not be adequate for tissue need if the oxidative stress is prolonged. In the schizophrenic subjects who's disease state is chronic, correlation between vitamin E and cholesterol (figures 44 & 45) is poor and may indicate a different mechanism is operating in this condition.

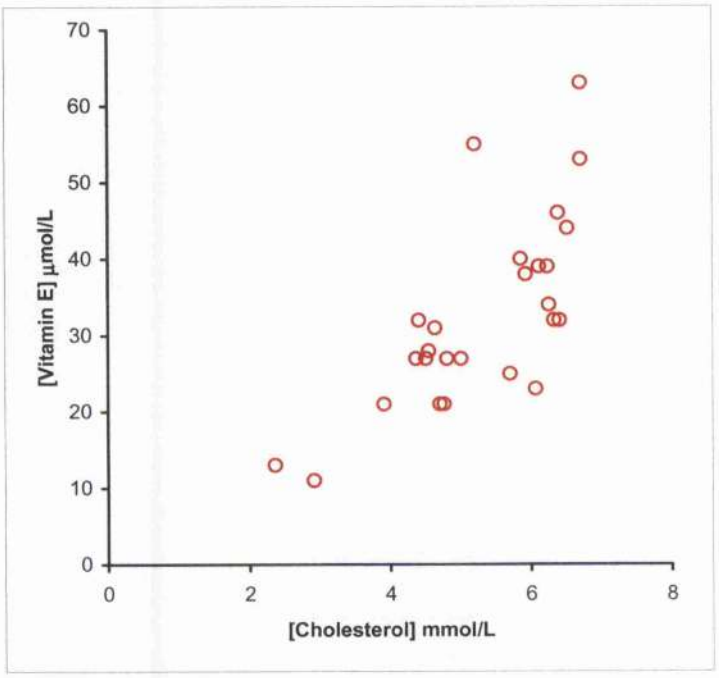


Figure 36: Normal Control Vitamin E vs Cholesterol Correlation r; 0.73 p: <0.0001

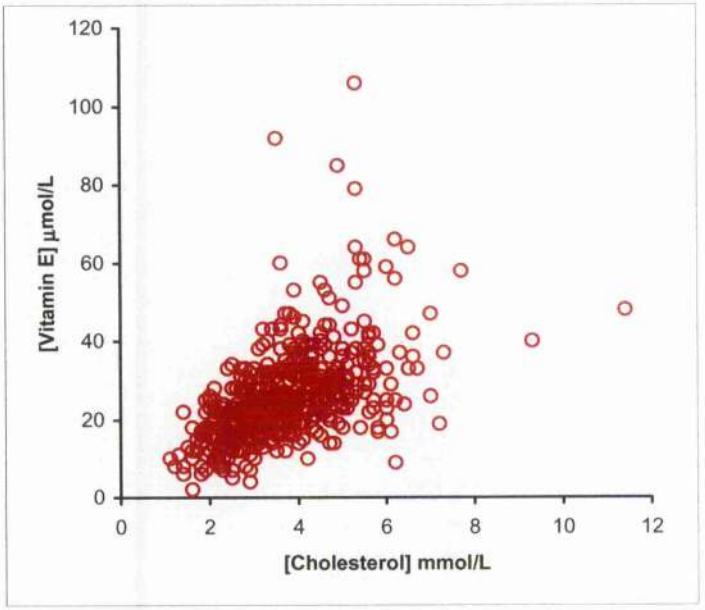


Figure 37: Ward Vitamin E vs Cholesterol Correlation r; 0.52 p: <0.0001

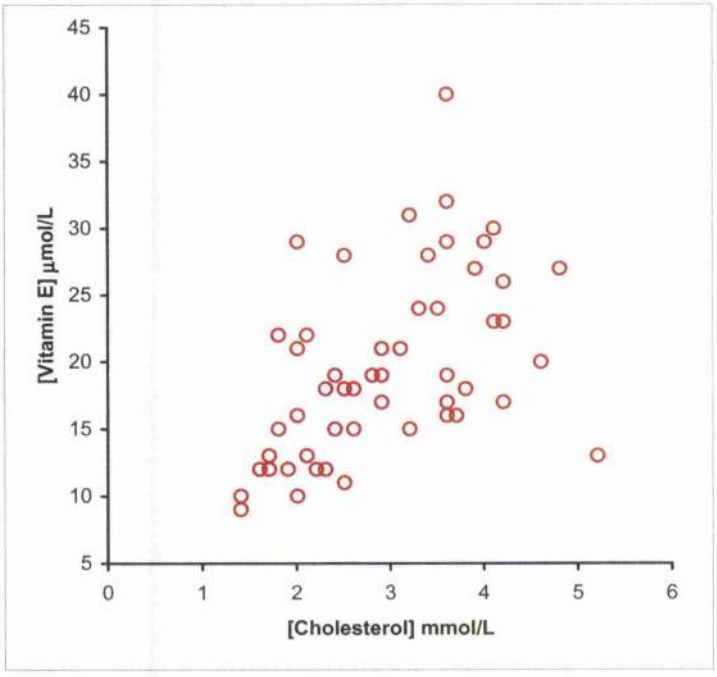


Figure 39: HDU Vitamin E vs Cholesterol Correlation r; 0.53 p: <0.0001

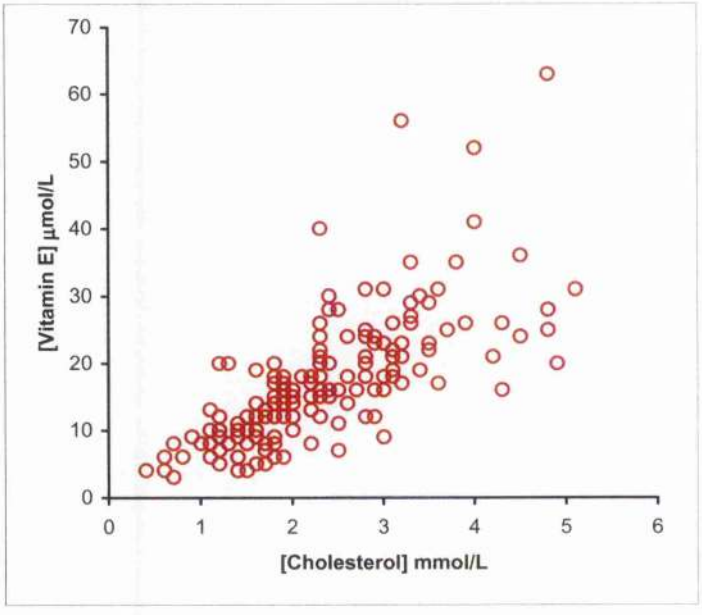


Figure 40: ICU Vitamin E vs Cholesterol Correlation r; 0.73 p: <0.0001

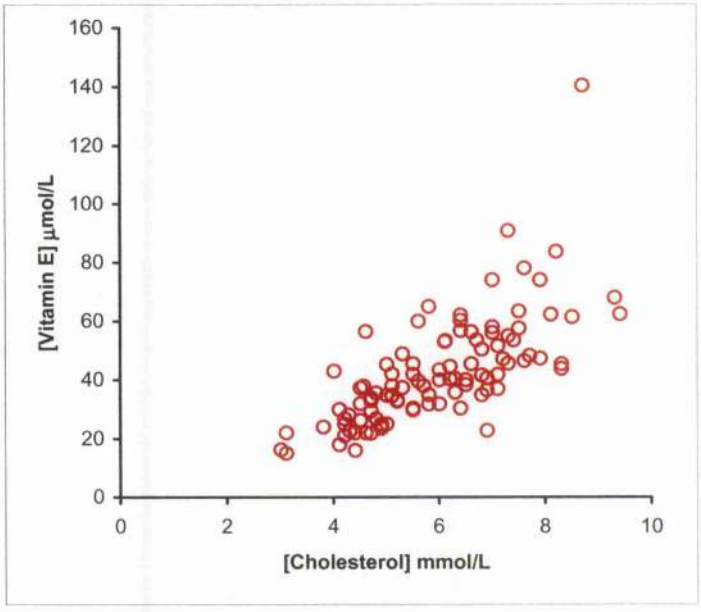


Figure 41: GP Study Vitamin E vs Cholesterol Correlation r; 0.73 p: <0.0001

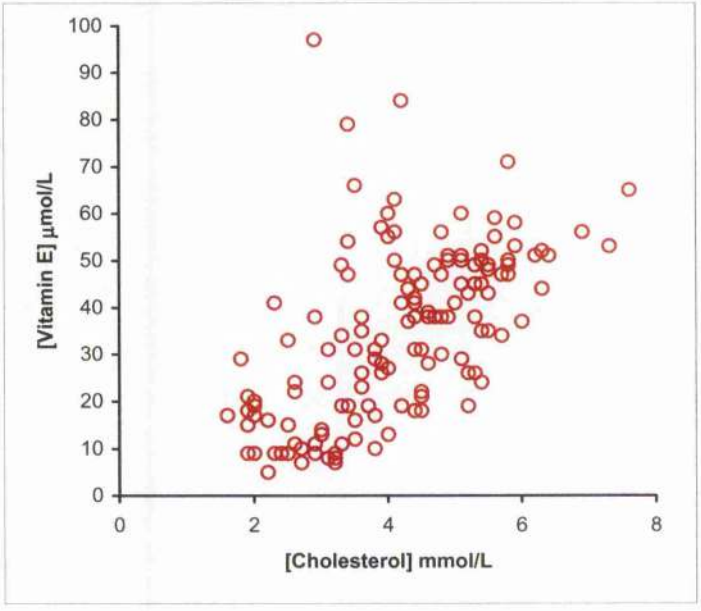


Figure 42: Home Nutrition Patients Vitamin E vs Cholesterol Correlation r: 0.58 p: <0.0001

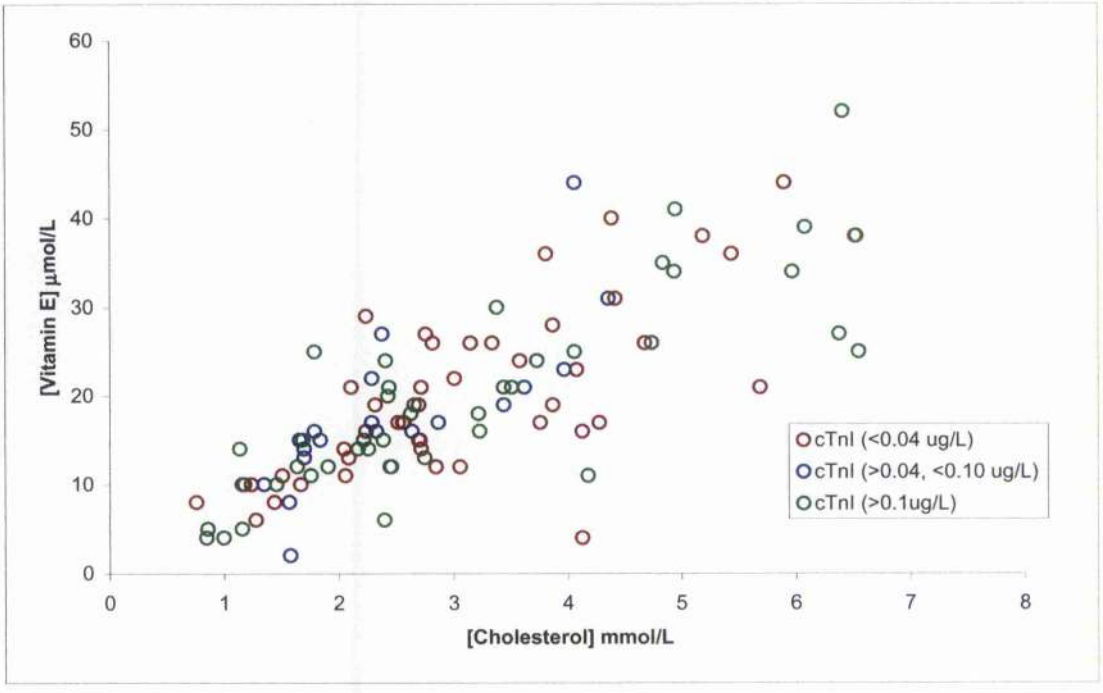


Figure 43: ICU Cardiac Injury Study Vitamin E vs. Cholesterol Correlation cTnI (<0.04 ug/L) r; 0.72 p; <0.0001. cTnI(>0.4 <0.1 ug/L) r; 0.77 p; <0.0001 cTnI (>0.1 ug/L) r; 0.84 p; <0.0001

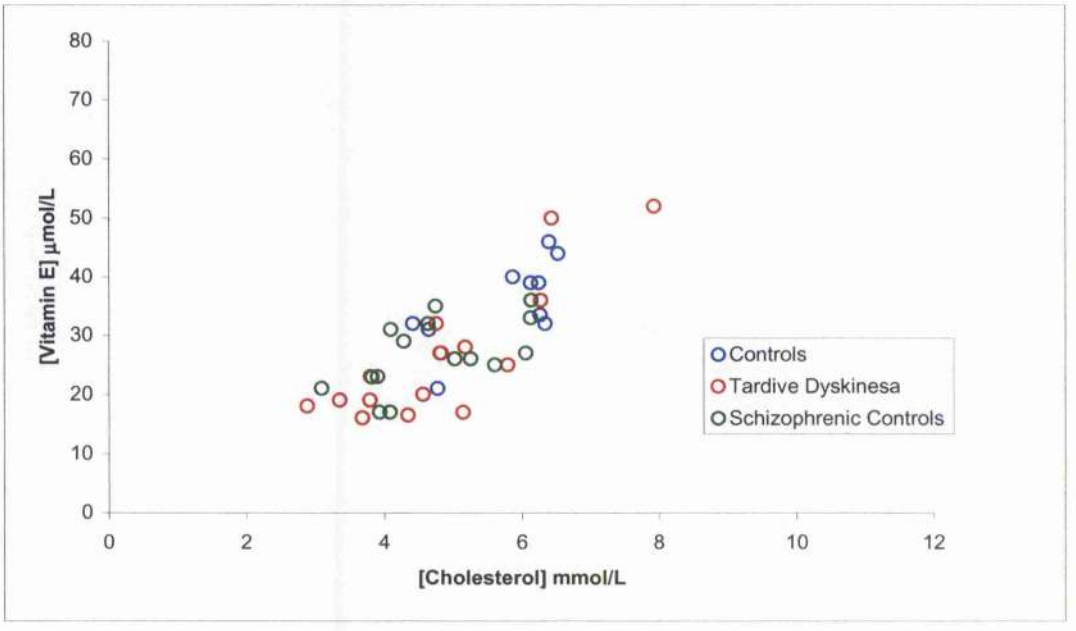


Figure 44: Tardive Dyskinesa Study: Vitamin E vs. Cholesterol Correlation Controls: r; 0.70, p;0.0248. Tardive Dyskinesa Group: r; 0.85, p; <0.0001. Schizophrenic Control Group: r; 0.58, p; 0.0243.

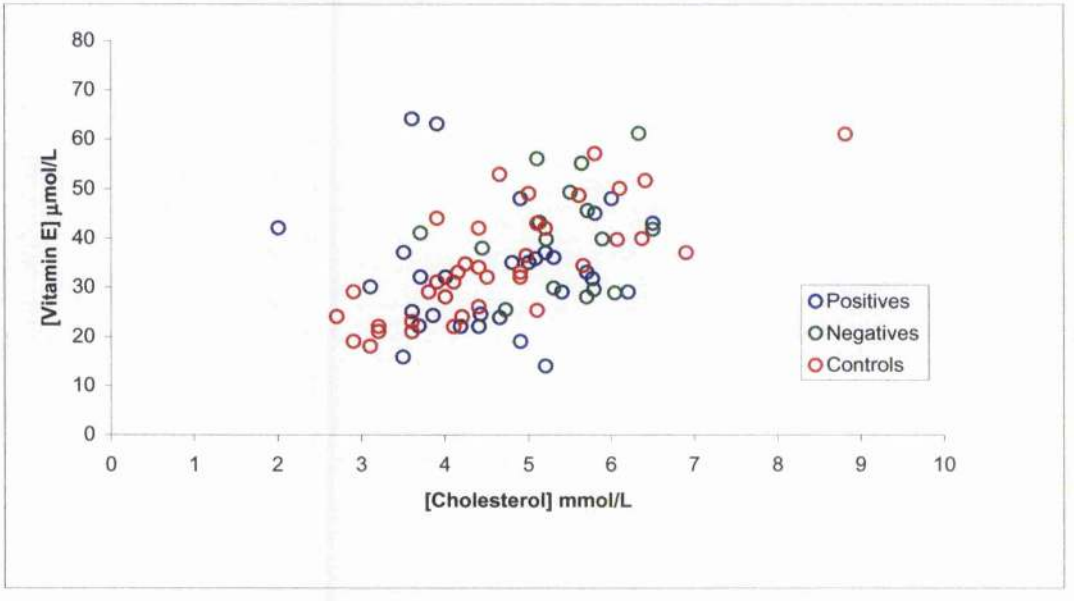


Figure 45: Schizophrenia Patient Study: Vitamin E vs. Cholesterol Correlation Controls: r; 0.76, p;<0.0001. Negatives: r; 0.16, p; 0.5565. Positives: r; 0.05, p; 0.8061

As previously discussed in 1.5.2 the mechanism of vitamin E absorption is unclear. All forms of vitamin E are taken up by intestinal cells and released into the circulation with chylomicrons where they reach the liver via chylomicron remnants. In the liver, a specific protein, α-Tocopherol Transport Protein (α-TTP) selectively sorts α-tocopherol for incorporation into VLDL and hence LDL via IDL. The capacity of plasma to increase vitamin E concentration is limited [289] and is apparently not due to limited absorption [289]. In addition, newly absorbed vitamin E (in the form of α-tocopherol) replaces old vitamin E in plasma lipoproteins which may be the limiting step in overall incorporation [289,290]. The underlying reasons are unclear but may include variations in α-TTP activity, metabolic rate, lipid content and composition, the status of other micronutrients that recycle vitamin E and environmental conditions. It seems likely that an adequate or optimal supply can vary between individuals. The results from the studies carried out in this thesis suggest, with the exception of some subjects in ICU and HDU, most are likely to have adequate absorption and transport of vitamin E evidenced by their normal plasma vitamin E levels (figure 35a). Subjects from ICU have greatest percentage (33.3%) of vitamin E levels < 13 μmol/L with HDU subjects and ward subjects showing percentages of 18.6% and 8.3% respectively. This suggests that the levels of vitamin E of these subjects may be insufficient to allow protection of the lipids in cell membranes against peroxidation or to enable possible secondary modulation actions such as anti-inflammatory effects or inhibition of platelet aggregation as previously discussed. It should be noted that the subjects in ICU (Table 7 and Table 24) and HDU (Table 7) show the lowest cholesterol levels of all the groups studied. This may be due in part to the effects of the acute phase (figure 31) on cholesterol levels but also possibly decreased absorption and liver transport. A low vitamin E may indicate deficiency but not its cause. However depending on the cholesterol and CRP levels (indicates acute phase response) some indication may be given to the cause of low vitamin E levels. For example a low

cholesterol with a low vitamin E could in the absence of an elevated CRP indicate decreased absorption and normal cholesterol with a low vitamin E may indicate impaired liver transport. It seems obvious that the subjects in ICU and HDU are at greatest risk as they are unable to absorb, transport and deliver to tissue membranes the vitamin E required to directly neutralise the damage to membranes lipids by free radicals or modulate secondary responses which may lead to the initiation of cellular defences to radical attack. Whether these patients would benefit from supplementation is open to debate as the newly absorbed vitamin E would only replace the old vitamin E in plasma lipoproteins and there may be variation in α-TTP activity. There is a requirement to measure patients' lipid levels, acute phase reactants such as CRP and liver function to assess the capacity to transport vitamin E.

In the schizophrenia studies the subjects with positive and negative symptoms show no correlation between cholesterol and vitamin E (Figure 45) and are in fact the only two groups to do so. This lack of correlation may be due to dietary effects or subjects taking vitamin E supplements. It should be noted that these studies are limited because of the number and demographic makeup of the subjects. Although vitamin E cholesterol ratios in the schizophrenia study and the tardive dyskinesia study show no significant difference between their respective study control groups and the schizophrenia patient subjects, there are significant differences with regard to vitamin E. The negative schizophrenics gave a statistically significantly lower vitamin E in relation to the positive schizophrenic group but not the normal control group. Both the schizophrenic control and tardive dyskinesia groups show a statistically significantly lower vitamin E than their normal control group. These findings in schizophrenic groups may be due to disease process, dietary effects or possibly deficiencies in liver α-TTP activity.

It was postulated that the patients in the ICU cardiac injury study would be under greater free radical insult and therefore show lower vitamin E levels in

patients with troponin I levels indicating cardiac muscle injury. Although some subjects had low vitamin E this was also the finding for the study group with no evidence of cardiac muscle injury. It therefore appears from these data that there is no relationship between vitamin E and cardiac injury.

The findings of a close correlation between vitamin E and cholesterol suggests that in non-acutely ill patients a corrected vitamin E cholesterol ratio may be used as an indicator of absorption and transport via liver α-Tocopherol Transport Protein into the plasma. Adequate levels may indicate the availability of vitamin E to the tissues. It is inappropriate to correct for lipid in the acutely ill patient due to the unpredictable effects on absorption, liver α-Tocopherol Transport Protein activity and lipoprotein content. For these subjects vitamin E should not be corrected for lipid but should also not be treated in isolation. Due regard to should be paid to effects of the acute phase response, decreased absorption and liver disturbance.

5 Conclusion

Low levels of vitamin A are relatively common in the hospital population. However plasma vitamin A level interpretation may be complicated by the effects on retinol binding protein by co-existing liver disease and acute phased response. It may be more appropriate to measure vitamin A in tissues, such as erythrocytes, that may not be affected by the acute phase response.

A low plasma vitamin E is a rare occurrence in the general population, however it is found more commonly in the hospital in-patient population. The concept of correcting vitamin E with lipid is appropriate when dealing with subjects in the general population but should not be used when assessing nutritionally depleted in-patients or in subjects who have low cholesterol levels and evidence of acute phase response. Tissue vitamin E measurements may provide the only reliable means of indicating deficiency.

Further larger studies on schizophrenia and tardive dyskinesia subjects are required to confirm the findings of this thesis. The definition of patients with positive and negative symptoms of schizophrenia is less well recognised now than when these studies were completed and will require review when the causes of positive and negative symptoms become clearer.

There are currently many studies looking into the effects of vitamin E supplementation and ischaemic heart disease which have still to report. However the MRC/BHF Heart protection study has reported no benefit in vitamin E supplementation. It is interesting that the ICU myocardial injury study showed no significant difference in the vitamin E levels in patients with myocardial injury indicated by cardiac troponin I levels. There is some thought that vitamin E, as an antioxidant, is not directly responsible for the protective effects associated with ischaemic heart disease but that it modulates cellular functions[292, 293].

Using direct measurements of lipoprotein fractions and oxidised LDL would have been more appropriate in helping assess deficiency of the fat soluble vitamins investigated in this thesis. It may be appropriate to examine other components such as cytosolic phospholipase A_2[293,294] or 5-lipoxygenase[295] as it has been shown to regulate the expression of these enzymes.

Although the title of this thesis indicates a study of a wide range of determinants of free radical activity the work has concentrated on vitamin E. In this thesis vitamin E measurement either alone or with lipid correction have been examined in a wide range of clinical situations. The use of lipid correction for vitamin E measurement should be used with caution. For the majority clinical applications the most reliable and available assessment of the vitamin is the plasma measurement without lipid correction.

As new hypotheses of vitamin E actions on the modulation of cellular function are investigated an indication of a patients vitamin E level will be still be required. The last word on actions of vitamin E is still yet to be written.

Appendix 1

Suppliers of Reagents, Equipment and Software

Alpha Laboratories Limited
40 Parham Drive, Eastleigh, Hampshire SO50 4NU

Capped Eppendorph tubes

Anachem Limited
Anachem House, Charles Street, Luton, Bedfordshire LU2 0EB

Gilson Micro Volume Pipettes

P100	Volume range:	20 – 100 µl
P200	Volume range:	50 – 200 µl
P1000	Volume range:	200 – 1000 µl

Analyse-It Software Ltd
PO Box 77, Leeds LS12 5XA

Analyse-it software a statistical analysis add in for Microsoft® Excel®.

Agilent Technologies UK Limited
Chemical Analysis Group, Lakeside, Cheadle Royal Business Park
Stockport, Cheshire SK8 3GR.

High Performance Liquid Chromatography 1100 Series
Chemstation Control and Integration Software

Beckman Coulter UK Limited
Oakley Court, Kingsmead Business Park, London Road,
High Wycombe, Buckinghamshire HP11 1JU.

Access Immunoassay System.

Cardiac Troponin I Reagent Pack,	Cat. No.: 33320.
Cardiac Troponin I Calibrators,	Cat. No.: 33325.
Cardiac Troponin I Quality Control	Cat. No.: 33329.

BDH Laboratory Supplies
McQuilkin & Co., 21 Polmadie Avenue, Glasgow G5 0BB

HPLC Grade Acetonitrile, Methanol, Water and Hexane

Bio-Rad Laboratories Limited
Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts, HP2 7TD.

Lyphocheck Immunoassy control 1 and 3 for vitamin internal Quality control.

Bio-Stat Diagnostics Systems
Biostat House, Pepper Road, Hazel Grove, Stockport, Cheshire SK7 5BW.

HDL-Cholesterol Kit Cat. No.: 915090.
Bio-Stat HDL-Cholesterol Calibrator, Cat. No.: 915084.

British Oxygen Company
BOC Gases and Gear Centre, 150 Polmadie Road, Glasgow

Oxygen Free Nitrogen.

Crawford Scientific
Holm Street, Strathaven, Lanarkshire ML10 6NB

5 ml Amber Vials and screw caps.
300 µl Glass Inserts for amber vials.
Butyl rubber seals

Diagnostic Scotland
Law Hospital, Carluke, ML8 5QZ

Low, Mid and High Quality Control Material.

DakoCytomation Ltd
Denmark House, Angle Drove, Ely, Cambs CB7 4ET

Rabbit Anti-human CRP antibody
CRP Assay Reaction Buffer
CRP Diluent Buffer

Hayman Ltd
Eastways Park, Witham, Essex CM8 3YE.

Ethanol

Microsoft® Corporation
One Microsoft Way, Redmond, WA 98052-6399 USA

Microsoft® Word® 2000
Microsoft® Excel® 2000

PerkinElmer Life Sciences
PerkinElmer House
204 Cambridge Science Park, Milton Road, Cambridge CB4 0GZ

Lamda 5 UV/VIS scanning spectrophotomer.

Phenomenex
Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2BN

Ultracarb 5μ ODS(20) 150 x 3.2 mm HPLC Column.
Ultracarb 5μ ODS(20) 30 x 4.3 mm HPLC guard Column.

Protein Reference Unit
Department of Immunology, PO Box 894, Sheffield S5 7YT.

C-reactive protein calibrant SPS-03
SPS-12 and SPS-13 control material

Roche Diagnostics Limited
Bell Lane, Lewes, East Sussex BN7 1LG

Hitachi 717 automatic analyser

Cholesterol Kit Cat. No.: 1489437
Cholesterol Standard, Precipath L, Cat. No.: 1285874.
Triglyceride Kit Cat. No.: 1488899
Triglyceride Standard, Precipath L, Cat. No.: 1285874

Sigma Chemical Company
Fancy Road, Poole Dorset BH17 7NH

Retinol (Cat. No. 95144)
Retinol Acetate (Cat. No. 46958)
α-tocopherol (Cat. No. T3257)
tocopherol acetate (Cat. No.T3001)

Techne (Cambridge) Limited
Duxford Road, Cambridge, England

Techne Dri-Block DB3 with SC3 Sample Concentrator.

Thistle Scientific Limited
DFDS House, Goldie Road, Uddingston, Glasgow G71 6NZ

MSE Microcentrifuge
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Glossary

$A^{-\bullet}$	Ascorbyl radical
$OH^{\bullet}$	Hydroxyl Radical
H_2O_2	Hydrogen Peroxide
$^1O_2$	Singlet molecular oxygen
$O_2^{-\bullet}$	Superoxide radical
AMI	Acute myocardial infarction
ADP	Adenosine Diphosphate
α-TTP	α-tocopherol transfer protein
ATP	Adenosine Triphosphate
ACAT	Acyl-cholesterol acyltransferase
APO CII	Apolipoprotein CII
APO B	Apolipoprotein B
CRP	C- Reactive Protein
CT	Catalase
cTnI	Cardiac troponin I
DHA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
EAR	Estimated Average Daily Requirement
GSH	Glutathione (Reduced)
GSH-PX	Glutathione peroxidase
HALS	4-hydroxyl alkanals
HAMA	Human anti-mouse antibodies
HDL	High Density Lipoprotein
HMG-CoA Reductase	3-hydroxy-3-methylglutaryl-CoA Reductase
HPLC	High pressure liquid chromatography
IDL	Intermediate density lipoprotein

LDL	Low Density Lipoprotein
LPO	Lipid peroxidation
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
mm-LDL	minimally modified Low Density Lipoprotein
NAD	Nicotinamide Adenine Dinucleotide
NADH	Dihydronicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Dihydronicotinamide Adenine Dinucleotide Phosphate
ox-LDL	Oxidised Low Density Lipoprotein
PAI-1	Plasminogen activator inhibitor–1
PET	Positron Emission Tomography
PLA_2	Phospholipase A_2
pO_2	Partial Pressure of Oxygen
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
RDA	Recommended Daily Allowance
ROS	Reactive oxygen species
RNI	Reference Nutrient Intake
SMR	Standardised mortality ratio
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TD	Tardive Dyskinesia
TF	Tissue factor
TG	Triglyceride
UV	Ultra Violet
VIS	Visible
VLDL	Very Low Density Lipoprotein

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